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IFPA Meeting 2011 workshop report III: Placental immunology; epigenetic and microRNA-dependent gene regulation; comparative placentation; trophoblast differentiation; stem cells[☆]

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Conflict of interest statement

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

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Abstract

Workshops are an important part of the IFPA annual meeting as they allow for discussion of specialised topics. At IFPA meeting 2011 there were twelve themed workshops, five of which are summarized in this report. These workshops related to various aspects of placental biology: 1) immunology; 2) epigenetics; 3) comparative placentation; 4) trophoblast differentiation; 5) stem cells.

Keywords

Immunology; Epigenetics; Comparative placentation; Trophoblast differentiation; Stem cells; Placenta

1. Placental immunology

Chairs: Larry Chamley and Tamara Tilburgs

Speakers: B Anne Croy, Judith N Bulmer, Ted Golos, Anne Husebekk, Guro M Johnsen, Ashley Moffett, Peter Parham, Margaret G Petroff, Jennifer H Southcombe, Tamara Tilburgs,

1.1. Outline

The placenta is one of nature's great mysteries. Since the fetus and its placenta are derived from both mother and father this organ is in essence a tissue transplant. In an uncomplicated pregnancy the placenta is a semi-allograft in which the paternally derived antigens are incompatible with the maternal immune system. In contrast, in a donor oocyte or surrogate pregnancy the placenta may be a full allograft which is genetically unrelated to the host mother. The immune paradox of pregnancy, in which the mother must tolerate the placenta and fetus yet not succumb to infection, was first elaborated by Sir Peter Medawar more than half a century ago. However, how much is actually known about how maternal tolerance is achieved and how mother and fetus survive what should be an immune conflict?

The workshop focused on three specific questions: 1) Are uterine natural killer (uNK) cells required for human pregnancy; 2) Where and what is the maternal-fetal interface: uterus, lymph, maternal blood?; 3) Antigen specific tolerance: can we generate tolerance - are there antigens to do this?

1.2. Summary

The Chairpersons opened the workshop by briefly presenting a case history of an abdominal pregnancy in a woman after total hysterectomy. Although 22 such pregnancies had been previously reported, this was the first in which the fetus was live-born. This appropriately introduced the first question – “in the light of this case are uNK cells essential for pregnancy”?

Anne Croy reviewed immune deficient mouse strains with very few to no uNK cells but successful gestation, indicating that uNK cells are not essential for murine pregnancy. The benefit of uNK cells, defined by murine studies, lies in protection of maternal and fetal hearts from abnormal compensation to the elevated cardiovascular demands of pregnancy. Judith Bulmer indicated that while the role of uNK cells in normal pregnancy has been intensively researched, decidual macrophages and T lymphocytes are receiving much less attention. Uterine NK cells potentially interact directly with extravillous trophoblast (EVT). In addition they secrete many cytokines, angiogenic growth factors and proteases, levels of which differ with gestational age. In tubal pregnancy EVT invades the fallopian tube wall and arteries, ultimately resulting in tube rupture/haemorrhage. Tubal implantation sites contain T cells and macrophages but, unless there is decidualisation of tubal mucosa, uNK cells are absent. In placenta accreta, decidua (and uNK cells) is at least focally absent but trophoblast invades myometrium and arteries. Uterine NK cells are present in ectopic decidua in pregnancy and in decidualised tissue. Successful extra-uterine pregnancy may reflect areas of extra-uterine decidualisation with uNK cells in ectopic decidua interacting with EVT. Alternatively, there is likely to be redundancy within the system and other cell types may fulfill uNK cell function at extra-uterine sites. Peter Parham described how interactions of killer cell immunoglobulin-like receptors (KIR) with human leukocyte antigen (HLA) class I molecules regulate these functions and are specific to simian primates, where co-evolution of receptors with ligands is evident. Emerging in hominoids as superior KIR ligands, the C1 and C2 epitopes of HLA-C are the only variable epitopes on trophoblast cells and thus able to bind uNK cell KIR. The combination of fetal C2 and maternal *KIR A* haplotypes correlates with pregnancy disorders that cause death, non-competitive offspring, and barrenness. The selection pressure they impose is writ in the impressive inverse correlation between C2 and *KIR A* frequencies worldwide. Human survival has clearly benefited from the contribution of uNK cells. This does not rule out rare occurrences of human reproduction without uNK cell function, but suggests it would not be a competitive evolutionary strategy in the long run. In some circumstances dramatic change can be effective, as illustrated in gibbons that lost HLA-C, HLA-G and all but one KIR. Such ‘simultaneous’ losses also point to the importance of uNK cell receptors and ligands in human reproduction. Ted Golos described how CD56⁺/perforin⁺ uNK cells in the rhesus monkey have morphological heterogeneity, including small compact cells, cells with expanded cytoplasm and compact perforin staining, and large cells of amorphous morphology, with widely dispersed staining. Immunodepletion of peripheral blood NK cells reduces uterine NK cell numbers, however abundant decidual Ki67⁺ NK cells suggest that trafficking from peripheral blood to the decidua only partly contributes to dramatic increases in the decidua. Immunodepletion in the luteal phase increases endometrial blood vessel wall thickness, and delays stromal maturation. NK cell function in the transition from secretory phase endometrium to decidua of pregnancy is targeted in this model.

