Half-time report

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Placental cell type-specific transcriptional responses to maternal endocrinological and metabolic conditions

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Literature review

Abstract

A well-established perspective states that noncommunicable diseases result from the gene-lifestyle interaction[1]. The developmental origin of health and disease (DOHaD) theory expanded this perspective into the early stage of life[2]. It underscores that the periconceptual, fetal and early infant exposures to environmental factors including maternal nutrition and stress influence long-term health outcomes. The placenta is an organ that sits at the fetal–maternal interface. It sustains fetal nutrition, acts as a protective barrier against teratogenic factors, and registers a record of the intrauterine environment. Therefore, the placental function lays the foundation of the fetal programming phase in the (DoHaD) theory. Understanding its compromised functions holds significant implications for understanding the link between maternal endocrinological and metabolic health with offspring development.

Maternal PCOS and obesity impose enivronmental disruptors such as oxidative stress[3,4], hyperinsulinmea[5,6], hyperlipidimea[7] (not necessarily perturbed in PCOS[8]), and specific amino acid levels[9] on the placenta, potentially perturbing its homeostasis. These stressors likely prompt adaptations within placental cells, leading to adjustments in RNA[10] and protein synthesis[11-14], cytoskeletal[3] and membrane integrity[15], as well as metabolic states[16,17]. Additionally, organelles within these cells autonomously signal and communicate stress, triggering stress-specific transcriptional responses.

This report reviews the literature focusing on the relationship between placental transcriptional responses and offspring outcomes in maternal PCOS and obesity, elucidating the implications of these conditions on placental function and fetal development.

Placenta at the maternal-fetal interface and its development

Structure and development of the maternal-fetal interface

The maternal-fetal interface represents the exchange site between the mother's body and the developing fetus. In humans, this interface is formed by the chorioallantois placenta, which interacts with the decidualized maternal uterus (Fig 1). The human placenta at term consists of cotyledons, groups of villi that serve as the functional units. Within these cotyledons, ciliated mesenchymal stromal cells (MSCs)[18] and blood vessels receive fetal blood through the umbilical cord, connecting to the fetus. These vessels, composed of vascular endothelial cells and surrounded by perivascular cells, branch off into capillaries[19]. The capillaries are circumscribed by the trophoblast layers. The layers feature a core of cytotrophoblasts (CTB) surrounded by an outermost layer of syncytiotrophoblasts (STB). Another essential trophoblast type, the extravillous trophoblast (EVT), penetrates the decidualized uterus. EVTs serve a pivotal function in adhering to the uterus and reducing the resistance of uterine spiral arteries. This remodeling ensures adequate blood supply around the STB, facilitating exchanges with fetal blood in the capillaries.

The human placenta consists of various cell types serving crucial functions. MSCs are distributed across different placental regions, including the villi, human basal decidua[20], chorionic plate[21], cotyledons[22], and the intervillous space[23], contributing to the placental structure and support. Fibroblasts and myofibroblast, akin to those found in other connective tissues, form a fundamental component of the placenta. Among the immune

cells present, Hofbauer cells (HB), identified as placental macrophages, emerge around 18 days post-conception[24], preceding the onset of embryonic blood circulation. These cells are locally generated within the placenta and play a significant role in its development[25]. Maternal immune cells, such as macrophages, natural killer (NK) cells, mast cells, and dendritic cells, inhabit various placental regions, including the decidua, myometrium, and maternal blood in the intervillous space. These immune cells interact with STB surface[26] and contribute to immune regulation, trophoblast invasion, angiogenesis, and the remodeling of spiral arteries. Collectively, these diverse cell types collaborate to fulfill the multifaceted functions of the placenta.

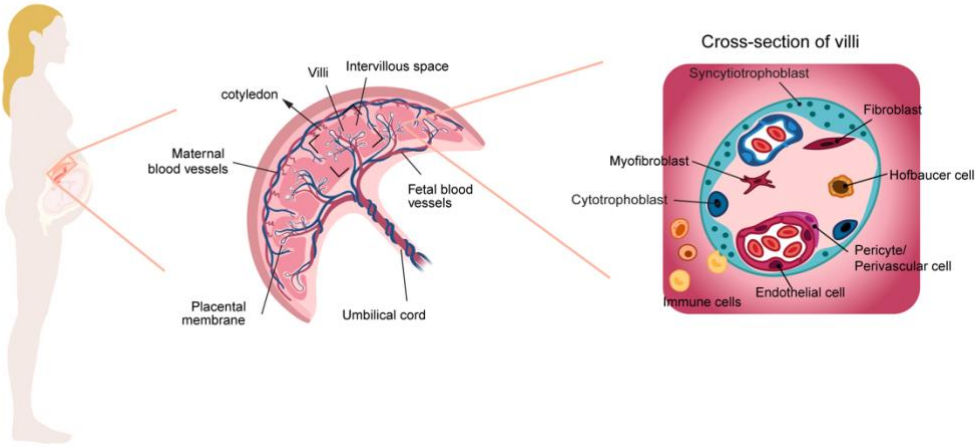

Fig1. Illustration of human placenta at the maternal-fetal interface. In the center is the depiction of the human placental structure at full term, accompanied on the right by a cross-sectional view of villi with labeled major cell types.

In the center is the depiction of the human placental structure at full term, accompanied on the right by a cross-sectional view of villi with labeled major cell types. Understanding the impact of PCOS and obesity on placental pathology necessitates examining these conditions within the context of normal placental development. The first trimester (0-13 weeks) witnesses significant trophoblast proliferation and differentiation, culminating in the formation of immature chorionic villi. Stem villi extend into the uterine wall, establishing the vital connection between the mother and the developing embryo[27]. These early processes are closely intertwined with embryonic and fetal growth[28], reaching a peak in trophoblast activity and differentiation around weeks 10-14. This intensive activity renders the placenta particularly susceptible to environmental influences during this critical phase. Throughout the second trimester, placental growth continues, marked by the differentiation and maturation of newly formed villi[29]. More mature intermediate and terminal villi emerge, characterized by a thinner stroma. Notably, second-trimester placental volumes and growth rates serve as potential predictors of birth weight in healthy pregnancies[29]. The third trimester is characterized by further expansion of placental mass through distal villous growth and angiogenesis. Intermediate and terminal villi become more prevalent, albeit smaller than in the second trimester, featuring thin stroma and vasculosyncytial membranes (fused fetal capillaries with syncytiotrophoblasts). Hormones synthesized by the villous syncytiotrophoblast, such as insulin-like growth factor, peak toward the end of this trimester. Normal human placental growth exhibits a relatively linear pattern throughout the second and third trimesters[30] (Table 1), sustaining an essential growth rate that is pivotal for effectively supporting fetal development.

Gestational age	< 28 weeks	28 - 32 weeks	33 - 36 weeks	37 - 40 weeks
Placenta weight	253 g	314 g	391 g	456 g

Table 1. The approximate placenta weight during the gestation[30].

Maternal and fetal factors influent placental development

Accordant with its position at the maternal-fetal interface, the placenta is at the confluence of two interlinked feedback from both maternal and fetal sides, collectively shaping the phenotypes. This assumption is valid from genetic and in-utero environmental exposure perspectives.

