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IFPA Meeting 2008 Workshops Report

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Abstract

Workshops are an important part of the IFPA annual meeting. At the IFPA meeting 2008 diverse topics were discussed in 12 themed workshops. Topics covered included: immunology of placentation; galectins and trophoblast invasion; signaling in implantation and invasion; markers to identify trophoblast subpopulations; placental pathology; placental toxicology; stereology; placental transport of fatty acids; placental mesenchymal stem cells; comparative placentation; trophoblast and neoplasia; trophoblast differentiation. This report is a summary of the various topics covered.

Keywords

Placenta; Trophoblast

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1. Introduction

The IFPA meeting 2008/12th EPG conference was held at Seggau Castle, Austria, 10–13th September 2008. One of the major aims of the IFPA meetings is to promote discussion and debate on a wide range of topics relating to the placenta. To this end 12 diverse 2-h long workshops were held. The following is a summary of the topics covered and discussions held.

2. Workshop 1: Immunology of placentation

Organizer: Surendra Sharma, Brown University, USA.

Speakers: B. Anne Croy, Queen's University, Canada; Lakshmi Krishnan, University of Ottawa, Canada; Andrea Kruse, University of Luebeck, Germany; Surendra Sharma, Brown University, USA.

2.1. Aim

It is increasingly clear that immunity, placenta and hormones play a pivotal role in orchestrating *in utero* embryonic development. Thus, the workshop explored the temporal and regulatory role of uterine immune cells and cytokines/chemokines at the maternal–fetal interface. The focus was on cutting edge research on immune–vascular choreography at the maternal–fetal interface.

2.2. Summary

It is becoming increasingly clear from both animal models and human studies that maternal immune cells play a role in maternal vascular changes associated with pregnancy. Much of the evidence for the role of uterine natural killer (uNK) cells in spiral artery remodeling in mice comes from the work done by Anne Croy's group. In this workshop data was presented from an intriguing set of observations on independent phenotypes of murine gestational blood pressure and spiral arterial modification. She has hypothesized that a temporal presence of lymphocytes during pregnancy contributes to the regulation of gestational mean arterial pressure (MAP) and to gestational age-dependent maternal and fetal tissue cross-talk. This is the first report demonstrating a link between MAP and gestational age-dependent kinetics of uNK cells. The study used cutting edge technology combining radio-telemetry with different well-established murine phenotypes. Fully implantable, miniature radio-telemetry probes were used to record time-phased hemodynamic data in wild type, alymphoid (NK-, T-, B-), normoglycemic and non-obese diabetic (NOD) and immunodeficient (T-, B-) NOD-scid mice. A novel, multiple stage pattern of mouse gestational MAP starting from pre-implantation stage through delivery was presented that correlated with stages of placental development. In NOD-scid mice gestational MAP and timing of spiral artery modification did not overlap. These findings may prove valuable for characterizing immunoregulatory effects on MAP in human pregnancy.

Various factors have the ability to influence uNK cell growth and functional properties. Andrea Kruse presented data suggesting that murine uterine dendritic cells and their cytokine products, IL-12 and IL-15 can alter uNK cell growth and functional properties. Defective recruitment of dendritic cells to the pregnant uterus in mice that are deficient in vascular addressins such as MAdCAM-1, P-selectin and ICAM-1 results in uNK cell anomalies. Confocal microscopic studies confirmed direct interactions between uterine dendritic cells and uNK cells. In mice with limited access to dendritic cells, uNK cells exhibited decreased frequency, size and functional activity. However, administration of IL-15 and IL-12 could overcome these uNK cell defects. In addition, uterine dendritic cells have been shown to be a major source of these cytokines.

Surendra Sharma described data on immune–vascular coupling leading to non-cytotoxic phenotype of $CD56^{bright}CD16^{-}$ uNK cells. Despite being replete with cytotoxic machinery, uNK cells remain immune tolerant at the maternal–fetal interface. However, the mechanisms that facilitate the non-cytotoxic phenotype of uNK cells remain poorly understood. In primary and cloned human peripheral blood NK and uNK cells, uNK cell-produced VEGF-C appears to be the regulatory factor that modified target endothelial and trophoblast cells to become resistant to uNK cell-mediated killing. Cytoprotection by VEGF-C is related to induction of "transporter associated with antigen processing (TAP)-1" expression and MHC class I assembly in target cells. siRNA-mediated silencing of TAP-1 expression abolished VEGF-C-imparted protection. In addition, in mice uNK cells can be altered in response to inflammatory triggers. These observations should help us understand how uNK cells induce tolerance to the fetal tissue, with implications for the role of not only uNK cells but also peripheral blood NK cells in adverse pregnancy outcomes, tumor surveillance, autoimmunity and immunity against infections.

Lakshmi Krishnan described data on the role of *Salmonella typhimurium* (ST) virulence in modulating inflammation and placental immunopathology. Intrauterine infections have garnered a great deal of attention because of their role in programming of adverse pregnancy outcomes and maternal health. Mouse strains that are otherwise resistant to ST acute infections rapidly succumb to the infectious agent during pregnancy. This phenomenon of pregnancy-associated infection is associated with a 1000-fold increase in systemic bacterial burden relative to non-pregnant control mice. Heavy bacterial burden in the placenta was found to be associated with cytokine storm controlled by inflammatory cytokines such as G-CSF, INF-gamma, TNF and chemokines. This also appeared to result in massive infiltration of polymorphonuclear lymphocyte lineage cells in the labyrinthine trophoblast tissue. In addition, an avirulent *aroA* ST mutant did not induce fetal loss or maternal illness, despite massive placental infection. These data could provide important insights into the pathogenesis and management of infection-induced adverse pregnancy conditions.

This workshop highlighted the potential importance of immune cells in vascular development and cross-talk in early pregnancy. Although mouse models were used for many of the studies, data presented are likely to provide important insights into the immunology of human placentation and intrauterine infections.

3. Workshop 2: Galectins, chemokines and trophoblast invasion

Organizers and Speakers: Harvey Kliman, Yale University, USA; Hamutal Meiri, Diagnostic Technologies Ltd., Yoknean, Israel; Marei Samar, Ort Brauda College and Diagnostic Technologies Ltd., Yoknean, Israel.

3.1. Aim

To explore the regulatory role that galectins and chemokines play in trophoblast invasion and maternal vascular conversion in the human placental bed. By obtaining a detailed understanding of molecular structure, function and placental differentiation pathways we may be able to determine the specific pathologic consequences of abnormalities in these processes.

3.2. Summary

Marei Samar gave a general overview of galectin biology. The galectin family proteins contain one or more carbohydrate recognition domains (CRDs) with affinity to beta-galactosides. Galectins can be shuttled between the nucleus and sub-cellular compartments, and are capable of being secreted by non-classical pathways from the cytosol. They can modulate cell–cell and cell–extracellular matrix adhesion, cell growth and differentiation, apoptosis, and immune

expression as well as intracellular functions in regulating metabolism, signaling and RNA processing. Under physiological conditions, galectins can be found as monomers, homodimers or multimers which dictates their ability to cross-link carbohydrate residues of glycoproteins, glycolipids, glycoconjugates and mucins. Galectins 1, 3, 9, 13 and 15 have been identified in the placenta where they have diverse functions. Galectin-13, also called placental protein 13 (PP13), is uniquely expressed in placental syncytiotrophoblast. Polymorphism in the PP13 LGALAS13 DNA has been found in a South African black and colored pregnant women cohort to include the A-to-G replacement at intron position −98 (−98C) and exon 3 deletion at position 222 (222delT). A significant association was also found between the 222delT locus and early onset pre-eclampsia (<34 weeks). PP13 molecular mutants have been cloned, expressed and purified. The purified molecular mutants have been used to raise antibodies that are currently being used as probes for the identification of potential PP13 molecular mutants in the placenta and body fluids.