The focus of the workshop then moved to the question “where and what is the maternal–fetal interface?” Jennifer Southcombe discussed the immunomodulatory role of syncytiotrophoblast microvesicles. In the later stages of pregnancy, the syncytiotrophoblast layer of the placenta is in direct contact with maternal blood. Vesicles less than 1 micron in size, syncytiotrophoblast microvesicles (STBM), are continually shed into the circulation.

They engage B cells and monocytes, and trigger the release of, or inhibit, various cytokines and chemokines. By altering cytokine responses the STBM may contribute to the changes in maternal immunity that are important for a successful pregnancy. STBM extend the local placental syncytiotrophoblast-maternal blood interface as they can interact with immune cells systemically. Guro Mørk Johnsen discussed the distribution of different T cell populations in decidua and maternal peripheral blood from normal 3rd trimester pregnancy as determined by flow cytometry. The percentages of activated Tregs ($CD4^+CD45RA^-FOXP3^{++}$) and cytokine-secreting T cells ($CD4^+CD45RA^-FOXP3^+$) were increased in decidua compared to blood, while resting Tregs ($CD4^+CD45RA^+FOXP3^+$) were more abundant in blood than in decidua. Both $CD4^+CD45RA^-FOXP3^-$ and $CD8^+CD45RA^-$ memory cells were significantly increased in decidua compared to blood, whereas $CD4^+CD45RA^+$ and $CD8^+CD45RA^+$ naive T cells were more abundant in the circulation. This suggests a role for Tregs in 3rd trimester decidua in pregnancy.

The workshop then addressed the final question “Can we generate tolerance - are there antigens to do this?” Anne Husebekk reminded the workshop that fetal/neonatal alloimmune thrombocytopenia is caused by antibodies to fetal human platelet antigen 1a in greater than 80% of the cases. These antibodies cross the placenta and sensitize fetal platelets, which are phagocytosed, rendering the fetus at risk of bleeding. HPA-1a is an epitope on the $\beta 3$ integrin of the fibrinogen receptor on platelets. $\beta 3$ integrin is also present on invading trophoblast cells as part of the vitronectin receptor. Immunization may occur during the first trimester. Recent data show an association between maternal anti-HPA-1a antibody level and reduced birth weight. As the vitronectin receptor carrying the HPA-1a epitope is also present on activated endothelial cells and trophoblast cells, it is likely that these cells may be influenced by the antibodies and reduced birth weight may indicate placental dysfunction. Tamara Tilburgs discussed HLA-C, which is the only polymorphic classical major histocompatibility antigen expressed by fetal trophoblast cells. There is increased activation of decidual T cells in pregnancies containing a fetal-maternal HLA-C antigen mismatch. However the increase in activated $CD4^+CD25^{dim}$ T cells coincides with an increase in functional $CD4^+CD25^{bright}$ regulatory T cells that can suppress this response. In addition, preliminary data show the presence of Y chromosome specific $CD8^+$ T cells in decidual tissue obtained from women carrying a male fetus. These observations indicate that maternal T cells actively recognize and tolerate fetal alloantigens. The future challenge is to determine their role in pregnancy complications. Peggy Petroff reminded the workshop that in pregnancy, T cells that recognize paternally-inherited major and minor histocompatibility antigens are expanded. Less certain, however, are the cellular sources of these antigens and the mechanisms by which they access maternal lymphoid organs. It has been shown that the human placenta is replete with minor histocompatibility antigens and that cellular debris exiting the placenta also possesses these antigens. The presence of minor antigens in the trophoblast changes the way we should view its immunologic potential: rather than regarding the syncytiotrophoblast as immunologically inert due to the absence of major histocompatibility complex (MHC), they may instead account for the fetal antigen-specific response in women.

1.3. Conclusion

The first part of the workshop emphasized the unique role uNK cells play during pregnancy. No definitive conclusion was reached as to whether or not uNK cells are essential for pregnancy; their importance may differ from species to species. The discussion and data presented supported the idea that uNK cells may not be necessary in an individual pregnancy. However, uNK cells may emerge as important and naturally selected components of successful pregnancy when considered on a long-term population scale. In the second part of the workshop there was general agreement that the fetal-maternal

interface extends beyond the decidua, to the lymph nodes, the syncytiotrophoblast layer and the syncytiotrophoblast microvesicles that are continually shed into the maternal circulation. Novel data was presented that both extravillous trophoblast cells and syncytiotrophoblasts express paternally-inherited minor histocompatibility antigens and both may be able to elicit an antigen specific response by maternal immune cells. The presence of decidual CD4⁺CD25^{bright}FOXP3⁺ regulatory T cells and their potential to generate antigen specific immune suppression may position these cells as crucial immune regulators during pregnancy. This data was mainly obtained in uncomplicated pregnancies and implications for pregnancy pathology are unclear. The future challenge is to determine the role of antigen specific immune recognition and regulation of fetal trophoblast cells by maternal immune cells during pregnancy complications.

