DNA METHYLATION DIFFERENCES BETWEEN CHILDREN CONCEIVED IN VITRO AND IN VIVO ARE ASSOCIATED WITH ART PROCEDURES

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ABSTRACT

Epidemiological data indicate that children conceived *in vitro* have a greater relative risk of low birth-weight, major and minor birth defects, and rare disorders involving imprinted genes, suggesting that epigenetic changes may be associated with assisted reproduction. DNA methylation and gene expression differences have been found in cord blood and placenta comparing children conceived *in vitro* using assisted reproductive technology (ART) and children conceived *in vivo*. The source of these differences (the effect of ART *versus* underlying infertility) has never been identified in humans. In order to determine what fraction of the DNA methylation and gene expression difference is attributable to the ART procedure and what fraction is attributable to underlying infertility, quasi-transcriptome-wide DNA methylation profiles were compared between (1) *in vitro* ART children of mothers who are infertile as a result of a physical impediment to fertilization (tubal blockage) or children conceived with the aid of donor oocytes as a group (tubal and donor egg group), and (2) children of parent(s) who have idiopathic infertility (infertility group). Both groups were compared to children conceived *in vivo*. Our data suggest strongly that many of the DNA methylation and gene expression differences observed between the *in vitro* and *in vivo* conceptions are associated with some aspect of ART procedure, rather than underlying infertility.

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LIST OF ABBREVIATIONS

°C	Centigrade
AF1	Amino terminal A/B domain
AQPEP Q/ Laeverin	Aminopeptidase Q
ART	Assisted Reproductive Technology
AS	Angelman Syndrome
Ascl2	Achaete-scute complex homolog 2 (mouse)
Avg	Average
bp	base pair
BWS	Beckwith – Wiedemann Syndrome
C-sections	Caesarean sections
CCDC62	Coiled-coil Domain Containing 62
CCDS	Consensus Coding Sequences
cm^3	cubic centimetres
CP	Cerebral Palsy
CRTAM	Class-I MHC-restricted T cell Associated Molecule
CT	Cycle Threshold
DBD	DNA Binding Domain
DLX5	Distal-less homeobox 5
DNA	Deoxyribonucleic Acid
DOR	Diminished Ovarian Reserve
E1A	adenovirus Early region 1A
E2F1	E2F transcription factor 1

ER	Estrogen
ERA75	Estrogen Receptor Alpha 75
EVT	Extravillous Trophoblast
FET	Frozen Embryo Transfer
FLJ10260	SLFN12, Schlafen family member 12
FLJ90650	Aminopeptidase Q/ Laeverin
G1 phase	Gap 1 phase
GAS2	Growth Arrest-specific protein 2
GRB10	Growth factor Receptor-bound protein 10
GRIN2C	Glutamate Receptor, Ionotropic, N-methyl D-aspartate 2C
H19	imprinted maternally expressed transcript (non-protein coding)
HDL	High-density Lipoprotein
HSPB3	Heat Shock 27kDa Protein 3
I.R.B.	Institutional Review Board
ICSI	Intracytoplasmic Sperm Injection
IFI16	Interferon, gamma-inducible protein 16
IGF	Insulin-like Growth Factor
IGF2R	Insulin-like Growth Factor 2 Receptor
IL5	Interleukin 5 (colony-stimulating factor, eosinophil)
IVF	*In Vitro* Fertilization
IVM	*In Vitro* oocyte Maturation
LBD	Ligand Binding Domain
LBW	Low Birthweight
LOI	Loss Of Imprinting
LYST	Lysosomal Trafficking regulator
MEST	Mesoderm Specific Transcript homolog (mouse)
min	minutes

ml	millilitre
mM	micromoles
NDN	Mouse Necdin homolog (human)
ng	nanogram
NMDA	N-methyl- D-aspartate
NR	Nuclear Receptor
PCDHGB7	Protocadherin Gamma subfamily B, 7
PCK2	Phosphoenolpyruvate Carboxykinase 2 (mitochondrial)
PCOS	Polycystic Ovary Syndrome
PCR	Polymerase Chain Reaction
PEG1	Paternally-expressed Gene 1 protein
Peg3	Paternally-expressed gene 3 protein (mouse)
pmol	picomole
PTPN20B	Protein Tyrosine Phosphatase, Non-receptor type 20B/A
PWS	Prader–Willi syndrome
Rb	Retinoblastoma protein
RNA	Ribonucleic acid
RT-PCR	Reverse Transcription Polymerase Chain Reaction
S phase	Synthesis phase
SAOS-2	Sarcoma Osteogenic
SD	Standard Deviation
SDF-1	Stromal-derived Factor-1
SNRPN	Small Nuclear Ribonucleoprotein Polypeptide N
SV40	Simian vacuolating virus 40
SVD	Spontaneous Vaginal Delivery
TCF2	Transcription Factor 2, hepatic
TrisCl	Tris (hydroxymethyl) amino methane

TTR	Transthyretin
U/µl	Units/microlitre
μ g	microgram
µl	microlitre
Xist	X-inactive specific transcript

CHAPTER 1
INTRODUCTION

1.1 Assisted Reproductive Technology (ART)

The first Assisted reproductive technology birth in 1978 represented a new era of infertility treatment for millions of couples previously unable to conceive. In 2009, ART resulted in 60,190 infants, contributing to >1% of annual births in the United States (Centers for Disease Control and Prevention). The number of children conceived by ART continues to increase worldwide. As this effective and advanced technology continues to evolve, more and more scientists and researchers are devoting attention toward the safety and efficacy of this technology.

ART consists of all treatments or procedures that include the *in vitro* handling of both human oocytes and sperm or of embryos for the purpose of establishing a pregnancy (Zegers-Hochschild *et al*., 2009). The first and most commonly used technology is *in vitro* fertilization (IVF). The IVF treatment cycle, including hormonal stimulation of multiple ovarian follicles, the process of oocyte retrieval and spermatozoa preparation, IVF, culture of embryos in medium for several days, and embryo transfer into the uterus

instead of the oviduct, is substantially different from natural conception (Buitendijk, 1999). It has been indicated that these distinct procedures might have tremendous effects on the developing conceptus.

1.2 Perinatal and Long-term Outcomes of Pregnancies by ARTs

During the last decade, the possible consequences and adverse effects of ARTs have been investigated increasingly. Both short- and long- term adverse outcomes of ARTs have been reported by multiple studies in recent years. The safety of the procedures and the health of children conceived by ARTs have been questioned (Powell, 2003).

ARTs have been associated with pregnancy complications (preeclampsia and placental complications), multiple births, adverse perinatal outcomes (low birth weight, preterm birth, perinatal mortality and congenital malformations) and epigenetic alternations (Iliadou *et al.*, 2011). Another explanation of pregnancy complications and adverse outcomes are multiple births and their associated problems, because multiple births are over-represented in ART pregnancies. In most developed countries, 30 – 50% of all twin pregnancies result from ARTs (Ombelet *et al*., 2005; Pandian *et al*., 2009; Sunderam *et al*., 2006; Gelbaya *et al*., 2010). However, the role of ARTs as an essential factor in the increased risk of preterm birth and low birth weight has been questioned (Williams *et al*., 1991; Draper *et al*., 1999; Basso & Baird, 2003; Ludwig, 2009) suggesting that background subfertility may be more important.

1.2.1 Neurological Outcomes

Neurological abnormalities appear to happen more commonly in ART-conceived children. Several studies have found an increased risk of cerebral palsy (CP) or neurological abnormalities among the ART population (Ericson *et al*., 2002; Strömberg *et al*., 2002; Pinborg *et al*., 2004; Lidegaard *et al*., 2005; Hvidtjørn *et al*., 2006; Hvidtjørn *et al*., 2010). Children of subfertile parents have been included as a control group to ART conceived children in recent studies, finding no difference in adverse neurological outcomes (Middelburg *et al*., 2009; Middelburg *et al*., 2010). Nevertheless, a recent population study indicated that ART may be a more important factor than parental subfertility in CP risk (Zhu *et al*., 2010). Further studies are required to clarify the possible contribution of background infertility to neurological outcomes in ART conceived children.

1.2.2 Growth of ART Conceived Children

A well-recognized adverse consequence of cultured embryos in cattle and sheep is "large offspring syndrome" (Young *et al*., 1998). It is proposed to be due to altered expression of insulin-like growth factor (IGF)-II receptor, which is imprinted in cattle and sheep but not humans (Young *et al*., 2001). These studies raised interest in the growth patterns of ART conceived children. The studies that have examined the growth and weight patterns of ART conceived children show conflicting results. However, there have been a handful of studies that have corrected for prematurity and birth weight to determine any actual effects of the ART process itself on growth (Miles *et al*., 2007; Makhoul *et al*., 2000; Green *et al*., 2009). In a matched control study, Miles *et al.*

analyzed the growth and metabolic parameters of 69 ART conceived children from fresh embryo transfers (Miles *et al*., 2007). Compared with controls, ART-conceived girls were taller, had higher IGF binding protein 3 levels and displayed a trend towards higher IGF-I levels. ART children also had higher high-density lipoprotein (HDL) and lower triglyceride levels than controls. The findings were particularly relevant as it was the first study to correct for parental heights and exclude children born premature or with low birth weight. The same group also assessed ART-conceived children from frozen embryo transfers, again showing that ART-conceived girls were taller than matched controls (Green *et al*., 2009). Recently, Makhoul et al. published findings on the follow-up of children with very low birth weight aged 6 to 10 years conceived by ART or fertility medications (Makhoul *et al*., 2009). This group accounted for the genetic height and the low birth weight status in their analyses, and found that the ART and fertility medication-conceived groups were significantly taller than controls (Makhoul *et al*., 2009). Currently, there is no clear explanation for the taller stature or the sex specificity of the effect observed.

Many of the studies on the growth of children conceived by ART had confounding factors and methodological weaknesses. Larger studies that include appropriately matched groups are needed to confirm these findings.

1.2.3 Epigenetics and Imprinting Disorders in ART Conceived Children

It is well known that the phenotype of an individual is not exclusively determined by genotype. In 1942, Conrad Waddington introduced the term epigenetics, originally defined as ‘‘the branch of biology which studies the causal interactions between genes

and their products which bring the phenotype into being" (Waddington, 1942) . Today, epigenetics is generally defined as ‘‘the study of changes in gene function that are mitotically and/or meiotically heritable and that do not entail a change in DNA sequence" (Wu & Morris, 2001). There are many different types of epigenetic modifications that are known to affect gene expression, including changes in nucleosome positioning and conformation, and histone modifications such as acetylation, phosphorylation, methylation and ubiquitinylation (Berger, 2007). DNA methylation is the most thoroughly studied epigenetic modification. In mammals, it is almost exclusively restricted to CpG dinucleotides. Clusters of CpG dinucleotides, i.e. CpG islands, a region with at least 200 bp, and a GC percentage that is greater than 50%, and with an observed-to-expected CpG ratio that is greater than 60% (Gardiner-Garden & Frommer, 1987), are often found within promoter regions of genes where depending on methylation level they either permit or silence transcription. The methylation of CpG dinucleotides can repress transcription either by blocking the binding of transcription factors to the promoter or by recruiting histone-modifying protein complexes that repress transcription through the formation of a more condensed chromatin structure. During cell division, the maintenance DNA methyltransferase 1 is up-regulated and recruited to the replication fork where it methylates CpG dinucleotides on the newly synthesized unmethylated daughter strand. By the symmetric methylation of the newly synthesized strands, methylation patterns and cellular identity are preserved through mitosis (Berger, 2007).

In mammalian development, there are two critical periods of epigenetic modification: gametogenesis and early preimplantation development. During

gametogenesis, genome-wide demethylation occurs, which is followed by remethylation before fertilization. Early embryogenesis is characterized by a second genome-wide demethylation event, a process that is sensitive to environmental factors (Santos *et al*., 2002). Following implantation, methylation is re-established early in embryonic life.

Diploid organisms possess two copies of each autosomal gene, one from each parent. Most autosomal genes are expressed simultaneously from both alleles. However, in a small subset of genes, one allele is silenced in a parent-specific manner by the process called genomic imprinting. Genomic imprinting is a process of chemical modification of nucleotides in which only one allele of specific genes is transcribed and the other allele is silenced based on the parent-of-origin. There are currently more than 80 imprinted genes in mammals, of which approximately 40 are known to be imprinted in humans (Morison *et al*., 2005). A majority of the imprinted genes have important roles in the control of embryonic and placental growth and development. Abnormalities in genomic imprints in human are known to cause 10 syndromes (Manipalviratn *et al*., 2009). Among imprinted genes, those that are paternally expressed tend to promote growth whereas those that are maternally expressed tend to suppress growth, leading to the hypothesis that genomic imprinting may have evolved as a parental 'battle of the sexes' to regulate the maternal allocation of resources to the offspring (Moore & Haig, 1991).

Given the extent of epigenetic reprogramming that occurs during gametogenesis and embryogenesis and the vulnerability of the process, it is not difficult to understand

how alteration in reprogramming could be associated with adverse clinical outcomes. Assisted reproductive techniques involve manipulation of several steps involved in conception that might alter the normal epigenetic reprogramming or imprinting processes: use of hormones to down-regulate pituitary function and to stimulate multiple oocyte production, *in vitro* maturation of oocytes, use of immature sperm, use of ICSI, *in vitro* culture of preimplanted embryos and cryopreservation of either gametes or embryos. Accordingly, scientists and researchers have attempted to determine whether ART conceived children carry epigenetic reprogramming defects (especially for imprinted genes).

It is now about ten years since a series of clinical studies raised concern about the association between ARTs and two imprinting syndromes, Beckwith – Wiedemann syndrome (BWS) (DeBaun *et al*., 2003; Gicquel *et al*., 2003; Maher *et al*., 2003; Halliday *et al*., 2004) and Angelman syndrome (AS) (Cox *et al*., 2002; Orstavik *et al*., 2003). However, the magnitude of this risk and the extent of imprinting disorder to which the risk applies remains unknown. Ten human imprinting syndromes have been identified but current evidence links ART with only three: Beckwith – Wiedemann syndrome, Angelman syndrome and the newly described maternal hypomethylation syndrome. There is increasing evidence of a link between ART and dramatic changes in methylation that lead to the same rare imprinting disorders, BWS and AS (Chang *et al*., 2005; Bowdin *et al*., 2007; Doornbos *et al*., 2007; Paoloni-Giacobino, 2007; Lim *et al*., 2009; Choufani *et al*., 2010) BWS has an estimated incidence of 1 in 13 700 live births in the general population (Choufani *et al*., 2010), but the risk is estimated to be 6 – 9 times higher

among ART offspring (Manipalviratn *et al*., 2009). While this would represent a dramatic increase in relative risk, the actual incidence of BWS in the ART population remains low (~1 in 4000 – 5500) (Manipalviratn *et al*., 2009). It is significant that the rate of methylation defects as the cause of BWS in the general population is ~60%, whereas this figure approaches 100% among the ART population (Manipalviratn *et al*., 2009). AS has an estimated incidence of 1 in 12 000 with imprinting abnormalities the aetiological factor in only 5% of cases (Steffenburg *et al*., 1996). Thus, the rate of imprinting abnormalities as a cause of AS in the general population is ~1 in 240 000. In contrast, 5 of the 7 reported cases of AS born after ART had an imprinting defect as the cause (Manipalviratn *et al*., 2009). However, it is worth noting that subfertility also appears to be associated with an increased risk of AS (Doornbos *et al*., 2007). There is emerging evidence for an increased relative risk of BWS and AS in the ART population. However, it is possible the ascertainment of BWS and AS among ART offspring may be higher due to under reporting in the general population, and more intense scrutiny and follow-up of ART-conceived children (Bowdin *et al*., 2007).