Genetically, recent research revealed both fetal insulins associated with single nucleotide polymorphisms (SNPs) and maternal glucose or insulin resistance associated SNPs predispose to a higher placenta weight, which is a proxy of placental development[31]. The in-utero environment, such as the maternal undernutrition or overnutrition[32], the chemical exposure[33], the maternal stress levels[34] were reported to affect placental development. The fetus can also influence placental development through hormone secretion[35]. Notably, fluctuations in insulin concentration within the fetoplacental circulation demonstrate discernible effects on both the structure and function of the placenta[36].The placenta's structural and functional development were coordinated to meet fetus's growth demands[37].

Throughout pregnancy, the roles of these maternal and fetal factors dynamically evolve. For instance, early in gestation, insulin receptors primarily reside on the microvillous membrane of the syncytiotrophoblast (STB). Conversely, by term, these receptors are predominantly located on the endothelium of fetoplacental vessels[38]. This shift suggests a transition in the control of insulin-dependent processes from the mother in early pregnancy to the fetus towards the end.

Placenta's functions and impact on mother and fetus

As the normal mammalian placenta is an apposition of the fetal membranes to the uterine vasculation, the primary function of the maternal-fetal interface to be noticed is for the physiological exchange[39]. At this interface, various transport mechanisms, including passive transport, facilitated transport, active transport, and endocytosis, facilitate the transfer of nutrients, drugs, and other substances between maternal and fetal circulation. The placenta expresses an array of transporters responsible for this transfer. Among these, solute carriers (SLC) and ATP-binding cassette proteins (ABC) stand as major superfamilies of mammalian transporters[40]. The SLC superfamily, consisting of 55 families and 300 members, primarily functions as ATP-independent uptake transporters for a wide range of substrates. Notably, extensive research has focused on characterizing amino acid SLCs due to their regulatory role in fetal growth[41]. On the other hand, ABC proteins play an active role in transporting various physiologically relevant compounds, including inorganic ions, glucose, amino acids, metal ions, cholesterol, and phospholipids[40]. Additionally, these proteins are pivotal in pumping pharmaceuticals and xenotoxicants out of the fetal circulation, contributing to the protection of the developing fetus[42]. Apart from the efflux transporters, the placenta also enzymatically degrades the potentially harmful molecules such as cortisol, vitamin D, and thyroid hormone[43].

By regulating the transporters, the placenta can fine-tune the exchange process. This exchange management is crucial for orchestrating maternal physiological adaptations to pregnancy and allocating resources according to fetal demand signals. Several mechanisms enable the human placenta to exert control over this process. Primarily, it influences maternal supply by modulating the secretion of hormones like placental growth hormones (pGH)[44] and signaling factors into the maternal circulation. This

alteration can impact the release of growth factors, such as insulin-like growth factor I (IGF-1)[45], from maternal tissues. These modifications reverberate back to influence placental growth and transport functions, forming an interconnected regulatory loop.

The placentas can also affect maternal and fetal conditions via releasing extracellular vesicles. STBs are particularly noteworthy for releasing macrovesicles known as syncytial nuclear aggregates (SNAs). These SNAs, ranging from 20 μm to several hundred microns in size, contain a diverse array of molecules, including fetal DNA, proteins, RNA, and lipids[46-48], and often contain multiple nuclei within each aggregate. Additionally, the placenta generates microvesicles, although their exact origins remain unclear. These microvesicles could potentially stem from various sources, such as blebs on the surface of SNAs, apoptotic blebs from STB, villous cytotrophoblasts exposed to maternal blood after STB denudation, or EVT. Furthermore, nanovesicles, comprising a mixture of exosomes and other vesicles with diameters ranging from ~20 to 100 nm, are actively secreted by STB during normal pregnancies. These vesicles are released into the maternal circulation through a process termed trophoblast deportation[49]. In addition to trophoblasts, placental MSCs have demonstrated the capability to secrete exosomes in vitro, although their precise role in vivo remains less clear. The placental extracellular vesicles are widely studied in their interactions between maternal immune cells and endothelial cells[50] in vitro (reviewed in[50,51]). Most studies were in non-physiological settings, where the researchers isolated the vesicles from placenta and added them to the invitro culture system of other cells. The results regarding their effects on immune cells are with little consensus, possibly depending on the method of isolation and preparation. Similarly, the trophoblast macrovesicles could either inhibit the endothelial cells proinflammatory signals if they have features of apoptosis or promote the secretion of proinflammatory cytokines if they undergo secondary necrosis induced by freeze-thawing. Placental microvesicles could inhibit the proliferation or increase apoptosis of the endothelial cells and promote the proinflammatory states of them[52,53]. These effects on endothelial cells could then induce maternal vascular dysfunction[50].

Placenta pathology in PCOS

Diagnosis of PCOS and consequences on maternal and fetal health

PCOS is typically diagnosed based on symptoms including (1) oligo-anovulation, (2) clinical or biochemical hyperandrogenism, and (3) polycystic ovarian morphology (PCOM) detected by ultrasound or anti-Müllerian hormone (AMH) levels in adults[54]. In a cross-sectional study recruiting 392 women, the prevalence of PCOS diagnosed with (1) and (2) under National Institutes of Health criteria was 6.1%, with (1) and either (2) or (3) under Androgen Excess and PCOS Society criteria was 19.9% and with any two of the three symptoms under Rotterdam criteria was 15.3%[55].

PCOS was associated with gestational diabetes mellitus[56], gestational hypertension[57,58], and higher miscarriage (in 30 to 50% compared to 10 to 15% of normal women)[59] although age and BMI are significant cofounding factors[60]. Babies born to mothers with PCOS are at an increased risk of macrosomia, *i.e.*, being larger than average at birth[61] and higher risk of developing PCOS[62].

PCOS and placenta pathology

PCOS can impact the placental structure and function. In PCOS women following in-vitro fertilization[63], the placentas exemplified anatomic abnormalities, inflammation, villous maturation issues, and vascular malperfusion features. Compared to healthy controls, women with PCOS who conceive naturally exhibit decreased placental volume and weight, while higher occurrences of inflammation, villous immaturity, infarction, and the presence of nucleated fetal red blood cells[64]. The EVT invasion is defective in PCOS women[65].

Proteomics profiling revealed that estrogen receptor- β was elevated in the placenta of women with PCOS[66]. The mechanisms of how androgen excess induces these effects were studied with mouse models (reviewed in [67-69]). The choice of models should match the research questions[63]. To study the placental and fetal exposure to the maternal hyperandrogenic conditions in PCOS, the pregnancy period and offspring outcome of the peripubertal dihydrotestosterone (DHT) model are suitable to be examined. This model establishes PCOS-like phenotypes in F0 mice by excess androgen but not transgenic manipulations, and the fetus and placenta during the pregnancy period of the mice model are exposed to hyperandrogenic conditions. The oocytes from the peripubertal DHT model were transcriptionally less affected compared to the perinatal androgenized model[70], thus, this model features the in-utero environment.

Placenta pathology in obesity

Definition of obesity and consequences on maternal and fetal health

Obesity is characterized by excessive adipose tissues. The body mass index (BMI) is correlated with the gold-standard methods for measuring body fat[71] and is used to categorize adults into underweight (<18.5), normal weight (18.5–24.9), overweight (25.0–29.9), obese class I (30–34.9), obese class II (35–39.9), and obese class III (≥40.0) classifications[72].

