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Sexually dimorphic effects of in vitro fertilization (IVF) on offspring growth and metabolism: a mouse model

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Sexually dimorphic effects of in vitro fertilization (IVF) on offspring growth and metabolism: a mouse model

by

Sky K. Feuer

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

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in the

GRADUATE DIVISION

of the

[redacted]

This work represents a substantial portion of my life, passion & devotion to science, research, & the field of assisted reproductive technology. I've nonchalantly joked about dedicating my thesis to every person who has kindly granted a favor over the past 3.5 years (and there have been many, indeed). However, this final product has manifested unquestionably because of Rhodel Simbulan. Thank you for being such a significant part of my life these past few years—I am proud to write this in your name.

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~

And above all, this is for You. For the strength inspired, the growth enthused, the joie de vivre, and the feeling of Home. Let us always be Us. (Yes, Hank, you too.)

Contributions

The contents of the Introduction are modified and reproduced from the following publications:

• Feuer S, Rinaudo P (2012) Preimplantation stress and development. Birth Defects Res C Embryo Today 96: 299-314.

• Feuer SK, Camarano L, Rinaudo PF (2013) ART and health: clinical outcomes and insights on molecular mechanisms from rodent studies. Mol Hum Reprod 19: 189-204.

• Bloise E, Feuer SK, Rinaudo PF (2014) Comparative intrauterine development and placental function in ART concepti: implications for human reproductive medicine and animal breeding. Hum Reprod Update 20: 822-839.

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• Feuer SK, Donjacour A, Simbulan R, Liu X, Maltepe E, Rinaudo PF (2014) Sexually dimorphic effect of *in vitro* fertilization on adult mouse liver and fat metabolomes. Endocrinology 155: 4554-4567.

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Abstract

The Developmental Origins of Health and Disease hypothesis holds that during critical periods in development, organisms exhibit an enhanced plasticity enabling them to fine-tune their metabolism and patterns of gene expression in accordance with environmental cues. Such changes often confer immediate survival advantages, however, transient stresses experienced *in utero* may induce inappropriate adaptive changes that conflict with postnatal environments and consequently impair adult metabolic health. Preimplantation development has been recognized as a window of notable environmental sensitivity, and several animal studies have reported that nutritional, oxidative, and *in vitro* stresses restricted exclusively to this period are sufficient to alter developmental, growth and metabolic trajectories, leading to pathologies such as hypertension, dyslipidemia, and β-cell dysfunction in adulthood. This is particularly relevant to the over 5 million children conceived by *in vitro* fertilization (IVF), although it remains controversial whether *in vitro* embryo manipulation will have lasting effects on offspring health. Although IVF is considered safe, these children display modest changes in growth kinetics, fasting glucose levels, fat deposition and vascular function, which may signify a lasting and possibly dangerous effect of IVF on subsequent development and metabolic homeostasis.

To address this controversy, we developed a mouse model to assess the long-term effects of IVF and found that even clinically optimized IVF conditions are sufficient to reprogram adult metabolism. In particular, female animals exhibit latent overgrowth, increased fat deposition and evidence of β-cell dysfunction, including impaired glucose-stimulated insulin secretion, basal hyperglycemia and insulinemia. Interestingly, males display no overt phenotype. Integrated microarray and metabolomics analyses of adult insulin-sensitive tissues identified sex- and tissue-specific IVF molecular signatures characterized by systemic oxidative stress, mitochondrial dysfunction, impaired insulin and adipogenic signaling, indicating that preimplantation stress can have unique and lasting developmental consequences. Additionally,

female-specific increases in adipogenic and oxidative stress markers may explain the predisposition of IVF females to more severe phenotypes.

Our data support the vulnerability of preimplantation embryos to environmental disturbance and demonstrate that conception by IVF can permanently reprogram growth trajectory and metabolic homeostasis through transcriptional and metabolic mechanisms with lasting consequences for adult growth and energy homeostasis. This has wide clinical relevance, underscoring the importance of continued follow-up of IVF offspring and future attention towards increasing the safety and efficacy of assisted conception.

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Chapter 1: Introduction

"We are not creating life. We have merely done what many people try to do in all kinds of medicine—to help nature."

- *Patrick Steptoe, July 1978, on the birth of the first baby by* in vitro *fertilization.*

Opening remarks

Manipulation of preimplantation embryos by means of assisted reproductive technologies (ART) is widely used for breeding livestock and to treat infertility in humans. In fact, ART has successfully contributed to the birth of over five million individuals worldwide, and now accounts for approximately 60,000 births annually in the United States [1,2]. Although ART is considered safe and the majority of children conceived via *in vitro* fertilization (IVF) appear healthy, the slightly increased prevalence of birth malformations, cancer, and imprinting disorders reflects possible dangers associated with these methods [3]. More recently, reports of metabolic and cardiovascular irregularities in IVF adolescents—including modest increases in blood pressure, fasting glucose, fat deposition, and growth velocity—may signify a lasting effect of IVF on postnatal growth and metabolic health [4-7]. Because IVF offspring are at most 36 years of age, the long-term effects of this procedure on the later stages of development and adult disease susceptibility are uncertain. Whereas the primary outcome of IVF is a live, healthy infant, these data highlight the importance of elucidating the mechanisms by which developmental stress can impact long-term health.

The Developmental Origins of Health and Disease hypothesis

Over the past several decades, research has begun to emerge in support of a clear biological basis for health and disease. More specifically, there is now appreciable evidence that exposure to stressful environments during critical periods of development can increase an organism's susceptibility to a variety of diseases later in life. The administration of thalidomide to alleviate morning sickness in the late 1950s and early 1960s—resulting in an epidemic of phocomelia and

other related birth defects—was paramount to demonstrating that events *in utero* could affect developmental outcomes [8,9]. Although previous researchers had suggested that developmental experiences could inform postnatal health outcomes [10-12], David Barker and colleagues expanded this theory in the late 1980s by correlating nutritional stress *in utero*, manifested by low birth and infancy weights, with heightened risk of adult cardiovascular disease in particular regions of England and Wales [13]. Their fetal origins of adult disease hypothesis posited that fetal undernutrition could reprogram gene pathways to permanently affect body composition and metabolism. Today, this conceptual framework has crystallized into the Developmental Origins of Health and Disease (DOHaD) hypothesis.

The DOHaD hypothesis postulates that during critical periods in development, organisms exhibit an enhanced plasticity that enables them to fine-turn patterns of gene expression in accordance with environmental cues. This ability of developing cells, tissues or organs to adapt to diverse surroundings thus provides one genotype the potential to engender a variety of morphological, physiological or metabolic phenotypes, often in anticipation of similar conditions during postnatal life. When confronted with unexpected or novel stimuli, adaptive changes in gene expression or fine-tuning of signaling networks may facilitate evolutionarily beneficial responses, better preparing an individual for early survival and reproductive success [14]. However, while re-shaping developmental trajectories can confer immediate survival advantages, it can also lead to deleterious outcomes long-term: transient stresses or suboptimal uterine environments may induce inappropriate adaptive changes that conflict with postnatal environments and impair adult health [15,16]. Most frequently, this manifests in latent cardiometabolic pathologies, including ischemic heart disease, stroke, diabetes and obesity. Depending upon the timing of the perturbation, developing organs can be differentially affected and undergo unique changes, leading to slightly different metabolic derangements (**Figure 1-1**).

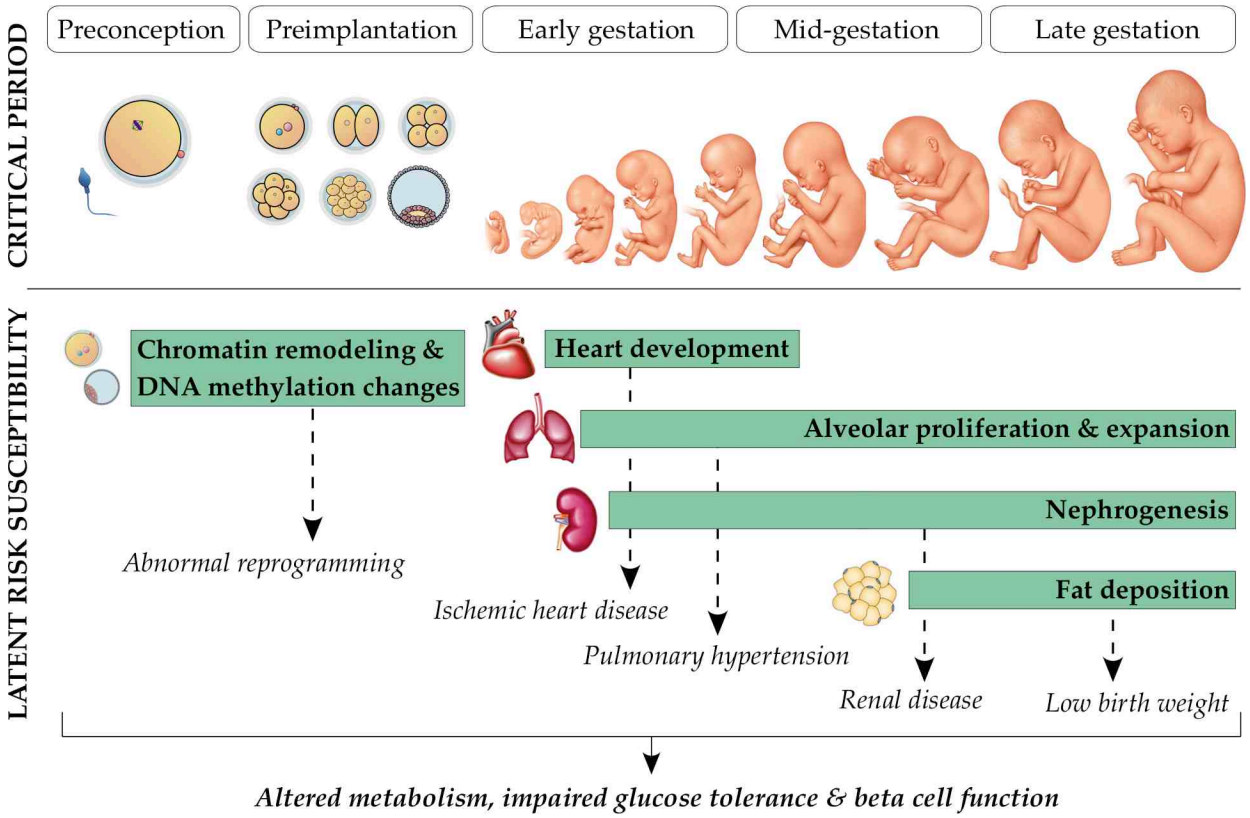


Figure 1-1. Developmental sensitivity in human pregnancy. Metabolic pathologies are common outcomes of stress *in utero*, but environmental insult during different windows of vulnerability can lead to distinct components of the metabolic syndrome in adulthood. Because preimplantation development is a time of extensive epigenetic remodeling, this period may be particularly susceptible to stress-induced metabolic complications.

Preimplantation development

Mammalian preimplantation development is a period of elegantly orchestrated molecular events, extending from fertilization of the oocyte to invasion of the uterine epithelium by the blastocyst (reviewed in [17]). More recently, it has been demonstrated that these early stages are remarkably sensitive to environmental states [18,19], which stimulate different signaling events and changes in gene regulatory networks [20].

Embryo development

Preimplantation development encompasses fertilization through the invasion of the hatched blastocyst into the uterus (**Figure 1-2**). After the oocyte is fertilized by a spermatozoan to form a single-celled zygote, the cellular constituents are replicated and partitioned over several rounds

of cleavage without increasing whole embryo volume [17]. At eight cells, embryo compaction facilitates polarization, providing a foundation for establishing two distinct cell lineages: the trophectoderm, precursor to extraembryonic tissues like the placenta, and the inner cell mass, which will give rise to the embryo proper as well as part of its extraembryonic membrane. These lineages evolve over subsequent asymmetric cleavage divisions, continuing through the formation of the blastocoel cavity. Cavitation permits full expansion of the blastocyst, which hatches from the protective zona pellucida before implantating into the uterus. The first attachment of the human blastocyst to the uterus (apposition stage) occurs on day 8 post-fertilization [21].

Metabolism

The transiton from zygote to blastocyst is marked by diverse nutritional requirements, which are satisfied by a balance between aerobic and anaerobic respiration co-functioning to meet the changing energy demands of early embryos [22]. Progression from the zygote to 2-cell stage has an absolute requirement for pyruvate, and further cleavage stages are synergistically supported by lactate availability [23,24]. Glucose cannot exclusively sustain development until the late 4-cell/early 8-cell stage, but becomes the principal carbohydrate metabolized beginning around compaction [25]. These requirements are relatively conserved across different mammalian species, with slight variations in the time of increased glucose oxidation.

Further, preimplantation development occurs over a dynamic range of conditions as the embryo travels from the oviduct to uterus. Cleavage staged embryos are exposed to mildly alkaline conditions (pH ~7.5-7.8 in different mammalian species) and higher oxygen tensions (~8%) before crossing the uterotubal junction into the more acidic (pH ~6.96-7.1) and less oxygenated (~1-2%) uterine environment [26-28]. Stratification of the reproductive environment reflects the stage-specific metabolic requirements throughout preimplantation development; entry into the uterus occurs coincident with the burst in glucose uptake and oxidation, as well as embryo polarization. This transition in energy substrate preference is further influenced by

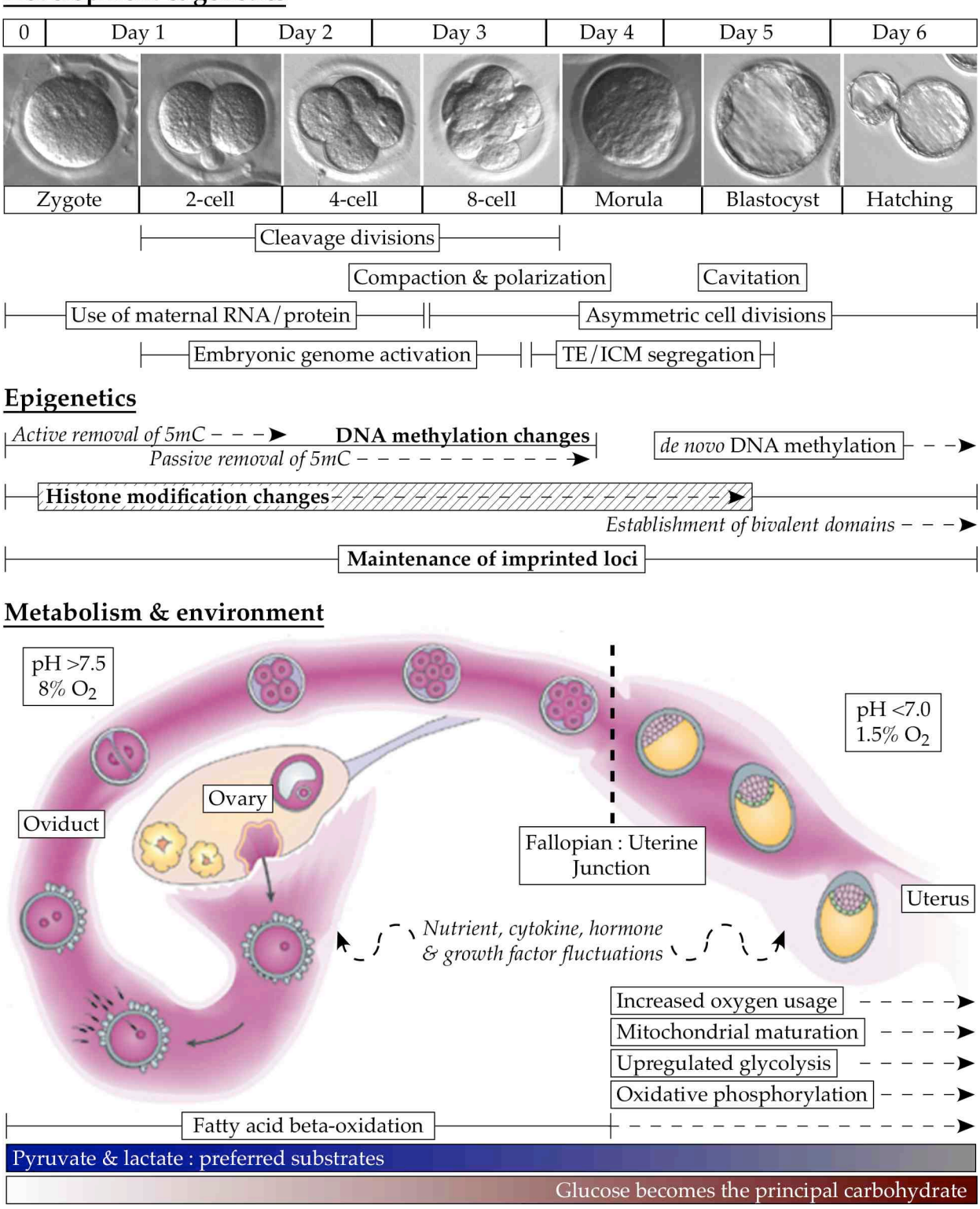


Figure 1-2. Summary of the developmental, epigenetic and metabolic milestones occurring during preimplantation development. These transitions are present in all mammalian species, but the figure depicts mouse development.

fluctuating availabilities of carbohydrates, amino acids, electrolytes, growth signals and other nutrients [17,22,29]. Importantly, disruptions to this dynamic metabolic environment may lead to the deprivation of crucial nutrients at specific points during preimplantation development, with potential consequences for proliferation and growth [27,30,31].

To compensate for nutrient flux—naturally or irregularly occurring—embryos demonstrate strong metabolic plasticity through the differential management of glycolytic activity, oxidative phosphorylation and membrane transport [30-34]. For example, *in vitro* experiments have shown that the absence of glucose in culture prevents the characteristic decline of pyruvate uptake after compaction [24]. Separately, membrane-bound HCO_3^-/Cl^- exchangers and Na^+/H^+ antiporters provide intracellular pH buffering capacity by organizing electroneutral exchange at respective thresholds of 7.2 and 6.9, thereby regulating internal pH within a narrow range and permitting recovery from intracellular alkalosis and acidosis [35,36]. Although the mechanisms by which embryos sense energy status and coordinate developmental plasticity have yet to be fully elucidated, they likely involve a careful integration of several cellular metabolic, transcriptomic, and epigenetic processes.

Epigenetics

Mammalian preimplantation development is additionally characterized by coordinated reprogramming of the genome and establishment of epigenetic marks that are maintained after birth [37]. Epigenetic regulation occurs at the DNA level via methylation of cytosine bases residing in CpG dinucleotides [38], or by modification of histone proteins through methylation, acetylation, ubiquitination, sumoylation or phosphorylation, amongst others [39]. These covalent moieties combine into cooperative epigenetic signatures that affect gene expression through a variety of mechanisms, including (but not limited to) control of gene or promoter accessibility by relaxation or condensation of chromatin, the recruitment of chromatin remodeling enzymes, and occlusion of transcriptional machinery [40-43].

Shortly after fertilization, the complementary parental genomes undergo dramatic changes in DNA methylation status, allowing for subsequent re-methylation to establish somatic differentiation patterns after implantation [44]. Both pronuclei are actively demethylated through an incompletely understood mechanism involving iterative oxidation of 5-methylcytosine via the ten-eleven translocase (TET) family of dioxygenases, as well as passively demethylated over the course of several cell divisions by consecutive cycles of chromatin replication and segregation [45,46]. Imprinted genes escape methyl group removal and are involved in governing normal embryonic and placental development. Concurrent with the epigenetic rearrangements, embryonic genome activation via the transfer from maternal to zygotic transcriptional control initiates a new developmental program [47]. Novel methylation signatures are largely re-established by the peri-implantation stage, and changes in methylation status are believed to control pluripotency, permitting cells to adopt different fates.

Preimplantation exposure to unusual or non-physiological environments

The embryo response to preimplantation stress highlights its remarkable plasticity and capacity for survival. Although the preimplantation environment already presents with a myriad of fluctuations in nutrients, oxygen and pH, these changes occur coincident with the embryo's evolving developmental and metabolic requirements. As a result, perturbation to these environments can have widespread effects on growth and metabolism.

Analysis of the literature reveals that the embryo's response to stress manifests as changes in cell number, lineage ratio, growth velocity and/or gene expression patterns, with subsequent influence on fetal growth. This occurs secondary to many different types of disturbance, including manipulation of pH, glucose or other carbohydrate levels, amino acid content, environment rigidity, and oxygen tension; each will impact developmental competence with distinct effects on blastocyst identity [30,31,48-51]. Stress can divert resources away from networks involved in coordinating optimal growth and development, re-routing them down

alternative metabolic branches. For example, increased amino acid turnover is associated with aneuploidy and embryo inviability [52,53].

The quiet embryo hypothesis postulates that stressed embryos will require more resources and energy in an attempt to alleviate molecular damage, leading to greater mitochondrial activity [54]. Normally, embryos and other rapidly proliferating cells substantially upregulate glycolysis, yet shunt its intermediates away from the TCA cycle [55,56]. Although inefficient for energy production at the ATP level, incomplete glucose oxidation is beneficial for generating biosynthetic intermediates required for cell growth and proliferation. Healthy blastocysts therefore employ mechanisms that slow glycolysis or divert glucose byproducts away from the TCA cycle [49,56,57], resulting in the accumulation of glycolytic intermediates as an anabolic feed for lipid and nucleotide synthesis. However, less viable embryos exhibit increase oxidative phosphorylation [29,53,54,58], reflecting slowed growth and a requirement for ATP in order to recover from cellular stress. An increase in mitochondrial respiration to fuel cell repair machinery would additionally be associated with an increase in free oxygen radicals, leading to aberrant signaling or added cell damage.

Due to its extensive chromatin reorganization, the preimplantation embryo may be particularly vulnerable to perturbation. Suboptimal or inappropriate environmental conditions could cause epigenetic errors or mutations, or affect the programming of cell states and response pathways. Cellular defense strategies against transient stress exposure may be inappropriately programmed as homeostasis, leading to atypical responses to normal physiological signals, exhaustion of cell machinery, and long-term phenotypes. Further, instead of redirecting resources toward damage responses, subtle metabolite fluctuations or atypical nutrient concentrations might instead be sensed as 'normal'—inevitably eliciting an 'appropriate' programming reaction in order to maximize metabolic fitness based on the environment available. In accordance with the DOHaD hypothesis, these reprogramming events are likely strategic for early survival, but may be inadequate or deleterious postnatally.

Assisted reproductive technologies

Human *in vitro* fertilization (IVF) as a treatment for infertility, defined as >1 year of regular unprotected intercourse without conception [59], is regarded as one of the most outstanding accomplishments of the 20th century, leading to the awarding of a Nobel Prize to its visionaries Sir Robert Edwards and Patrick Steptoe in 2010. Assisted conception has been a great source of joy to a number of families and has contributed to the birth of over five million children worldwide, with its use continuing to increase dramatically [1]. However, IVF has been viewed with some skepticism since it deviates rather significantly from natural conception. The dynamic conditions existent in the genital tract are not only lost with IVF, but the procedure introduces many additional variables that could be regarded as stressful to the embryo.

Overview of ART procedures & their potential basis for stress

Before fertilization, preimplantation development, and implantation can occur, the gametes and embryos must overcome a series of obstacles that can sometimes prevent successful pregnancy. In addition to the barriers and maturation points naturally occurring in the reproductive tract, which ensure that only the most dominant and competent gametes attain merely the *opportunity* for fertilization, infertility can complicate these processes with disease, imbalances or blockages. IVF circumvents several of these barriers by combining the gametes (oocytes and sperm) in a laboratory dish and transferring selected resulting fertilized embryos to the uterus several days later (**Figure 1-3**). ART treatments can also involve donor gametes or a gestational carrier.

First, oocytes are collected following controlled ovarian hyperstimulation, which refers to the daily administration of gonadotropins to induce multiple follicles to develop and mature in ovaries, from which mature oocytes are retrieved using transvaginal ultrasound aspiration. Alternatively, immature oocytes may be harvested (for example, from pre-adolescent girls preparing for chemotherapy) and matured *in vitro* at a later time. Sperm is separated from semen obtained from ejaculate or directly from the testicle, epididymus or vas deferens, and allowed to capacitate prior to fertilization. Fertilization may be accomplished either by

overnight co-incubation of gametes, or via intracytoplasmic sperm injection (ICSI). Originally developed to treat severe male infertility in 1990, ICSI uses a micromanipulation device to pierce the zona pellucida of a denuded oocyte and inject a single spermatozoan into the ooplasm; today ICSI is used in approximately two-thirds of ART fertilizations worldwide [60].

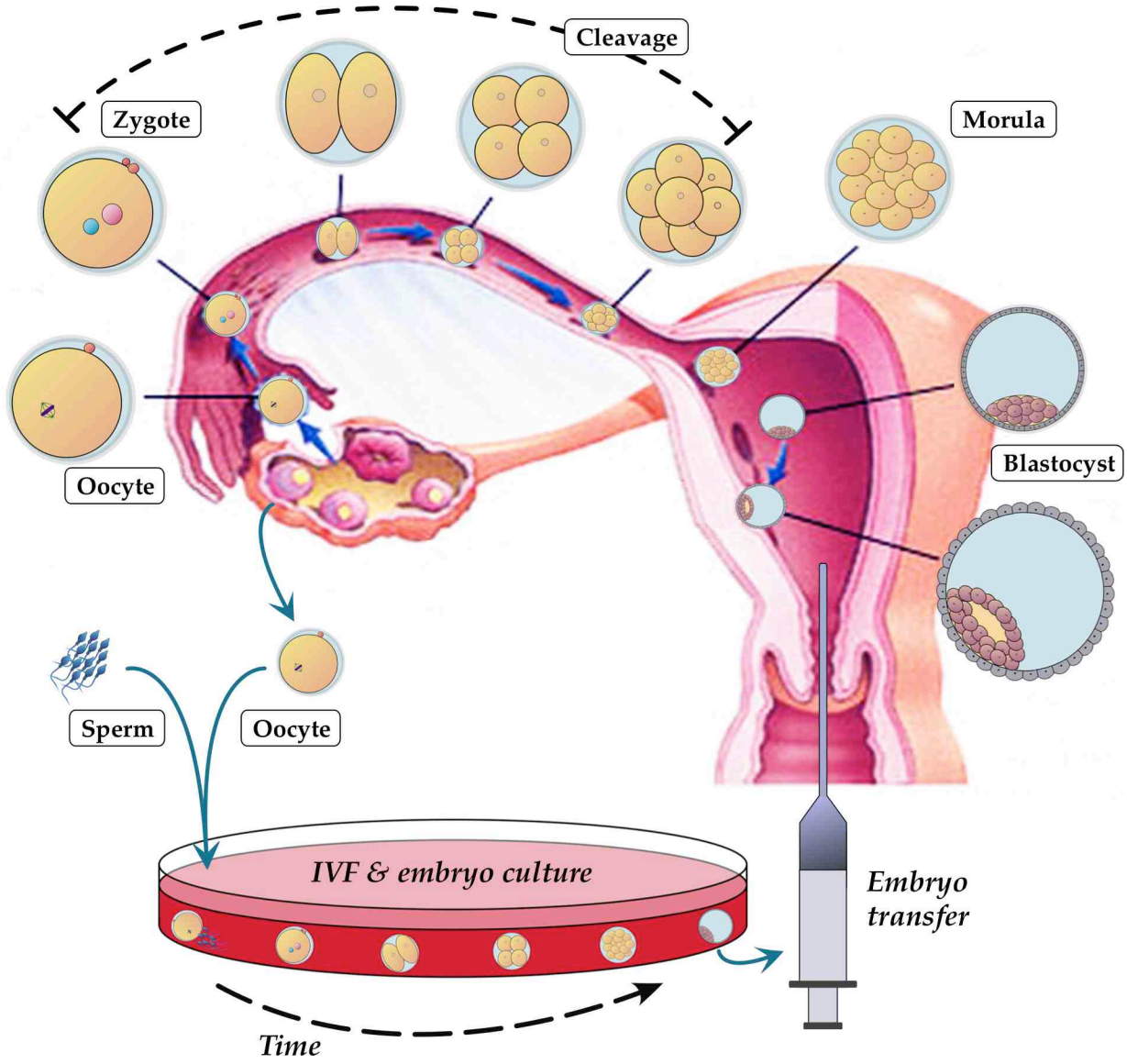


Figure 1-3. Preimplantation development and IVF. Overview of the stages of preimplantation embryo development from ovulation and fertilization of the oocyte in the oviduct, through cleavage divisions and embryo polarization, to blastocyst formation and implantation in the uterus. *In vitro*, gametes are isolated for co-incubation or oocyte fertilization by ICSI, and fertilized zygotes are cultured 3-5 days prior to transfer back into the uterus at either the 8-cell or blastocyst stage.

Following fertilization, zygotes are cultured in a defined chemical medium to the 8-cell or blastocyst stage (3 or 5 days) in a petri dish before transfer into the uterus. Historically, embryos were cultured at 37°C and 5% CO_2 in ambient oxygen (~20% in air), although mounting evidence supports the beneficial effects of performing culture at 5% oxygen tensions on embryo-fetal development and pregnancy, better reflecting the conditions present in the female reproductive tract [61]. The concentrations of many components in culture media differ markedly from their physiological quantities [62], which is important because embryo metabolism relies heavily on feedback mechanisms [63]. Fortunately, significant advances in culture medium recipes have improved embryo development, pregnancy and live birth rates: the earliest years of human IVF were conducted using a high glucose medium lacking amino acids, similar to the suboptimal Whitten's medium used in mouse studies. In 1993, potassium simplex optimization medium (KSOM) was developed for improved embryo culture, and with the addition of an amino acid cocktail in 1995 (KAA) is now considered optimal for mouse embryo development and serves as the basis for several human IVF media used today [3,64]. Recent evidence has demonstrated that different culture conditions lead to unique outcomes, with culture medium formulae correlating with blastocyst rates, cell numbers and gene expression signatures, as well as specific gestational lengths, birth rates and weights [48,65]. This further indicates the importance of the embryo culture environment and the vulnerability of preimplantation embryos to the nutritional milieu available prior to implantation.

