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IFPA meeting 2018 workshop report I: Reproduction and placentation among ocean-living species; placental imaging; epigenetics and extracellular vesicles in pregnancy

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ABSTRACT

Workshops are an important part of the IFPA annual meeting as they allow for discussion of specialized topics. At IFPA meeting 2018 there were nine themed workshops, four of which are summarized in this report. These workshops discussed new knowledge and technological innovations in the following areas of research: 1) viviparity in ocean-living species; 2) placental imaging; 3) epigenetics; and 4) extracellular vesicles in pregnancy.

1. Reproduction and placentation among ocean-living species

Chairs: Hiroaki Soma and Anthony M. Carter.
Speakers: Anthony M. Carter, Charles F. Cotton, Satoshi Hayakawa, Mari Kawaguchi, Keiichi Sato, Hiroaki Soma, Takateru Tomita, and Camilla M. Whittington.

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1.1. Outline

Anthony Carter opened by noting that many teleosts and a majority of sharks are viviparous. They employ various strategies for the supply of nutrition to the embryos of marine vertebrates. These range from histotrophic nutrition, as in the brood pouch of male sea horses and pipefish or the uterus of the great white shark, to true placentation as in requiem sharks.

1.2. Summary

Camilla Whittington said there are at least 23 independent origins of viviparity in fish, but syngnathid fish (seahorses and pipefish) are unique in exhibiting male pregnancy. Male seahorses and pipefish have evolved specialized brood pouches that provide protection, gas exchange, osmoregulation, and limited nutrient provisioning to developing embryos. Her work had focused on identifying the genetic and physiological changes underpinning male pregnancy in seahorses, which have the most complex brood pouch morphology (*Hippocampus abdominalis*). The brood pouch facilitated a close apposition of paternal and fetal tissues to form a placenta. She had identified paternal changes during pregnancy associated with brood pouch remodeling, nutrient and waste transport, gas exchange, osmoregulation, and immunological protection of developing embryos, as well as parturition. These genetic data provided testable hypotheses about the functions of the seahorse brood pouch during pregnancy, which she followed up with physiology-based experiments. Key shared mechanisms underpinning pregnancy and birth in seahorses and other vertebrates suggest a common toolkit of genes regulating pregnancy in divergent evolutionary lineages.

Mari Kawaguchi described how the brood pouch was formed during the development of male seahorses from juvenile to adult. The primordium emerged as linear projections at the ventro-lateral sides of the body. These projections then elongated and fused at the body midline. Finally, a baggy structure was formed. The brood pouch specific tissue or pseudoplacenta, which plays important roles during incubation, then developed to surround the lumen, ready to incubate embryos.

Satoshi Hayakawa said that though viviparity evolved several times in invertebrate animals, placenta associated immune regulation including non-classical major histocompatibility complex (such as HLA-G), PD-1/PD-L1 system, and FoxP3 + regulatory T cells first appeared in jawed vertebrates. Together with Wahei Yoshida and Kiyoshi Asahina, he had observed sequential changes in yolk sac umbilical cord and placental structure of the blacktip reef shark (*Carcharhinus melanopterus*) with immunohistochemical methods and also searched for immune-related genes from vertebrate and non-vertebrate genome databases (Global Invertebrate Genomics Alliance). This work had revealed that co-evolution of placental viviparity and the adaptive immune system was the fruit of two rounds of gene duplication which took place 500 million years ago.

Charles F. Cotton noted that most viviparous elasmobranchs lack a connection with the mother. Thus, the embryos must acquire oxygen from the surrounding uterine fluid for a period ranging from several months to two years. To test the hypothesis of uterine-supplied oxygen delivery, he had applied a “gas diffusion model” to the uterine wall of two dogfish species (*Squalus* cf. *mitsukurii and S. cubensis*) and compared theoretical delivery to the theoretical demand of developing embryos. This model showed that oxygen supply via diffusion through the uterine wall contributed less than 15–30% of the total oxygen demand of late-stage embryos, suggesting an alternate mode of oxygen delivery, likely uterine flushing.