Obesity in women is increasing worldwide[73] (prevalence in each country from 1975-2016: https://www.ncdrisc.org/obesity-prevalence-ranking.html). In 2016, the prevalence in Chile was 32.1%[73]. The prevalence of obesity in women of reproductive age (20–39 years) in the United States (US) reached 39.7% in 2018 based on the National Health and Nutrition Examination Survey[74]. Its detrimental effects on various pregnancy outcomes have been acknowledged (reviewed on the US population in [75]). As highlighted, obese mothers face increased risks of preeclampsia, type 2 diabetes, and gestational diabetes. Maternal obesity is associated with higher rates of stillbirth and premature birth. Infants born to obese mothers face elevated risks of congenital malformations, large for gestational age[76] (or increased birth weight[77]), and dystocia oxidative stress[77]. Furthermore, these babies face higher risk of developing obesity in their childhood and metabolic, cardiovascular, and neurological disorders later in life.

Obesity and placenta pathology

Obesity has been linked to larger placental size, an increased placenta-to-fetal weight ratio[78], and low-grade inflammation[79] .

During the first trimester, obese pregnant individuals exhibit lower vascular indices[80] and reduced concentrations of PAPP-A, indicating diminished early placental growth. Indirect evidence suggested that a decrease in uterine NK cells among obese mothers might contribute to delayed spiral artery remodeling[81,82]. Effective placentation and spiral artery remodeling significantly impact blood flow and the increase of oxygen tension in the intervillous space. In obese women's placentas, higher levels of the negative cell cycle regulator BRCA1 have been observed, potentially impeding cell proliferation and placental growth[83]. The obese women were also reported to experience reduced trophoblast insulin sensitivity[84], especially with the number of insulin-sensitive genes being reduced by 30-fold[85]. This potentially diminishes the growth-promoting effect of hyperinsulinemia.

In later stages, the placenta has been configured and formed in shape. Macroscopic assessments have reported longer cords in the placentas from obese individuals[86]. Histological examination reveals varied villous vascularity in obese women's placentas, ranging from normal vascularization to hypovascular or unusually numerous, dilated, and

congested villous vessels[87]. Chronic villitis[88] and increased concentrations of erythropoietin in cord blood at birth[86] have also been associated with pregestational obesity. Some studies note abnormal villous maturation in placentas from pre-pregnancy obese or overweight women, with reports of both accelerated[88] and immature villous development[89], although other studies do not corroborate these findings[86].

Placental cell types in response to maternal obesity

The aforementioned pathological phenotypes in development, vascularization, and immune functions may be mediated by specific cell types.

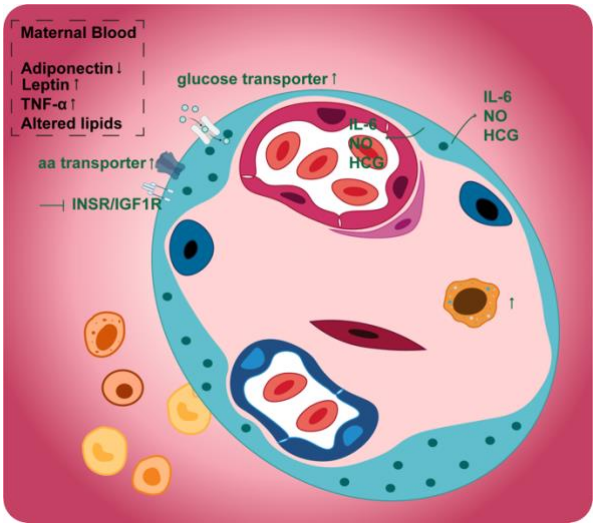

Figure2. The summary of the pathological changes in placental villi in response to maternal obese conditions from in vivo studies.

Trophoblast cells

Maternal obesity promotes the nutrient uptake and transport of trophoblast cells by altering metabolites, hormones, growth factors, and cytokines that can be sensed by trophoblast cells.

Altered adipokine levels in the bloodstream, like reduced adiponectin and increased leptin[90], were linked to maternal obesity[91]. This alteration may promote amino acid absorption in trophoblast cells. Studies on cultured primary trophoblast cells showed that adiponectin reduces the expression of amino acid and glucose transporters[92], while leptin increases system A amino acid transport (SA1) activity. Leptin could also trigger the release of IL-6, nitric oxide, and human chorionic gonadotrophin from term trophoblast cells[90].

Elevated IGF-1 concentrations were observed in the circulation of obese mothers[93], stimulating trophoblast proliferation[94] and enhancing glucose and amino acid uptake in trophoblast cells[95].

Certain cytokines, specifically IL-6[96,97] and Tumor Necrosis Factor-α (TNF-α)[98], found at higher levels in maternal circulation, could influence lipid and amino acid transport and metabolism in trophoblasts. IL-6 and TNF-α also stimulate SA1 by STAT3[99] and MAPK[100] signaling respectively. TNF-α's activation of phospholipase A2 in human trophoblast cells was proposed as a mechanism favoring excess fetal fat accumulation and neonatal adiposity[101]. It may hinder insulin actions on trophoblasts by inhibiting the signals from the IGF-1/insulin hybrid receptors[102].

Fatty acids can impact trophoblasts differently based on chain length and saturation. For instance, oleic acid (OA) at physiological levels stimulates mTOR signaling and amino acid uptake while promoting specific protein expression in trophoblast cells. In contrast, docosahexaenoic acid (DHA) decreases mTOR signaling and System A amino acid uptake[103]. Different fatty acids' effects range from altering cytokine release to influencing trophoblast invasion and downstream signaling, with saturated lipotoxic fatty acids like palmitic acid (PA) stimulating cytokine release and inhibiting trophoblast invasion. In obese mothers, an elevated downstream JNK/EGR-1 signaling of PA and TNF-α was reported.

Mesenchymal stromal cells

The maternal obesity's effects on placental stroma cells were less studied and many reports observed no molecular differences in obese mothers from normal-weight mothers. As to the question of whether the abundance of leptin receptors on villous stroma cells is a proxy for maternal obesity, the answer is no[104]. The placental stroma cells also express GLUT4, but the mRNA and protein levels are not significantly affected by the hyperinsulinemia in mothers and fetuses[105]. This suggested a possible placenta-specific insulin resistance in those mothers.

Innate immune cells

The obese women appear to have increased quantity and altered composition of immune cells in their placentas. Notably, the macrophages were reported to accumulate within the placental villous stroma, while STB or fetal vascular endothelium quantities had no discernible changes[89]. These macrophages might originate from activated monocytes in the maternal circulation[106] or primitive fetal macrophages[25,107]. Furthermore, it's been suggested that maternal obesity might reduce the count of NK cells, potentially contributing to complications like pre-eclampsia[82].