2. Epigenetic and microRNA-dependent gene regulation

Chairs: Rosalind John and Yoel Sadovsky.

Speakers: Richard Chaillet, Edward Chuong, Rosalind John, Joana Moreira de Mello, Wendy Robinson, Yoel Sadovsky.

2.1. Outline

Exciting new research findings implicate epigenetic mechanisms as central regulators of placental development and function. Post-translational modifications of histones, nucleosome remodeling, changes in DNA methylation, and the imprinted marking of specific parental alleles are different epigenetic mechanisms of transcriptional control that affect a wide variety of cellular processes. Many of these mechanisms likely contribute to fetoplacental developmental defects. Chromatin remodeling complexes and cytosine methyltransferases facilitate epigenetic reprogramming during pre-implantation development. Correct reprogramming produces developmentally competent embryos and trophoblast cells. Defects in reprogramming, such as loss of critical genomic imprints, lead to placental dysfunction as evidenced by specific changes in gene expression of imprinted genes in the mouse placenta. Trans-generational inheritance of both normal and disease states may be mediated by maintenance of epigenetic modifications through meiosis. In addition, non-heritable expression patterns of microRNA transcripts modulate gene expression in the developing placenta, promoting stability of the transcriptomic and proteomic landscape as well as providing a mechanism to dynamically respond to exogenous stimuli. Many aspects of this diverse collection of epigenetic controls seem unique to the placenta, with mis-expression possibly contributing to diseases. The recent development of high-throughput analysis of chromatin modifications, RNA, microRNA and protein output, has enabled comprehensive determinations of the cellular epigenome in distinct physiological and pathological conditions. The identification of changes in this operational form of the epigenome may lead to a better understanding of underlying cellular pathophysiology.

This workshop aimed to provide state-of-the-art presentations on DNA methylation, imprinting and microRNA-dependent gene regulation in the placenta, and their impacts on placental development and fetal growth.

2.2. Summary

Richard Chaillet discussed the role of DNA (cytosine-5)-methyltransferase 1 (DNMT1) enzymes in imprinting mechanisms. Genomic imprinting is a process of gene regulation in which parental alleles of imprinted genes are distinguished by DNA methylation. Imprinted genes are formed through a developmental reprogramming process. A cardinal feature of

this process is the inheritance of unique gametic imprinted methylation patterns by the action of maternal and zygotic DNMT1 cytosine methyltransferase enzymes. These enzymes co-ordinately function during pre-implantation to ensure the uniform presence of methylation imprints in all embryonic and extraembryonic cells.

Rosalind John discussed the epigenetic regulation of maternal adaptation to pregnancy. Placenta-derived factors act to modify the maternal–fetal interface and to regulate maternal adaptation to pregnancy for optimal fetal benefit. By directly regulating specific placental lineages, genomic imprinting can modulate the supply of nutrients to the embryo. For example, the imprinted *Igf2* gene acts within the labyrinth to regulate the diffusion exchange characteristics of the mouse placenta. Imprinting could also modulate adaptations to pregnancy by controlling cell types that secrete pregnancy-specific factors into the maternal blood stream. *Phlda2* specifically regulates a key endocrine lineage of the mouse placenta, the spongiotrophoblast, which produces many factors that are critical for successful pregnancy including prolactin-related hormones and pregnancy-specific glycoproteins. Consequently, *Phlda2* may regulate adaptation to pregnancy by manipulating the amount of these factors secreted into the mothers system. *Esx1*, *Cited1* and *Nrk*, which also regulate this lineage, are maternally expressed by virtue of their location on the X-chromosome, which is preferentially paternally inactivated in the mouse placenta. It has recently been reported that the imprinted *Ascl2* gene negatively regulates both the spongiotrophoblast and the parietal trophoblast giant cell lineages of the mouse placenta. Thus deregulated imprints in the placenta can act directly to restrict the growth of the fetus through restricting the flow of nutrients but may also cause inappropriate adaptations to pregnancy, with additional adverse consequences for maternal health.

Yoel Sadovsky discussed the search for function of placental microRNAs in relation to studies on trophoblast response to injury. MicroRNAs are germane for modulation of gene expression through RNA degradation and inhibition of translation. Human trophoblast cells express common and unique microRNA species, but the functional targets of most microRNAs remain unknown. The search for relevant targets of trophoblast microRNAs includes high-throughput expression assays, *in silico* analyses for integration of complex data with or without *a priori* assumption of target complementarity, reporter assays, and mutagenesis of microRNA binding elements.

Wendy Robinson discussed clonal patterns of placental development as determined by evaluating X-chromosome inactivation profiles. The data support a model whereby each villous tree represents a clone derived from one or very few progenitor cells. Trophoblast also shows distinct developmental origin from amnion and chorion, as expected. Comparing patterns of clonal growth in the placenta to the distribution of other types of genetic and epigenetic variation can help distinguish the relative contribution of variation that arises in the pre-implantation embryo from that arising predominantly after tissue differentiation.

