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IFPA Meeting 2013 Workshop Report III: Maternal placental immunological interactions, novel determinants of trophoblast cell fate, dual *ex vivo* perfusion of the human placenta

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A R T I C L E I N F O

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A B S T R A C T

Workshops are an important part of the IFPA annual meeting as they allow for discussion of specialised topics. At IFPA meeting 2013 there were twelve themed workshops, three of which are summarized in this report. These workshops related to various aspects of placental biology but collectively covered areas of placental function, cell turnover and immunology: 1) immunology; 2) novel determinants of placental cell fate; 3) dual perfusion of human placental tissue.

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Fetoplacental vascular tone
Syncytiotrophoblast microvesicles
Immunology
Placental cell fate

1. Maternal placental immunological interactions: advances in the field

Chairs: Caroline Dunk and Michelle Letarte
Speakers: Mohamed Abumaree, Christian Castillo, Anne Croy, Caroline Dunk, Sylvie Girard, Alison Wallace, Cristian Zenerino

1.1. Outline

This workshop aimed to discuss some of the latest findings and techniques that have contributed to our expanding knowledge of the interactions between the maternal decidual leukocytes and fetal trophoblast and their effects.

1.2. Summary

Caroline Dunk discussed the dynamic changes in decidual leukocyte populations across gestation. The uterine natural killer (uNK) cells are known to play important roles in spiral artery angiogenesis, trophoblast invasion and vascular remodeling. However little is known about the signaling pathways regulating these effects. It has recently been shown that activation of the Sphingosine S1P pathway via S1PR5 on uNK cells can downregulate vascular endothelial cell growth factor (VEGF) expression and decrease uNK cell mediated trophoblast migration and *in vitro* angiogenesis. In the second trimester S1PR5 expression levels are decreased in uNK cells. It has been further shown that the second trimester decidua is an immune tolerant environment characterized by uNK cells incapable of mounting a cytotoxic response, M2 macrophages, and the appearance of a novel N2 angiogenic neutrophil population.

Anne Croy addressed the heterogeneity of uNK cell activation pathways. During mesometrial decidualization in mice, a transient population of uNK cells is established and expands until mid-pregnancy. Initially these cells enhance mesometrial angiogenesis, new vessel pruning and uterine lumen closure and thereby the rate of conceptus development and growth. Uterine NK cells then participate in induction of endothelial tip cells and in initiation of spiral arterial remodeling. Uterine NK cell surface receptors that recognize MHC ligands and those that recognize other ligands are both independently essential in these processes and play different roles in regulation of uNK cell-produced VEGF.

Alison Wallace presented a study investigating uNK cells in pregnancies with poor spiral artery remodeling. Uterine artery Doppler resistance index (RI) in the first trimester of pregnancy can be used as a proxy measure of the extent of remodeling of the uterine spiral arteries. Using this technique uNK cells were isolated from pregnancies with normal (normal RI) or impaired spiral artery remodeling (high RI). Their receptor expression phenotype and contribution to trophoblast chemotaxis and invasion were determined. High RI uNK cells displayed decreased expression of the receptors ILT2 and KIR2DL1/S1, and were less able to chemoattract trophoblast and induce extravillous trophoblast outgrowth from explants. This may contribute to poor placentation in high RI pregnancies.

Christian Castillo discussed how *Trypanosoma cruzi* (*T. cruzi*) induces cellular proliferation and differentiation in the trophoblast. The congenital transmission rate of *T. cruzi* is low, suggesting the existence of local placental antiparasitic mechanisms; of which epithelial turnover of trophoblast may be one. In order to determine whether the parasite is able to induce cellular proliferation and differentiation in the trophoblast, BeWo cells and chorionic villi explants were incubated in the presence and absence of *T. cruzi* and respective positive controls. *T. cruzi* induced a significant increase of BrdU incorporation into DNA, in the number of mitosis, nucleolar organizer regions, expression and secretion of hCG. It was concluded that *T. cruzi* may increase trophoblast cell turnover.