Several supplementary ART procedures are available, including the cryopreservation of gametes and/or remaining embryos, assisted hatching of the embryo from the zona pellucida (mechanically, chemically, or using a laser), as well as genetic screening for inherited diseases in 1-2 cells removed from the developing embryo. Most recently, spindle-chromosomal complex transfer—developed to combat mitochondrial disease inheritance by relocating the maternal nuclear DNA to an enucleated donor oocyte, which is then fertilized [66]—was approved in the United Kingdom in February 2015, and could lead to the first birth as early as 2016. Of the ~165,000 ART cycles performed in 2012 (when data are most recently available), the live birth

success rates in the United States ranged from ~40% in women under the age of 35, to ~30% in women 35-37 years old, and were dramatically reduced after age 43 (<5%) [67].

In spite of the advancements in IVF procedures over the past several decades, ART still imparts several potentially stressful factors, which could affect embryo growth and viability (reviewed in [27]). For example, the use of exogenous gonadotropins to stimulate the maturation of multiple oocytes rescues non-dominant follicles and possibly developmentally incompetent oocytes. Both sets of gametes must be handled to assess maturity, and with ICSI oocytes undergo enzymatic and mechanical removal of the protective surrounding cumulous and coronal cells. Moreover, direct penetration of the zona pellucida is physically destructive and could either result in cytoplasmic leakage or contaminate the ooplasm with culture medium. Cryopreservation and thawing procedures may be damaging to intracellular structures, membrane and spindle integrity. Interestingly, petri dishes are six orders of magnitude stiffer than the uterine epithelium [51], and several studies have shown that the rigidity of an environment can alter cell fate transitions and patterns of stem cell differentiation [68]. Finally, the embryos are often exposed to temperature fluctuations and ambient oxygen levels, even when cultured under 5% oxygen tensions. Given that *in vitro* manipulation of embryos introduces vastly novel environments, the preimplantation embryo may have limited mechanisms for maintaining metabolic homeostasis throughout these stimuli.

Complications associated with ART

Because the preimplantation environment established *in vitro* deviates significantly from the female reproductive tract, it is not entirely surprising that numerous health complications are present following manipulation of embryos *in vitro*. To this end, a large number of studies have evaluated the outcome of ART pregnancies and will be briefly described here (reviewed in [3]).

Adverse perinatal outcomes

It is clear that the most significant and consistent risk associated with the use of ART is the increased incidence of multiple pregnancies [2]. Multiple gestations are strongly correlated with greater risk of preterm delivery and low birth weight, which themselves are linked to negative neonatal outcomes such as necrotizing entercolitis, cerebral palsy, cognitive/neuromotor dysfunctions and behavioral difficulties. However, it is unclear whether the results of ART twin pregnancies are more severe than in spontaneous twin conceptions [69]. Unfavorable obstetric outcomes have also been observed in ART singleton gestations, including antepartum hemorrhaging, gestational diabetes, pregnancy hypertensive disorders, preterm delivery, Cesarian section, perinatal mortality, and low birth weight [70].

Low birth weight (defined as <10th percentile) is an established marker of intrauterine stress and has been associated with coronary heart disease, hypertension and hyperlipidemia [71]. Lower birth weights are further correlated with increased blood pressure, fasting insulin concentrations, insulin resistance, and incidence of type 2 diabetes mellitus [72]. While low birth weight is a well-described complication of ART pregnancy [69], there are also marked changes in intrauterine growth trajectory: ART procedures precipitate fetal growth restriction in early to mid-pregnancy, followed by significant increases in placental size and rapid fetal growth toward the end of gestation (reviewed in [73]). The early growth deficits may occur secondary to impaired placentation, which subsequently induce placentomegaly as a compensatory mechanism. ART placentae additionally exhibit histopathological irregularities, which may reflect impaired establishment of the maternal:fetal interface and insufficient fetal support. Further, there is evidence that growth restriction followed by rapid postnatal accelerated growth kinetics can predispose cardiometabolic complications in adulthood [74,75].

Pediatric outcomes

Postnatally, ART has been associated with modest increases in birth defects, most frequently for cardiovascular, urogenital and gastrointestinal malformations. Incidence of the rare imprinting disorders Beckwith-Wiedeman syndrome and Angelman syndrome, along with DNA

methylation discrepancies, are additionally slightly higher in ART children [76,77]. Most studies have found no evidence of ART-induced pediatric neurodevelopmental or behavioral issues, including cognitive function, autism, reduced IQ, or other neurological sequellae [78-80]; a link between ART and autism was recently reported, although the association was largely attributed to adverse prenatal/perinatal outcomes such as multiple births [81]. Any relationship between ART and cancer susceptibility is controversial, and has yet to be confirmed [82].

The long-term health of ART children is probably the most pressing question of the field today [83], particularly in the shadow of the DOHaD hypothesis. However, because the eldest IVF individual is in her mid-thirties (Louise Brown, born July 1978), the relationship between preimplantation embryo manipulation and adult-onset metabolic pathologies is elusive. There are limited investigations into the growth and metabolic features of ART children, and arguably the best-conducted metabolic studies available compared a small cohort of IVF pre-adolescents to children conceived spontaneously by subfertile parents—a valuable control, and the optimal way to remove the potential effects of infertility. ART children (mean age 12.6) displayed modest changes in growth kinetics [4], fat deposition [5], blood pressure and glucose levels [6]. Similar studies have verified ART-induced differences in systemic circulation, artery structure and pulmonary vascular function, highlighting early symptoms of systemic and pulmonary vascular dysfunction [84]. Importantly, these changes are subtle and at best indicate a predisposition for metabolic irregularities in adulthood, which will only be revealed in time.

Limitations and confounding factors

Because ART patients represent a rather exclusive population, reliable data is often confounded by variables such as increased maternal age, infertility, or lack of appropriate control groups. Couples seeking ART are frequently older than those conceiving spontaneously, and therefore more likely to enter ART pregnancies with pre-existing medical conditions, such as diabetes [85]. ART procedures additionally are used for the most part by individuals who fail to conceive spontaneously. Unfortunately, the majority of epidemiological studies compare ART outcomes

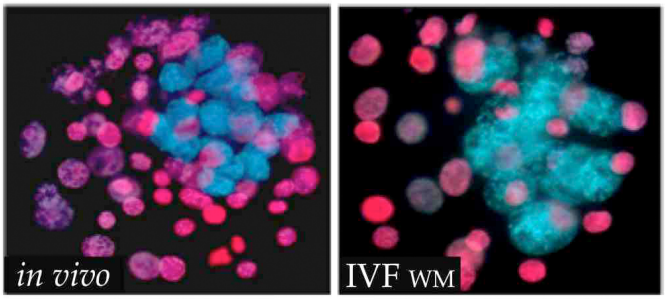
with those of fertile populations, rather than to the results of natural conceptions by subfertile patients. This is important, as infertility *per se* is a credible risk factor for multiple health complications, including cardiovascular disease, depression, and certain reproductive cancers—each of which might affect gestation [86-88]. Further, subfertile women are at increased risk for perinatal complications such as hypertension, pre-eclampsia, antepartum hemorrhage, preterm delivery and low (or very low) infant birth weight, with both prolonged and different etiologies of infertility more significantly affecting perinatal outcomes (reviewed in [3]). It is therefore difficult to parse an effect of embryo manipulation *in vitro* from outcomes secondary to the infertility status of the patients themselves, which underscores the importance and value of using animal models to address these questions.

Previous studies by the Rinaudo Laboratory

Numerous reports have demonstrated an effect of preimplantation environment on a variety of embryo characteristics, including morphology, developmental potential, growth velocity, cell number and lineage ratio, as well as transcriptional signature (reviewed in [3,27]). In particular, our laboratory has evaluated the effects of mouse *in vitro* culture with specific attention to the impact of culture medium and/or oxygen tension. We have published that compared to *in vivo*-generated embryos, a stressful culture medium (Whitten's medium, containing 5mM glucose and lacking amino acids) more significantly impairs cell number, blastocyst rate and gene expression profile than an optimized medium, KSOM supplemented with amino acids (KAA, **Figure 1-4**). The effects of culture medium composition are significantly exacerbated by higher oxygen tensions (20% vs. 5%). In particular, superior *in vitro* culture conditions (KAA, 5% O_2) lead to the misexpression of 29 genes in the blastocyst compared to *in vivo*, but embryos derived from suboptimal conditions (WM, 20% O_2) display a nearly 40-cell reduction and misexpression of over 1,000 genes [49,50].

Condition	# ICM cells	# Cells	%Blastocysts
WM 20% O_2	11.7±0.5	52.5±2.5	61.4%
KAA 20% O_2	11.2±0.4	54.6±1.8	69.2%
WM 5% O_2	16.4±0.5	73.6±2.1	70.3%
KAA 5% O_2	23.5±0.8	90.0±2.3	81.1%
in vivo	12.6±0.6	65.2±1.8	

Stress



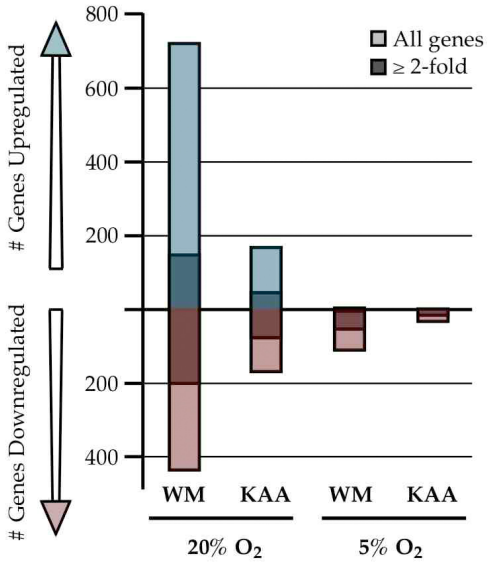


Figure 1-4. Effect of mouse *in vitro* culture under assorted conditions with varying degrees of stress on (A) cell number and blastocyst rate and (B) gene expression. (C) Dual stain of ICM (blue) and TE (pink) comparing IVF and *in vivo* blastocysts. WM, Whitten's medium; KAA, KSOM with amino acids; ICM, inner cell mass.

To investigate whether an altered blastocyst phenotype would affect fetal growth and postnatal health, we developed a mouse embryo transfer model to determine if ART procedures predispose adult metabolic pathologies. In an outbred model (CF1 x B6D2F1), blastocysts generated under stressful IVF conditions (WM, 20% O_2) were transferred to pseudopregnant recipients and the offspring were maintained until adulthood [89]. Compared to control mice derived from naturally conceived blastocysts also transferred to pseudopregnant recipients, IVF_{WM} male mice were significantly heavier and glucose intolerant by 19 weeks of age (**Figure 1-5**). Optimized culture conditions (KAA, 5%O_2) normalized postnatal growth and adult glucose homeostasis. The insulinogenic index (a measure of islet function, calculated by dividing $\Delta_{insulin}$ at 30min of the GTT by $\Delta_{glucose}$ at the same interval [90]) was lower in both IVF_{WM} and IVF_{KAA} mice, indicating a reduced ability of IVF mice to secrete insulin in response to glucose, possibly due to β-cell insensitivity to glucose (**Figure 1-5**) [89].

Because genetic background can influence phenotype [91], and given the value of the genetic homozygosity and decreased phenotypic variability in inbred strains for reproducibility of results [92], we chose to repeat the experiments using an inbred model. In a preliminary

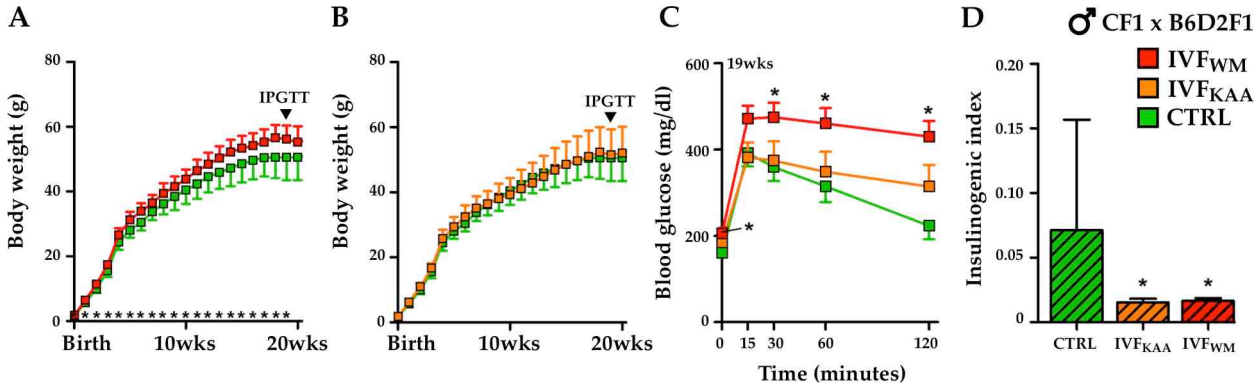
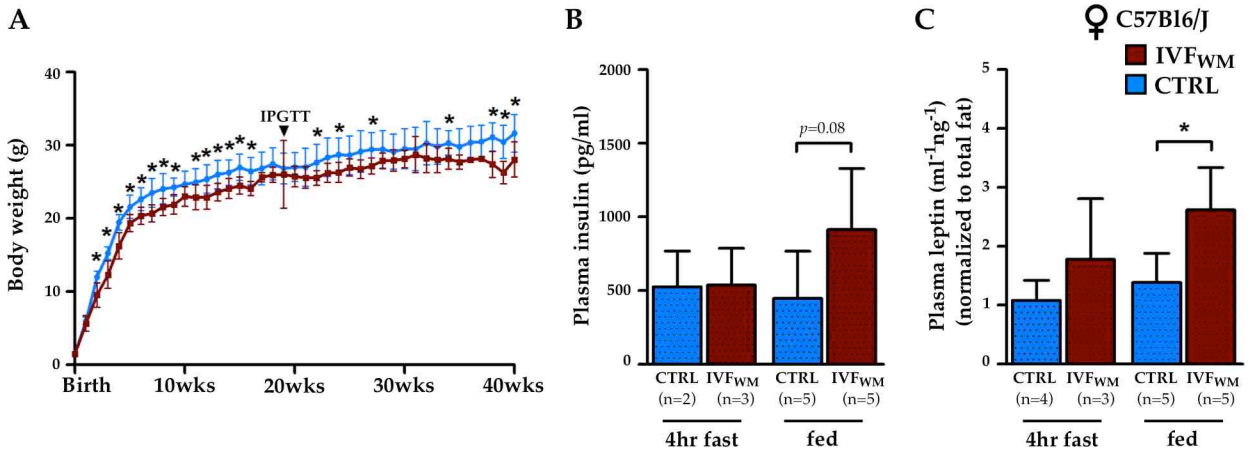


Figure 1-5. Impaired β-cell function in outbred CF1 x B6D2F1 male IVF mice. Postnatal growth curves showing (A) increased body weight in IVF$_{WM}$ males compared to flushed blastocyst controls, which is normalized in (B) optimized IVF$_{KAA}$ conditions. (C) Impaired glucose tolerance in IVF$_{WM}$ males, and (D) significantly lower insulinogenic index for both IVF$_{WM}$ and IVF$_{KAA}$ males versus controls. Error bars depict standard deviation, $^{*}p<0.05$.

study of C57Bl6/J blastocysts generated under suboptimal IVF conditions, we found that IVF$_{WM}$ significantly impaired postnatal growth in both males and females by 2 weeks of age, which remained restricted throughout the postnatal period until time of sacrifice at 35-40 weeks. Adult C57Bl6/J mice (>19wks) displayed blunted glucose tolerance and modest increases in circulating insulin and leptin, with females exhibiting a more severe phenotype (**Figure 1-6**, males not shown). Body morphometry at birth was not different between IVF and controls, indicating that external physiology of IVF conceptuses is not a reliable indicator of postnatal growth or adult glucose homeostasis [93].



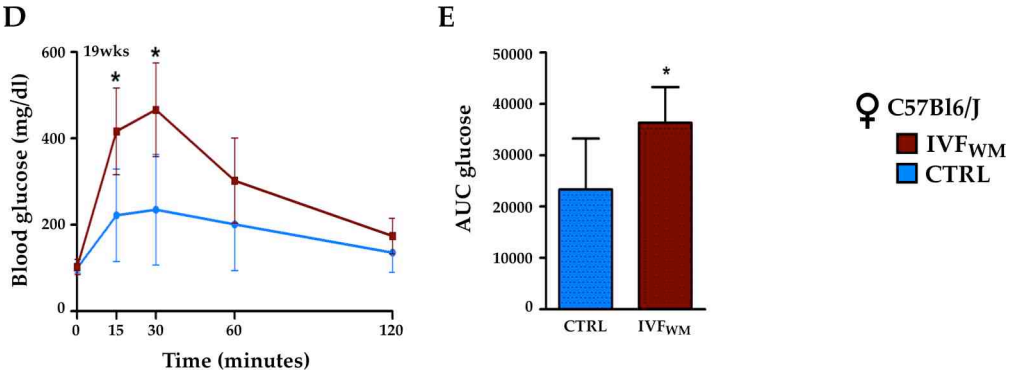


Figure 1-6. Conception by suboptimal IVF_{WM} reduces growth and impairs glucose tolerance in C57Bl6/J mice. (A) Postnatal growth curves depicting average body weight for female control (blue) and IVF_{WM} (red) mice. (B, C) Serum (B) insulin and (C) leptin levels in 4hr-fasted and fed 19wk old mice. (D) IPGTT samples at 19wks for a clearance of a 1.5mg/g glucose bolus over 120min, with (E) corresponding area under the curve (AUC). For all measurements, sample size was n=6 control and n=8 IVF females, unless otherwise indicated. Error bars depict standard deviation, *p<0.05.

Aims and findings of the dissertation

In accordance with the DOHaD hypothesis, embryo manipulation *in vitro* may alter developmental and metabolic trajectories, predisposing pathologies such as obesity and β-cell dysfunction in adulthood [94]. This is particularly relevant to the over 5 million children conceived by IVF, although it remains controversial whether ART procedures will affect long-term metabolic health. Our preliminary evidence that suboptimal IVF conditions significantly perturb growth and glucose tolerance in mouse offspring necessitates a more thorough and clinically applicable investigation of long-term IVF outcomes. Therefore, we developed and present here a mouse model assessing the effects of clinically optimized IVF conditions on postnatal growth and adult metabolic physiology.

In Chapter 2, we demonstrate that improved IVF and embryo culture conditions alter postnatal growth trajectory, fat accumulation, and glucose metabolism in adult mice. Specifically, female mice exhibit latent overgrowth, fat deposition, and impaired β-cell function, but remain phenotypically indistinguishable from controls until approximately 17 weeks of age. Males do not display any overt phenotype.

In Chapter 3, we perform an integrated 'omics study combining microarray and nontargeted mass spectrometry to more clearly interrogate the transcriptional, metabolic and

sexually dimorphic changes associated with IVF in adulthood. Microarray analysis of pancreatic islets and insulin sensitive tissues (liver, skeletal muscle, and gonadal fat) revealed broad changes in metabolic homeostasis, with evidence of systemic oxidative stress and mitochondrial dysfunction. Unbiased metabolic profiling in serum, liver and gonadal fat identified a sex- and tissue-specific effect of IVF on adult metabolite signatures, including female-specific increases in markers of oxidative stress and adipogenesis. Additionally, there was a striking effect of IVF on adult sexual dimorphism, with exaggerated sex bias in hepatic IVF tissue and significantly reduced male-female differences in IVF adipose tissue. Together, these analyses describe IVF-associated molecular changes that reflect the observed physiological phenotypes.

Chapter 4 evaluates the data and integrates it into the existing fields of developmental stress, sexual dimorphism, and the current status of IVF outcomes. In the concluding remarks (Chapter 5), we return to the preimplantation stages and evaluate potential mechanisms underlying the latent metabolic phenotypes induced by IVF, which maybe extrapolated to whole fields of developmental reprogramming, obesity and diabetes. We also provide suggestions for future work, as well as a brief commentary on assisted reproductive policy and IVF social issues.

Chapter 2: Effect of optimized *in vitro* fertilization conditions on postnatal growth, fat deposition and glucose homeostasis in mice

It is becoming increasingly apparent that the conditions present during preimplantation development can affect embryo growth and metabolism, but also subsequent placentation, fetal growth, postnatal and adult metabolic health. This is particularly relevant to the over 5 million individuals conceived *in vitro*, as their long-term health is indeterminate. Because the primary objective of assisted reproductive procedures is delivery at term of a live, healthy infant, postnatal aftereffects occurring outside of the neonatal period are often overlooked. Consequently, it is imperative to expand the evaluation of IVF outcomes to include postnatal growth and adult metabolic health.

We and others have demonstrated that embryo culture differentially affects developmental velocity, blastocyst rate and cell number, placentation, fetal growth, and gene expression signatures, with more stressful conditions predisposing more severe phenotypes [48-50]. To expand these analyses and evaluate postnatal outcomes, in a previous and preliminary study our laboratory found that IVF under suboptimal conditions (Whitten's medium and 20% oxygen) affects growth, glucose homeostasis, leptin and corticosterone levels in a sex-specific manner (see **Figure 1-4**, **1-5**) [93].

The observation that IVF and embryo culture under stressful conditions perturbs adult metabolism led us to design additional experiments using optimized conditions. We selected potassium simple optimized medium containing amino acids (hereafter referred to as KAA), with 5% oxygen levels. Compared with Whitten's medium and a 20% oxygen tension, these parameters improve blastocyst rate, cell number and competence for implantation [50,95], and thus are considered optimal for mouse embryo culture [48]. Importantly, many IVF clinics utilize media derived from KSOM, making its use clinically relevant. In this chapter, we provide evidence that preimplantation embryo manipulation can alter postnatal growth trajectory and impair adult glucose and lipid metabolism in a sex-, tissue-, and culture

condition-specific fashion. This indicates that individual stresses experienced transiently during development may have unique and lasting consequences for adult metabolism.

Results

To investigate the impact of *in vitro* fertilization and embryo culture on subsequent development and postnatal physiology, we developed a mouse model of IVF, embryo culture, and embryo transfer (**Figure 2-1**).

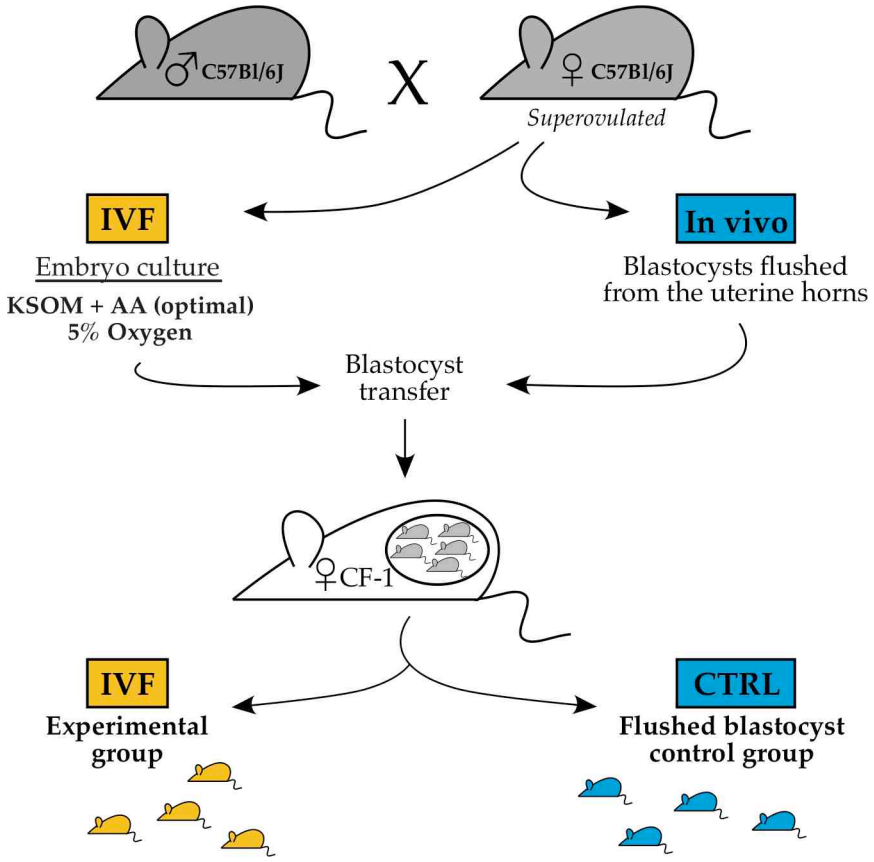


Figure 2-1. Experimental paradigm for generating postnatal IVF and control cohorts.

Briefly, cumulous-oocyte-complexes were isolated from superovulated C57Bl/6J females and incubated with previously capacitated cauda epididymal sperm. Following fertilization, zygotes were washed and cultured in selected conditions (for this study, KAA medium and 5% oxygen,

IVF group). Blastocysts were transferred into pseudo pregnant CF-1 recipients, and resulting offspring were maintained up to 30 weeks for physiological and molecular analyses. As a control, C57Bl/6J females were superovulated and mated overnight with C57Bl/6J males. *In vivo*-generated blastocysts were isolated 96hrs post-fertilization and immediately transferred to recipients (flushed blastocyst/FB control group).

Decreased survival of IVF litters and growth restriction in IVF female pups

Litter size was reduced following conception using optimized IVF conditions (e.g. IVF_{KAA}, **Figure 2-2a**), with a decreased percentage of transferred embryos surviving to term (**Figure 2-2b**). There was no effect of IVF on male:female ratio (**Figure 2-2c**).

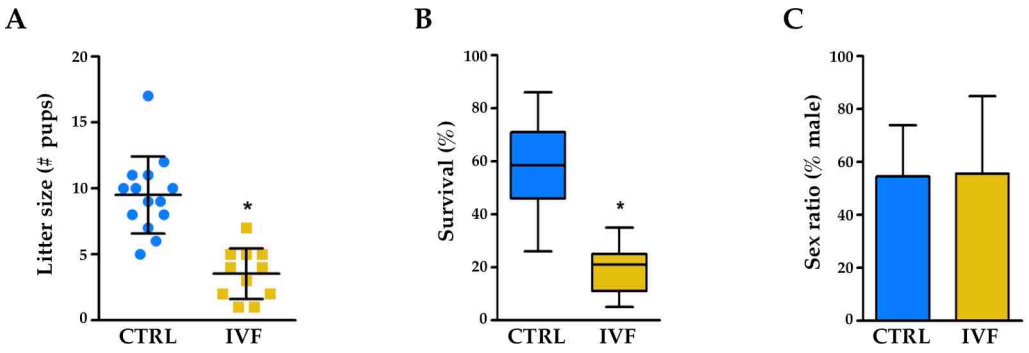


Figure 2-2. Litter and survival parameters measured in control and IVF pups at birth. For all analyses, samples size was n=14 control litters and n=11 IVF litters. Error bars depict standard deviation, *p<0.05.

Because litter size can influence postnatal growth and glucose homeostasis [96,97], only litters of 4-8 animals were maintained to remove any potential confounding effects of litter size. In this cohort, we observed a marked sexually dimorphic effect of IVF, beginning with body morphometry at birth. In females: birth weight, anogenital distance (a measure of prenatal androgen exposure [98]), crown-rump length, and head diameter were all significantly lower in IVF pups compared to controls, whereas anogenital index (anogenital distance standardized to body weight), abdominal diameter, and body mass index were not affected (**Table 2-1**).

Conversely, male IVF and control pups were indistinguishable for all parameters examined, although the IVF group displayed a trend toward a longer anogenital distance (p=0.08).

	Males		Females	
Physiological parameter	**FB (n=9)**	**IVF (n=15)**	**FB (n=8)**	**IVF (n=10)**
Birth weight (g)	1.51 ± 0.12	1.50 ± 0.24	1.65 ± 0.08	**1.40 ± 0.18****
Anogenital distance (mm)	1.84 ± 0.17	2.07 ± 0.33*	1.33 ± 0.17	**1.14 ± 0.16****
Anogenital index (mm/g)	1.23 ± 0.14	1.43 ± 0.41	0.80 ± 0.10	0.84 ± 0.19
Head diameter (mm)	7.64 ± 0.23	7.63 ± 0.36	8.06 ± 0.45	**7.55 ± 0.46****
Abdominal diameter (mm)	9.92 ± 1.41	9.16 ± 1.13	8.80 ± 0.73	8.46 ± 1.40
Crown-rump length (mm)	28.92 ± 1.27	29.24 ± 1.49	31.10 ± 0.86	**29.04 ± 1.11****
Body mass index (g/cm^2)	0.182 ± 0.02	0.175 ± 0.02	0.170 ± 0.01	0.165 ± 0.02

Table 2-1. Morphometric parameters measured in FB and IVF pups at birth. Values are ±SDM, with p<0.05 indicated by ** in bold and p<0.1 by *.

Female IVF animals exhibit latent overgrowth & increased fat deposition

After birth, weights were similar between the IVF and control cohorts until 17 weeks of age, at which point IVF females became heavier than controls (**Figure 2-3a**). Because latent growth alterations have been associated with metabolic disease [99], we probed the consequences of nutritional stress by challenging both cohorts with a high fat diet beginning at 24 weeks and lasting until sacrifice at 30 weeks (6wks total). All animals displayed comparable changes in weight. IVF females consumed more food at 7 and 20 weeks of age, but the average chow intake at 28 weeks—after placement on the high fat diet—equaled control amounts (**Figure 2-3b**). For analyses of body composition, dual X-ray absorptiometry (DEXA) scanning at 8, 16, 21 and 28 weeks showed increased bone mineral density in IVF females at 8, 21 and 28 weeks (**Figure 2-3c**). We additionally observed continuous accumulation of fat throughout postnatal life: body fat percentage was initially lower in IVF than FB females, but the IVF animals steadily accrued fat mass and surpassed control levels by age 21 weeks (**Figure 2-3d**, **2-3e**). Interestingly, this increase in fat deposition occurred prior to the nutritional challenge posed by the high-fat diet.