Joana Moreira de Mello discussed a candidate gene for X-linked genomic imprinting in human full-term placenta. Genomic imprinting and X-chromosome inactivation are major epigenetic phenomena in mammals. Female humans display random X inactivation in every somatic tissue but the existence of imprinted genes on the X, which is subject to transcriptional inactivation, remains enigmatic. Imprinted X-linked genes have been described in mice, but there is only indirect evidence in humans. A human X-linked gene that undergoes genomic imprinting has been recently reported. This gene is not clustered with imprinted genes and the methylation pattern of one of its promoter's CpG islands does not correlate with preferential maternal monoallelic expression. This is likely the first human X-linked gene that is subject to imprinting in the term placenta.

Edward Chuong discussed the epigenomics of trophoblast differentiation. Chromatin immunoprecipitation and high-throughput sequencing (ChIP-Seq) was used to identify genome-wide epigenetic changes that occur during *in vitro* differentiation of mouse trophoblast stem cells to primary trophoblast giant cells. These data highlighted thousands of genomic regions that were classified into active/inactive regulatory elements based on enrichment of specific histone modifications. Focusing on regulatory elements that become active in trophoblast giant cells may provide insight into how epigenetic modifications help establish trophoblast lineage commitment. These types of analyses are being extended to trophoblast derived from rat and rabbit to investigate the evolutionary conservation of trophoblast gene regulation.

2.3. Conclusions

Deciphering the role of imprinting, epigenetic marks and post transcriptional miRNA-based regulation of RNA and protein expression is clearly essential in order to understand normal placental development and function. Moreover, placental trophoblast cells exhibit unique types of epigenomic and miRNA pathways that do not exist in embryo-derived lineages. Recent developments in high throughput and sequencing technologies enable new insights into mechanisms by which environmental stressors impact trophoblast epigenomic mechanisms to induce placental injury. Aberrant patterns of DNA methylation or miRNA expression may suggest biomarkers of placental dysfunction that contribute to clinically relevant diseases during pregnancy. Of equal importance, these advances allow us to gain a better insight into the role that epigenetic and miRNA-related processes might have played in placental evolution, resulting in the diversity of placental types that exist in modern day mammals.

3. Comparative placentation

Chairs: Anthony Carter and Mandi de Mestre.

Speakers: Anthony Carter, Hayley Dickinson, Allen Enders, Carolyn Jones, Mandi de Mestre, Martha Ramirez-Pinilla, Hiroaki Soma.

3.1. Outline

The focus of this workshop was on placental diversity in vertebrates ranging from fish to mammals. Laboratory rodents and domesticated mammals having been well covered at previous meetings, the intention was to widen the net to lesser known species.

3.2. Summary

Amanda de Mestre explained that placentation in the horse (*Equus caballus*) had the unique feature of combining a noninvasive epitheliochorial placenta for nutrient and gaseous exchange with invasive trophoblast that performs endocrine functions. Invasive trophoblast cells establish themselves in the endometrial stroma in structures termed endometrial cups. Trophoblast cells survive despite a hostile uterine immunological environment. When transplanted to sites outside the uterus in non-pregnant mares, invasive trophoblast cells survive and function for a similar period to normal endometrial cups. This observation, together with *in vitro* studies of trophoblast–lymphocyte interactions, suggests invasive trophoblast possesses innate immunomodulatory capabilities essential to their survival. Furthermore, molecules specific to the uterine environment are not critical to the survival of invasive trophoblast.

Hayley Dickinson characterized the placenta of the spiny mouse (*Acomys cahirinus*), a small rodent species native to North Africa and the Middle East. Precocial offspring are born after

a 39-day gestation and litter sizes are small (1–4). The placenta is discoid hemotrichorial with emergence of the labyrinth at mid-gestation. The junctional zone is made up of glycogen cells, spongiotrophoblast, giant cells and large maternal blood spaces. Unlike conventional laboratory rodents, micro-CT imaging revealed a large vascular contribution to the fetal membranes originating from the umbilical vessels close to the fetal surface of the placenta. Sex differences in placental development have also been noted.

Anthony Carter introduced the Laotian rock rat (*Laonastes aenigmamus*), a relict species of rodent. First described in 2005, this species belonged to a lineage basal to the hystricognaths that had been thought to have gone extinct in the Miocene. Unlike in hystricognaths, the labyrinth was neither folded nor lobulated and there was no subplacenta. These two characteristics had been regarded as adaptations related to the reproductive strategy of hystricognaths. Therefore it is noteworthy that *Laonastes* also carried a single fetus which was born in a precocial state. The implication might be that the reproductive strategy shared by *Laonastes* and hystricognaths evolved before the placental adaptations characteristic of the latter group.

Allen Enders said the endotheliochorial placenta of the three-toed sloth (*Bradypus tridactylus*) was generally considered similar to that of the short tailed shrew (*Blarina brevicauda*) because of the perforated trophoblast. Both placentas have a highly irregular heterogeneous interstitial lamina and both have openings into intratrophoblast spaces from the maternal side. However maternal and fetal endothelia show several differences and the sloth did not have complete discontinuities as seen in shrew trophoblast. It is possible that such discontinuities might develop in the sloth placenta later in pregnancy since only a relatively early pregnancy specimen had been examined by electron microscopy.