Mohamed Abumaree discussed modulation of macrophage differentiation from inflammatory M1 to anti-inflammatory M2 macrophages by placental cells. During pregnancy the mother must adapt her immune response to foreign paternal antigens, possibly by signals given by the pregnancy to the mother's immune cells. Such tolerogenic signals may include the interaction between immune cells and trophoblast debris which are shed from the placenta into the maternal blood during normal pregnancy. It was shown that trophoblast debris can shift macrophage differentiation from the inflammatory M1 into an anti-inflammatory M2 phenotype. This result suggests a new immunosuppressive property of trophoblast debris that may be employed to protect the fetal allograft from the maternal immune system in normal pregnancy.

Sylvie Gerard addressed how inflammation may be a cause of placental dysfunction in high-risk pregnancies. The mechanism underlying the association between inflammation during human pregnancy and developmental abnormalities in the fetus were discussed. Evidence was provided of a distinct inflammatory profile in placenta from human high-risk pregnancies associated with reduced fetal movements. Using an *in vitro* model of human term placental explants a direct modulation of placental function by inflammatory cytokines was shown. These data give new insights into the mechanisms linking prenatal inflammation, placental dysfunction and altered fetal development and give possible therapeutic targets aimed to protect the placenta and subsequently the developing fetus.

Cristian Zenerino presented evidence on the anti-inflammatory effect of low molecular weight heparin (LMWH) being mediated by placental high mobility group box 1 (HMGB1) modulation. LMWH has been widely used for treating pre-eclampsia since several trials described its anti-inflammatory effect, although its mechanisms of action on the placental tissue are still unclear. HMGB1 is a transcription factor with extracellular cytokine-like functions able to induce pro-inflammatory molecules. LMWH binds to HMGB1 *in vitro*, thus changing its structural conformation and inhibiting its activity. It was investigated whether anti-inflammatory LMWH activity is mediated by HMGB1 modulation. Higher levels of HMGB1 were found in pre-eclamptic placenta compared to controls. In addition, decreased HMGB1, TNF-α and IL-6 gene expression and increased HMGB1 protein was found in LMWH-treated villous explants. These data suggest that LMWH could exert its anti-inflammatory effect on the placental tissue by changing HMGB1 structural conformation thus impairing its function.

1.3. Conclusions

The immune system is increasingly recognized as an integral part of a successful pregnancy. Transient inflammation is essential for any immune response and also for normal placental function; however sustained inflammation is associated with pregnancy

complications. Specialized subsets of immune cells, such as uNK cells play a critical role in feto–maternal interactions, and even contribute to angiogenesis. This workshop detailed an increasing focus on the functionality of uNK cell receptors demonstrating the involvement of Sphingosine signaling in the angiogenic phenotype, and showed that uNK cell populations are heterogeneous in terms of the pathways that regulate VEGF expression. A contribution of defective uNK cells to the development of utero-placental insufficiency was also suggested. The beneficial interaction of the placenta with maternal immunology was demonstrated by both the effects of the parasite *T. cruzi,* which can stimulate trophoblast proliferation and differentiation, and the observation that trophoblast debris can induce the differentiation of anti-inflammatory M2 macrophages, perhaps to protect the fetal allograft from the maternal immune system. Finally a molecular mechanism contributing to the anti-inflammatory effect of LMWH in pre-eclampsia was identified. This workshop covered a wide range of groundbreaking research in maternal-placental immunology and offers great hope for the future and a number of enticing questions for the research community.

2. Novel determinants of trophoblast cell fate

Chairs: Sascha Drewlo and Andrea Jurisicova
Speakers: Graham Burton, Larry Chamley, Andrea Jurisicova, Mana Parast, Dan Rappolee, Sussanah Varmuza

2.1. Outline

In the last decade, substantial evidence indicates that a compromised *in utero* environment can dramatically influence fetal development and health, impacting both early and late postnatal life. Altered placental development resulting in placental insufficiency is believed to be responsible for a large proportion of cases of fetal mortality in humans. In addition, growth-compromised babies resulting from fetal growth restriction following placental insufficiency are at increased risk for adverse, long-term outcomes such as hypertension, obesity and type II diabetes. Thus, it is crucial to gain an understanding of the basic cellular and molecular events responsible for proper placental development and the origins of cellular defects observed in placental insufficiency.

