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Placental Contribution to Fetal Neurodevelopment: Differential Expression of Neurogenesis Mediators in Physiological and Preeclamptic Placenta-derived Mesenchymal Stromal Cells

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Abbreviations

BDNF: Brain Derived Neurotrophic Factor

BM: Bone Marrow

DCX: Doublecortin

EVT: Extravillious Trophoblast

FGR: Fetal Growth Restriction

HMGB1: High Mobility Group Box 1

HPA axis: Hypothalamic-Pituitary-Adrenal axis

IDO-1: Indoleamine-2,3-Dioxygenase 1

IVS: Intravillous Space

Kyn: Kynurenine

MSCs: Mesenchymal Stromal Cells

MVs: Microvesicles

NCAM: Neural Cell Adhesion Molecule

NGF: Nerve Growth Factor

NMDA: N-Methyl-D-Aspartate

NT-3: Neurotrophin 3

NT-4: Neurotrophin 4

PDMSCs: Placenta-derived Mesenchymal Stromal Cells

PE: Preeclampsia

PlGF: Placental Growth Factor

PSA-NCAM: Polysialic Acid Neural Cell Adhesion Molecule

sFlt-1: Soluble Vascular Endothelial Growth Factor Receptor 1

STB: Syncithiotrophoblast

TDO: Tryptophan-2,3- Dioxygenase

TPH-1: Tryptophan Hydroxylase 1

TrkB: Tropomyosin-related Kynase B

Trp: Tryptophan

VEGF-A: Vascular Endothelial Growth Factor A

VEGFR-2: Vascular Endothelial Growth Factor Receptor 2

5-HT: 5-Hydroxytriptamine, Serotonin

ABSTRACT

A healthy and functional placenta, able to support fetal growth and development, is pivotal for a successful pregnancy and future health of the newborn. The syncithiotrophoblast is directly involved in the modulation of fetal neurodevelopment, metabolizing fetal neurotransmitters from maternal circulating precursors until the second trimester of pregnancy. Among placenta cellular populations, Placenta-derived Mesenchymal Stromal Cells (PDMSCs) represent a peculiar cell type with stem cell-like features resident in the chorionic villi. They have absent immunogenicity and unique immunomodulatory properties as well as the ability to regulate different cellular processes through a paracrine activity. Importantly, PDMSCs play a key role in placental pathophysiology. We recently reported that PDMSCs produce important neuroactive molecules as Brain-Derived Growth Factor (BDNF) and Neurotrophins -3 and -4 (NT-3, NT-4). In particular, we demonstrated that these molecules are over-expressed in PDMSCs isolated from pregnancies complicated by Preeclampsia (PE) and Fetal Growth Restriction (FGR), severe placenta-related disorders associated with neurological impairment and intellectual disability for the newborn. PDMSCs contribution to fetal neurodevelopment has never been investigated. Since they are mainly localized nearby fetal vessels, an ideal position to release molecules directly in the fetal circulation, we hypothesize that they could be directly involved in the placental modulation of fetal neurodevelopment and that the aberrant PE-FGR PDMSCs neurotrophins production could contribute to the cognitive defects typical of PE-FGR newborns.

For the first time to our knowledge, we reported a differential expression of neurogenesis-related molecules in normal and PE-FGR PDMSCs. In particular, PE-

FGR PDMSCs overexpress neurogenesis activators Neural Cell Adhesion Molecule (NCAM), Doublecortin (DCX), Nestin and High Mobility Group Box 1 (HMGB1), as well as they display a downregulation of neurogenesis inhibitors Polysialic Acid NCAM, Indoleamine-2,3-dioxygenase 1 (IDO-1), and miRNA-25, 124, -134, -137 and -181a. Moreover, we demonstrated PDMSCs contribution and differential regulation of IDO1-mediated tryptophan catabolism, with a decrease of neurotoxic kynurenine (Kyn) release by PE-FGR relative to physiological PDMSCs.

Our data strongly suggest that in pregnancy affected by PE-FGR PDMSCs could activate compensation mechanisms in order to protect fetal nervous system development.

INTRODUCTION

Introduction

Since earliest times, the placenta has been recognized as being of great importance and at the same time quite mysterious, even somewhat mystical. The Greeks recognized the importance of the placenta in fetal nutrition, and named the outermost embryonic membranes *chorion* (membrane) and the innermost membrane encompassing the fetus *amnion* (bowl). The Greek philosopher and biologist Aristotle (384-322 B.C.E.) may have been the first to use the term *chorion*, and he also recognized the yolk sac of lower vertebrates. Nonetheless, he did much to establish the science of the study of fetal membranes in his great embryological treatise *"*D*e generatione animalium"* ("On the generation of animals", *Opera, edidit Academia Regia Borussica*. 5 vols. Reimer, Berolini, 1831-1870). In 1667, Walter Needham, a Cambridge medical graduate who was the first to report chemical experiments on the developing mammalian embryo, claimed that the uterine arteries must supply nutrients to the uterus and developing fetus. In Chapter Two of his *"Disquisitio anatomica de formato foetu"* ("Disquisition on the anatomy of the formation of the fetus"), he demonstrated the *chorion frondosum* (leafy membrane) and *chorion leave* (disappearing membrane), and confirmed that the placenta consists of separate maternal and fetal portions with individual circulations [1]. Nowadays, the human placenta has become a focus of interest, being a unique structure of maternal and fetal origin and a temporary organ that performs crucial functions in sustaining a successful pregnancy. It is responsible for the import of nutrients, exchange of gases and removal of waste products from the fetus. In addition, it synthesizes several peptides and synthetic hormones regulating many functions required for a healthy pregnancy.

Placenta Development

Placentation occurs during the first trimester of pregnancy and involves the development of the villous tree structure with the simultaneous transformation of uterine arteries which supply maternal blood to the placenta [2, 3].

The placenta begins to develop at 6 to 7 days postconception when the blastocyst attaches to the endometrium [4, 5]. At this stage the blastocyst consists of an inner cell mass (the future embryo) and an outer cell layer from which the placenta arises (Langman, J., *Langman's Medical Embryology* 2003, Lippincott, Williams and Wilkins). Between days 8 and 12 following conception, the trophoectoderm differentiates into two trophoblast layers, an outer multinucleated syncytiotrophoblast and an inner layer of single cell cytotrophoblasts [5]. The trophoblast layer is now divided into three zones: the outer trophoblastic shell adjacent to the endometrium, the primary chorionic plate facing the embryo with the lacunae and trabeculae in between [5]. The primary chorionic plate consists of extraembryonic mesoderm and the inner layer of the trophoblast. The lacunae and trabeculae will eventually form the intravillous space (IVS) and the anchoring villi, whilst the primary chorionic plate and trophoblastic shell form the basis of the chorionic and basal plates respectively. Some of the cytotrophoblasts penetrate the syncytiotrophoblast and invade into the endometrium, forming a layer known as the extravillous cytotrophoblast (EVT) [6]. The cytotrophoblasts within the trabeculae proliferate and by day 13 develop side branches which project into the lacunae [2, 3]. These side branches are called primary villi and are the first villous structures to develop. The extraembryonic mesenchyme of the primary chorionic plate invades the trabeculae and primary villi turning them into secondary villi [5]. Haemangioblastic progenitor cells within the mesenchyme

differentiate into the first fetal capillaries and once the villi are vascularized they are termed tertiary villi [5, 7, 8]. Each trabecula develops into a thick villous stem which branches repeatedly, giving rise to a villous tree that continues to develop, forming the highly branched placenta [9]. The trophoblastic plug maintains the early placenta in a hypoxic state (PO_2< 15mmHg) relative to the endometrial stroma which is believed to protect the embryo from free-radical damage and promote trophoblast proliferation and capillary angiogenesis [10, 11]. Towards the end of the first trimester, the trophoblast plugs disappear creating maternal arterial flow into the IVS, and raising PO_2 to 60mmHg [12]. Remodeling of the uterine spiral arteries describes the transformation of small muscular spiral arteries in the decidua and inner third of the myometrium into vessels [13]. This physiological conversion is necessary to maintain high volume, low resistance maternal blood flow to the IVS which is essential to meet fetal growth demands in late gestation.

The first generation of placental villi are vascularized by the local *de novo* formation of capillaries, known as vasculogenesis. Vasculogenesis starts 21 days after conception as string-like collections of haemangioblastic cells appear within villi [8]. Vascular endothelial growth factor A (VEGF-A) and its receptor, vascular endothelial growth factor receptor 2 (VEGFR-2), are highly expressed in early pregnancy and are thought to be responsible for the recruitment and aggregation of endothelial precursor cells into cord-like structures [14]. The villous capillaries fuse together and with the allantoic vessels thereby establishing a connection between the placental and embryonic circulations, the umbilical cord, between days 32 and 35 [15]. Subsequent development of the feto-placental circulation is by angiogenesis. Formation of the capillary networks occurs from day 32 until the end of the second trimester predominately by branching

angiogenesis and lateral sprouting. From the end of the second trimester to term, villous vascular growth switches to non-branching angiogenesis to form the terminal capillary loops. At this stage, capillary growth exceeds trophoblast growth resulting in coiling of the capillaries within terminal villi [15]. Each terminal villus has one or two capillary loops and these loops bulge towards the trophoblast surface thereby creating a thin, vasculo-syncytial membrane for diffusion exchange. The gestational switch from predominately branching to non-branching angiogenesis might be mediated by a reduction in VEGF-A and an increase in placental growth factor (PlGF) expression and both these factors may be regulated by oxygen [16, 17].

Fetal Programming and Neurodevelopment

The intrauterine environment has an important influence on lifelong health and babies who grew poorly in the womb are more likely to develop chronic diseases in later life. Placental function is a major determinant of fetal growth and is therefore also a key influence on lifelong health. The capacity of the placenta to transport nutrients to the fetus and regulate fetal growth is determined by both maternal and fetal signals. The way in which the placenta responds to these signals is subjected to evolutionary selective pressures [18]. The idea that disease risk in adulthood is affected by prenatal and early life events emerged during the twentieth century [19]. Barker and colleagues published a number of papers in Lancet from 1986 and onwards that came to be the foundation of the developmental origins of health and disease [20-22]. In these studies they showed how undernutrition in fetal life affected developmental processes and permanently changed the body's structure and function, and how these changes

subsequently were linked to risk of ischemic heart disease in adult life [23, 24]. Later referred to as the fetal origins hypothesis, it was supported by experimental data in animals indicating that maternal nutrition can program long term effects on the offspring without necessarily affecting size at birth.

Placental Tryptophan Metabolism

During pregnancy, the essential amino acid tryptophan (Trp) is actively transported to the fetus by the placenta [25]. Besides being utilized for protein synthesis by the placenta and fetus, another fate of Trp is degradation via the serotonin (5-hydroxytriptamine, 5-HT) and kynurenine (Kyn) pathways. There is growing interest in the role of Trp and its metabolites in a diverse range of disease processes including inflammatory brain diseases [26, 27]. By analogy with the human adult brain, some of Trp metabolites have been implicated in producing fetal neurological damage during pregnancy, especially in association with in utero infection [28]. However, to date only limited information regarding tryptophan, its kynurenine metabolites, and the kynurenine pathway enzymes in pregnancy has been published [29, 30].

Tryptophan hydroxylase-1 (TPH-1) is known to be highly expressed in the placenta of several species [31], and in mice placental synthesis of serotonin is important for early brain development [32]. Serotonin may also be important for maintaining vasodilation of the uterine circulation in the vicinity of the implanted placenta [33]. A recent study showed that the fetal forebrain accumulates placenta-derived 5-HT during early pregnancy, a period during which axons experience active outgrowth and guidance [32]. The serotonergic system steers neurodevelopment through the key modulation of

neurogenesis, cell migration, and brain wiring that give rise to proper brain function. With a diversity of molecular targets on which to focus, it makes sense that perturbations of 5-HT signaling have been implicated in the pathogenesis of diverse neurodevelopmental disorders. The perturbations of the 5-HT neurotransmitter system during development, whether directly on the fetal brain or on its placental modulation during early gestation, may have long-lasting developmental and physiological consequences. Risk factors, both genetic and environmental, that alter 5-HT concentration in the fetal brain tissue may thus ultimately pose far-reaching functional consequences throughout life [34].

Trp is also oxidized by enzymes tryptophan-2,3-dioxygenase (TDO) and indoleamine 2,3- dioxygenase 1 (IDO-1), both of which are expressed in the human placenta. In early murine pregnancy IDO-1 activity has been implicated in suppression of the maternal immune response to the conceptus [35, 36]. The role of this pathway in human pregnancy during early and late gestation is unclear. IDO-1 activation results in decreased blood concentrations of Trp and increased blood concentrations of kyn pathway metabolites [37]. This is relevant to pregnancy because many kynurenine metabolites have actions within the central nervous system and might impact on the fetal brain. For example, kynurenine is a convulsant [37], whereas kynurenic acid, a N-methyl-D-aspartate (NMDA) receptor antagonist, has anti-convulsant properties [38]. Quinolinic acid, a NMDA receptor agonist and an excitotoxin, has been implicated in many neurological disorders, such as Alzheimer's and Huntington's diseases. It has been proposed that placenta-derived kyn metabolites may be involved in the etiology of perinatal brain damage and all of them have been found in umbilical cord blood at term. Little is known about circulating levels of tryptophan metabolites throughout normal

physiological events such as pregnancy and labour, or of the ability of the placenta to produce kynurenines during stress, such as the hypoxia that often arises in late gestation and at parturition [39].

Neurotrophins and Placentation

Neurotrophins, a family of closely related proteins, were originally identified as growth factors for survival, development, and function of neurons in both the central and peripheral nervous systems [40]. Subsequently, neurotrophins have been shown to have functions in immune and reproductive systems. In particular, Nerve Growth Factor (NGF) and Brain-derived Neurotrophic Factor (BDNF) are known to play an important role during pregnancy in the process of placental angiogenesis and maturation [40], however little is known about the mechanisms through which they influence the growth and development of the placenta and pregnancy outcome.