Evidence for trophoblast secretory activity in non-eutherian epitheliochorial placentae was presented by Carolyn Jones. Epitheliochorial placentae from the Tammar wallaby (*Macropus eugenii*) and the three-toed skink (*Chalcides chalcides*) exhibit microvillous surfaces on both the trophoblast and uterine epithelium that do not appear to interdigitate closely, unlike those of eutherian epitheliochorial placentae. Trophoblast cells containing heavily glycosylated granules are present in both species and it is speculated that some of these might be secretory in nature, possibly for the production of material to enhance intercellular contact.

Martha Ramírez-Pinilla said that the chorioallantoic placenta of the Andean skink (*Mabuya* sp.), a squamate reptile, exhibited the highest placental complexity and the highest level of placentotrophy known among Reptilia, which meant that virtually all of the nutrients for embryonic development were obtained across the placenta. A massive nutrient transfer (water, lipids, proteins, inorganic ions) was known to occur during embryonic development, especially when the chorioallantoic placenta was mature. It possessed areas functionally related to gas exchange and highly specialized structures related to nutrient transfer (placentome, paraplacentome, chorionic areolas, and absorptive plaques). Some of these morphological specializations showed an impressive convergence with those of eutherian mammals.

Hiroaki Soma compared placentation in the scalloped hammerhead shark (*Sphyrna lewini*) and the manta ray (*Manta birostris*). The scalloped hammerhead shark has a gestational period of approximately 1 year and bears about 20 pups nourished by yolk sac placentation. The chorionic epithelium of hammerhead shark placenta contains immunopositive placental-like proteins when anti-human antibodies are used. A ductus vitelointestinalis was positioned between two umbilical cord vessels. On the other hand, the Manta ray has a 1 year long gestation but bears only one pup nourished by placental analogues such as

trophonemata that are characterized by crypts with vascularized villous strings. There are abundant secretory vesicles with the granules of proteins and lipids being released from the uterine epithelial surface into the uterine cavity for the pup.

3.3. Conclusions

Features of placental types that occur in nine animals ranging from the domestic horse to reptiles and fish were presented. This workshop highlighted not only placental morphological diversity that exists across species but also illustrated interesting examples of convergent evolution at either the cellular or functional level. A number of species displayed specific placental and/or pregnancy adaptations in placental structure and gestation length that could be exploited to investigate basic mechanisms of placental function. For example, the spiny mouse has a longer gestation period when compared with conventional laboratory rodents and could be utilised to study immunological responses in pregnancy that involve memory. In summary, expanding our understanding of structure and function to a wider range of placental types will result in a better appreciation of the evolutionary aspects of placentation and may provide a novel approach for future studies of placental function and pathology.

4. Trophoblast differentiation and tools for studying it – with a focus on human villous trophoblast

Chairs: Alex Heazell and Berthold Huppertz.

Speakers: William Ackerman, Sarah Coleman, Gavin Collett, Jennifer Ducray, Norah Fogarty, Martin Gauster, Sandra Haider, Olivia Holland, Aline Lorenzon, Guillaume Pidoux.

4.1. Outline

Trophoblast differentiation remains a major topic in placental research. The last decade has seen a number of discoveries shedding new light on the different subtypes of trophoblast, including villous cytotrophoblast and syncytiotrophoblast, extravillous trophoblast with its interstitial, intramural, endovascular and endoglandular subtypes plus the multinucleated trophoblast X-cells. Although it has been a major focus of placental research for decades, regulation of trophoblast differentiation is still a mystery and a large number of groups present an even larger number of different points of view on how trophoblast differentiates and what is the fate of the different subtypes. This is often due to different methodological approaches with one of the major issues in this field being the cell types and culture conditions used to analyze trophoblast differentiation.

This workshop aimed to discuss the latest developments in achieving insight into the differentiation pathways of villous trophoblast, including morphological changes of the cells, direct insights into molecular pathways, changes in expression and localization patterns of proteins, etc. The workshop was divided into three main themes: 1) cytotrophoblast proliferation, differentiation and apoptosis; 2) cytotrophoblast fusion; 3) syncytiotrophoblast differentiation and syncytial knots.

4.2. Summary

Sandra Haider discussed the role of Notch signalling in human placental development. Notch signalling is a highly conserved pathway controlling proliferation, cell death and differentiation. The localization of all Notch receptors (Notch 1–4) and their ligands (Jagged1 and 2, DLL1, 3, 4) was investigated in first trimester human placenta. In addition, the role of Notch signalling in trophoblast proliferation and invasion was studied. Notch

receptors and ligands were widely expressed in different trophoblast subtypes. Notch1 and Notch4 were specific markers for a subset of proliferative cell column trophoblast cells and villous cytotrophoblast, respectively. Inhibition of Notch signalling enhanced outgrowth of villous explant cultures and stimulated cytotrophoblast migration. Additionally, inhibition of Notch signalling induced proliferation of cell column trophoblast cells and villous cytotrophoblast.