Introducing a section on reproduction in the great white shark (*Carcharodon carcharias*), **Keiichi Satoh** noted that gestation in viviparous sharks and the maternal input to intrauterine embryos can be very complex. In lamniform sharks, including the great white shark, oophagy was one of the primary modes of embryonic nutrition. However, the nutrition of embryos appeared to be more complex than thought previously as embryos probably relied on a changing source of nutrition over the course of their development. Lipid-rich fluid was secreted from the uterine epithelium only in early gestation before the onset of oophagy; the embryos probably used the abundant uterine fluid, and then encased nutrient eggs, for nutrition at this stage of their development; but the uterine fluid was the major source of embryonic nutrition before oophagy onset. Histochemical staining suggested that the villous strings of the uterine epithelium were implicated in the secretion of lipid droplets and at least two types of PAS-positive granular and fluid substances. Lipid secretion in the white shark was a novel mode in shark reproduction, and resembled that from the trophonemata of pregnant manta rays.

Hiroaki Soma then described findings on the fine structure of the pregnant uterus of a great white shark weighing 1526 kg caught by drift-net fishing in the Okinawa Islands Sea. There were three embryos on each side of the uterus without placentation. The uterus contained a large amount of milk and egg-shells. The uterine specimens were investigated by histochemistry and electron microscopy. The thickened uterine endometrium stained well histochemically with PAS, hPL and SP1 in addition to GLUT-1 and GLUT-3. The surface ultrastructure of the uterine endothelium showed mosaic sheet patterns. In the endometrial gland surface of uterine epithelium, very active milk-like proteins were produced and released to the uterine cavity for nourishment of the fetuses. It was concluded that uterine endometrium served as an alternative to placentation for fetal nutrition in the pregnant uterus of the great white shark.

Takateru Tomita added that one of the mysteries of great white shark reproduction is how the embryo acquires oxygen in utero without a placental connection. His group had applied the “gas-diffusion model” to the great white shark uterus, and revealed that it had a high capacity for oxygen exchange, which was almost comparable to that of fish gills. This result supported the hypothesis that, unlike in dogfish, embryonic respiration was fully supported by oxygen diffusing from the uterine wall. The study shed novel light on the mechanism of oxygen transfer from mother to embryo in non-mammalian vertebrates.

1.3. Conclusions

Viviparity is an important biological innovation that has evolved convergently many times in mammals, reptiles, fish, amphibians, and invertebrates. It is therefore an ideal model to study evolutionary innovations, offering the opportunity to compare and contrast naturally replicated evolutionary experiments. Seahorses are unique in their mode of reproduction as the male, not the female, carries embryos in a brood pouch located on the ventral surface of the tail. Viviparity in sharks is instructive because it is seldom associated with true placentation. Alternative strategies have been adopted to supply the developing embryos with oxygen and nutrients. Thus, consideration of viviparity in these ocean-going species offers a unique opportunity to study the convergent evolution of matrotrophy, both with and without a placenta.

2. Placental imaging

Chair: Ganesh Acharya and Junichi Hasegawa.

Speakers: Ganesh Acharya, Junichi Hasegawa, Shoichi Magawa, and Anne Sørensen.

2.1. Outline

Different modalities of placental imaging are used to study its structure and function from molecular/subcellular to organ/system level. Some of them are emerging new techniques, whereas others are a refinement of conventional imaging modalities that has been possible with the advancement in technology. This workshop presented recent

advances in some of the most important methods of placental imaging (ultrasound, magnetic resonance imaging and microscopy) applicable to basic, clinical and translational research in placentology.

2.2. Summary

Ganesh Acharya discussed the application of high-resolution live cell imaging in placental research. Light microscopy has the advantage of live cell imaging compared to other techniques, such as electron microscopy, but lower resolution has been its major limitation. Recent developments in optical nanoscopy, such as structured illumination microscopy (SIM), have allowed high-resolution imaging of the smallest human cells, such as spermatozoa, and their subcellular structures without the use of electron microscopy. However, it requires the use of fluorescent labeling which may be toxic to cells. On the other hand, quantitative phase microscopy (QPM) can be utilized for label-free imaging, and phototoxicity can be avoided as the phase information is obtained from a single recorded intensity pattern. Morphological changes in the trophoblasts and other placental cells exposed to different conditions can be studied and tracked *ex vivo* using these imaging methods. Combining SIM and QPM can be useful as fluorescence microscopy provides excellent morphological information with subcellular resolution, while phase microscopy provides quantitative information. Multimodal microscopic imaging modalities may become standard techniques of evaluating cellular structure and function in trophoblast research in the near future.