BDNF is involved in cerebral morphogenesis, synaptic plasticity and learning, mental health, degenerative central nervous system diseases and energy metabolism [41, 42]. During development, BDNF plays a fundamental role in cerebral morphogenesis, governing neuronal migration and survival and regulating neuronal connectivity [43]. Knowledge about production sites within the fetal compartments is lacking, likewise, reference intervals for different gestational ages have not yet been established. Umbilical cord blood analyses of premature neonates allow the assumption that levels are increasing with advancing gestation [44], but concentrations in fetal blood samples have not yet been determined. In 2011, Fujita and colleagues investigated the correlation of neurotrophins and their receptor in human placenta with uterine

environment and fetal growth [45]. They reported that neurotrophin receptor tropomyosin– related kynase B (TrkB) mRNA was expressed on decidual and villous tissue and increased with gestational age. Villous TrkB mRNA was significantly increased in pregnancies complicated by preeclampsia than in controls and was higher in the normotensive small for gestational age (SGA) placentae. It was also significantly increased in the small twin of discordant twin pregnancies. BDNF, the main ligand of TrkB, was expressed in membranous chorion and villous tissue and was significantly higher in maternal plasma in normotensive SGA and preeclampsia than in controls. TrkB mRNA expression was up-regulated on cultured villous tissue explants and on JEG-3, a choriocarcinoma cell line, by H_2O_2 treatment. BDNF decreased apoptotic cells in H_2O_2-treated JEG-3, indicating that BDNF/TrkB signaling had anti-apoptotic effects against oxidative stress in JEG-3 and suggesting a protective role of BDNF/TrkB in human villous tissue under unfavorable conditions in utero [45].

Neurotrophins-3 and -4 (NT-3 and NT-4) are also members of neurotrophin family. NT-3 plays an essential role in the development of both the neural-crest derived peripheral nervous system and the central nervous system [46]. Neurotrophin 4 (NT-4) bind to the TrkB receptor corresponds with the onset of neurogenesis in the neural tube during brain development and is differentially regulated in later development [47]. There are very few studies which have discussed the role of NT-3 and NT-4 in the development of the feto-placental unit [48]. It has been hypothesized that NT-3 function in the regulation of placental and fetal brain development and for the maternal inflammatory responses have demonstrated the expression of neurotrophin 4 and its receptor TrkB in trophoblast cells and placentas during different stages of pregnancy in mice [49] (Figure 1). The altered level and expression of these neurotrophins have been also indicated in

complicated pregnancies such as fetal growth restriction, preeclampsia and preterm delivery. It has been suggested that BDNF and NGF could be markers for the presence of central nervous system abnormalities, infectious insults in utero or both [50]. Study suggests that NT-3 may be playing a regulatory function on placenta and fetal brain development and maternal inflammatory response. It has been reported that the circulating NT-3 levels increased in early neonatal life, possibly due to exposure to various stimuli soon after birth [51]. NT-3 and NT-4 have been documented to act at early stages of neuronal development and to decrease after hypoxia-ischaemia [52].

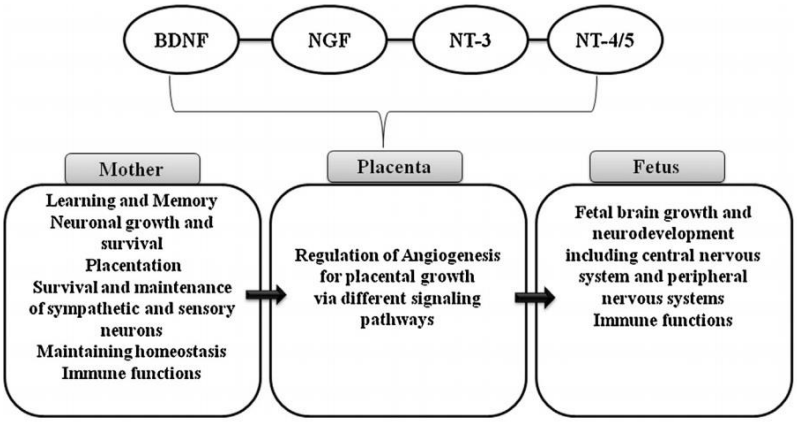

Figure 1. Role and function of neurotrophins during pregnancy [48].

Placental Mesenchymal Stromal Cells

The first reports on the possible existence of a population with a mesenchymal progenitor are attributed to Friedenstein and colleagues [53]. Indeed, they identified and defined these cells as plastic-adherent fibroblast colony-forming units with clonogenic capacity [54]. Later, these cells were also named as marrow stromal cells, on the basis of their possible use as a feeder layer for hematopoietic stem cells [55]. Mesenchymal Stromal Cells (MSCs) are pluripotent progenitor cells which divide many times and

whose progeny eventually gives rise to different tissues. By definition, these stem cells are not governed by or limited to a fixed number of mitotic divisions [56]. Their progeny is affected by a number of factors, however, as they become tracked into very specific developmental pathways in which both intrinsic and extrinsic factors combine to control the molecular and cellular pattern of expression that results in specific tissues that perform specific functions based on their molecular repertoire. Indeed, the progression from stem cell to final end phenotype is marked by discrete stages with transit from one stage to the next dependent on local cuing from surrounding cells (paracrine regulation) as well as signals emitted by the cell itself and the reception of its own signaling (autocrine regulation) [56]. Multipotent stromal cells were initially identified in the Bone Marrow (BM) and their basic characteristics are spindle shaped and plastic adherence [57]. Many clinical applications using adult progenitor cells in cellular therapy and regenerative medicine have been showing promising results [58]. Regenerative medicine is always in demand for new available sources of MSCs as their remarkable plasticity progressively reveals itself to the scientific community. Stromal cells present different potential as far as cell therapies are concern, mostly due to their ability to differentiate into all categories of specialized cells [59]. However, harvesting mesenchymal cells from human adult BM presents several problems: the use of invasive techniques, difficult access and low cell number as the percentage of MSCs in the BM is very low (0.001–0.01%) and it decreases with age [60].

A possible alternative source of MSCs for regenerative medicine use is represented by fetal tissue samples [61]. Among fetal tissues, the placenta represents a highly specialized, transient organ with fundamental role in fetal protection and development [60]. With so many functions that are essential to human life, placenta may also

represent a remarkable source of MSCs considering its increased cell population. In addition, the necessary procedures to harvest stromal cells are non-invasive and free of any ethical issues since human placenta is discarded after birth [61]. In the last decades, placental MSCs are been studied elaborately for their potency in immuno-modulating and promising therapeutic applications. Several reports have shown that multipotent cells obtained from umbilical cord blood, the subendothelial layer, the amniotic epithelial layer, Warton's jelly and the matrix of umbilical cord have the ability to differentiate into mesenchymal, neural and endodermal lineages, such as hepatic progenitor cells, under specific conditions [62-64]. The existence of MSCs in chorionic villi of the human placenta is of more recent investigations. In 2004, Igura K. and colleagues reported the protocol to isolate chorionic villi MSCs, their differentiation potential *in vitro* and their possible application for fetal tissue engineering [65]. Recently, our group demonstrated elevated production of pro-inflammatory cytokines by chorionic villi MSCs isolated from placentae complicated by Preeclampsia (PE) relative to MSCs isolated from physiological placentae [66]. Importantly, preeclamptic MSCs are able induce a PE-like phenotype in physiological villous placental explants, proving for the first time that MSCs from chorionic villi are central players in placental pathophysiology [66].

Placental MSCs and Central Nervous System Pathophysiology

The central nervous system is one of the most limited systems in the human body in terms of regeneration and recovery after cell loss [67]. MSCs can give rise to a wide range of mature cell types, however, the finding of a reliable and abundant source for

the replacement of missing neurons in neurodegenerative diseases is a controversial issue. The use of placenta-derived stromal cells from fetal membrane including the chorion or amnion have recently emerged as alternative sources of MSCs for use in regenerative medicine [68]. As the chorion is the largest part of the placental outer membrane, the collection of chorion MSCs (C-MSCs) discarded at the time of birth has been proposed as an abundant, ethically acceptable, easily accessible and less expensive cell source compared with the collection of MSCs from bone marrow aspirates [69].

In 2016, Katsiani and colleagues isolated MSCs-like cells from human first trimester placental chorionic villi [70]. They identified a cell population which share common features with adult counterparts and retain them during long-term culturing, express typical surface antigens of multipotent stem cells over the entire culture period (20 passages), while the expression of stemness genes *Oct4* and *Nanog*, regulators of pluripotency, were reserved only during the twelve early passages. Moreover, these cells expressed Nestin and Doublecortin (DCX), specific markers of neural stem/progenitors cells, after induction, underlying the plasticity of chorionic villi MSCs [70]. C- MSCs have been described for their ability to integrate into degenerative environment, release neurotrophic cytokines, contrast oxidative stress and an inherent differentiation potential towards site-appropriate phenotypes in an *in vitro* model of 6-hydroxydopamine induced Parkinson's disease [71]. In addition, C-MSCs express several specific neural stem/ progenitor markers, like Nestin and Connexin 43. Importantly, C- MSCs- derived conditioned media (CM) significantly reduced neurotoxin-induced apoptosis in a Parkinson's disease cell model [71]. Recently, also MSCs isolated from both human umbilical cord blood and amniotic membranes have been found to significantly improve neurodegenerative diseases symptoms, such as

learning and memory decline in an Amiloid Precursor Protein (APP) and Presenilin-1 (PS-1) double-transgenic mouse model of Alzheimer's disease, one of the most important neurodegenerative irreversible and progressive disorder with observable memory impairment [72-74].

It is becoming increasingly accepted that the regenerative effects promoted by MSCs are mainly associated with their secretome. MSCs secretome is composed by a proteic soluble fraction, constituted by growth factors, cytokines and a vesicular fraction composed by microvesicles (MVs) and exosomes, which are involved in the transference of proteins and genetic material like microRNAs to other cells [75]. The protective actions promoted by MSCs secreted molecules are closely related with therapeutic plasticity in the central nervous system [76]. In 2013, our group described placenta-derived MSCs (PDMSCs) secretome, reporting that these cells are able to spontaneously release BDNF, NT-3 and -4 in the cell culture media without induction [66]. Importantly, we described a differential expression of these neurogenic-related molecules in PDMSCs isolated from preeclamptic placentae [66]. During pregnancy, they are involved in critical processes as placental invasion, trophoblast cell proliferation, fetal growth and development, as well as stimulates cytokines production [77], but their potential release into the fetal circulation or their possible implication in the modulation of fetal neurodevelopment has not yet been elucidated.

Placental Pathology

Placental dysfunction can lead to a number of adverse fetal outcomes [78]. Moreover, because the placenta reflects the metabolic milieu of both mother and fetus, it serves as a valuable tool for studying the metabolic perturbations that may take place during pregnancy [79]. Evaluation of the placenta requires a good understanding of the questions and issues concerning both the fetus/infant and the mother. Information from placental pathology can be critical in early neonatal care and in reproductive planning for the family, and it can provide risk assessment for neurologic outcome of the infant [80].

The placenta has long been recognized as the necessary component for the genesis of PE [81]. PE begins to abate with the delivery of the placenta and can occur in the absence of a fetus but with the presence of trophoblast tissue with hydatiform moles [82]. The unique feature of the placenta proposed to result in PE is its exposure to reduced placental perfusion. To explain the relationship of the placenta to the maternal syndrome several studies propose that the placenta in response to reduced perfusion produces molecules that act upon the mother to bring about the clinical findings of PE [83].

Definition and Pathophysiology of Preeclampsia

PE is usually defined as a pregnancy-specific disorder with multisystem involvement characterized by hypertension (systolic blood pressure > 140 mmHg and diastolic pressure of > 90 mmHg) and proteinuria (> 0.3 g/24h) occurring after the 20th week of pregnancy in previously normotensive women [84-87]. In 2013, the ultimate guidelines

from The American College of Obstetricians and Gynecologists (ACOG) stated that PE may be diagnosed in the absence of proteinuria, when new-onset hypertension is accompanied by serum creatinine increase, thrombocytopenia (platelet count less than 100.000/microliter), impaired liver function (elevated blood levels of liver enzymes to twice the normal concentration), pulmonary edema, or central symptoms (ACOG website, *Hypertension in pregnancy,* 2013). PE complicates 2%–10% of pregnancies worldwide and constitutes a major source of morbidity and mortality [88, 89]. Overall, 10%–15% of maternal deaths are directly associated with PE [90]. In the mother, PE may cause premature cardiovascular disease, such as chronic hypertension, ischemic heart disease, and stroke later in life, while children born after preeclamptic pregnancies and who are relatively small at birth, have an increased risk of stroke, coronary heart disease, and metabolic syndrome in adult life [91-93]. The only curative treatment is delivery, management must continuously balance the risk–benefit ratio of induced preterm delivery and maternal–fetal complications. Screening women at high risk and preventing recurrences are also key issues in the management of PE [86].

PE has a complex pathophysiology, the primary cause being abnormal placentation (Figure 2) [94]. After implantation, the spiral arteries are invaded and plugged by cytotrophoblast until about 8 weeks of gestation. Unplugging starts where placentation is shallowest and the plugs are smallest, at the pole opposite the cord insertion, and then progresses circumferentially around the chorionic sac. At 8-9 weeks the chorionic villi are not mature enough to sustain the oxidative stress that comes with their first contact with oxygenated blood and atrophy to form the chorion laeve. In the later phases (10-12 weeks), the more mature villi withstand the oxidative stress and survive to form the definitive placenta [95].