Aline Lorenzon discussed stromal cell derived factor-2 (SDF-2) and trophoblast differentiation. It has been shown that SDF-2 is widely expressed in human placenta, although its function is still unknown. Primary cytotrophoblast cells were cultured for 30 min, 24 and 48 h for differentiation assays in 21% O₂, and for 24 h in 21% O₂ followed by 1 h or overnight in 2% O₂. Immunoblotting demonstrated that SDF-2 was downregulated in hypoxia, with hypoxia inducible factor (HIF) and β -hCG confirming hypoxia and differentiation conditions, respectively. The focus is to now understand the role played by SDF-2 and its biological meaning at the maternal–fetal interface.

Martin Gauster discussed lysophosphatidylinositol as a putative inducer of villous trophoblast differentiation. Bioactive lipids have been suggested to be involved in placental growth and differentiation. One such lipid is lysophosphatidylinositol (LPI), a lysophospholipid generated by enzymatic hydrolysis of phosphatidylinositol. The ability of LPI to induce villous trophoblast differentiation was tested in BeWo and ACH-3P cell lines. LPI induced increased β -hCG secretion, downregulated expression of E-cadherin and impaired BrdU incorporation. These results suggest LPI as an endogenous inducer of villous trophoblast differentiation. The underlying mechanisms have not been defined so far, but the detection of the LPI receptor, a G-coupled-protein receptor, in human placental tissues and primary trophoblast suggests a possible role for receptor mediated signalling.

Jennifer Ducray discussed increased cytotrophoblast fusion in pre-eclampsia. Using term placentae from normotensive and pre-eclamptic pregnancies, double immunofluorescence was employed to compare the distribution of cytotrophoblast cells together with staining of these cells for cleaved caspase 8. Initial findings suggest a higher rate of cleaved caspase 8 positive cytotrophoblast cells in the pre-eclamptic cohort. If not matched by downstream caspase activity, a dysfunctional trophoblast turnover could result. In addition, punctate autofluorescence was prevalent in the syncytiotrophoblast of pre-eclamptic samples. It is tempting to speculate that these could be lipofuscin-like material resulting from inadequate autophagic processes of trophoblast turnover in pre-eclampsia which in turn may result in necrotic shedding.

Guillaume Pidoux discussed the role of ezrin and gap junctions in trophoblast fusion. A limited number of human cell types can fuse to form a multinucleated syncytium. In the differentiation of human placenta, mononuclear cytotrophoblasts aggregate and fuse to form a syncytiotrophoblast. The cAMP/phosphokinase A (PKA) signaling pathway is indispensable for syncytial formation and in particular a role for the AKAPs (A-kinase anchoring proteins) has been implicated. Ezrin was shown to be an AKAP required for hCG regulation of cell–cell fusion. Ezrin organizes a supramolecular complex containing PKA, connexin 43 and zona occludens 1 (ZO1), and targets PKA to connexin 43 and controls its phosphorylation to enhance gap junctional communication, which is a prerequisite for trophoblast cell–cell fusion.

William Ackerman discussed cellular events involved in syncytialization. The syncytiotrophoblast is a remarkably specialized epithelium that regulates many functions crucial to the human placenta. Following fusion of a progenitor cytotrophoblast with the syncytiotrophoblast, the newly merged cellular organelles undergo dramatic modifications.

To gain initial insights into the complicated cellular events that accompany syncytialization a BeWo model has been used. These cells can be induced to undergo fusion events that, in many respects, recapitulate those that occur *in vivo*. Dramatic structural and biochemical alterations in a number of organelles, including the Golgi/trans-Golgi system, endocytotic pathways, mitochondria, and endoplasmic reticulum were discussed.

Gavin Collett discussed the role of RhoE in regulating fusion of BeWo choriocarcinoma cells. The role of Rho GTPases in trophoblast differentiation and fusion is not known. Treatment of BeWo cells with dibutyryl cyclic AMP (dbcAMP) resulted in a protein kinase A-dependent upregulation of RhoE concomitant with cell fusion. Silencing of RhoE expression by siRNA inhibited dbcAMP-induced fusion but had no effect on functional differentiation, as determined by expression of human chorionic gonadotrophin and placental alkaline phosphatase. Upregulation of RhoE by dbcAMP was attenuated under hypoxic conditions in which cell fusion was impaired.

Norah Fogarty discussed differences between syncytial knots and sprouts. It is pertinent that features of syncytial knots and sprouts are defined so that researchers can distinguish between these functionally different regions. In serial sections of normal term placenta, syncytial nuclear aggregates were traced through to identify sprouts, false knots and true knots. Sprouts contained PCNA-positive nuclei whereas knots did not. Knots contained nuclei with heterogeneous DNA damage. Nuclei that were 8'OHdG or TUNEL-positive also displayed morphological features of degeneration, including a dramatically smaller nuclear area and a heavily condensed ring of DNA around the periphery. These data suggest that knots may be formed by shepherding of aged nuclei, which may drag in functional nuclei as collateral. Alternatively, functional nuclei are gathered into knots prior to condensation of their chromatin.