Recent technologic developments have facilitated the interrogation of many genes simultaneously, and these methods have been used to test for widespread epigenetic changes in phenotypically normal children conceived with ART. Katari et al. (Katari *et al*., 2009) , using an Illumina array platform, examined methylation differences in CpG sites of >700 genes in samples of placenta and cord blood obtained from *in vitro*– and *in vivo*–conceived children. Cord blood samples exhibited greater CpG-site methylation than placental samples in both ART and non-ART children, suggesting that CpG

methylation may play a role in determining cell type in humans. This lower level of methylation in the placenta is consistent with observations for imprinted genes in the placenta of mouse embryos exposed to *in vitro* culture (Doherty *et al*., 2000; Li *et al*., 2005). Additionally, methylation differences were observed between in *vivo*– and in *vitro*–conceived children in a number of genes known or suspected to be imprinted (Katari *et al*., 2009). ART conceived children displayed overall lower average methylation levels at specific CpG sites in placenta and higher methylation levels in cord blood (Katari *et al*., 2009). This may be related to the response of ‘‘outer’’ and ‘‘inner’’ cells of the blastocyst to *in vitro* culture, as in mouse studies (Mann *et al*., 2004). Overall, *in vitro* conception was associated with statistically significant differences in CpG methylation which resulted in gene expression differences at both imprinted and nonimprinted loci (Katari *et al*., 2009). Of note, several of the genes whose expression differed between the *in vitro* and *in vivo* groups are known to affect adipocyte development and differentiation, insulin signaling, and obesity (Katari *et al*., 2009).

1.3 A Confounding Factor – Underlying Parental Infertility or Sub-infertility

ART conceived children, as a group, are different from children conceived *in vivo* in their phenotype (low birth weight for example) or potential phenotype (long-term outcomes) and epigenotype (DNA methylation level for example). However, the source of these differences has been questioned since the study of this field started. Because ARTs are the treatment for infertility, these two factors coexist in almost all samples in the reported human studies. It is difficult to distinguish the role of ARTs or underlying

parental infertility in the phenotype and epigenotype outcomes of ART conceived children.

In animal models, in which fertile animals were used, underlying parental infertility or sub-infertility is not an issue. ART-associated errors in imprinting have been identified in animals. Several studies have shown that superovulation is associated with methylation changes in maternal and paternal alleles in both oocytes and blastocysts, especially for imprinted genes (Fauque *et al*., 2007; Fortier *et al*., 2008; Sato *et al*., 2007; Shi *et al*., 2002). Methylation errors have also been demonstrated after *in vitro* oocyte maturation (IVM) protocols with associated fetal overgrowth and pathologic endocrine changes in cattle (Hiendleder *et al*., 2006). Aberrant methylation has likewise been demonstrated in human oocytes after IVM (Borghol *et al*., 2006). Embryo transfer alone has been shown to result in imprinting errors in the yolk sac and placenta in mice (Rivera *et al*., 2008). Culture media have been implicated in imprinting alterations (Fauque *et al*., 2007; Doherty *et al*., 2000). Epigenetic alterations of the imprinted, maternally expressed H19, and IGF2R have been noted after IVF (Fauque *et al*., 2007; Li *et al*., 2005; de Waal *et al*., 2012), with a more profound loss of methylation in vitrified embryos (Wang *et al*., 2010). In addition to evidence of specific epigenetic alterations of a single gene, or genes, associated with ART procedures, several studies in animals have suggested that ART may have a more widespread or global impact on imprinting. For example, Zaitseva *et al*. (Zaitseva *et al*., 2007) found a higher overall methylation level in embryos developed *in vitro* than in those conceived *in vivo*. Loss of imprinting has been shown to continue into the postimplantation period, particularly in the placenta (Fortier *et*

al., 2008; Khosla *et al*., 2001; Mann *et al*., 2004). It has been suggested that mechanisms for maintenance of imprinted genes are not as resilient in trophoblastic tissues (Fortier *et al*., 2008; Lewis *et al*., 2004) compared with the developing embryo, raising the possibility that more direct contact with the environment may make the trophectoderm and placenta more vulnerable to imprinting errors (Fortier *et al*., 2008).

Nevertheless, results from studies in sperm suggest that underlying male infertility may contribute to epigenetic abnormalities in pregnancies through ARTs. Altered methylation patterns at imprinted loci have been reported from infertile men (both those with abnormal protamine expression and oligozoospermic patients), compared with fertile donors (Carrell & Hammoud, 2010; Hammoud *et al*., 2010; Jenkins & Carrell, 2011; Nanassy & Carrell, 2011a; Nanassy & Carrell, 2011b). However, there were differences in the statistical significance of the results between the infertile groups, suggesting that the risk of transmission of epigenetic alterations may differ according to the underlying cause of male infertility. Furthermore, the methylation imprinting marks of two oppositely imprinted genes, *H19* and *MEST/PEG1*, were studied in human testicular spermatozoa from patients with azoospermia of different aetiologies. It was found that spermatozoa from men with abnormal spermatogenesis carry methylation defects in the *H19* imprinted gene, which also affect the CCCTC-binding factor-binding site, further supporting an association between the occurrence of imprinting errors and disruptive spermatogenesis (Marques *et al*., 2010). However, it should be noted that these studies do not provide a causal link for epigenetic inheritance of imprinting diseases.

1.4 A Reasonable Approach for Isolating the Confounding Factor

Although results from animal studies suggest that ARTs are associated with altered epigenetic modifications, to distinguish the role of ARTs or underlying parental infertility in the epigenotype outcomes of ART conceived children in human studies is still essential. If some forms of infertility are also associated with altered epigenetic modifications, then the uncoupling of any particular ART procedure from any particular epigenetic alteration becomes problematic. In order to determine what fraction of epigenotype (DNA methylation) and gene expression difference is attributable to the ART procedure and what fraction is attributable to underlying parental infertility, two subgroups of ART conceived children are likely to be useful:

The first group consists of children born to mothers who are infertile as a result of a physical barrier to fertilization. Females who have fallopian tube occlusion are unable to conceive naturally because sperm cannot reach the ovulated oocyte and oocytes cannot reach the uterus. Despite this infertility-causing anatomical condition, there is no reason, *a priori,* to suspect that the oocytes of these females are any less capable of fertilization and development to term than the oocytes of any woman selected at random. In fact, a significant fraction of women with tubal occlusion are known to be fertile because they have already given birth, often more than once, and have undergone voluntary tubal ligation as a sterilization procedure. Subsequent treatment of such women for infertility involves all of the standard ART procedures in the absence of any likelihood that there are other causes of infertility that might be associated with abnormal epigenetic modifications.

The second group consists of children conceived with the aid of donor oocytes. The use of donated eggs can avoid the drastic decline in IVF success with age. In general, egg donors are young, healthy women and birth rates using their eggs vary little as a function of the age of the recipient. For the majority of donor-egg cycles, live birth rates are consistently above 50%, highlighting the effect of age on ovarian function and egg quality, whereas endometrial receptivity seems to be unaffected (**Figure. 1**). As a result, the epigenetic reprogramming of children conceived with donor eggs is unlikely to be influenced by the underlying infertility issues of the recipient.

Because there is no reason to suspect underlying epigenetic problems in the oocytes that resulted in either group of children, they may serve as one form of internal control for the effect of ART *versus* the effect of infertility, *per se.* In my study, quasi-transcriptome-wide DNA methylation profiles were compared between children conceived *in vivo* and *in vitro*. The most differently methylated genes in the quasi-transcriptome-wide DNA methylation profiles were then compared in a larger number of samples between children conceived *in vivo* and *in vitro*. The methylation levels of these gene were also compared between the internal control group (Tubal and donor egg group) and Control group (children conceived *in vivo*), or between ART conceived children of parent(s) who have idiopathic infertility (Infertility group) and Control group.

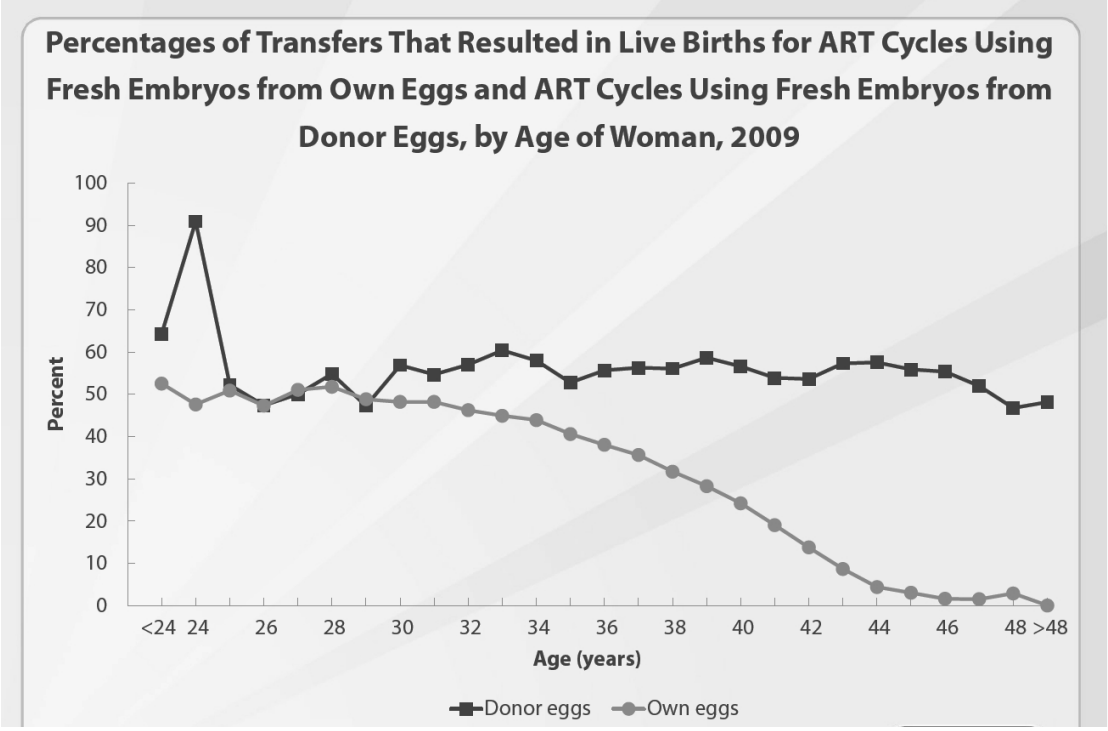


Figure 1. Percentages of transfers that resulted in live births for ART cycles using fresh embryos from own eggs and ART cycles using fresh embryos from donor eggs, by age of woman, 2009. Cited from Centers for Disease Control and Prevention.

1.5 Hypothesis

Animal models suggest that a number of ART procedures (ovarian hyperstimulation (Fauque *et al*., 2007; Fortier *et al*., 2008; Sato *et al*., 2007; Shi *et al*., 2002), embryo culture media (Fauque *et al*., 2007; Doherty *et al*., 2000) and embryo transfer (Rivera *et al*., 2008)) result in altered epigenetic modificantions in the developing embryo. Considering the increasing number of studies of epidemiological association of human ART with adverse phenotypic outcomes, I hypothesize that the treatment-induced environment plays a role in the ART-associated DNA methylation and gene expression

differences. I also hypothesize that the parental underlying infertility rarely plays a role in the majority of DNA methylation and gene expression differences in children conceived *in vitro*.

CHAPTER 2

MATERIALS AND METHODS

2.1 Ethics Statement and Samples

Written, informed consent was obtained in advance from the mother of each newborn (University of Pennsylvania I.R.B. approved protocol no. 804530).

We have provided the demographic data summary showing maternal age, gestational age, and birth weight (at delivery), fetal sex, mode of birth delivery and race for all the individuals and ART procedures' information for ART children in the pyrosequencing assays in **Table 1**.

2.2 Sample Collection and Processing

Placenta samples were collected from each newborn. Tissue samples were collected and processed within five hours of delivery (Fajardy *et al*., 2009). Placental tissue (1.5-2.5 cm^3) was excised from the fetal surface of the placenta, directly behind the cord insertion site. The sample was rinsed extensively with sterile saline solution to minimize maternal blood contamination. Half of the tissue sample was sectioned into smaller pieces (0.5 cm^3), transferred to a 15 ml Falcon tube and immersed in RNALater RNA Stabilization Reagent (Ambion, USA), following the manufacturers guidelines. The

remaining tissue was transferred to a 15 ml Falcon tube for DNA extraction. All tissue DNA and RNA samples were initially stored at 4°C, and nucleic acid extractions were performed within 2-4 days of collection. Approximately 4-5 mg of tissue was used to extract genomic DNA and RNA. The remaining tissue was stored at -80°C.

2.3 DNA and RNA Isolation

Placenta genomic DNA was extracted using the PureLink™ Genomic DNA Mini Kit (Invitrogen Corporation, USA), following the manufacturer's guidelines. The isolated DNA was redissolved in TrisCl (10 mM, pH 8.0) and stored at -80°C until further use. Placenta total cellular RNA was extracted using TRIzol® Reagent (Invitrogen Corporation, USA), following the manufacturer's guidelines. The isolated RNA was redissolved in Milli-Q water and stored at -80°C until further use. Isolated DNA and RNA were analyzed by agarose gel electrophoresis and quantified using a NanoDrop ND1000 spectrophotometer(Thermo Fisher Scientific, USA).

2.4 Bisulfite Conversion

Unmethylated cytosine in genomic DNA (0.5-1 µg) was converted to uracil by treatment with sodium bisulfate using the EZ DNA Methylation Kit™ (Zymo Research Corp., USA), following the manufacturer's guidelines. The bisulfite-converted DNA was redissolved in 20 µl TrisCl (10 mM, pH 8.0) buffer and stored at -20°C until further use. All converted DNA samples were used within three months of the bisulfite conversion.

2.5 Infinium Methylation Assay

Site-specific CpG methylation was analyzed in the bisulfate-converted placenta DNA template for 48 individuals using Illumina's HumanMethylation27 Bead-Chip array, following the manufacturer's guidelines (Illumina, USA). The array contained probes for 27,578 CpG dinucleotides located predominately in the proximal promoter regions of over 14,000 consensus coding sequences (CCDS) genes throughout the genome. In addition, the array included 110 miRNA promoters and imprinted genes. Four bead chips were used, and these were processed simultaneously. Briefly, 1 μg of bisulfite converted DNA was isothermally amplified at 37°C overnight. The amplified DNA product was fragmented by an endpoint enzymatic process and the fragmented DNA was precipitated, redissolved and applied to the array and hybridized overnight. A single-base extension reaction was carried out and the fluorescently stained chip was imaged using the Illumina Bead Array Reader and the Bead Scan Software (Illumina, USA). The assay contained controls to assess the following parameters: staining, hybridization, target removal, extension, bisulfite conversion, G/T mismatch, as well as negative controls and non-polymorphic controls. The experiments passed all quality controls successfully (Please see Illumina's "GenomeStudio Methylation Module User Guide" manual for greater details regarding the criteria used to assess the controls). Illumina's GenomeStudio Methylation Module v1.0 was used to analyze the data to assign site-specific DNA methylation beta-values to each CpG site. The extent of methylation (beta-value) at each CpG site was determined by comparing the proportion of signal from methylated and unmethylated alleles in the DNA sample.