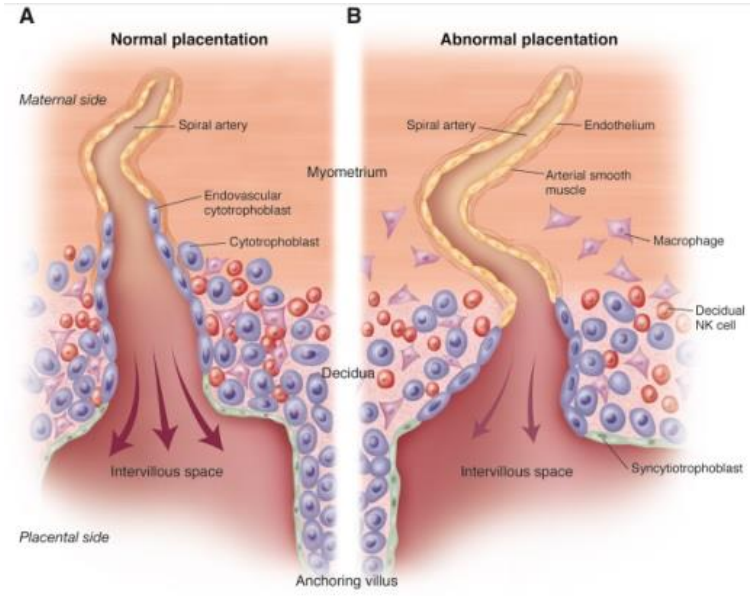

Figure 2. Normal and PE placentation at 15 to 16 weeks of pregnancy. During normal placentation (**A**), cytotrophoblasts (blue) invade the maternal decidua and adjacent spiral arteries. They penetrate the walls of the arteries and replace part of the maternal endothelium (yellow), stimulating remodeling of the arterial wall. In the preclinical stage of preeclampsia, invasion is restricted (**B**) with impaired arterial remodeling [96].

Deep endovascular invasion of the spiral arteries by trophoblast is associated with full artery remodeling. When remodeling fails, the quality of utero-placental perfusion is altered from a constant low pressure flow to one that is more pulsatile, at higher pressure, determining in time an ischemia-reperfusion injury [97]. These defects may be related to the nitric oxide pathway, which contributes substantially to the control of vascular tone. Moreover, inhibition of maternal synthesis of nitric oxide prevents embryo implantation [98]. Chronic placental ischemia causes fetal complications, including Fetal Growth Restriction (FGR) and intrauterine death. In parallel, oxidative stress induces release into the maternal circulation of free radicals, oxidized lipids and

cytokines [99], all responsible for endothelial dysfunction that lead to the clinical signs observed in the mother. Oxidative and other stresses cause syncytiotrophoblast (STB) dysfunction, which stimulates release of multiple factors, which are not yet fully defined [96]. Many are proinflammatory, including STB microvesicles, and some contribute to the angiogenic balance, as well as stimulating vascular inflammation. Two major proangiogenic players are VEGF and PlGF [100]. Both have several isoforms and splice variants. STB secretes PlGF and a soluble decoy receptor for VEGF and PlGF (named soluble VEGF receptor-1 or sFlt1), which inhibits their activity. Hence sFlt1 is a major antiangiogenic factor. Preeclampsia is characterized by excessive release from STB of sFlt1 and soluble endoglin, which is indirectly antiangiogenic [101, 102]. As a consequence, circulating PlGF is reduced (Figure 3) and sFlt1/PlGF ratio in maternal serum is higher in pregnant PE patient relative to normotensive pregnant women [103].

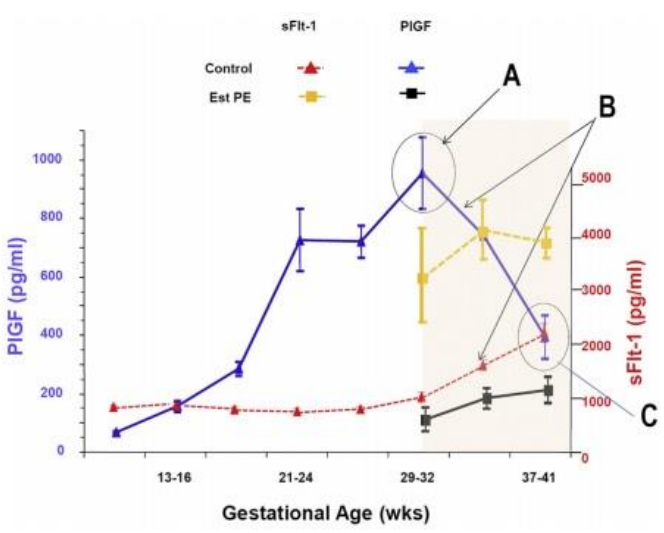

Figure 3. In normal pregnancy, PlGF (blue triangles) rises steadily to 29-32 weeks (**A**) and falls thereafter until delivery (**B**). On the same time course, sFlt1 (red triangles) increases (**B**). By term, normal values of both biomarkers are close to those of established PE (yellow and black squares) (**C**) [94].

Definition and Etiology of Fetal Growth Restriction

The term Fetal Growth Restriction (FGR) is used to describe fetuses with an estimated fetal weight that is less than the 10^{th} percentile for gestational age [104]. However, this definition does not take into account the individualized growth potential of each fetus, and its use may fail to identify larger fetuses that have not achieved their growth potential and may be at risk of adverse outcomes. Conversely, this definition will result in the misdiagnosis of FGR for some constitutionally small fetuses [105, 106].

The etiology of fetal growth restriction can be broadly categorized into maternal, fetal, and placental. Although the primary pathophysiologic mechanisms underlying these conditions are different, they often have the same final common pathway: suboptimal uterine–placental perfusion and fetal nutrition. Maternal factors varies from clinical conditions (which include infectious disease [107] or any chronic disorder that is associated with vascular disease, such as pregnancy-related hypertensive diseases, and immuno-mediated antiphospholipid syndrome [108-110]) to malnutrition [111]. Also maternal abuse of tobacco, alcohol or narcotics during pregnancy [112, 113] as well as teratogen exposure [114] and multiple gestations [115] are correlated to an increased risk of delivery a growth restricted fetus. FGR is also associated with confined placental mosaicism [116] and certain chromosomal abnormalities: at least 50% of fetuses with trisomy 13 or trisomy 18 have fetal growth restriction [117].

Early screening to predict the likelihood of a FGR fetus include medical and obstetric history, uterine artery Doppler and maternal serum parameters [118]. Utero-placental Doppler is the most powerful predictor of the clinical deterioration and the circumstances surrounding delivery [119, 120]. The systematic review and meta-analysis conducted by Cnossen et al. in 2008 established uterine artery Doppler

ultrasonography as a predictor of FGR, providing a more accurate prediction when performed in the second trimester than in the first-trimester [121]. Numerous studies have also shown that some maternal biochemical markers (pregnancy associated plasma protein-a, α-fetoprotein, human chorionic gonadotropin, inhibin A) are associated with placental function and fetal growth, and their levels are altered in FGR pregnancies [122].

Neurodevelopmental Outcomes of Placental Pathology

Early growth restricted fetuses by definition carry at least one of the major risk factors for perinatal morbidity and mortality that are low birthweight and prematurity. Early and late-onset FGR are both associated with poor short- and long-term neurodevelopmental disorders and also with cardiovascular and metabolic complications [123].

A neurodevelopmental disorder is characterized by impairments in the functioning of the brain, which affect the child's behavior, memory or ability to learn [124]. Disorders believed to be of neurodevelopmental origin range on a broad continuum from rare and very severe to more frequent and less disabling conditions, encompassing diseases such as cerebral palsy, schizophrenia, intellectual disability (mental retardation), autism spectrum disorders, attention deficit/hyperactivity disorder, tic disorders, speech disorders, dyslexia and learning disabilities. The common characteristic of these disorders are that they are believed to be the outcome of some abnormal developmental processes of the brain, in the unborn or very young child [125].

Brain development in the fetus starts already within a few weeks of conception, and proceeds all the way into adulthood. The brain is however thought to be particularly vulnerable while the child is still within the womb, as fundamental structures are formed and because of the immense growth. (Figure 4). Severe neurodevelopmental impairment is estimated to affect approximately 2.5% of the population, while around 15-20% is believed to be affected by less disabling neurodevelopmental disorders [126]. Animal studies have shown that changes in fetal endocrinology and metabolism can have long term consequences for postnatal neuroendocrine functions, metabolism, and organ structure and function [127]. There is good evidence from several prospective studies that maternal stress, anxiety or depression during pregnancy is associated with several types of adverse neurodevelopmental outcomes in the child. Outcomes linked so far with prenatal stress or anxiety include autism, schizophrenia, emotional/behavioral problems and reduced cognitive abilities.

The range of prenatal maternal stressors that predict child outcomes is quite wide and includes minor stresses and disorders of anxiety and depression [128]. Several *in vivo* studies have shown an effect of prenatal stress in reprogramming the function of the Hypothalamic- Pituitary- Adrenal (HPA) axis in the offspring, often resulting in a more prolonged and greater cortisol/corticosterone response to stressors later in development [129].

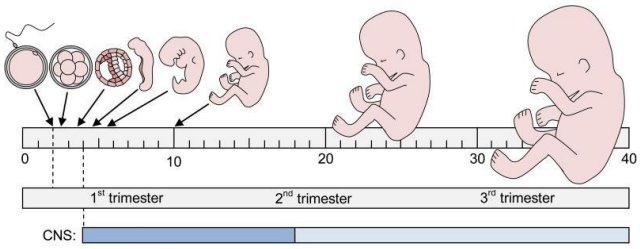

Figure 4. Critical or sensitive periods in human development. Most developing organs are sensitive to teratogens during the embryonic period from 4-10 weeks of gestation. An exception is the central nervous system (CNS), which remains sensitive throughout the fetal period from 10-40 weeks of gestation. Dark blue bar indicates major structural defects, while light blue bar refers to minor structural or functional defects [130].

The placenta plays a crucial role in moderating fetal exposure to maternal cortisol and in preparing the fetus for the environment in which it is going to find itself. The developing fetus is normally protected from the high levels of circulating maternal cortisol by the placental barrier enzyme 11β-hydroxysteroid dehydrogenase Type II (11β-HSD2), which is located at the syncytiotrophoblast level and converts cortisol to the inactive cortisone. In general, human 11β-HSD2 mRNA increases to term and remains relatively stable in late gestation [131]. Prenatal stress, pregnancy pathological conditions (preeclampsia, maternal asthma, preterm birth, fetal growth restriction) and maternal diet can affect 11β-HSD2 expression levels, increasing fetal exposure to maternal cortisol/corticosterone and causing a decrease of fetal Adenocorticotropic Hormone which impedes fetal adrenal growth and maturation [132].

Studies conducted on animal models of calories restriction (50% of *ad libitum*) from day 14 of pregnancy through to weaning demonstrate an altered structural development of the hypothalamus and hippocampus, by changing the production of BDNF and cell proliferation during development [133]. These early modifications in cerebral structure

may have long-lasting consequences on the regulation of neuroendocrine activity, energy metabolism and cognition [134]. Uterine artery ligation, resulting in low-birth weight offspring, impairs sensorimotor gating, but enhances motor function and spatial memory in adult offspring [135]. Good nutrition is particularly important for pregnant animals as well as fetuses to maintain overall health during pregnancy and the requirement for nutrients increases during pregnancy compared with the normal state. Therefore, information regarding safe levels of particular nutrients as well as the amounts that increase the risk of adverse effects is required. In pregnant rodents, fetal body weight was shown to be lower in animals fed a high Trp diet compared with rodents fed a normal diet [136]. In addition, excess Trp in the placenta may restrict not only fetal growth but also impair placenta development, determining a higher rate of fetal death [137].

There are very few studies which have reported the levels of neurotrophins and their role in FGR complicated pregnancies. Malamitsi-Puchner et al. (2007) showed no differences in the circulating levels of BDNF, NT-3 and NT-4 in FGR pregnancies. NGF was the only neurotrophin that higher in the maternal and fetal plasma and further positively correlated with the infant centiles and birth weights. This no change in the neurotrophins could possibly be attributed to the activation of the brain-sparing effect [138]. A rat model of FGR showed less expression of BDNF and NT-3 in the cerebral cortex than controls, alterations may be related to the delay of neuronal migration [139]. Lower maternal and higher cord BDNF levels were indicated in women with PE compared to normotensive women and suggested a possible role for BDNF in the pathophysiology of PE. BNDF levels were significantly higher also in umbilical cord blood from preeclamptic pregnancies [140].

Hypothesis of the Study

Since placenta-derived mesenchymal stromal cells play a key role in placental pathophysiology and they are localized nearby fetal vessels within the placental villi, an ideal position to release molecules directly in the fetal circulation, we hypothesize that they could be directly involved in the placental modulation of fetal nervous system development. Importantly, the aberrant PE-PDMSCs neurotrophins production could contribute to the cognitive defects typical of PE-FGR newborns.

In the present study, we focused on the role of PDMSCs in physiological and pathological fetal neurogenesis by investigating the expression of main neurogenic molecules Neural Cell Adhesion Molecule (NCAM), Polysialic Acid NCAM, Doublecortin, Nestin and High Mobility Group Box 1 in normal and PE-FGR PDMSCs, as well as we described microRNA-25, -124, -134, -137 and -181a differential release in normal and pathological cells. Moreover, we elucidated placental contribution to fetal neurodevelopment in both physiological and preeclamptic conditions. In particular, we determined whether PE-FGR PDMSCs have alterations in IDO-1 expression and/or tryptophan catabolism mediated by IDO-1.