Anne Sørensen reviewed T2* weighted placental MRI as a promising marker of placental dysfunction. The potential of detecting placental dysfunction *in vivo* has increased interest in placental MRI over the last decade. In particular, T2* weighted MRI has proven to be a simple and useful method of assessing placental function. Previous studies have demonstrated that the dysfunctional placenta has an increased hyperoxic response in T2* signal intensity, which is mainly caused by a low baseline T2* relaxation time. From a clinical perspective, this method may be a simple tool to discriminate between constitutionally small fetuses and fetuses suffering from intrauterine growth restriction and placental hypoxia.

Junichi Hasegawa discussed the application of Superb Microvascular Imaging (SMI) with high frequency ultrasound transducers in placental evaluation. The technological improvement of high frequency linear ultrasound transducers offers significant clinical benefits since the anatomical structures and hemodynamics of minute vessels can be delineated. SMI is a new blood flow imaging technique that employs a unique algorithm to minimize motion artifacts. This improved imaging technique is useful for evaluation of structural placental abnormalities, such as placental infarction, hematoma, and abnormally invasive placentation, as well as placental vascularity and blood flow in fetal growth restriction.

Shoichi Magawa presented the findings of his research using non-invasive blood oxygen level dependent magnetic resonance imaging (BOLD-MRI) to investigate human placental intravillous capillary and fetal brain oxygenation during maternal oxygenation. Magawa and colleagues evaluated the placenta and fetal brain in late pregnancy of healthy Japanese women by BOLD using their own protocol. In all cases of normal pregnancy, the BOLD value ($\Delta T2^*$) increased due to maternal oxygen administration, and it will be possible to compare the BOLD value of normal and abnormal pregnancies in the future. The BOLD value of the fetal brain did not change even in late pregnancy, due to auto-regulation of fetal cerebral blood flow. They also used BOLD in cases with intrauterine fetal death and discussed placental hemodynamics after fetal demise.

2.3. Conclusion

Major advances are happening in imaging technologies that are applicable to study placental structure and function in research settings. However, it is important to identify strengths, limitations, and pitfalls of using different imaging techniques to evaluate placenta. Defining indications regarding their application for screening and diagnostic purposes, standardizing protocols and improving interpretation of findings are important for optimal use of these techniques both in research as well as clinical settings.

3. Epigenetics

Chairs: Leslie Myatt and Kiyonori Miura.

Speakers: Marisa Bartolomei, Chaini Konwar, Kiyonori Miura, Hidenobu Soejima, and Victor Yuan.

3.1. Outline

Placental function is known to be affected significantly by the intrauterine environment, which in turn is influenced by amount and type of nutrition, maternal stress, hormonal and inflammatory milieu among others. These varying environmental signals influence the placenta epigenome but we lack detailed information related to effects on specific placental cell types, differences across gestational age, and whether or how the changes seen at the epigenetic level relate mechanistically to differences in transcription and ultimately in placental function. In this workshop we discussed interpretation of epigenetic data, and current knowledge regarding the influence of sex, ethnicity, cellular composition, gestational age and environmental conditions on placental epigenetics and how this relates to placental function.

3.2. Summary

Victor Yuan and **Chaini Konwar** presented their research on population-specific DNA methylation differences and their involvement in placental pathologies. DNA methylation (DNAm) is an epigenetic modification that can affect gene expression and can be influenced by genetic and environmental factors. As in other tissues, our group has identified significant population-specific variation in placental DNAm. We also found that differences in placental allele frequencies of immune-system genes such as *IL6* were associated with chorioamnionitis only in specific populations. Additionally, DNAm was altered in chorioamnionitis-affected placentas and the *IL6* genotype significantly influenced DNAm levels, which negatively correlated with gene expression. Therefore, placental DNAm studies should account for population specific variability, as differences in population structure can confound the variable of interest, and thus may drive DNAm differences between groups.