Harvey Kliman discussed more specifically the role of PP13 in trophoblast biology. When invasive trophoblast fail to invade and convert maternal spiral arterioles during human pregnancy, decreased maternal perfusion of the placenta results. It has been proposed that this decreased perfusion triggers placental-induced maternal hypertension, and in severe cases, leads to second- or third-trimester pre-eclampsia. Many studies have focused on markers of endothelial damage, platelet activation, vasoconstriction, end-organ ischemia and oxidative stress, but these observations have not elucidated the first-trimester pathogenesis of the disease. It has previously been demonstrated that women who are likely to develop pre-eclampsia have low levels of serum PP13 between 6 and 13 weeks of gestation. However, the connection between decreased first-trimester PP13 and third-trimester pre-eclampsia has not yet been established. It has been hypothesized that PP13 plays a role in early trophoblast conversion of maternal spiral arterioles. Interestingly, syncytiotrophoblast-derived PP13 is secreted into the intervillous space, diffuses out of the decidual veins and precipitates, creating a PP13 diversion to lure maternal immune cells away from the maternal spiral arterioles. This decoy function may be the mechanism that allows invasive trophoblast to convert the decidual arterioles without hindrance from maternal immune surveillance. Thus, this work may both explain why women with low first-trimester PP13 levels go on to develop pre-eclampsia and how PP13, acting as a galectin, may facilitate trophoblast invasion and conversion of the maternal spiral arterioles in normal pregnancy.

Hamutal Meiri discussed the clinical utility of PP13 in identifying women at risk for pre-eclampsia. The efficacy of PP13 analysis has been intensively examined in clinical studies. The results of seven studies with >350 patients who subsequently developed pre-eclampsia compared to 5000 controls have indicated that maternal blood PP13 levels in women with pre-eclampsia are significantly lower during the first trimester of pregnancy, reach normal levels during the second trimester and become higher than normal toward the clinical presentation of the disease. Receiver operating characteristic (ROC) analysis has shown an 80–85% sensitivity for 15% false positive rate for a single first-trimester test. The false positive rate decreased to 6% when the slope of increase from the first to the second trimester was combined with the first trimester level. These very early changes emphasize placental signaling for the underlying pre-eclampsia pathology, presumably associated with impaired release of PP13 from the differentiating syncytiotrophoblast or genetic polymorphism associated with primary defects in protein structure. The later increase is most likely associated with placental deterioration and the release of syncytiotrophoblast microparticles carrying an abundance of the protein amount into the maternal blood. The benefit of early testing for pre-eclampsia includes improved antenatal and perinatal care; enabling early surveillance to avoid patient neglect, the development of a risk/care strategy; adjusting patient lifestyle to the anticipated risk; encouraging frequent visits to optimize-timed delivery; patient eligibility to enroll in clinical trials to evaluate various therapies; and enabling the development of new therapeutics. In

addition, experiments were discussed whereby placenta explants have been cultured to assess the therapeutic impact on PP13 release of drugs widely used in prevention, stabilization and management of pre-eclampsia. Explants are immortalized by cryoprotection to enable utilization by remote centers with preliminary verification studies being performed with an overseas partner to compare the impact of $MgSO_4$ and oxygen levels.

PP13 may prove to be an efficient marker for early detection of women at risk of developing pre-eclampsia. However, much more needs to be understood of its role in early pregnancy development.

4. Workshop 3: Signaling in implantation and invasion

Organizers: Asgi Fazleabas, University of Illinois, Chicago, USA; Claire Roberts, University of Adelaide, Australia.

Speakers: Asgi Fazleabas, University of Illinois, Chicago, USA; Guiying Nie, Prince Henry's Institute for Medical Research, Melbourne, Australia; Claire Roberts, University of Adelaide, Australia; Melissa Westwood, University of Manchester, UK.

4.1. Aim

To present current concepts on the mechanisms associated with embryo–maternal interactions and the establishment of pregnancy. The focus was on the role of the embryo in modulating its environment during the establishment of pregnancy and the clinical implications of these processes.

4.2. Summary

Asgi Fazleabas introduced the concept of endometrial receptivity which was first established in rodents and then extended to other species. Although estrogen and progesterone have long been believed to be essential for developing an appropriate endometrial environment for blastocyst implantation, it is now evident that peptide hormones, such as chorionic gonadotropin (CG), and growth factors secreted by a variety of cell types within the uterine endometrium further modulate these effects. In a non-human primate model, the baboon, studies have demonstrated a direct role for CG in the uterine endometrium. In preparation for the implanting blastocyst, the endometrium becomes increasingly vascular, inducing α-smooth muscle actin (α-SMA) in stromal fibroblasts. In addition, endometrial glands respond to CG by displaying an enhanced secretory activity and a plaque response develops in the luminal epithelium. CG also alters endometrial morphology and gene expression in preparation for implantation. In addition to the quality of the embryo, inhibition of stromal cell apoptosis by CG and subsequent cell differentiation into fully differentiated decidual cells are critical for successful implantation. The decidualization process is important for control of endometrial vascularization and involves vasodilation and angiogenesis in the endometrium. During this process endometrial stromal fibroblasts transform morphologically and biochemically into polygonal, secretory cells and begin to express specific decidual proteins such as prolactin and insulin-like growth factor binding protein-1 (IGFBP-1). Although the morphological changes are useful predictors of successful implantation, the molecular mechanisms underlying them are largely unknown and remain to be elucidated. Infusion of CG into the uterine cavity of cycling baboons, in a manner which mimics normal blastocyst transit, induces the induction of α-SMA in stromal fibroblasts. Disruption of α-SMA leads to apoptosis and a decrease in Notch1 and work from his laboratory has shown that treatment with CG rescues these cells from apoptosis and induces Notch1 expression pointing toward the critical role of both CG and Notch1 processes in implantation.

Guiying Nie has previously demonstrated that proprotein convertase 5/6 (PC6), a member of the proprotein convertase (PC) family, is a critical endometrial factor for implantation. PC6 is up-regulated in the endometrium specifically at the time of implantation in association with epithelial differentiation (in human and monkey) and stromal cell decidualization (in the mouse, human and monkey). PCs convert a range of precursor proteins into their bioactive forms; they are thus regarded as critical "master switch" molecules. It has been hypothesized that PC6 exerts its critical roles in the endometrium by regulating a cohort of proteins of diverse functions essential for implantation. Recent work has focused on the role of PC6 in human endometrial epithelial cells in preparation for implantation. PC6 expression is dysregulated in endometrial epithelium of infertile women, and knockdown of PC6 production in human endometrial epithelial cells significantly reduces the capacity of these cells to adhere to blastocysts in an *in vitro* model. These data strongly suggest that PC6 signaling in endometrial epithelium is critical for establishing endometrial receptivity for implantation. Proteomics technology is being used to identify the exact pathways of PC6 signaling in the endometrium. Two clinical implications of this research have been proposed: (1) PC6 may provide a biomarker for endometrial fertility/infertility (2) Because PC6 is also involved in HIV infection, PC6 could possibly be targeted to stop embryo implantation and reduce HIV infection at the same time in women, with the ultimate goal of developing dual-role female contraceptives.

Pregnancy success is determined by a complex interaction of a number of factors. The quality of implantation and placentation impacts significantly on growth and function of the placenta and hence differentiation and growth of the embryo and fetus. The embryo and placenta are genetically identical and comprise a unique combination of the maternal and paternal genomes. Indeed, pregnancy outcome may be influenced by paternity which is a known risk factor for pregnancy complications including miscarriage and pre-eclampsia. Impaired placental trophoblast invasion of the uterus and its vasculature and utero-placental insufficiency have been implicated in these and other pregnancy complications. Insulin-like growth factor (IGF)-II has been shown to promote placental trophoblast invasion *in vitro* and placental transport function. Up until recently it was thought that IGF-II acts in an autocrine paracrine manner but work in Claire Roberts' laboratory has shown that maternal circulating IGF-II deficiency associated with undernutrition or elevated plasma IGF-II following infusion in early to mid pregnancy are associated with poor or enhanced placental differentiation and function, respectively, and are also correlated with fetal weight. IGF-II has been shown to promote placental trophoblast invasion via a complex interaction with the type 2 IGF receptor (IGF2R), urokinase plasminogen activator and plasminogen which is enhanced by low oxygen tension. IGF-I does not however act in the same way. Specific actions of IGF-II following binding to IGF2R warrant further investigation to determine whether a signal transduction cascade ensues or if some other pathway is activated.

