CTR Annual Trophoblast Meeting 9 – 10th July, 2012

Day One Presentations

14.15 – 15.30 Placental Development
Chaired by: Peter Rugg-Gunn, University of Cambridge

Roberta Hannibal, Stanford University
Genomic copy number variation during trophoblast giant cell endoreplication
Roberta L. Hannibal, Edward Chuong, & Julie C. Baker
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The placenta is a mammalian specific organ crucial for fetal development. A key feature of the placenta is a polyploid trohoblast cell type that invades and remodels the uterus to promote flow of blood and nutrients to the fetus. In rodents, these are called trophoblast giant cells (TGCs) and have up to 1,000N DNA content due to endoreplication. Recent work has shown that placental specific defects in TGC endopolyploidy cause impairment to fetal growth, resulting in perinatal death. However, the function of endopolyploidy in TGCs remains unknown. Two hypotheses are that 1) polyploidy acquires the necessary quantity of genes while saving materials and time and 2) polyploidy regulates gene expression by selectively amplifying certain genomic regions. We examined the genomic organization of TGCs using array comparative genomics hybridization (aCGH). We found that certain regions of the genome are preferentially underreplicated (UR domains). To further investigate, we compared our aCGH data to our data on RNA expression and the histone modifications H3K27ac, H3K4me1 and H3K4me3 in cultured TGCs and their progenitors, trophoblast stem cells (TS cells). We found that UR domains anticorrelate with RNA expression and active histone marks in both TGCs and TS cells. As active histone marks anticorrelate with late replicating DNA in other 2N cell types, we are pursuing the hypothesis that replication timing in TS cells causes UR domains in TGCs. Endocycles may be progressing with such speed that they do not have time to replicate late-replicating DNA. This suggests that endocycle speed is crucial to TGC function, supporting the hypothesis that polyploidy is important for acquiring the necessary quantity of genes while saving time.

Sandra Haider, University of Vienna
Notch signaling controls column proliferation, trophoblast invasion and extravillous trophoblast differentiation
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Objective: Notch signalling is a highly conserved pathway controlling development, tissue homeostasis, differentiation and cell death. Here, we investigated for the first time localization of all Notch receptors (Notch1-4), their activated Notch intracellular domains (NICD) as well as the Notch ligands (Jag1 and 2, and Dll1,3,4) in early human placentae. Moreover, the role of Notch signalling in cell column (CC) proliferation, trophoblast invasion and extravillous trophoblast (EVT) differentiation was analyzed by using different trophoblast model systems.

Methods: Protein expression patterns were analyzed in tissue sections of normal first trimester placentae, placentae of complete hydatidiform mole (CHM) pregnancies, and different trophoblast models by using immunofluorescence and Western blotting, respectively. Notch activity was evaluated in the trophoblast cell line SGHPL-5 and in primary EVT using a luciferase reporter containing either wildtype or mutant binding sites for RBPJκ, the NICD-dependent, key-regulatory transcription factor in Notch signalling. Migration and invasion were studied in transwell assays of SGHPL-5 cells (uncoated) and primary EVT (Matrigel-coated), respectively, as well as in villous explant cultures seeded on collagen-I. Proliferation was measured by BrdU labelling of floating villous explant cultures. Notch target gene expression was evaluated by real-time PCR. Experiments were performed in the absence or presence of the Notch/γ-secretase inhibitors DAPT and L-685458. Cross-talk to Wingless (Wnt) signalling was analyzed by investigating a canonical Wnt reporter in NICD1-overexpressing SGHPL-5 cells.

Results: Immunofluorescence revealed subtype-specific combinations of distinct Notch receptors and ligands in villous cytotrophoblasts (vCTB), CC, endovascular trophoblasts and interstitial EVT, the latter expressing Dll-1,-4, Jag1, -2, Notch2 as well as nuclear NICD2. Notch1 is specifically expressed in a subset of proliferative CC trophoblasts likely representing progenitors of the invasive differentiation pathway. Moreover Notch1 is expressed in all vCTB of CHM placentae, but absent from vCTB of age-matched normal placentae. RBPJκ reporter assays revealed basal Notch activity in SGHPL-5 cells and primary EVT which could be inhibited upon DAPT or L-685458 treatment. Notch reporter activity increased in primary EVT upon in vitro differentiation on fibronectin. Inhibition of Notch signalling increased invasion and migration of the different trophoblast model systems, but also elevated proliferation in cell columns. Moreover, total numbers of EVT as well as markers of EVT differentiation (integrin α1, HLA-G, MMP-2, TCF-4) were elevated upon addition of DAPT or L-685458. Activation of the pathway through NICD1 overexpression suppressed Wnt signalling.