2.6 Pyrosequencing Methylation Assays

Site-specific CpG methylation was analyzed in the bisulfate converted placenta DNA template for AQPEP (FLJ90650), CCDC62, CRTAM, DLX5, FLJ10260, GAS2, GRIN2C, H19, HSPB3, IFI16, IL5, LYST, MEST, NDN, PCDHGB7, PCK2, PTPN20B, SNRPN, TCF2, and TTR using custom designed bisulfite pyrosequencing assays (Qiagen, USA). The assays were designed to target the same CpGs interrogated by the Infinium arrays. Briefly, 500 ng bisulfite converted DNA was used for generating PCR amplified templates for pyrosequencing.

The primer sequences are following:

AQPEP forward (5' GTAGTTTTGGAAGAGGTTATGT 3'), reverse (5' ATCTTCCCCTCCCCTCAA 3') and sequencing (5' TTTGGAAGAGGTTATGTA 3'); CCDC62 forward (5' ATTGGGGGTTTTTAAGTGGTTTAG 3'), reverse (5' AACAACCTTTATTAACTTCTTCATTCTCTA 3') and sequencing (5' ATTCTTTCAAACCTTCCA 3'); CRTAM forward (5' ATGTTTAAATTATGTATTTGAGGGATGTT 3'), reverse (5' AAAACCCACAAAAACACACACT 3') and sequencing (5' ATGTATTTGAGGGATGTTA 3'); DLX5 forward (5' GGAATTGATTGAGTTGGTTGTATTTG 3'), reverse (5' CTCAACCACCACCCTCATA 3') and sequencing (5' TATTTGTGTATTAGGATGTAGAGT 3'); FLJ10260 forward (5' TGGTTATTTGGTGTGTATATAATGTAAA 3'), reverse (5'

ATAACTTCACTTCAACCTCCCATTA 3') and sequencing (5'

AGATTAGTTTGAATGGTTGTA 3'); GAS2 forward (5'

AGAGTTTGGAGTAAAGAAGTATAGG 3'), reverse (5'

ACAAAAACACCCATTTAACATCC 3') and sequencing (5'

TTGTGAATTTTAGGGGAT 3'); GRIN2C forward (5'

GGGGTTAGGGATTGAATTAAGA 3'), reverse (5' CCCTAATACCCACCCCATTC

3') and sequencing (5' GGTTAGGGATTGAATTAAGATA 3'); H19 forward (5'

AAGAAGGGGGAGTTAGGTATTTA 3'), reverse (5'

AAACCTCCTTCTTTCAAAAAACTAA 3') and sequencing (5'

GTGGTAGTTGGTTGGA 3'); HSPB3 forward (5'

GGGTGAAGTTTTTTGAGATAAAAT 3'), reverse (5'

AAAACAAATCCCACTTTCAAATC 3') and sequencing (5'

GTGTTTATTTATTTTGGT 3'); IFI16 forward (5'

TGTTGAGTAGAGGATTAAATAGATTT 3'), reverse (5'

ACTATCCTCTTAACCATAACTAAATTACT 3') and sequencing (5'

TTAGTGTATTTTTTAGGAT 3'); IL5 forward (5'

GGTTTGAATATATATGATGGAGGTGTATGT 3'), reverse (5'

TATATCTACCTCCCAACTACCTCTACT 3') and sequencing (5'

ATGTTGTAGTTTAGAAGTTTGAT 3'); LYST forward (5'

AGTTAAAAGGTTATTGGGATGGT 3'), reverse (5'

AACTTAATTTACCAAATTCCCCATATAA 3') and sequencing (5'

ATTGGGATGGTTTTTTAGA 3'); MEST cg02490034 forward (5'

TTGGTTTTTGTTGAGTATGTGATG 3'), reverse (5'

ATAAAAACCAAAAAAATAACATTTTAATAA 3') and sequencing (5' AAATTAGGGGAAGGG 3'); MEST cg09059945 forward (5' GAAATTAGGGGAAGGGTTGAAA 3'), reverse (5' CCTTCTCCCTACCAAAC 3') and sequencing (5' TATTTTATATTTTTTGTAATAGGTGG 3'); NDN forward (5' GGGTTTAGAGGAGGG 3'), reverse (5' CAAAAACCCTACCCTTACCAA 3') and sequencing (5' AGATTTTTATTTTGTTTTGATATG 3'); PCDHGB7 cg23563234 forward (5' GGTAATTTGGTGTGTTGGGTAAG 3'), reverse (5' ATCCCTCAACCTCTAACCTA 3') and sequencing (5' GTTGGGTAAGGTTTG 3'); PCDHGB7 cg14011639 forward (5' GGTGTGTTGGGTAAGGTTTG 3'), reverse (5' CCCTCAACCTCTAACCTAAAAT 3') and sequencing (5' CAACCTCTAACCTAAAATTCCCT 3'); PCK2 forward (5' AAGAGTGGATTTAGTTTTTTAATGG 3'), reverse (5' AAACCTAATAAAACTAACACTAACTT 3') and sequencing (5' AGGGAGTTGGTTTGT 3'); PTPN20B forward (5' GTTTGGGAGAGGGGGATT 3'), reverse (5' CCCCAACCACCTTTCCTCA 3') and sequencing (5' CAAAACACTAAAAACCCAT 3'); SNRPN cg25978208 forward (5' TGAGGTTGGAGGATTTAGGTTGTG 3'), reverse (5' ATATTTCCCTACACATCACTCT 3') and sequencing (5' GATTTAGGTTGTGAGGTA 3'); SNRPN cg26033681 forward (5' TGGTTGAAAAGGAGAGGGTTAGA 3'), reverse (5' ATTAATTCCTTATACACCTTCCACAA 3') and sequencing (5' AGGTTTAAAGAAGTTATGATAG 3'); TCF2 forward (5' ATTTAATTTTTGGATTTGTTAAGTTAGTGT 3'), reverse (5'

CCCTTCCTAAATAATCAATTTCTCTT 3') and sequencing (5' ATTTGTTAAGTTAGTGTTTTGTA 3'); TTR forward (5' GTTTTGGTTTTTTAAAGTGTTGGGATTA 3'), reverse (5' CAACTATCAACCTTATTCCTACATACCC 3') and sequencing (5' TTTTTTAAAGTGTTGGGATTAT 3'). For CCDC62, PCDHGB7 cg14011639 and PTPN20B the 5'-biotin modification is on the forward primer, whereas for AQPEP, CRTAM, DLX5, FLJ10260, GAS2, GRIN2C, H19, HSPB3, IFI16, IL5, LYST, MEST, NDN, PCDHGB7 cg23563234, PCK2, SNRPN, TCF2, and TTRthe 5'-biotin modification is on the reverse primer.

The PCR reactions (25 μl) contained the following: 25 ng of bisulfite DNA, 0.75 U HotStar Taq Polymerase (Qiagen, USA), 1× PCR buffer, 1.5 mM MgCl2, 200 μM of each dNTP, and 140 pmol of each forward and reverse primer. Recommended PCR cycling conditions were: 95°C for 15 min; 45 cycles (95°C for 30 s; 60°C for 30 s; 72°C for 30 s); 72°C for 5 min. The biotinylated PCR product (10 μl) was used for each assay with 1× the respective sequencing primer. Pyrosequencing was done using the PSQ96HS system using the PyroMark Gold Reagent Kit, following the manufacturer's guidelines (Qiagen, USA). Methylation was quantified using PyroMark Q-CpG Software (Qiagen, USA), which calculates the ratio of converted C's (T's) to unconverted C's at each CpG and expresses this as a percentage methylation.

2.7 Digestion of Genomic DNA with *HpyCH4IV*

Genomic DNA (500 ng) from samples was digested one hour at 37°C with 10 units *AluI* (It is a four-cutter that does not have a recognition site between the primers used for this assay.). After the first digestion, the enzyme *AluI* was heat inactivated for 20 minutes at 65°C.The *AluI*-digested DNA was then digested with an excess of a methyl-sensitive restriction endonuclease *HpyCH4IV* (10 units) or same volume of Milli-Q water as the non-methyl-sensitive restriction endonuclease control to measure the total amount DNA molecules. All prepared samples were incubated at 37˚C for 16 hours, following by heat inactivation at 65˚C for 20 minutes.

2.8 Quantitative Real-time PCR for Methylation Status

Quantitative Real-time PCR for methylation status was performed using PerfeCTa® SYBR® Green FastMix®, ROXTM on a Applied Biosystems StepOnePlusTM(Applied Biosystems, USA). The primers for promoter region of GRB10 were designed by Primer3 (version 0.4.0) on line. The primers to bracket HpyCH4IV cleavage site in the promoter region of GRB10 were (5’ GGTGTCTTTCCTCATTGTTCTTTT 3’) (forward) and (5’TTCTTTACCACCTTTCTCTGGAGT 3’)(reverse), yielding a PCR product of 259 bp. Melting curves were obtained following thermal cycles Non-methyl-sensitive restriction endonuclease controls for each sample were run in the same plate with enzyme-treated DNA samples in 4 replicates.

2.9 Quantitative Real Time RT-PCR

First-strand cDNA was obtained using Superscript™ III Reverse Transcriptase (RT) (Invitrogen Corporation, USA). To produce cDNA from total RNA, a mixture containing 1 µg extracted total RNA, 0.5 µg oligo (dT)18 primer and 1 µl dNTP mix (10 mM each base) in final 13 µl of solution was heated to 65°C for 5 min, cooled down on ice for 2 min, and then added to a 7 µl of reaction mixture (4 µl Superscript™ III RT buffer (10×), 1 µl DTT (0.1 M), 1 µl RNaseOUT™ Recombinant RNase inhibitor (40 U/µl; Invitrogen Corporation, USA) and 1 µl Superscript™ III M-MLV reverse transcriptase (200 U/µl), for reverse transcription at 50°C for 60 min. Reactions were terminated at 70°C for 15 min. RT products were stored at -20°C until use. Quantitative real time RT-PCR assays were carried out using a Applied Biosystems StepOnePlus™ (Applied Biosystems, USA). All probes spanned exon/intron boundaries to prevent genomic DNA amplification.

Steady state mRNA levels of AQPEP (laeverin), CCDC62, GRIN2C, MEST, NDN, PTPN20B and housekeeping gene TBP were measured using gene-specific TaqMan probes (Applied Biosystems, USA, product numbers: Hs01060572_m1, Hs00261486_m1, Hs0106626_m1, Hs01040913_g1, Hs00267349_s1, Hs00944181_m1 and Hs00920497_m1, respectively). Taqman PCR reactions were performed by mixing 1 µl of cDNA (50 ng/µl) with 19 µl of reaction mixture (10 µl Taqman Master Mix (2×), 1 µl Taqman primer (20×), and 8 µl nuclease free dH2O) and amplified under the following conditions: 50°C for 2 min, 95°C for 10 min, followed by 45 cycles of 95°C for 15 s and 60°C for 60 s.

Table 1. Demographic data summary for the individuals used in the pyrosequencing assays

	Control (n=49)	Infertility (n=45)	Tubal +donor egg (n=45)	Tubal only (n=21)
Maternal age (Avg±SD years)	34.5 ±5.0	35.8±3.9	38.2±5.9	34.4 ±2.9
Gestational age (Avg±SD weeks)	39.3±1.0	39.1±1.4	38.0±2.3	38.0±2.5
Birth weight (Avg±SD g)	3639.6±459.1	3467.2±529.3	3132.4±684.7	3136.7±756.3
Males	25	23	19	9
Females	24	22	26	12
C-sections	28	19	28	12
SVD	21	26	17	9
multiple gestation	2	13	14	7
Parents' Race				
Asian/ Asian	12	3	3	3
black /black	1	1	1	1
Hispanic/White	1	1	2	2
white/ Asian	1	2	0	0
white/white	33	34	35	13
White - Hispanic /white	1	0	0	0
white /other	0	2	1	0
Asian /Asian-White	0	0	2	2
Hispanic / Hispanic	0	0	1	0
white/black	0	2	0	0
Day 5 transfers	-	6	8	5
Day 3 transfers	-	39	37	16
Conventianl IVF	-	36	41	19
ICSI	-	9	4	2
FETs(frozen embryo transfer)	-	5	9	3
donor sperm	-	3	0	0
donor egg	-	0	24	0

CHAPTER 3

RESULTS

3.1 Selection of Candidate Genes from Infinium Methylation Array

High quality array data sets were generated for children conceived *in vivo* (Control group) and children conceived *in vitro* (ART group) from the Illumina's HumanMethylation27 Bead-Chip array. The array panel contained probes for 27,578 CpG dinucleotides located predominately in the proximal promoter regions of over 14,000 consensus coding sequences (CCDS) genes throughout the genome. In addition, the array included 110 miRNA promoters and imprinted genes. On average, two assays were selected per CCDS gene and from 3-20 CpG sites for imprinted genes (Illumina, USA). The fraction of molecules methylated (beta-value) at each CpG site was determined by comparing the proportion of total signal from methylated and unmethylated molecules in the DNA sample. A beta value of "0" represents a completely unmethylated CpG site, and a beta-value of "1" represents a completely methylated CpG site. We observed that more than 95% genes in the array had two CpG sites measured, so if both CpG sites for a specific gene are differently methylated in the same direction between groups, the possibility of this gene being differently methylated between groups by chance is much lower. I identified candidate genes whose regulation may be altered in children conceived *in vitro* by applying biological criteria – *bona fide* candidates were required to show differential methylation of two or more CpG sites in the same gene.

This criterion has been followed in several published studies from my lab (Katari *et al*., 2009; Turan *et al*., 2010a; Turan *et al*., 2010b), and the results from these studies has been validated.

To validate the data from the Infinium methylation array by pyrosequencing technology, average CpG site methylation levels (Infinium beta values) of ART and Control samples were compared (Student's unpaired T-test (two tails) - ART (n=24) vs. Control (n=24)). Subsequently, genes with at least 2 CpG sites significantly differently methylated (p<=0.05) or strongly suggestive of differential methylation (PCK2, p=0.059, 0.069) were selected. These candidates were then

1) Sorted by magnitude of mean difference; genes with mean difference of beta value for at least 2 CpG sites >0.07 or <-0.07 were selected. Then, genes with mean beta-values appropriate to validate by pyrosequencing (0.7>beta-val>0.1) were selected. Under these criteria, nine genes (*GRIN2C, PCK2, PCDHGB7, PTPN20B, MEST, TCF2, HSPB3, FLJ10260, NDN*) were selected. (Additional genes fulfilling these criteria were available, however, high quality pyrosequencing assays could not be designed or the assays failed), or;
2) Sorted by magnitude of mean difference; genes with mean difference of beta value for one CpG site >0.07 or <-0.07 were selected. Last, genes with mean of beta-value appropriate to validate by pyrosequencing (0.7>beta-val>0.1) were selected. Under these criteria, Nine genes

(*DLX5, CRTAM, TTR, SNRPN, IL5, LYST, IL5, IFI16, AQPEP)* were selected. Although, there is only one CpG site with mean difference of beta value >0.07 or <-0.07 for each selected gene, but at least 2 CpG sites were significantly differently methylated (p<=0.05) for each selected gene (mean difference of beta value for the other CpG site is not >0.07 or <-0.07). (Additional genes fulfilling these criteria were available, however, high quality pyrosequencing assays could not be designed or the assays failed).