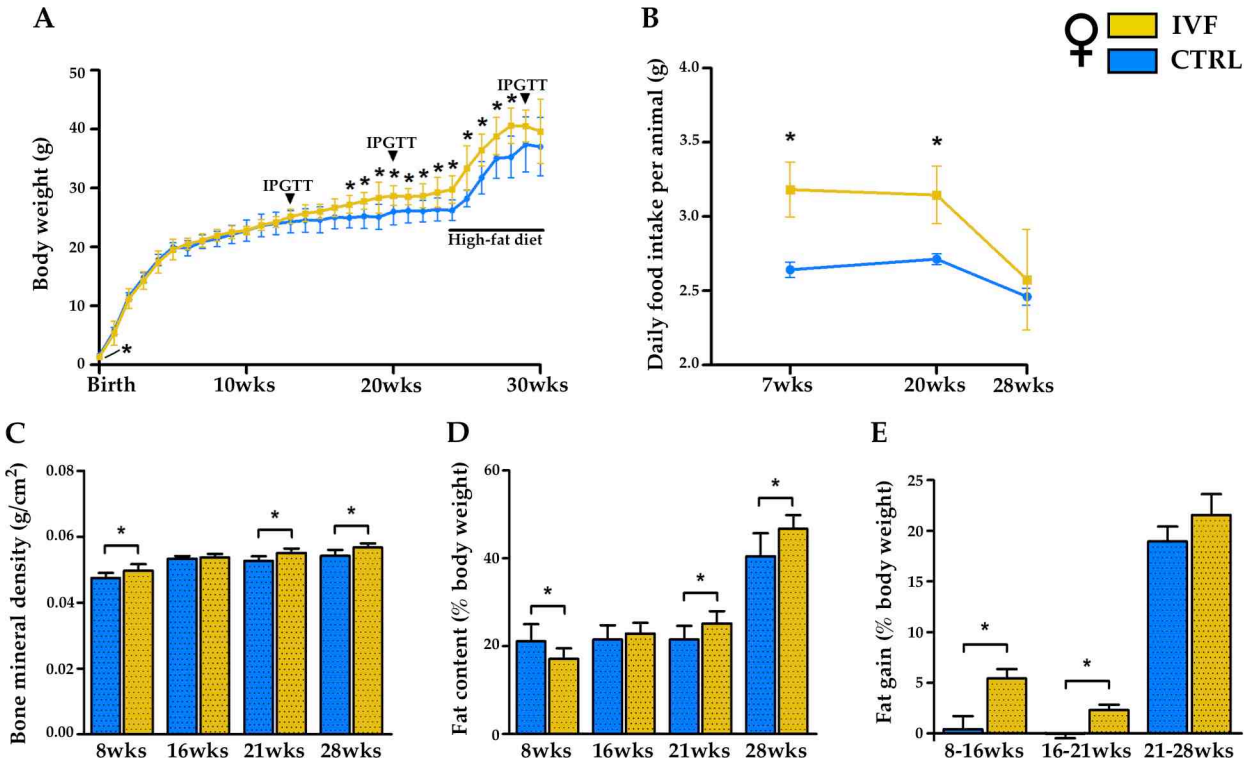
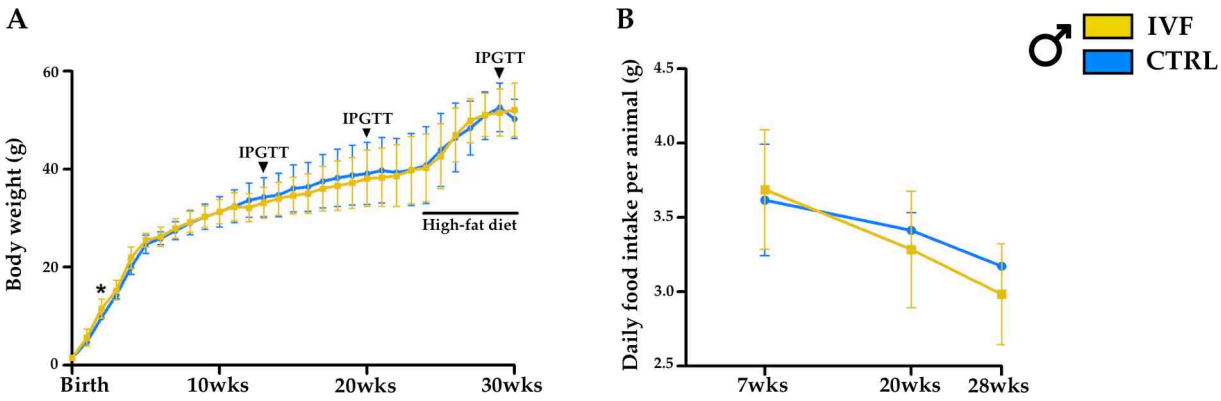


Figure 2-3. Evidence of altered growth and body composition in IVF female mice. (A) Postnatal growth of control (blue) and IVF (yellow) females, showing periodic sampling of IPGTT (see Figure 2-5) and the administration of a high-fat diet beginning at 24wks. (B) Average daily food intake monitored over selected 5-day periods throughout postnatal development. (C-E) Periodic DEXA scanning of (D) bone mineral density and (E) fat content, with calculated (F) percent fat gain in control and IVF females. For all analyses, sample size was n=8 control and n=7-9 IVF females. Error bars depict standard deviation, *p<0.05.

In contrast, there was no effect of IVF on growth (**Figure 2-4a**), food intake (**Figure 2-4b**), or body composition (**Figure 2-4c – 2-4e**) in male animals, including after placement on the high-fat diet.



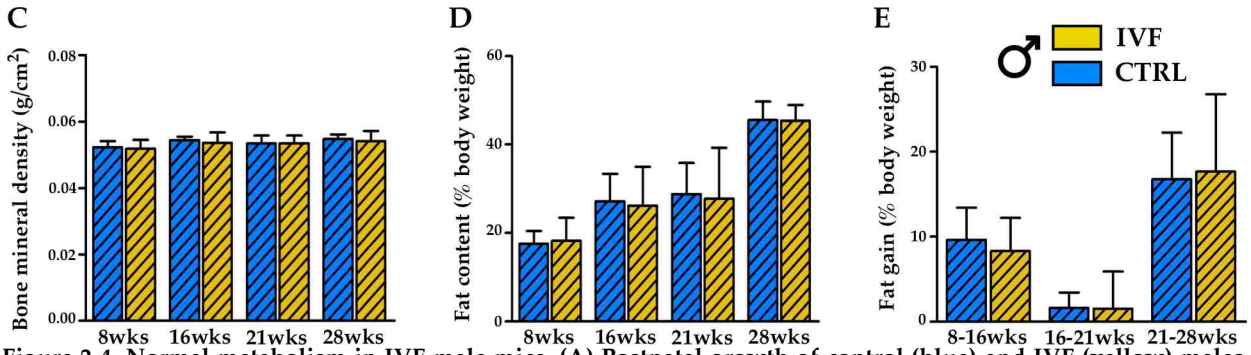
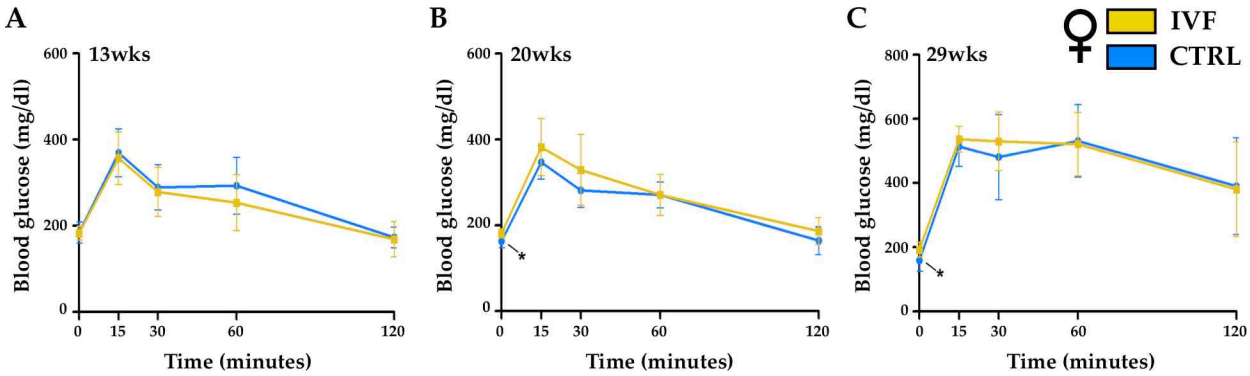


Figure 2-4. Normal metabolism in IVF male mice. (A) Postnatal growth of control (blue) and IVF (yellow) males, showing periodic sampling of IPGTT (see Figure 2-6) and the administration of a high-fat diet beginning at 24wks. (B) Average daily food intake monitored over selected 5-day periods throughout postnatal development. (C-E) Periodic DEXA scanning of (D) bone mineral density and (E) fat content, with calculated (F) percent fat gain in control and IVF males. For all analyses, sample size was n=8 control and n=14 IVF males. Error bars depict standard deviation, *p<0.05.

Increased fasting glucose levels but normal glucose tolerance in IVF female mice

Because low birth weight and accelerated neonatal catch-up growth are linked to impaired glucose homeostasis and the development of type 2 diabetes [100-102], we examined glucose metabolism by intraperitoneal glucose tolerance tests (IPGTT) at 13, 20 and 29 weeks. At all time points analyzed, there were no changes in glucose handling between female IVF and control animals (**Figure 2-5a – 2-5c**), although fasting glucose levels were significantly higher in IVF females at 20 and 29 weeks (**Figure 2-5d**). Glucose tolerance was markedly impaired in both cohorts at 29 weeks compared to 13 and 20 weeks with corresponding increases in the area under the curve (AUC, **Figure 2-5e**), likely secondary to the effects of aging and/or the high-fat diet.



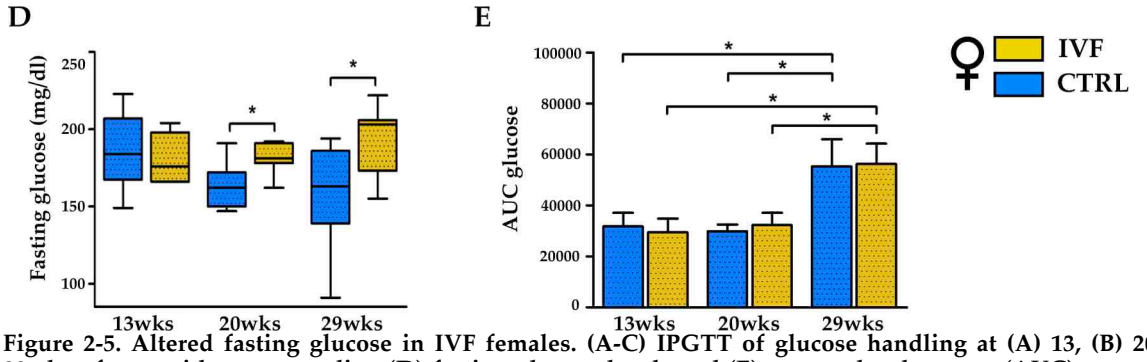


Figure 2-5. Altered fasting glucose in IVF females. (A-C) IPGTT of glucose handling at (A) 13, (B) 20 and (C) 29wks of age, with corresponding (D) fasting glucose levels and (E) area under the curve (AUC) measured from total glucose levels during entire IPGTT course. For all analyses, sample size was n=8 control and n=7 IVF females. Error bars depict standard deviation, *p<0.05.

There were no changes in glucose handling, fasting glucose levels, or AUC between male IVF and control animals (**Figure 2-6**), although IVF males exhibited a trend toward decreased fasting glucose at 13wks (p=0.06, **Figure 2-6a**, **2-6d**). Similar to the females, all males showed evidence of glucose intolerance at 29 weeks versus 13 and 20 weeks (**Figure 2-6e**).

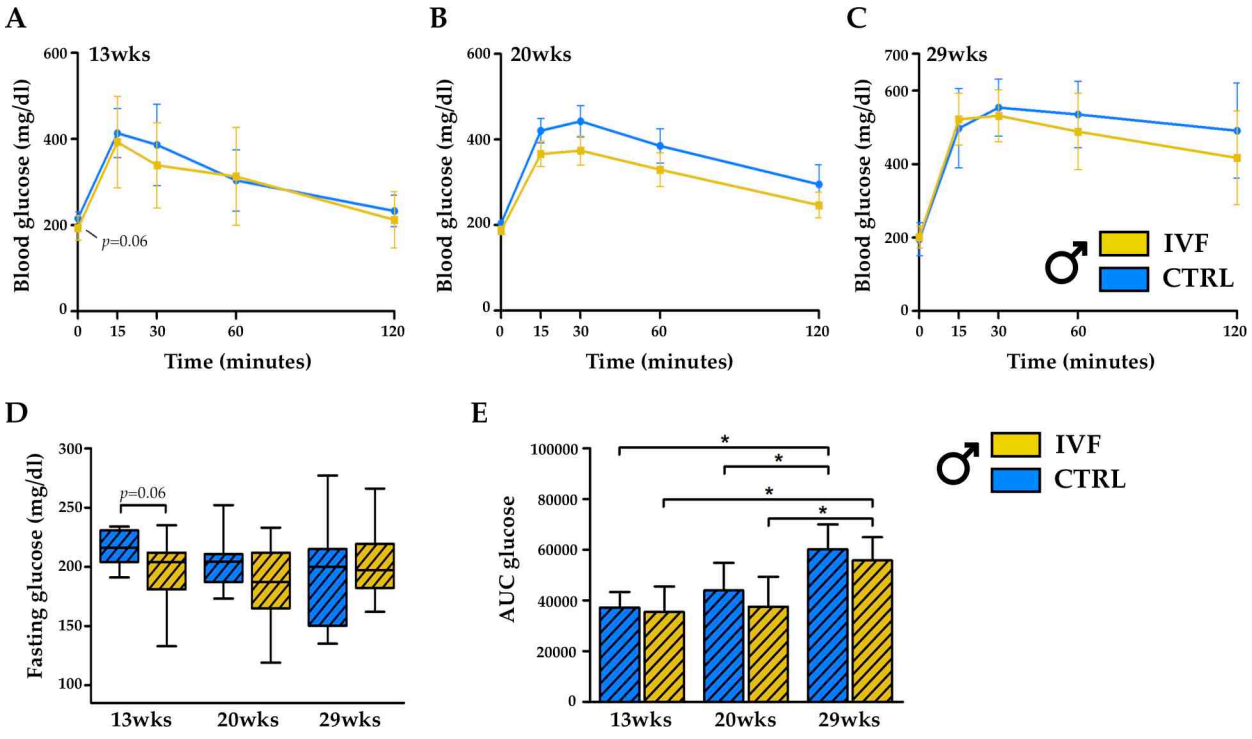


Figure 2-6. Normal glucose handling in IVF males. (A-C) IPGTT of glucose handling at (A) 13, (B) 20 and (C) 29wks of age, with corresponding (D) fasting glucose levels and (E) area under the curve (AUC) measured from total glucose levels during entire IPGTT course. For all analyses, sample size was n=8 control and n=11-14 IVF males. Error bars depict standard deviation, *p<0.05.

Evidence of pancreatic β-cell dysfunction in adult IVF female mice

To further interrogate β-cell function, we performed glucose-simulated insulin secretion assays in female IVF and control pancreatic islets isolated and cultured *in vitro*. Preliminary experiments in islets from mice aged 12 weeks revealed normal insulin secretion in response to basal and stimulatory levels of glucose for both conception conditions (**Figure 2-7a**). At 29 weeks, the insulin secretory response to glucose was normal in control animals, even after 5 weeks on the high fat diet. However, 29-week old IVF females exhibited basal hyperinsulinemia, and the effect of glucose on islet insulin secretion was ablated. This indicates these animals are susceptible to β-cell dysfunction following nutritional stress (**Figure 2-7b**).

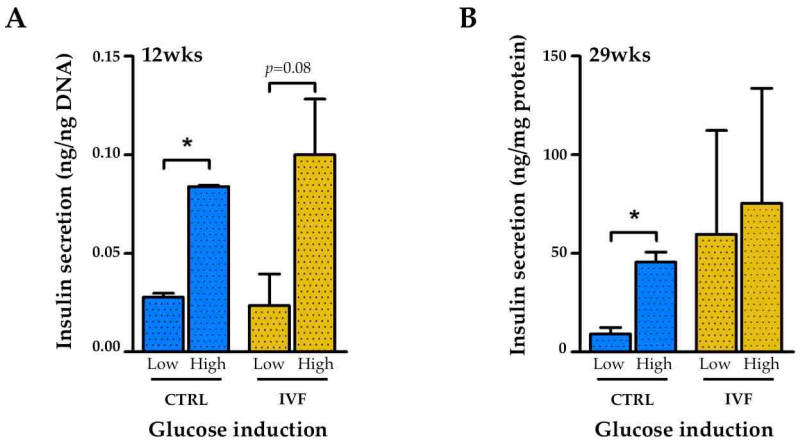


Figure 2-7. Latent β-cell dysfunction in IVF females. Glucose-stimulated insulin secretion assays conducted on isolate pancreatic islets from (A) 12wk (n=2 control and n=2 IVF) and (B) 29wk (n=3 control and n=5 IVF) mice, depicting normalized insulin secretion in response to basal (3mM) and stimulatory (28mM) glucose challenges. Error bars depict standard deviation, *p<0.05.

Altered anogenital parameters in adult IVF females

Animals were sacrificed at 30 weeks of age, at which point the IVF females were moderately heavier than controls (p=0.08), with shorter anogenital distances and smaller anogenital indices (**Table 2-2**). BMI, crown-rump length, and biparietal diameter were normal. Analysis of organ weights revealed that IVF females had smaller pancreata (weight-standardized) compared to FB controls (**Figure 2-8a**). For all morphometric parameters examined, male IVF animals were statistically indistinguishable from FB controls, although they displayed a trending decrease in relative adrenal weight (p=0.06, **Table 2-2** and **Figure 2-8b**).

	Males		Females	
Physiological parameter	**FB (n=8)**	**IVF (n=14)**	**FB (n=7-8)**	**IVF (n=7)**
Sacrifice weight (g)	50.25 ± 4.10	50.22 ± 4.85	36.64 ± 5.21	40.90 ± 2.85*
Anogenital distance (mm)	10.66 ± 0.77	10.58 ± 1.02	3.89 ± 0.58	**2.90 ± 0.70****
Anogenital index (mm/g)	0.21 ± 0.03	0.21 ± 0.02	0.11 ± 0.02	**0.07 ± 0.02****
Biparietal diameter (mm)	11.73 ± 0.68	11.49 ± 0.85	11.71 ± 0.97	11.04 ± 0.53
Crown-rump length (mm)	100.25 ± 3.69	99.06 ± 4.91	97.04 ± 5.91	99.86 ± 2.54
Body mass index (g/cm^2)	0.500 ± 0.02	0.514 ± 0.06	0.394 ± 0.09	0.411 ± 0.04

Table 2-2. Morphometric parameters measured in FB and IVF mice at sacrifice (29-30wks). Values are ±SDM, with $p<0.05$ indicated by ** in bold and $p<0.1$ by *.

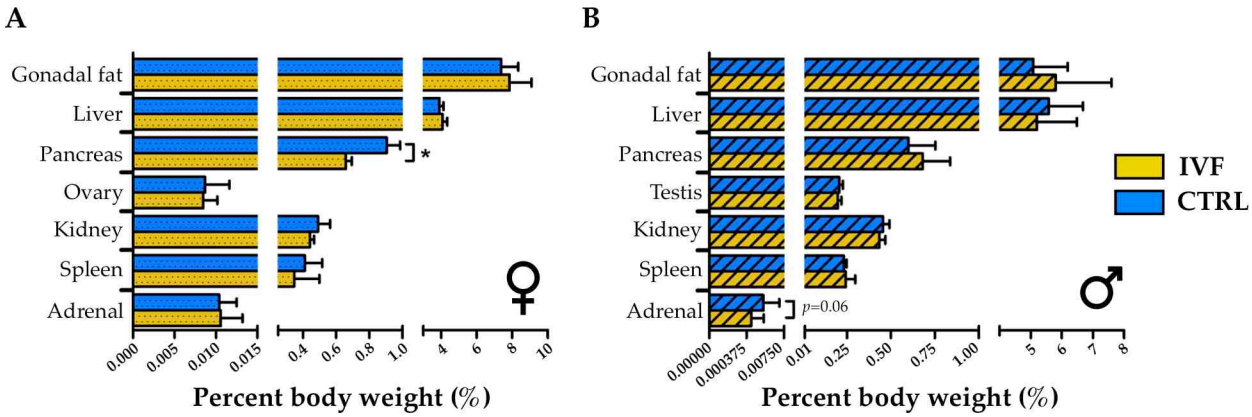


Figure 2-8. Organ sizes depicted as relative body weights for (A) female and (B) male control and IVF animals at sacrifice (29-30wks). For all measurements, sample size was: n=5-8 control females, n=5-8 IVF females, n=8 control males, n=8-14 IVF males. Error bars depict standard deviation, *$p<0.05$.

Comments on litter size: no effect of litter size on postnatal growth

Although the vast majority of studies examining litter size versus adult metabolism have compared very small litters (<3 pups) with larger ones (>8 pups), there is some concern that neonatal programming can be affected by litter size within the range of 4-8 animals [103]. To address this, we evaluated growth in IVF and control litters with further size breakdown: litters were separated as small (4 pups) versus large (≥5 pups). As described in **Table 2-3**, the only group containing a small litter was the IVF female cohort, and those specific animals were sacrificed at 12 weeks for the preliminary islet insulin secretion assays (see **Figure 2-7a**). We performed a comparison of growth until this point and did not observe any significant changes

	Cohort size	Litter sizes	Small (4 pups)	Large (5-8 pups)
Control males	n=8 mice	5, 8 pups	n=0	n=8
Control females	n=8 mice	5, 6 pups	n=0	n=8
IVF males	n=12 mice	5, 5, 5, 7 pups	n=0	n=12
IVF females	n=9 mice	4, 5, 7 pups	n=2	n=7

Table 2-3. Summary of litter size contributions to the IVF and FB cohorts used in this study.

in growth between the small- and large-sized litters (**Figure 2-9a**). To ensure no differences existed between the small group and the largest-sized litters (n=7-8 pups), we additionally evaluated postnatal growth between these two groups, again only until time of sacrifice of the smallest-sized litter at 12 weeks. Growth curves were significantly different between the IVF and control females at ages 4-6 weeks but for not other times points (**Figure 2-9b**). As a result, we did not believe the differences were large enough to warrant stratified analyses by litter size.

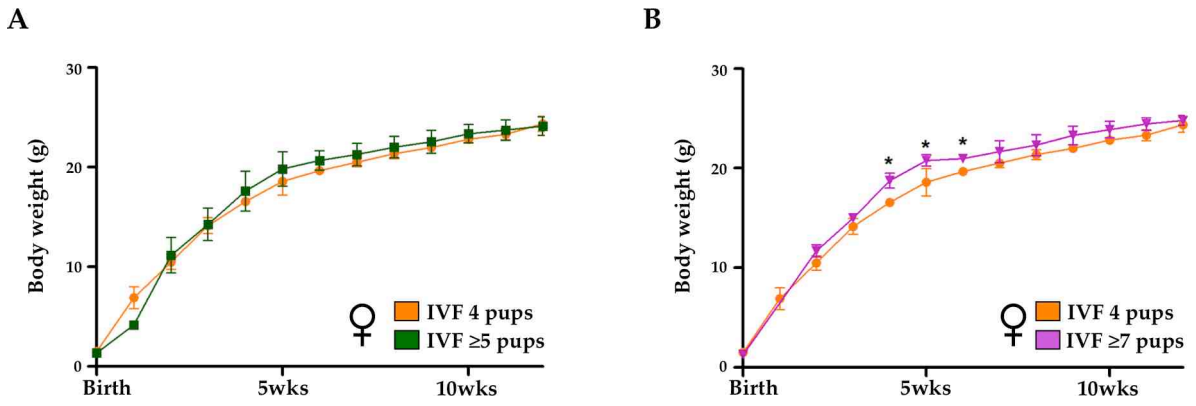


Figure 2-9. No differences in growth between IVF mice derived from small (4 pups, n=2) vs large (5-8 pups, n=7) or largest (7-8 pups, n=3) litters. Error bars depict standard deviation, *p<0.05.

Taken together, these data indicate a sex-specific effect of IVF on postnatal growth, fat deposition, and beta cell function in adulthood.

Chapter 3: Molecular, metabolic, and sexually dimorphic effects of *in vitro* fertilization on adult insulin-sensitive tissues in mice

The data presented in Chapter 2 demonstrate that *in vitro* fertilization and embryo culture, even under optimized conditions, lead to long-term sexually dimorphic growth and metabolic alterations in a mouse model. This both confirms the sensitivity of the fertilization and preimplantation stages to environmental influence on postnatal physiology, and extends the assessment of embryo culture outcomes into late postnatal life. However, it is not unclear what molecular and metabolic changes are present in IVF postnatal tissues, and how they might contribute to the female-specific alterations in growth, fat deposition, and glucose handling.

Sex-based differences are present throughout the majority of mammalian physiologies, behaviors and diseases, arising from a variety of immunological, hormonal, genetic and epigenetic mechanisms [104]. Sexual dimorphism is particularly common to several metabolic hallmarks in adulthood—including glucose homeostasis, insulin sensitivity, β-cell function, adipose tissue depots—and therefore can influence disease susceptibility and progression [105]. Because up to one-third of transcripts are differentially expressed between males and females by the blastocyst stage [106], it is not surprising that models of developmental programming frequently exhibit sex bias, although this phenomenon is poorly understood [107].

In this chapter, we adopt an integrated omics approach, using microarray and non-targeted metabolomics analyses to further interrogate the metabolic changes associated with IVF in adulthood, as well as dissociate some of its sex-specific physiology. Recent advances in metabolomics technology have permitted comprehensive and systematic analyses of the biochemical fingerprints within cells and tissues, thus providing an immediate compendium of cellular metabolic processes [108]. Coupling cell functional status (metabolomics) with events occurring more upstream (transcriptomics) thereby provides a richer and more expanded understanding of the complex IVF phenotypes. To begin, microarray profiling was conducted on female IVF- and *in vivo*-conceived pancreatic islets, liver, gonadal fat, and skeletal muscle

isolated from the mice generated and described in Chapter 2. Global metabolite signatures were then evaluated in both male and female liver and gonadal adipose tissues from each conception condition. Serum profiling was additionally performed in adult IVF and control females. Here we describe the transcriptional, metabolic, and sexually dimorphic alterations associated with IVF, including female-specific evidence of increased adipogenesis and oxidative stress.

Results

Microarray profiling and global metabolomics were conducted on tissues harvested from the mice produced in Chapter 2 [93]. As described earlier, these mice were generated by IVF under conditions considered optimal for mouse embryo culture and reflective of current IVF clinical practices [48], and control *in vivo*-produced blastocysts were isolated 96 hours post-fertilization for transfer to recipients [89]. To probe the consequences of nutritional stress, all animals were placed on a high fat diet beginning at 24 weeks of age until time of sacrifice at 30 weeks (6 weeks total), at which point tissues were harvested for experiments. For the subset of animals used in the molecular studies described in this chapter: birth weight was initially lower in female IVF animals, then body weights were similar between IVF and control mice up through 16 weeks, at which point IVF females showed a statistical increase in body weight (prior to administration of the high fat diet) that lasted through 28 weeks of age. At time of sacrifice, there were no significant changes in body weight or weight-standardized organ sizes between the two groups. Dual energy X-ray absorptiometry at 8, 16, 21 and 28 weeks revealed that IVF females had initially lower percent adiposity but then statistically surpassed control levels of body fat by 21 weeks of age. These weight and fat percent findings might be partially explained by the increased food consumption by IVF females at 7 and 20 weeks, but not at 28 weeks. **Figure 3-1** describes the physiological changes present between IVF and control animals.

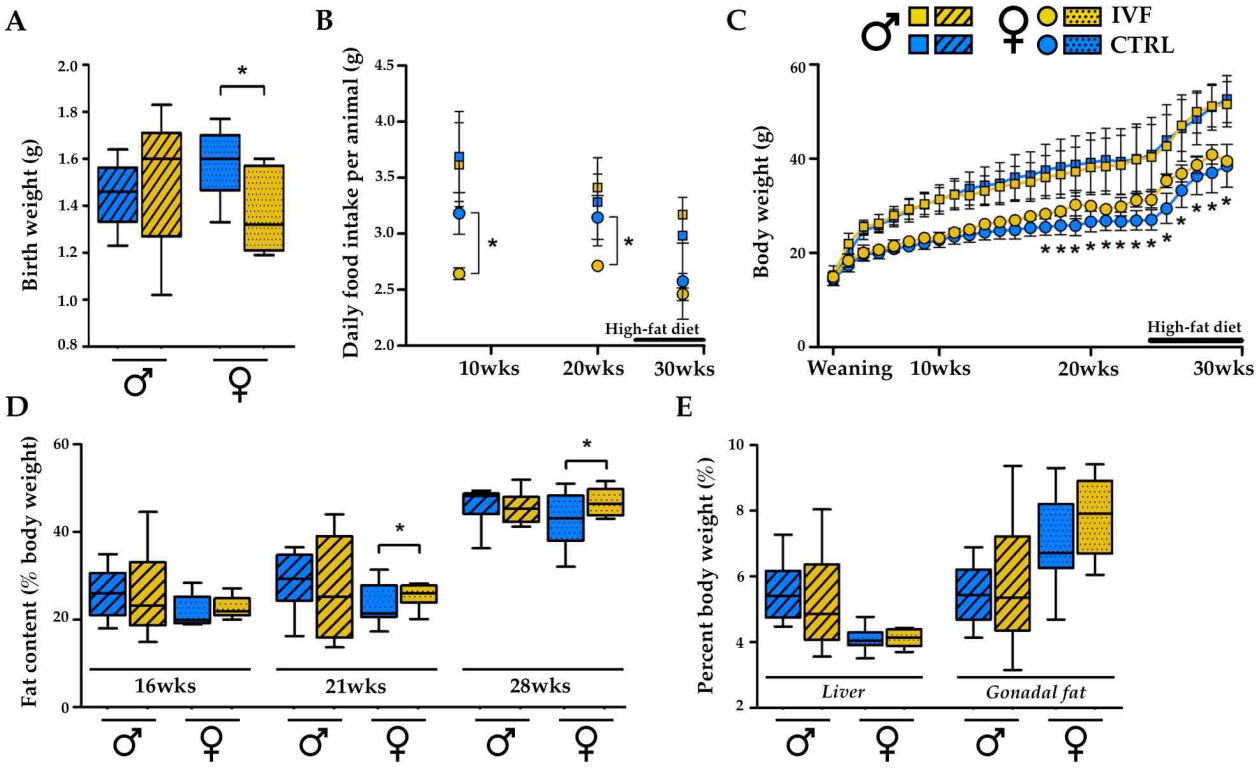


Figure 3-1. Physiological parameters of the subset of IVF (yellow) and control (blue) mice used in the microarray and metabolomics studies, including (A) birth weight, (B) periodic food intake, (C) postnatal growth through 30wks of age, (D) periodic DEXA scanning, and (E) relative organ weights at sacrifice. Error bars depict standard deviation, *p<0.05.