The maternal environment has profound effects on placental and hence fetal development and pregnancy outcome. Consequently, supplementing maternal growth factors has been suggested as a potential treatment for pregnancy complications such as fetal growth restriction. Work in Melissa Westwood's laboratory has utilized an explant model of human first-trimester placenta to show that maternal IGF can activate PI-3K/Akt and MAPK signaling pathways to influence proliferation and survival of cytotrophoblast in the epithelial bilayer of the placenta. If maternal growth factors are to influence cytotrophoblast kinetics from the syncytial surface, pathways that transduce signals from syncytium to cytotrophoblast must exist. This might be achieved either by a syncytioplasmic kinase relay which is activated by ligand binding to receptors on the microvillus membrane or by trancytosis of IGF with exocytosis at the basal syncytial surface in order to activate receptors on underlying cytotrophoblast. Understanding how maternal growth signals to the placenta are mediated is important as the expression/activation

of many signaling molecules is abnormal in fetal growth restriction and, therefore, simply elevating maternal growth factors may not result in enhanced placental and fetal growth.

This workshop highlighted advances being made toward understanding some of the key factors in the regulation of endometrial receptivity and early implantation. These are complex, highly regulated processes and deeper understanding of the factors involved could lead to important clinical implications for early pregnancy success.

5. Workshop 4: Trophoblast: markers to identify subpopulations

Organizers: Graham Burton, University of Cambridge, UK; Larry Chamley, University of Auckland, New Zealand.

Speakers: Martin Bilban, Medical University of Vienna, Austria; Graham Burton, University of Cambridge, UK; Sascha Drewlo, Samuel Lunefeld Research Institute, Toronto, Canada; Daniele Evain-Brion, INSERM U 767, Paris, France; Joanna James, St. George's Hospital, London, UK; Ashley Moffett, University of Cambridge, UK; Padma Murthi, Royal Women's Hospital, Melbourne, Australia.

5.1. Aim

To determine whether there are morphological, immunohistochemical or molecular markers that can be used to characterise subpopulations of human cytotrophoblast cells. The workshop addressed the question 'What do we know about trophoblast subpopulations in the human, and how can they be identified?'

5.2. Summary

Graham Burton introduced the various trophoblast subpopulations that can be recognized on a morphological basis. Villous cytotrophoblast cells are heterogenous in appearance depending on their state of differentiation. Those cytotrophoblast displaying large round nuclei, which in the past have been considered 'resting' cells, often show evidence of necrotic or apopnecrotic changes at the ultrastructural level. The cells of the cytotrophoblast columns form a distinct subpopulation characterised by their accumulation of glycogen, which is often eluted during routine fixation. These cells spread laterally to form the cytotrophoblast shell at the materno-fetal interface and are continuous with the endovascular extravillous trophoblast (EVT) where spiral arteries come into contact with the cytotrophoblast shell. By contrast, interstitial EVT differentiate from the maternal surface of the shell, where they can be identified by their pleiomorphic shape and intense staining characteristics.

Ashley Moffett discussed the immunological markers of trophoblast cells, which are known to express an unusual repertoire of human leukocyte antigen (HLA) molecules. Close homology between, and extreme polymorphism at, the classical HLA class-I (HLA-I) loci has made it difficult to generate locus-specific monoclonal antibodies (mAbs). However, using commercially available single HLA-I antigen beads the reactivity of a panel of mAb to 96 common HLA-I allotypes has been characterised, and used to analyse HLA expression of normal trophoblast; choriocarcinoma cells JEG-3 and JAR; and the placental cell lines HTR-8/SVneo, Swan-71 and TEV-1. Villous trophoblast is HLA-null, whilst EVT cells express HLA-C, HLA-G and HLA-E, but not HLA-A, HLA-B or HLA-DR. Moreover, JEG-3 and JAR cells do reflect extravillous and villous trophoblast HLA phenotypes, respectively. By contrast, HLA antigens detected in the placental cell lines were not representative of either of the *in vivo* trophoblast phenotypes.

Padma Murthi discussed the expression pattern of a homeobox transcription factor gene (HLX) in different trophoblast subpopulations. In early human pregnancy, HLX is localised to villous

cytotrophoblast nuclei and to the extravillous cytotrophoblast nuclei in the proximal regions of cell columns. In first-trimester placental bed biopsies, HLX was not detected in the nuclei but was instead found only in the cytoplasm of interstitial EVT. HLX was not detected in syncytiotrophoblast at any stage of placental development but was detected in the nuclei of residual villous cytotrophoblast cells in term placenta.

Martin Bilban considered the value of microarray technologies to distinguish between trophoblast subpopulations on the basis of their mRNA transcript profiles. Due to the limited access to primary trophoblast cells, cell lines are often used as model systems. Although these cell lines share some marker expression with their primary counterparts, it is unknown to what extent trophoblast cell lines recapture the invasive phenotype of extravillous trophoblast. Affymetrix U133A GeneChip analysis of >20,000 genes was performed on the widely used trophoblast-like cell lines SGHPL-5, HTR-8/SVneo, BeWo, JEG-3, the novel cell line ACH3P, EVT and villous cytotrophoblast cells. Hierarchical clustering and principal component analysis showed that (1) large differences exist between primary trophoblast (both villous and extravillous) and trophoblast cell lines, (2) BeWo, JEG-3 and ACH3P differ from immortalized HTR8/SVneo and SGHPL-5 and (3) among the cell lines investigated, SGHPL-5 resemble most closely the EVT signature.

Daniele Evain-Brion discussed endocrine markers of trophoblast, illustrating by *in situ* immunohistochemistry that cultured primary invasive EVT, as well as syncytiotrophoblast, produce hPL, placental GH, leptin and PAPP-A. hCG appears to be a potent endocrine marker to differentiate the various trophoblast subpopulations. Indeed hCG is immunodetected not only in villous trophoblast but also in EVT all along their invasive pathway, except in the proliferative zone at the proximal end of the cytotrophoblast column. However, the forms of hCG produced by invasive cells differ in their gycosylation state and bioactivity. Most importantly, invasive EVT and JEG3 choriocarcinoma cells produce hyper-glycosylated hCG (H hCG) that stimulates trophoblast invasion, while the syncytiotrophoblast does not.

Sascha Drewlo reported on the differences in trophoblast subpopulations between normal and pathological placentae. During placental development different trophoblast subpopulations have different proliferative capacities. Glial cell missing-1 (Gcm1) was shown to have a direct impact on the differentiation of cytotrophoblast cells in mice. GCM1 additionally influences the proliferation of these cells in the human placenta by balancing cell division and terminal differentiation. Stereological and molecular investigations showed that in severe IUGR and pre-eclampsia GCM1 and proliferation are altered in opposite directions, suggesting an early loss of proliferative cytotrophoblast cells in severe IUGR.

Joanna James questioned the longstanding dogma that the villous cytotrophoblast is a single bipotent population and proposed that two populations of villous cytotrophoblast exist as early as 8 weeks of gestation; (1) monolayer villous cytotrophoblasts committed to syncytiotrophoblast differentiation and (2) EVT progenitors located in the mulitilayered cell islands in villous tips committed to EVT differentiation. The basis for this is that monolayer villous cytotrophoblast and cells of the mesenchymal core are no longer viable after 48 h in an explant model, whereas cytotrophoblast in the multilayered cell islands of villous tips remains viable and able to produce new EVT outgrowths for up to 3 weeks in culture, but do not regenerate the syncytiotrophoblast. These EVT progenitors could be isolated from 10-day-old explants by sequential trypsin digestion, resulting in a 95% pure trophoblast population that proliferated in culture and did not syncytialise. 20% of the EVT progenitors differentiated into EVT over 4 days in culture as shown by immunohistochemistry for HLA-G, MHC-I and the EVT specific antibody Bo1D11, and EVT progenitors expressed CD9 and the unique marker alphavbeta6 integrin. Contrary to previous reports, FGF-4 did not have any effect on EVT outgrowth or trophoblast differentiation from first-trimester villous explants.