The imprinted gene *H19* was also selected because 7 of the 16 CpG sites on the Infinium array were significantly hypermethylated in ART group, with the largest beta value mean difference between two groups ART (n=24) vs. Control (n=24) being 0.067. Some of the genes that had the largest variance in beta values between the individuals (maximum beta-minimum beta >0.4) showed good correlations between the Infinium array and pyrosequencing data (for example, *GRIN2C* R^2>0.75 (**Table 2.**)). *CCDC62* was selected 1) because of the large variance in beta values between the individuals (maximum beta-minimum beta>0.4) and 2) the mean difference (-0.079 and -0.062) between the ART and Control groups for both CpG sites tested in the array. However, the difference between the groups was not significant (P=0.117 and 0.135), but this may be because there were only 24 samples per group on the array. As a result, we selected 20 genes in total (**Table 2.**).

The growth factor receptor adaptor protein (*GRB10)* was also selected for investigation. *GRB10* interacts with insulin receptors and insulin-like growth-factor receptors. Overexpression of some isoforms of the encoded protein inhibits tyrosine kinase activity and results in growth suppression. *GRB10* is imprinted in a highly tissue-specific manner, with expression observed from the paternal allele in the brain, and from the maternal allele in the placental trophoblasts. *GRB10* was hypomethylated at several CpG sites in both placenta and cord blood between *in vitro* and *in vivo* conceived children (Katari *et al*., 2009). *GRB10* has been identified as one of the 23 genes whose methylation levels explain 70-87% of the variance in birth weight and it is associated with growth phenotypes in human or mouse models. In addition, *GRB10* is correlated with transcriptional control of at least seven genes reported to be involved in fetal or placental growth (Turan *et al*., 2012).

Thus, it is interesting to study the DNA methylation levels of CpG sites in *GRB10* in ART and other subgroups and the source of the DNA methylation level differences. Basically, there are two CpG islands in gene *GRB10*, CpG Island I is almost unmethylated in placenta and CpG Island II is hypothesized to be the imprinting control region of *GRB10* (Monk *et al*., 2009). The average DNA methylation level of CpG Island II is about 50%, because the maternal allele is methylated and the paternal allele is unmethylated.

The data of the Infinium array showed that 8 of 12 CpG sites of *GRB10* were significantly hypomethylated in children conceived by ART. Among these CpG sites, six are from CpG Island I, the other two are CpG Island II (**Table 3.**).

Table 2. Comparison of methylation levels between children conceived *in vivo* (Control n=24) and *in vitro* (ART n=24) of candidate CpG sites in Infinium array and pyrosequencing

			ART (n=24) vs. Control (n=24)			
			Infinium		Pyrosequencing	
CpG ID	Gene	$R^{2(1)}$	MD(2)	*P* (3)	MD	*P* (4)
cg09169283	**GRIN2C**	0.7517	-0.0966	***0.0036***	-0.0627	***0.0051***
cg03251655	**FLJ10260**	0.6116	0.1133	***0.0003***	0.0943	***0.0106***
cg25978208	**SNRPN**	0.7052	0.0946	***0.0057***	0.0791	***0.0116***
cg09059945	**MEST**	0.8064	0.0903	***0.0050***	0.0658	***0.0129***
cg02490034	**MEST**	0.7595	0.0689	***0.0286***	0.0629	***0.0161***
cg12532169	**NDN**	0.7225	0.1184	***0.0003***	0.0896	***0.0133***
cg13828758	**NDN**	0.5501	0.1119	***0.0002***	0.0638	***0.0178***
cg12788467	**TCF2**	0.4479	0.0771	***0.0066***	0.0301	***0.0309***
cg26081812	**IL5**	0.0037	0.0968	***<10^{-5}***	0.0304	***0.0348***
cg04008843	**TTR**	0.1392	0.0832	***<10^{-6}***	0.0179	***0.0428***
cg25044651	**FLJ90650**	0.4328	0.1009	0.1294	0.0525	0.0541
cg03547797	**GAS2**	0.3999	0.0775	***0.0030***	0.0432	0.0600
cg26402828	**PCK2**	0.6563	-0.0966	0.0688	-0.0932	0.0621
cg26033681	**SNRPN**	0.0462	-0.0546	***0.0005***	0.0088	0.1292
cg16192575	**PTPN20B**	0.1574	0.0751	***0.0355***	0.0426	0.1697
cg08775774	**CCDC62**	0.6947	-0.0617	0.1351	-0.0261	0.1729
cg10977115	**CRTAM**	0.3345	0.0808	***0.0007***	0.0173	0.1934
cg23563234	**PCDHGB7**	0.6408	0.1071	***0.0174***	0.0387	0.2068
cg14011639	**PCDHGB7**	0.4603	0.0703	***0.0334***	0.0244	0.2296
cg02829654	**LYST**	0.0815	0.0918	***0.0007***	-0.0078	0.2318
cg12041387	**DLX5**	0.1749	0.0533	***0.0068***	0.0143	0.2732
cg07342901	**H19**	0.0658	0.0671	***0.0003***	0.0037	0.3018
cg21406461	**IFI16**	0.2267	0.0971	***0.0007***	0.0080	0.3649
cg11391732	**HSPB3**	0.1485	0.1184	***0.0002***	-0.0007	0.4898

(1) correlation between Infinium Methylation Array data and pyrosequencing data for the same 48 samples

(2) mean difference of beta-values between groups (pyrosequencing data were also converted to represent beta-values)

(3) Student's unpaired t-Test, two-tailed, P<0.05 are in bold

(4) Student's unpaired t-Test, one-tailed , P<0.05 are in bold

Table 3. Comparison of methylation levels between children conceived *in vivo* (Control n=24) and *in vitro* (ART n=24) of *GRB10* CpG sites in Infinium array

			ART (n=24) vs. Control (n=24)	
CpG ID	Gene	CpG island	MD(1)	*P*(2)
cg15774495	**GRB10**	CpG Island I	-0.0100	***<10^{-8}***
cg24183958	**GRB10**	CpG Island I	-0.0117	***<10^{-7}***
cg20651681	**GRB10**	CpG Island I	-0.0111	***<10^{-6}***
cg06386517	**GRB10**	CpG Island I	-0.0039	***<10^{-5}***
cg06790324	**GRB10**	CpG Island I	-0.0028	***0.0003***
cg24302095	**GRB10**	CpG Island I	-0.0059	***0.0050***
cg08835688	**GRB10**	CpG Island II	-0.0319	***0.0060***
cg12903171	**GRB10**	CpG Island II	-0.0288	***0.0186***
cg25915982	**GRB10**	CpG Island II	0.0136	0.1817
cg03104936	**GRB10**	CpG Island II	-0.0300	0.2486
cg09150232	**GRB10**	CpG Island II	0.0305	0.3068
cg26163537	**GRB10**	CpG Island I	-0.0006	0.9011

(1) mean difference of beta-values between groups

(2) Student's unpaired t-Test, two-tailed, P<0.05 are in bold

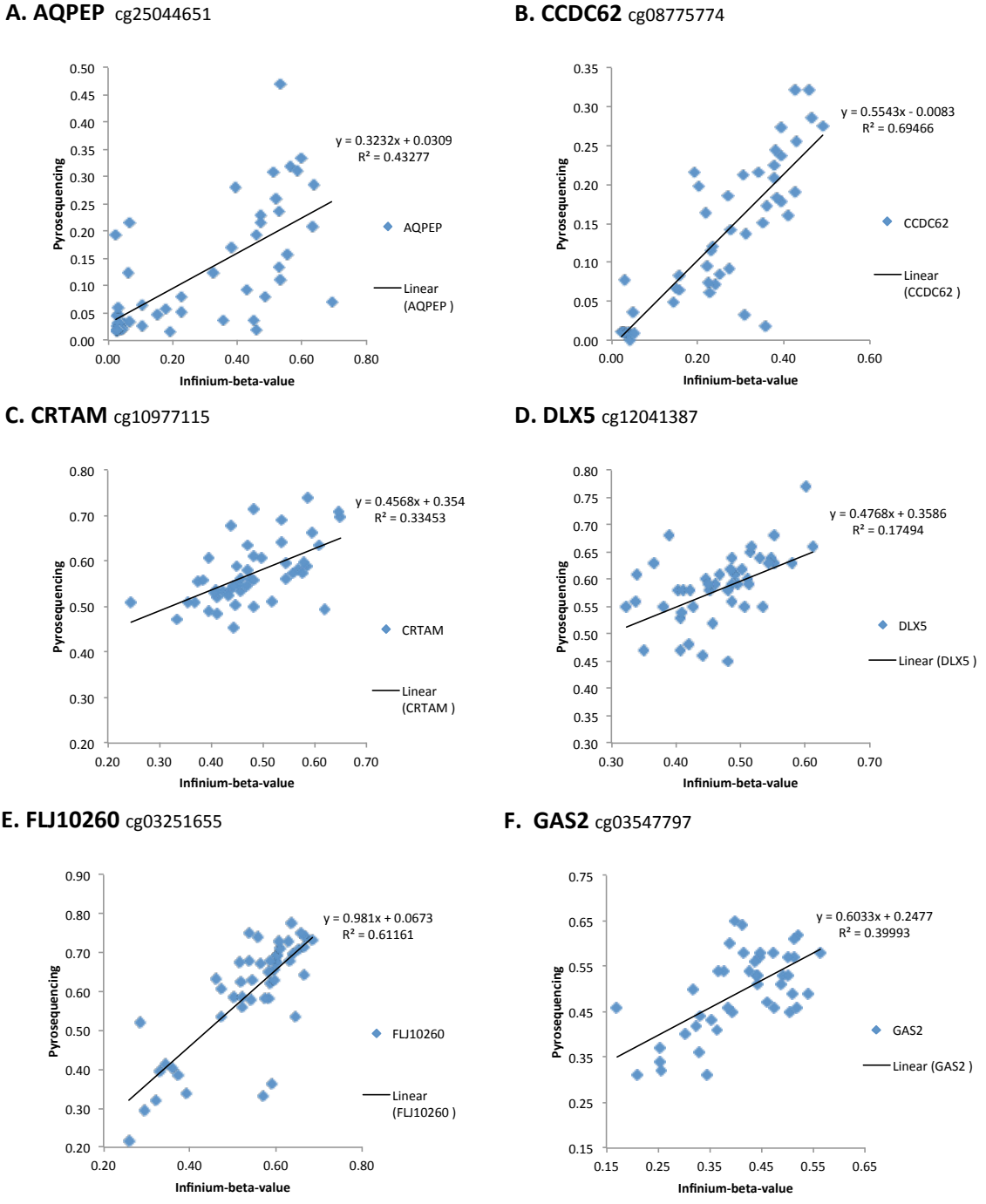
3.2 Validation of Infinium Methylation Array Differences by Pyrosequencing Using the Same 48 Samples

To validate the differences found on the Infinium Methylation Array between ART children and children conceived *in vivo* in placenta, bisulfite pyrosequencing assays for the selected 20 candidate genes were performed on the same 48 samples used in the Infinium Methylation Array. The pyrosequencing data were converted to the form of beta-value, which is convenient for comparing the results between two technologies of measuring DNA methylation level of the identical CpG sites. Among the 24 pyrosequencing assays for 20 candidate genes, the data of 9 assays for 8 genes showed strong correlation with the Infinium methylation array data (*GRIN2C, FLJ10260, SNRPN, MEST, NDN, PCK2, CCDC62, PCDHGB7* R^2>0.50, **Table 2.**, **Figure 2.**); the data of 8 assays for 8 genes showed a weaker correlation with the Infinium methylation array data (*TCF2, FLJ90650, GAS2, PTPN20B, CRTAM, DLX5, IFI16, PCDHGB7* 0.15<R^2<0.50, **Table 2.**, **Figure 2.**); the data of 4 assays for 4 genes showed poor correlation with the Infinium methylation array data (*IL5, SNRPN, LYST, H19* R^2<0.10, **Table 2.**, **Figure 2.**). The data for 10 CpG sites of 8 genes (*GRIN2C, FLJ10260, SNRPN, MEST, NDN, TCF2, IL5, TTR*) were validated by pyrosequencing and data for both assays were significantly different in the same direction between ART children and children conceived *in vivo*, hyper- or hypomethlated. However, the results from pyrosequencing show less mean difference of beta-value between the ART children and

the children conceived *in vivo*, and the mean differences of beta-value for gene *TCF2, IL5, TTR* using pyrosequencing were much smaller than the differences in Infinium array (**Table 2**.). Data for another three genes (*FLJ90650, GAS2, PCK2*) were suggestive of being significantly different between ART children and children conceived *in vivo* ($p<0.065$, **Table 2**.). Pyrosequencing results for the other 11 CpG sites did not show statistically significant differences, including the results for gene *CCDC62* as expected.

Because of the limitations of pyrosequencing technology, we could not reliably measure DNA methylation levels less than 5 %. Unfortunately, we could not design any pyrosequencing assays for the other two targets in CpG Island II for *GRB10*.

As a result, we utilized another method –methylation sensitive restriction enzyme digestion and real-time PCR-to measure the DNA methylation levels of CpG sites in CpG Island II. We used the methylation sensitive restriction enzyme HpyCH4IV to digest purified DNA. Unmethylated sites can be digested and methylated sites cannot be digested. By using the designed pair of primers to amplify the region around the enzyme digestion site, we can measure the different amount of DNA molecules depending on the proportion of methylated and unmethylated template by comparing HpyCH4IV digested template with undigested template. We did the same comparisons as for the pyrosequencing data, when we using the same samples in the Infinium array (ART (n=24) vs. Control (n=24)), this CpG site was significantly hypomethylated (**Figure 3.**) in ART group. Interestingly, this site is the first CpG site in the transcribed region.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Pyrosequencing
Infinium-beta-value
B. CCDC62 cg08775774
y = 0.5543x - 0.0083
R² = 0.69466
CCDC62
Linear (CCDC62)
Pyrosequencing
Infinium-beta-value
C. CRTAM cg10977115
y = 0.4568x + 0.354
R² = 0.33453
CRTAM
Linear (CRTAM)
Pyrosequencing
Infinium-beta-value
D. DLX5 cg12041387
y = 0.4768x + 0.3586
R² = 0.17494
DLX5
Linear (DLX5)
Pyrosequencing
Infinium-beta-value
E. FLJ10260 cg03251655
y = 0.981x + 0.0673
R² = 0.61161
FLJ10260
Linear (FLJ10260)
Pyrosequencing
Infinium-beta-value
F. GAS2 cg03547797
y = 0.6033x + 0.2477
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GAS2
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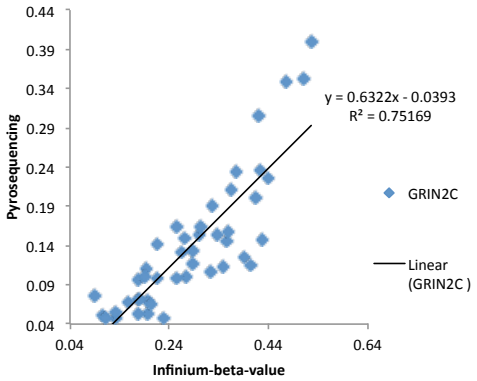
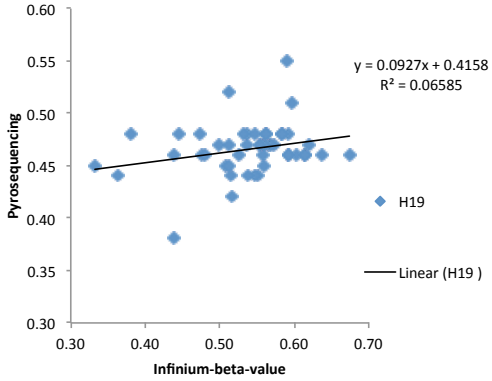
Pyrosequencing
Infinium-beta-value

Figure 2.