IVF metabolic defects are reflected in global gene expression signatures

To elucidate the molecular mechanisms underlying the impairments in IVF energy metabolism, we used Affymetrix Mouse Gene 1.0 ST microarrays to probe gene expression profiles in pancreatic islets and insulin sensitive tissues (liver, skeletal muscle, gonadal fat) isolated from three IVF and three control female animals aged 29 weeks and derived from at least two separate litters of 5-7 pups per condition. This analysis identified 1657 liver transcripts (1355 unique genes), 2724 muscle transcripts (2148 genes), 678 fat transcripts (588 genes), and 906 islet transcripts (756 genes) differentially expressed (p<0.05) between the two conception conditions (**Figure 3-2a**). Interestingly, the degree of concordance in gene expression changes between IVF tissues was minimal (**Figure 3-2b**, **3-2c**) and not robustly associated with any particular biological theme, indicating that the impact of IVF on the adult transcriptome varies in a tissue-specific manner.

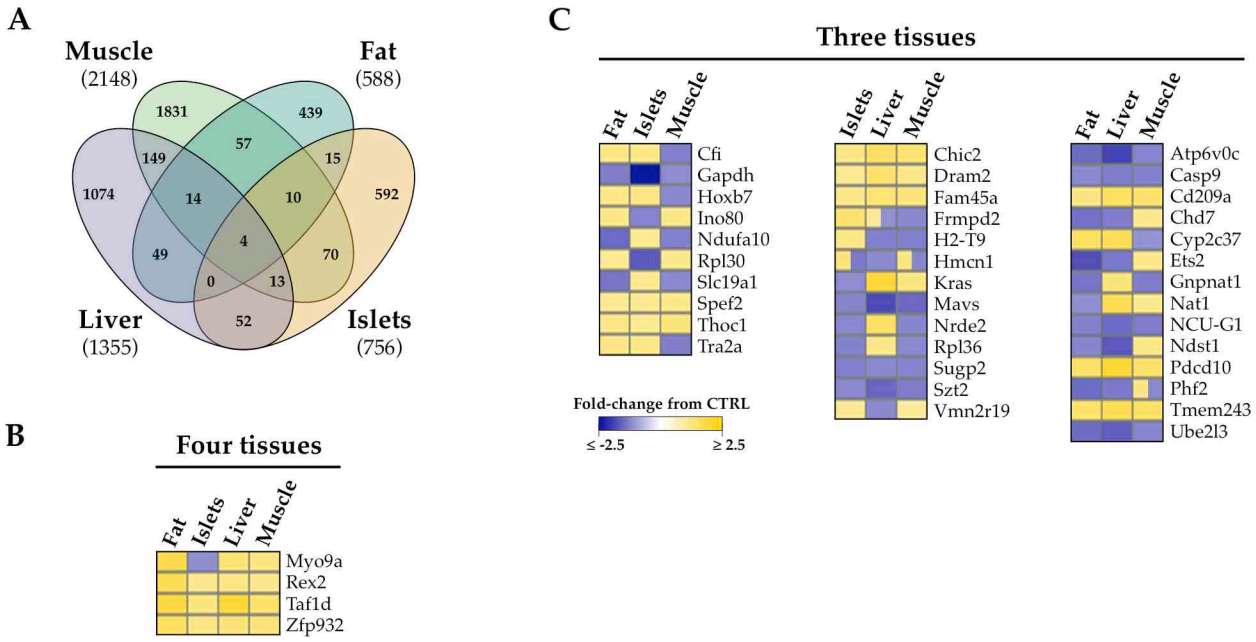


Figure 3-2. IVF transcriptional signatures are tissue-specific. (A) Venn diagram comparing concordance of gene misexpression in adult female IVF versus control across the 4 insulin-sensitive tissues. Numbers in brackets are the total number of genes in the signature list (B, C) Heat map representation of the genes misexpressed in 3 or more IVF tissues, and their directionality of change.

Processing of gene lists by Ingenuity Pathway Analysis identified multiple glucose, lipid, and transcriptional regulatory pathways altered in all four tissues that might contribute to the metabolic defects associated with IVF, indicating that patterns of gene expression were consistent with our physiological observations (**Table 3-1**). A full list of the significantly misregulated pathways and their associated genes for each tissue is shown **Appendix 1**. To distinguish the most prevalently and significantly altered pathways, *p*-values associated with individual dysregulated pathways were negatively log-transformed (here, -log(p<0.05) corresponds to values >1.3) and ranked according to highest combined log score across the four tissues. Liver X receptor/retinoid X receptor (LXR/RXR) activation, mitochondrial dysfunction, and type I diabetes mellitus signaling were among the most prominent pathways globally altered following IVF (**Figure 3-3**), demonstrating the widespread energy defects in IVF metabolic tissues. Together, these data indicate that although the IVF-associated gene misexpression is tissue-specific, the gene lists are associated with similar pathways.

PANCREATIC ISLETS - Canonical pathway	*p*-value	# Genes
Inositol phosphate signaling & biosynthesis	0.0001	6
Lipoate biosynthesis & incorporation	0.0129	1
Role of hyper(cyto/chemo)kinemia in influenze pathogenesis	0.0131	2
Systemic lupus erythematosus signaling	0.0184	4
Oxidized GTP and dGTP detoxification	0.0193	1
L-serine degradation	0.0193	1
EIF2 signaling	0.0196	4
Role of cytokines in mediating communication between immune cells	0.0214	2
Docosahexaenoic acid (DHA) signaling	0.0238	2
ILK signaling	0.0249	4

LIVER - Canonical pathway	*p*-value	# Genes
Nur77 signaling in T lymphocytes	0.0004	8
Antigen presentation pathway	0.0006	6
Complement system	0.0007	6
Spermine/spermidine biosynthesis	0.0016	2
Protein ubiquitination pathway	0.0042	12
Role of PKR in interferon induction & antiviral response	0.0049	4
LXR/RXR activation	0.0054	4
Assembly of RNA polymerase II complex	0.0020	10
Bile acid biosynthesis	0.0023	2
Methylglyoxal degradation	0.0029	4

SKELETAL MUSCLE - Canonical pathway	*p*-value	# Genes
Hypoxia signaling in the cardiovascular system	0.0098	6
Selenocysteine biosynthesis	0.0125	2
UDP-N-acetyl-D-galactosamine biosynthesis	0.0282	2
Folate transformations	0.0282	2
RhoGDI signaling	0.0295	10
Glutamate removal from folates	0.0301	1
Xanthine/xanthosine salvage	0.0301	1
CXCR4 signaling	0.0310	9
Glucocorticoid receptor signaling	0.0333	13
Sphingosine-1-phosphate signaling	0.0340	7

GONADAL FAT - Canonical pathway	*p*-value	# Genes
LXR/RXR activation	0.0001	10
Atherosclerosis signaling	0.0004	9
Ephrin receptor signaling	0.0006	11
Nicotine degradation	0.0006	5
Estrogen biosynthesis	0.0006	5
Buproprion degradation	0.0010	4
Acetone degradation	0.0012	4
RhoGDI signaling	0.0020	10
Tyrosine degradation	0.0023	2
Methionine degradation	0.0029	4

Table 3-1. The top 10 most significantly altered pathways associated with IVF transcriptional signatures ($p<0.05$, based on a 1.2 fold-change cutoff).

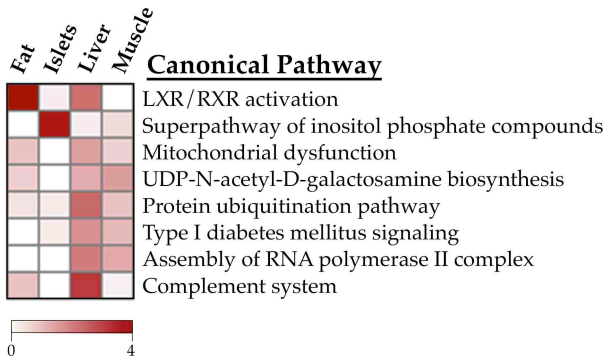


Figure 3-3. Evidence of metabolic dysfunction across multiple IVF tissues. *P*-values associated with each misregulated canonical pathway were negatively log-transformed and ranked according to highest combined log score across the 4 tissues. The heat map shows the top scoring pathways most commonly disrupted in adult IVF females. LXR, liver X receptor; RXR, retinoid X receptor.

Metabolic sexual dimorphism in adult fat tissue of control mice

Unbiased metabolomic investigation of IVF and control fat samples (n=10 male and n=7 female IVF, n=5 male and n=7 female control,) identified a total of 231 endogenous biochemicals comprising all major metabolic groups (**Figure 3-4a**). A complete list of relative metabolite concentrations may be found in **Appendix 2**. Pearson correlations between metabolite concentrations and either percent adiposity or fasting glucose levels at sacrifice revealed no significant relationships. Due to the sex-biased effect of IVF on adult metabolism [89,93], we first compared profiles between control males and females and observed significant sexual dimorphism for 57 metabolites (24.7%, $p<0.05$, **Figure 3-4b**). The dataset was particularly enriched for small molecules involved in lipid and amino acid metabolism. Males exhibited broad increases in metabolite concentration relative to females (**Figure 3-4c**). We performed metabolite set enrichment analysis (MSEA) to determine if any biologically meaningful pathways were overrepresented by the altered metabolites [109], which revealed that metabolites involved in glycerolipid metabolism, the urea cycle, and sphingolipid metabolism exhibited the most significant sex bias in the control fat samples (**Figure 3-4d**).

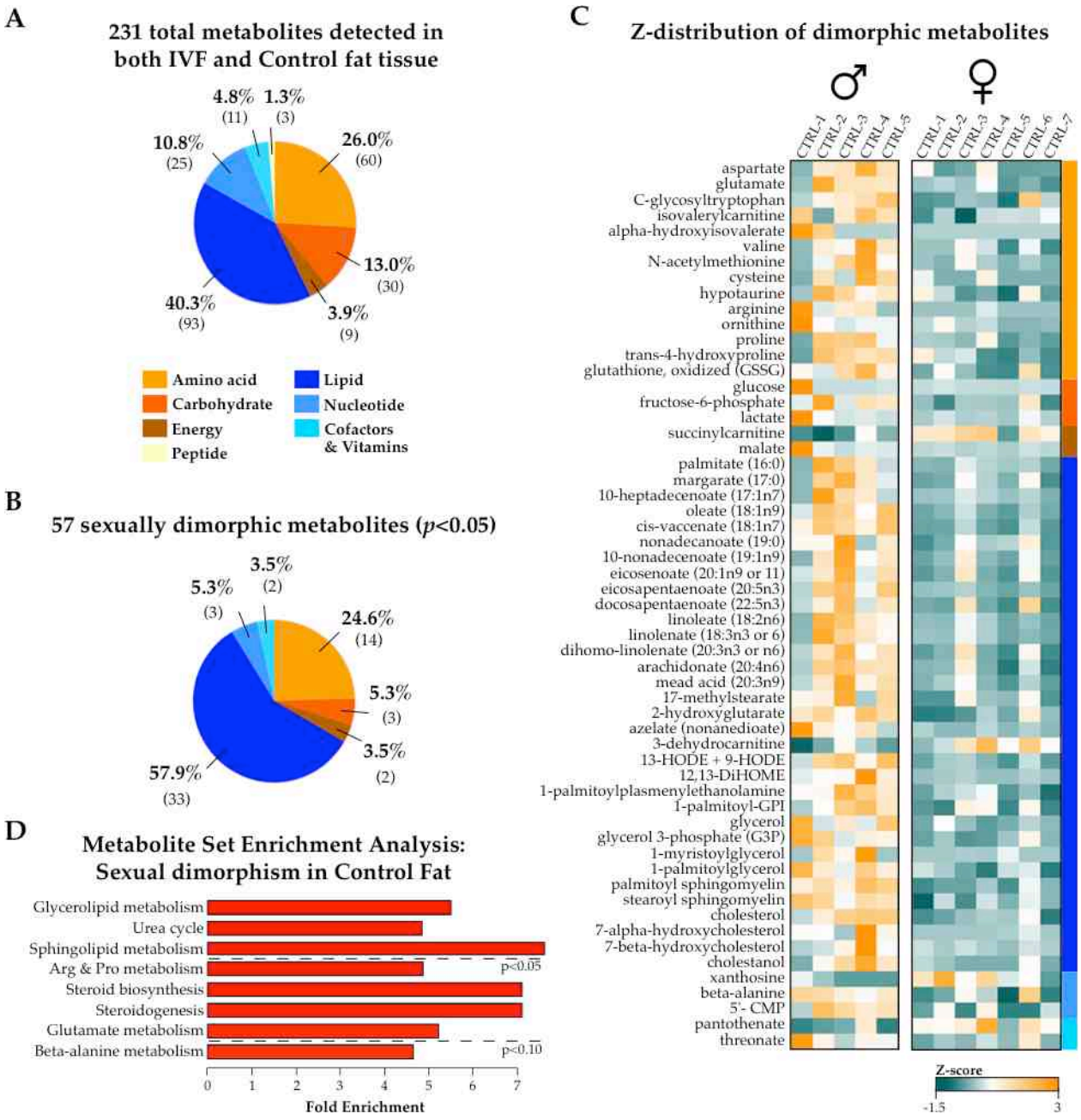
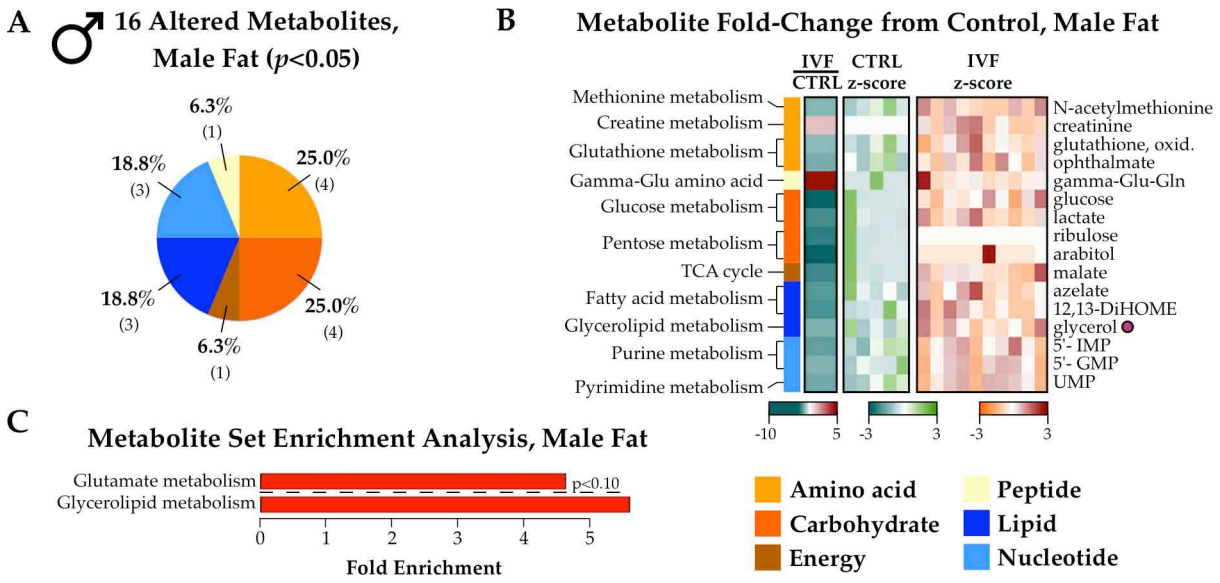


Figure 3-4. Metabolic sexual dimorphism in adult adipose tissue. (A) Nontargeted mass spectrometry profiling of 29wk IVF and control fat tissue (n=5 male and n=7 female controls; 10 IVF male and n=7 IVF females; 29 animals total) identified 231 named metabolites comprising all major metabolic groups. (B) The concentrations of 57 metabolites (24.7%) exhibited significant sex bias (p<0.05 males versus females), consisting predominantly of lipid and amino acid derivatives. (C) Z-distribution of each biochemical across all control samples. Apart from succinylcarnitine and 3-dehydrocarnitine, sexually dimorphic metabolites displayed uniformly increased concentrations in males. (D) Summary plot for MSEA, where pathways are ranked by Bonferroni-corrected p-value with hatched lines depicting p-value cutoffs. CMP, cytidine monophosphate; DiHOME, hydroxyoctadec-9(Z)-enolate; GPI, glycerophosphoinositol; HODE, hydroxyoctadecadienoic acid.

Sex-specific effect of IVF on the adult fat metabolome

In males, 16 metabolites were significantly altered between IVF and control fat samples ($p<0.05$, 2 molecules increased and 14 decreased), and 9 approached significance ($0.05<p<0.1$, 0 increased and 9 decreased) (**Figure 3-5a**, **3-5b**). This included a dramatic reduction in levels of the glycolytic metabolites glucose and lactate, the pentose phosphate pathway metabolites ribulose and arabitol, as well as the nucleotide precursors inosine 5'-monophosphate, guanosine 5'-monophosphate and uridine monophosphate, suggesting a decreased shunting of glycolytic intermediates through the pentose phosphate pathway toward nucleotide synthesis. MSEA highlighted an involvement of the altered metabolites with these pathways, although the associations were not significant following post-hoc correction (**Figure 3-5c**).

Comparatively, female IVF fat tissue differed from female controls by 19 metabolites ($p<0.05$, 15 increased and 4 decreased), and 21 showed a trend toward significance ($0.05<p<0.1$, 13 increased and 8 decreased) (**Figure 3-5d**, **3-5e**). Concentrations of several amino acids were increased in IVF mice, including urea cycle intermediates. Multiple long chain (18C) fatty acids were increased, whereas levels of the glycerophosphoethanolamines (GPE) 1-arachidonoyl-GPE and 2-docosahexaenoyl-GPE were decreased. Further, decreased maltotetraose could reflect changes in glycogenolysis. There was also evidence of increased oxidative stress in female IVF



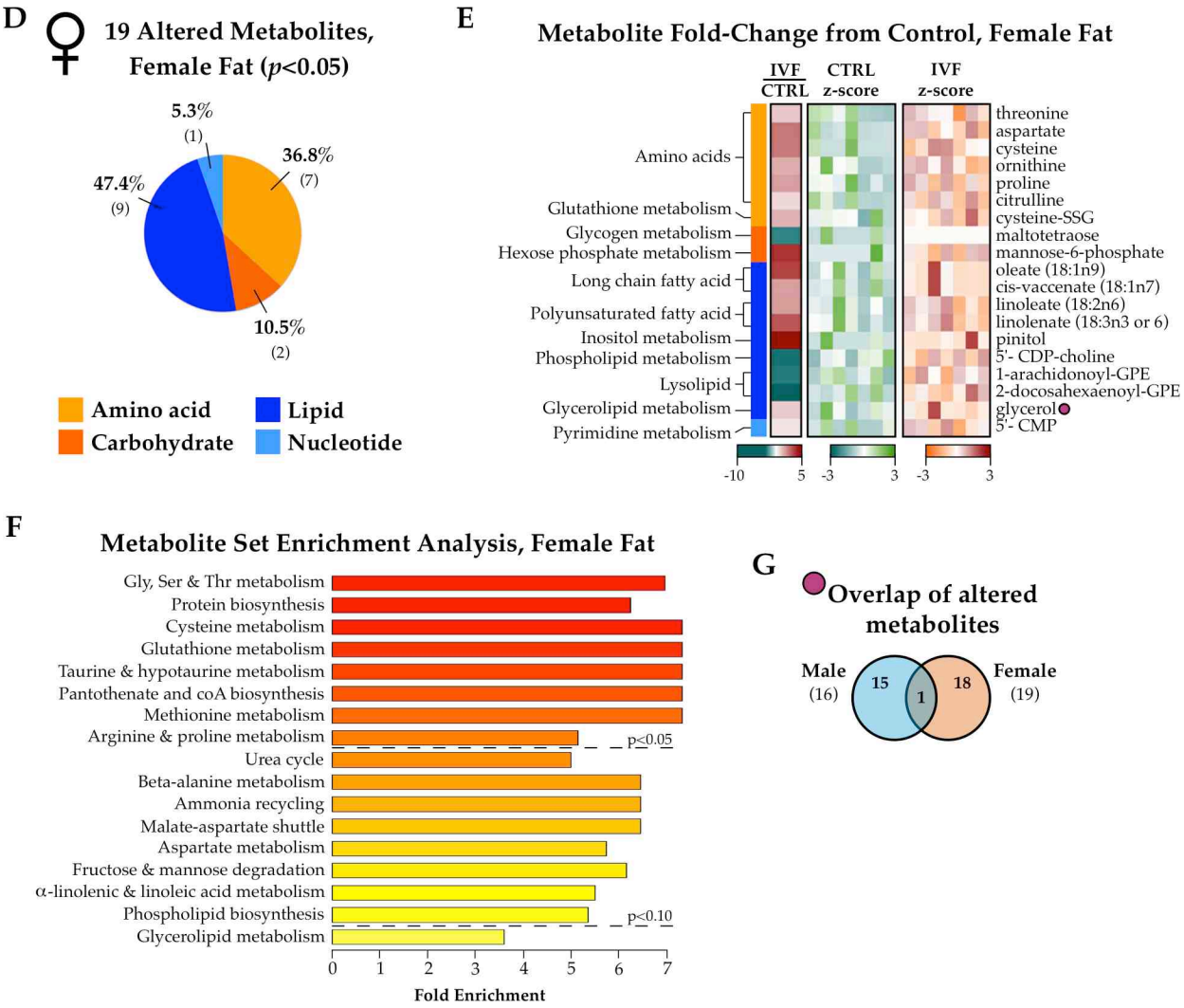


Figure 3-5. Effect of IVF on the adult fat metabolome. (A) Categorical distribution of the 16 metabolites significantly altered in IVF males from controls. (B) Heat map depicting fold-change in metabolite concentration between IVF and control metabolite values in male fat samples, including Z-distribution of individual control (blue) and IVF (red) values relative to their respective population means. (C) MSEA summary of Bonferroni-corrected pathways associated with the metabolite changes. (D-F) Same as A-C but for the 19 metabolites altered in female IVF fat samples. (G) Venn diagram showing overlap in altered metabolites (purple symbols) between male and female IVF cohorts. CDP, cytidine disphospho; CMP, cytidine monophosphate; Glu-Gln, glutamylglutamine; IMP, inosine monophosphate; SSG, glutathione disulfide; UMP, uridine monophosphate.

fat, evidenced by depleted levels of (reduced) glutathione (p=0.088, **Appendix 2**) and a corresponding increase in its oxidized form cysteine-glutathione disulfide (p=0.042). MSEA showed significant alterations to many amino acid and protein synthesis pathways, as well as a trend toward changes in the malate-aspartate shuttle, urea cycle, ammonia recycling, and fructose/mannose degradation (**Figure 3-5f**).

The effect of IVF on adult fat metabolite composition was strikingly sex-specific: glycerol was the only metabolite significantly different in IVF versus control samples for both males and females. However, the changes occurred in different directions (1.8-fold decrease from control males, 1.8-fold increase in females, **Figure 3-5g**).

Reduced sexual dimorphism in IVF adult fat tissue

We next investigated the effect of IVF on metabolic sexual dimorphism, and observed a striking depletion in the number of metabolites that differed in concentration between IVF male and IVF female fat samples. Compared with the 24.7% (57 of 231 metabolites) sex bias in controls, just 33 (14.3%) showed significant male-female differences ($p<0.05$), predominantly for metabolites involved in lipid metabolism (**Figure 3-6a**, **3-6b**). Only 14 metabolites retained significant sexual dimorphism between the control and IVF cohorts (purple symbols), indicating that male-female differential concentrations were lost for 43 metabolites and gained for 19 (**Figure 3-6c**). Of these, sexual dimorphism in amino acid and lipid molecules were the most affected by IVF (**Figure 3-6d**). MSEA additionally showed that only steroidogenesis was different between sexes (**Figure 3-6e**). Overall, there was a dramatic reduction in IVF metabolic sexual dimorphism in gonadal fat tissue. Of note, there were IVF female-specific increases in the inflammation- and reactive oxygen species-associated metabolites opthalmate, corticosterone and urate, as well as the adipogenic marker palmitoylethanolamide, which might be involved in the female-specific changes in fat deposition and metabolic homeostasis.

Sex-specific effect of IVF on the adult liver metabolome

We next profiled liver samples (n=6 per sex and per conception condition) and detected a total of 373 endogenous biochemicals (**Figure 3-7a**), of which 53 (14.2%) exhibited significant male-female differences in concentration. As with the control fat tissue, dimorphic metabolites were predominantly comprised of lipid and amino acid derivatives (**Figure 3-7b**), but increases or decreases in metabolite levels were metabolite- and pathway-specific (**Figure 3-7c**). MSEA

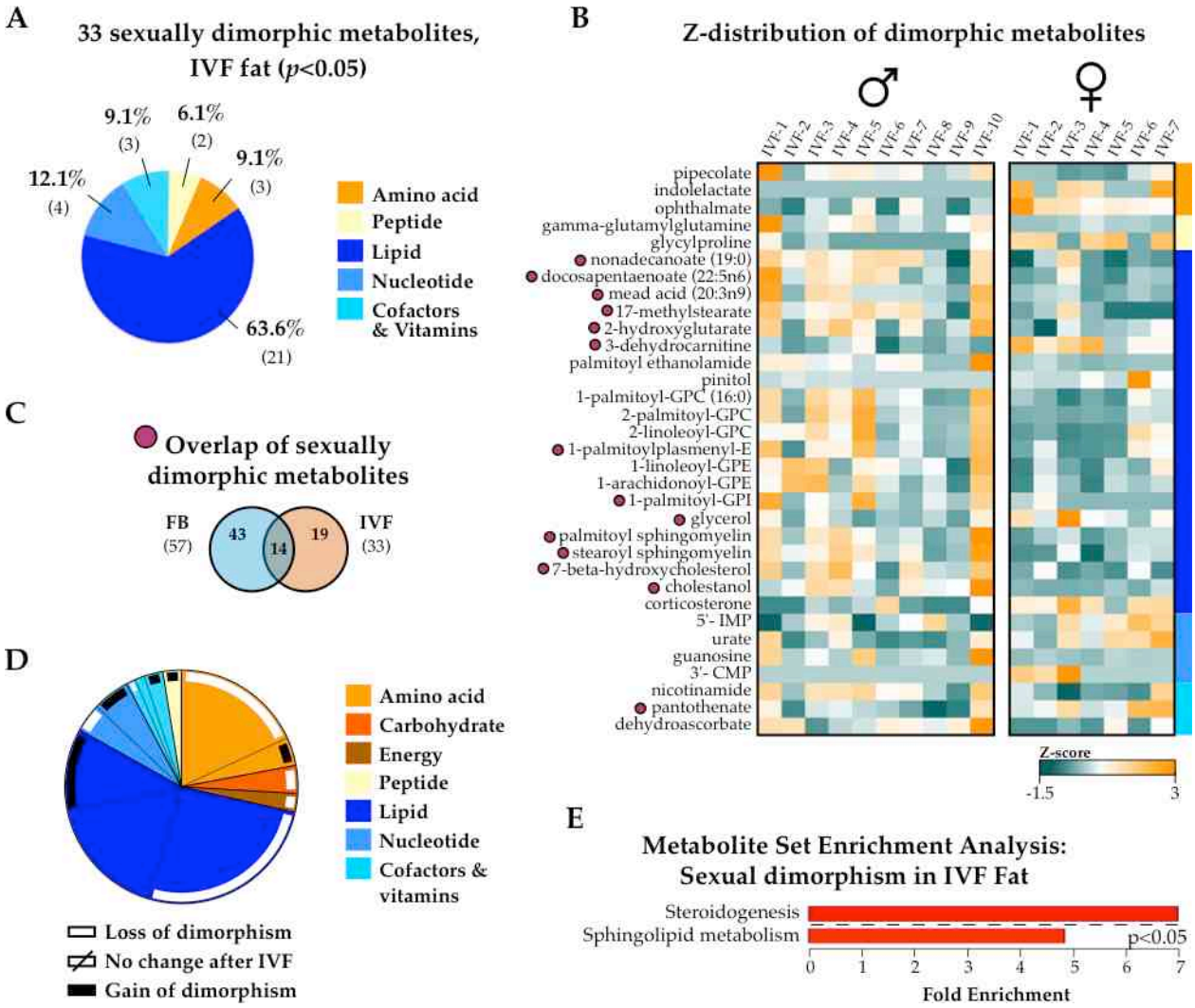


Figure 3-6. Reduced sexual dimorphism in IVF fat tissue. (A) A total of 33 metabolites (14.3%) were sexually dimorphic (p<0.05 between males and females) in fat samples from IVF animals (n=10 males and n=7 females). (B) Z-distribution of the metabolites exhibiting sex bias, with (C) purple symbols identifying those retaining male vs. female differences in both IVF and control samples. (D) Categorical distribution of metabolites with altered sexual dimorphism: white bars indicate metabolites no longer exhibiting significant male-female differences after IVF, and black bars show metabolites with acquired sex bias, compared to controls. For example, of the 41 lipid-categorized metabolites with dimorphism in at least 1 conception condition (dark blue section), 21 are no longer dimorphic in IVF tissues (black bar), 12 maintain sex bias in both control and IVF, and 8 exhibit dimorphism only in IVF samples (white bar). (E) MSEA of IVF fat dimorphism. CMP, cytidine monophosphate; E, ethanoloamine; GPC, glycerophosphocholine; GPI, glycerophosphoinositol; IMP, inosine monophosphate.

classified sphingolipid metabolism, and glycine, serine and threonine metabolism as the pathways most affected by sex (**Figure 3-7d**). Pearson correlations did not reveal any significant relationships between metabolite concentration and percent adiposity. However, levels of pyridoxal (one of the 3 forms of vitamin B6) displayed both a significant sex bias and a high correlation with fasting glucose levels ($r = 0.67$, $p = 0.008$).