The gene expression aspect in the molecular pathologies of PCOS and obesity

The molecular mechanisms of the above-mentioned observations are better resolved with high-throughput unbiased functional molecular profiling. The placenta's transcriptome, or the complete set of RNA transcripts, is an essential aspect of placental development. It contains the genetic information to be translated and needed for proper fetal development and pregnancy outcomes. However, the human placenta has recently gotten into the spotlight of second-generation RNA-sequencing[108-111] and so far, the transcriptomic profiling of placentas in PCOS and obese women is scarce. Nevertheless, these profiles have still raised some targets underlying the pathological changes. For example, integrated analysis of transcriptome and DNA methylome in trophoblasts from PCOS compared to healthy mothers and functional validation in mouse models revealed FOS insufficiency (neutrophins) might be the mediator of higher risk of neurological diseases in offsprings from PCOS women[112]. The RNA-sequencing on term placentas from lean and obese women confirmed the dysregulation of genes related to lipid metabolism, angiogenesis, hormone activity, and inflammation and highlighted the central role of activation of MAPK-JNK signaling in it[113].

The aspiration to develop targeted treatments for PCOS and obesity-related maternal and fetal morbidities concerning the placenta is hindered by a significant knowledge gap in understanding the underlying mechanisms of these conditions. In terms of transcriptional profiling, the RNA transcripts sampled from each placenta are confounded with the composition of cell types. Specifically, we are facing the questions stated in Box 1.

Box 1. Questions confronted in revealing the mechanisms of the placental pathology in PCOS and obesity.

1. The data readouts are confounded with varied cell-type compositions. This is an issue considering that tissue extraction from each of the relatively large placentas is nearly impossible to identical.

2. What are the placental cell types that are affected by maternal PCOS or obesity and

3. How are the cell types affected, in terms of molecular characterization:

3.1 What genes' expression are affected?

3.2 What signaling and metabolic pathways are affected?

3.3 What cellular functions are exerted by those DEGs?

4. If trophoblasts are affected, whether they are immature or accelerated in terms of differentiation progress

5. How are the nuclei in the STBs response to maternal PCOS or obesity and how do they communicate and co-opt with each other?

6. How do trophoblasts take in information from fetal vascular cells or the maternal cells?

To our advantage, single cell sequencing technology can generate data to answer these questions with proper study design. In single-cell or single-nuclei sequencing (scRNA-seq/ snRNA-seq), cells or nuclei are isolated, and the reads from a cell or nuclei are ligated to a barcode sequence for their identities. In this way, the relative abundance of different transcript species in cells or nuclei is measured. Given the cell ontology assumption, in which the same cell type or cell states exemplify similar gene expression patterns, the cell types and their contributed transcripts can be delineated by transcriptomic data clustering and/or classification, and the first two questions can be resolved.

In the third question regarding the differences in the interested pathological samples to the control samples, there are cluster-based or cluster-free differential expression analysis strategies. The cluster-based strategies are based on the consideration that the intrinsic difference of scRNA-seq is that in bulk sequencing, the samples in a group are independent samples from a population distribution while the scRNA-seq data from each sample are from multiple cell type populations. It is nature then to calculate the cluster-based pseudo-bulk expression matrices, then in each cluster, the data structure becomes the same as the bulk RNA-seq data. After standard normalization, the differential expression test using regression models as known in edgeR[114], DESeq2[115] , limma[116] can be applied. Other than the gene expression values, the factors that are divergent between groups and influence the estimated variance and coefficients of gene expressions are often used as covariates. This usually includes age, sex, day of the experiment, percentages of cells in the cell-cluster or cell-type in the total of cells and, GEM batch (defined by nuclear isolation, GEM generation, and barcoding performed for all samples in one batch in the same 10X GEM generation run, v2 or v3 chemistry). Another strategy is to compare the expression in individual cells, using statistical tests such as t-test, Wilcoxon rank-sum test, logistic regression[117], negative binomial and Poisson generalized linear models, likelihood ratio test[118], and the two-part hurdle model implemented by MAST[119]. However, in a benchmark study used the differential expression (DE) results from eighteen bulk RNA-seq datasets matching cell populations

as gold standard, the pseudo bulk methods outperformed such single-cell methods measured by the concordance between DE results in bulk versus scRNA-seq datasets[120].

For questions 3.2 and 3.3 regarding the perturbed pathways and cellular functions, there are two streams of the analysis: pathway-related gene set test and pathway activity inference. The first is to test whether certain pathways are over-represented in one condition compared to the others. This can be achieved by hypergeometric tests or Fisher's exact tests to identify over-represented gene sets[121]. Or one could adopt gene set enrichment analysis algorithms based on pre-ranked gene-level test statistics using fgsea[122] or GSVA[123]. As in the DE test, bulk enrichment tests can also be utilized after creating pseudo-bulk samples. Such methods as *fry* and *camera* implemented in limma[124], are compatible with the linear model and empirical Bayes moderation, thus, they can accommodate experimental designs by including studied factors in the design matrix. The *camera* and *roast* tests can also account for inter-gene correlation. The pathway activity inference, on the other hand, is to score the activity of a pathway in individual cells. Some of the widely used tools for pathway activity inference include VISION[125], AUCell[126], Pagoda2[127] or simply constructing z-score[128]. There's no comprehensive benchmark on the accuracy of these methods so far, so one should try the methods on the data at hand and select according to biology and design further validations.

For question 4 regarding the differentiation of trophoblasts, the single-cell sequencing covers a vast "landscape" of possible states[129], the powerful high-covered sampling on many cells in a static time, given the ergodic theory[130], could reflect the distribution of cellular heterogeneity in transcriptome within a short time, i.e., the order of the sampled cells in pseudo-time[131]. This question is even better resolved given the transcription[132] or post-transcription kinetics[133] that shapes the cellular dynamic landscape in terms of the transcriptome. In the placenta studies, one recent study which used StOrder leveraging both spatial and scRNAseq data to infer trajectory[134]. Four other studies that didn't retain the spatial information and used Monocle 2 to infer trajectory from scRNA-seq data[135-138]. Monocle2 orders cells along the pseudotime without prior knowledge and can analyze linear or branched trajectories but not cyclic trajectories[139]. In trophoblast differentiation, the differentiation is connected from CTBs and cannot be reversed, thus, Monocle can be optimal here.

For the question regarding the multi-nucleated STB, single nuclei sequencing is especially promising. As shown cases, previously snRNA-seq has been leveraged to profile skeletal muscle and cardiac myonuclei and identified subtypes that are concordant with previous reports but that are difficult to capture by scRNA-seq[140]. One limitation, however, is that the syncytium identity is lost in the snRNA-seq. Therefore, whether the myonuclei subsets are related to multinucleation and specialization of nuclei in one syncytium remains elusive.

Lastly, to investigate which ligand-receptor interactions influence the intracellular signaling of target cells, prior knowledge of ligand-to-target signaling paths is necessary and the key to solving the problem. The complementary data sources of ligand-receptor and signaling include Guide2Pharma[141] as ligand-receptor databases, InWeb_InBioMap[142] for protein-protein interaction, PhoshoSite[143] for post-translational modification, EVEX (http://evexdb.org/) from text mining and PathwayCommons[144] for pathways. The gene regulatory data sources to infer the regulatory networks in the target cells include ChIP-seq data from ENCODE[145], perturbation results from transcription factor knockout studies in GEO[146], motifs from TRANSFAC[147] or JASPAR[148], and also information from EVEX and PathwayCommons[144]. Then, utilizing this knowledge, computational methods such as NicheNet[149] can be applied.