MATERIALS AND METHODS

Materials and Methods

Study Population

The study groups included singleton pregnancies complicated by severe PE with FGR (n= 15) and physiological control term pregnancies (n= 18). The diagnosis of PE was made according to the following criteria: presence of pregnancy-induced hypertension (systolic ≥140 mmHg, diastolic ≥90 mmHg) and proteinuria (≥300 mg/24 h) after the 20th weeks of gestation in previously normotensive women [84-87]. FGR was defined as birth weight below the fifth centile according to the Italian growth curves normalized for gestational age and sex accompanied by pathological umbilical artery Doppler waveforms (absent or reverse end diastolic flow, AREDF) and increased resistance to flow in maternal uterine arteries (early diastolic notch or pulsatility index more than 0.58) [104].

Control patients were healthy women with singleton term physiological normotensive pregnancies and no signs of preeclampsia or FGR. Patients with diabetes, infections, kidney disease, congenital malformations and chromosomal anomalies (number and/or structure) were excluded.

PDMSCs Isolation and Characterization

Normal and PE-FGR placentae were collected immediately after delivery. The amniotic membranes were mechanically removed and the decidua was peeled off from the basal plate in order to avoid maternal cell contamination. Next, membranes were removed and 30 g of villous tissue were sampled from the chorionic plate for each pathological and physiological placenta. Placental tissue was washed several times with Hank's

Buffered Salt Solution (HBSS, Gibco, Life Technologies, Italy) in order to remove the excess of blood and next it was mechanically minced and digested for 3 hours with 100 U/mL collagenase type I (Gibco, Life Technologies, Italy) plus 5 mg/mL DNAse I (Gibco, Life Technologies, Italy). Finally, cells were separated by gradient using 1.073 Ficoll Paque Premium (GE Healthcare Europe, Italy). The mononuclear cells ring was collected, washed and PDMSCs were resuspended in Dulbecco's modified Minimum Essential Medium (DMEM, Gibo, Life Technologies, Italy) supplemented with 10% Fetal Bovine Serum (FBS Australian origin, Italy) and maintained at 37°C and 5% CO_2. Importantly, the culture media was not supplemented with basic fibroblast growth factor (bFGF) in order to maintain the original biochemical and molecular features of PDMSCs. After passages three to five, physiological and PE PDMSCs were characterized by flow cytometry for the expression of the following antigens: HLA-I, HLA-DR, CD105, C166, CD90, CD34, CD73, CD133, CD20, CD326, CD31, CD45 and CD14 (Miltenyi Biotech, Italy). Normal and PE-PDMSCs were analyzed by semi-quantitative PCR to assess gene expression levels of stem cell markers Oct-4 and Nanog. Primers sequences were the following: Oct4 FW(5'- CGT GAA GCTG GAG AAG GAG AAG CTG-3'), RV(5'-CAA GGG CCG CAG CTT ACA CAT GTT C-3'); Nanog FW(5'- AAT ACC TCA GCC TCC AGC AGA TG-3'), RV(5'-CTG CGT CAC ACC ATT GCT ATT CT-3').

Immunocytofluorescence

Normal and PE-FGR PDMSCs were treated by Trp 100 μg/mL for 48h or cultured in basal medium and plated in 4 well chamber slides (Thermo Fisher Scientific, Cat. No.

177399, Milano, Italy). Cells were incubated overnight at 37°C and 5% CO_2 and, the day after, they were fixed in 4% paraphormaldehyde. PDMSCs monolayers were pre-incubated in 10% horse serum in PBS (containing 0.1% Triton X-100) to block nonspecific binding and incubated with primary antibodies overnight at 4°C. Mouse monoclonal antibodies anti-human IDO-1 (1:50 dilution; Merck S.p.A., Cat. No. 05-840), PSA-NCAM (1:200 diluition; Merck S.p.A., Cat. No. MAB5324) and Nestin (1:200 diluition; Abcam, Cat. No. AB22035) were used. Control IgG were used as negative controls. Monolayers were treated with 0.4% DAPI (4',6-diamidino-2-phenylindole) for nuclear detection. Fluorescence images were viewed and captured using a fluorescence microscope (Leica DM LB2, Leica Microsystems, Milano, Italy).

Tryptophan-treated PDMSCs Cell Cultures

Physiological and PE-FGR PDMSCs were plated in 6 well plates at a density of $1x10^5$ cells/mL and treated by tryptophan (Trp) 100 μg/mL or cultured in basal medium without FBS supplementation at 37°C and 5% CO_2. After 48 and 72 hours, RNA and protein isolation was performed and cell cultures supernatants were collected and stored at -80°C.

Lactate Dehydrogenase (LDH) Cytotoxicity Colorimetric Assay

Cell death or cytotoxicity was evaluated in PDMSCs cell cultures treated by Trp by the quantification of plasma membrane damage using the LDH Cytotoxicity Assay Kit II (BioVision, cat. No. K313-500, USA). Lactate dehydragenase (LDH), the most studied cytotoxicity marker, is a stable enzyme, present in all cell types, and rapidly released

into the cell culture medium upon damage of the plasma membrane. This kit utilizes WST reagent for a fast and more sensitive detection of LDH released from damaged cells. The assay is based on an enzymatic coupling reaction: LDH oxidizes lactate to generate Nicotinamide Adenine Dinucleotide (NADH), which then reacts with WST to generate yellow color. The intensity of the generated color correlates directly with the cell number lysed. LDH activity was quantified by a microplate reader (xMark Microplate Sprectrophotometer, Biorad, USA) set to 450 nm. Percent cytotoxicity values were determined based on the amount of LDH released from 30 minutes readings, as follows: (test sample – low control)/ (high control-low control) x 100. The cut-off values for the LDH assay was determined by the 2xStandard Deviation (SD) rule where the threshold is defined as 2xSD beyond the mean of the screened samples. Values equal or exceeding the threshold were considered as sign of cytotoxicity.

RNA and miRNA Isolation and Real Time PCR

Total RNA was extracted from PDMSCs using TRIzol reagent according to manufacturer instructions (Sigma Aldrich, Italy) and treated with DNAse I to remove genomic DNA contamination. miRNA isolation was performed using *mirVana*^{TM} miRNA Isolation Kit (Life Technologies, Cat. No. AM1506, Italy), following manufacturer's instruction. The kit employs an organic extraction followed by immobilization of RNA on glass-fiber filters to purify total RNA enriched for small pieces from cells. Briefly, normal and PE-FGR PDMSCs were plated in 6 well plates at a density of $1x10^5$ cells/mL and cultured in DMEM without FBS supplementation at 37°C and 5% CO_2. Sample were then lysed in a denaturing solution which stabilizes

RNA and inactivates RNases. The lysate was extracted once with Acid-Phenol: Chloroform which removes most of the other cellular components, leaving a semi-pure RNA sample. This was further purified over a glass-fiber filter. The glass-fiber filter procedure uses solutions formulated specifically for miRNA retention to avoid the loss of small RNAs that is typically seen with glass-fiber filter methods. Ethanol was added to samples and they were passed through a Filter Cartridge containing a glass-fiber filter which immobilizes the RNA. The filter was then washed a few times, and finally the RNA was eluted with a low ionic-strength solution.

Five µg of total RNA was reverse transcribed using random hexamers approach with the RevertAid H Minus First Strand cDNA Synthesis kit (Fermentas Europe, St. Leon-Rot., Germany). The resulting templates were quantified by Real-time PCR (StepOneTM Real-Time PCR System, Applied Biosystems, Carlsbad, California). TaqMan primers and probes for ribosomal 18S, Doublecortin, NCAM, IDO-1, HMGB1 and miRNA-25, 124, -134, -137 and -181a were purchased from Applied Biosystems as TaqMan Gene Expression Assays. For the relative quantitation, PCR signals were compared among groups after normalization using 18S as internal reference. Relative expression and fold change was calculated according to Livak and Schmittgen (Livak KJ, Schmittgen TD, Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) *Method. Methods 2001*, 25: 402–408).

Gene Promoter Region *in Silico* Analysis

Sequence of gene promoter region was determined *in silico* for IDO-1 (identifier, ID: 3620), NCAM (ID: 4684) and HMGB1 (ID: 3146) with “Gene Regulation” menu of

Genomatix online software, using sequences found in the online National Center for Biotechnology Information (NCBI) gene database.

Protein Isolation and Western Blot Analysis

Total proteins were isolated from PDMSCs using 1x Radio Immunoprecipitation Assay (RIPA) buffer. Forty µg of total protein from PDMSCs were processed by SDS-page on 10% polyacrylamide pre-cast gradient gels (Life Technologies, Cat. No. NW00100BOX, Milano, Italy). Next, proteins were transferred on PVDF membranes and probed with primary antibodies at 4°C over night. Primary antibodies were mouse monoclonal anti-human PSA-NCAM (1:1000 dilution; Merck S.p.A., Cat. No. MAB5324) and mouse monoclonal anti-human HMGB1 (1:1500, Sigma Aldrich, Cat. No. WH0003146M8-100UG). Biotinylated secondary antibody was goat anti-mouse (1:5000 diluition for PSA-NCAM and 1:20000 diluition for HMGB1, Santa Cruz Biotechnology, Cat. No. sc-2005, USA). Protein expression levels were normalized to β-actin by blotting with mouse monoclonal anti-human β-actin antibody (1:5000, Sigma Aldrich, Italy) after stripping with 10x ReBlot Plus Strong Antibody Stripping Solution (Merck Millipore, Italy). Protein expression levels were assessed by chemo-luminescence using Luminata^{TM} Classico Western HRP reagent (Merck Millipore, Italy), followed by densitometry with ImageJ software.

Enzyme-Linked Immunosorbent Assay (ELISA)

Kynurenine (Kyn) concentration in Trp-treated PDMSCs cell culture supernantants was determined by an ELISA assay (Cusabio, USA, Cat. No. CSB-E13659h). The microtiter

plate provided in this kit has been pre-coated with the antigen. Briefly, standards or samples were added to the plate wells, each in duplicate, with a rabbit monoclonal antibody against human Kyn and Horseradish Peroxidase (HRP) conjugated goat anti-rabbit secondary antibody. The competitive inhibition reaction was launched between pre-coated Kyn and Kyn in samples. A substrate solution was added to the wells and the color developed in opposite to the amount of Kyn in the samples. The color was stopped and its intensity was measured using a microplate reader (xMark Microplate Sprectrophotometer, Biorad, USA) set to 450 nm, with wavelength correction set to 540 nm. KYN concentration was expressed in pmol/mL.

Statistical Analysis

Data are represented as median and range or mean ± standard error (SE). For comparison of data between multiple groups we used one-way analysis of variance (ANOVA) with posthoc Bonferroni's test. For comparison between 2 groups we used paired and unpaired Student's t-test as appropriate. Categorical variables are presented as frequencies (percentages) and the comparison among different groups was done with c^2 by means of a 2x2 or 2x3 contingency table; Fisher's exact test was used for small sample sizes. Statistical tests were carried out using SPSS Version 18 statistical software and significance was accepted at $P < 0.05$.

RESULTS

Results

Study Population

The clinical features of the study population were reported in Table 1. Control (n=18) and PE-FGR (n=15) pregnancies were comparable for maternal age, while gestational age ($p<0.01$), neonatal weight ($p=0.02$) and placental weight ($p=0.03$) at delivery were, as expected, significantly lower in PE-FGR group relative to controls. These differences are explained by the higher number of preterm deliveries in the PE population due to the worsening of the clinical picture. Systolic blood pressure ($p<0.01$) and proteinuria ($p=0.02$) were higher in PE-FGR relative to normal pregnancies, accordingly to the definition of PE [84-87]. Moreover, we observed a significant increase in the use of antihypertensive drugs in the PE relative to the physiological population ($p=0.045$), as well as a higher rate of antibiotics ($p=0.02$) and corticosteroids ($p=0.02$) administration.

All PE pregnancies presented fetal-placental compromise with abnormal umbilical artery (44%, $p=0.02$) and/or uterine artery (88%, $p<0.01$). Doppler velocimetry and birth weight centiles were significantly lower relative to those of the control group ($p<0.01$). A trend of increase in the percentage of caesarean section (CS) was observed in the PE-FGR relative to the physiological group, which had elective caesarean deliveries in case of previous CS, breech fetal position and/or maternal therapeutic indications. Magnesium sulfate ($p<0.01$) and oxygen administration ($p<0.01$) at delivery were significantly increased in the PE relative to control population.

	Controls (n=18)	PE-FGR (n=15)	P value
Nulliparae (%)	20	33.3	
Gestational Age at Delivery (weeks)	39.4 (39.1- 41.1)	32.7 (28.8- 33.7)	p<0.01
Maternal Age at Delivery (years)	34 (29- 37)	33.5 (32- 41)	
Ethnicity (%)			
Caucasia	85.7	100	
African	-	-	
Asian	14.3	-	
Prenatal Medications (%)			
Antihypertensive	-	66.7	p<0.01
Antihistamine	-	-	
Antinflammatory	-	50	
Antispasmodic	-	-	
Antiemetic	-	-	
Albumin	-	-	
Antibiotics	20	100	p= 0.02
Diuretics	-	33.3	
Folic Acid	80	83.3	
Heparin	-	16.7	
Iron	-	16.7	
Proton Pump Inhibitors	-	-	
Corticosteroids	-	66	p= 0.02
Acetaminophen	-	-	
Smokers (%)	20	-	
Alcohol (%)	40	-	
Previous Prenatal Admissions (%)	-	33.3	
Systolic Blood Pressure (mmHg)	115 (110- 120)	140 (130- 175)	p<0.01
Diastolic Blood Pressure (mmHg)	80 (70- 85)	90 (85- 100)	
Proteinuria (g/24h)	absent	1,325 (0.15-2.5)	p= 0.02
Pathological Umbilical Doppler (%)	-	44	p= 0.02
Pathological Uterine Doppler (%)	-	88	p= 0.013
Placental Weight (g)	580 (525- 640)	261,8 (200- 323)	p= 0.03
Neonatal Weight (g)	3715 (3300- 4020)	1070 (758- 1180)	p= 0.02
Birth Weight Centile	76 (73- 96)	3.5 (2- 9)	p<0.01
Fetal Sex (%)			
Male	80	16.7	
Female	20	83.3	
AREDF (%)	0	33.3	
Labor (%)	60	17	
Antiobiotics in Labour (%)	20	100	
Delivery to Processing (range in hours)	0.08- 0.4	-	
Caesarian Section (%)	40	83	
Anesthesia (%)			
Local	50	-	
Spinal	50	83.3	
Epidural	-	-	
General	-	16.7	
Maternal Oxygen at Delivery (%)	-	50	p<0.01
Magnesium Sulfate (%)	-	50	p<0.01

Table 1. Clinical features of the study population.