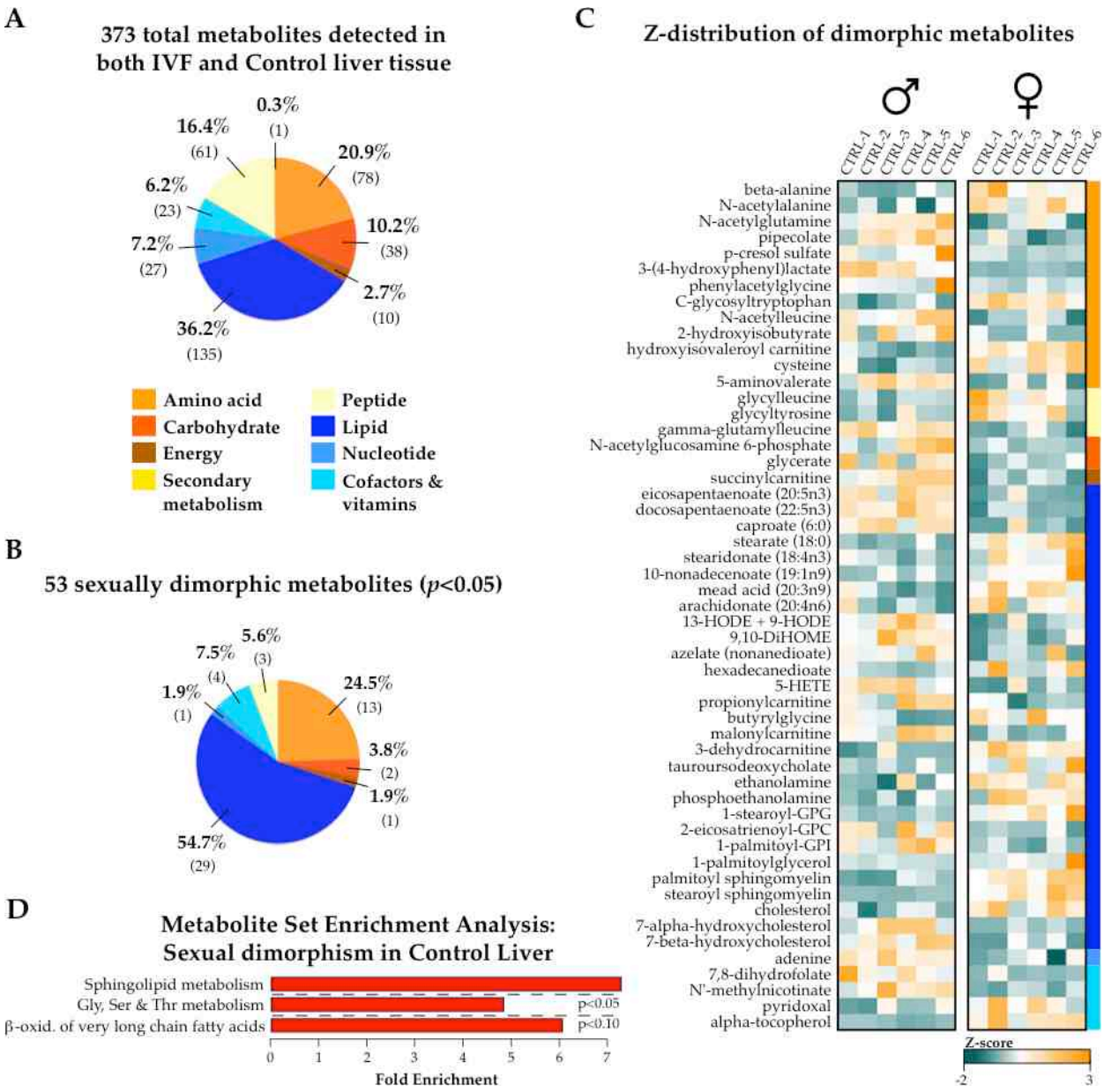


Figure 3-7. Metabolic sexual dimorphism in adult liver tissue. (A) Nontargeted mass spectrometry profiling of 29wk IVF and control livers (n=6 per sex and conception condition; 24 animals total) identified 373 named biochemicals comprising all major metabolic groups. (B) Categorical distribution of the 53 metabolites exhibiting significant sex bias in control samples (p<0.05), and (C) their Z-distribution within the control population. Of note, concentrations of metabolites were not uniformly changed, as was observed in fat tissue. Instead, the changes were more segmented and pathway-specific. (D) MSEA summary with pathways ranked by Bonferroni-corrected p-value. GPC, glycerophosphocholine; GPG, glycerophosphoglycerol; GPI, glycerophosphoinositol.

Investigation of IVF liver tissue revealed significant changes in levels of 32 metabolites between IVF and control male livers (p<0.05, 25 molecules increased and 7 decreased), with an additional 30 approaching significance (0.05<p<0.1, 20 increased and 10 decreased, **Figure 3-8a**). A

complete list of relative metabolite concentrations may be found in **Appendix 3**. The most striking change was widespread incorporation of dipeptides into IVF livers, particularly for leucine-, alanine-, glycine-, and isoleucine-based dipeptides **Figure 3-8b**). There was also a strong depletion of the bile acid metabolites cholate, beta-muricholate, and alpha-muricholate. Other notable decreases in male IVF livers relative to controls included the ketone body 3-hydroxybutyrate and the active form of folic acid, 5-methyltetrahydrofolate. Comparative increases consisted of 3-dehydrocarnitine, the nicotinamide adenine dinucleotide (NAD) metabolism precursor nicotinamide riboside, as well as the purine metabolites 5'AMP, 5'GMP, and guanosine. None of the changes were significantly associated with any MSEA pathways.

In contrast, female IVF livers differed from female controls by 31 metabolites ($p<0.05$, 13 increased and 18 decreased), and 32 showed a trend toward significance ($0.05<p<0.1$, 12 increased and 20 decreased). These were enriched for fatty acid metabolites (**Figure 3-8c**, **3-8d**), including long chain fatty acids and acylglycines—in conjunction with a strong depletion of glutamine in all IVF samples, this may reflect changes in mitochondrial fatty acid catabolism. Higher levels of the glycogen intermediates maltotetraose, maltopentaose and maltohexaose suggests an increase in liver glycogen breakdown, which combined with the changes in lactate and ribose-5-phosphate concentration indicate an alternative fate for glucose in these livers. None of the altered metabolites were significantly associated with any metabolic pathways after post hoc correction, although MSEA did identify changes in gluconeogenesis, the pentose phosphate pathway, and long chain fatty acid β-oxidation, among others (**Figure 3-8e**).

As was observed in fat samples, the IVF-associated changes were relatively sex-specific. Five metabolites were significantly different in IVF versus control samples for both males and females, including N-acetylalanine, the dipeptides glycylleucine, glycylphenylalanine and glycltyrosine, as well as nicotinamide riboside (**Figure 3-8f** and purple symbols in **3-8b**, **3-8d**).

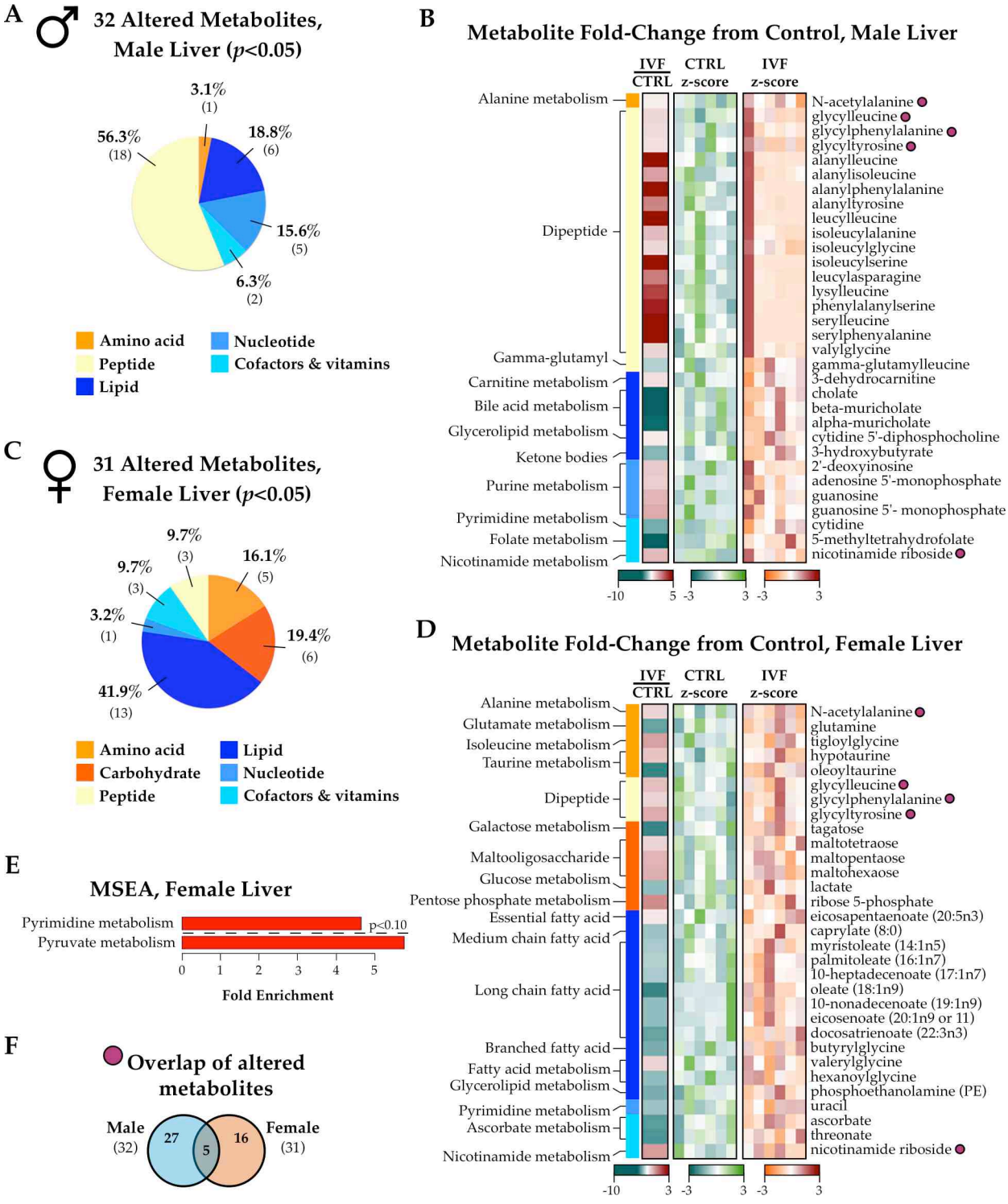


Figure 3-8. Effect of IVF on the adult liver metabolome. (A) Categorical distribution of the 32 metabolites significantly altered in IVF males from controls. (B) Heat map depicting fold-change in metabolite concentration between IVF and control metabolite values in male liver samples, including z-distribution of individual control (blue) and IVF (red) values relative to their respective population means. (C, D) Same as A, B but for the 31 metabolites altered in female IVF liver samples. (E) MSEA summary of Bonferroni-corrected pathways associated with the metabolite changes in females. (F) Venn diagram showing overlap in altered metabolites (purple symbols) between male and female IVF cohorts.

Exaggerated sexual dimorphism in the IVF adult liver

In contrast to IVF fat tissue, IVF liver sexual dimorphism was increased compared to controls. A total of 75 metabolites (20.1% vs. 14.2% in controls) displayed significant male-female concentration differences, similarly enriched for lipid and amino acid metabolites (**Figure 3-9a**, **3-9b**). Relative to control samples, 26 metabolites lost significant dimorphic concentrations (p>0.05), sex differences were maintained in 27 molecules, and 43 new metabolites exhibited significantly different male-female concentrations (purple symbols and **Figure 3-9c**). Of these, the sex bias was increased particularly for amino acid metabolites, and altered in lipids, with dimorphic concentrations shifting away from sterol and fatty acid metabolism and increasing for glycerophosphocholines and other lysolipids (**Figure 3-9b**, **3-9d**). MSEA processing revealed more significant dimorphism for methionine, one carbon folate, and pyrimidine metabolism. Pathways with novel dimorphism included betaine and glutamate metabolism, ammonia recycling, and other non-significant glucose handling pathways (**Figure 3-9e**).

Evidence of metabolic stress in the IVF female serum metabolome

To further characterize the metabolic consequences of IVF in females, we profiled IVF and control serum. This analysis detected 280 distinct metabolites (**Figure 3-10a**), of which 35 were significantly altered in IVF plasma (**Figure 3-10b**, **3-10c**; p<0.05, 9 metabolites increased and 26 decreased), with 21 approaching significance (0.05<p<0.1, 9 increased and 12 decreased). **Appendix 4** provides a comprehensive list of all serum biochemicals identified by the analysis. Although it is unclear which tissues(s) contributed to the metabolite differences observed in IVF females, the IVF metabolic signature reflected widespread shifts in energy metabolism. IVF females were clearly distinguishable from controls by a dramatic reduction in serum glycerophosphocholines, short-chain acyl-carnitines and acyl-glycines. Branched amino acid oxidation was restricted, as evidenced by the elevation of 3-methyl-2-oxobutyrate, 3-methyl-2-oxovalerate and 4-methyl-2-oxopentanoate, in conjunction with a decrease in their derivatives isobutyrylglycine and beta-hydroxyisovalerate. Additional noteworthy differences included

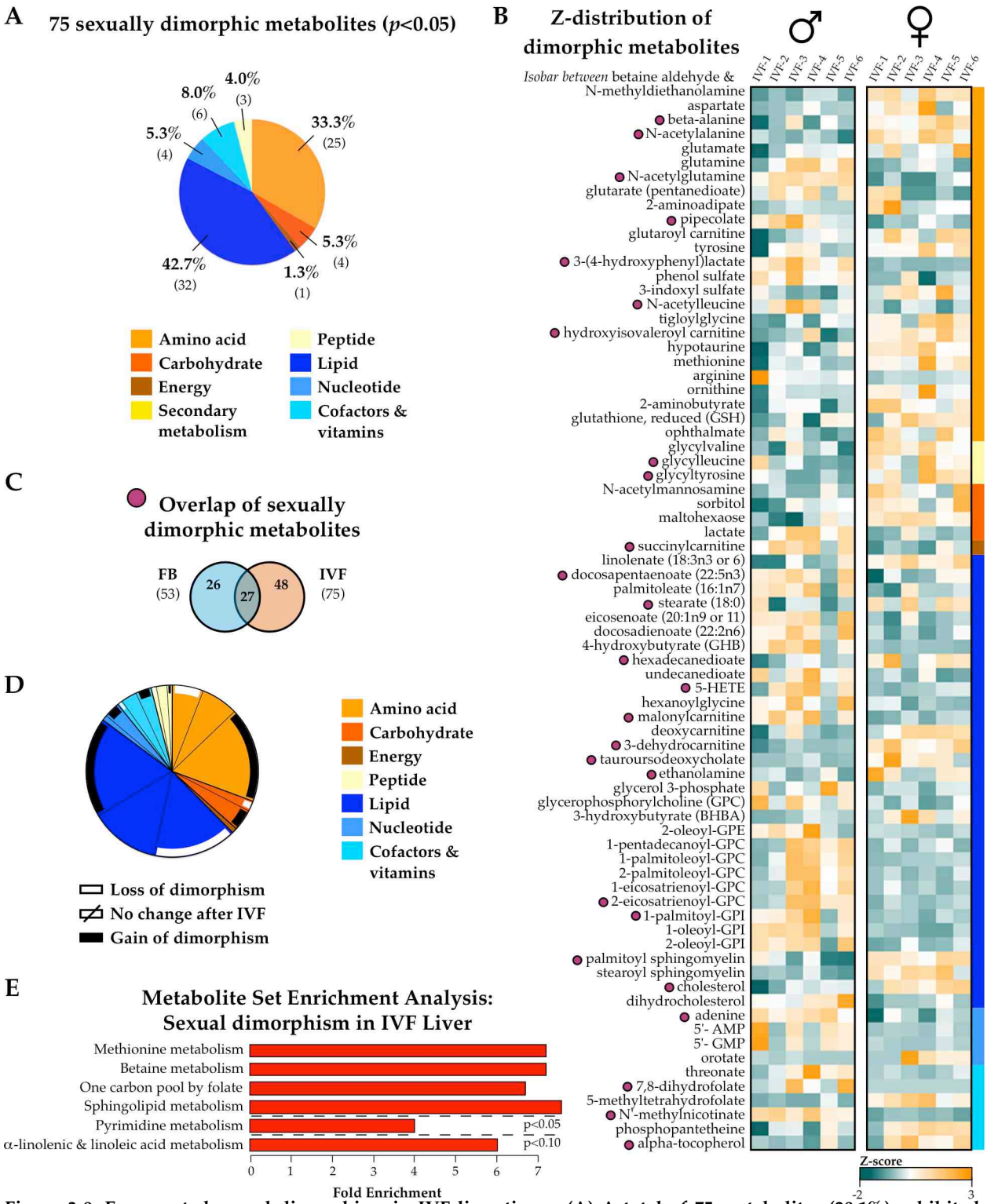


Figure 3-9. Exaggerated sexual dimorphism in IVF liver tissue. (A) A total of 75 metabolites (20.1%) exhibited sexually dimorphic concentrations ($p<0.05$ between males and females) in liver samples from IVF animals (n=6 each sex). (B) Z-distribution of the IVF-associated metabolites with sex bias, with (C) purple symbols indicating retained dimorphism versus control samples. (D) Categorical distribution of the metabolites exhibiting altered sexual dimorphism, with white and black bars indicating metabolites with lost or acquired sex bias following IVF, respectively, as discussed in Figure 3-6. (E) MSEA summary of the sexually dimorphic pathways in IVF liver. GPC, glycerophosphocholine; GPI, glycerophosphoinositol.

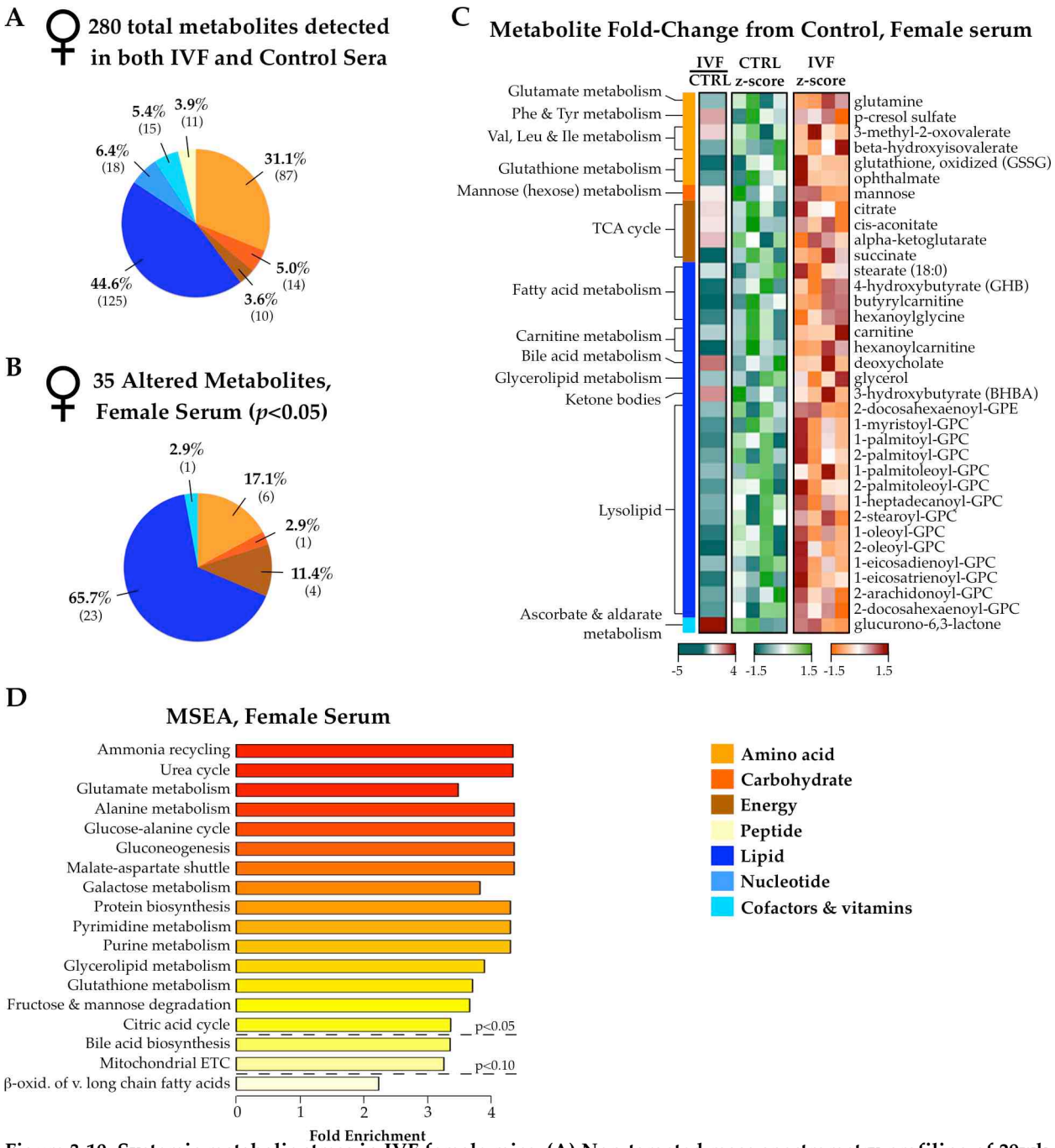


Figure 3-10. Systemic metabolic stress in IVF female mice. (A) Non-targeted mass spectrometry profiling of 29wk IVF and control serum samples (n=4 per conception condition; 8 animals total) identified 280 named biochemicals comprising all major metabolic groups. (B) Categorical distribution of the 35 metabolites with significantly different concentrations between IVF and control females (p<0.05). (C) Heat map depicting fold-change in metabolite concentrations, including Z-distribution of individual control (blue) and IVF (red) values relative to their respective population means. (D) MSEA summary with pathways ranked by Bonferroni-corrected p-value. GPC, glycerophosphocholine; GPE, glycerophosphoethanolamine; ETC, electron transport chain.

changes in glycolytic and TCA cycle intermediates, increased levels of the ketone 3-hydroxybutyrate, and depleted systemic glutamine and glutamate. Higher corticosterone levels

in IVF bordered statistical significance (p=0.06, **Appendix 4**). MSEA of serum metabolite signatures identified multiple pathways significantly affected in IVF sera (**Figure 3-10d**), including shifts in ammonia recycling and the urea cycle, glucose oxidation, nucleotide metabolism, and mitochondrial function. Pearson correlation did not identify any relationship between metabolites affected by IVF and percent adiposity.

Taken together, these results demonstrate a sex- and tissue-specific effect of IVF on adult metabolism (summarized in **Figure 3-11**).

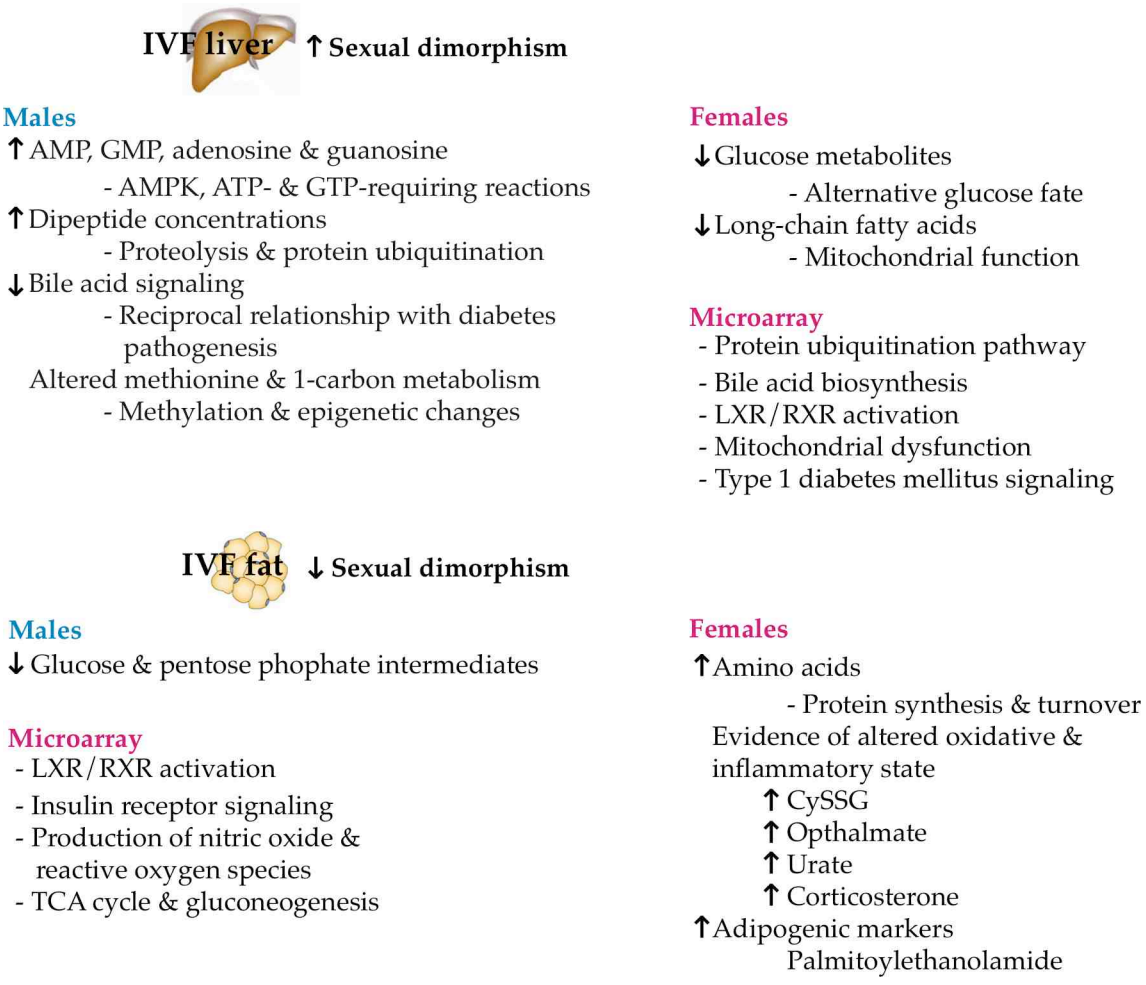


Figure 3-11. Integrated metabolic and gene expression changes observed in adult IVF liver and fat tissues.

Chapter 4: Discussion

"The beginning of medical ethics, however, is primum non nocere*; this permits alleviation of infertility, but would it permit more remote techniques? … Yet the desire to have children must be among the most basic of human instincts, and denying it can lead to considerable psychological and social difficulties."*

- Robert Edwards & David Sharpe, 1971, on social values & human embryology research [110].

Our data demonstrate that *in vitro* fertilization and embryo culture lead to long-term metabolic alterations in a mouse model, confirming the sensitivity of the fertilization and preimplantation stages to environmental influence on postnatal growth, physiology, glucose tolerance and redox homeostasis. Reassuringly, the long-term IVF phenotypes were modest, but the data presented in this dissertation indicate that even clinically optimized IVF conditions are sufficient to reprogram adult mouse growth, fat deposition and glucose homeostasis, leading to widespread sex- and tissue-specific molecular and biochemical alterations in multiple regulatory pathways.

The mouse model

Animal models of ART offer an alternative way to develop and improve ART procedures, as well as investigate their influence on health. Importantly, the use of animal models removes fertility as a confounding factor, permitting in-depth analyses of the potential effects of ART techniques without the variability caused by subfertility. Because the eldest IVF individuals are in their mid-30s, the longer-term outcomes of ART are unclear, making the mouse model particularly valuable for our study due to the short generation time to produce adult animals. A caveat is that requirements for fertilization and preimplantation development can vary across different mammalian species; caution must therefore be exercised because animal models might provide an incomplete or inaccurate view of the demands of the human embryo and fetus [111]. For example, rodents carry large litters (n=8-12 pups) for short gestations (~21 days), reflecting energy requirements distinct from human pregnancy. Rodents additionally exhibit unique physiology during development: mice have functional brown adipose tissue throughout life,

and maturation of metabolic tissues and hormonal regulatory networks occur at different times than in humans [112]. However, the studies conducted using mammalian models of fertilization and preimplantation development impart enormous promise for further optimizing ART techniques and minimizing potential hazards.

Importantly, evidence in support of the DOHaD hypothesis is not limited to animal models. During the winter of 1944-1945, a German blockade obstructed food and fuel shipments to western portions of the Netherlands, resulting in a significant famine (400-800 calories per day) lasting approximately 5 months [113]. Although famine is unfortunately frequent in the world, the Dutch Hunger Winter is unique because the shortages both originated and concluded rather suddenly and to a previously healthy population—resulting in a cohort of individuals exposed to caloric restriction exclusively during gestation. This provides a well-constructed survey of the effects of intrauterine nutrient deprivation on the progression of disease pathologies in adulthood, with individuals born directly prior to or shortly after the caloric restriction functioning as control comparisons. The conclusion: nutrient deprivation *in utero* is associated with glucose intolerance, obesity, and cardiac dysfunction in adulthood [114-118]. Further, the famine was not confined to a specific socioeconomic rank, compared with Barker's studies highlighting social strata as a key determinant in adverse health outcome.