Prospects toward the placenta pathological niche in PCOS and obesity

The placenta's development and function are convoluted with the fetus and decidua, apparently, the positional context is not negligible to analyze the cells in the placenta. As the fetus grows asymmetrically anchored to the implantation side, the location of the villi close to the implantation side or opposite to it makes a difference. Also, at the interface in the dual perfusion of maternal and fetal circulation, the placental cells' depth relative to the maternal or fetal side partly determines the molecules and cell types that are in the niche[150]. The placenta-maternal interaction extends into the decidua and myometrium, the biological profiling registered with the depth of this invasion is crucial to understand the physiological link between placental and maternal cells. It's therefore one of the limitations of only profiling the transcriptomics in cells in randomly picked small regions of placentas. In this regard, one solution is to profile the spatial information with spatial sequencing technology. There is already spatial transcriptomics coupled with snRNA-seq and snATAC-seq performed on the human implantation site in the first trimester[151]. More endeavors should be delivered to cover the gestational age and pathologies. Another in-vitro manipulable solution is to establish multi-channel microfluidics with placenta-on-chip connected to another organ-on-chip. Placenta-on-a-chip models primarily emulate transport functions of the placenta villi[152]. These models can utilize the trophoblasts derived from human pluripotent stem cells (hPSCs)[153]. The implementation of such 'placenta-on-a-chip' models alongside primary cell types and other hPSC-derived cells likely replicate the communication across the maternal, placental, and fetal cells. Relying on synthetic biology to develop an artificial placenta is also promising[154]. These resolutions also allow the quest to the key question regarding the interplay of the placenta to maternal and fetal conditions, which is infeasible to studies in which only placentas are under scope. Clinically, the co-measurement of hormones and metabolites from maternal and fetus circulation is valuable to figure out the mechanisms that indeed are linked to either side.

Apart from the clinical relevance to the fetus or mother's conditions and outcomes, the upstream molecular mechanism of the transcriptomic changes in the placenta, including the signaling cascades in response to the conditions and the transcription regulation network rewiring is of importance to the targeted therapy or creation of a wholesome reproductive system. To decipher these, the multi-omics technologies will be of great help.

Status report of the doctoral education project

Overall aim and introduction of the doctoral project

Several environmental factors that increase offspring susceptibility to disease have been demonstrated. One mechanism underlying this epidemiology is fetal programming. Fetal programming mechanism proposes that early adaptations to suboptimal conditions in utero could translate into adverse health outcomes in later life. The placenta is the organ encompassing the fetal-maternal interface and it is hypothesized to mediate the maternal conditions to fetal programming and long-term ill-health in offspring.

To examine this hypothesis, my research question is to illustrate how maternal conditions such as preeclampsia, PCOS, and obesity affect the placenta and thereby affect fetal development. To interpret the cellular and molecular changes of the placenta in different maternal conditions, we will generate and compare single-cell transcriptome and whole-genome DNA methylation data sampled from the placenta of women with different health conditions and corresponding mouse models. To investigate the pathological changes in the placenta's interplay with fetal development, we will generate and link transcriptome and DNA methylation profiles from mouse embryonic tissues with offspring tissues alongside the placenta samples.

Aim 1: Use prepubertal PCOS-like mouse model to study molecular mechanisms of PCOS-induced placental dysfunction and fetal impair

The aim of the first project was to reveal the mechanisms by which maternal hyperandrogenism in women with polycystic ovary syndromee (PCOS) affects offspring health. To achieve this, we resorted to mouse models. The commonly used models include the transgenic nerve growth factor model, the prenatal androgenized, peripubertal androgenized, and a diet-induced obesity model. Our questions include: How are the tissues in the hypothalamic–pituitary–gonadal (HPG-axis) affected in PCOS? Which mouse model available is suitable for studying the fetal programming of the in-utero environment of PCOS? How were the placenta, fetus, and offspring affected in this mice model? If the phenotypes from high systematic dihydrotestosterone were mediated via an androgen receptor? What were the transcriptional and DNA methylation mechanisms that were involved in these effects? These questions were answered with the following designs:

- Profile the transcriptome from the tissues in the HPG-axis and the oocytes from the transgenic nerve growth factor model, the prenatal androgenized, peripubertal androgenized, and the diet-induced obesity model
- Characterize the placental and embryonic developmental phenotypes of the PCOS-mice and a competitive inhibitor flutamide cotreated mice
- Infer the biological processes in placenta affected in PCOS-like mice through analyzing the RNA and whole-genome bisufite sequencing
- Infer the metabolic rewiring in placenta from PCOS-like mice
- Link the molecular profile to the cellular phenotypes by correlation with the cell type proportions, which are proxy for placenta development

Aim 2: Systematic review the cell types in human placenta

To identify and characterize cell types in human placenta is still a complex endeavour requiring understandings of the biology and methodology one adopt. Single cell RNA sequencing is one of the methods that could help characterizing cells in human placenta. Here, we intended to review the knowledge gained regarding placental structure and function from the use of single-cell RNAseq (scRNAseq) followed by comparing

scRNA-seq analyses, highlighting their similarities and differences. Then, we aimed at identifying consensus marker genes for the various trophoblast cell types across studies.

Aim 3: Revealing cell-type-specific signature of gene expression and signalling pathways in human placentas from obese mothers by single-cell RNA-seq

The main scope is to reveal the interactions between maternal obese conditions and placental cellular transcriptional response. We'd explore the responses relating the fetal healthy to our most. The strategies include:

- Use single-cell RNA-seq to profile the placenta containing the villous trees from obese or normal weight women. Then to analyse the cell type composition variations, gene modules underlying the variation and cell communication, and hub genes that are important in mediating the cellular process.
- Use placenta-adipose co-culture organ-on-chip to evaluate the potential cellular and gene mechanisms suggested by the maternal single-cell analysis
- For the clinical relevance, we'd compare the transcriptional responses related to average or large-for-gestational age babies. For the hub genes, we'd analyse the characteristics in UK biobank and SweGen mutations.

Aim 4: Revealing cell-type-specific signature of gene expression and signalling pathways in human placentas from PCOS patients and the effects of metformin treatment

Metformin can lower insulin and blood sugar levels in women with PCOS. It was reported to stimulate ovulation, encourage regular menstrual cycle, and lower the risk of miscarriage, and with long-term health benefits, such as lowering high cholesterol levels and reducing the risk of heart disease. The aim of this project is to investigate how placental cells response to PCOS conditions and how metformin affects the placental cells. In the end, this can provide more molecular pharmacology of metformin to treat PCOS and in turn help with the license and practical usage of metformin.

Methods

Systematic review and meta-analysis. For a systematic review, we followed the PRISMA-DTA Checklist. Specifically, we reported the eligible criteria including study and report characteristics. Our information source was Pubmed. The full search term was constructed with the guidance of placenta pathologist. We manual inspected all the information sources and specified the process in PRISMA flowchart. We extracted the single cell raw count tables sequenced from placental villi of healthy women from the sources. We assessed the risk of bias by measuring the alignment scores between individual studies. We synthesized the results by Scanorama and normalize them together by analytical Pearson residuals. After the cell type identities were annotated, the differentially expressed genes were found using MAST and intersected with the DEGs reported from the individual studies.