Conclusion: Notch signalling could play a major role in controlling EVT differentiation since inhibition of the pathway increased cell column proliferation, invasion and EVT marker expression. In proliferative trophoblasts Notch signalling may negatively affect Wnt signalling, the latter being associated with EVT differentiation. However, complex expression patterns of Notch and ligands suggest trophoblast subtype-specific roles of the pathway warranting further functional studies on individual Notch receptors and ligands. Notch1 expression in vCTB of CHM placentae could indicate aberrant maintenance of a stem cell-like phenotype possibly associated with the pre-malign conditions of the gestational disease.

Ina Leinweber, Leibniz Institute for Zoo and Wildlife Research, Berlin

The marsupial yolk-sac placenta expresses MHC class Ia homologues

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Pregnancy in eutherians and marsupials requires that fetal and maternal tissues reside in close apposition to each other. In well studied eutherian mammals, there is evidence for silencing of classical class I MHC molecules at the fetal-maternal interface. However, this specific regulation of class I molecules may not be necessary in the Marsupialia where the conceptus is enclosed in a protective shell coat for up to 80% of gestation, and the period of placental/uterine interaction is relatively short. To better understand this process in marsupials we have characterised mRNA and protein expression of the recently described MHC class I loci of the tammar wallaby *(Macropus eugenii)* from the period of shell coat rupture (day 18) through to the last days (25/26) of pregnancy. We found prevalent expression of class Ia molecules in both tri- and bi-laminar yolk sac tissues throughout the attachment phase of pregnancy. These data suggest that the short-lived placenta of marsupials expresses the same complement of class I MHC molecules as normal nucleated cells and that there is no silencing of gene expression during the main period of fetal-maternal interaction. The presence of MHC class I molecules suggests that the fetus is either not affected by the maternal immune system, or that the short pregnancy of marsupials allows birth prior to any maternal immune challenge. The elaboration of lactation instead of placentation in many marsupials may be a strategy to avoid immunological conflict.

Margaret Brosnahan, Cornell University

Interleukin 22 is expressed by the invasive trophoblast of the equine (*E. caballus*) chorionic girdle

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Invasive trophoblast cells of the equine chorionic girdle migrate through maternal endometrium to form endometrial cups, dense accumulations of trophoblast cells that produce equine chorionic gonadotropin (eCG) between days 40 and 120 of pregnancy. The invading trophoblast cells are distinctive in their ability to evade maternal immune destruction; these mechanisms are not well defined. We performed gene expression microarray analysis on placental tissues obtained at day 34 of gestation and observed greater than 900-fold upregulation of mRNA encoding the cytokine interleukin 22 in chorionic girdle relative to non-invasive chorion. We then used quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) to verify high expression of IL22 in chorionic girdle. Additional qRT-PCR analysis showed a striking increase in IL22 mRNA expression in chorionic girdle from days 32 to 35, and

absence of IL22 expression in other conceptus tissues. Bioinformatic analysis and cDNA sequencing confirmed the predicted length of horse IL22, and demonstrated a 3' extension absent in IL22 genes of humans and mice but present in the cow and pig. Our discovery of IL22 in the chorionic girdle is a novel finding, as this cytokine previously has been reported in immune cells only. IL22 has immunomodulatory functions, with primary action on epithelial cells. IL22 receptor mRNA was detected in pregnant endometrium at levels similar to other equine epithelia. Based upon these findings we hypothesize that IL22 cytokine produced by the chorionic girdle binds IL22R1 on endometrium, serving as a mechanism of fetal-maternal communication by modulating endometrial responses to trophoblast invasion.

Stephanie Kaiser, University of Essen

TFAP2c dependent trophoblast lineage differentiation during placental development

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The placenta is the first organ to be formed during pregnancy and impairment of placental formation leads to various pregnancy complications. It has been shown that the highly conserved family of Activator Protein-2 (AP-2/TFAP2) is important for placenta development. We could demonstrate a marked downregulation of TFAP2c expression level in lineage development of trophoblast stem cells (TSCs) lacking connexin 31 (CX31) which could cause the placental phenotype observed in CX31 deficient mice. Here we investigated if the failure of an appropriate lineage development in CX31 deficient TSCs is probably due to a reduced expression level of TFAP2c. Because complete TFAP2c deficiency causes early embryonic death on day 7.5 of gestation due to a loss of extraembryonic cell lineage development we investigated mice heterozygous for TFAP2c.

Placentas of TFAP2c heterozygous mice which express reduced levels of TFAP2c revealed enhanced resorption sites at ED 14.5 (52% compared to 5.7% in wild type matings). Phenotypic analysis of ED 14.5 placentas showed a reduced spongiotrophoblast layer which was identified by using the marker gene *tpbpa*. Moreover development of the labyrinth was impaired indicated by higher proliferation rates and large clusters of undifferentiated cells, both signs of delayed differentiation. The clusters showed staining for tpbpa, PAS and KI67 which indicates an accumulation of glycogen rich and proliferative cell populations. In addition, staining for CD31 revealed less and poorly branched embryonic vessels in the labyrinth.