PDMSCs Antigens Profile

PDMSCs were isolated from both physiological term and PE-FGR placentae and, starting from passages 3 to 5, they were characterized for the expression of typical MSCs markers by flow cytometry. All PDMSCs lines investigated were positive for CD105, CD166, CD90 and CD73, while they were negative for HLAII, CD34, CD133, CD20, CD326, CD31, CD45 and CD14, thus showing proper mesenchymal stem cell phenotype and excluding contamination from trophoblast/epithelial cells and hematopoietic progenitors (Figure 5) [66]. Moreover, all PDMSCs properly expressed both Oct4 and Nanog mRNA [66].

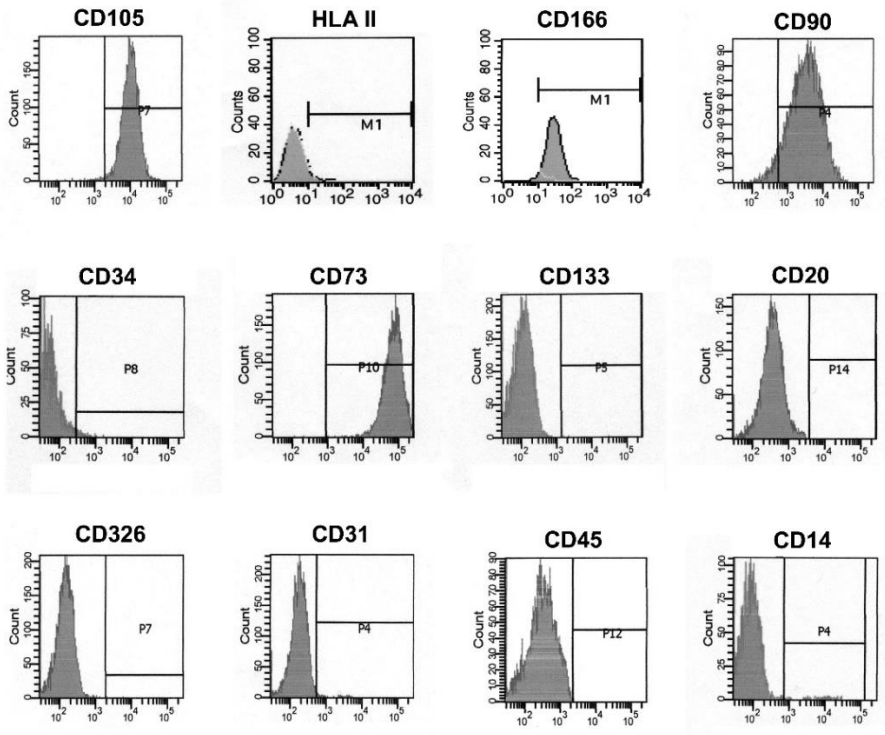

Figure 5. Representative phenotype of chorionic PDMSCs at passage 5 as assessed by flow cytometry. All cells were positive for CD166, CD105, CD90, CD73 and negative for HLA II, CD34, CD133, CD20, CD326, CD31, CD45 and CD14, thus displaying proper mesenchymal profile and no contamination from epithelial, hematopoietic, immune or endothelial cells [66].

Neuroactive Molecules Expression in Normal and PE-FGR PDMSCs

We previously demonstrated that PDMSCs are able to secrete neurotrophins BDNF, NT-3 and -4, important neuroactive molecules that drives the first stages of Neural Progenitor Cells (NPCs) differentiation and synaptogenesis, and that these peptides are all over-expressed in mesenchymal cells isolated from PE pregnancies [66]. We next investigated the expression of main neurogenesis mediators Neural Cell Adhesion Molecule, Doublecortin, IDO-1 and High Mobility Group Box 1 in order to complete the characterization of PDMSCs neurogenic profile. We reported that neural progenitors markers NCAM, DCX and HMGB1 ($p= 0.004$), which has a double biological activity of both neuroinflammation marker and promoter of neurogenesis after brain damage, mRNA levels were increased in PE-FGR relative to normal PDMSCs (Figure 6, panels A, B and D). Gene expression of IDO-1, the main enzyme in Trp catabolism responsible for the production of both neurotoxic and neuroprotective compounds, was significantly down-regulated in PE-FGR relative to physiological PDMSCs ($p<0.001$) (Fig. 6, panel C).

Immunofluorescent staining showed presence of positive immunoreactivity for Polysialic acid NCAM (Figure 7, panel A), a post-translational isoform of NCAM which inhibits NPCs survival, in normal relative PE-FGR PDMSCs, while spatial localization of IDO-1 (Fig. 7, panel B) and Nestin (Fig. 7, panel C), a type VI intermediate filament implicated axon radial growth, was mainly detected in PE-FGR relative to physiological PDMSCs. Finally, PSA-NCAM down-regulation in PE-FGR relative to normal PDMSCs was confirmed at the protein level by Western Blot analysis (Figure 8).

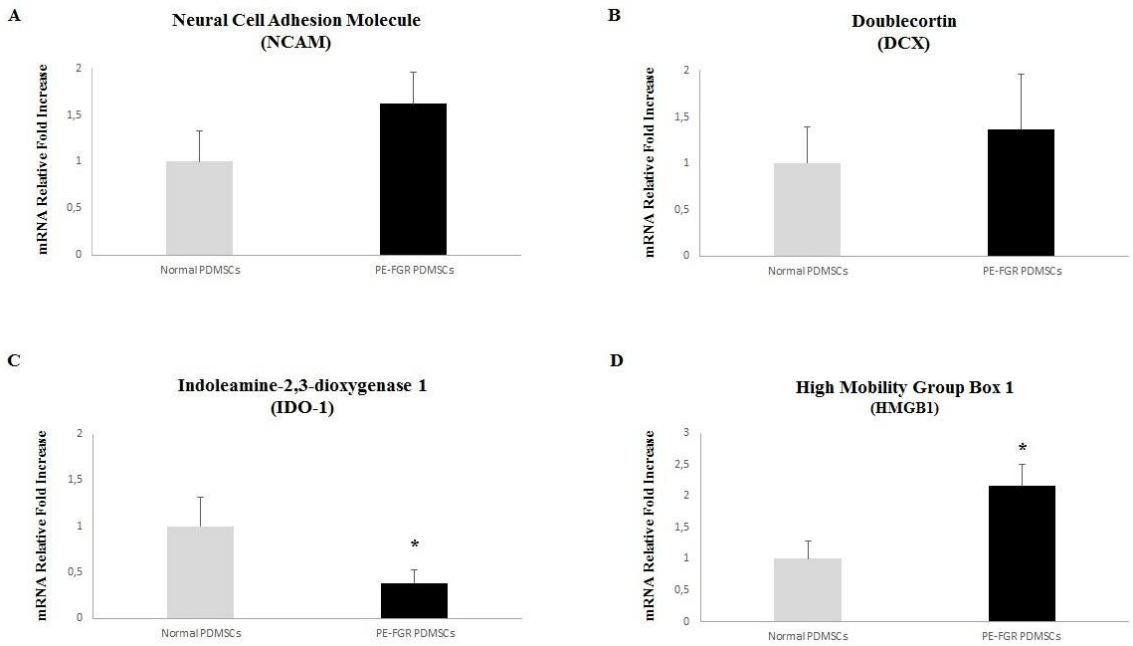

Figure 6. Real Time PCR analysis showed an over-expression of NCAM (**A**) and DCX (**B**) and HMGB1 (**D**) mRNA levels, while IDO-1 (**C**) mRNA expression was significantly decreased in PE-FGR vs normal PDMSCs. Statistical significance (*) has been considered as $p< 0.05$.

Since mesenchymal stromal cells use microRNAs as main intercellular tissue-specific signaling system to modulate gene expression, thus being part of diverse biological processes including neurogenesis, we focused on normal and PE-FGR PDMSCs miRNAs contribution to the onset and development of fetal neurological impairment. In particular, we described a down-regulation of miRNA-25 ($p=0.02$), -124, -134, -137 ($p=0.02$) and -181a ($p=0.02$), known to be key modulators of neurodevelopment, in PE-FGR relative physiological PDMSCs (Figure 9).

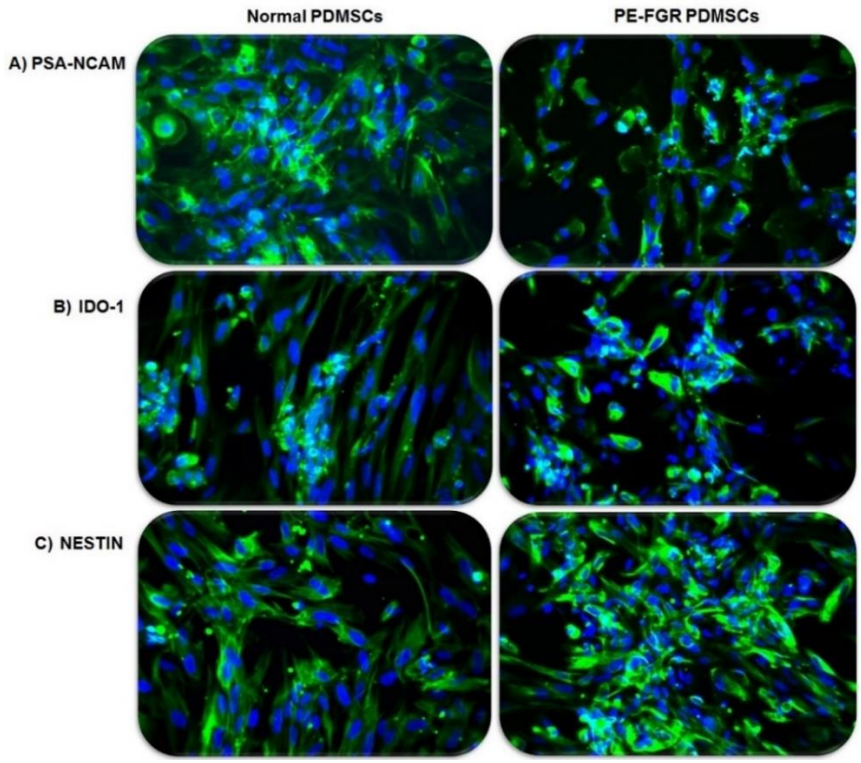

Figure 7. Immunofluorescent staining showed presence of positive immunoreactivity for PSA-NCAM (**A**, green) in normal vs PE-FGR PDMSCs, while spatial localization of IDO-1 (**B**, green) and Nestin (**C**, green) was detected mainly in PE-FGR vs physiological PDMSCs. Cell nuclei are showed in blue by DAPI signal. Original magnifications x20.

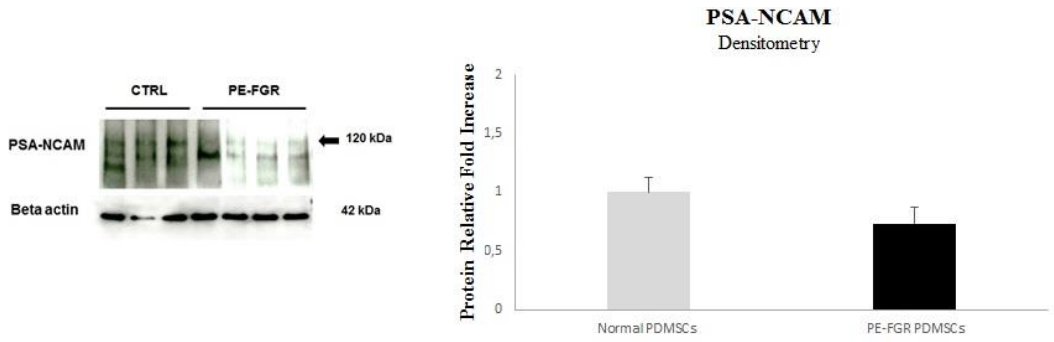

Figure 8. Western Blot analysis showed decreased PSA-NCAM protein levels in PE-FGR vs normal PDMSCs.