RNA-seq analysis. From the RNA quantification matrices, we filtered out the low-expressed genes. Principal component analysis and Spearman correlation were calculated based on log2 normalized variance stabilized transformed counts.

For differentially expressed analysis, the RNA quantification matrices were analyzed using DESeq with the treatment condition as the variable of interest.

For Cell type deconvolution analysis, the single nuclei, or single cell RNA count matrix from E10.5 placenta with the annotated cell type information and our RNA-seq count

matrix was processed using the CIBERSORTx to impute the placental or trophoblast cell type fractions.

Whole genome bisulfite sequencing data processing. For WGBS data, the illumina and library specific adapters were trimmed using bbduk (v38.98, BBMap - Bushnell B. - sourceforge.net/projects/bbmap/). The reads after trimming were mapped to mouse genome GRCm38, deduplicated and methylated Cs were called using bismark. Coverage was calculated as using a customed script. Differentially methylated sites and regions were analyzed using MethylSig package in R. Further annotation was made using AnnotationHub package In R.

Single nuclei RNA-seq preprocess. Gene counts were obtained by aligning reads to the GRCh38 genome using Cell Ranger software (10x Genomics). We used SCANPY to process and cluster the expression profiles and infer cell identities of major cell classes. To remove doublets and poor-quality cells, cells were excluded from subsequent analysis if. In addition, we called doublets using Scrublet and flagged and removed cells that were labeled as doublets.

For the UMAP visualization of individual major cell type classes, the analytical pearson residue workflow was used to find the highly variable genes from individual batches. We selected the set of relevant principal components based on Elbow plots. High resolution cell clusters were identified using the Leiden clustering algorithm.

We annotated cell types using previously published marker genes and single-cell RNA-sequencing data.

We performed differential expression analyses using the MAST enclosed in R package Seurat. Lowly expressed genes were excluded and only genes with more than one count in at least x cells were considered. For the analysis between N-AGA, O-AGA, and O-LAGA, we included sex, and log transformed number of genes as covariates in the differential expression analysis.

Gene Ontology enrichment analyses were performed using decoupler.

Project 1: Use the mouse model to study molecular mechanisms of PCOS-induced placental dysfunction and fetal defects

Transcriptomic survey of key reproductive and metabolic tissues in mouse models of polycystic ovary syndrome.

Pei Yu†, Sanjiv Risal†, Hong Jiang, Haojiang Lu, Eva Lindgren, Elisabet Stener-Victorin*, Qiaolin Deng*

Published on Communications Biology.

Manuscript 2: Targeting androgen receptor against developmental programming by maternal androgen exposure in utero and on offspring

Haojiang Lu†, Hong Jiang†, Congru Li, Emilie Derisoud, Han-Pin Pui, Sanjiv Risal, Yu Pei, Eva Lindgren, Theresa Maxian, Claes Ohlsson, Anna Benrick, Sandra Haider, Elisabet Stener-Victorin*, Qiaolin Deng*

In preparation for submission.

Manuscript abstract: Maternal hyperandrogenism in women with polycystic ovary syndrome (PCOS) exerts a discernible impact on offspring health. Yet, the precise mechanisms are unknown. In dihydrotestosterone-induced PCOS-like mice, impaired

placental and embryonic development were prevented by the androgen receptor blocker, flutamide. Comprehensive analysis using whole-genome bisulfite and RNA sequencing revealed a correlation between the diminished proportion of trophoblast precursors and decreased expression of Cdx2. Moreover, the absence of Gcm1, Synb, and Prl3b1 resulted in decreased numbers of syncytiotrophoblasts and sinusoidal trophoblast giant cells. The compromised formation of the placenta labyrinth formation likely contributes to the observed mid-gestation lethality. Human trophoblast organoids treated with androgens exhibited analogous alterations, substantiating impaired trophoblast differentiation as a key factor in PCOS-pregnancy complications. Importantly, all deleterious effects were mediated through the androgen receptor pathways, as placental development and the health of female and male offspring exposed to androgens in utero were comparable to controls when treated with flutamide. These findings provide novel insight into the complexities of PCOS-related pregnancy complications, paving the way for prospective directions in treatment strategies.

Project 2: Systematic review the cell types in human placenta

Revealing the molecular landscape of human placenta: a systematic review and meta-analysis of single cell sequencing studies

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Submitted to Human Reproductive Update.

Manuscript abstract:

Background: With increasing significance of developmental programming effects associated with placental dysfunction, more investigations are devoted to improving characterization and understanding of placental signature in health and diseases. The placenta is a transitory but dynamic organ adapting to the shifting demands of fetal development and available resources of the maternal supply throughout pregnancy. Trophoblasts (cytotrophoblasts, syncytiotrophoblasts and extravillous trophoblasts), are placental-specific cell types responsible for the main placental exchanges and adaptations. Transcriptomic studies with single-cell resolution have led to advances in understanding the role of placentas in health and diseases. These studies, however, often show discrepancy in characterization of the different placental cell types.

Objective and rationale: We aim to review the knowledge regarding placental structure and function from the use of single-cell RNAseq (scRNAseq) followed by comparing cell-type specific genes, highlighting their similarities and differences. Moreover, we intend to identify consensus marker genes for the various trophoblast cell types across studies. Finally, we will discuss the contributions and potential applications of scRNAseq in studying pregnancy-related diseases.

Search methods: We conducted a comprehensive systematic literature review to identify different cell types and their functions in human maternal-fetal interface, focusing on all original scRNA-seq studies on placentas published before March 2023 and published reviews (total of 28 studies identified) using PubMed search. Our approach involved curating cell types and subtypes that had been defined using scRNA-seq and comparing the genes used as markers or identified as potential new markers. Next, we reanalyzed expression matrices from the six available scRNA-seq raw datasets with cell annotations (4 from first trimester and 2 from term), using Wilcoxon rank-sum tests to compare gene expression among studies and annotate trophoblast cell markers in both 1st trimester and term placentas. Furthermore, we integrated scRNAseq raw data available from 18 healthy 1st trimester and 9 term placentas, performed clustering and differential gene expression

analysis. We further compared our annotated and integrated analyses and curated markers from the literature to obtain a common signature gene list for major placental cell types.

Outcomes: Variations in the sampling site, gestational age, fetal sex and subsequent sequencing and analyzing methods were observed between the studies. Although their proportions varied, the three trophoblast types were consistently identified across all scRNAseq studies unlike other non-trophoblast cell types. Notably, no marker genes were shared by all studies for any of the investigated cell types. Moreover, most of the newly defined markers in one study were not observed in other studies. These discrepancies were confirmed by our analysis on trophoblast cell types, where hundreds of potential marker genes were identified in each study but with little overlap across studies. From 35,461 and 23,378 cells of high quality in the 1st trimester and term placentas, respectively, we obtained major placental cell types, including perivascular cells that previously had not been identified in the 1st trimester. Importantly, our meta-analysis provides marker genes for major placental cell types based on our extensive curation.

Wider implications: This review emphasizes the need for establishing a consensus for annotating placental cell types from scRNAseq data. The marker genes identified here can be deployed for defining human placental cell types, thereby facilitating, and improving the reproducibility of trophoblast cell annotation.