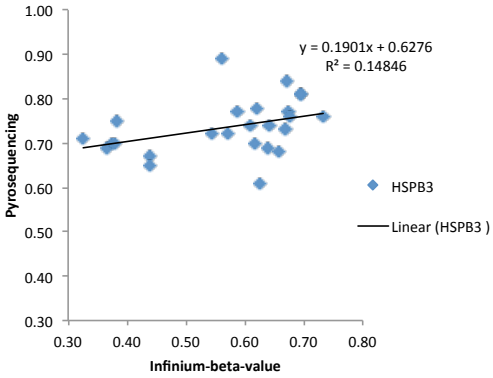
G. GRIN2C cg09169283



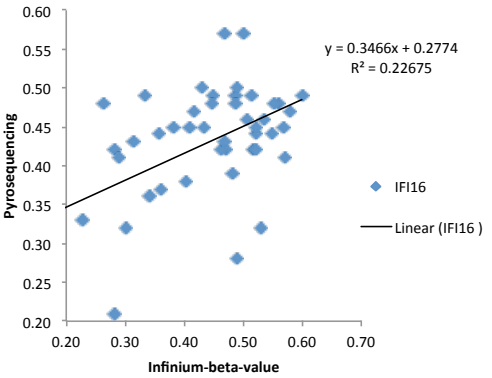
H. H19 cg07342901



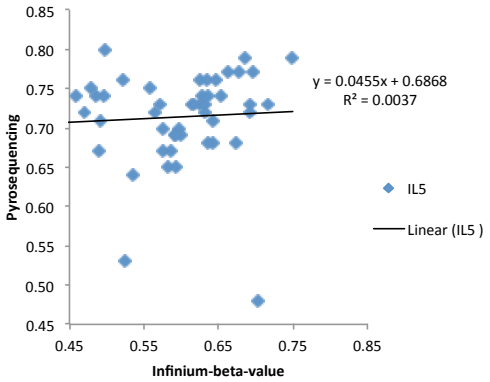
I. HSPB3 cg11391732



J. IFI16 cg21406461



K. IL5 cg26081812



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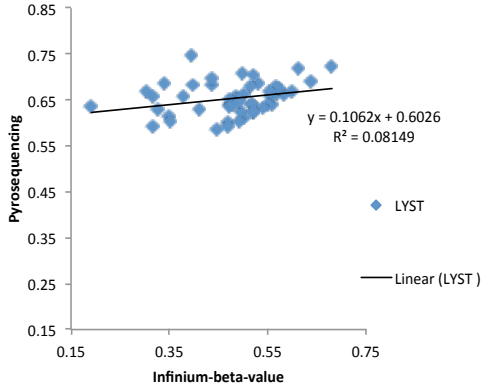
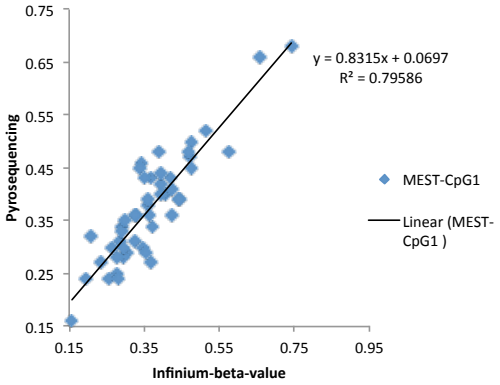
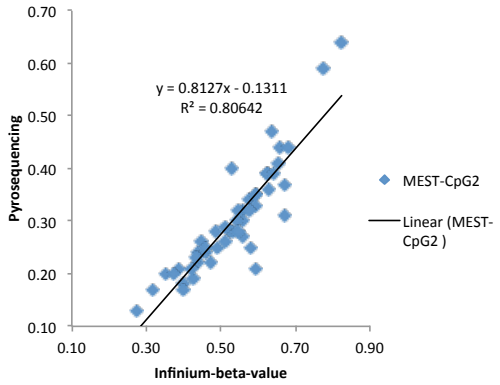


Figure 2. (continued)

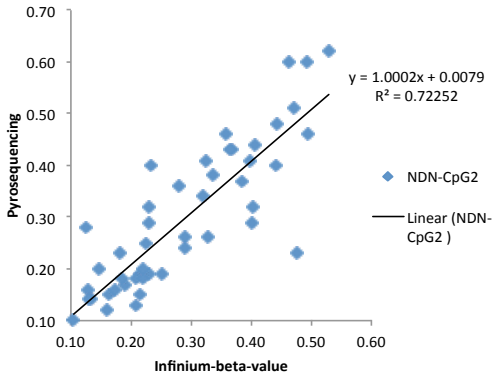
M. MEST cg02490034



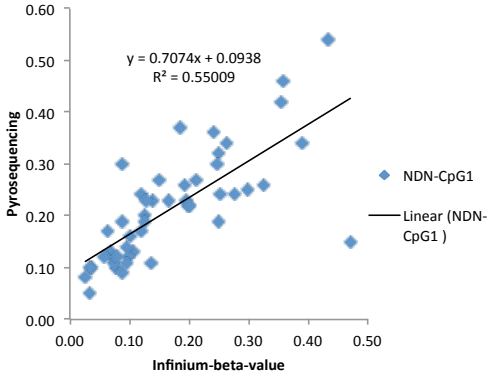
N. MEST cg09059945



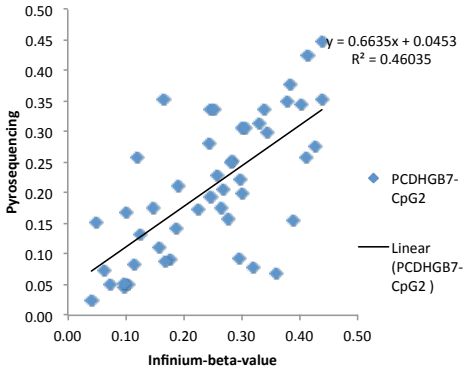
O.NDN cg12532169



P. NDN cg13828758



Q. PCDHGB7 cg14011639



R. PCDHGB7 cg23563234

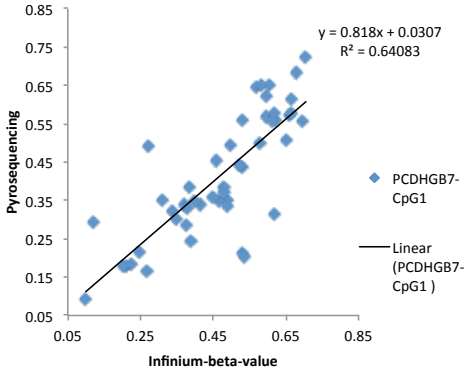


Figure 2. (continued)

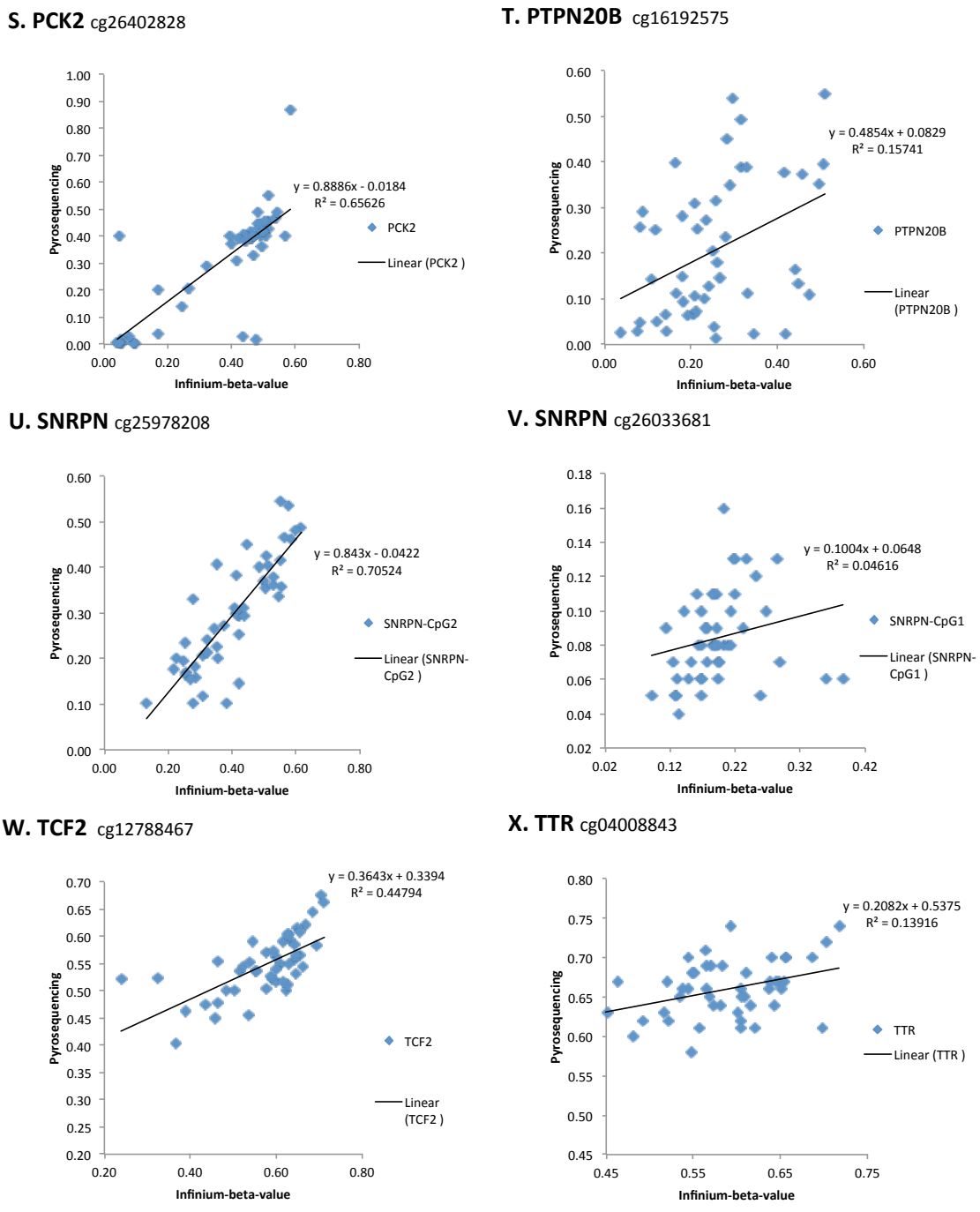


Figure 2. Correlation between Infinium methylation array data and pyrosequencing data for the selected 20 genes in the same ART (n=24) and Control (n=24) samples. More than one CpG site of *MEST*, *NDN*, *PCDHGB7* and *SNRPN* has been measured. *GRIN2C, FLJ10260, SNRPN, MEST, NDN, PCK2, CCDC62* and

PCDHGB7 R^2>0.50; *TCF2, FLJ90650, GAS2, PTPN20B, CRTAM, DLX5,* IFI16 and *PCDHGB7* 0.15<R^2<0.50; *IL5, SNRPN, LYST* and *H19* R^2<0.10. A. *AQPEP*, B. *CCDC62*, C. *CRTAM*, D. *DLX5*, E. *FLJ10260*, F. *GAS2*, G. *GRIN2C*, H. *H19*, I. *HSPB3*, G. *IFI16*, K. *IL5*, L. *LYST*, M. *MEST* cg02490034, N. *MEST* cg09059945, O. *NDN* cg12532169, P. *NDN* cg13821758, Q. *PCDHGB7* cg14011639, R. *PCDHGB7* cg23563234, S. *PCK2*, T. *PTPN20B*, U. *SNRPN* cg25978208, V. *SNRPN* cg26033681, W. *TCF2*, X. *TTR*.

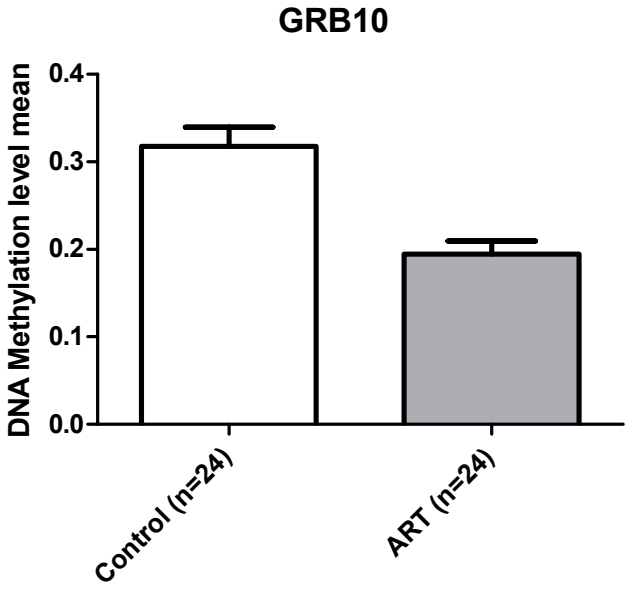


Figure 3. DNA methylation sensitive restriction enzyme HpyCH4IV digestion and realtime-PCR results for *GRB10* measuring samples (ART (n=24) vs. Control (n=24)) used in the Infinium Methylation Array. Mean Difference of beta value = -0.1231, $P<10^{-4}$, Student's unpaired t-TEST, one-tailed.

3.3 Validation of Infinium Methylation Array Differences by Pyrosequencing between ART and Control Samples

To validate the differences found on the Infinium Methylation Array between ART children and children conceived *in vivo* in placenta in more samples, same bisulfite pyrosequencing assays for the selected 15 candidate genes were performed on 90 ART samples and 49 Control samples (including the samples used in the Infinium array). Pyrosequencing can measure the DNA methylation level of not only the identical target CpG sites in Infinium Methylation Assay but also the CpGs sites near the target if there are any in the same pyrosequencing assay. As a result, we measured methylation levels at more than one CpG site in several pyrosequencing assays (**Table 4.**). As shown in **Table 4.**, the CpG ID for additional CpG sites measured by pyrosequencing was named as the target's CpG ID plus a letter (i.e., for *GRIN2C*, one extra CpG site was measured and named as cg09169283-a). Among the 33 CpG sites for the selected 15 candidate genes, CpG sites of 5 genes (*GRIN2C*, *FLJ90650* (*AQPEP*), *CCDC62*, *PTPN20B*, *NDN*) were significantly differently methylated ($P<0.05$, **Table 4.**) in the ART children. CpG sites for gene *CCDC62* and *GRIN2C* were hypomethylated in ART children, and CpG sites for gene *FLJ90650* (*AQPEP*), *PTPN20B* and *NDN* were hypermethylated in ART children (**Figure 4.**, **Figure 5.**). CpG site for *FLJ10260* was suggestive of being hypermethylated in ART children ($P=0.0639$). Although the CpG sites for *TTR* and *LYST* were significantly or suggestively significantly hypomethylated in ART children from the pyrosequencing data, they were hypermethylated from Infinium Methylation Array data.