Sarah Coleman discussed nuclear condensation in syncytial nuclear aggregates. Syncytiotrophoblast nuclei show variable amounts of heterochromatin, hypothesised to be more condensed in syncytial nuclear aggregates. Heterochromatin was analysed in an archive of electron micrographs. Nuclei in syncytial nuclear aggregates were more often pyknotic than those in syncytiotrophoblast; one possibility is that as nuclei condense they are gathered into syncytial nuclear aggregates. The frequency of apoptosis was assessed in different types of syncytial nuclear aggregates by immunohistochemistry of the cytokeratin M30 neoepitope. Knots were more likely to be M30 positive than bridges suggesting that these syncytial nuclear aggregate structures may have different origins and functions.

Olivia Holland discussed the morphology of syncytial nuclear aggregates released from first trimester syncytiotrophoblast. Syncytial nuclear aggregates shed from first trimester placenta *in vitro* were analysed by three-dimensional reconstruction of confocal Z-stack images. Morphologies, dimensions and nuclear content broadly agreed with the limited published *in vivo* estimates. Fifty-five percent of the syncytial nuclear aggregates exhibited heterochromatic nuclei and/or blebbing of the plasma membrane, suggesting they were undergoing programmed death, but almost half of the syncytial nuclear aggregates had euchromatic nuclei and some possessed nucleoli. The observed differing gross and nuclear morphologies of syncytial nuclear aggregates may reflect different points along a continuum of programmed cell death. Alternatively, they may represent structures of entirely different origins.

4.3. Conclusions

This workshop highlighted the whole spectrum of villous trophoblast differentiation. Within the cytotrophoblast factors inducing differentiation such as stromal cell derived factor-2 (SDF-2) and lysophosphatidylinositol were described as potential new players, while the

Notch signaling pathway was shown to force differentiation. Prior to fusion activity of caspase 8 still remains an important step that may be accelerated in cases suffering from pre-eclampsia. During fusion ezrin, gap junctions and RhoE play crucial roles while at the same time the organelles of fusing cells undergo dramatic morphological changes. On the level of the syncytiotrophoblast from term placenta, an important step towards understanding of morphological structures has been made. It is becoming more accepted that syncytial sprouts are means for the generation of new villi; this is why they may contain nuclei that are still PCNA positive. On the other hand, syncytial knots are means for the accumulation of aged and late-apoptotic nuclei, which may be released into the maternal circulation. Interestingly, syncytial structures released from first trimester placenta show a new phenotype that needs to be further elucidated. In conclusion, expanding our knowledge of factors, pathways, players and structures in combination with relating findings from cell lines to primary cells may provide a way to understand trophoblast turnover in health and disease.

5. Stem cells

Chairs: Bill Kalionis and Ornella Parolini.

Speakers: Chie-Pein Chen, Edward Chuong, Bill Kalionis, Julia König, Anna Maria Nuzzo, Ornella Parolini, Alessandro Rolfo.

5.1. Outline

The workshop on placental stem cells was conceived as an opportunity to consider the placenta not only for its key role in supporting life in the womb, but also as a possible source of stem/progenitor cells with the potential to be exploited for regenerative and reparative medicine. In recent years great strides have been made in understanding the basic biology of placenta-derived stem cells, as well as their differentiation potential and immunological characteristics. Encouraging results have been obtained by investigating placental cell-based therapy in pre-clinical animal models but much more work needs to be done in this area. This workshop therefore represented an opportunity for interaction and discussion of the many unanswered questions regarding placental stem cells. These include the need for better definition of the cells being used in terms of their exact location in the placental tissues, phenotype, stem cell potential, as well as the need to have comparative studies to verify whether placental cells are better than those isolated from other sources in terms of therapeutic applicability. Specific discussion points included: 1) which cells are we identifying in placenta?; 2) do these cells all have the same potential, can we call them stem or progenitor cells?; 3) what is their differentiation capability?; 4) are immunomodulatory features the same for stem/stromal cells from different parts of the placenta?

5.2. Summary

Ornella Parolini discussed paracrine effects of amniotic membrane and amnion-derived cells. Transplantation of fetal membrane-derived cells, or conditioned medium from these cells, reduces lung fibrosis in bleomycin-challenged mice. Amniotic membrane patching promotes ischemic rat heart repair and reduces liver fibrosis in the BDL rat model. These positive outcomes were observed despite low or absent levels of donor cells in host tissues, suggesting that amniotic membrane-derived cells act by secreting paracrine soluble factors. An emerging concept from the study of transplanted placental cells is that important therapeutic actions result from the immunomodulatory effects they exert on the surrounding host environment, rather than their differentiation.

Bill Kalionis discussed migration of placental mesenchymal stem cells in a placental vessel perfusion model. Human placental mesenchymal stem/stromal cells (pMSCs) are migratory

cells but pMSC migration in the placenta is poorly understood. A transformed stem cell line (called PRCFP) was generated from primary pMSCs. PRCFP retained important stem cell properties including multipotent differentiation and migration. PRCFP cells were labelled with a live-cell fluorescent stain, perfused into placental vessels and their migration across the endothelial cell barrier assessed. PRCFP cells crossed the endothelium of the vessel but remained within the perivascular stem cell niche and did not migrate into the stroma. It was concluded that pMSC migration is tightly regulated by the perivascular stem cell niche.