Project 3 (ongoing): Revealing cell-type-specific signature of gene expression and signalling pathways in human placentas from obese mothers by single-cell RNA-seq

Summary

Biological groups. To study the maternal obesity's effects on placental and fetal growth, we stratify the placenta samples into three groups: samples from normal-weighted mothers who gave birth to normal-weighted babies (N-NGA, n=4), samples from obese mothers who gave birth to normal-weighted babies (O-NGA, n=4) and obese mothers who gave birth to larger-than-normal weighted (O-LNGA, n=4).

Cell types recovered from placenta exposed in maternal obese or normal weight conditions.

The selected placentas were sequenced with droplet-based transcriptomic assays (Chromium v3) for single nuclei sequencing. The analysis was performed on 37,408 high-quality nuclei that covered balanced numbers of nuclei from N-NGA, O-NGA, and O-NAGA samples). Unsupervised clustering led to the discovery of major placental cell types: trophoblasts, endothelial cells, stromal cells, and leukocytes, which were annotated to 14 cell types with the canonical cell marker expression. Trophoblasts accounted for the majority (78.7%) of all conditions and were predominantly composed of syncytiotrophoblast (STB) (65.7%), with smaller contributions from villous cytotrophoblast (CTB) (9.6%). Notably, we also captured the relatively rare proliferative CTB (pCTB) (2.1%) which was in the cell cycle, and early STB (eSTB) (0.9%). The eSTB nuclei expressed both Syncytin-1 (*ERVW-1*), which binds to SLC1A5 expressed by a small proportion of CTB, and Syncytin-2 (*ERVFRD-1*) which binds to MFSD2A mainly expressed by STB.

Logically according to our sampling in the villous part, the extravillous trophoblast (EVT) only accounted for 0.5% of nuclei in our data. We recovered two subtypes of endothelial cells (Endo-1, Endo-2). Except for conventional fibroblast (5.2%), we captured 2% nuclei as myofibroblast that concurrently expressed collagens and actin or myosin subunits. Among the immune cells in the placental villi, Hofbauer cells were the most abundant (4.7%) and the other leukocytes only accounted for 1.44%.

To gain an overview of the impact on cell types by maternal obesity or large baby weight, we compared the proportion and observed a drop in STB in O-LNGA samples. We then utilized the number of differentially expressed genes (DEGs) as a proxy of the degree of response to the maternal conditions. In general, in trophoblasts, CTB was most responsive, and the O-NGA group deviated more to the N-NGA. In stroma cells, one subtype of endothelial cells responded considerably to O-NGA, while fibroblast was more responsive to O-LNGA. Among the immune cells in the placental villi, only Hoffbauer cells had an appreciable response. Other immune cells had nearly no DEGs.

Adaptation in cytotrophoblast and syncytiotrophoblast function induced by maternal obesity. We utilized pseudotime inference to dissect the trajectories of trophoblast adaptation. The pseudotime ordering of the trophoblasts confirmed the path from pCTB to SCB: the pCTB exited cell cycle and differentiated into eSTB which could fuse with CTB and fuse into the STB in term placenta. The testifiable hypothesis could be spatial location of the eSTB should be under the STB layer where the syncytiation happens. In placentas from obese mothers, the trophoblast nuclei were more paused at the eSTB state approaching to becoming mature STB nuclei. This suggested problems in the fusion process. Indeed, the one gene that was differentially expressed in both O-NGA and O-LNGA samples compared to N-NGA samples was SMOC1, a glucose-responsive hepatokine as a therapeutic target for glycemic control.

Project 4 (ongoing): Revealing cell-type-specific signature of gene expression and signalling pathways in human placentas from PCOS patients and the effects of metformin treatment

To analyze PCOS etiology in placenta and the effects of metformin, we got acess to PregMet2 cohort and received 33 snap-frozen placentas from PCOS women taking placebo (placebo group) and 28 from PCOS women taking metformin (metformin group). We excluded women with smoking or other pathologies and performed Prime-seq on 15 samples from placebo group and 16 to profile the transcripts in the samples. Then we selected 9 samples in each group with no prior metformin treatment, not extreme obesity and no gestational diabetes, and meeting age (19-39) criteria. These sampled were sequenced at the single nucleus level. The preliminary results include:

We then established ethical permit to recruit healthy women from Karolinska hospital to collect placenta samples. We successfully collected 12 samples and are on the way to sequencing and analyzing the data.

Status report of progress towards the degree outcomes

A. Knowledge and understanding

Outcome ***A1***

For the Degree of Doctor the student is required to demonstrate broad knowledge and systematic understanding of his/her research field as well as deep and current specialist knowledge in a particular aspect of this field.

My present status:

I read preprints and peer-reviewed research articles and reviews on a daily basis, especially with keywords including “placenta”, “metabolic syndrome”, “PCOS”, or “transgenerational inheritance”. I attend journal clubs, and seminars and take doctoral courses relevant to my project.

Outcome ***A2***

For the Degree of Doctor the student is required to demonstrate familiarity with scientific methodology in general and with the methods of his/her specific field of research in particular.

My present status:

The methodologies I mostly need are bioinformatic strategies. In this regard, I took courses from Medbioinfo Graduate School. I learned how to design projects so that I can have the right data examine the right variables conforming to the statistical rules and cut to my scientific questions. I developed good working habits, especially the good practice of writing project-level codes and version control when building up pipelines for my lab. When choosing algorithms to use, I pay more attention to the CPU consumption, paralleled computation efficiencies. I learned advanced statistics and utilized the correct and appropriate statistical methodology for analysing my multivariate omics data and constructing good statistical models for my data. I also learned Machine learning and Deep learning to deal with the tensors and manifolds in biological data. These weapons help me reveal the network from the molecular features to phenotypes. I learned to interpret the different variations, such as the time, pathologies, species, and different and common molecular mechanisms underlying my data so that I can draw more clear and convincing conclusions from my data.

B. Proficiency and ability

Outcome ***B1***

For the Degree of Doctor the student is required to demonstrate a capacity for scientific analysis and synthesis and the independent critical review and assessment of new and complex phenomena, issues and situations.

My present status:

My present status:

I have developed my scientific reasoning in several ways during my project. I generally synthesize opinions from reading and data-generated hypotheses. I recorded them by writing them down. Presenting my research at lab meetings and conferences has given me the chance to get insights and guidance. Engaging in discussions at seminars and at meetings provides input and new ways of reasoning from colleagues in the field.

Outcome ***B2***

For the Degree of Doctor the student is required to demonstrate an ability to identify and formulate research questions critically, independently, creatively and with scientific rigour, and to plan and conduct research and other advanced tasks using appropriate methods and within given time frames as well as to review and evaluate such work.

My present status:

We planned together how to develop the projects at hand with colleagues ans supervisors. I wrote project proposals and will revise and evaluate them with my supervisor. I carry the responsibility of keeping time frames and goals and continuously report my progress

to my supervisor. When problems arise, I notify my supervisor of them and how I plan to solve them. I analyse data and present results to my group and supervisor, together with the conclusions I draw from them, and revise from the feedback I receive.

Outcome ***B3***

For the Degree of Doctor the student is required to demonstrate through the writing of a thesis the ability to make a significant contribution to the development of knowledge through his/her own research.