Although very few human studies have evaluated postnatal IVF outcomes past birth, the assorted phenotypes illustrated by animal models—including those presented in this work—have begun to materialize in ART children as well. Mouse models of IVF have described intrauterine growth restriction followed by accelerated perinatal growth velocity [73]; ART babies are born low birth weight and exhibit rapid catch-up growth in the first year of life [4]. Fasting glucose levels are increased in IVF mice [89,93,119] and humans (mean age 13 years) [6]. There is evidence of impaired insulin homeostasis in IVF mice [120], and IVF young adults (~20 years) also display decreased peripheral insulin sensitivity [119]. One group reported an increase in biochemical markers of early insulin resistance in ICSI children aged 7 years, but due to the poor design of the study this data remains controversial [121]. An analysis of systemic

(flow-mediated dilation of the brachial artery, pulse-wave velocity and carotid intima-media thickness) and pulmonary (pulmonary artery pressure at high altitude by Doppler echocardiography) vascular function in 11-year old ART children identified alterations reflecting a predisposition to exaggerated hypoxic pulmonary dysfunction, although it is unclear how this might evolve [84]. Systolic and diastolic blood pressures, as well as circulating triglyceride content are reportedly increased in IVF children, including after correction for birth weight and multiple gestations [7]. A separate study of cardiac geometric morphology revealed left ventricular cardiac hypertrophy and diastolic dysfunction in 128 IVF versus 100 control children (mean age 4 years) [122], and our laboratory has shown that IVF outbred (CF1 x B6D2F1) mice have lower systolic blood pressure and larger left ventricle wall size [89]. Interestingly, a different mouse study observed increased blood pressure in FVB x NMRI IVF animals [123], which could be attributable to mouse strain or *in vitro* culture conditions. Finally, fat deposition is increased in IVF mice [89,93]; IVF pre-adolescents (~13 years) have significantly increased peripheral body fat and a trend towards increased total body fat [5]. Importantly, these differences are neither attributable to current/early risk factors (age, gender, BMI), nor parental factors (subfertility cause). Although many of the changes observed in IVF children are modest and not yet pathological in nature, together this points to an increased risk for cardiometabolic anomalies in adulthood.

Increasing phenotypic severity with more stressful IVF conditions

It is well known that chemically defined culture conditions can influence blastocyst competence for post-implantation development in both mice [48] and humans [124]. Our group and others have previously reported that fertilization and culture of preimplantation embryos *in vitro* affects rates of blastocyst development, cell number and lineage allocation, gene expression, and placental function in a manner sensitive to both culture medium composition and oxygen tension [19,20,49,125,126], indicating that modest fluctuations in preimplantation environment can impact embryo metabolism and subsequent viability. Several groups have corroborated the

detrimental effects of Whitten's medium as compared to other culture media [49,127], and we have shown that embryos cultured from zygote to blastocyst at 5% oxygen in Whitten's versus KAA medium results in the misregulated expression of 114 versus 29 genes, respectively, compared to *in vivo*-generated flushed blastocyst controls [50]. These effects are exacerbated by higher oxygen tensions: the same conditions at ambient oxygen alter 1159 and 354 genes [49], indicating a dose-response where increasing embryo stress leads to more severe phenotypes.

For postnatal examinations, we therefore designed experiments to evaluate whether a graded degree of preimplantation disturbance would differentially alter metabolic health in adult mice. Our preliminary physiological studies revealed that suboptimal IVF conditions (Whitten's medium and 20% oxygen) impaired offspring growth and glucose tolerance (**Figure 1-5**). Indeed, the degree of IVF stress was reflected in the IVF_{WM} versus IVF_{KAA} animals, with IVF_{WM} females exhibiting more significant glucose intolerance and growth retardation. The dosage phenomenon has been confirmed in an outbred (CF1 x B6D2F1) mouse model using the same IVF conditions as presented in this study. IVF_{WM} more significantly affected growth curves and glucose tolerance than IVF_{KAA}, although interestingly, phenotypes were stronger in male mice [89]. This suggests that greater deviations from the natural preimplantation environment provided by the female reproductive tract (in terms of oxygen, glucose, amino acids, etc.) engender more significant changes not only to the embryo but also throughout postnatal life.

Altered growth and glucose intolerance are common outcomes of developmental stress

We found that the growth curves of both IVF_{WM} and IVF_{KAA} mice were disturbed, but in different fashions. Male and female IVF_{WM} mice had normal birth weights, but their growth failed to keep pace with controls and by two weeks of age the IVF_{WM} mice were significantly retarded and remained smaller through puberty and most of adulthood. On the contrary, only the growth kinetics of IVF_{KAA} female mice, but not male, were altered. IVF_{KAA} females were significantly smaller at birth, exhibited an accelerated catch-up growth in the first week of postnatal life, and surpassed weights of their FB counterparts in adulthood. Other authors have demonstrated that

different preimplantation conditions uniquely affect postnatal growth patterns. For example, Banrezes et al. reported that transferring 2-cell embryos after manipulating redox potential during the pronuclear stage impacted postnatal body weight [128]. They determined that restricting the zygote energy source exclusively to pyruvate resulted in offspring with significant and persisting growth retardation, compared to control embryos cultured in standard M16 medium. Comparatively, exogenous lactate produced offspring with growth restriction prior to weaning but subsequent weight normalization to controls, whereas zygote exposure to cystolic alkalization significantly increased offspring size after birth. Although neonatal growth abnormalities are well-described markers of adult cardiometabolic disease [129], the authors did not investigate metabolic physiology or glucose tolerance in these mice.

We additionally observed altered glucose homeostasis following clinically optimized IVF_{KAA}, but the perturbations were significant only in female mice. These animals had greater fasted circulating glucose levels at 20 and 29 weeks, after they became heavier than their control counterparts. However, the IPGTT showed no impairment of glucose tolerance. Because higher fasting glucose levels can reflect insulin resistance, we evaluated β-cell function via glucose-stimulated insulin secretion assays on pancreatic islets isolated and cultured *in vitro*. At 12 weeks, prior to the development of the metabolic phenotypes, the insulin secretory response to high glucose was normal in IVF islets. Conversely, at 29 weeks, baseline insulin secretions were significantly elevated and the IVF islets failed to respond with an appropriate increase in insulin secretion following high glucose exposure (**Figure 2-7**). Basal hyperinsulinemia is sufficient to maintain high fasting plasma glucose concentrations, which in turn can further stimulate β-cell insulin secretion [130]. Because persistent hyperglycemia prompts insulin oversecretion and leads to a progressive loss of β-cell function [130], it would be interesting to maintain these mice until a later age (~1 year) to further evaluate glucose tolerance, toxicity, and the insulin secretory response. IVF mice also had higher BMD, a feature that is associated with type II diabetes mellitus in humans (29).

Changes to growth trajectory and glucose handling have similarly been observed in mice generated by somatic cell nuclear transfer [120], and in rodent models of intrauterine growth retardation [131], caloric restriction [132], and maternal diet-induced obesity [133]. It appears therefore that the early embryo senses and responds to the environment with a "memory" that endures into adulthood. Interestingly, although each form of developmental stress influences growth kinetics and glucose homeostasis, the specific outcomes are distinct. In our study, neither the birth weight nor the accelerated growth in IVF mice was associated with glucose intolerance in the ways predicted by previous calorie restriction models. This suggests that while altered glucose homeostasis is a common response to environmental perturbation during development, the timing at which the stress occurs (i.e. preimplantation or *in utero*) can result in unique phenotypic changes.

Sexually dimorphic outcomes of IVF

It is well known that sex differences between males and females vastly affect mammalian physiologies, behaviors, diseases and phenotypes through a variety of hormonal, immunological, genetic and epigenetic mechanisms. One of the novel findings of our study is an expanded description of the naturally-occurring metabolic sexual dimorphism in liver and adipose tissues. This is important, as the majority of published metabolomics-based investigations are either restricted to one sex or not stratified by sex. We observed sexual discordances in sterol metabolism, redox state, fatty acid mobilization and oxidation; similar findings have been reported in transcriptomic male-female comparisons in both murine liver and adipose tissue [134]. Our data showed that in control fat, 24% of the detected metabolites (57 of 231) were differentially concentrated between the sexes, compared with 14% (53 of 373) in the liver, suggesting that fat tissue is a preferential locus of sexual dimorphism. Indeed, fat mass is largely divergent between sexes [135], and is a location of sexually dimorphic transcriptional changes in response to nutritional reprogramming [136]. Although we are not aware of any analogous human metabolomics-based studies in these tissues, serum metabolites are markedly

different between the sexes [137], although it is undocumented which tissue(s) contribute to these dissimilarities. Unfortunately, we did not profile serum in males, which will be a valuable future addition to the study. Interestingly, apart from succinylcarnitine and 3-dehydrocarnitine, concentrations of the sexually dimorphic metabolites in fat tissue were uniformly increased in males and decreased in females (**Figure 3-4c**); by comparison the sex bias in liver tissue was more segmented and network-specific (**Figure 3-7c**). Of note, unsupervised hierarchical clustering revealed no correlation between metabolite profile and estrus cycle at time of sacrifice. Overall, the significant sex bias present in control tissues highlights the importance of controlling for sex in metabolic investigations.

Based on the naturally present sexual dimorphism, it is not surprising that models of developmental stress and embryo manipulation also frequently report sex-specific phenotypes. For example, administration of a low-protein diet exclusively during the preimplantation period is associated with postnatal hypertension in both sexes, but weight gain only in female mice [138]. The occurrence of a diabetic or glucose intolerant state is often sexually dimorphic as well: in the non-obese diabetic mouse model, females develop diabetes more rapidly and with increased severity [139]. Conversely, *in utero* caloric restriction is associated with low birth weight, accelerated postnatal catch-up growth and glucose intolerance predominantly in male mice [102]. Interestingly, in the C57Bl/6J strain used in this study, males are generally more prone to glucose intolerance [140]. In a separate model of IVF (outbred CF1 x B6D2F1 mice), suboptimal IVF conditions (Whitten's medium and 20% oxygen) precipitated overgrowth and glucose intolerance specifically in males [89], highlighting the fact that phenotypes are additionally influenced by genetic background [91]. Although the mechanisms underlying this sexual dimorphism remain unclear, they may be related to sex-specific regulatory pathways, gonadal hormone differences or hypothalamic-pituitary-adrenal axis control [141,142].

Another important finding of this study is the sex-specific effect of IVF on the adult liver and fat metabolomes. We did not observe uniform or consistent patterns of change between genders or across tissues that would suggest a common IVF signature present in both males and

females. One possible explanation is that male and female IVF blastocysts are differentially affected by the environments they encounter during early development. Subsequently, the additional numerous and complex developmental steps occurring within maturing liver or adipose tissue could be further altered in accordance with new, tissue-specific developmental cues. The net result would be each tissue adopting a unique and sex-specific metabolic signature of the developmental stress encountered, rather than a singly uniform pattern. Indeed, male-female disparities are apparent even prior to gonadal formation and are therefore partially independent of sex hormone quality and quantity. For example, differential expression of several X-linked transcripts including the metabolic genes glucose-6-phosphate dehydrogenase (*G6pd*) or phosphoglycerate kinase (*Pgk*) may be observed as early as the preimplantation stages [143]. Moreover, up to one-third of transcripts are differentially expressed by sex in the blastocyst, in particular for glucose and protein metabolic pathways [106,144]. This indicates that the preimplantation embryo is poised already to disparately respond to environmental changes in a sex-specific fashion, which may explain the frequent sex bias observed in various models of DOHaD and metabolic reprogramming [37].

Unexpectedly, we observed a striking effect of IVF on adult metabolic sexual dimorphism, which was increased in IVF liver (**Figure 3-9**) and decreased in IVF adipose tissue (**Figure 3-6**). Fat was more susceptible to change: only 24.6% of the metabolites exhibiting sex bias in control tissues (14 of 57 metabolites) maintained that dimorphism in IVF fat, compared to IVF liver samples in which dimorphism was preserved for 50.9% of the metabolites (27 of 53). The majority of the changes in sex bias occurred in lipid and amino acid metabolites. Male-female differences in amino acid concentrations were almost completely abrogated in IVF adipose tissue, whereas IVF livers displayed a shift toward increased dimorphism for these metabolites, particularly for compounds involved in glutamine, lysine, and taurine metabolism, as well as the urea cycle. Sexually dimorphic concentrations of glycerolipids and lysolipids were increased in both tissues, with IVF females displaying significantly lower levels than males. This is particularly relevant as these female animals display similarly strong decreases in

serum concentrations of both glycero- and lysolipids, which has also been observed in metabolomics-based analyses of impaired fasting glucose [145] and diet-induced obesity [146].

Absence of a unique IVF fingerprint in adult tissues

It is widely believed that epigenetic changes mediate developmental plasticity and contribute significantly to the programming of environmental signals [147]. Given that epigenetic modifications are extensively remodeled in the preimplantation embryo [44], and because culture conditions can affect chromatin marks [127], it is possible that IVF-induced changes in transcriptional and epigenetic regulation are responsible for propagating the adult metabolic phenotypes. We therefore hypothesized that some of the transcriptional changes present in IVF embryos might be maintained throughout development and similarly misexpressed in multiple adult IVF tissues. To more clearly define the molecular consequences of IVF and embryo culture, we used microarray to globally assess the transcriptomes of adult female IVF pancreatic islets and insulin-sensitive tissues, as these mice had a distinct metabolic phenotype. Surprisingly, we did not observe a universal IVF fingerprint, and the transcriptional changes associated with IVF were highly tissue-specific with negligible overlap between tissues. Interestingly, the genes exhibiting concordant misexpression in all four IVF tissues were predominantly involved in transcriptional regulation.

Although each IVF tissue transcriptome was unique, the individual gene lists were associated with common cellular pathways (**Figure 3-3**). Overall, the gene expression changes were subtle yet the IVF signatures confirmed changes in glucose handling and mitochondrial function. The most pervasive differences were to genes involved in sterol and fatty acid metabolism (LXR/RXR activation) [148], as well as glucose metabolism, including inositol phosphate metabolism (i.e. PI3K/Akt signaling), type I diabetes mellitus signaling, and mitochondrial dysfunction. Moreover, the significance behind the additional pathway changes in IVF mice (UDP-N-acetyl-D-galactosamine biosynthesis, assembly of RNA polymerase II complex, complement system) is unclear and will require further investigation. Other models of

developmental environmental perturbation have similarly reported tissue-distinct transcriptional profiles implicated in similar cellular processes. For example, in a study of transgenerational inheritance following gestational vinclozolin exposure, F3 generation rats exhibited unique and tissue-specific changes in gene expression with concordant, systemic pathway dysregulation [149].

The reasons for the lack of a uniform IVF signature, and tissue-specific maintenance of the IVF-induced transcriptional changes throughout *in utero* and postnatal development are unknown. One explanation is that the molecular changes induced in blastocysts following preimplantation disturbance are differentially affected by organogenesis, growth factors, or sexually dimorphic signals occurring during later stages of development. As a result, cell physiology and metabolism within each developing tissue may be altered in accordance with new, tissue-specific developmental cues. Several critical periods of increased vulnerability have been described during development and it is possible that some IVF-induced changes are resolved at these times. Fitting within this framework, we have shown tissue-specific maintenance of transcriptional and epigenetic changes in IVF embryos and offspring: the glucose-sensitive gene *Txnip* is significantly increased in IVF blastocysts, and selectively increased in female IVF fat and muscle tissues [93]. Importantly, the *Txnip* dysregulation was associated with enriched H4 acetylation at the *Txnip* promoter, suggesting that adipose tissue may be a locus where preimplantation stress-induced reprogramming is maintained. This is particularly relevant, as fat is now regarded as a primary driver of systemic metabolic dysfunction [150].

Integrated IVF profiles & metabolic disease

We next investigated if the metabolites differentially measured between IVF and *in vivo* tissues could be used as biomarkers to predict chronic disease susceptibility, as our physiologic studies indicated that IVF mice are predisposed to glucose intolerance [89,93]. Indeed, the serum metabolomics study was particularly valuable (**Figure 3-10**): because the IVF metabolic

signature has not yet been described and long-term IVF outcomes are unknown, it is unclear which metabolite changes might contribute to the metabolic phenotypes. However, several of the observed biochemical differences have been similarly noted in serum metabolomics-based investigations into obesity and diabetes. For example, blocked branched-chain amino acid oxidation and increased ketogenesis is strongly associated with obesity-related insulin resistance in both rats and humans [151]; decreased circulating glutamine is a marker of glucose intolerance and insulin resistance in mice and humans [152]; and a significant global reduction in serum glycerophospholipid content is correlated with both impaired fasting glucose [145] and diet-induced obesity [146]. Overall, the altered concentrations of systemic glycolytic, TCA cycle and fatty acid derivatives reflected widespread shifts in nutrient usage. Importantly, the IVF gene expression profiles were mirrored in serum metabolite signatures, indicating a complementation between the microarray and metabolomics experiments.

There were also several biochemicals present in IVF liver and fat tissues that have been linked to metabolic disease. Levels of the purine metabolites AMP, GMP, adenosine, and guanosine were increased in IVF liver, more so in males. AMP is the principal activator of AMP kinase (AMPK), which functions in regulating energy metabolism and glucose homeostasis in the liver and other tissues [153]. Specifically, activated AMPK conserves cellular resources by promoting ATP-generating mechanisms and inhibiting anabolic pathways. The elevation of multiple supporting metabolites suggests that increased AMP and GMP generation is not derived exclusively from energy-requiring cellular reactions involving ATP and GTP (respectively), but additionally through *de novo* synthesis and salvage pathways. These pathways support growth and proliferation through the provision of nucleotides necessary for RNA and DNA synthesis, which may represent another relationship between broad metabolic reprogramming and the disruption of glucose handling in mice conceived by IVF.

Aggregate comparison of all IVF to control liver samples (males and females) showed a striking depletion of several bile acids and salts, with males more severely affected (**Figure 3-8b** and **Appendix 3**). Bile acid levels have a reciprocal relationship with both glucose and insulin

[154], and it has been demonstrated that impaired bile acid synthesis and subsequent reduction in bile acid pool size significantly decreases energy expenditure and contributes to the pathogenesis of obesity and diabetes [155]. Changes in bile acid metabolism may therefore be directly connected to the perturbed glucose handling in IVF mice. This hypothesis is supported by the microarray data from IVF livers revealing transcriptional changes associated with bile acid biosynthesis (altered expression of hepatic *Akr1c4, Baat, Cyp27a1* in IVF mice) and diabetes mellitus signaling (including *Casp9, Cycs, Fcer1g, HlaB, HlaC, Ikbkb, Il1rap, Irf1, Nfkb2*) [93].

Other examples of IVF transcriptional and biochemical profiles synergizing with metabolic disease include the prominent increases in dipeptide concentrations present in male IVF livers coupled with hepatic misexpression of protein ubiquitination pathway genes (*Cul1, Cul2, Dnajb1, Dnajb4, Dnajb9, Dnajb14, Dnajc19, Dnajc21, HlaB, HlaC, Pan2, Psmc6, Psmd12, Sugt1, Tap1, Ube2l3, Uchl3, Usp16, Usp46*). Dipeptides can regulate protein ubiquitination [156], the activity of which affects hepatic lipid production, insulin resistance and secretion [157]. Separately, altered expression of genes involved in mitochondrial function (*Casp9, Cox6c, Cox7a2, Cycs, Gpx4, Ndufa5, Ndufb4, Ndufb6, Ndufs4, Uqcrb*) in conjunction with decreased levels of long chain fatty acids, glutamine, lactate, and increased ribose-5-phosphate in IVF female liver may reflect changes in mitochondrial activity and the use of alternative anabolic branches, such as the shunting of glucose through the pentose phosphate pathway.

Potential etiology of the IVF female phenotype

Under the particular conception conditions used in our study, female IVF animals were predisposed to increased fat accumulation. In separate experiments, we have found that IVF offspring (both males and females) exhibit fat-exclusive maintenance of epigenetic alterations present in IVF blastocysts versus *in vivo* [93], suggesting that adipose tissue is a locus for sex- and tissue-specific changes associated with IVF. Because fat is a primary driver of metabolic dysfunction [150], it is possible that the acquired sex bias in this IVF tissue contributes to the sex-specific metabolic phenotypes. Comparison of IVF adipose metabolic profiles points toward

altered redox homeostasis, with female-specific increases in ophthalmate, cysteine glutathione disulfide (CySS), urate and corticosterone. Glutathione (GSH) is the primary source of antioxidant reducing power in animals, and both ophthalmate and CySS are formed under oxidative stress of GSH. Further, urate and corticosterone can induce pro-inflammatory signaling and increase the production of reactive oxygen species in adipocytes and other tissues [158-160]. The relationship between adipogenesis and redox state is complex, and emerging evidence suggests that adipogenesis is accelerated by oxidizing conditions. For example, reactive oxygen species and antioxidant activity show parallel increases with fat accumulation through adipogenic transcription factor-dependent mechanisms [161-163]. It is therefore plausible that the female-specific oxidization in IVF fat tissue is in part responsible for the increased adiposity in these animals. Fitting with this hypothesis are the reported transcriptional changes in IVF female fat associated with the production of reactive oxygen species (*Alb*, *Akt2*, *ApoB*, *ApoC3*, *ApoF*, *Cat*, *SerpinA1*, *Ppp1Ca*).

Limitations

A few factors in this study merit acknowledgement. Our control group was designed to specifically test the impact of IVF and embryo culture, while removing variables such as superovulation and the embryo transfer procedure. The former strategy results in significantly different litter sizes [89], and neonatal programming studies demonstrate unmistakable changes in growth pattern associated with substantial differences in litter size [164]. Use of the flushed blastocyst cohort allowed us to generate litters with equivalent—although not identical—litter sizes, and we were careful to select mice from appropriately sized litters for the molecular analyses. Importantly, comparison of control male versus female mice in the metabolomics studies may therefore not accurately depict the natural sexual dimorphism present in adult tissues of non-superovulated mice.

Separately, the administration of a high-fat diet makes the interpretation of the results more complex. The DOHaD hypothesis is an expansion of the life course hypothesis, which

proposes that early developmental plasticity establishes a life course trajectory that influences offspring response to later challenges, such that pathological conditions develop from a gradual accumulation of stressful exposures [165]. In this model, a developmental insult (i.e. IVF) primes a vulnerability to a later-occurring stressor (nutritional stress) that can trigger disease. Because the increase in weight and fat deposition in IVF females preceded the change in diet, we can conclude a nutrition-independent effect of IVF on postnatal growth and body composition, and that IVF does not alter the susceptibility of offspring to diet-induced obesity. This is supported by a report that 3 days of overfeeding significantly increases fasting glucose and insulin levels in both IVF and naturally-conceived young adults (mean age 20 years) compared to baseline parameters, but not between the two conception conditions [119]. We therefore do not believe the high-fat diet is a confounding variable, as both the experimental and control groups received and responded similarly to the diet (regarding weight gain, fat gain, food intake, etc.). However, an important consideration is that the molecular analyses were performed in adult tissues, thus it is unclear if the IVF-associated changes were uncovered or masked by the administration of the high-fat diet, or occurred secondary to *in utero* and developmental experiences.

Other factors worth acknowledging are that white adipose tissue depots can vary by adipocyte size, protein composition, gene expression, and response to gonadal hormones [166,167], such that our evaluation of gonadal fat represents a circumscribed analysis of sexual dimorphism and conception impact on metabolism, and cannot necessarily be extrapolated to other adipose depots. Further, the metabolomics data could be influenced by outlying values—for example, one of the male liver samples contributed extreme values. We consequently displayed the fold-change and Z-score data in heat map form to evaluate variability. Metabolomics technology has not yet achieved total metabolite coverage, thus creating an intrinsic and unavoidable bias toward known compounds. This study should subsequently be regarded as hypothesis generating, rather than providing a cause-effect relationship. Because a number of mechanisms may contribute to the observed changes in metabolite pool size, future

studies must focus on transporter activity and enzyme kinetics to better describe the causes of the metabolite flux, as well as which aspects of IVF metabolic signatures are relevant to the underlying etiology of the outward phenotypes.

In summary, in accordance with the DOHaD hypothesis our data support the view that preimplantation development is an environmentally sensitive period during which environmental disturbance can induce permanent changes to adult metabolism and energy use. We show that *in vitro* fertilization and embryo culture can permanently reprogram growth trajectory and energy homeostasis in the developing and adult individual through transcriptional, epigenetic, and metabolic mechanisms. Comparison of the IVF metabolic and transcriptional signatures indicated several areas of overlap, thus establishing a relationship between molecular alterations and physiological phenotypes. It remains unclear why females specifically are susceptible to a more severe metabolic phenotype and increased evidence of oxidative stress, but this may be related to the particular IVF conditions and/or the significant sexual dimorphism already present in early embryos. This study underscores the importance of increasing the safety and efficacy of ART procedures, as well as continued, sex-specific follow-up of IVF-conceived offspring beyond early postnatal life.

Chapter 5: Concluding remarks

In this final section, we provide a commentary on the potential mechanisms contributing to the development of the adult IVF phenotypes, as well as suggestions for future work and recommendations for assisted reproductive policy in the United States.

Comments on mechanism

The mechanisms by which embryos sense energy status and coordinate developmental plasticity have yet to be elucidated, in part due to paucity of embryo experimental material and difficulty in measuring the intrauterine environments. However, it is widely believed that the mechanism of reprogramming is epigenetic in nature [168]. Because of its extensive chromatin reorganization, the preimplantation embryo may be particularly vulnerable to perturbation. Suboptimal conditions could inappropriately affect epigenetic programming of metabolic gene networks, such that specific adaptations conferring immediate survival advantages under stress (such as embryo culture *in vitro*) may be somatically maintained and contribute to a variety of postnatal consequences [147,169]. Therefore, investigating the relationship between energy sensing, acute metabolic plasticity, and epigenetic programming is crucial for determining the basis of how embryonic energy status establishes long-term energy homeostasis.

This is a challenging and costly endeavor. Ideally, the best way to elucidate the metabolic programming mechanism(s) would be to perform global epigenetic analyses in embryos after exposure to an assortment of environments *in vitro* to precisely describe how individual components of the IVF procedure (oxygen tension, culture medium recipe, rigidity of the culture substrate) affect embryo metabolism, transcription and epigenetics, and repeat the experiments throughout development and in postnatal tissues. This would consist of both ChIP-seq analysis of a variety of histone modifications, including activating, repressive, and enhancer marks (H3K4me3, H3K27ac; H4K20me1, H3K9me2/3; H3K4me1), as well as comprehensive DNA methylation analysis using whole genome bisulfite sequencing or reduced representation bisulfite sequencing. Identifying alterations induced by IVF that persist in postnatal tissues

would distinguish candidate molecular changes that may underlie the metabolic phenotypes. However, this would have to be confirmed by rescuing the IVF molecular phenotypes in embryos to determine if adult metabolism normalizes (as discussed below).

New evidence has emerged revealing that the expression of particular metabolic enzymes can affect chromatin remodeling to regulate gene expression [170]. Wellen et al. elegantly linked metabolism and epigenetics by demonstrating that ATP citrate lyase, which catalyzes the production of acetyl-coA from citrate, can localize to the nucleus to become the chief provider of acetyl moieties for histone acetylation [171]. The presence of ATP citrate lyase and resulting acetylation events affect cell cycle progression and adipocyte differentiation in a glucose-dependent manner, indicating a key role for cellular metabolic state in developmental processes. Within this framework, if 1) the availability of different metabolites affects chromatin organization, and 2) the critical preimplantation stages of gene reprogramming are disturbed due to the unusual developmental circumstances imposed by ART, this offers a reasonable mechanism by which embryo conditions could influence metabolic programming and alter the future cellular metabolic potential irreversibly [3]. To date, the localization and activity ATP citrate lyase, as well as its impact on histone acetylation, have not been describe in embryos.

It remains unclear which proteins are functioning at the focal point of stress-induced reprogramming in the IVF embryo, although evidence points to enzymes for which nutritional and redox states, epigenetics and stress response pathways converge. For example, Ingenuity Pathway Analysis identified AMPK as a major upstream regulator of IVF- and *in vitro* culture-induced changes in blastocyst gene expression. AMPK is a conserved Ser/Thr kinase complex that functions as a gatekeeper of cellular energy homeostasis. Under conditions of metabolic stress, activation of AMPK drives conservation of ATP by promoting catabolic pathways including autophagy and mitochondrial biogenesis, as well as inhibiting anabolic processes such as mTORC1-dependent protein synthesis and glucose-derived manufacturing of biosynthetic intermediates [172,173]. Recently, it was demonstrated that under conditions of stress, activated AMPK promotes transcription through direct association with chromatin [174],

thus linking metabolic with epigenetic state and providing a potential mechanism of programmed cellular adaption to stress. It is therefore possible that AMPK is a key component of the environment- and stress-sensing mechanism in preimplantation embryos. In response to *in vitro* stress, AMPK would coordinate embryo developmental plasticity by adapting embryo metabolic physiology to promote growth and survival, and/or facilitate both acute and long-term transcriptional changes through epigenetic mechanisms.

Alternatively, the microarray experiments in adult IVF pancreatic islets uncovered a 2-fold decrease in expression of glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*), which we confirmed in IVF blastocysts (data not shown). Gapdh is a focal point in maintaining cellular homeostasis, with multiple roles spanning glycolysis, intracellular trafficking, regulation of gene expression, and the stress response [175]. Gapdh coordinates several switches between metabolic function, redox state, and apoptosis to orchestrate regulation of and recovery from different insults or stressors. Given its multifunctional roles, it is not surprising that Gapdh can play a critical role in the manifestation of disease. Indeed, GAPDH is downregulated in patients with diabetes, and GAPDH inhibition is the key pathway behind diabetes-induced cellular damage in peripheral tissues and β-cells [176]. Subnormal levels of Gapdh additionally resist recovery following conditions of transient hyperglycemia, such that Gapdh may be a key player in the 'metabolic memory' phenomenon observed in diabetes [177]. It is therefore possible that Gapdh downregulation in IVF blastocysts alters intracellular glucose metabolism and instigates a reprogramming cascade, which is maintained throughout development to impair adult metabolic health and islet function in IVF offspring.

Because the *in vitro* stress transducers and reprogramming effectors are currently elusive, a key area of investigation will be to uncover which principal upstream events or stimuli initiate the reprogramming cascade. According to the 'unifying mechanism' theory of diabetes, hyperglycemic induction of reactive oxygen species (ROS) is the central event precipitating diabetes pathogenesis [178,179]. Although IVF embryos are not exposed to hyperglycemia (Whitten's medium has a glucose concentration of 5.5mM, while KSOM is

0.2mM), there is ample evidence in the literature that embryo culture induces an increase in ROS [180-182]. It is therefore likely that increased ROS from *in vitro* culture is the initial stimulus that reprograms the embryo. If this is confirmed, there is valuable therapeutic potential in decreasing ROS levels via the addition of antioxidants to culture media—for example, Vitamin C or N-acetyl-L-cysteine (NAC)—which could minimize or even avoid the potential adverse outcomes of ART.