These findings point to an impaired trophoblast lineage differentiation in heterozygous TFAP2c mice leading to reduced spongiotrophoblast formation combined with a delay in labyrinthine development which could be the reason for embryonic lethality.

15.45-17.00 Placental Metabolism and Transport
Chaired by: Abby Fowden, University of Cambridge

Rohan Lewis, University of Southampton

Placental amino acid metabolism: does the placenta feed the mother?

Placental metabolism can alter both the quantity and composition of amino acid within the placenta and thus modify the amino acid pool available for transport to the fetus. Placental synthesis of the metabolically important amino acid glutamine has been observed in several species and we have now demonstrated this in humans.

While the presumption has been that placental amino acid synthesis creates amino acids for transport to the fetus our recent data suggest that this may not be the case. Glutamine synthesised by the isolated perfused human placenta was found to be predominantly transported to the maternal circulation. This raises questions about the biological role of placental amino acid synthesis and the metabolic interactions between the placenta, mother and fetus.

Transfer of placentally derived glutamine to the mother could play several different roles. Placental release of glutamine into the maternal circulation may lessen the demand on maternal organs to make glutamine for inter-organ cycling at a time when placental demand is stressing the system. It may provide a mechanism by which ammonia generated by placental amino acid catabolism can be safely transported to the mother. It may drive placental uptake of maternal amino acids by exchange, increasing the availability of key amino acids in the placental amino acid pool and increasing their transfer to the fetus.

This work suggests that synthesis of glutamine by the human placenta may have multiple roles which influence both maternal and fetal metabolism.

Modou Jobarteh, University of Aberdeen

Effect of nutritional supplementation on placental transport function in Gambian women

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Nutritional deficiencies are common in developing countries. These become more significant during pregnancy. This is more of a problem in relation to multiple micronutrient (MMN) deficiency and in energy supply. Supplementation with MMN and energy has been shown to be beneficial, but little is known about the mechanisms involved. The placenta plays a central role in providing nutrition to the developing fetus, and it may be that supplements act, at least in part, by improving placental function. This project will examine placental transport function in MMN and protein energy supplemented women.

In a double blind randomised control trial, placenta samples were collected from pregnant women given either iron-folate, iron folate plus MMN, plus high energy supplements or both. Supplements were started at less than 20 weeks gestation. Generally, placentas were collected as quickly as possible after birth and were transferred to the laboratory on ice. The time from collection to delivery in the lab was recorded. Samples were taken, stored in RNA later, fixed for histology and frozen in -80ºC as appropriate.

Preliminary analysis of data collected at birth shows boys have a higher mean birth weight (3.087kg Vs 2.986kg, p-value=0.02) and placental length (18.87cm Vs 18.37cm, p-value= 0.01) than girls. The placental fetal weight ratio was not significantly different between boys and girls (0.1694 and 0.1733. p value= 0.25 respectively). Low birth weight babies have lower mean placental weigh (427.4g vs. 526g, p value 0.0001) and gestational length (38.93 weeks vs. 40.05 weeks, p value 0.0001). However, in this cohort placental/neonatal weight ratio is not changed in low birth weight babies compared to normal. There was no effect of season on birth weight, placental weight or placental/neonatal weight ratio. Placenta samples collected and processed within 90minutes of delivery had a better RNA integrity measured with Agilent bioanalyzer.

The results are preliminary and functional parameters remain to be measured. However, the data do suggest that the placenta may have a role in regulating growth in utero. This study will be measuring transporters of iron, zinc amino acid and glucose on both gene and protein level in placenta from the different supplemented group. We also aim to study actual uptake of analogues of iron, glucose and zinc in the placental villous samples.

Michelle Desforges, University of Manchester

Reduced placental taurine transporter activity and nitrative stress in pregnancies complicated by pre-eclampsia

Authors: Chloe Hirst, Susan L Greenwood & Michelle Desforges

Taurine is a conditionally essential amino acid in human pregnancy, necessary for fetal organogenesis. Fetal requirements for taurine are met by uptake from maternal blood into the syncytiotrophoblast via the taurine transporter protein, TauT. We have shown that siRNA-mediated TauT knockdown in primary trophoblast compromises their differentiation and increases apoptotic susceptibility, suggesting an important role for taurine in maintaining the syncytiotrophoblast. Pre-eclampsia (PE) is associated with fetal growth restriction (FGR) and abnormal maintenance of the syncytiotrophoblast. We measured syncytiotrophoblast TauT activity in PE and found that it was lower than in normal pregnancy but there was no difference in transporter protein expression. PE is also associated with placental oxidative and nitrative stress, the latter of which is known to alter the function of certain proteins by nitration of tyrosine residues. In several non-placental tissues, TauT is inhibited by nitration. We propose that post-translational modification of TauT through tyrosine nitration down-regulates transporter activity in PE. Our recent data demonstrates that nitration is higher in PE placentas, previously shown to have reduced TauT activity, than in normal pregnancy. Furthermore, inducing placental nitrative stress with SIN-1 *in vitro* significantly reduces TauT activity. We speculate that reduced TauT activity by nitration could contribute to abnormal syncytiotrophoblast maintenance and FGR in PE.