2.2. Summary

Andrea Jurisicova discussed whether key determinants of trophoblast stem cell fate are conserved. Derivation of murine trophoblast stem cells (TS) 15 years ago opened new studies in the field of placental biology. Yet, human TS cells have not been successfully established. TS cells were attempted to be derived from human blastocysts in a variety of conditions, including classical FGF-dependent environment, yet establishment of a stable line was not successful. It was found that stem cell factor (SCF), but not FGF improved the quality of blastocyst outgrowths *in vitro*, evidenced by an increase in β-HCG secretion. The trophectoderm of human blastocysts express the trophoblast determinant, CDX2, but also maintain expression of the OCT4 and NANOG. Unlike their murine counterparts, the trophectoderm of fully expanded human blastocysts does not express FGF receptors. However, significant FGF receptor expression was found in the first trimester cytotrophoblast, with decreased expression by 12 weeks of gestation. It was concluded that FGF dependent signaling is unlikely to play a role in the maintenance of human TS cells in preimplantation embryos, but may be involved in proliferation of cytotrophoblast post implantation.

Mana Parast shared data on BMP4-directed trophoblast differentiation of human embryonic stem cells being mediated through a dNp63 + cytotrophoblast stem cell state. Little is known about early trophoblast differentiation in the human embryo, due to lack of a proper *in vitro* model system. Human embryonic stem cells (hESCs) differentiate into functional trophoblast following BMP4 treatment in presence of feeder-conditioned media; however, this model has not been widely accepted, in part due to lack of proof for a trophoblast progenitor population. By direct comparison to primary human placental tissues and isolated cytotrophoblast, through gene expression profiling, it was demonstrated that BMP4-treated hESC differentiate into cytotrophoblast cells. In primary cytotrophoblast cells, p63 levels are reduced as cells differentiate into syncytiotrophoblast, and that forced expression of p63 maintains cyclin B1 and inhibits syncytiotrophoblast differentiation. It was demonstrated that, similar to *in vivo* events, hESC differentiation into trophoblast is characterized by a $p63^{+}/KRT7^{+}$ cytotrophoblast stem cell state, followed by formation of functional $KLF4^{+}$ syncytiotrophoblast and $HLA\text{-}G^{+}$ extravillous trophoblast cells. These results establish that BMP4-treated hESCs are an excellent model of human trophoblast differentiation, closely mimicking the *in vivo* progression from $p63^{+}$ cytotrophoblast stem cells to terminally-differentiated trophoblast subtypes.

Sussanah Varmuza discussed the epigenetic programming of trophoblast lineages and the role of Sfmbt2. The Polycomb group gene Sfmbt2 encodes a member of a class of chromatin proteins involved in stem cell maintenance. They typically act as part of multiprotein complexes that target large suites of genes for epigenetic silencing. It has been shown that loss of Sfmbt2 in mice impairs both trophoblast stem cell maintenance *in vitro*, and impairs development of all trophoblast compartments in the placenta. Defects in both quantity and subcellular distribution of human SFMBT2 correlates with pathological placentation. Preliminary interaction analysis reveals that SFMBT2 protein binds at least two different transcription factors, which combined would represent a robust set of target genes. These data are consistent with the hypothesis that SFMBT2 is required for the proliferation of trophoblast progenitors, and that it acts as a regulator of the trophoblast epigenome.

Dan Rappolee addressed stress pathways in trophoblast determination and placental development. Stress causes differentiation and can be dominant over the FGF4 signaling that maintains multipotency and proliferation of mouse trophoblast stem cells (TSCs). Protein kinase inhibitor screens indicate that stress-activated protein kinase (SAPK) is the most important enzyme for mediating stress-induced decreases in TSC growth. SAPK is necessary to mediate stress-induced TSC differentiation via induction of Hand1 and Hand1-dependent PL1 hormone (first lineage). For most stresses at 20% O_2, increasing doses increase SAPK activity in an S-shaped response curve, with a corresponding reverse S-shaped curve for growth. The adaptive response to increasing stress is to deplete the stem cell population by inducing differentiation. If stress continues this creates depletion of TSCs and imbalance of differentiation that prioritizes the first lineage. Unlike most stresses, decreasing oxygen from 20% to 0% leads to a U-shaped response curve for SAPK activity with highest TSC growth at 2% O_2. Hypoxic stress at 0.5% oxygen diminishes growth and induces differentiation of the first lineage while suppressing later lineages. However, hypoxic stress induces a rapid differentiation only through 1–2days despite the presence of FGF4. From 4 to 7 days, differentiation at 2%–20% oxygen increases from a common 3-fold to 10- to 25-fold, respectively. But, from 4 to 7 days at 0.5% oxygen differentiation stalls due to mitochondrial insufficiency even in the absence of FGF4. Thus hypoxic stress creates a third pathogenic hit by diminishing TSC growth, further depleting stem cells by

inducing differentiation, but then not sustaining differentiation after a rapid initiation. Marker analysis coupled with analysis of ATP usage, and studies with mitochondrial agonists and antagonists suggest that the two lineages most requiring higher oxygen and energy are at the surface of the labyrinthine placenta.