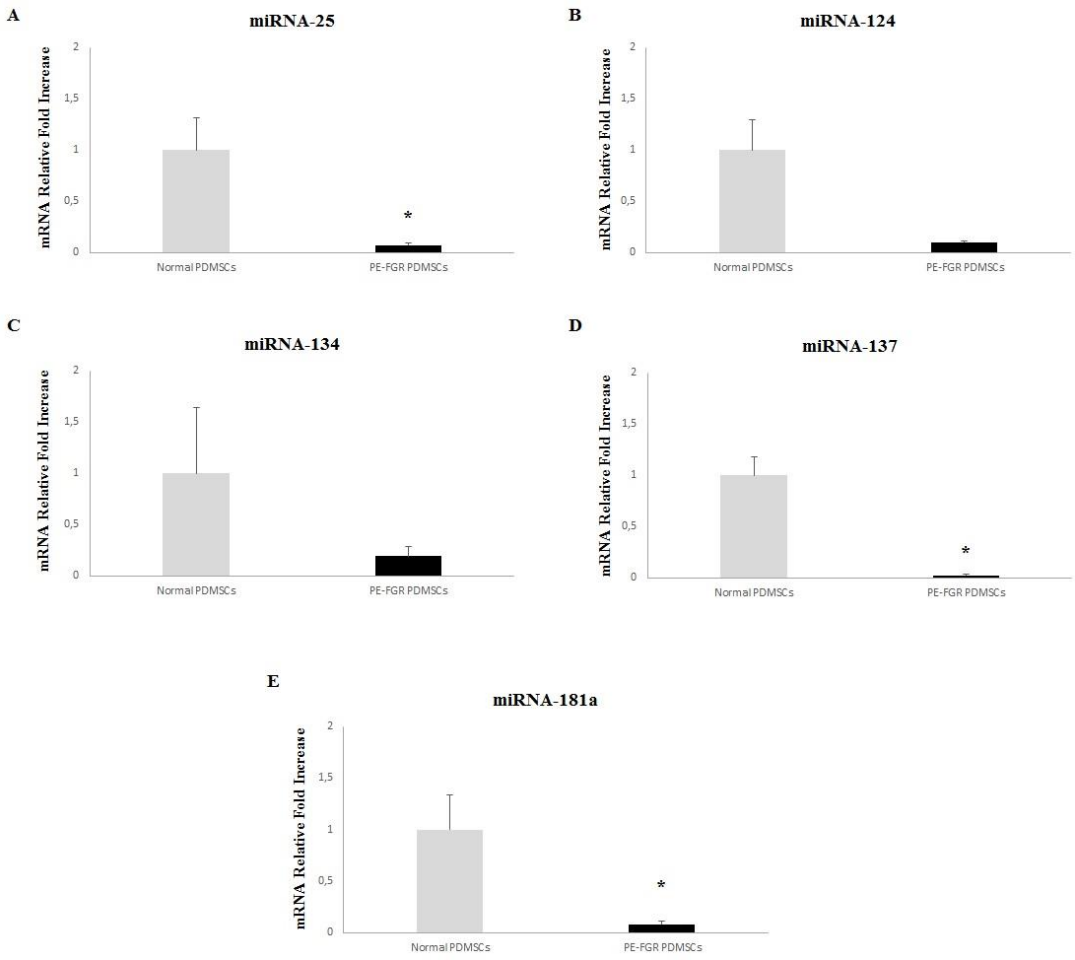

Figure 9. Real Time PCR analysis showed a significant down-regulation of miRNA-25 (**A**), -137 (**D**) and -181a (**E**) expression in PE-FGR vs normal PDMSCs. A trend of decrease was observed in miRNA-124 (**B**) and -134 (**C**) levels in PE-FGR vs normal PDMSCs. Statistical significance (*) has been considered as $p < 0.05$.

Tryptophan Administration Did Not Alter Normal and PE-FGR PDMSCs Cultures Viability

In order to evaluate whether Trp affect cell viability of physiological and PE-FGR mesenchymal cell cultures, the lactate dehydrogenase assay, which assessed the cell membrane integrity by measuring the LDH leakage from cells, was performed. After 48h ($p=0.03$) and 72h ($p=0.03$) of Trp treatment we observed that the relative LDH

amount released into the media was comparable among PDMSCs cultured in basal conditions and those treated by Trp (Figure 10). Importantly, all Trp LDH levels were lower compared to the considered cytotoxicity cut-off of 0.66% (Fig. 10).

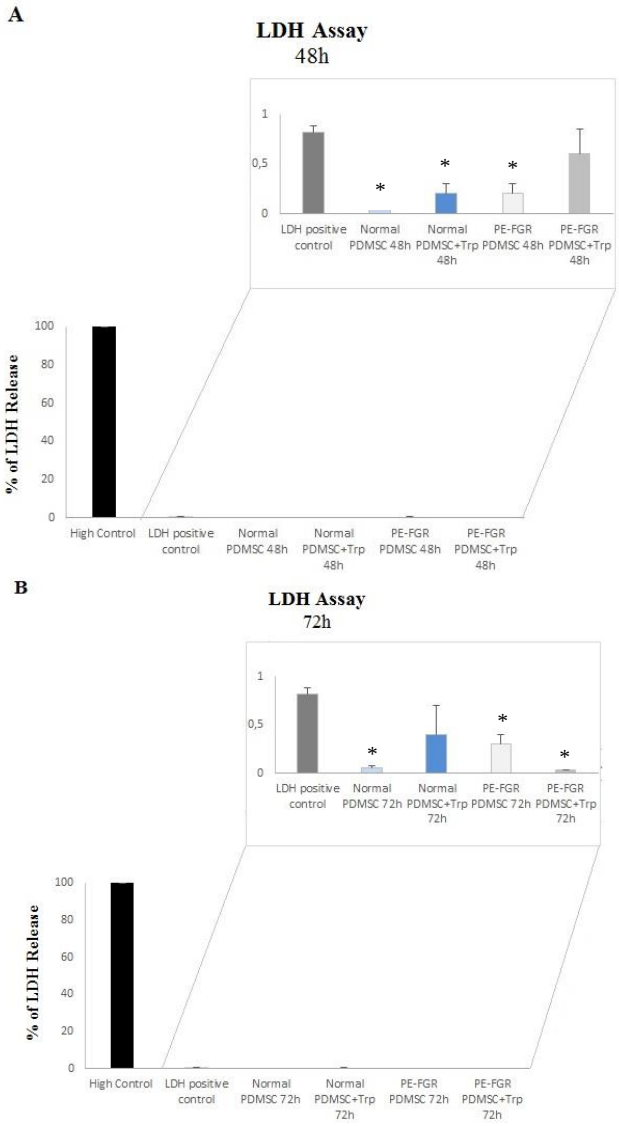

Figure 10. Tryptophan administration effect on normal and PE-FGR PDMSCs viability at 48h (**A**) and 72h (**B**). LDH positive control was provided by the kit. PDMSCs treated by Triton X-100 for 5 minutes were used as high control. Cytotoxicity in 48h and 72h supernatant of normal and PE-FGR treated with basal culture media or Trp was assessed by LDH assay. Statistical significance (*) has been considered as $p < 0.05$.

Tryptophan Mediates IDO-1, NCAM and HMGB1 Expression in Normal and PE-FGR PDMSCs

Since both normal and PE-FGR PDMSCs express IDO-1, a major Trp-degrading enzyme, we investigated their contribution to tryptophan catabolism treating cell cultures by 100 µg/mL of Trp for 48h and 72h. We first performed an *in silico* analysis of IDO-1 gene promoter region in order to evaluate Trp administration effects on its expression and we observed the presence of an Interferon Regulatory Factor (IRF) modulated by Trp concentration (Figure 11, panel A) [141, 142]. We determined also NCAM and HMGB1 gene promoter region sequences to understand how Trp and its metabolism regulate neurogenesis. NCAM (Fig. 11, panel B) and HMGB1 (Fig. 11, panel C) presented an IFR element on their promoters, underlying tryptophan importance in neurogenic processes.

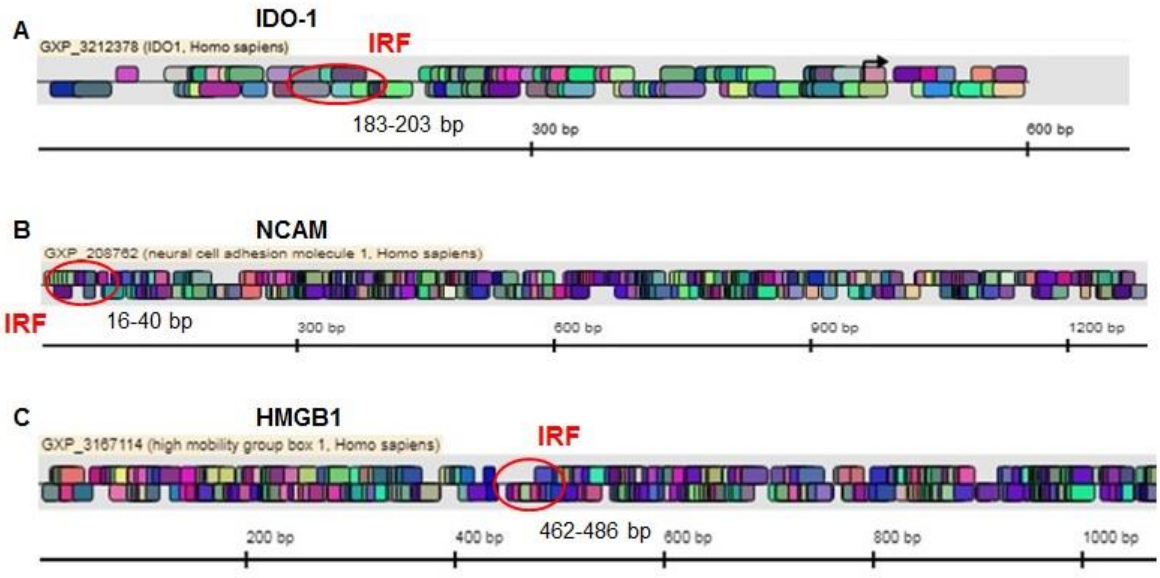

Figure 11. *In silico* analysis of IDO-1 (**A**), NCAM (**B**) and HMGB1 (**C**) gene promoter region revealed the presence of an Interferon Regulatory Factor (IRF).

IDO-1, NCAM and HMGB1 modulation by Trp was confirmed at both the gene and protein levels. In particular, we reported a significant increase in IDO-1 gene expression in both normal (p=0.03) and PE-FGR PDMSCs (p=0.03 vs normal PDMSCs; p=0.03 vs untreated PE-FGR PDMSCs) after 48h of Trp treatment relative to normal cells cultured in basal medium (Figure 12, panel A). As expected by our previous data, IDO-1 mRNA levels were decreased in PE-FGR relative to normal PDMSCs, while NCAM gene expression was upregulated (Fig. 12, panels A and B). NCAM mRNA levels were increased in Trp-treated PDMSCs (p=0.03 Trp-treated PE-FGR vs untreated PE-FGR PDMSCs) (Fig. 12, panel B). At 72h, Trp treatment did not have effect on both IDO-1 and NCAM expression (Fig. 12, panels A and B). We described a significant increase of HMGB1 gene levels in both Trp-treated normal and PE-FGR PDMSCs relative to normal untreated cells (Fig. 12, panel C) at both time points. HMGB1 mRNA expression maintained increased levels in PE-FGR relative to physiological PDMSCs both when cells were cultured in basal medium or treated by Trp (Fig. 12, panel C).

At the protein level, we demonstrated the presence of positive immunoreactivity for PSA-NCAM in normal relative to PE-FGR PDMSCs both cultured in basal medium or with Trp administration (Figure 13). Western Blot analysis showed increased HMGB1 protein levels in PE-FGR relative to normal PDMSCs at 48h and 72h (Figure 14). Trp treatment determined its upregulation in normal (p=0.04) and PE-FGR PDMSCs (p= 0,04 vs normal PDMSCs; p=0.04 vs normal Trp-treated PDMSCs) relative to normal untreated cells at 48h, while at 72h HMGB1 expression decreased in both groups (Fig. 14).

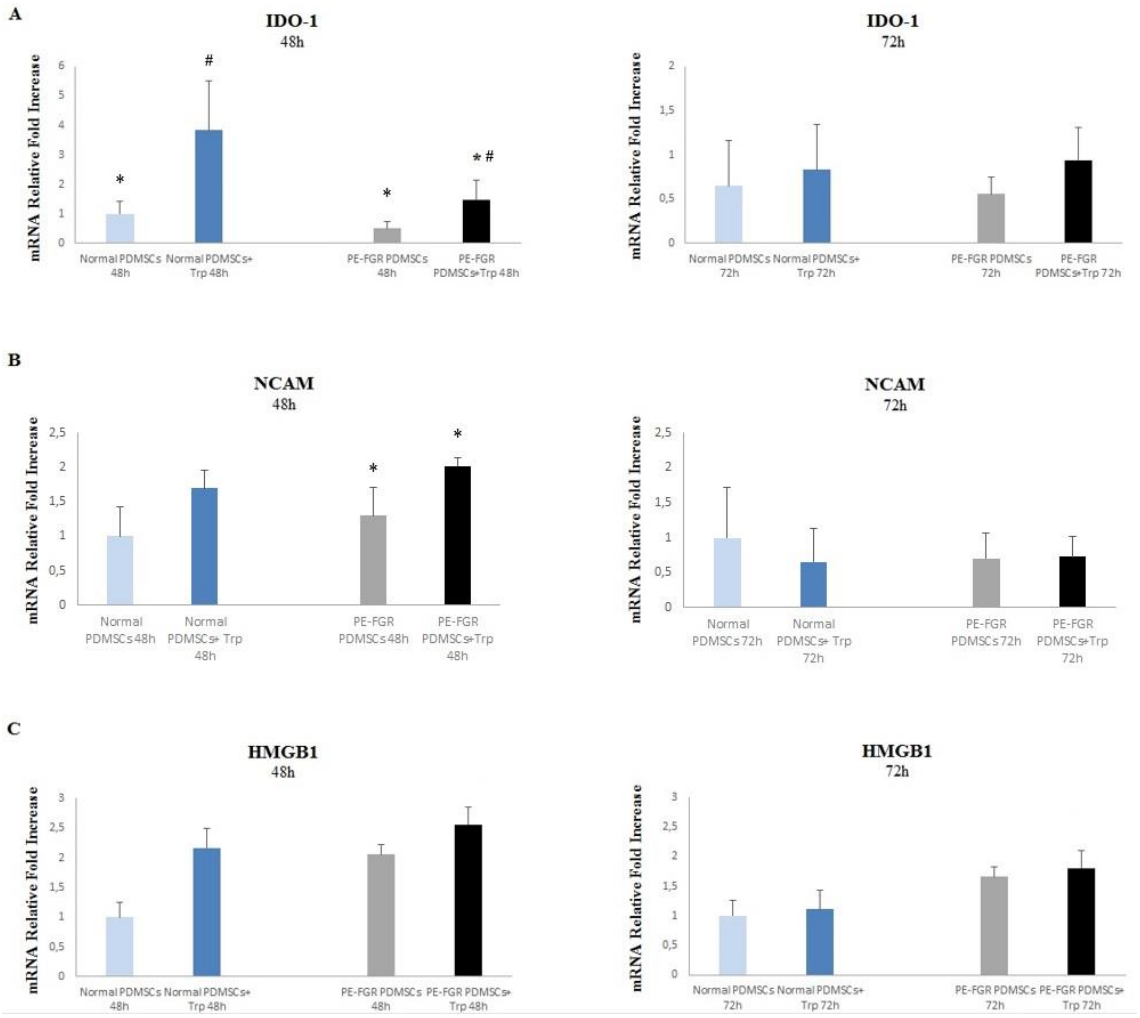

Figure 12. Real Time PCR analysis showed a significant down-regulation of IDO-1 (**A**) mRNA levels in PE-FGR vs normal PDMSCs at 48h, while Trp administration determined an increase in both normal and PE-FGR treated PDMSCs vs the untreated control. On the contrary, NCAM (**B**) and HMGB1 (**C**) gene expression was increased in PE-FGR vs normal PDMSCs at 48h. After Trp treatment at 48h, NCAM gene levels were decreased in normal PDMSCs vs the untreated control, while there was an upregulation in PE-FGR vs untreated physiological PDMSCs. HMGB1 mRNA levels were increased in both normal and PE-FGR PDMSCs at 48h and 72h after Trp administration. IDO-1 and NCAM mRNA levels showed no significant differences at 72h between untreated and Trp-treated cells. Statistical significance (*, $^{\#}$) has been considered as $p< 0.05$.