Because we cannot judge which assay is most reliable in these instances, we cannot conclude whether these sites differ in a consistent manner in these samples. The beta value mean difference are subtle, and DNA methylation levels for different CpGs sites have different extent of individual variance, as a result less differences were seen after measuring a large number of samples.

We also measured the DNA methylation levels of the same 90 ART samples and 49 Control samples for *GRB10* by the DNA methylation sensitive enzyme based method. The CpG site measured using this method was significantly hypomethylated in the ART children (**Table 4**., **Figure 4 - F**.)

Table 4. Comparison of methylation levels between children conceived *in vivo* (Control n=49) and *in vitro* (ART n=90) of candidate CpG sites in pyrosequencing or enzyme based method (*GRB10*)

		ART (n=90) vs. Control (n=49)	
CpG ID	Gene	MD[1]	*P*[2]
cg09169283	**GRIN2C**	-0.0620	***<10^{-6}***
cg09169283-a	**GRIN2C**	-0.0315	***<10^{-4}***
cg25044651	**FLJ90650**	0.0530	***0.0025***
cg25044651-a	**FLJ90650**	0.0437	***0.0124***
cg25044651-b	**FLJ90650**	0.0630	***0.0017***
cg25044651-c	**FLJ90650**	0.0366	***0.0356***
cg08775774	**CCDC62**	-0.0385	***0.0073***
cg08775774-a	**CCDC62**	-0.0377	***0.0080***
cg16192575	**PTPN20B**	0.0588	***0.0143***
cg16192575-a	**PTPN20B**	0.0594	***0.0178***
cg16192575-b	**PTPN20B**	0.0480	***0.0353***
cg16192575-c	**PTPN20B**	0.0545	***0.0374***
cg16192575-d	**PTPN20B**	0.0629	***0.0305***
cg13828758	**NDN**	0.0381	***0.0226***
cg12532169	**NDN**	0.0471	***0.0248***
HpyCH4IV+realtime	**GRB10**	-0.0522	***0.0094***
cg04008843	**TTR**	-0.0158	0.0252
cg02829654	**LYST**	-0.0171	0.0556
cg03251655	**FLJ10260**	0.0335	0.0639
cg14011639	**PCDHGB7**	0.0261	0.0981
cg23563234	**PCDHGB7**	0.0273	0.1596
cg23563234-a	**PCDHGB7**	0.0204	0.2229
cg09059945	**MEST**	0.0166	0.1571
cg09059945-a	**MEST**	0.0204	0.1309
cg02490034	**MEST**	0.0026	0.4370
cg02490034-a	**MEST**	0.0105	0.2945
cg07342901	**H19**	0.0089	0.1551
cg25978208	**SNRPN**	0.0108	0.3034
cg25978208-a	**SNRPN**	-0.0204	0.1549
cg26033681	**SNRPN**	0.0061	0.2251
cg12788467	**TCF2**	-0.0065	0.2902
cg12788467-a	**TCF2**	-0.0055	0.3300
cg26081812	**IL5**	0.0039	0.3543
cg10977115	**CRTAM**	-0.0019	0.4435

(1) mean difference of beta-values between groups

(2) Student's unpaired t-Test, one-tailed, P<0.05 and validated Infinium methylation array data are in bold

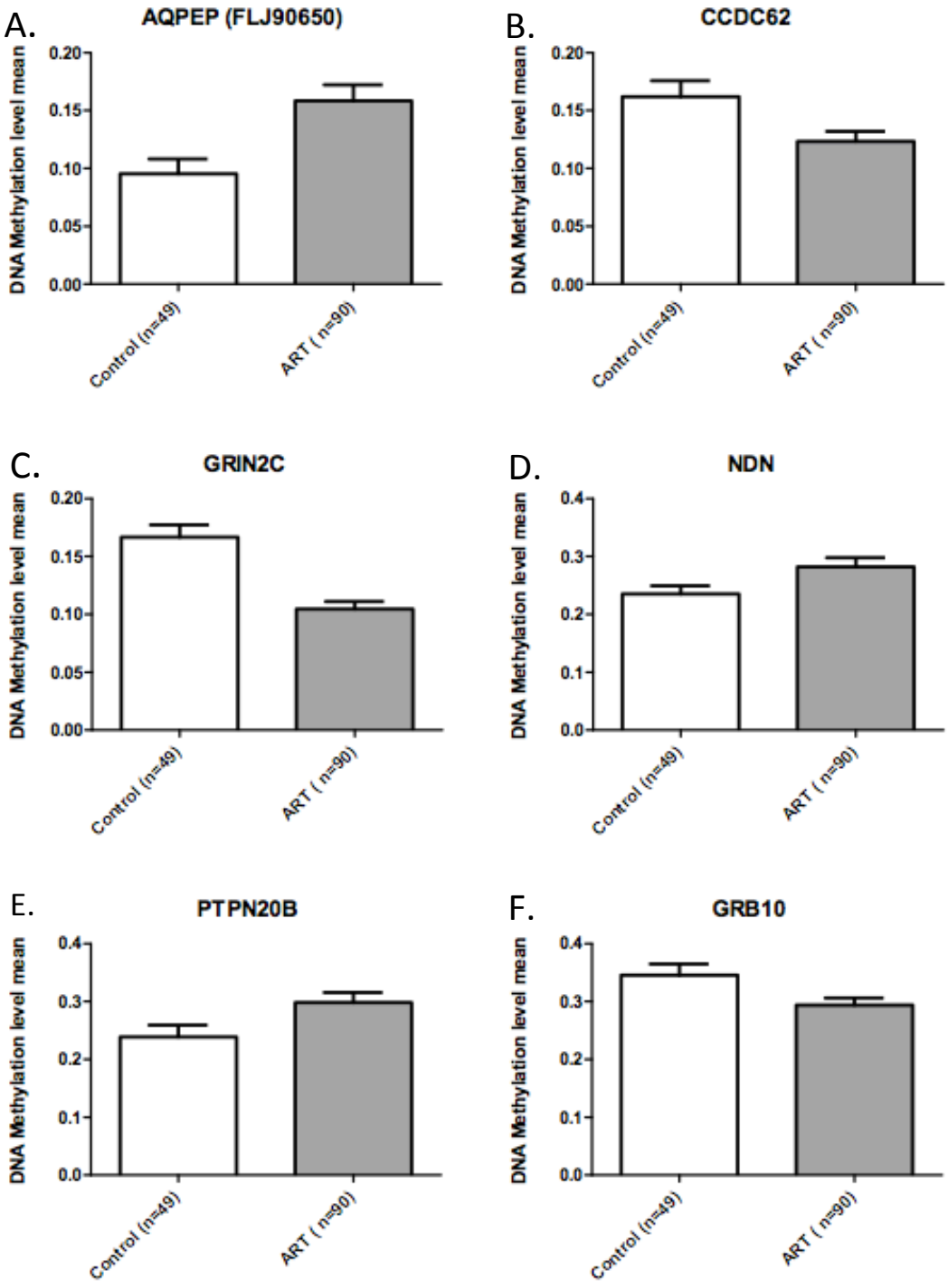
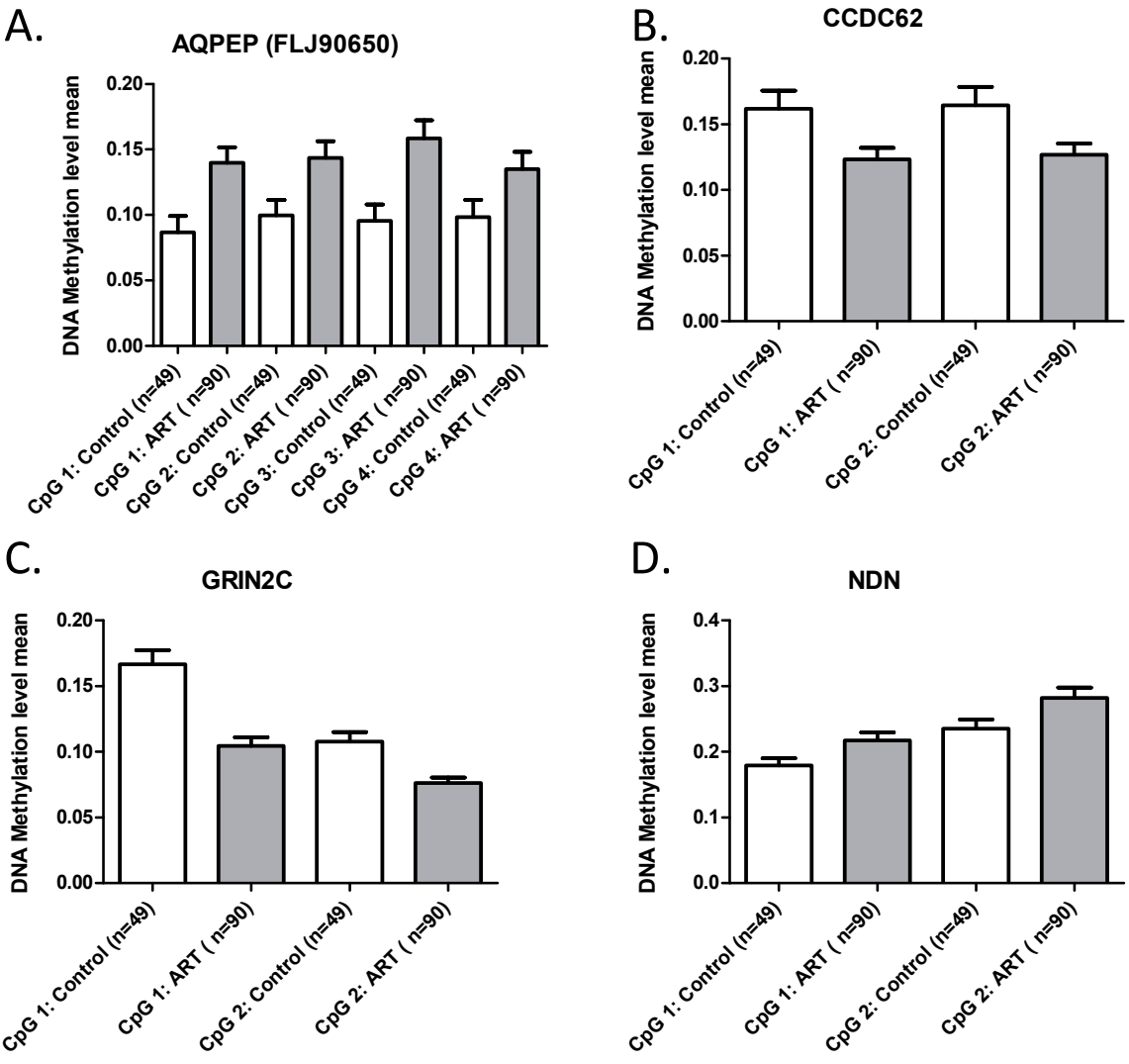


Figure 4. Bar graphs of DNA methylation level mean results represented as beta-values between ART (n=90) and Control (n=49) by pyrosequencing or restriction enzyme based method. Pyrosequencing results for A. *AQPEP*, B. *CCDC62*, C. *GRIN2C*, D. *NDN* and E. *PTPN20B*. Restriction enzyme based method for F. *GRB10*. Mean difference of beta-values between groups and P values for student's unpaired t-Test are shown in Table 4.



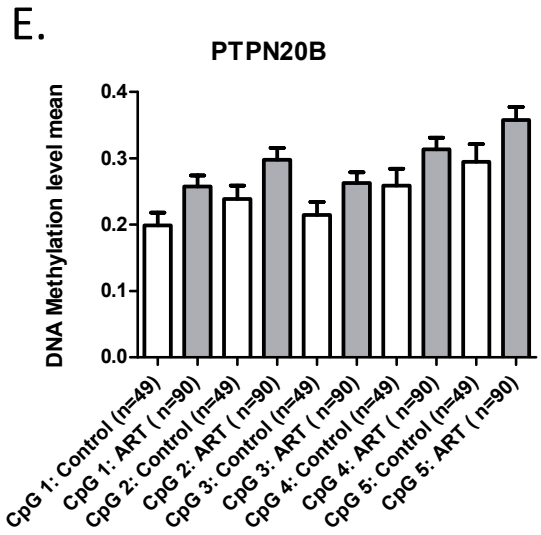


Figure 5. Bar graphs of DNA methylation level mean results represented as beta-values for multiple CpG sites of each gene between ART (n=90) and Control (n=49) by pyrosequencing. A. *AQPEP* (4 CpG sites), B. *CCDC62* (2 CpG sites), C. *GRIN2C* (2 CpG sites), D. *NDN* (2 CpG sites) and E. *PTPN20B* (5 CpG sites). Mean difference of beta-values between groups and P values for student's unpaired t-Test are shown in Table 4.

3.4 Validation of Infinium Methylation Array Differences by Pyrosequencing Using Independent ART Samples

To validate the differences found on the Infinium Methylation Array between ART children and children conceived *in vivo* in placenta in independent samples, we analyzed the bisulfite pyrosequencing assay results of the 69 independent ART samples (not used in Infinium Methylation Array, including Tubal, donor egg, and infertile samples) and all 49 Control samples (including 25 independent Control samples and 24 Control samples also used in the Infinium Methylation Array). CpG sites of 4 genes *(GRIN2C, FLJ90650 (AQPEP), CCDC62, PTPN20B)* were significantly differently methylated ($P<0.05$, **Table 5**.) in the independent ART cohort. CpG sites for gene *CCDC62* and *GRIN2C* were hypomethylated in the independent ART cohort, and CpG sites for *FLJ90650* and *PTPN20B* were hypermethylated in the independent ART cohort (**Figure 6.**). CpG sites for *NDN* were almost significantly hypermethylated in the independent ART cohort ($P=0.0826$). Although the CpG sites for *TTR* and *LYST* were significantly hypomethylated in the independent ART cohort from the pyrosequencing data, they were hypermethylated from Infinium Methylation Array data. Because of this discrepancy in direction of difference, we cannot conclude that the differences observed are consistent. To easier observe the inter-individual variance for each gene and determine if outliers exists in Controls or independent ART samples, Whiskers box plots were made in **Figure 6.**.

The comparison results for *GRB10* by the restriction enzyme based method are also shown in **Table 5.**. Although the measured CpG site is also hypomethylated in the independent ART cohort, the difference was not statistically significant when only the 69 independent samples are considered (although the result is significant when all 90 ART samples are considered).