Graham Burton discussed endoplasmic reticulum (ER) stress and its impact on placental disease. The ER is the organelle responsible for synthesis of all secreted and membrane proteins. It is also involved in initial post-translational modifications before the proteins are trafficked to the Golgi apparatus. Protein synthesis is heavily energy demanding, and there must also be strict quality control as misfolded proteins can aggregate and initiate pathologies. Overloading the ER functional capacity, or reducing energy or nutrient supplies, activates a set of three highly conserved signaling pathways collectively referred to as the ‘unfolded protein response’. The principal aim of these is to restore homeostasis within the ER, but they are now realised to have wider functions in integrating cell responses to stress. The impact of these pathways on stem cell function is only just beginning to be explored, and, although the precise molecular details are unclear, there are at least three possible mechanisms. First, the transcription factor XPB-1 produced through ER stress may activate unexpected target genes. Second, the inhibition of protein synthesis that occurs in response to ER stress may lower levels of key regulatory proteins. Third, aberrant post-translational processing secondary to ER stress may adversely affect the bioactivity of secreted growth factors.

Larry Chamley presented data on the role of antiphospholipid antibodies in damaging the syncytiotrophoblast. Antiphospholipid antibodies are autoantibodies that are associated with several pregnancy complications. Exactly how they cause these clinical problems is not clear. It has been shown that these antibodies are rapidly internalized into the syncytiotrophoblast via an antigen-dependent receptor-mediated process whereas, control antibodies are not. Once inside the syncytiotrophoblast antiphospholipid antibodies target the mitochondria inducing changes in the control of cell death leading to necrotic cell death and an increase in numbers of multinucleated syncytial nuclear aggregates extruded from the placenta into the maternal blood. These finding may explain, in part, how antiphospholipid antibodies contribute to diseases of pregnancy.

2.3. Conclusions

From work presented and discussed it is obvious that trophoblast cells, whether at stem cell or at differentiation stages respond to unique environmental cues (e.g growth factors, control by key transcriptional regulators or stress agents). Integrating cellular signaling and transcriptional responses to these stimuli leads to cell decisions—differentiation, senescence or death. Overlaying molecular outcomes of these triggers with molecular changes detected in placental pathologies will be crucial to determine their relevance to altered human development.

3. Dual *ex vivo* perfusion of an isolated cotyledon of human placenta

Chairs: Paul Brownbill, Stefan Hansson, Henning Schneider
Speakers: Sarah Jones, Rohan Lewis, Caroline Pehrson, Dionne Tannetta, Christian Wadsack

3.1. Outline

The workshop focused on *ex vivo* dual perfusion of human placental tissue. This technique was originally designed to study the variety of mechanisms involved in transplacental transport of different compounds. Great progress has been made in this area and sophisticated mechanisms involving specific transporters localized in the microvillous and basal surfaces of trophoblast, as well as in the endothelial cells of the villous capillaries have been identified. However, over the years the spectrum of applications has widened considerably, including the role of trophoblast metabolism, use of the perfusion system as a physiological model for the generation of syncytiotrophoblast micro- and nanovesicles, exposure of the trophoblast and its response to infectious as well as toxic agents, the assessment of the villous vasculature and differences in hemodynamic responses of normal as well as pathological placentae.

3.2. Summary

Caroline Pehrson discussed use of the *ex vivo* dual placental perfusion model to study adhesion of malaria parasites. In placental malaria (PM) *Plasmodium falciparum* infected erythrocytes sequester in the placenta through specific binding of the *P. falciparum* VAR2CSA antigen to chondroitin sulfate A. Anti-PM vaccine development is focused on hindering the placental parasite accumulation by identification of sub-units of VAR2CSA that induce antibodies inhibiting the binding of VAR2CSA expressing infected erythrocytes to chondroitin sulfate A. The *ex vivo* dual placental perfusion model is currently implemented to investigate the biology of infected erythrocyte adherence in the placenta and the inhibitory capacity of antibodies from immunized rodents and humans in phase I clinical trials.