Marisa Bartolomei discussed the regulation of DNAm in the placenta from an imprinted gene perspective. Imprinted genes comprise a small number of genes in mammals and are expressed from a single parental allele. These genes, which are found in clusters, are the main block to uniparental development. That is, uniparental maternal embryos develop into tissues of embryonic origin with a failure of extraembryonic development and uniparental paternal embryos develop into extraembryonic/placental derivatives, with a failure of embryonic development. Imprinted genes are regulated by DNAm at imprinting control regions (ICRs). DNAm at ICRs is acquired in the maternal or paternal germline, maintained when the embryo undergoes post-fertilization reprogramming, and erased during gametogenesis to prepare for the next generation. DNAm erasure employs both active and passive DNAm strategies and deletion of *Tet1*, an enzyme that oxidizes methylcytosine, results in defects in DNA methylation reprogramming and imprinted gene perturbations.

Hidenobu Soejima presented their data on the association between the imprinting disorder Beckwith-Wiedemann syndrome and placental mesenchymal dysplasia. Beckwith-Wiedemann syndrome (BWS) is caused by aberrant expression of imprinted genes due to several genetic or epigenetic abnormalities at 11p15.5. A subset of placental

mesenchymal dysplasia (PMD), a morphological aberration of the placenta defined by placentomegaly and multicystic changes, is associated with infants with BWS and androgenetic/biparental mosaicism (ABM), suggesting disrupted imprinting. Soejima and colleagues analyzed PMD tissues genetically and epigenetically and found that most PMDs showed ABM, but some had normal biparental inheritance. In biparental cases, aberrant methylations at several imprinted genes were found.

Kiyonori Miura discussed the clinical significance of C19MC and C14 MC microRNA in perinatal management. Pregnancy-associated microRNAs (miRs) on the chromosome 19 miR cluster (C19MC) region are imprinted in the placenta with expression from the paternally inherited chromosome. The pregnancy-associated, but not placenta-specific, miR-323–3p is located on the chromosome 14 miR cluster (C14MC) region, which is imprinted in embryonic and placental tissues with expression from the maternally inherited chromosome. The plasma concentration of miRs from the C19MC and C14MC regions can be measured by quantitative real-time reverse transcription (RT)-PCR, and aberrant levels have been reported in various pregnancy-associated diseases and abnormal pregnancies (e.g. preeclampsia, molar pregnancy, ectopic pregnancy).

3.3. Conclusions

This workshop highlighted the role of DNA methylation in gene expression in different settings. The influence of differences in population structure on driving differences in methylation between groups was clearly illustrated. The role of DNA methylation at imprinting control regions in regulation of imprinted genes was highlighted and it appears both active and passive DNA methylation strategies are involved. A subset of placental mesenchymal dysplasia is associated with disrupted imprinting in infants with Beckwith Wiedemann Syndrome with some showing androgenetic/biparental mosaicism but some with normal biparental inheritance with aberrant methylation at several imprinted genes, illustrating the complexity of the phenomenon. Imprinting also regulates expression of microRNAs on chromosome 19 (paternal) and 14 (maternal) with differential expression and appearance of these miRNAs in maternal plasma with pregnancy complications.

4. Extracellular vesicles in pregnancy

Chairs: Carlos Salomon and Hirotaka Nishi.

Speakers: Larry Chamley, Yuri Hasegawa, Carlos Salomon, and Hironori Takahashi.

4.1. Outline

During the past decade, there has been an extraordinary explosion of research in the field of extracellular vesicles (EVs), especially in the specific type of EV originating from endosomal compartments called exosomes. EVs are released from a wide range of cell including the human placenta and are capable of transferring their contents (e.g. proteins and miRNAs) to other cells, a process that is thought to be essential to several biological processes including immune response, cell metabolism and intercellular communication during pregnancy. Unfortunately, progress in the field has been hindered by a lack of standardized protocols relating to the taxonomy and isolation of exosomes. This has confounded data interpretation within the current body of literature. This workshop discussed the heterogeneity, isolation, purification and characterization of placental exosomes and their capacity to interact and deliver bioactive molecules to target cells during pregnancy.