Larry Chamley summarised the workshop by concluding that molecular techniques are beginning to illustrate the differences between trophoblast subpopulations, and between primary cytotrophoblast cells and experimental cell lines. It was emphasized that as yet there is no perfect model for cytotrophoblast cells, but that researchers must select the most appropriate for their needs and be careful when extrapolating to the *in vivo* situation.

6. Workshop 5: Clinical correlates of placental pathologies

Organizers: Sarah Keating, University of Toronto, Canada; Mark Turner, University of Liverpool, UK.

Speakers: Sabine Dekan, Medical University of Vienna, Austria; Jan Jaap Erwich, University of Groningen, Netherlands; Victoria Geenes, Imperial College London, United Kingdom; Fusun Gundogan, Brown University, USA; Sarah Keating, University of Toronto, Canada; Alveen O'Malley, Trinity College Medical School, Ireland; Meiri Robertson, The Canberra Hospital, Australia.

6.1. Aim

To present "state of the art" clinical interpretation of placental pathology by illustrating how placental histopathology contributes to: (a) identifying novel disease processes; (b) improving our understanding of clinical problems; (c) synergies with other methods.

6.2. Summary

Sarah Keating gave an overview of placental histopathology describing current histopathologic grading systems that are used for amniotic infection syndrome and maternal and fetal vascular underperfusion. The fact that interobserver variability between perinatal pathologists is often suboptimal was also discussed. The rest of the presentations in the workshop were taken from submitted abstracts to the meeting and as such are only briefly summarised here. Jan Jaap Erwich presented data from a study of placental pathology in 750 singleton intrauterine deaths at >20 weeks using the TULIP classification. Placental subcategories were defined as placental bed pathology: inadequate spiral artery remodeling and/or spiral artery pathology leading to utero-placental vascular insufficiency; developmental pathology: morphologic abnormalities that arise because of abnormal developmental processes; parenchyma: acquired placenta parenchyma disorders of the villi or intervillous space; abnormal localisation, umbilical cord complication or not otherwise specified multiple placental causes. Placental causes accounted for 65% of intrauterine fetal deaths. A need was identified for clearer definitions of pathologic entities including further subclassifications. Fusun Gundogan discussed a study which had investigated placental pathology at the maternal–fetal interface in *in vitro* fertilisation (IVF) pregnancies via egg donation (ED–IVF) and non-egg donation. In ED–IVF placentae there were increased syncytial knots, increased fibrin/fibrinoid deposition and increased numbers of immune cells. Taken together, ED–IVF placentae were associated with potentially immune regulated pathologies such as chronic deciduitis more often than non-egg donation IVF.

Meiri Robertson presented a case report of a woman with two consecutive second-trimester losses with a previous history of chronic histiocytic intervillositis. The pathophysiology of the rare entity chronic histiocytic intervillositis (CHIV) was discussed. Chronic intervillositis is a rare placental lesion characterised by lymphohistiocytic intervillous infiltration and fibrin deposition. The possibility of early diagnosis using chorionic villous biopsy and amelioration of pregnancy outcome following CHIV by administration of aspirin and corticosteroids was also discussed. Alveen O'Malley presented data from a stereological study of placentae from women with a range thrombophilias. Stereological parameters investigated were: star volume, surface area measurement of terminal villi and their capillaries, and syncytial knot counts.

Significant differences in stereological parameters were found, some of which were specific to inherited vs. acquired thrombophilias. Placental morphology was abnormal despite apparently normal pregnancy outcomes. Sabine Dekan presented data from a study correlating prenatal MRI placental assessment with post-natal/postabortion pathological and histopathological findings. Abnormalities observed by MRI predominantly reflected thrombus and infarction as determined by pathological assessment. The study concluded that MRI may provide useful information about the placenta in cases with oligohydramnios or maternal obesity. Victoria Geenes presented a study on the *in vitro* effects of bile acid on human placenta morphology. Intrahepatic cholestasis of pregnancy is associated with increased maternal serum bile acids and increased risks of fetal complications. In a placental explant model a bile acid incriminated in the aetiology of obstetric cholestasis increased the rate of abnormal findings on histological assessment. In addition, these abnormalities could be abrogated by exposure to ursodeoxycholic acid, a common treatment for women with intrahepatic cholestasis of pregnancy.

The workshop demonstrated the variety of uses for placental histopathological assessment. The consensus of the workshop was that in order to develop widely used and clinically relevant pathologic classification systems our understanding of pathogenesis of many conditions needs to become better developed and that computer-assisted image analysis may contribute to this process. Pathologic assessment of the placenta remains “the gold standard” and is essential following high-risk pregnancies.

7. Workshop 6: Toxicology testing using the human placenta for evaluation of human health risk

Organizers: Richard K. Miller, University of Rochester, USA; Kathleen Shiverick, University of Florida, USA.

Speakers: Alessia Giovannelli, University of Sienna, Italy; Antoine Malek, Zurich University Hospital, Switzerland; Richard K. Miller, University of Rochester, USA; Kathleen Shiverick, University of Florida, USA; Angela Woodall-Gray, University of Rochester, USA.

7.1. Aim

To examine agents which produce developmental and reproductive toxicities in humans and may impact the placenta. In addition, how experimental models can be used to determine such toxicity in the placenta will be explored.

7.2. Summary

The workshop concentrated on three aspects of reproductive and developmental toxicology, which directly involve the placenta and have only recently become issues for human health.

Nanoparticles—Nanoparticles (NPs) are defined as particles less than 100 nm in diameter. Their composition varies widely, with characteristics of NPs not only dependent upon size and shape but also the materials of the inner core, charge and surface coating. NPs are found in the environment (e.g. burning of fossil fuels), in manufacturing (e.g. coatings for clothing, metals and electronics) and in medicine (e.g. nanomedicine for therapy and diagnosis). Inhalation of NPs, e.g. gold, can result in their appearance in the body, e.g. brain, blood, liver. There is limited published information concerning the distribution to the feto-placental unit or effects upon the fetus due to maternal exposures. Richard Miller reviewed reports of 5 nm gold NPs and Quantum Dots in the developing embryos of mouse and rat. Passage of NPs across the human placenta may be dependent upon the surface coating of the NPs, e.g. albumin vs. IgG, will either reduce or facilitate the movement of NPs across the human placenta because

maternal albumin is catabolized and the IgG is transferred into the fetus via IgG receptors. 5 nm gold NPs coated with serum albumin were not transferred into the fetal circulation of the perfused human placenta *in vitro* after 12 h of maternal exposure. In addition, since the workshop, it has been reported that 10–30 nm gold PEGylated NPs are also not transferred within 6 h following human placental perfusion. Antoine Malek described a current series of studies where polystyrene particles ranging from 50 to 240 nm could be detected in the fetal circulation of the perfused human placenta, while 500 nm particles were not. Questions arose regarding the nature of polystyrene particles and the importance of the surface bound FITC protein as well as charge; however, the contrast between the passage of the 50–240 nm polystyrene FITC particles and the absence of passage of gold 5–0 nm NPs left those in attendance with many questions unresolved. However, the polystyrene itself was not studied but rather the marker – FITC positive staining originally associated with polystyrene. Given that viruses, e.g. HIV, CMV, Cocksackie B and Echo 11, do not transit the human placenta under *in vitro* perfusion conditions and are much smaller than 240 nm, the identification of FITC staining in the fetal circulation may not reflect the presence of polystrene particles. In contrast, the studies with gold NPs coated with human albumin or PEGylated, gold did appear in the placenta (trophoblast), but did not appear in the fetal endothelium or fetal circulation even after 12 h of perfusion as demonstrated by electron microscopy. It was concluded that more detailed investigations were required to resolve these divergent reports.

This field of nanotechnology is just at its birth concerning reproductive and developmental potential, and many more insightful investigations will be required to understand the toxicokinetics and toxicity of extensive variety of NPs.