Christian Wadsack addressed how dual perfusion can provide new insights into bidirectional lipid transport across the placenta. Data obtained from recent studies in humans, rodents and cell culture demonstrate that circulating maternal lipids may be transported to the fetus. Dual perfusion of a single cotyledon is the only experimental model to study human placental transfer of lipids in organized tissue. The discussed perfusion results indicate that the placenta exhibits the ability to manipulate the mass of maternal lipids that is taken up by the placenta and thereby possibly impact upon the growth and development of the fetus.

Dionne Tannetta presented data on isolation and fractionation of placental perfusion derived syncytiotrophoblast micro- and nanovesicles. Placental perfusion medium harvested from the maternal circuit yields mixed populations of syncytiotrophoblast derived microvesicles (pSTBM). Sequential centrifugation and 0.22 μm filtration were used to enrich pSTBM for either exosomes or microvesicles, assessed using five color flow cytometry, nanoparticle tracking analysis and Western blotting. Fresh perfusates were firstly centrifuged at $1500\times$ g $2\times$ to remove red blood cells (RBC; $\sim$68%). Incubating the resultant supernatant with magnetic dynabeads coated with anti-glycophorin A/B (RBC), anti-CD41 (platelets) and anti-pan HLA (platelets/leukocytes) antibodies effectively removed the remaining low level contaminating platelets, RBC and platelet and RBC vesicles. Microvesicles were then pelleted by $10{,}000\times$ g centrifugation ($\sim$390 nm modal size). Finally 0.22 μm filtration of the microvesicle depleted supernatant and $150{,}000\times$ g centrifugation pelleted nanovesicles ($\sim$160 nm modal size), enriched for exosome markers Alix and CD63. This method yields enriched syncytiotrophoblast exosome and microvesicle preparations from placental perfusate which will aid studies into their role in normal and pathological pregnancies.

Sarah Jones discussed the use of *ex vivo* placental perfusion to assess fetoplacental vascular function in normal and fetal growth restricted pregnancies. Elevated resistance and pulsatility indices (RI and PI) in the umbilical arterial circulation measured clinically by Doppler waveform velocimetry are indicative of poor pregnancy

outcome; additionally elevated indices are assumed to represent increased resistance to flow in the placental vasculature. In normal pregnancy, PI and RI values measured 2–4 h prior to delivery displayed a strong positive correlation with fetal-side inflow hydrostatic pressure (FIHP) measured *ex vivo* during placental perfusion. Flow mediated vasodilatation (FMVD), observed with incremental fetal-side flow ramping, also correlated well with RI and PI values, demonstrating that placentas which exhibited the most effective FMVD responses *ex vivo* were those with the lowest resistance measurements *in vivo*. Using *ex vivo* placental perfusion to investigate vascular function in healthy versus fetal growth restricted pregnancies, revealed that vascular resistance in fetal growth restriction is increased and FMVD reduced. This vascular dysregulation in fetal growth restriction provides direct functional evidence for the fetoplacental circulation as an important locus for elevated resistance to flow. These data provide further supportive evidence that *ex vivo* dual placental perfusion is a valid and robust model for investigating vascular resistance in the fetoplacental circulation of healthy and diseased pregnancies.

Rohan Lewis shared data on the circulating amino acid milieu and placental amino acid transfer. Amino acid transport across the placenta provides essential substrates for fetal growth. However, it is possible that the transport of amino acids may be an important determinant of placental function in other ways. Building on observations made in the *ex vivo* perfused human placenta, it has been suggested that the maintenance of amino acid gradients within the placenta may be important drivers of placental uptake as well as transport to the fetus of both amino acids and a diverse range of other organic molecules.

3.3. Conclusions

This workshop highlighted the wide range of uses for *ex vivo* dual perfusion of human placental tissue, a technique which was originally designed for the study of transplacental transport. It was demonstrated that a variety of different aspects of placental biology may be studied using this technique and that due to the maintenance of the complex tissue structure study conditions are closer to the *in vivo* situation than in other *ex vivo* techniques.

Conflict of interest statement

None of the authors have any conflict of interest to declare.