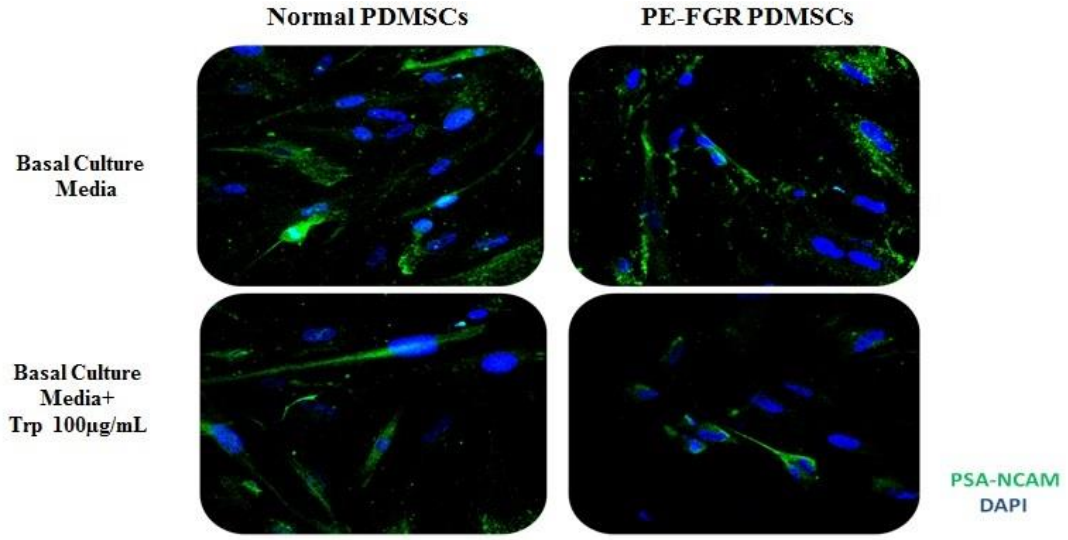

Figure 13. Immunofluorescent staining showed presence of positive immunoreactivity for PSA-NCAM (green) in normal vs PE-FGR PDMSCs, both cultured in basal conditions or treated by Trp for 48h. Cell nuclei are showed in blue by DAPI signal. Original magnifications x20.

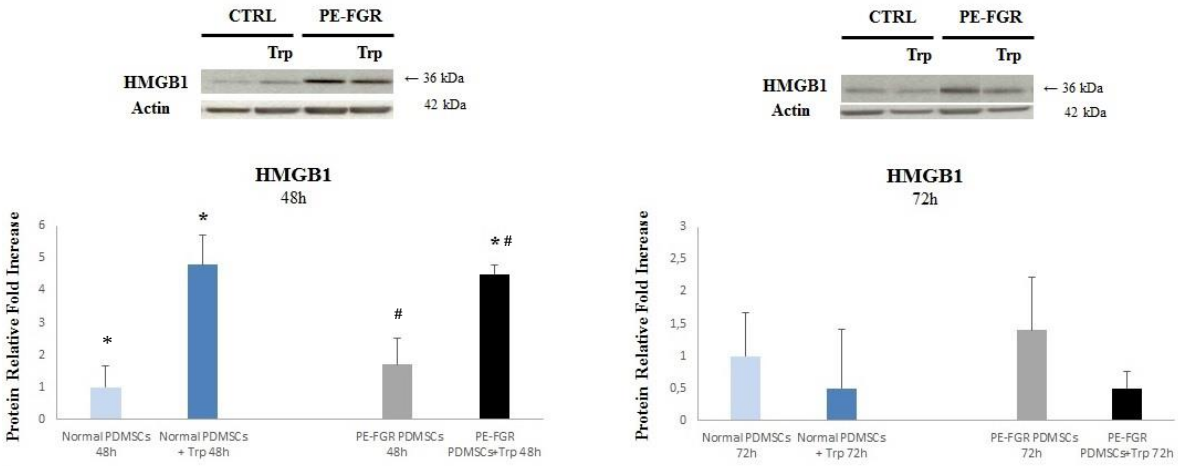

Figure 14. Western Blot analysis showed an increase of HMGB1 protein levels in PE-FGR vs normal PDMSCs at 48h. Trp treatment induced a significant upregulation of HMGB1 in both normal and PE-FGR vs untreated physiological PDMSCs. At 72h, a trend of increase of HMGB1 protein levels was observed in PE-FGR vs normal PDMSCs. Statistical significance (*, #) has been considered as $p < 0.05$.

Differential IDO-1 Enzymatic Activity in Normal and PE-FGR PDMSCs

In order to understand PDMSCs contribution to Trp metabolism and whether IDO-1 enzymatic activity is differentially regulated in physiological and PE-FGR mesenchymal cells we investigated kynurenine release in the cell culture media of Trp-treated PDMSCs. Kyn is the direct product of IDO-1 activity and has neurotoxic properties being a convulsant compound. Its concentration was decreased in PE-FGR relative to normal culture medium at 48h and 72h (Figure 15). Tryptophan administration induced a decreased of Kyn release in normal PDMSCs relative to untreated physiological cells at 48h, while at 72h there was an upregulation (Fig. 15). On the contrary, Kyn production was increased in PE-FGR Trp-treated PDMSCs relative to untreated normal cells at both time points (Fig. 15). Kyn levels in PE-FGR PDMSCs culture supernatans remained lower relative to those released by normal PDMSCs both when cells were cultured in basal medium or treated by Trp (Fig. 15).

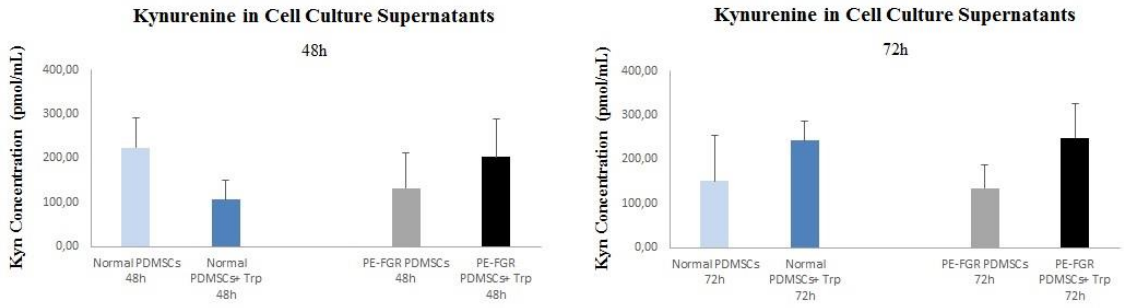

Figure 15. ELISA assay showed a downregulation of Kyn release in cell culture supernatants of PE-FGR vs normal PDMSCs at both 48h and 72h. After Trp administration, there was a decrease in Kyn production in normal PDMSCs vs the untreated control at 48h, while Kyn release was increased in PE-FGR at both time points and in normal PDMSCs at 72h vs the untreated control.

DISCUSSION

Discussion

The placental syncythiotrophoblast is involved in the modulation of fetal neurodevelopment, metabolizing fetal serotonin from maternal circulating tryptophan until the second trimester of pregnancy. We recently demonstrated that Placenta-derived Mesenchymal Stromal Cells express BDNF and Neurotrophins -3 and -4, key modulators of neurogenesis, which are over-expressed in PDMSCs isolated from pregnancies complicated by Preeclampsia [66], a placenta-related disorder associated with neurological impairment for the newborn. PDMSCs contribution to fetal neurodevelopment has never been investigated.

In the present study we described, for the first time to our knowledge, a differential expression of neurogenesis-related molecules in normal and PE-FGR PDMSCs. In particular, PE-FGR PDMSCs overexpress neurogenesis activators Neural Cell Adhesion Molecule, Doublecortin, High Mobility Group Box 1 and Nestin, as well as they downregulate neurogenesis inhibitors Polysialic Acid NCAM, Indoleamine-2,3-dioxygenase 1 and miRNA-25, 124, -134, -137 and -181a expression. Importantly, we reported PDMSCs contribution to IDO1-mediated tryptophan catabolism, with a decrease of neurotoxic kynurenine release by PE-FGR relative to physiological PDMSCs.

The literature of the last decades is focused on the use of MSCs from different origins, like bone marrow, umbilical cord and adipose tissue, in the therapy of neurodegenerative diseases [143]. Several studies aim at using MSCs ability to release pro-neurogenic factors and to differentiate into astrocytes, oligodendrocytes and microglia cells after *in vitro* induction or after injection into a neurogenic niche [144] to improve neuronal death and aberrant synaptic signaling. In particular, BDNF is

considered a lead candidate for the treatment of Huntington's disease (HD), an autosomal dominant neuropsychiatric disability that causes neuronal death progressive striatal and then widespread brain atrophy. In 2016, Pollock and colleagues used a lentiviral vector to genetically engineer BM MSCs to overexpress BDNF in a rat model of HD [145]. MSCs were chosen as neurotrophin delivery platform since they are known to secrete a variety of factors that reduce inflammation and programmed cell death, enhance connections between neurons and decrease cell toxicity. Moreover, they have been shown to be readily engineered using viral transduction and do not require immunosuppression following allogeneic transplantation. Our group reported PDMSCs release of BDNF [66], which occurs without previous genomic manipulation or use of culture media supplemented with differentiating growth factors. Aberrant neurotrophins secretion by mesenchymal cells isolated from PE placentae could be a direct consequence of the PE pro-inflammatory microenvironment, a mechanism previously suggested by Qiao and colleagues. They described increased peripheral BDNF production by sensory neurons located in the dorsal root ganglia after inflammatory stimuli of the visceral organs [146], with a concomitant upregulation of BDNF receptor TrkB synthesis that result in enhanced cellular growth, survival and differentiation in order to replace damaged cells. Similarly, BDNF secreted by PDMSCs can have a double role, the first as an attempt to counteract aberrant placentation typical of PE, and the second to try to ameliorate brain injury related to the hostile intrauterine environment and premature birth that often occurs for PE-FGR newborns [147]. D'Souza et al. showed placental BDNF gene expression lower in women with PE than in normotensive women [148], contributing to PE pathophysiology. These evidences are in contrast with our data of increased PE PDMSCs BDNF release, mainly because

methodologic differences since we analyzed a single cellular population in contrast to a homogenate of total placental tissue. In our model, BDNF overexpression may not be enough to counteract PE onset alone, but since placental mesenchymal cells are located nearby fetal vessels within placental villi we can hypothesize that these peptides could be directed to the fetal circulation to improve negative neonatal outcomes in terms of nervous system development. In the past years, it was shown that whilst MSCs cannot make hematopoietic stem cells, they do physically support them and promote their differentiation [149]. Similarly, neuroactive molecules contained in PDMSCs conditioned medium can be a potential help to fetal neurogenesis also due to the fact that the fetal brain-blood barrier is more permeable, not being yet completely developed.

In line with neurotrophins upregulation in PE relative to normal PDMSCs we found increased levels of neurogenic markers NCAM and DCX reporting, for the first time to our knowledge, mesenchymal cells neuron-like phenotype in the absence of genetic manipulation or differentiating growth factors. Expression of pro-neurogenic molecules can be a consequence of increased inflammatory cytokines Interleuchin-6 (IL-6), Interferon γ (IFN γ) and Tumor Necrosis Factor α (TNF α) secreted by PE PDMSCs [66]. These cytokines can affect cell proliferation, differentiation and migration and hence modulate cell repair potential [150]. High levels of cytokines are also detected in the fetal brain, suggesting a possible role during development [151]. In particular, INF γ has been implicated in neuronal fate determination of human hippocampal neural progenitor cells [152]. IL-6 is known to participate in neurogenesis, influencing both neurons and glial cells, and in the response of mature neurons and glial cells in normal conditions and following a wide range of injury models. It behaves in a neurotrophin-

like fashion and seemingly makes understandable why its cytokine family is known as neuropoietins [153]. According to these evidences, we can suggest that IL-6, INF γ and TNF α can promote neuronal markers expression in PE-FGR PDMSCs via paracrine signaling.