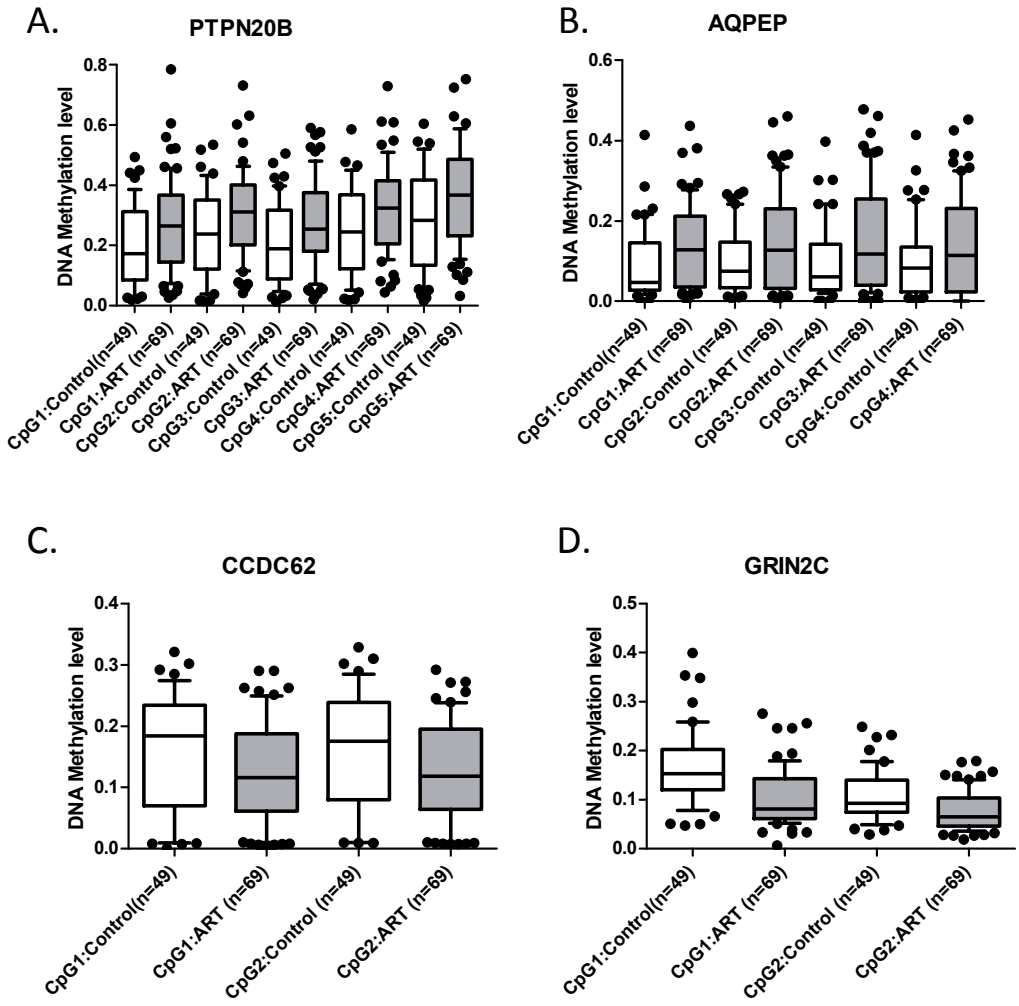
Table 5. Comparison of methylation levels between children conceived *in vivo* (Control n=49) and *in vitro* (independent ART n=69) of candidate CpG sites in pyrosequencing or enzyme based method (*GRB10*)

		ART (n = 69) vs. Control (n = 49)[3]	
		Pyrosequencing	
CpG ID	Gene	MD[1]	*P*[2]
cg09169283	**GRIN2C**	-0.0626	***<10^{-3}***
cg16192575	**PTPN20B**	0.0726	***0.0045***
cg25044651	**FLJ90650**	0.0484	***0.0045***
cg08775774	**CCDC62**	-0.0394	***0.0091***
cg13828758	**NDN**	0.0270	0.0826
cg12532169	**NDN**	0.0298	0.1053
cg04008843	**TTR**	-0.0235	***0.0032***
cg02829654	**LYST**	-0.0234	***0.0243***
cg03251655	**FLJ10260**	0.0288	0.1071
cg12788467	**TCF2**	-0.0149	0.1193
cg14011639	**PCDHGB7**	0.0236	0.1363
cg26033681	**SNRPN**	0.0089	0.1608
cg07342901	**H19**	0.0084	0.1995
cg23563234	**PCDHGB7**	0.0227	0.2175
cg10977115	**CRTAM**	-0.0076	0.3002
cg02490034	**MEST**	-0.0060	0.3561
cg09059945	**MEST**	0.0038	0.4050
cg25978208	**SNRPN**	-0.0032	0.4409
cg26081812	**IL5**	-0.0006	0.4787
HpyCH4IV+realtime	**GRB10**	-0.0213	0.1734

(1) mean difference of beta-values between groups (pyrosequencing data were also converted to represent beta-values)

(2) Student's unpaired t-Test, one-tailed, P<0.05 are in bold

(3) comparison between new ART samples (n=69) not measured in the Infinium Methlation Assay and all Control samples (n=49)



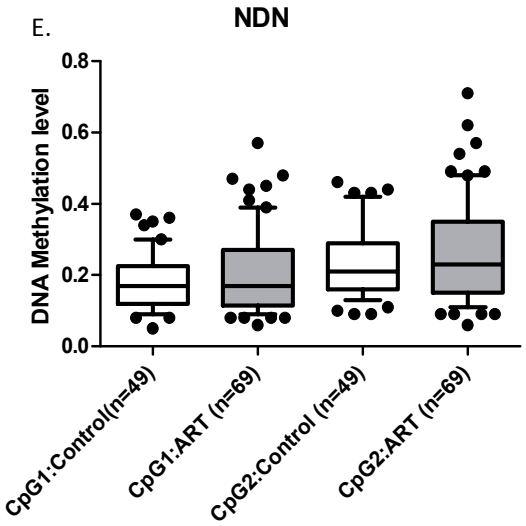


Figure 6. Whiskers box plots of DNA methylation level results represented as beta-values for multiple CpG sites of each gene between independent ART (n=69) and Control (n=49) samples by pyrosequencing. A. *PTPN20B* (5 CpG sites), B. *AQPEP* (4 CpG sites), C. *CCDC62* (2 CpG sites), D. *GRIN2C* (2 CpG sites) and E. *NDN* (2 CpG sites). Mean difference of beta-values between groups and P values for student's unpaired t-Test are shown in Table 5.

3.5 Comparison of ART Subgroup (Tubal +Donor Egg Group (n=45) or Infertility Group (n=45)) with Control Group (n=49)

We noticed that the DNA methylation level (beta-value) mean difference of the same CpG site between ART group and Control group were often subtle, with the largest absolute mean differences of about 0.1. This is as expected, because the vast majority of

children conceived by ART are normal and healthy, and rarely have adverse phenotypes. To confirm the subtle DNA methylation level difference between ART subgroup and Control group, and to assess whether the differences were attributable to ART procedures; we put 24 samples conceived with the aid of donor egg and 21 tubal samples together as one group, and compared them with the control group. In 33 CpG sites of 15 genes measured by pyrosequencing, 14 CpG sites of 5 genes (*GRIN2C*, *PTPN20B*, *CCDC62*, *NDN*, *AQPEP (FLJ90650)*) were significantly differently methylated in Tubal and donor egg group (**Table 6**., **Figure 7.**). Among the measured CpG sites, 2 CpG sites of Gene *GRIN2C* and 2 CpG sites of *CCDC62* were significantly hypomethylated in Tubal and donor egg group; 5 CpG sites of Gene *PTPN20B*, 3 CpG sites of *AQPEP (FLJ90650)*, and 2 CpG sites of Gene *NDN* were hypermethylated in Tubal and donor egg group (**Figure 8.**). In addition, one CpG site of Gene *FLJ10260*) was suggestively hypermethylated (P=0.0539) in Tubal and donor egg group. The CpG site measured by restriction enzyme based method for *GRB10* was also significantly hypomethylated in Tubal and donor group (**Table 6.**, **Figure 7.**).

In addition to the 45 tubal and donor egg samples, we compared the other 45 ART samples – Infertility samples with the controls. The "Infertility group" was comprised of children conceived by ART whose mothers, fathers or both were diagnosed as infertile. The details of the infertility diagnosis were complicated, including DOR (Diminished Ovarian Reserve) for 8 samples, endometriosis for 2 samples, fibroid uterus for one sample, hypothalamic amenorrhea for 2 samples, infertility resulting from age for 2

samples, male factor for 5 samples, PCOS (Polycystic Ovary Syndrome) for 5 samples, unexplained infertility for 5 samples, and multiple infertility factors for 15 samples.

Comparing the data between ART group and Control, and Tubal + donor egg group and Control, one can predict a role of ART procedure in affecting the DNA methylation levels of some of CpG sites in the children conceived by ART. Next, we compared the DNA methylation levels of infertility group to Control group. Our purpose was to check a general role of infertility rather than the role of a specific kind of infertility condition in influencing the DNA methylation levels of some CpG sites in children conceived by ART, as a result we chose the samples in different infertility background as a group. In 33 CpG sites of 15 genes measured by pyrosequencing, 6 CpG sites of 2 genes (*GRIN2C*, *AQPEP (FLJ90650)*) were significantly differently methylated in the infertility group (**Table 6.**, **Figure 7.**). Among the measured CpG sites, 2 CpG sites of Gene *GRIN2C* were hypomethylated in infertility group; 4 CpG sites of *AQPEP (FLJ90650*) were hypermethylated in infertility group (**Figure 8.**). In addition, two CpG sites of Gene *MEST* were almost significantly differently hypermethylated in infertility group (**Table 6.**). The CpG site measured by restriction enzyme based method for *GRB10* was also not significantly hypermethylated in infertility group (**Table 6.**, **Figure 7.**) .

Interestingly, for genes *CCDC62*, *GRIN2C*, *NDN*, *PTPN20B* and *GRB10*, the DNA methylation levels of all the CpG sites of infertility group were between the ones of Tubal + donor egg and Control group. This means that the DNA methylation levels of Tubal +donor egg group were closer to the other ART samples than the Control group.

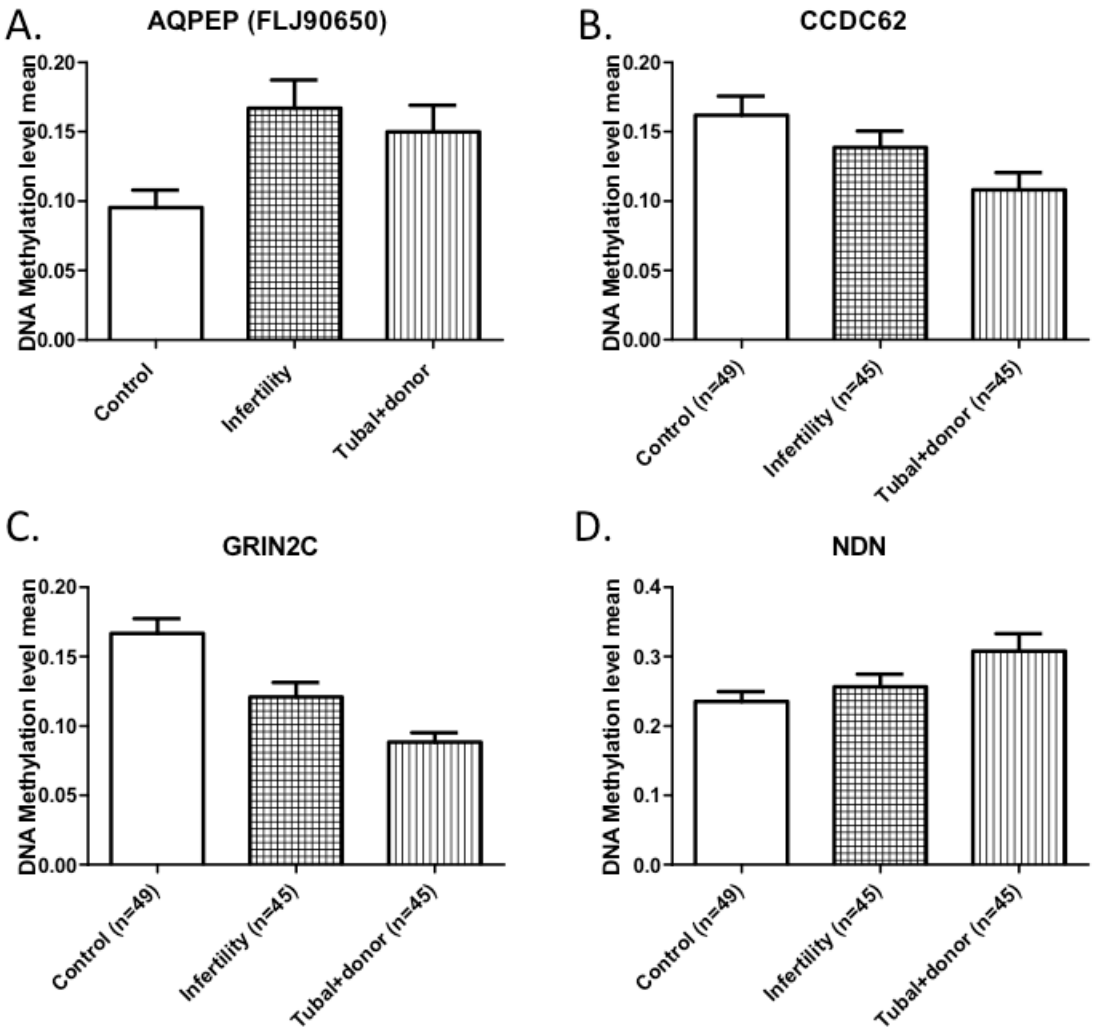
Because the DNA methylation levels of Tubal + donor egg group, a group whose parents are not involved in any idiopathic infertile issue, were considered only affected by one of or more steps in ART procedure, it strongly suggests that the DNA methylation level differences of CpG sites in specific genes are associated with the different environmental factors of ART procedure compared to the natural conceiving environment provided by the mother. Even for gene *FLJ90650* (*AQPEP*), the DNA methylation levels of all the CpG sites of the infertility group were more different from the Control group than from the Tubal + donor egg group. In addition, the DNA methylation levels of Tubal + donor egg group are not significantly different from the infertility group ($P>0.2$). These data suggest that ART procedures are the major factor affecting the DNA methylation levels of gene *FLJ90650* (*AQPEP*).