Phthalates—Phthalates (PHT) are a family of plasticizers and their metabolites, which have been associated with birth defects in humans based upon monitoring of PHT/metabolite levels in the mothers. Pregnancy loss and birth defects have been observed in animal studies following treatment with PHT/metabolites. Angela Woodall-Gray and Alessia Giovannelli presented complementary studies suggesting the possibility that different PHT metabolites were associated with increased miscarriage rates in women. Angela Woodall-Gray reported a dose related increase of both villous cytotrophoblast and EVT apoptosis in early human placental explants following *in vitro* culture in mono-butyl phthalate. Alessia Giovannelli reported a relative risk of 1.58-fold for women with miscarriage compared to controls based upon di(2-ethylhexyl) phthalate (DEHP) and mono-ethylhexyl phthalate (MEHP) serum levels. There was a substantial discussion about the importance of these clinical associations and need for reductions in such human health exposures to phthalates. Questions arose because the PHTs and metabolites reflect different types of commercial usage, e.g. dibutyl phthalate (DBP) is widely used in women's cosmetics, creams, nail polishes, while DEHP is more widely used in children's toys, medical devices and food packaging. All women do have detectable levels of each of these PHTs and their metabolites. The fascinating aspect of the clinical association with miscarriage and DEHP and MEHP is that these PHTs and their metabolites do not bioaccumulate and have half-lives in the range of 3–6 h. Thus, it would appear that humans are chronically exposed to these plasticizers and even though the best estimate of exposure is monitoring blood or urine levels of PHTs/metabolities on a regular basis, the capture of the analytical specimen at the time of diagnosis of pregnancy loss reflects recent exposure. Collection of multiple specimens from a population of women in early pregnancy would be required to validate the continuing exposures, which are assumed.

Therefore, based upon this clinical association between PHTs serum levels and miscarriage rates in human and the dose related increases in apoptosis in early human placental explants exposed to MBP, that growing concerns should be focused not only on birth defects but also on reproductive loss for phthalates.

Dental infections: smoking is a major risk factor for oral periodontal infections. Periodontal disease and its associated bacteria are recognized to be involved with systemic conditions such as cardiovascular disease. Kathleen Shiverick reviewed the literature supporting a link between periodontal disease and adverse pregnancy outcomes. Recent evidence suggests that oral bacteria could impact pregnancy following oral-hematogenous spread to gestational tissues and human epidemiological studies support a link between preterm birth and low birth weight with periodontal disease and its associated pathogens. In addition, animal studies have demonstrated colonization of maternal and fetal tissues by oral microorganisms, resulting in premature delivery and pregnancy loss. Evidence indicates that pathogenic mechanisms of circulating periodontal bacteria involve the ability to cross the placenta, as well as stimulating an inflammatory response in the mother. *In vitro* invasion of HTR-8/SVneo cells in culture by *Porphyromonas gingivalis*, an oral anaerobic bacterium, was demonstrated using immunofluorescence confocal microscopy. *P. gingivalis* invaded HTR-8/SVneo cells efficiently and in high numbers, in a pattern similar to gingival epithelial cells. Transcriptional profile analysis showed that *P. gingivalis* significantly regulated 380 genes in HTR-8/SVneo cells. Gene ontology pathway analysis further revealed that the host pathways most significantly impacted included the cell cycle, apoptosis, Wnt signaling and TGF-beta signaling pathways. In addition, exposure to *P. gingivalis* decreased long-term viability of the HTR-8/SVneo cells. Thus, the ability of *P. gingivalis* to invade trophoblast cells has relevance to the potential ability of the organism to colonize the placenta and to adversely impact on pregnancy outcomes.

Discussion focused on mechanisms of bacterial injury to cells, including microorganism-specific secreted LPS vs. invasion of host cells, and the role of toll-like receptors on host cells. The role of cigarette smoking as a risk factor for pregnancy complications was further discussed in terms of increasing the risk of entry of oral microorganisms into the blood, as well as direct effects on placental cells to increase susceptibility to infection by oral pathogens.

This workshop highlighted the use of placental perfusion and trophoblast cell line models in investigating the potential impact of environmental toxins and bacteria on placental function and potential exposure to the fetus.

8. Workshop 7: How to quantify placental structure using stereology?

Organizers and Speakers: Tahera Ansari, Imperial College London, UK; Terry Mayhew, University of Nottingham, UK.

8.1. Aim

To present a ‘hands-on’ practical class introducing basic principles for quantifying placental structures using stereological methods. The idea was to offer a simple introduction to stereological estimation with a strong focus on application. This was supported by considerations of sampling and relevant biomedical contexts (e.g. villous growth, angiogenesis, trophoblast turnover, diffusive transport in normal, complicated and experimental pregnancies).

8.2. Summary

The workshop was novel in that it was a hands-on practical class designed to introduce participants to basic principles for quantifying placental structures (human and murine) using stereological methods. Terry Mayhew began the workshop by introducing the principle of random tissue sampling and its importance for estimating relevant structural quantities with minimal bias and high precision. The basic principles of stereological estimation using tissue probes (sections, lines and points) were described together with some possibilities for

describing key processes (villous growth, angiogenesis, trophoblast turnover, diffusive transport) in normal, complicated and experimental pregnancies. Tahera Ansari followed with a prepared practical exercise which gave participants the opportunity to estimate quantities (villous and feto-placental capillary volumes) from microscopic images of human placenta using simple test grids and pocket calculators. Participants were able to familiarise themselves with procedures under the guidance of teachers and had opportunities to raise practical issues.

Immediately after the workshop, and up until the end of the meeting, participants provided extremely favourable feedback on the quality of instruction and value of the session. There was considerable interest in additional courses exploring other stereological possibilities. Both instructors are grateful to all the participants for their enthusiasm and feedback.

9. Workshop 8: Placental transport of fatty acids: mechanisms and clinical implications

Organizers: Gernot Desoye, Medical University of Graz, Austria; Thomas Jansson, University of Cincinnati, USA.

Speakers: Asim Duttaroy, University of Oslo, Norway; Irene Cetin, University of Milan, Italy; Susanne Lager, University of Gothenburg, Sweden; Yoel Sadovsky, University of Pittsburgh, USA.

9.1. Aim

To review current knowledge on mechanisms of placental fatty acid transport and discuss clinical implications of changes in these transport systems. The workshop focused on current controversies and clinical implications in order to identify critical basic and clinical research questions for future studies.

9.2. Summary

Obese and overweight pregnant women have elevated circulating levels of pro-inflammatory cytokines and free fatty acids and often give birth to larger babies. Susanne Lager presented preliminary data which suggests that incubation of isolated human trophoblast cells with elevated free fatty acids (400 μM oleic acid) results in an increase in IL-6 and IL-10 release and stimulated System A amino acid transporter activity. The uptake of fatty acids into cultured trophoblast cells increased after 24 h incubation in the presence of high concentrations of IL-6 (2–20 ng/ml). These data suggest that maternal hyperlipidemia and elevated IL-6 stimulate placental fatty acid and amino acid uptake. Overall, this will result in an increased supply of nutrients available for transfer to the fetus. Therefore, the metabolic environment of the obese pregnant woman stimulates both lipid and amino acid transport by the placenta, however, the cellular mechanisms responsible for the increased transport remain to be investigated. These findings may contribute to an understanding of the mechanisms underlying increased placental nutrient delivery and fetal overgrowth associated with high maternal BMI.

Lipids are essential as feto-placental fuel, building blocks and signals for organ development. Yoel Sadovsky presented data on lipid trafficking within trophoblast and demonstrated the central role of PPARγ in this process. The nuclear receptor PPARγ plays a pivotal role in placental development and function. PPARγ-null mouse embryos exhibit fetal growth restriction and subsequently fetal death, associated with diminished placental fat accumulation. PPARγ is expressed in human placental villi and plays a role in ligand-dependent modulation of trophoblast differentiation and attenuation of trophoblast apoptosis. In addition, ligand-activated PPARγ stimulates trophoblast uptake of fatty acids as well as expression of proteins that modulate placental accumulation and trafficking of fatty acids, including the lipid droplet-

associated protein adipophilin and fatty acid transport proteins. PPARγ may also play a role in placental adaptation to hypoxia-induced lipotoxicity.