Additional suggestions for future work

There are many avenues for future investigation, as both an expansion of the project presented in this dissertation, as well as within the broader context of the DOHaD and ART fields.

Continuation of the project

Several paths forward for expanding the current study are available. The **metabolic analyses** could be expanded to additional tissues, such as gastrocnemius and soleus muscle, to complement the muscle microarray and further investigate the sexually dimorphic changes present in IVF adults. Another vital component of an integrated 'omics study is an overview of **epigenetic changes** and chromatin architecture. To this end, DNA methylation profiles and histone modification signatures would complete the global molecular description of IVF in adult tissues. Given that chromatin landscapes stem from a combination of histone marks, ChIP-seq analysis of several activating and repressive modifications would be an ideal method of capturing epigenetic profiles in IVF mice versus controls.

Next, the molecular analyses presented in this work were conducted in 29 week-old IVF mice, after both the administration of a high-fat diet and the manifestation of the adult phenotypes. It is therefore unclear which of the changes contributed to the metabolic alterations, or occurred secondary to them. As a result, it would be valuable to explore the transcriptional, epigenetic and metabolic differences present in younger (~12 week) IVF versus

control animals, to better understand which molecular alterations might play a more causal role in the development of the increased fat and impaired glucose handling phenotypes.

Correspondingly, at 29 weeks, female IVF offspring displayed evidence of insulin resistance and islet dysfunction (basal hyperinsulinemia and higher fasting glucose levels) but normal glucose tolerance. Preliminary histological analysis found an increased number of islets in these mice as shown by significantly more insulin-positive tissue ($p<0.05$, data not shown). Increased β-cell mass is a sign of insulin resistance and an early marker of diabetes pathogenesis [183]. Therefore, it is possible that the glucose and insulin phenotypes could evolve into frank diabetes, which should be confirmed in older (~1 year) animals.

Confirmation of animal data in humans

The data presented in this dissertation provide the first global analysis of IVF-associated molecular changes in mice, and the next step will be to validate these animal findings in human IVF offspring. Importantly, IVF mouse serum metabolic profiles contained several biomarkers that have previously been correlated with metabolic disease in humans:

1. blocked branched-chain amino acid oxidation and ketogenesis, which are strongly associated with obesity-related insulin resistance in both rats and humans [151];
2. decreased circulating glutamine, a known marker of glucose intolerance and insulin resistance in mice and humans [152];
3. a significant global reduction in serum glycerophospholipid content, previously correlated with both impaired fasting glucose [145] and diet-induced obesity [146] in humans;
4. altered glycolytic, TCA cycle and fatty acid derivatives, reflecting widespread shifts in nutrient usage.

Today, many of the physiological metabolic phenotypes present in animal models of IVF have begun to emerge in human IVF offspring—it is therefore of great interest to define the IVF metabolic signature in humans.

Metabolomics is becoming increasingly appreciated as an optimal tool for exploring the genetic-environment-health paradigm and identifying diagnostic biomarkers of future disease, and thus has growing importance in pediatric research for predicting physiological status in adulthood [184]. Moreover, analysis of urine is an ideal method of non-invasively capturing the metabolic fingerprint of an individual. Given the realities and challenges of patient recruitment, metabolomics-based urinalysis is the most plausible mode of investigation (more optimal that serum). Under these conditions, we would hypothesize that metabolic urinalysis of IVF offspring would uncover an increased occurrence of predictive biomarkers of diabetes and the metabolic syndrome that have previously been described in humans, including changes in glutamine-glutamate ratio [152], branched-chain keto-acids [185], and the increased presence of 2-aminoadipic acid [186].

Any evaluation of human IVF will require a particularly thoughtful control group, as couples seeking ART often represent a rather narrow population. Ideally, control children would be age- and gender-matched naturally conceived singletons born to subfertile parents, with subfertility defined as not achieving conception following at least 1 year of frequent unprotected intercourse. As a result, the targeted control group would be either the naturally conceived siblings of IVF participants, or the offspring of parents who previously visited a fertility clinic but ultimately conceived without the use of IVF. In addition to age- and gender-matching, other parameters including birth weight, gestational age, BMI, maternal BMI during pregnancy, pregnancy complications, and subfertility diagnoses must be recorded for later statistical adjustments.

Assessing the length of embryo culture impact on long-term health outcomes

It remains controversial if the length of time spent in culture during the preimplantation period might affect both short- and long-term health, and arguably one of the most discussed controversies in reproductive medicine today is the efficacy and safety of cleavage (Day 3) versus blastocyst (Day 5) stage transfer of human embryos.

To date, human reports have focused exclusively on obstetric and neonatal outcomes, and the majority of studies have identified significant increases in pregnancy and live birth rates in favor of blastocyst transfer [187-189]. However, Day 5 transfer is associated with increased prenatal complications, including a greater incidence of preterm birth, low birth weight, low APGAR score, respiratory diagnoses, and congenital malformations [190-193]. Further, a recent study reported that the length of embryo culture was a highly significant independent factor determining birth weight (p=0.007), with the percentage of babies born LGA nearly doubling for blastocyst transfers compared with Day 2 or 3 transfer [194]. This was independently corroborated by a meta-analysis observing that the risk of prenatal growth restriction is lower following blastocyst transfer [191].

Several animal studies have investigated long-term outcomes following preimplantation culture, but no study has formally compared Day 3 versus Day 5 embryos with adult metabolic health. Rexhaj, *et al*. reported vascular dysfunction in ART mice generated by implantation of both 2-cell embryos and blastocysts [123]. Banrezes, *et al*. exposed mouse zygotes to different types of severe nutritional depletion for 10-15 hours and observed adult body weight changes [128]. Separately, Scott, *et al*. reported glucose and insulin defects in IVF mice aged 8 weeks derived 2-cell transfer; however, this group transferred 1-5 embryos yielding 1-4 pups per surrogate relative to naturally mated control litters of 2-8 animals [120]. Due to the influence of litter size on postnatal growth and adult metabolism, it is unclear if these metabolic defects were significant to the IVF procedure or secondary to the intrauterine or pre-weaning environments [103,195]. Litter size was not reported in the Rexhaj, *et al*. study.

As a result, a fundamental and clinically relevant inquiry is the assessment of the effect of different lengths of *in vitro* culture on postnatal growth and metabolic physiology in a mouse model, in order to clarify if reduced time in culture would improve adult phenotypes. Because epigenetic changes begin soon after fertilization and continue through the blastocyst stage [44], we would anticipate that earlier transfer would minimize the reprogramming effects on postnatal physiology (altered growth, fat deposition, and β-cell dysfunction). Although other

groups have reported changes associated with earlier transfers, the data is obscured by suboptimal culture conditions or small litter sizes. However, the specific timing of preimplantation sensitivity is unknown, and it is possible that both Day 3 and Day 5 transferred offspring would exhibit similar metabolic and molecular changes. This result would be equally important, as it would indicate that blastocyst culture is not more unsafe than cleavage stage transfer, and that the process of culture *in vitro* (rather than the length of culture) is responsible for reprogramming the embryo.

Discovering markers of embryo stress and health

Finally, increased research efforts are needed toward investigating the developmental, growth and metabolic requirements of an embryo prior to implantation, which may be used to improve identification strategies for distinguishing favorable outcomes of ART. Although blastocyst morphology and birth weight are the most ubiquitous, non-invasive means of measuring embryonic and fetal heath (respectively) today, they may not be reliable predictors of future health. To this end, there is a need for better markers of ART 'success'—this would not only affect embryo selection protocols before transfer, but also aid in the detection of disease susceptibilities that might originate during periods of early development.

Approaching this task is challenging: it requires identifying changes induced by IVF and embryo culture in the blastocyst that are both maintained throughout development and associated with the adult metabolic phenotypes. Our laboratory has evidence that both Txnip and the Parp1/Gapdh pathways are altered in IVF embryos with persisting changes in adult tissues, but additional experiments are necessary to determine that these alterations are indeed correlated with and contribute to the phenotypes. This could be addressed using genetic models to normalize Txnip levels in the embryo, such as the commercially available B6N;129S-*Txnip*^{tm1Rlee}/J mouse containing loxP sites flanking exon 1 of the *Txnip* gene. Similarly, Gapdh inhibition with IVF could be restored using an Oct4 promoter-driven GAPDH construct to compensate for the reduced Gapdh specifically during the preimplantation window. If embryo

expression of Txnip/Gapdh is rescued in the IVF model, leading to an alleviated phenotype, these may be used as early markers by which to evaluate the future health of IVF offspring.

An appeal for community outreach & public policy

Although human *in vitro* fertilization was initially developed to treat infertility, it has provided great joy and procreative opportunities for many other demographics, including members of the LGBT community, single parents, young cancer patients, and individuals (or couples) wishing to delay conception until middle age. Tech giants such as Facebook and Apple now compensate oocyte freezing and storage costs for female employees desiring to postpone family plans in favor of career advancement [196]. With the recent introduction of cytoplasmic transfer for three-donor reproduction in the United Kingdom, combined with the novel possibilities of editing the human germline to combat inherited diseases, it appears that advances in IVF technology will continue to dramatically increase its frequency of use.

Very few studies have evaluated the postnatal outcomes of ART in humans, yet there is mounting evidence that IVF adolescents exhibit metabolic abnormalities that predispose adult cardiometabolic pathologies. Although *in vitro* fertilization is not currently considered an environmental exposure, it introduces many unusual variables (high oxygen, different nutritional milieu, a rigid plastic substrate) that may be perceived as stressful to the developing embryo and thus beget adult health abnormalities. This is particularly disconcerting because 1) this information has not been widely disseminated to either the public or to legislature, and 2) there are no formal registries or standardized mechanisms of monitoring the health of IVF offspring past infancy.

To this end, there is a growing need for the creation of assisted reproductive technology policy in the United States, specifically regarding the establishment of mechanisms for increasing the safety and efficacy of embryo culture procedures, as well as continued follow-up of IVF offspring beyond birth. Further, the IVF community would benefit from an organization providing education and resources about these procedures, functioning as a platform for

translating the most recent scientific findings into a more accessible form for a lay audience. There are now legitimate, science-based concerns over the long-term health of IVF children; the time has come for a public, political and scientific dialogue about these consequences.

Chapter 6: Materials and Methods

Animals

All animals were maintained according to institutional regulations, under a constant 12 hr light/dark cycle with *ad libitum* access to water and standard chow (PicoLab® #5058; 23% protein, 22% fat, 55% carbohydrate). To probe the consequences of nutritional stress, all animals were placed on a high fat diet beginning at 24 weeks of age (Research Diets, Inc. #D12492; 20% protein, 60% fat, 20% carbohydrate [197]) until time of sacrifice at 30 weeks.

IVF, embryo culture and transfer

In vitro fertilization, embryo culture, and embryo transfer experiments were performed as previously described [93]. Briefly, C57Bl/6J females aged 6-8 weeks were injected with 5 IU pregnant mares serum gonadotropin (PMSG) followed 46-48 hours later by 5 IU human chorionic gonadotropin (hCG) to induce superovulation. 13-15 hours post-hCG administration, cumulous-oocyte-complexes were isolated from ampullae and incubated 4-6 hours in human tubal fluid (HTF) medium (Millipore, MR-070-D) with previously capacitated (1 hour) cauda epididymal sperm from C57Bl/6J males. Fertilized zygotes were washed and cultured to the blastocyst stage in potassium simplex optimization medium (KSOM, containing 0.2mM pyruvate, 10mM lactate, 0.2mM glucose, 1mM glutamine; Millipore, MR-106-D) supplemented with amino acids [198], at 37°C under Ovoil™ (Vitrolife, #10029) with 5% CO_2 and 5% oxygen in a modular humidified chamber. To generate post-implantation cohorts, pseudopregancy was induced by mating naturally cycling CF-1 females to vasectomized CD-1 males, confirmed by the presence of a copulation plug the following morning (considered day 0.5). Late-cavitating blastocysts were transferred to the uterine horns of recipients (8-10 embryos per horn) on day 2.5 of pseudopregnancy. For control experiments, C57Bl/6J female mice were superovulated as described above and mated to C57Bl/6J males overnight. Embryonic day 3.5 blastocysts (96 hours post-hCG administration) were flushed from the oviducts and transferred immediately to

the uterine horns of CF-1 recipients, thus controlling for litter size and the embryo transfer procedure. Recipients had similar weights at the time of transfer, and comparable weight gain during pregnancy. To rule out possible litter size effects [103], we chose only to maintain litters comprised of 4-8 animals for each conception group, and only animals derived from litters of 5-7 pups were used in the microarray and metabolomics analyses.

Body weight, morphometrics, food intake and body composition analyses

Animals were weighed weekly beginning at birth. Food intake was measured daily for 1 week at ages 7, 20 and 28 weeks. Fat and lean body mass, percent fat, whole-body areal bone mineral density and bone mineral content were determined at 8, 16, 21 and 29 weeks of age by dual-energy X-ray absorptiometry (DEXA) using a Lunar PIXImus II mouse densitometer. Organ weights were recorded at sacrifice, and body morphometrics including body length, body mass index (body weight normalized to crown-rump length), anogenital distance, anogenital index (equal to anogenital distance divided by body weight), biparietal diameter, and abdominal diameter were measured both at birth and time of sacrifice (30 weeks).

Glucose tolerance and plasma measurements

Glucose tolerance was assessed at approximately 13, 20 and 29 weeks of age. Animals were fasted for 6 hours (compared to overnight), as this protocol is more sensitive for detecting abnormalities in glucose handling [199]. After fasting with *ad libitum* access to water, baseline glucose values were individually established using a handheld glucometer, and 50μl blood samples were collected from tail tips for plasma composition analyses. Clearance of a 1.5mg/g glucose bolus injected intraperitoneally was subsequently monitored at 15, 30, 60 and 120-minute intervals post-injection. The sum of the trapezoidal areas between the 0, 15, 30, 60 and 120-minute time points corresponding to each animal were summed to obtain the area under the curve (AUC).

Islet isolation and in vitro *insulin secretion assay*

Islets of Langerhans were isolated from surrounding exocrine pancreas in mice aged 12 weeks and 29 weeks by the UCSF Islet Production Core Facility according to standard procedures [200]. Briefly, pancreata were distended with 3ml cold M199 medium (Gibco BRL) containing 1.5mg/dl collagenase (type P; Boehringer Mannheim), excised, and incubated in a 37°C stationary bath. Islets were separated by density gradients (Histopaque-1077; Sigma), handpicked under a stereomicroscope, and pooled into samples of 250 and 500 for RNA and protein isolation, respectively. Remaining islets in groups of 500 were cultured free-floating for 24 hours in 5ml RPMI 1640 medium supplemented with L-glutamine and benzyl penicillin (100 U/ml), exposed to basal and stimulatory concentrations of glucose (3mM and 28mM), and the supernatant was collected for insulin determination as described above. Insulin levels were normalized to total islet DNA or protein content.

Microarray preparation and analysis

Microarray experiments were conducted using 4 tissues (liver, gastrocnemius muscle, gonadal fat, pancreatic islets) derived from 3 adult FB and 3 IVF female mice (24 independent microarrays). Animals contributing to this analysis were derived from at least two separate litters of 5-7 pups per condition, and to minimize variation, each animal provided the 4 tissues. Total RNA was extracted and purified from previously frozen liver, muscle and fat using the RNeasy mini kit (Qiagen), then submitted to the Gladstone Genomics Core Facility at UCSF for labeling, hybridization and scanning of the microarrays. Whole frozen islets were also sent to the core facility for RNA isolation and amplification prior to microarray processing. Extracted RNA was hybridized to Affymetrix Mouse Gene 1.0 ST arrays, consisting of more than 770,000 unique oligonucleotide features representing 28,853 genes. Microarray data was analyzed with GeneSpring GX 12.1 software (Agilent Technologies) using a 30% cutoff and significance threshold of $p<0.05$ without correction. Post-processing of the resulting gene lists was conducted using Ingenuity Pathway Analysis (Ingenuity® Systems,

http://www.ingenuity.com), which uses Fischer's exact test to identify significant functional associations represented within selected gene sets.

Metabolomic profiling

Non-fasted animals were sacrificed by CO_2 exposure followed by cervical dislocation in the morning, and tissues were harvested from animals generated by 5 separate IVF and 5 control experiments. At least 3 independent cohorts contributed to each analysis of liver/fat and sex. Serum samples were obtained from 1 IVF and 2 control litters. Estrous cycle was monitored using vaginal smear. Immediately after collection, whole liver (24 samples: n=6 for each sex and conception condition), gonadal fat (29 samples: n=7 IVF and n=7 control females, n=10 IVF and n=5 control males) and serum (8 samples: n=4 females for each conception condition) samples were snap-frozen for unbiased metabolomic profiling by Metabolon, Inc., as described in detail elsewhere [201,202]. Briefly, samples underwent a series of organic and aqueous extractions optimized for small molecule recovery, and were split into equal parts for gas chromatography-mass spectrometry (GC/MS) and liquid chromatography-tandem mass spectrometry (LC-MS/MS) analyses. For the latter platform, samples were again divided for profiling in both positive (acidic) and negative (basic) ionization modes.

Bioinformatics and statistics

All data are presented as the mean plus or minus the standard deviation (SDM), unless otherwise specified. Either a one-way ANOVA or a two-tailed student's *t*-test was used for statistical analysis as appropriate. Tukey's post hoc test was applied to assess differences between groups when a one-way ANOVA was significant. $^*p<0.05$ was considered significant.

Mass spectrometry profiles were processed using software developed by Metabolon, Inc. [203]. Peaks were called against a library of 2,500 named biochemicals comprised of amino acids, lipids, carbohydrates, nucleotides, peptides, vitamins, cofactors, and xenobiotics. For statistical interpretation of detected metabolites, ANOVA contrasts were performed to identify

biochemicals that differed significantly between (a) the *in vivo* and IVF conception conditions, and (b) males versus females, with two-way ANOVA analyses to describe biochemicals exhibiting a significant interaction between sex and conception parameters. A Welch's two-sample *t*-test was performed to identify biochemicals that differed significantly between the *in vivo* and IVF conception conditions in serum samples (female only). For all comparisons, $^{*}p<0.05$ was considered significant. Unsupervised Pearson correlations were used to evaluate the relationship between metabolite concentrations and both percent adiposity and fasting glucose levels at time of death. For moderately or strong coefficient values (defined as $|r|>0.6$), additional correlation analyses were conducted with segregation by sex, conception condition, or both.

Heat maps were generated using GENE-E software developed by the Broad Institute available at: http://www.broadinstitute.org/cancer/software/GENE-E/. The metabolomics heat maps depict the fold-change difference in metabolite concentration between mean IVF and control values, or the z-score (calculated as $z=(x-\mu)/\sigma$; where x=the individual scaled metabolite value for an animal, μ=the mean value of the metabolite for the defined population, and σ=the standard deviation of that population) comparing either metabolite concentrations between male and female animals, or individual control and IVF values to their respective population means.

The web-based metabolomic data processing tool MetaboAnalyst was used for tissue metabolite data analysis [204,205]. Detailed methodology may be found at http://www.metaboanalyst.ca. Metabolite set enrichment analysis (MSEA) was conducted on metabolite data mapped according to Human Metabolome Database (HMDB) or Kyoto Encyclopedia of Genes and Genomes (Kegg) identifiers, using the metabolite pathway associated metabolite set library (currently 88 entries).

References

1. ICMART. International Committee Monitoring Assisted Reproductive Technology (ICMART) World Report: Preliminary 2008 Data; 2012; Istanbul, Turkey.

2. Technology SoAR (2012) SART 2010 National Clinic Summary Report.

3. Feuer SK, Camarano L, Rinaudo PF (2013) ART and health: clinical outcomes and insights on molecular mechanisms from rodent studies. Mol Hum Reprod 19: 189-204.

4. Ceelen M, van Weissenbruch MM, Prein J, Smit JJ, Vermeiden JP, et al. (2009) Growth during infancy and early childhood in relation to blood pressure and body fat measures at age 8-18 years of IVF children and spontaneously conceived controls born to subfertile parents. Hum Reprod 24: 2788-2795.

5. Ceelen M, van Weissenbruch MM, Roos JC, Vermeiden JP, van Leeuwen FE, et al. (2007) Body composition in children and adolescents born after in vitro fertilization or spontaneous conception. J Clin Endocrinol Metab 92: 3417-3423.

6. Ceelen M, van Weissenbruch MM, Vermeiden JP, van Leeuwen FE, Delemarre-van de Waal HA (2008) Cardiometabolic differences in children born after in vitro fertilization: follow-up study. J Clin Endocrinol Metab 93: 1682-1688.

7. Sakka SD, Loutradis D, Kanaka-Gantenbein C, Margeli A, Papastamataki M, et al. (2010) Absence of insulin resistance and low-grade inflammation despite early metabolic syndrome manifestations in children born after in vitro fertilization. Fertil Steril 94: 1693-1699.

8. Brent RL, Holmes LB (1988) Clinical and basic science lessons from the thalidomide tragedy: what have we learned about the causes of limb defects? Teratology 38: 241-251.

9. McBride DE (1961) Septic abortion followed by septic shock. J Am Osteopath Assoc 60: 545-552.

10. Kermack WO, McKendrick AG, McKinlay PL (1934) Death-rates in Great Britain and Sweden: Expression of Specific Mortality Rates as Products of Two Factors, and some Consequences thereof. J Hyg (Lond) 34: 433-457.

11. McCance RA, Widdowson EM (1974) The determinants of growth and form. Proc R Soc Lond B Biol Sci 185: 1-17.

12. Wadsworth ME, Cripps HA, Midwinter RE, Colley JR (1985) Blood pressure in a national birth cohort at the age of 36 related to social and familial factors, smoking, and body mass. Br Med J (Clin Res Ed) 291: 1534-1538.

13. Barker DJ, Osmond C (1986) Infant mortality, childhood nutrition, and ischaemic heart disease in England and Wales. Lancet 1: 1077-1081.

14. Moczek AP, Sultan S, Foster S, Ledon-Rettig C, Dworkin I, et al. (2011) The role of developmental plasticity in evolutionary innovation. Proc Biol Sci 278: 2705-2713.

15. Wadhwa PD, Buss C, Entringer S, Swanson JM (2009) Developmental origins of health and disease: brief history of the approach and current focus on epigenetic mechanisms. Semin Reprod Med 27: 358-368.

16. Bateson P, Barker D, Clutton-Brock T, Deb D, D'Udine B, et al. (2004) Developmental plasticity and human health. Nature 430: 419-421.

17. Cockburn K, Rossant J (2010) Making the blastocyst: lessons from the mouse. J Clin Invest 120: 995-1003.

18. Kwong WY, Wild AE, Roberts P, Willis AC, Fleming TP (2000) Maternal undernutrition during the preimplantation period of rat development causes blastocyst abnormalities and programming of postnatal hypertension. Development 127: 4195-4202.

19. Ecker DJ, Stein P, Xu Z, Williams CJ, Kopf GS, et al. (2004) Long-term effects of culture of preimplantation mouse embryos on behavior. Proc Natl Acad Sci U S A 101: 1595-1600.

20. Fernandez-Gonzalez R, Moreira P, Bilbao A, Jimenez A, Perez-Crespo M, et al. (2004) Long-term effect of in vitro culture of mouse embryos with serum on mRNA expression of imprinting genes, development, and behavior. Proc Natl Acad Sci U S A 101: 5880-5885.

21. Dey SK, Lim H, Das SK, Reese J, Paria BC, et al. (2004) Molecular cues to implantation. Endocr Rev 25: 341-373.

22. Wilding M, Coppola G, Dale B, Di Matteo L (2009) Mitochondria and human preimplantation embryo development. Reproduction 137: 619-624.

23. Biggers JD, Whittingham DG, Donahue RP (1967) The pattern of energy metabolism in the mouse oocyte and zygote. Proc Natl Acad Sci U S A 58: 560-567.

24. Brinster RL (1965) Studies on the development of mouse embryos in vitro. IV. Interaction of energy sources. J Reprod Fertil 10: 227-240.

25. Brinster RL, Thomson JL (1966) Development of eight-cell mouse embryos in vitro. Exp Cell Res 42: 308-315.

26. Zhao Y, Baltz JM (1996) Bicarbonate/chloride exchange and intracellular pH throughout preimplantation mouse embryo development. Am J Physiol 271: C1512-1520.

27. Feuer S, Rinaudo P (2012) Preimplantation stress and development. Birth Defects Res C Embryo Today 96: 299-314.

28. Bavister B (2004) Oxygen concentration and preimplantation development. Reprod Biomed Online 9: 484-486.

29. Leese HJ (2012) Metabolism of the preimplantation embryo: 40 years on. Reproduction 143: 417-427.

30. Lane M, Gardner DK (2000) Lactate regulates pyruvate uptake and metabolism in the preimplantation mouse embryo. Biol Reprod 62: 16-22.

31. Lane M, Gardner DK (2003) Ammonium induces aberrant blastocyst differentiation, metabolism, pH regulation, gene expression and subsequently alters fetal development in the mouse. Biol Reprod 69: 1109-1117.

32. Edwards LJ, Williams DA, Gardner DK (1998) Intracellular pH of the mouse preimplantation embryo: amino acids act as buffers of intracellular pH. Hum Reprod 13: 3441-3448.

33. Gardner DK, Leese HJ (1988) The role of glucose and pyruvate transport in regulating nutrient utilization by preimplantation mouse embryos. Development 104: 423-429.

34. Horsthemke B, Ludwig M (2005) Assisted reproduction: the epigenetic perspective. Hum Reprod Update 11: 473-482.

35. Zhao Y, Chauvet PJ, Alper SL, Baltz JM (1995) Expression and function of bicarbonate/chloride exchangers in the preimplantation mouse embryo. J Biol Chem 270: 24428-24434.

36. Phillips KP, Leveille MC, Claman P, Baltz JM (2000) Intracellular pH regulation in human preimplantation embryos. Hum Reprod 15: 896-904.

37. Bermejo-Alvarez P, Rizos D, Lonergan P, Gutierrez-Adan A (2011) Transcriptional sexual dimorphism during preimplantation embryo development and its consequences for developmental competence and adult health and disease. Reproduction 141: 563-570.

38. Ooi SK, O'Donnell AH, Bestor TH (2009) Mammalian cytosine methylation at a glance. J Cell Sci 122: 2787-2791.

39. Zhang Y, Reinberg D (2001) Transcription regulation by histone methylation: interplay between different covalent modifications of the core histone tails. Genes Dev 15: 2343-2360.

40. Khorasanizadeh S (2011) Recognition of methylated histones: new twists and variations. Curr Opin Struct Biol 21: 744-749.

41. Wang Z, Zang C, Rosenfeld JA, Schones DE, Barski A, et al. (2008) Combinatorial patterns of histone acetylations and methylations in the human genome. Nature Genetics 40: 897-903.

42. Burdge GC, Lillycrop KA (2010) Nutrition, epigenetics, and developmental plasticity: implications for understanding human disease. Annu Rev Nutr 30: 315-339.

43. Fischer JJ, Toedling J, Krueger T, Schueler M, Huber W, et al. (2008) Combinatorial effects of four histone modifications in transcription and differentiation. Genomics 91: 41-51.

44. Reik W (2007) Stability and flexibility of epigenetic gene regulation in mammalian development. Nature 447: 425-432.

45. Guo F, Li X, Liang D, Li T, Zhu P, et al. (2014) Active and passive demethylation of male and female pronuclear DNA in the Mammalian zygote. Cell Stem Cell 15: 447-458.

46. Saitou M, Kagiwada S, Kurimoto K (2012) Epigenetic reprogramming in mouse pre-implantation development and primordial germ cells. Development 139: 15-31.

47. Schultz RM (2002) The molecular foundations of the maternal to zygotic transition in the preimplantation embryo. Hum Reprod Update 8: 323-331.

48. Schwarzer C, Esteves TC, Arauzo-Bravo MJ, Le Gac S, Nordhoff V, et al. (2012) ART culture conditions change the probability of mouse embryo gestation through defined cellular and molecular responses. Hum Reprod 27: 2627-2640.

49. Rinaudo PF, Giritharan G, Talbi S, Dobson AT, Schultz RM (2006) Effects of oxygen tension on gene expression in preimplantation mouse embryos. Fertil Steril 86: 1252-1265, 1265 e1251-1236.

50. Rinaudo P, Schultz RM (2004) Effects of embryo culture on global pattern of gene expression in preimplantation mouse embryos. Reproduction 128: 301-311.

51. Kolahi KS, Donjacour A, Liu X, Lin W, Simbulan RK, et al. (2012) Effect of substrate stiffness on early mouse embryo development. PLoS One 7: e41717.

52. Picton HM, Elder K, Houghton FD, Hawkhead JA, Rutherford AJ, et al. (2010) Association between amino acid turnover and chromosome aneuploidy during human preimplantation embryo development in vitro. Mol Hum Reprod 16: 557-569.

53. Leese HJ (2002) Quiet please, do not disturb: a hypothesis of embryo metabolism and viability. Bioessays 24: 845-849.

54. Leese HJ, Baumann CG, Brison DR, McEvoy TG, Sturmey RG (2008) Metabolism of the viable mammalian embryo: quietness revisited. Mol Hum Reprod 14: 667-672.

55. Vander Heiden MG, Locasale JW, Swanson KD, Sharfi H, Heffron GJ, et al. (2010) Evidence for an alternative glycolytic pathway in rapidly proliferating cells. Science 329: 1492-1499.

56. Krisher RL, Prather RS (2012) A role for the Warburg effect in preimplantation embryo development: metabolic modification to support rapid cell proliferation. Mol Reprod Dev 79: 311-320.

57. Redel BK, Brown AN, Spate LD, Whitworth KM, Green JA, et al. (2012) Glycolysis in preimplantation development is partially controlled by the Warburg Effect. Mol Reprod Dev 79: 262-271.