NCAM plays an active role in bringing axons and glial cells into close apposition, but also in transducing the signals between these cells [154]. There are several isoforms of this molecule, which result from differential splicing and post-translational modifications and whose synthesis is developmentally regulated [155]. Polysialic acid moieties on NCAM not only prevent homophilic NCAM–NCAM adhesion, but also serve more generally as negative regulators of cell to cell interactions. PSA-NCAM expression is developmentally down-regulated and is abundantly present on all growing fiber tracts in the developing central nervous system, persisting in certain areas of the adult brain known to exhibit plasticity [156]. Considered its function to inhibit cell to cell signaling, PSA-NCAM decrease in PE-FGR relative to normal cells can preserve mesenchymal cells crosstalk via both autocrine and paracrine pathways, maintaining their biology and unique modulatory properties unchanged.

Overexpression of neuronal markers DCX and Nestin in PE-FGR relative to physiological PDMSCs can make these cells pleasant candidate in the treatment of certain central nervous system pathologies, like it has been described by Lindsay et al. [157]. They proposed that olfactory mucosa MSCs may be derived from the neural crest and are an easily accessible source of cells which may be therapeutically advantageous over BM-derived MSCs for the treatment of demyelinating conditions such as multiple sclerosis [157]. This is a controversial matter since we reported that PDMSCs secretome owns a remarkable inflammatory profile, playing a key role in PE pathophysiology [66].

Enhanced pro-inflammatory cytokines secretion can also be a determinant of the neurogenic activity of PE-FGR mesenchymal cells [151], leading us to speculate that the microenvironment in which PDMSCs conditioned medium acts can results in different biological outcomes. We demonstrated that PE PDMSCs secretome is able to induce a PE-like phenotype in physiological villous placental explants [66], but if or how it can modulate neurons regeneration and repair in the context of neurodegenerative disorders is not yet been elucidated.

Nestin is defined as a class VI intermediate filament protein reported to be upregulated in most mitotic cells and downregulated in all cells upon differentiation [158]. Tondreau's group initially reported that BM-derived MSCs expressed Nestin before differentiation *in vitro* [159]. Wiese et al. showed that Nestin was enriched in embryonic stem-derived progenitor cells that could develop into neuroectodermal, endodermal, and mesodermal lineages [160]. Thereafter, Méndez-Ferrer et al. presented abundant evidence to confirm that bone marrow MSCs could be identified by Nestin [161]. Accordingly, Nestin synthesis in PDMSCs occurs without any *in vitro* stimulation and identify their undifferentiated state. Since several groups reported Nestin participation to many bone diseases development, when they contribute to mesenchymal niche maintenance and tissue growth and regeneration [162], we can suppose that in PE-FGR it plays a similar role.

There are evidences that MSCs isolated from chorion and amnion derived from preeclamptic pregnancies are comparably suitable for neuro-regeneration as the ones from uncomplicated pregnancies [163-165]. Although Joerger-Messerli and colleagues found significant differences in the expression of the MSCs markers CD105, CD90 and CD73 between membranes, the neural differentiation potential was not altered between

PE and healthy controls [165]. They lately showed that undifferentiated Warton's Jelly (WJ) derived-MSCs from preterm birth expressed the neural progenitor markers Musashi-1 and Nestin and the mature neuroglial markers MAP-2, MBP and GFAP, similarly to term controls, indicating that preterm delivery has no detrimental impact on the quality of WJ-MSCs [164]. Preeclampsia leads to oxidative stress in the placenta, resulting in the release of inflammatory molecules, including cytokines that activate the nuclear factor kappa-light-chain-enhancer of activated B cells(NF-kB) and Toll-like receptor 4 (TLR4) pathways [166]. NF-kB signaling has been shown to mediate the asymmetric division and differentiation of neural stem cells into neural progenitor cells (NPC) at very early stages of neurogenesis [167]. Taken together, these premises can make us conclude that PDMSCs from PE-FGR placentae are more committed to neuronal-like phenotype relative to mesenchymal cells isolated from healthy controls through activation of the pathways involved in the pathophysiology of the disease.

Herein, we demonstrated High mobility group box 1 increased expression in PE-FGR relative to normal PDMSCs, as expected because of the inflammatory microenvironment that characterizes PE. HMGB1 is widely expressed in cells of vertebrates in two forms, a nuclear factor that regulates BDNF transcription and a secreted inflammatory cytokine [168]. During early brain development, HMGB1 displays a complex temporal and spatial distribution pattern in the central nervous system, where facilitates neurite outgrowth and cell migration during critical processes such as forebrain development. During adulthood, HMGB1 serves to induce inflammation after injury like lesions in the spinal cord and brain. Receptor for advanced glycation end products and Toll-like receptors signal transduction pathways mediate HMGB1-induced neuroinflammation and necrosis. Increased levels of

endogenous HMGB1 have also been detected in neurodegenerative diseases, thus HMGB1 plays important and double-edged roles during neural development and degeneration [168]. In our system, HMGB1 increase can correspond to BDNF upregulated secretion in PE mesenchymal cells. A part from being a marker of tissue damage, we can suppose that HMGB1 can mediate the potential neuroprotective role of PDMSCs in a pathologic context like that of PE with fetal growth restriction, both signaling adverse conditions and then participating in the activation of rescue pathways.

MicroRNAs represent the new dimension of gene regulation discovered in recent years. They are abundantly expressed in the central nervous system, with about half of known species detected in the human brain, implicating their significant contribution in neural development and function [169]. In the present study we observed miRNA-25, 124, -134, -137 and -181a downregulation in PE-FGR relative to PDMSCs isolated from healthy placentae. miR-25 acts inducing proliferation in differentiated neurons, maintaining them in the post-mitotic state through inhibition of p57 synthesis [170]. miR-124 and -137, which display parallel changes in expression as cells differentiate, are among the top expressed miRNAs in the adult brain and have been implicated in neuronal differentiation, neurodegenerative diseases, and neurological disorders [171]. Alterations in miR-124 have been shown to be associated with social behavioral deficits in frontotemporal dementia. Its expression is repressed in brains of patients with HD and mouse models [172]. miR-137 has been determined to be a gene related to schizophrenia susceptibility and to be downregulated in brain tissue from patients with depression and suicidal behavior [173]. Moreover, miR-137 has been shown to be deregulated in a cellular model of HD, as miR-124 [174]. Our findings of decreased miR-25, -124 and -137 expression in PE-FGR relative to normal mesenchymal cells can

be referred to the role of this small molecules in neuronal differentiation, since these cells are in an undifferentiated state, although they display neuron-like features.

microRNA-134 is a brain-specific miRNA and is differently expressed in brain tissues subjected to ischemic injury, however the underlying mechanism of miR-134 in regulating cerebral ischemic injury remains poorly understood [175]. Recently, overexpression of miR-134 was shown to inhibit the expression of the downstream gene of CREB, including BDNF, suggesting that downregulation of miR-134 alleviates ischemic injury through enhancing CREB expression and downstream genes [176]. Since we proved increased BDNF secretion by PE-FGR relative to healthy PDMSCs, miR-134 downregulation in pathological cells is in line with enhanced CREB-downstream genes transcription reported by other groups. Furthermore, its importance in ameliorating ischemic or hypoxic injuries is confirmed in our model.

Hu and colleagues previously reported that miR-181a expression was significantly increased in placental samples from women with severe PE [177]. Additionally, it is strongly expressed in bone marrow and thymus, it can modulate hematopoietic lineage differentiation and has an important role during T- and B-cell development [178]. In the central nervous system, miR-181a is highly abundant in cerebellar cortex and its expression is increased in autism patients as well as in an animal model of autism [179]. Importantly, it is a regulator of IDO-1 gene expression [178]. Contrarily to Hu et al. findings, we observed a downregulation in miR-181a synthesis in MSCs from PE pregnancies with FGR relative to those isolated from control placentae. Over being a result conditioned by the different sample type, it is concordant with IDO-1 mRNA decrease in pathological placental MSCs, as well as an indicator of the possible

protective role of PDMSCs towards post-natal development of autism spectrum disorders for which FGR represents a severe risk factor [180].

Tryptophan is a precursor for serotonin, a neurotransmitter that may impact energy metabolism during gestation given that recent studies have demonstrated increased 5-HT production is crucial for the modulation of maternal insulin secretion [181]. Trp modulation of neurogenesis markers NCAM and HMGB1 was not studied before. Herein, we described how Trp administration lead to an increased transcription of both NCAM and HMGB1 genes, demonstrating its importance in neurogenic pathways. Excess of Trp during pregnancy may however cause complications for both the mother and the fetus, as observed in a mouse model of casein-rich diet. Pregnant dams fed 5% trp diet displayed lower placental and fetal weigh at the 17th day of gestation, with a higher rate of fetal reabsorption relative to dams fed a 2% trp diet [137]. This is explained by the fact that mice fed 7.0 g Trp/kg during gestation experienced accumulation of non-proteinaceous form of tryptophan in the maternal body, placenta, and fetus. Enhanced IDO-1 enzymatic activity in PE-FGR PDMSCs can be aimed at avoiding placental accumulation of this toxic trp form, protecting fetal brain development.

Studies on an animal model of FGR showed that dams submitted to dietary protein restriction throughout gestation, who received intravenous infusions of stable Trp isotope, maintained active Trp transfer from mother to fetus, as well as *de novo* synthesis of 5-HT in the fetus [182]. In growth restricted fetuses, maternal–fetal tryptophan transfer remained unaffected, but conversion to quinolinic acid, a neurotoxic compound, was impaired [182]. We developed an *in vitro* model to mimic the *in vivo* maternal-placental-fetal Trp axis during pregnancy. The placental tissue is completely

dependent on maternal dietary intake of tryptophan, being an essential amino acid that we are not able to synthetize, for both tissue metabolism and transport to fetal brain. Our results demonstrate that PDMCs are actively involved in Trp metabolism being able to produce kynurenine through IDO-1 enzymatic activity. Importantly, IDO-1 kinetics is changed between normal and PE-FGR PDMSCs. In fact, kyn concentration in physiological mesenchymal cells supernatants is higher relative to that found in PE-FGR culture medium, but they respond differentially to Trp administration, which seems to enhance IDO-1 activity as demonstrated by IRF sequences found on its gene promoter region. Accordingly, we can propose that biological microenvironment typical of PE-FGR alters Trp catabolism and production of kynurenine pathway metabolites. From *in silico* analysis of IDO-1 gene promoter we know that also hypoxia and pro-inflammatory cytokines, typical features of PE-FGR syndromes, can induce its transcription. Further investigations are required to understand how Trp pathways in PDMSCs can modulate both placental growth and functionality and fetal neurodevelopment. Stressful events during pregnancy adversely affect brain development and may increase the risk of psychiatric disorders later in life. Early changes in the kynurenine pathway, which contains several neuroactive metabolites, may constitute a molecular link between prenatal stress and delayed pathological consequences. Notarangelo et al. reported increased levels of kynurenine, a neuroactive protein with convulsant properties, in placental tissue, fetal plasma and fetal brain of the litter of pregnant mice that experienced a single physical stressful event at the end of pregnancy [183]. Our findings differ since PE-FGR represent a chronic biological stress for the fetus, with the establishment of a hypoxic and pro-inflammatory uterine environment in which the fetus itself grows, and not an acute single event. Moreover,

our data refers to a single placental cell population and not to tissue biopsies that contain more cell types.

Conclusions

In summary, we showed that undifferentiated PDMSCs derived from pregnancies complicated by preeclampsia and fetal growth restriction display enhanced neuronal markers expression relative to cells isolated from healthy placentae. Several evidences indicate collateral effects of magnesium sulfate [184] and corticosteroids [185, 186] on fetal brain formation. Magnesium sulfate is commonly used in the treatment of sever PE-induced hypertension [187], while corticosteroids improve lung maturation in case of preterm delivery, which often occurs in case of maternal and/or fetal conditions worsening in PE-FGR pregnancies [186]. Our preliminary investigations give new insight in the clinical use of such drugs, since placenta neuroprotective role towards fetal brain can balance risk associated with magnesium sulfate and corticosteroids administration.

Our clearly depict mesenchymal cells active involvement in the modulation of fetal neurodevelopment, with a possible neuroprotective role of PE-FGR PDMSCs towards inflammation, hypoxia and oxidative stress, all typical biological features of the above mentioned placenta-related syndromes that could lead to impaired fetal neurogenesis and/or cognitive and behavioral deficits in the post-natal life.

Future Directions

Our findings suggest chorionic placental mesenchymal stromal cells as a valuable therapeutic tool in terms of improving neurodevelopmental fetal/neonatal outcomes, given the autologous nature of the graft and its availability after birth, thus opening to new intriguing perspectives for the treatment of brain disorders associated with preeclampsia and fetal growth restriction. These results will lead to the identification of new biomarkers to early diagnose neurological damage typical of newborns from PE pregnancies and to prevent cognitive impairment development.

Further analysis is needed to asses PDMSCs conditioned medium effects on central nervous system development. The next steps will be to develop an animal model of PE-FGR that will allow us to characterize tryptophan maternal dietary intake during pregnancy and its placental transport and metabolism in order to better understand PDMSCs role in the production of neuroactive molecules of the kynurenine and serotonin pathways, the two main Trp catabolic routes. *In vivo* experiments will be focused also on tracking normal and PE-FGR PDMSCs secretome to effectively demonstrate that it can reaches fetal circulation. Finally, both animal experimentation and clinical follow up of newborns from PE-FGR pregnancies will be useful to identify a proper therapeutic strategy to prevent cognitive disorders establishment in the post-natal and adult life.

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