Asim Duttaroy discussed transfer of long chain polyunsaturated fatty acids (LCPUFA) across the human placenta. Essential fatty acids and their LCPUFA derivatives are also critical for fetal growth and development. Although long chain fatty acids (LCFA) can enter the cell via passive diffusion, emerging reports indicate that LCFA uptake is tightly regulated by several plasma membrane-located transport/binding proteins such as fatty acid translocase (FAT/CD36), plasma membrane fatty acid binding protein (FABPpm), fatty acid transport protein (FATP) and intracellular FABPs in several tissues including human placenta. Fatty acid-activated transcription factors (PPARs, LXR, RXR, and SREBP-1) have been demonstrated to regulate these fatty acid transport/binding proteins, and placental functions. p-FABPpm, located exclusively on the maternal-facing membranes of the placenta, may be involved in the sequestration of maternal LCPUFAs. Preferential transfer of LCPUFA across BeWo cell monolayers has now been established and can be used for further understanding of placental transfer of fatty acids. However, further studies are required on p-FABPpm, which allows the preferential binding of LCPUFAs over non-essential fatty acids. Maternal fatty acids may regulate their own placental transport as well as placental function via several fatty acid-activated transcription factors. A focus for future studies will be to further establish the complex inter-relationships of the many multiple proteins influencing cellular LCFA uptake and retention by the placenta.

Irene Cetin presented data on arachidonic acid (AA) and docosahexaenoic acid (DHA) that have been reported to be significantly decreased in cord blood of neonates of women with type 1, type 2 or gestational diabetic (GDM) pregnancies. The degree of fatty acid abnormality is less and less pronounced with the reduction of pathologic severity. However, not all studies have reported similar results and both arterial and venous cord blood have been sampled in different reports. Therefore, in a recent study the percent of fatty acids in umbilical vein and arterial plasma from GDM pregnancies was evaluated in order to assess whether AA and DHA are reduced in fetuses of well controlled GDM and whether this is the result of impaired placental transfer or endogenous fetal metabolism. The results show a significant decrease of AA and DHA as well as of total $n-6$ and total $n-3$ in the umbilical artery but not in the umbilical vein of GDM fetuses. Therefore, it appears that altered handling or metabolism of LCPUFA by the fetus rather than impaired placental transfer seems to be responsible for the lower proportion of these fatty acids in plasma of neonates of GDM mothers.

This workshop highlighted the importance of fatty acid transport and metabolism by the placenta for fetal growth and development, and how alterations in these processes may contribute to pregnancy complications.

10. Workshop 9: Human placental mesenchymal stem cells

Organizers: Ingrid Lang, Medical University of Graz, Austria; Dirk Strunk, Medical University of Graz, Austria.

Speakers: Bill Kalionis, Melbourne University, Australia; Antoine Malek, University Hospital Zurich, Switzerland; Ornella Parolini, Fondazione Poliambulanza Instituto Ospedaliero, Brescia, Italy; Dirk Strunk, Medical University of Graz, Austria; Geok Chin Tan, National University of Malaysia, Kuala Lumpur, Malaysia; Wolfgang Wagner, University of Heidelberg, Germany.

10.1. Aim

To present an overview of mesenchymal stem cell (MSC) research, biology and function. Based on the critical discussion of current knowledge about MSC multi-potency compared to other stem cells, the workshop focused on placental and umbilical cord MSCs and a brief introduction into clinical scale MSC propagation under experimental as well as GMP defined conditions.

10.2. Summary

Wolfgang Wagner presented an overview of mesenchymal stem cells (MSCs) that represent a type of adult stem cell that can easily be isolated from various tissues and expanded *in vitro*. Past reports on their pluripotency and possible clinical applications have raised hopes and interest in MSCs. However, MSCs represent a heterogeneous mixture of cell types and the composition of subpopulations is affected by the initial steps of cell preparation. Furthermore, reliable molecular markers for multipotent cell fractions are still elusive. MSCs are usually culture expanded prior to their application and the effect of long-term *in vitro* culture is unclear. Studies have indicated that replicative senescence of MSC preparations is a continuous process starting from the first passage onwards. Within 43–77 days of cultivation (7–12 passages), MSCs demonstrated morphological abnormalities, enlargement, attenuated expression of specific surface markers and ultimately proliferation arrest. Adipogenic differentiation potential decreased whereas the propensity for osteogenic differentiation increased. mRNA expression profiling revealed a consistent pattern of alterations in the global gene expression signature of MSCs at different passages, these changes were not restricted to later passages, but were continuously acquired with increasing passages. Furthermore, microRNA expression profiling revealed an up-regulation of hsa-mir-371, hsa-mir-369-5P, hsa-mir-29c, hsa-mir-499 and hsa-let-7f upon *in vitro* propagation. It was concluded that more precise molecular and cellular markers to define subsets of MSCs and to standardize protocols for expansion of MSC are urgently needed.

Ornella Parolini presented a concise review on the characteristics and applications of placenta-derived stem cells. Given the ever-increasing evidence that cells derived from different regions of human term placenta display plasticity and immunomodulatory characteristics, discarding of this tissue after birth may soon be a thing of the past. Cells from the amniotic epithelial region express embryonic stem cell markers and differentiate *in vitro* toward lineages of all three germ layers. Cells from the amniotic and chorionic mesenchymal regions display phenotypic and functional characteristics similar to mesenchymal stromal cells of other origins, in particular differentiation toward mesodermal, ectodermal and endodermal lineages. Furthermore, *in vitro* studies show that amnion- and chorion-derived cells are non-immunogenic, with long-term engraftment observed after the xenotransplantation of these cells into different animal models. Pre-clinical studies show that cells from the amniotic epithelial region could aid in the functional restoration of neuronal, pancreatic and hepatic tissues and confer beneficial effects after transplantation into animal models of spinal cord injury, Parkinson's disease, middle cerebral artery occlusion and diabetes. At present, cells of the amniotic mesenchymal region have been shown to survive and differentiate into cardiomyocyte-like cells and improve cardiac function after transplantation into infarcted rat hearts.

Antoine Malek introduced an optimized method for the isolation and cultivation of multipotent placental stromal cells. Non-confluent culture is a critical parameter to maintain a 'stemness' profile during expansion. Geok Chin Tan reported that cultured human amniotic mesenchymal cells possess stem cell and angiogenic properties, which may imply their utility for angiogenic therapy and for the development of vascularized engineered tissues. Bill Kalionis and his group have identified homeobox genes, which are critical regulators of stem cell function, in villous

stroma of first-trimester placentae captured by laser microdissection and in cultured placental mesenchymal stem cells of term placenta.

Dirk Strunk introduced data from a clinical scale *ex vivo* expansion of human MSCs as a strict prerequisite for MSC therapy. A study was presented defining the minimum requirements for producing sufficient MSC numbers for therapeutic application in a completely animal serum-free manufacturing practice (GMP) standard system from small bone marrow aspiration volumes. Several application doses of MSCs without animal serum were produced in a standardized one-step procedure within 2 weeks supporting therapeutic approaches which depend on the fast and safe availability of sufficient MSC doses in the clinical setting. Future studies will be required to determine whether this technology can also be applied for the expansion of placenta-derived MSCs. Growing evidence suggests that therapeutic actions of placental cells result from immunomodulatory effects rather than differentiation of the cells themselves. In any case, further research is warranted to fully characterise the differentiation potential and immunomodulatory properties of these cells *in vivo*, to allow a full assessment of their potential applicability in different experimental and clinical settings.

This workshop highlighted the placenta as a potentially rich source of mesenchymal stem cells. However, much more work appears to be required to fully define these cells and their differentiation pathways.

11. Workshop 10: Different species with different placental solutions for fetal development and survival

Organizers: Vibeke Dantzer, University of Copenhagen, Denmark; Christiane Pfarrer, University of Veterinary Medicine Hannover, Germany.

Speakers: Douglas Antczak, Cornell University, New York, USA; Vibeke Dantzer, University of Copenhagen, Denmark; Amanda De Mestre, Royal Veterinary College, UK; Allen Enders, University of California, Davis, USA; Nina Hambruch, University of Veterinary Medicine Hannover, Germany; Gregory Johnson, Texas A&M University, USA; Maria Angelica Miglino, University of Sao Paulo, Brazil.

11.1. Aim

To present different placental concepts/strategies found in species economically or scientifically relevant to develop new ideas and strategies for animal reproduction.