Luz Garcia, University of Granada

The impact of maternal obesity on iron status, placental transferrin receptor expression and neonatal growth in pregnant women and their offspring

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Obesity during pregnancy is associated with a decrease in iron status, possibly due to a rise in the inflammatory protein, hepcidin, known to reduce iron absorption. Iron deficiency during pregnancy has deleterious consequences for the mother and her offspring. In animal experiments, we have shown that maternal iron deficiency is compensated for by increased expression of transferrin receptor in the placenta, which in turn results in a rise in iron transfer, at the cost of maternal iron stores. This project was designed to answer two questions: a) does maternal obesity result in reduced iron status? And 2) can the placenta compensate by increasing transferrin receptor levels?

Placentas were collected at term from women participating in the Preobe study. Iron status was measured by serum transferrin receptor and ferritin levels and transferrin receptor expression in placentas by Western blotting. Women of different BMI (normal - BMI<25 kg/m^2; n:126), overweight (25≤BMI<30 kg/m^2; n:47) and obese (BMI≥30 kg/m^2; n:43) were classified according to iron status (IS: Iron sufficiency, Hb>110g/L, Ferritin (F)>12mg/L; ID without anaemia, Hb>110g/L, F<12mg/L; IDA: ID with anaemia, Hb< 110g/L, F< 12mg/L) measured at 24, 34 weeks of gestation and at delivery.

As expected, obese women gave birth to heavier babies (3231±422, n=131 vs 3475±507, n=44; *p*=0.008) with heavier placentas (474±113, n=90 vs 570±133, n=31; *p*=0.001). The placental/neonatal ratio was changed (0.14±(0.13-0.17), n=86 vs 0.16±(0.14-0.18), n=28, p=0.005). Iron deficiency was more common in obese women than controls during pregnancy (serum iron: 84.66±33.08, n=126 vs 69.95±25.26, n=43, p=0.009; TSAT index: 18.86±8.44, n=126 vs 15.83±6.20, n=43, p=0.014 and at delivery (ferritin: 25.18±13.98, n=82 vs 16.53±6.14, n=20, p<0.0001; sTfR: 20.91±8.82, n=72 vs 25.68±11.46, n=21, p=0.045). Further, pTfR expression was inversely related to maternal iron status, irrespective of whether the mother was obese or not. pTfR expression was also associated with neonatal parameters of iron status. Importantly, there was a significant inverse relation between pTfR and placental weight, suggesting total iron transport capacity may be compensated for in smaller placentas, as has been reported for

System A and amino acid transport (Godfrey, 1998). These results show that it is iron status, rather than obesity as such, that drives placental transferrin receptor expression. These results support those found by ourselves in animal experiments and also suggest that it may be of value to consider prophylactic iron supplementation to obese women during pregnancy.

Jane Cleal, University of Southampton

The microRNAs miR-27a and miR-143 regulate placental amino acid transporter mRNA levels

Fetal growth depends on placental transfer of amino acids from maternal to fetal blood. We have shown that this process is mediated by the facilitated amino acid transporters TAT1 (slc16a10), LAT3 (slc43a1) and LAT4 (slc43a2) in human placenta. In placentas from the highly characterised Southampton Women's Survey (SWS) *TAT1* mRNA levels were positively related to measures of fetal growth.

It is therefore important to determine how TAT1 is regulated in placenta. In mice, a maternal high fat diet impairs fetal growth and also decreases placental *Tat1* mRNA levels compared to controls. A high fat diet in mice has previously been shown to alter expression of microRNAs (miRNA), which results in post-transcriptional silencing of specific genes.

The miRNAs miR-143 and miR-27a are involved in regulating lipid metabolism, and miR-27a is predicted to regulate T*AT1* expression. In the SWS placentas, miR-27a expression negatively correlated with *TAT1* in male but not female placentas. Although not related to *TAT1* expression, placental miR-143 correlated with measures of growth in the offspring. Interesting sex specific associations were also observed between these miRNAs and the amino acid transporters *ASC1* and *LAT2*.

This data suggest that these miRNAs are involved in the regulation of amino acid transporter mRNA expression and may influence the transfer of amino acids to the fetus. By influencing the supply of amino acids to the fetus placental miRNAs may regulate fetal growth and underpin sex differences in placental mRNA levels and function.