58. Baumann CG, Morris DG, Sreenan JM, Leese HJ (2007) The quiet embryo hypothesis: molecular characteristics favoring viability. Mol Reprod Dev 74: 1345-1353.

59. Fritz MA, Speroff L (2010) Clinical Gynecologic Endocrinology and Infertility; Seigafuse S, editor. Philadelphia, PA: LWW; Eighth Edition. 1488 p.

60. Ferraretti AP, Goossens V, de Mouzon J, Bhattacharya S, Castilla JA, et al. (2012) Assisted reproductive technology in Europe, 2008: results generated from European registers by ESHRE. Hum Reprod 27: 2571-2584.

61. Guo N, Li Y, Ai J, Gu L, Chen W, et al. (2014) Two different concentrations of oxygen for culturing precompaction stage embryos on human embryo development competence: a prospective randomized sibling-oocyte study. Int J Clin Exp Pathol 7: 6191-6198.

62. Gardner DK, Leese HJ (1990) Concentrations of nutrients in mouse oviduct fluid and their effects on embryo development and metabolism in vitro. J Reprod Fertil 88: 361-368.

63. Summers MC, Biggers JD (2003) Chemically defined media and the culture of mammalian preimplantation embryos: historical perspective and current issues. Hum Reprod Update 9: 557-582.

64. Wiemer KE, Anderson AR, Kyslinger ML, Weikert ML (2002) Embryonic development and pregnancies following sequential culture in human tubal fluid and a modified simplex optimized medium containing amino acids. Reprod Biomed Online 5: 323-327.

65. Zandstra H, Van Montfoort AP, Dumoulin JC (2015) Does the type of culture medium used influence birthweight of children born after IVF? Hum Reprod 30: 530-542.

66. Tachibana M, Amato P, Sparman M, Woodward J, Sanchis DM, et al. (2013) Towards germline gene therapy of inherited mitochondrial diseases. Nature 493: 627-631.

67. SART (2014) Society for Assisted Reproductive Technology 2012 National Clinic Summary Report.

68. DuFort CC, Paszek MJ, Weaver VM (2011) Balancing forces: architectural control of mechanotransduction. Nat Rev Mol Cell Biol 12: 308-319.

69. Schieve LA, Meikle SF, Ferre C, Peterson HB, Jeng G, et al. (2002) Low and very low birth weight in infants conceived with use of assisted reproductive technology. N Engl J Med 346: 731-737.

70. Jackson RA, Gibson KA, Wu YW, Croughan MS (2004) Perinatal outcomes in singletons following in vitro fertilization: a meta-analysis. Obstet Gynecol 103: 551-563.

71. Walker SK, Hartwich KM, Robinson JS (2000) Long-term effects on offspring of exposure of oocytes and embryos to chemical and physical agents. Hum Reprod Update 6: 564-577.

72. de Boo HA, Harding JE (2006) The developmental origins of adult disease (Barker) hypothesis. Aust N Z J Obstet Gynaecol 46: 4-14.

73. Bloise E, Feuer SK, Rinaudo PF (2014) Comparative intrauterine development and placental function of ART concepti: implications for human reproductive medicine and animal breeding. Hum Reprod Update 20: 822-839.

74. Armitage JA, Khan IY, Taylor PD, Nathanielsz PW, Poston L (2004) Developmental programming of the metabolic syndrome by maternal nutritional imbalance: how strong is the evidence from experimental models in mammals? J Physiol 561: 355-377.

75. Meas T (2010) Fetal origins of insulin resistance and the metabolic syndrome: a key role for adipose tissue? Diabetes Metab 36: 11-20.

76. Manipalviratn S, DeCherney A, Segars J (2009) Imprinting disorders and assisted reproductive technology. Fertil Steril 91: 305-315.

77. Katari S, Turan N, Bibikova M, Erinle O, Chalian R, et al. (2009) DNA methylation and gene expression differences in children conceived in vitro or in vivo. Hum Mol Genet 18: 3769-3778.

78. Wagenaar K, Ceelen M, van Weissenbruch MM, Knol DL, Delemarre-van de Waal HA, et al. (2008) School functioning in 8- to 18-year-old children born after in vitro fertilization. Eur J Pediatr 167: 1289-1295.

79. Hvidtjorn D, Grove J, Schendel D, Schieve LA, Svaerke C, et al. (2011) Risk of autism spectrum disorders in children born after assisted conception: a population-based follow-up study. J Epidemiol Community Health 65: 497-502.

80. Middelburg KJ, Heineman MJ, Bos AF, Hadders-Algra M (2008) Neuromotor, cognitive, language and behavioural outcome in children born following IVF or ICSI-a systematic review. Hum Reprod Update 14: 219-231.

81. Fountain C, Zhang Y, Kissin DM, Schieve LA, Jamieson DJ, et al. (2015) Association Between Assisted Reproductive Technology Conception and Autism in California, 1997-2007. Am J Public Health: e1-e9.

82. Kallen B, Finnstrom O, Nygren KG, Olausson PO (2005) In vitro fertilization in Sweden: child morbidity including cancer risk. Fertil Steril 84: 605-610.

83. Servick K (2014) Unsettled questions trail IVF's success. Science 345: 744-746.

84. Scherrer U, Rimoldi SF, Rexhaj E, Stuber T, Duplain H, et al. (2012) Systemic and pulmonary vascular dysfunction in children conceived by assisted reproductive technologies. Circulation 125: 1890-1896.

85. Schieve LA, Cohen B, Nannini A, Ferre C, Reynolds MA, et al. (2007) A population-based study of maternal and perinatal outcomes associated with assisted reproductive technology in Massachusetts. Matern Child Health J 11: 517-525.

86. Brinton LA, Westhoff CL, Scoccia B, Lamb EJ, Althuis MD, et al. (2005) Causes of infertility as predictors of subsequent cancer risk. Epidemiology 16: 500-507.

87. Wilkins KM, Warnock JK, Serrano E (2010) Depressive symptoms related to infertility and infertility treatments. Psychiatr Clin North Am 33: 309-321.

88. Parikh NI, Cnattingius S, Mittleman MA, Ludvigsson JF, Ingelsson E (2012) Subfertility and risk of later life maternal cardiovascular disease. Hum Reprod 27: 568-575.

89. Donjacour A, Liu X, Lin W, Simbulan R, Rinaudo PF (2014) In vitro fertilization affects growth and glucose metabolism in a sex-specific manner in an outbred mouse model. Biol Reprod 90: 80.

90. Sone H, Kagawa Y (2005) Pancreatic beta cell senescence contributes to the pathogenesis of type 2 diabetes in high-fat diet-induced diabetic mice. Diabetologia 48: 58-67.

91. Festing MF (1976) Phenotypic variability of inbred and outbred mice. Nature 263: 230-232.

92. Beck JA, Lloyd S, Hafezparast M, Lennon-Pierce M, Eppig JT, et al. (2000) Genealogies of mouse inbred strains. Nat Genet 24: 23-25.

93. Feuer SK, Liu X, Donjacour A, Lin W, Simbulan RK, et al. (2014) Use of a mouse in vitro fertilization model to understand the developmental origins of health and disease hypothesis. Endocrinology 155: 1956-1969.

94. Barker DJ (2007) The origins of the developmental origins theory. J Intern Med 261: 412-417.

95. Chatot CL, Ziomek CA, Bavister BD, Lewis JL, Torres I (1989) An improved culture medium supports development of random-bred 1-cell mouse embryos in vitro. J Reprod Fertil 86: 679-688.

96. Frolkis VV, Grigorov YG, Pisarchuk KL (1993) Long-term effects of litter size in early postnatal period on metabolism, aging and life span in rats. Arch Gerontol Geriatr 17: 65-73.

97. Waterland RA, Garza C (2002) Early postnatal nutrition determines adult pancreatic glucose-responsive insulin secretion and islet gene expression in rats. J Nutr 132: 357-364.

98. Dean A, Sharpe RM (2013) Anogenital distance or digit length ratio as measures of fetal androgen exposure: relationship to male reproductive development and its disorders. J Clin Endocrinol Metab.

99. Li CC, Young PE, Maloney CA, Eaton SA, Cowley MJ, et al. (2013) Maternal obesity and diabetes induces latent metabolic defects and widespread epigenetic changes in isogenic mice. Epigenetics 8.

100. Eriksson JG, Forsen TJ, Osmond C, Barker DJ (2003) Pathways of infant and childhood growth that lead to type 2 diabetes. Diabetes Care 26: 3006-3010.

101. Hernandez-Valencia M, Patti ME (2006) A thin phenotype is protective for impaired glucose tolerance and related to low birth weight in mice. Arch Med Res 37: 813-817.

102. Jimenez-Chillaron JC, Hernandez-Valencia M, Lightner A, Faucette RR, Reamer C, et al. (2006) Reductions in caloric intake and early postnatal growth prevent glucose intolerance and obesity associated with low birthweight. Diabetologia 49: 1974-1984.

103. Epstein HT (1978) The effect of litter size on weight gain in mice. J Nutr 108: 120-123.

104. Gabory A, Attig L, Junien C (2009) Sexual dimorphism in environmental epigenetic programming. Mol Cell Endocrinol 304: 8-18.

105. Geer EB, Shen W (2009) Gender differences in insulin resistance, body composition, and energy balance. Gend Med 6 Suppl 1: 60-75.

106. Bermejo-Alvarez P, Rizos D, Rath D, Lonergan P, Gutierrez-Adan A (2010) Sex determines the expression level of one third of the actively expressed genes in bovine blastocysts. Proc Natl Acad Sci U S A 107: 3394-3399.

107. Gilbert JS, Banek CT (2012) Sex Differences in the Developmental Programming of Adult Disease. In: Dubey RK, editor. Sex Hormones: InTech.

108. Kaddurah-Daouk R, Kristal BS, Weinshilboum RM (2008) Metabolomics: a global biochemical approach to drug response and disease. Annu Rev Pharmacol Toxicol 48: 653-683.

109. Xia J, Wishart DS (2010) MSEA: a web-based tool to identify biologically meaningful patterns in quantitative metabolomic data. Nucleic Acids Res 38: W71-77.

110. Edwards RG, Sharpe DJ (1971) Social values and research in human embryology. Nature 231: 87-91.

111. He K, Zhao H, Wang Q, Pan Y (2010) A comparative genome analysis of gene expression reveals different regulatory mechanisms between mouse and human embryo pre-implantation development. Reprod Biol Endocrinol 8: 41.

112. Rinaudo P, Wang E (2012) Fetal programming and metabolic syndrome. Annu Rev Physiol 74: 107-130.

113. Schulz LC (2010) The Dutch Hunger Winter and the developmental origins of health and disease. Proc Natl Acad Sci U S A 107: 16757-16758.

114. de Rooij SR, Painter RC, Phillips DI, Osmond C, Michels RP, et al. (2006) Hypothalamic-pituitary-adrenal axis activity in adults who were prenatally exposed to the Dutch famine. Eur J Endocrinol 155: 153-160.

115. de Rooij SR, Painter RC, Phillips DI, Osmond C, Michels RP, et al. (2006) Impaired insulin secretion after prenatal exposure to the Dutch famine. Diabetes Care 29: 1897-1901.

116. de Rooij SR, Painter RC, Phillips DI, Osmond C, Tanck MW, et al. (2006) Cortisol responses to psychological stress in adults after prenatal exposure to the Dutch famine. Psychoneuroendocrinology 31: 1257-1265.

117. de Rooij SR, Painter RC, Roseboom TJ, Phillips DI, Osmond C, et al. (2006) Glucose tolerance at age 58 and the decline of glucose tolerance in comparison with age 50 in people prenatally exposed to the Dutch famine. Diabetologia 49: 637-643.

118. de Rooij SR, Wouters H, Yonker JE, Painter RC, Roseboom TJ (2010) Prenatal undernutrition and cognitive function in late adulthood. Proc Natl Acad Sci U S A 107: 16881-16886.

119. Chen M, Wu L, Zhao J, Wu F, Davies MJ, et al. (2014) Altered glucose metabolism in mouse and humans conceived by IVF. Diabetes 63: 3189-3198.

120. Scott KA, Yamazaki Y, Yamamoto M, Lin Y, Melhorn SJ, et al. (2010) Glucose parameters are altered in mouse offspring produced by assisted reproductive technologies and somatic cell nuclear transfer. Biol Reprod 83: 220-227.

121. Gkourogianni A, Kosteria I, Telonis AG, Margeli A, Mantzou E, et al. (2014) Plasma metabolomic profiling suggests early indications for predisposition to latent insulin resistance in children conceived by ICSI. PLoS One 9: e94001.

122. Zhou J, Liu H, Gu HT, Cui YG, Zhao NN, et al. (2014) Association of cardiac development with assisted reproductive technology in childhood: a prospective single-blind pilot study. Cell Physiol Biochem 34: 988-1000.

123. Rexhaj E, Paoloni-Giacobino A, Rimoldi SF, Fuster DG, Anderegg M, et al. (2013) Mice generated by in vitro fertilization exhibit vascular dysfunction and shortened life span. J Clin Invest 123: 5052-5060.

124. Dumoulin JC, Land JA, Van Montfoort AP, Nelissen EC, Coonen E, et al. (2010) Effect of in vitro culture of human embryos on birthweight of newborns. Hum Reprod 25: 605-612.

125. Giritharan G, Talbi S, Donjacour A, Di Sebastiano F, Dobson AT, et al. (2007) Effect of in vitro fertilization on gene expression and development of mouse preimplantation embryos. Reproduction 134: 63-72.

126. Bloise E, Lin W, Liu X, Simbulan R, Kolahi KS, et al. (2012) Impaired placental nutrient transport in mice generated by in vitro fertilization. Endocrinology 153: 3457-3467.

127. Doherty AS, Mann MR, Tremblay KD, Bartolomei MS, Schultz RM (2000) Differential effects of culture on imprinted H19 expression in the preimplantation mouse embryo. Biol Reprod 62: 1526-1535.

128. Banrezes B, Sainte-Beuve T, Canon E, Schultz RM, Cancela J, et al. (2011) Adult body weight is programmed by a redox-regulated and energy-dependent process during the pronuclear stage in mouse. PLoS One 6: e29388.

129. Barker DJ (1998) Mothers, babies and health in later life. Glasgow: Churchill Livingston.

130. DeFronzo RA, Tripathy D (2009) Skeletal muscle insulin resistance is the primary defect in type 2 diabetes. Diabetes Care 32 Suppl 2: S157-163.

131. Simmons RA, Templeton LJ, Gertz SJ (2001) Intrauterine growth retardation leads to the development of type 2 diabetes in the rat. Diabetes 50: 2279-2286.

132. Sutton GM, Centanni AV, Butler AA (2010) Protein malnutrition during pregnancy in C57BL/6J mice results in offspring with altered circadian physiology before obesity. Endocrinology 151: 1570-1580.

133. Jungheim ES, Schoeller EL, Marquard KL, Louden ED, Schaffer JE, et al. (2010) Diet-induced obesity model: abnormal oocytes and persistent growth abnormalities in the offspring. Endocrinology 151: 4039-4046.

134. Yang X, Schadt EE, Wang S, Wang H, Arnold AP, et al. (2006) Tissue-specific expression and regulation of sexually dimorphic genes in mice. Genome Res 16: 995-1004.

135. Wells JC (2007) Sexual dimorphism of body composition. Best Pract Res Clin Endocrinol Metab 21: 415-430.

136. Guo C, Li C, Myatt L, Nathanielsz PW, Sun K (2013) Sexually dimorphic effects of maternal nutrient reduction on expression of genes regulating cortisol metabolism in fetal baboon adipose and liver tissues. Diabetes 62: 1175-1185.

137. Mittelstrass K, Ried JS, Yu Z, Krumsiek J, Gieger C, et al. (2011) Discovery of sexual dimorphisms in metabolic and genetic biomarkers. PLoS Genet 7: e1002215.

138. Watkins AJ, Ursell E, Panton R, Papenbrock T, Hollis L, et al. (2008) Adaptive responses by mouse early embryos to maternal diet protect fetal growth but predispose to adult onset disease. Biol Reprod 78: 299-306.

139. Makino S, Kunimoto K, Muraoka Y, Mizushima Y, Katagiri K, et al. (1980) Breeding of a non-obese, diabetic strain of mice. Jikken Dobutsu 29: 1-13.

140. Toye AA, Lippiat JD, Proks P, Shimomura K, Bentley L, et al. (2005) A genetic and physiological study of impaired glucose homeostasis control in C57BL/6J mice. Diabetologia 48: 675-686.

141. Gabory A, Roseboom TJ, Moore T, Moore LG, Junien C (2013) Placental contribution to the origins of sexual dimorphism in health and diseases: sex chromosomes and epigenetics. Biol Sex Differ 4: 5.

142. Gutierrez-Adan A, Perez-Crespo M, Fernandez-Gonzalez R, Ramirez MA, Moreira P, et al. (2006) Developmental consequences of sexual dimorphism during pre-implantation embryonic development. Reprod Domest Anim 41 Suppl 2: 54-62.

143. Wrenzycki C, Lucas-Hahn A, Herrmann D, Lemme E, Korsawe K, et al. (2002) In vitro production and nuclear transfer affect dosage compensation of the X-linked gene transcripts G6PD, PGK, and Xist in preimplantation bovine embryos. Biol Reprod 66: 127-134.

144. Fernandez-Gonzalez R, de Dios Hourcade J, Lopez-Vidriero I, Benguria A, De Fonseca FR, et al. (2009) Analysis of gene transcription alterations at the blastocyst stage related to the long-term consequences of in vitro culture in mice. Reproduction 137: 271-283.

145. Xu F, Tavintharan S, Sum CF, Woon K, Lim SC, et al. (2013) Metabolic Signature Shift in Type 2 Diabetes Mellitus Revealed by Mass Spectrometry-based Metabolomics. J Clin Endocrinol Metab 98: E1060-1065.

146. Kim HJ, Kim JH, Noh S, Hur HJ, Sung MJ, et al. (2011) Metabolomic analysis of livers and serum from high-fat diet induced obese mice. J Proteome Res 10: 722-731.

147. Gluckman PD, Hanson MA, Low FM (2011) The role of developmental plasticity and epigenetics in human health. Birth Defects Res C Embryo Today 93: 12-18.

148. Edwards PA, Kennedy MA, Mak PA (2002) LXRs; oxysterol-activated nuclear receptors that regulate genes controlling lipid homeostasis. Vascul Pharmacol 38: 249-256.

149. Skinner MK, Manikkam M, Haque MM, Zhang B, Savenkova MI (2012) Epigenetic transgenerational inheritance of somatic transcriptomes and epigenetic control regions. Genome Biol 13: R91.

150. Muoio DM, Newgard CB (2006) Obesity-related derangements in metabolic regulation. Annu Rev Biochem 75: 367-401.

151. Newgard CB, An J, Bain JR, Muehlbauer MJ, Stevens RD, et al. (2009) A branched-chain amino acid-related metabolic signature that differentiates obese and lean humans and contributes to insulin resistance. Cell Metab 9: 311-326.

152. Cheng S, Rhee EP, Larson MG, Lewis GD, McCabe EL, et al. (2012) Metabolite profiling identifies pathways associated with metabolic risk in humans. Circulation 125: 2222-2231.

153. Viollet B, Guigas B, Leclerc J, Hebrard S, Lantier L, et al. (2009) AMP-activated protein kinase in the regulation of hepatic energy metabolism: from physiology to therapeutic perspectives. Acta Physiol (Oxf) 196: 81-98.

154. Li T, Francl JM, Boehme S, Ochoa A, Zhang Y, et al. (2012) Glucose and insulin induction of bile acid synthesis: mechanisms and implication in diabetes and obesity. J Biol Chem 287: 1861-1873.

155. Watanabe M, Horai Y, Houten SM, Morimoto K, Sugizaki T, et al. (2011) Lowering bile acid pool size with a synthetic farnesoid X receptor (FXR) agonist induces obesity and diabetes through reduced energy expenditure. J Biol Chem 286: 26913-26920.

156. Du F, Navarro-Garcia F, Xia Z, Tasaki T, Varshavsky A (2002) Pairs of dipeptides synergistically activate the binding of substrate by ubiquitin ligase through dissociation of its autoinhibitory domain. Proc Natl Acad Sci U S A 99: 14110-14115.

157. Wing SS (2008) The UPS in diabetes and obesity. BMC Biochem 9 Suppl 1: S6.

158. Baldwin W, McRae S, Marek G, Wymer D, Pannu V, et al. (2011) Hyperuricemia as a mediator of the proinflammatory endocrine imbalance in the adipose tissue in a murine model of the metabolic syndrome. Diabetes 60: 1258-1269.

159. Sautin YY, Nakagawa T, Zharikov S, Johnson RJ (2007) Adverse effects of the classic antioxidant uric acid in adipocytes: NADPH oxidase-mediated oxidative/nitrosative stress. Am J Physiol Cell Physiol 293: C584-596.

160. Zafir A, Banu N (2009) Modulation of in vivo oxidative status by exogenous corticosterone and restraint stress in rats. Stress 12: 167-177.

161. Calzadilla P, Sapochnik D, Cosentino S, Diz V, Dicelio L, et al. (2011) N-acetylcysteine reduces markers of differentiation in 3T3-L1 adipocytes. Int J Mol Sci 12: 6936-6951.

162. Vigilanza P, Aquilano K, Baldelli S, Rotilio G, Ciriolo MR (2011) Modulation of intracellular glutathione affects adipogenesis in 3T3-L1 cells. J Cell Physiol 226: 2016-2024.

163. Imhoff BR, Hansen JM (2011) Differential redox potential profiles during adipogenesis and osteogenesis. Cell Mol Biol Lett 16: 149-161.

164. Aubert R, Suquet JP, Lemonnier D (1980) Long-term morphological and metabolic effects of early under- and over-nutrition in mice. J Nutr 110: 649-661.

165. Godfrey KM, Gluckman PD, Hanson MA (2010) Developmental origins of metabolic disease: life course and intergenerational perspectives. Trends Endocrinol Metab 21: 199-205.

166. Sackmann-Sala L, Berryman DE, Munn RD, Lubbers ER, Kopchick JJ (2012) Heterogeneity among white adipose tissue depots in male C57BL/6J mice. Obesity (Silver Spring) 20: 101-111.

167. Fuente-Martin E, Argente-Arizon P, Ros P, Argente J, Chowen JA (2013) Sex differences in adipose tissue: It is not only a question of quantity and distribution. Adipocyte 2: 128-134.

168. Sebert S, Sharkey D, Budge H, Symonds ME (2011) The early programming of metabolic health: is epigenetic setting the missing link? Am J Clin Nutr 94: 1953S-1958S.

169. Haaf T (2006) Methylation dynamics in the early mammalian embryo: implications of genome reprogramming defects for development. Curr Top Microbiol Immunol 310: 13-22.

170. Rathmell JC, Newgard CB (2009) Biochemistry. A glucose-to-gene link. Science 324: 1021-1022.

171. Wellen KE, Hatzivassiliou G, Sachdeva UM, Bui TV, Cross JR, et al. (2009) ATP-citrate lyase links cellular metabolism to histone acetylation. Science 324: 1076-1080.

172. Faubert B, Boily G, Izreig S, Griss T, Samborska B, et al. (2013) AMPK is a negative regulator of the Warburg effect and suppresses tumor growth in vivo. Cell Metab 17: 113-124.

173. Mihaylova MM, Shaw RJ (2011) The AMPK signalling pathway coordinates cell growth, autophagy and metabolism. Nat Cell Biol 13: 1016-1023.

174. Bungard D, Fuerth BJ, Zeng PY, Faubert B, Maas NL, et al. (2010) Signaling kinase AMPK activates stress-promoted transcription via histone H2B phosphorylation. Science 329: 1201-1205.

175. Sirover MA (2011) On the functional diversity of glyceraldehyde-3-phosphate dehydrogenase: biochemical mechanisms and regulatory control. Biochim Biophys Acta 1810: 741-751.

176. Brownlee M (2005) The pathobiology of diabetic complications: a unifying mechanism. Diabetes 54: 1615-1625.

177. Kanwar M, Kowluru RA (2009) Role of glyceraldehyde 3-phosphate dehydrogenase in the development and progression of diabetic retinopathy. Diabetes 58: 227-234.

178. Nishikawa T, Edelstein D, Du XL, Yamagishi S, Matsumura T, et al. (2000) Normalizing mitochondrial superoxide production blocks three pathways of hyperglycaemic damage. Nature 404: 787-790.

179. Sakai K, Matsumoto K, Nishikawa T, Suefuji M, Nakamaru K, et al. (2003) Mitochondrial reactive oxygen species reduce insulin secretion by pancreatic beta-cells. Biochem Biophys Res Commun 300: 216-222.

180. Goto Y, Noda Y, Mori T, Nakano M (1993) Increased generation of reactive oxygen species in embryos cultured in vitro. Free Radic Biol Med 15: 69-75.

181. Cebral E, Carrasco I, Vantman D, Smith R (2007) Preimplantation embryotoxicity after mouse embryo exposition to reactive oxygen species. Biocell 31: 51-59.

182. Martin-Romero FJ, Miguel-Lasobras EM, Dominguez-Arroyo JA, Gonzalez-Carrera E, Alvarez IS (2008) Contribution of culture media to oxidative stress and its effect on human oocytes. Reprod Biomed Online 17: 652-661.

183. Weir GC, Bonner-Weir S (2004) Five stages of evolving beta-cell dysfunction during progression to diabetes. Diabetes 53 Suppl 3: S16-21.

184. Moco S, Collino S, Rezzi S, Martin FP (2013) Metabolomics perspectives in pediatric research. Pediatr Res 73: 570-576.

185. Menni C, Fauman E, Erte I, Perry JR, Kastenmuller G, et al. (2013) Biomarkers for type 2 diabetes and impaired fasting glucose using a nontargeted metabolomics approach. Diabetes 62: 4270-4276.

186. Wang TJ, Ngo D, Psychogios N, Dejam A, Larson MG, et al. (2013) 2-Aminoadipic acid is a biomarker for diabetes risk. J Clin Invest 123: 4309-4317.

187. Papanikolaou EG, Camus M, Kolibianakis EM, Van Landuyt L, Van Steirteghem A, et al. (2006) In vitro fertilization with single blastocyst-stage versus single cleavage-stage embryos. N Engl J Med 354: 1139-1146.

188. Papanikolaou EG, Kolibianakis EM, Tournaye H, Venetis CA, Fatemi H, et al. (2008) Live birth rates after transfer of equal number of blastocysts or cleavage-stage embryos in IVF. A systematic review and meta-analysis. Hum Reprod 23: 91-99.

189. Blake DA, Farquhar CM, Johnson N, Proctor M (2007) Cleavage stage versus blastocyst stage embryo transfer in assisted conception. Cochrane Database Syst Rev: CD002118.

190. Dar S, Lazer T, Shah PS, Librach CL (2014) Neonatal outcomes among singleton births after blastocyst versus cleavage stage embryo transfer: a systematic review and meta-analysis. Hum Reprod Update.

191. Maheshwari A, Kalampokas T, Davidson J, Bhattacharya S (2013) Obstetric and perinatal outcomes in singleton pregnancies resulting from the transfer of blastocyst-stage versus cleavage-stage embryos generated through in vitro fertilization treatment: a systematic review and meta-analysis. Fertil Steril 100: 1615-1621 e1611-1610.

192. Kallen B, Finnstrom O, Lindam A, Nilsson E, Nygren KG, et al. (2010) Blastocyst versus cleavage stage transfer in in vitro fertilization: differences in neonatal outcome? Fertil Steril 94: 1680-1683.

193. Milki AA, Jun SH, Hinckley MD, Behr B, Giudice LC, et al. (2003) Incidence of monozygotic twinning with blastocyst transfer compared to cleavage-stage transfer. Fertil Steril 79: 503-506.

194. Makinen S, Soderstrom-Anttila V, Vainio J, Suikkari AM, Tuuri T (2013) Does long in vitro culture promote large for gestational age babies? Hum Reprod 28: 828-834.

195. Hahn P (1984) Effect of litter size on plasma cholesterol and insulin and some liver and adipose tissue enzymes in adult rodents. J Nutr 114: 1231-1234.

196. Farr C (2014) Apple, Facebook will pay for female employees to freeze their eggs. Reuters: Thomson Reuters.

197. Weisberg SP, Hunter D, Huber R, Lemieux J, Slaymaker S, et al. (2006) CCR2 modulates inflammatory and metabolic effects of high-fat feeding. J Clin Invest 116: 115-124.

198. Lawitts JA, Biggers JD (1993) Culture of preimplantation embryos. Methods Enzymol 225: 153-164.

199. Heikkinen S, Argmann CA, Champy MF, Auwerx J (2007) Evaluation of glucose homeostasis. Curr Protoc Mol Biol Chapter 29: Unit 29B 23.

200. O'Dowd JF (2009) The isolation and purification of rodent pancreatic islets of Langerhans. Methods Mol Biol 560: 37-42.

201. Evans AM, DeHaven CD, Barrett T, Mitchell M, Milgram E (2009) Integrated, nontargeted ultrahigh performance liquid chromatography/electrospray ionization tandem mass

spectrometry platform for the identification and relative quantification of the small-molecule complement of biological systems. Anal Chem 81: 6656-6667.

202. Lawton KA, Berger A, Mitchell M, Milgram KE, Evans AM, et al. (2008) Analysis of the adult human plasma metabolome. Pharmacogenomics 9: 383-397.

203. Dehaven CD, Evans AM, Dai H, Lawton KA (2010) Organization of GC/MS and LC/MS metabolomics data into chemical libraries. J Cheminform 2: 9.

204. Xia J, Psychogios N, Young N, Wishart DS (2009) MetaboAnalyst: a web server for metabolomic data analysis and interpretation. Nucleic Acids Res 37: W652-660.

205. Xia J, Mandal R, Sinelnikov IV, Broadhurst D, Wishart DS (2012) MetaboAnalyst 2.0--a comprehensive server for metabolomic data analysis. Nucleic Acids Res 40: W127-133.

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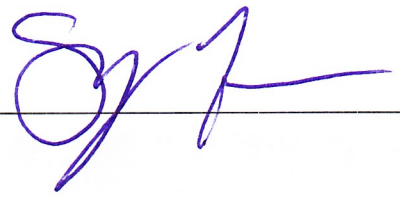
Author signature  Date 19 May 2015