11.2. Summary

Embryonic and fetal loss can occur at different times, although the highest frequency is prior to the full establishment of the placenta. In several species this loss, as well as perinatal mortality are of large economic relevance. In this workshop different experimental approaches that increase our knowledge of important placentation processes were discussed. Data from a broad range of placental types was presented to elucidate different events occurring in specifically sensitive phases of development. A large variation of species, their similarities and differences, were covered.

In the work of Carolyn Jones (University of Manchester, presented by Vibeke Dantzer), which is based on lectin histochemical studies, glycotypes at the feto-maternal interface were presented. This data demonstrated that the heavily glycosylated surfaces of an implanting blastocyst and receptive endometrium interact in a highly controlled and specific manner during implantation. In epitheliochorial placentation, this relationship is maintained throughout pregnancy, and closely related species, such as horse and donkey, pig and peccary have very

similar glycotypes/glycocodes at the feto-maternal interface. Recent results from interspecies hybrids between Old and New World camelids, the camel and llama, indicate that successful interbreeding requires the trophoblast to bear glycans that are complementary to those of the maternal host, whereas hybrid embryos showing incompatible glycotypes may fail to implant. The glycocode thus appears fundamental to successful implantation, maintenance of pregnancy and the development of new species.

Douglas Antczak discussed the similarities between the invasive, minor part, of the equine placenta to more invasive placental types of primates and rodents. Although the horse has an epitheliochorial placenta there are two clinically important aspects of early placental development that are unusual compared to other species. Up to the fourth week of gestation, the spherical equine conceptus moves freely. Between days 25 and 40, the invasive trophoblast cells accumulate at the chorionic girdle and then migrate and invade into the endometrium from day 36 to form the endometrial cups. These invasive equine trophoblast cells differentiate and secrete equine chorionic gonadotrophin (eCG) and express high levels of polymorphic major histocompatibility complex (MHC) class I molecules, which are different from the non-polymorphic MHC class I molecules expressed in human EVT. These parallel evolutionary paths in species as divergent as humans and horses may emphasize the importance of certain aspects of placental physiology that promote fetal well-being. In addition, Amanda De Mestre showed that equine trophoblast chorionic girdle cells may be used as a novel approach to cellular therapy. Ectopical transplantation of trophoblast cells from day 33 to 34 equine conceptuses into the vulva of non-pregnant mares lead to secretion of eCG thereby modulating the reproductive physiology and behaviour of the recipients. The lifespan of invasive trophoblast cells in non-pregnant recipients (42–105 days) was similar to that of these cells during pregnancy (44–84 days). Furthermore, oestrous heat behaviour was suppressed for up to 3 months in invasive trophoblast recipients and had profound effects on ovarian, uterine, hormonal and behavioural physiology. The ability of transplanted trophoblast cells to resist immune destruction, together with their secretory capacity, suggests transplanted trophoblast cells may be a viable approach to allogeneic cellular therapy.

Gregory Johnson demonstrated, by a number of methods, that the lysophingolipid sphingosine-1-phosphate (S1P) is a potent stimulator of angiogenesis, hypothesizing that this important S1P pathway is activated in order to stimulate endometrial angiogenesis during ovine pregnancy. *In vivo* (days 40–120) and *in vitro* studies provide strong support for roles of S1P, S1P receptors and S1P-regulated genes in angiogenesis, specifically for endothelial cell invasion and outgrowth. Collectively, these data indicate that the S1P signaling pathway is integral to mechanisms for mediating uterine angiogenesis necessary to support hematotrophic exchange of nutrients for fetal–placental development during pregnancy.

The definitive placenta of the hyena differs from that of other carnivores (e.g. mustelids) in being labyrinthine hemochorial rather than endotheliochorial. It is similar in being zonary and having hemophagous regions. Allen Enders demonstrated how primary fetal villi, from the earliest available hyena placental stages (days 25–27), with mesenchymal cores extend into the endometrial gland openings with subsequently initial invasion of the endometrium in a similar fashion as seen in mustelids, but unlike the latter, may be hemophagous as well as phagocytic of endometrial debris. The orderly arrangement seen in these early stages of hyena placental development leads to the labyrinthine arrangement formed by secondary and tertiary branches from the primary villi extending into maternal blood.

Maria Miglino presented unique data from a late placenta of the endangered species *Tamandua tetradactyla*, the lesser anteater of South America. In this species the discoidal, lobulated placenta occupies two-thirds of the tiny uterine cavity, the pregnant uterus simplex measures just 8 × 13 cm. It is hemochorial showing syncytiotrophoblast and a few cytotrophoblast cells

of the villi as well as many macrophages. A unique whitish gelatinous discoidal plaque of 7 cm in diameter covered the rest of the uterus and here blood extravasations between the plaque and the endometrium were seen. In the gelatinous discoidal plaque decidual structures were evident. This placenta thus has similarities to other hemochorial placentae, but also has a very special feature which needs further attention.

In vivo methods used so far are unsuitable to analyse isolated aspects of the feto-maternal interplay in the bovine placenta and morphological analysis of cell cultures has been shown to be insufficient. Nina Hambruch has, therefore, developed an *in vitro* model of an isolated bovine trophoblast cell line that was characterised over several passages according to the expression of specific trophoblast-characteristic molecules TIMP-2, PL, cytokeratin, smooth muscle actin, beta actin, laminin, integrin alpha 6 and vimentin. Contamination with endothelial cells and maternal epithelium was excluded by testing the uptake of low density lipoprotein (LDL) and Y chromosome-specific PCR. The resulting bovine trophoblast cell line of confirmed fetal origin responded to EGF stimulation suggesting that these cells are a valuable tool for further mechanistic studies elucidating feto-maternal interplay.

The workshop concluded that promising new approaches to reproductive disorders in large (farm) animals is well underway. However, classical studies still have their justification, as access to placentae of endangered species is restricted, and here especially different stages of gestation are needed.

12. Workshop 11: Trophoblast and neoplasia

Organizers: Paul Bischof, University of Geneva, Switzerland; D. Michael Nelson Washington University, USA.

Speakers: Paul Bischof, University of Geneva, Switzerland; Marie Cohen, University of Geneva, Switzerland; Irmgard Irminger, University of Geneva, Switzerland; Leslie Myatt, University of Cincinnati, USA; D. Michael Nelson Washington University, USA; Billy Yung, University of Cambridge, UK.

12.1. Aim

To describe some molecular mechanisms common to trophoblast and tumor invasion. The focus was on molecules that perturb the cell cycle such as p53, GRP 78/Bip, different oncogenes and anti-oncogenes.

12.2. Summary

Paul Bischof gave an overview of the function of p53 in cell biology and in the invasive human trophoblast. As a transcription factor and oncosuppressor, p53 is known to modulate expression of many matrix metalloproteinases (MMP), enzymes instrumental in remodeling the extracellular matrix during invasive processes. Although over-expressed in cytotrophoblast cells, endogenous p53 is incapable of transcriptionally regulating MMP-2 and MMP-9. This dysregulation may be due in part to the large proportion of p53 retained in the cytoplasm. Marie Cohen elaborated on the role of p53 in trophoblast describing studies of proteins involved in complexing p53 in cytotrophoblast. Glucose-regulated protein 78 (GRP78), a chaperone protein in the endoplasmic reticulum (ER), stabilizes p53 and thereby indirectly regulates MMP-2 and MMP-9 during invasion of trophoblast cells. Highly expressed in the membrane of cytotrophoblasts, GRP78 may regulate invasion of these cells by binding p53 in the cytoplasmic compartment, a hypothesis also posed for cancer cells. Michael Nelson described studies on enhanced p53 expression in term villous cytotrophoblast compared to syncytiotrophoblast. p53 interacts with Mcl-1 and Bak in both phenotypes, but the balance of these interactions favour apoptosis in cytotrophoblast. The higher level of apoptosis in

trophoblast of placentae from pregnancies with fetal growth restriction associates with higher p53 expression, suggesting p53 regulates term as well as first-trimester trophoblast biology.