Chie-Pein Chen discussed how human placental multipotent mesenchymal stromal cells may modulate trophoblast migration via Rap1 activation. The Rap1 regulatory pathway was examined in human trophoblast cells after stimulation with hepatocyte growth factor (HGF) secreted by human placental multipotent mesenchymal stromal cells (hPMSCs). HGF induced trophoblast cAMP production with the downstream pathways mediated through PKA and Epac1 to trigger trophoblast Rap1 and integrin β 1 activation. The total trophoblast cell integrin β 1 protein did not change, but the cell surface active form of integrin β 1 was significantly increased. hPMSC conditioned medium enhanced trophoblast migration, which was decreased by c-Met blocking antibody or conditioned medium containing lower levels of HGF from hPMSCs cultured in hypoxia. Thus, trophoblast cells may acquire signals for migration from villous hPMSCs.

Alessandro Rolfo discussed increased production of macrophage migration inhibitor factor and other pro-inflammatory cytokines by pre-eclamptic placental mesenchymal stem cells. Placenta-derived mesenchymal stem cells (PDMSCs) are well renowned for their unique anti-phlogistic activity. Since pre-eclamptic placentae are characterized by exacerbated inflammation and increased cytokine production, it was hypothesized that pre-eclamptic-PDMSCs possess altered anti-inflammatory properties. By cytokine array, it was demonstrated that there was increased release of pro-inflammatory mediators by pre-eclamptic-PDMSCs versus controls. In particular, significantly increased levels of pro-inflammatory MIF (macrophage migration inhibitory factor) and TNF- α (tumor necrosis factor α) were reported. Moreover, it was found that explants treated with pre-eclamptic-PDMSC conditioned media showed significantly increased MIF expression relative to controls. These data suggest that pre-eclamptic-PDMSCs possess an aberrant pro-inflammatory action.

Anna Maria Nuzzo discussed characterization of activator protein-1 (AP-1) family gene expression in PDMSCs derived from normal and pre-eclamptic pregnancies. Mesenchymal stromal cells control proliferation and apoptosis in neighbouring cells. Less is known about PDMSCs and their contribution to proper trophoblast development. It was found that there is differential expression of AP-1 family genes, key cell fate regulators, in PDMSCs and placental chorionic tissue from normal and pre-eclamptic pregnancies, characterized by excessive trophoblast apoptosis. Pre-eclamptic-PDMSCs presented increased pro-apoptotic JunB expression versus controls, while pre-eclamptic placentae showed decreased anti-proliferative JunD and increased pro-inflammatory Fra1 levels. These data suggest native differences in AP-1 gene expression among healthy, pre-eclamptic-PDMSCs and neighbouring chorionic tissue, thus indicating a contribution of PDMSCs to physiological and pathological placenta development.

Julia König discussed secretion of angiogenic growth factors by amnion-derived mesenchymal stromal cells. To investigate whether mesenchymal stromal cells (MSC) from the amnion of human term placenta (hAMSC) have angiogenic properties similar to bone marrow-derived MSC (BMSC), the paracrine effects of hAMSC on network formation and viability of endothelial cells were examined. hAMSC-conditioned medium stabilized endothelial networks in the Matrigel assay and enhanced endothelial viability as shown by a

lactate dehydrogenase (LDH) assay. Protein analysis of hAMSC-conditioned medium with an angiogenesis array kit revealed secretion of pro-angiogenic factors such as angiogenin and IL-8. This suggests that hAMSC might be a useful alternative to BMSC for therapeutic applications in vascular biology.

Edward Chuong discussed evolutionary aspects of trophoblast stem cell biology. The placenta has undergone significant morphological diversification during eutherian evolution. As a consequence, it has proven difficult to elucidate a conserved set of genes that establish trophoblast identity in all mammals. RNA-Seq was performed on the earliest trophoblast population, trophoblast stem cells, from mouse, rat, rabbit and rhesus macaque. By comparing the trophoblast stem cell transcriptomes of multiple mammals, it is possible to focus on which genes may have been expressed in the ancestral trophoblast, and have remained conserved throughout eutherian evolution.

5.3. Conclusions

This workshop highlighted the varied research into placental stem cells of varying origin. In particular human placental mesenchymal stem cells were shown to be a rich source of cytokines and angiogenic growth factors that may play roles in placental vascular stability and in regulating trophoblast invasion. In addition, their properties may be altered in pre-eclampsia, although whether this is a cause or effect of the condition is not clear. In animal models membrane-derived stem cells appear to play roles in immunomodulation of surrounding host tissue after transplantation. Trophoblast-stem cells also provide a species comparator for evolutionary studies. The encouraging data provided in this workshop, although preliminary, prompts further investigations from both stem cell researchers and placental biologists. Stem cell researchers need a better understanding of the basic biology of placental stem cells in order to assess, and improve, their potential utility in future clinical settings. Placenta biologists need to further explore these cells because abnormalities in their functions are likely to contribute to clinically significant placental pathologies.