My present status:

I wrote the manuscripts for which I was a co-first author. I read and revised the manuscripts for which I was a co-author.

Outcome ***B4***

For the Degree of Doctor the student is required to demonstrate an ability to present and discuss research and research results, orally and in writing and with authority, both in national and international contexts and in dialogue with the scientific community and society in general.

My present status:

I have presented posters on my research at international conferences.

Outcome ***B5***

For the Degree of Doctor the student is required to demonstrate an ability to identify the need for further knowledge.

My present status:

I need to put the learned and proposed suitable strategies in my projects. There are hypotheses generated from the preliminary data and the previous projects, which I could design experiments to testify.

Outcome ***B6***

For the Degree of Doctor the student is required to demonstrate an ability to contribute to the development of society and to support the learning of others in research, education and other advanced professional contexts.

My present status:

I have written public sciences blogs to broader audience. I submitted my scripts and pipelines to open-source GitHub repositories. I helped my friends in analysing and interpreting their scientific projects.

Judgement and approach

Outcome ***C1***

For the Degree of Doctor the student is required to demonstrate intellectual independence and scientific integrity as well as an ability to make ethical judgements in research.

My present status:

I carry out data analysis and planning of projects, which I discuss with my supervisor. I have been involved in peer-reviewing an article. I argue for what I believe to be right and criticize or questions results or conclusions that are insufficiently supported. We evaluated the ethical aspects in my projects and established proper agreement for the clinical data handling.

Outcome ***C2***

For the Degree of Doctor the student is required to demonstrate deeper insight into the possibilities and limitations of science, its role in society and the responsibility of the individual in its application.

My present status:

I'm aware of the limitations for the current projects. Science grows step by step and technical, resources, time limitations are substantial compared to the complexity of biological systems. We are at the cutting-edge of the human knowledge, and we should bear the responsibility to be integrity, not to retract the progress intentionally and restrain ourselves from misuse.

Plan for the remaining doctoral education

Now that study 1 and 2 were finished and the manuscripts were under review, I will optimize the studies until they got approved by peer-review. The next two years will be devoted to the resolving the scientific questions in study 3 and 4. I will write documents on methods, analysis, results along the way and summarize into manuscripts. From the falsifiable hypothesis generated from preliminary analysis, I will plan experiments to validate them. I'd like also to advance my bioinformatic expertise in formalizing and proposing projects with topics extended from the current projects, like Epigenetic rewiring in Obese placenta, comparing the cell-cell network or trajectory network and SNP-genes module.

Regarding learning activities, I've completed the courses with bearing required number of credits. I will take courses mostly suitable to my research plan, especially in advanced and practical bioinformatic courses. I would like to learn and challenge more in the scientific communication and discussion. Therefore, I plan to attend conferences and seminars more.

	Year 3	Year 4
Research	Review cycle for study 1 and 2. Complete Analyzing data for study 3. Continue writing manuscript for study 3. Analyze data from project 4.	Complete study 3 and 4, write manuscript and submit. Write thesis.
Learning	Variation interpretation and structural bioinformatics, 5HP (Mauno Vihinen, Lund Univ). Computational Systems Biology, 5 HP (Mika Gustafsson, Linköping Univ).	No course planned
Teaching	Supervising master student in validating hypothesis for project 3 and 4. Especially in smFISH or IHC.	Seek opportunity for delivering bioinformatic workshops.
Meeting	15–18 Apr, EMBO | EMBL Symposium The mechanics of life: from development to disease	TBD

Ethical considerations

Ethical Aspects:

Is this research project morally justifiable? This research project is justifiable based on the common morality and four principles. Its purpose is to benefit human well-being, particularly in reproductive health, without causing harm to others. The research team collects information from usual medical examinations and surgeries, and the tissue samples are typically discarded if not collected for research purposes. We do collect sensitive information regarding participants' names, medical history, addresses, contacts, and economic class, and we want more information from their blood tests.

Therefore, we have taken measures to protect the privacy rights of the participants by obtaining informed consent and establishing ethical agreements. The participants are women from Chile and Sweden, and they are free to withdraw from the study at any time, and the research team has no intention of profiting from the research. The first benefit party is just the research group to have research output, but not in a profitable way.

Are there any ethical problems within this research project, and how can they be reduced? The research project raises issues concerning justice. Although the researchers did not intentionally bias any group in recruiting participants, the fact remains that the women who go to the hospitals they collaborated with are generally from lower economic classes. In addition, the willingness to participate in this type of research may be influenced by factors such as values, religion, educational and cultural background, and political class. While willingness is biased in these regards, ethical principles and guidelines and practical measures such as informed consent can minimize any potential coercion.

To address concerns about justice, the researchers could sample from a wider range of hospitals and provide more detailed information about the study design and potential risks to potential participants. Additionally, to reduce the risk of privacy violations, the researchers should store sensitive information in secure locations and limit access to authorized personnel.

Is investing in the research project the right priority, or would it be better to put resources into something else? This question pertains to the importance of the scientific question and the efficiency and sufficiency of the methods used to answer it. The question at hand concerns the lack of knowledge about natural and healthy pregnancy, which is undoubtedly an important issue. However, the use of bioinformatics and sequencing to collect vast amounts of information may not always provide the best means of addressing critical questions. Therefore, adding embryo information to examine the supportive role and interplay between placenta and embryo development, even if not from transcriptomics, could offer valuable insights. However, such an approach may raise additional ethical issues.

Regarding animal warfare: We conducted the experiments by guidelines specified by the European Council Directive and controlled by Comparative Medicine, Karolinska Institutet, Stockholm, Sweden. The animals used are kept in IVC-GM 500 cages with maintained temperature (22℃) and humidity (55–65%) at a 12/12 h light/dark cycle and fed with in house chow diet. We use light isoflurane anesthesia when doing surgeries. All the procedures are conducted by qualified persons who are trained in handling animals. And for all the projects, we replace the animal experiments with human organoid studies if possible.

In a more public context: We should all abide by the consensus and principles. Except for ethics, we also considered pragmatically, that our research methods and results will harm the stability and sustainability of the society and ecosystem. When developing new techniques or pushing forward new scientific knowledge, we consider whether we could practically utilize them, how to safely practice those techniques, creation, and trials, and how to regularize and conform to the community when that technique is practical.

Ethical permit, animal experiments project 1: 20485-2020.

Ethical permit, organoids project 1: EK Nr. 084/2009.

Ethical permit, human data project 3: 2022-04167-01.

Ethical permit, human data project 4: Organoids study: EC ref no. 084/2009. Human placenta samples collected from healthy women: 2022-03542-01, 2023-02270-02. Human placenta samples from PCOS women: 2022-04167-01.

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Attachments to the half-time report

- Copy of the individual study plan[1]
- The research plan
- A Ladok transcript[2] showing completed courses
- Copy of ethical approval(s), if any (decision page only, not the application)
- Publications and/or manuscripts that will form part of the thesis, if any

Also to be sent to the half-time committee:

- The document *Information to the half-time committee* (see KI web page "Half-time review")
- Copy of *Form 5, Half-time review*, part 1 completed

[1] If using the digital ISP-system: print a pdf from the ISP-system after a new revision has been initiated

[2] A Ladok excerpt is not needed if using the digital ISP-system.