4.2. Summary

Larry Chamley discussed the interaction between extracellular vesicles secreted from the human placenta with maternal tissues. It has been known for more than a century that once deported from the placenta, trophoblast macrovesicles/syncytial nuclear aggregates are trapped in the capillaries of the maternal lungs but the much smaller placental micro and nanovesicles would intuitively be expected to pass through the maternal lungs and be distributed throughout her body. However, we have shown that is not the case and that both micro and nanovesicles show considerable tropism for the lungs and also are targeted to the liver while nanovesicles but not microvesicles also target the kidneys. Neither type of placental vesicles target to the other investigated organs including the spleen. Comparison of the interactions between placental microvesicles and leucocytes *in vitro* and *in vivo* suggests that *in vitro* experiments may overestimate this interaction.

Carlos Salomon discussed the variability of isolation methods for different types of extracellular vesicles with an emphasis on exosomes. The term extracellular vesicle is a non-specific classification that suits all membrane-bound vesicles of different sizes and biogenic origins (i.e., endosomal and plasma membrane origins). Exosomes are a subtype of extracellular vesicles that are defined explicitly by endosomal biogenesis and particle size (around 100 nm) and density (1.13–1.19 g.ml-1) in a sucrose gradient. Several reports have described the presence of exosomes and other types of extracellular vesicles in maternal circulation under normal and pathological conditions including preeclampsia, gestational diabetes, preterm birth, and fetal growth restriction. The levels of circulating exosomes seem to be pregnancy-condition specific and dependent on gestational age. To understand the role of extracellular vesicles during pregnancy several sources of vesicles have been used such us primary cell (e.g., trophoblast cells), cell lines (e.g., BeWo, JEG-3, and HTR-8/Svneo), placental perfusion, and placental explants. Finally, several methods to isolate extracellular vesicles and enrich a specific type such as exosomes have been used; however, inconsistency in these procedures might compromise the interpretation and reproducibility of the results.

Yuri Hasegawa discussed the association between placental microRNA and placental abnormalities. Hasegawa and colleagues identified aberrant circulating levels of pregnancy-associated placenta-specific miRNA in women with diseases caused by placental dysfunction (e.g. placenta previa and gestational trophoblastic disease). Several placental miRNAs on chromosome 19 miRNA cluster region (C19MC) are associated with the development of placental vessels. Therefore, miRNAs predominantly expressed in the placenta are probably involved in placental differentiation and in the maintenance of pregnancy.

Hironori Takahashi presented the potential role of exosomal placental-associated microRNA for extravillous trophoblast (EVT). EVT invasion into the decidua is essential for successful pregnancy, yet it is unclear how it is regulated. Takahashi and colleagues investigated whether placenta-associated miRNAs derived from C19MC are involved in EVT invasion. Placenta-associated miRNAs were significantly downregulated in EVTs compared with first-trimester chorionic villous trophoblasts (CVTs). Next, they hypothesized that CVT-derived exosomal placenta-associated miRNAs transferred to EVT. Using an *in vitro* model system, BeWo-derived exosomal miRNAs were internalized into the EVT cell lines with subsequently reduced cell invasion via target gene repression.

4.3. Conclusions

In the last ten years, we have seen an explosion in the extracellular vesicles field, and specific types of extracellular vesicles called exosomes have received the primary attention. The different types of extracellular vesicles can be classified as exosomes, microvesicles, and apoptotic bodies. Exosomes are small vesicles of around 100 nm originated from the endosomal compartment usually enriched in CD63,

TSG101, CD81, and CD9 proteins. Microvesicles or shedding vesicles are 50–1000 nm in size, budding from the plasma membrane, and are enriched in CD40 protein. Apoptotic bodies are 800–5000 nm in size and are fragments from dying cells.

All types of extracellular vesicles have been identified in maternal plasma; however, important questions about their biodistribution and interaction with maternal tissues have not yet been answered. Placental extracellular vesicles are packed with signaling molecules such as miRNAs that may regulate the activity of both proximal and distal target cells, including trophoblast migration and placental development. As such, exosomal signaling represents an essential pathway mediating intercellular communication. Finally, it is urgent that methods to isolate vesicles are standardized to increase the reproducibility of extracellular vesicle research.