Billy Yung presented data on how ER stress regulates AKT activity through attenuation of protein translation and by promotion of interaction between AKT and GRP78. The level of interaction between the two proteins is dependent upon the severity of the ER stress. Although the interaction between AKT and GRP78 occurs in both normal and carcinoma cells, there is differential regulation of AKT activity, with AKT suppressed in normal cells and enhanced in choriocarcinoma cells. In addition, Leslie Myatt noted that ER stress triggers the unfolded protein response that results from a variety of stimuli. ER stress causes the molecular chaperones Gp96 and GRP78 to dissociate from the proximal sensors ATF6, IRE1 and PERK and to bind to unfolded proteins. The proximal sensors in turn activate transcription of Gp96 and GRP78, inhibit protein synthesis, promote protein degradation, and reduce protein load. Both proteins are expressed in syncytiotrophoblast, EVT and placental vascular endothelium. Expression of both is increased in pregnancies complicated by pre-eclampsia as an indication of ER stress. In addition, ATF6 is diffuse in villous stroma and syncytiotrophoblast and expression is increased in pre-eclampsia, especially in the perivasculature.

Irmgard Irminger discussed the role of the multiple isoforms of the tumor suppressor BARD1. This protein forms heterodimers with the breast cancer gene product BRCA1, yielding E3 ubiquitin ligase activity and functions in DNA repair, mitosis and cell cycle control. BARD1 is expressed in first-trimester placenta, is induced by hCG and hypoxia and is under tight temporal and spatial control. Breast and ovarian cancers associate with truncated and deletion-bearing BARD1 isoforms that have lost tumor suppressor functions, while retaining pro-proliferative and oncogenic functions. Interestingly, these same isoforms are expressed in cytotrophoblast and may regulate invasion. BARD1 isoforms exemplify the insights into trophoblast biology that can be obtained from the study of cancer cells.

The workshop presentations emphasized the multiple regulators and pathways that are involved in normal trophoblast invasion and turnover. The complexity involved highlighted the opportunities for dysregulation to yield trophoblast neoplasia or placental dysfunction.

13. Workshop 12: Trophoblast: regulation of differentiation

Organizers: Ian Crocker, University of Manchester, UK; Sascha Drewlo, Samuel Lunenfeld Research Institute, Canada.

Speakers: Tereza Cindrova-Davies, University of Cambridge, UK; Daniele Evain-Brion, INSERM U 767, Paris, France; Martin Knöfler, Medical University of Vienna, Austria; Padma Murthi, The Royal Women's Hospital, Australia; Neal Rote, University Hospitals of Cleveland, USA.

13.1. Aim

To explore the impact of environmental and molecular factors on trophoblast differentiation and possible contribution to placental pathologies.

13.2. Summary

Trophoblast differentiation arguably dictates fetal and placental development. Trophoblast cells of the early blastocyst differentiate into the constituent cells of the human placenta. These cytotrophoblast cells can then differentiate into extravillous anchoring cell columns, invasive intermediate trophoblast, or fuse to form the hormonally active villous syncytiotrophoblast. Knowledge of the molecular triggers for initiation and control of trophoblast differentiation is expanding rapidly, particularly through the use of knockout and knockdown experiments. In

addition to reviewing current knowledge, this workshop introduced new factors which contribute to trophoblast differentiation and discussed their potential role in placental-related pathologies.

Padma Murthi discussed the importance of the homebox gene transcription factor DLX3 in normal placental development and placental pathology. Transient over-expression studies of DLX3 *in vitro* in BeWo cells using syncytin, 3 β-hydroxysteroid dehydrogenase and human β-chorionic gonadotrophin (β-hCG) as markers of differentiation confirmed the importance of this homeobox gene in trophoblast differentiation. In cases of fetal growth restriction DLX3 was over-expressed (at both the mRNA and protein level) in syncytiotrophoblast, with residual expression in villous cytotrophoblast and capillary endothelium. It has been proposed that increased DLX3 may be a contributing factor in abnormal trophoblast differentiation, as previously defined in fetal growth restriction.

Danielle Evain-Brion described recent findings on the identification of membrane proteins directly involved in trophoblast cell fusion and differentiation in human chorionic villi. In trophoblast from trisomy (T) 21-affected placentae, fusion and differentiation are temporally and multifactorially regulated processes, involving gap junction communications, connexin 43, ZO-1 and the fusogenic retroviral envelope proteins syncytins 1 and 2. It was also demonstrated that syncytin 2 was differentially distributed in T21, and proposed that defective syncytiotrophoblast formation was associated with the abnormal secretion and bioactivity of hCG. A marked decrease in surface hCG receptors (LH/CG-R) on cytotrophoblast in T21, unrelated to either specific deletions or mutations, was also observed. siRNA for LH/CG-R in cytotrophoblast cultures confirmed its role in syncytiotrophoblast formation and recombinant hCG overcame the *in vitro* T21 phenotype, allowing cytotrophoblast to fuse and syncytialise. It was concluded that in T21 abnormal endogenous hCG signaling, in addition to other fusogenic elements, has the potential to impair trophoblast differentiation.

Tereza Cindrova-Davies revisited a controversial topic and one of fundamental importance to trophoblast biology, notably that of transcriptional activity within the syncytiotrophoblast. Past evidence of transcriptional inactivity was questioned, a surprising observation given its active secretory role. Data was presented from four different experimental approaches: (i) the detection of RNA polymerase II by immunohistochemistry, using an antibody specific for its active form, (ii) a functional assay measuring uptake and incorporation of fluorouridine, a nucleoside analogue, (iii) the immunohistochemical detection of histone modifications associated with gene activation, and (iv) additional markers for active and repressed chromatin. Each method confirmed transcriptional activity in a portion of syncytiotrophoblast nuclei, a finding which re-establishes a role for transcription regulators in the syncytiotrophoblast and provides a means of manipulating *de novo* RNA synthesis. Qualitative evidence suggested that transcription was more abundant in the first trimester than at term.

Neal Rote provided a broad overview as well as a more detailed discussion of the role of apoptosis in trophoblast differentiation and specifically fusion events, again highlighting the potential for anomalies in pathologic situations. In fusogenic BeWo cells there is an up-regulation of anti-apoptotic (Bcl-2) and suppression of pro-apoptotic elements (Bax, p53, and caspases 3 and 8) at the transcriptional level following fusion, a result potentiating increased apoptotic resistance. There are significant differences between induced apoptosis in BeWo cells as compared to forskolin-induced differentiation. The importance of caspase 8 in BeWo cell fusion, using a sub-line in which the proform of the enzyme had been silenced, was confirmed. More specifically, externalisation of phosphatidylserine (PS) was suppressed following cAMP treatment, whilst hCG expression remained unaffected. The potential role of Annexin-A5 in regulating PS externalisation, a requirement for trophoblast fusion, was examined using a series of siRNA transfections, evaluating hCG production and the loss of E-

cadherin. Although Annexin-A5 externalisation was not obligatory for trophoblast fusion, a role in protecting the syncytiotrophoblast from a maternal inflammatory and coagulatory response, a feature potentially disrupted in pre-eclampsia, was proposed.

Martin Knöfler discussed the potential role of transcription factors such as Snail and TCF in controlling the epithelial to mesenchymal transition (EMT) during tumourogenesis. Invasive differentiation of human trophoblast resembling cancer cell invasion is promoted by numerous growth factors produced by trophoblast and decidua. Whereas several critical signaling cascades promoting trophoblast invasion have been elucidated, how the differentiated trophoblast phenotype is initiated and maintained is still not clear. Snail and TCF, the latter being regulated by Wnt signaling, are predominantly expressed in EVT and are likely to play a role in controlling trophoblast invasion. In addition, initial experiments identifying AP-2 proteins as regulators of trophoblast motility were described.

This workshop brought together several diverse areas of trophoblast differentiation from cytotrophoblast fusion into the syncytiotrophoblast to cytotrophoblast differentiation to extravillous trophoblast.

14. Conclusion

This workshop report highlights the strength and breadth of research being undertaken in the placental field at the present time. This report also highlights the willingness of researchers within this field to share new and novel data and experimental paradigms within a semi-formal setting designed to stimulate discussion and debate.