Table 6. Comparison of methylation levels between children conceived *in vivo* (Control n=49) and *in vitro* subgroups (Tubal +donor egg n=45, Infertility n=45) of candidate CpG sites in pyrosequencing or enzyme based method (*GRB10*)

		Tubal +donor egg (n=45) vs. Control (n=49)		Infertility (n=45) vs. Control (n=49)	
CpG ID	Gene	MD[1]	*P*[2]	MD	*P*
cg09169283	**GRIN2C**	-0.0782	***<10^{-7}***	-0.0458	***0.0016***
cg09169283-a	**GRIN2C**	-0.0443	***<10^{-6}***	-0.0187	***0.0296***
cg16192575	**PTPN20B**	0.0919	***0.0015***	0.0257	0.1934
cg16192575-a	**PTPN20B**	0.0811	***0.0054***	0.0173	0.2876
cg16192575-b	**PTPN20B**	0.0959	***0.0043***	0.0149	0.3002
cg16192575-c	**PTPN20B**	0.0931	***0.0085***	0.0131	0.3528
cg16192575-d	**PTPN20B**	0.0362	***0.0392***	0.0327	0.2006
cg08775774	**CCDC62**	-0.0538	***0.0025***	-0.0232	0.1037
cg08775774-a	**CCDC62**	-0.0510	***0.0036***	-0.0244	0.0926
cg13828758	**NDN**	0.0579	***0.0037***	0.0182	0.1733
cg12532169	**NDN**	0.0729	***0.0059***	0.0213	0.1801
cg25044651	**FLJ90650**	0.0435	***0.0141***	0.0625	***0.0027***
cg25044651-a	**FLJ90650**	0.0362	***0.0391***	0.0512	***0.0109***
cg25044651-b	**FLJ90650**	0.0545	***0.0093***	0.0715	***0.0016***
cg25044651-c	**FLJ90650**	0.0252	0.1227	0.0480	***0.0200***
HpyCH4IV+realtime	**GRB10**	-0.0724	***0.0020***	-0.0321	0.1198
cg04008843	**TTR**	-0.0167	***0.0372***	-0.0149	0.0428
cg03251655	**FLJ10260**	0.0432	0.0539	0.0237	0.1816
cg02829654	**LYST**	-0.0177	0.0778	-0.0164	0.0881
cg26033681	**SNRPN**	0.0130	0.1160	-0.0008	0.4496
cg14011639	**PCDHGB7**	0.0240	0.1503	0.0282	0.1099
cg07342901	**H19**	0.0110	0.1815	0.0068	0.0915
cg02490034	**MEST**	-0.0145	0.2075	0.0197	0.1643
cg02490034-a	**MEST**	-0.0099	0.3219	0.0308	0.0931
cg12788467	**TCF2**	-0.0104	0.2399	-0.0026	0.4143
cg12788467-a	**TCF2**	-0.0070	0.3269	-0.0039	0.3822
cg23563234	**PCDHGB7**	0.0208	0.2659	0.0338	0.1387
cg23563234-a	**PCDHGB7**	0.0210	0.2608	0.0198	0.2550
cg26081812	**IL5**	0.0066	0.2983	0.0011	0.4623
cg25978208	**SNRPN**	0.0093	0.3493	0.0124	0.3050
cg25978208-a	**SNRPN**	-0.0218	0.1727	-0.0190	0.1966
cg09059945	**MEST**	0.0025	0.4474	0.0307	0.0610
cg09059945-a	**MEST**	0.0060	0.3888	0.0349	0.0578
cg10977115	**CRTAM**	0.0003	0.4934	-0.0041	0.3929

(1) mean difference of beta-values between groups (pyrosequencing data were also converted to represent beta-values)

(2) Student's unpaired t-Test, one-tailed, $P<0.05$ and validated Infinium methylation array data are in bold.



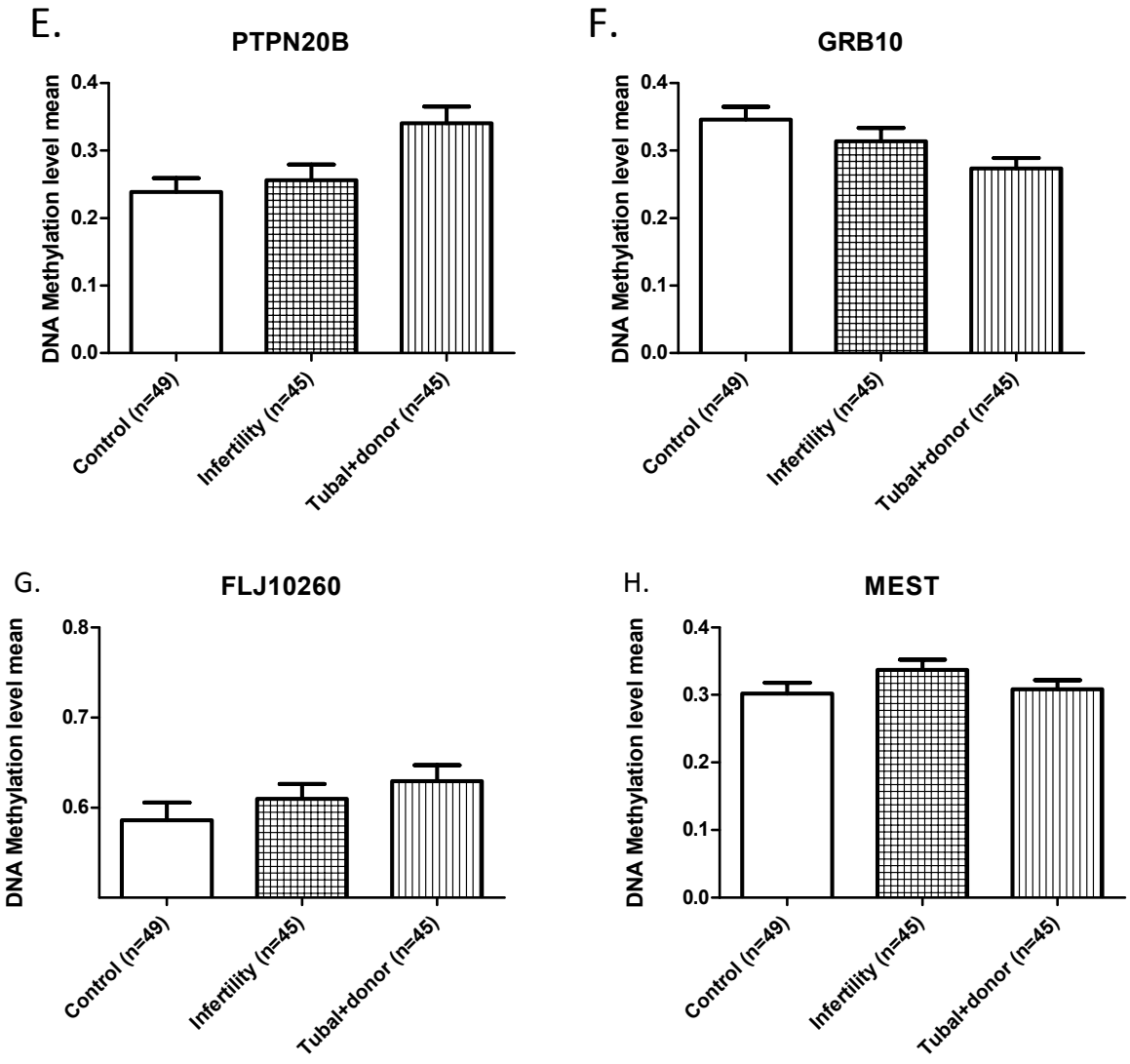


Figure 7. Bar graphs of DNA methylation level mean results represented as beta-values of Infertiltiy (n=45), Tubal + donor egg (n=45) and Control (n=49) group by pyrosequencing or restriction enzyme based method. Pyrosequencing results for A. *AQPEP*, B. *CCDC62*, C. *GRIN2C*, D. *NDN*, E. *PTPN20B* G. *FLJ10260* and H. *MEST*. Restriction enzyme based method for F. *GRB10*. Mean difference of beta-values between groups and P values for student's unpaired t-Test are shown in Table 6.

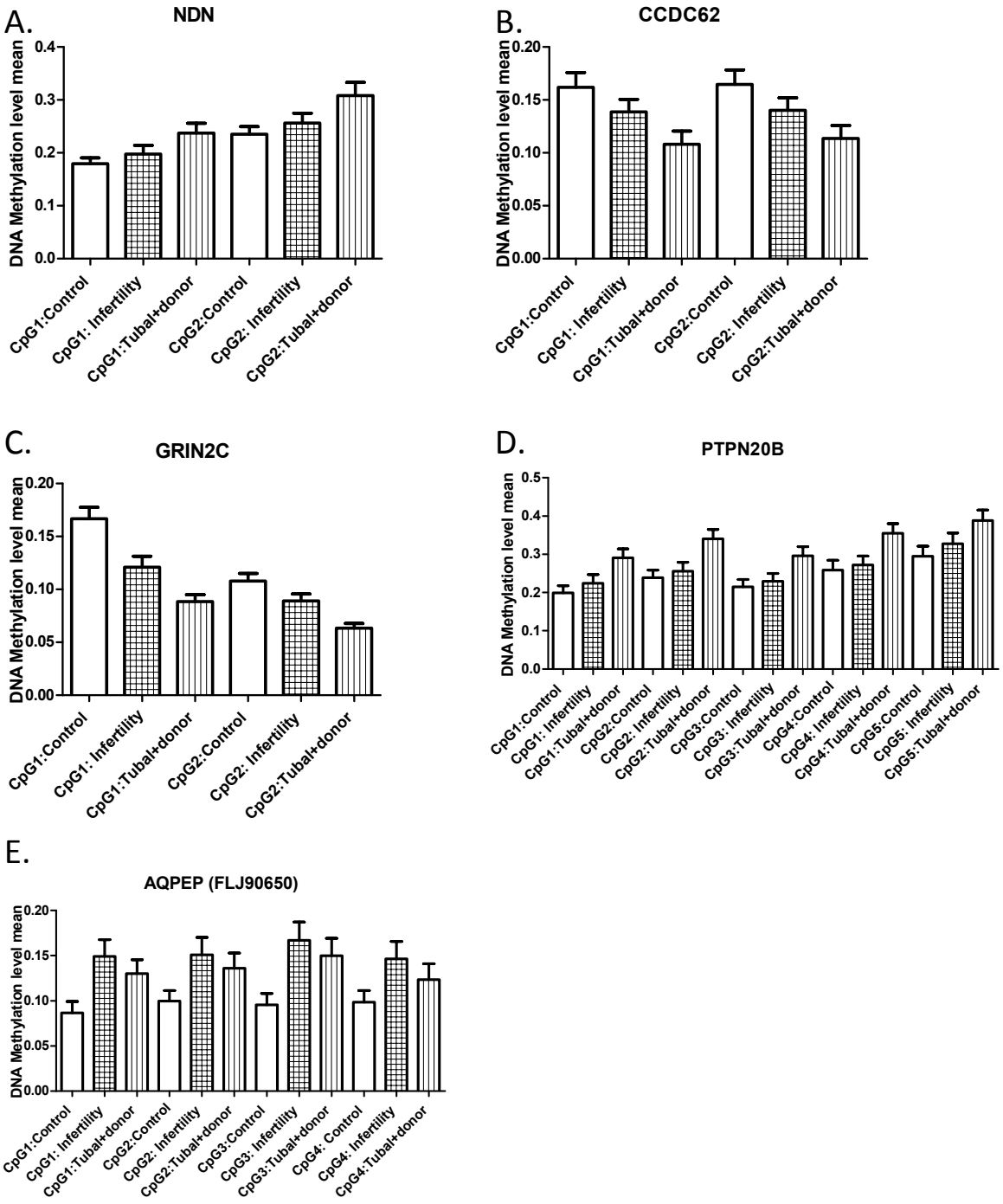


Figure 8. Bar graphs of DNA methylation level mean results represented as beta-values for multiple CpG sites of each gene of Infertiltiy (n=45), Tubal + donor egg (n=45) and Control (n=49) group. A. *NDN* (2 CpG sites), B. *CCDC62* (2 CpG sites), C.

GRIN2C (2 CpG sites), D. *PTPN20B* (5 CpG sites) and E. *AQPEP* (4 CpG sites). Mean difference of beta-values between groups and P values for student's unpaired t-Test are shown in Table 6.

3.6 Comparison of Tubal Individuals with Controls: 21 Tubal Only Compared with 49 Controls

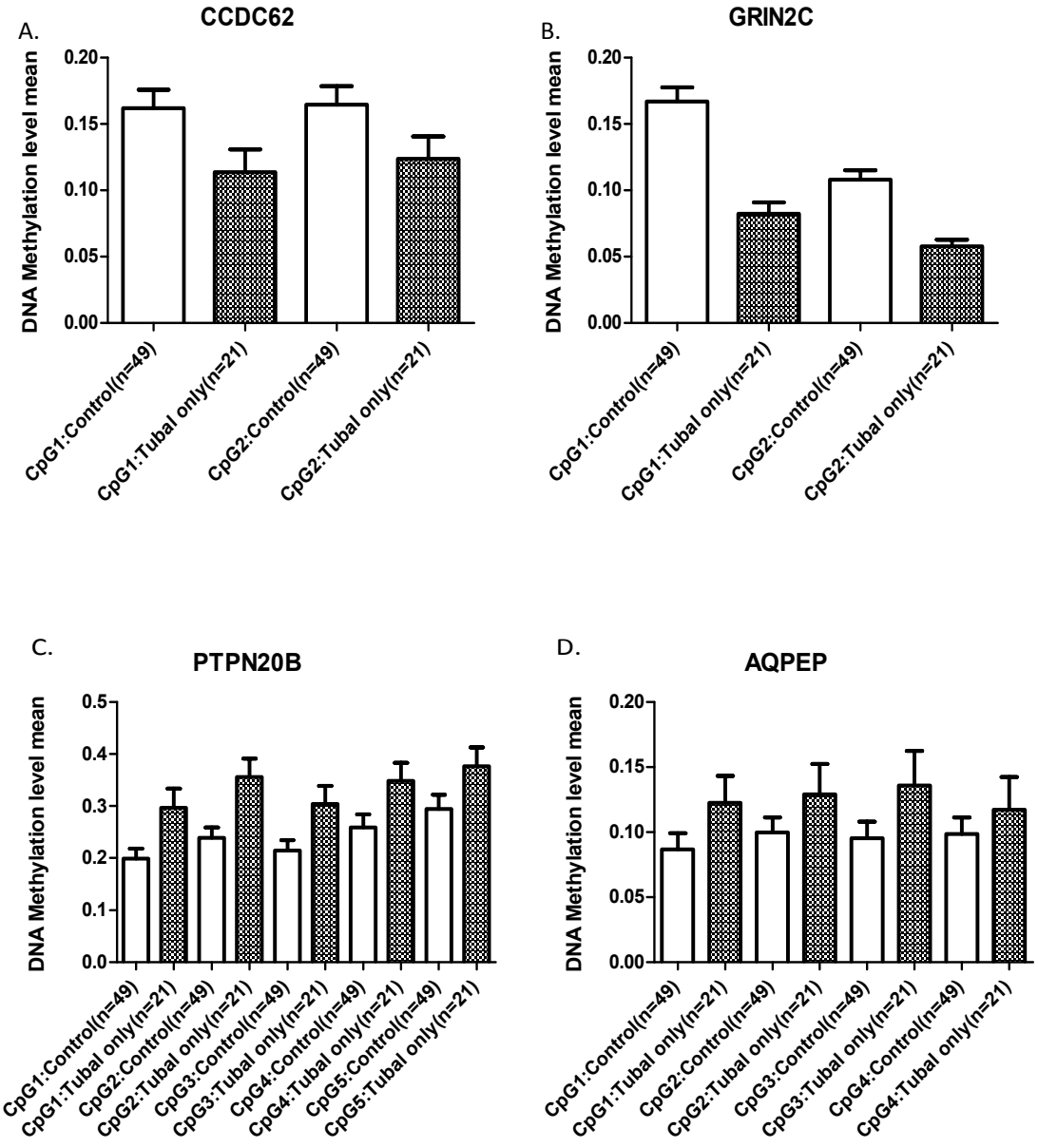
To study the epigenetic effect of ART procedures to the ART conceived children, the tubal group has fewer interventions than the donor egg group, whose mothers might provide problematic or potentially problematic maternal environments to fetal development. In our study, because of sample limitations we obtained only 21 tubal samples, with three using frozen embryo transfer (FET) cycles. We compared the pyrosequencing data of DNA methylation levels between the tubal only group (n=21) and Control group (children conceived *in vivo*, n=49) for 33 CpG sites of 15 genes. CpG sites of 5 genes (*GRIN2C*, *NDN*, *PTPN20B*, *CCDC62*, *H19*) were significantly differently methylated in the Tubal only group. Among the measured CpG sites, 2 CpG sites of Gene *GRIN2C* and 2 CpG sites of *CCDC62* were hypomethylated in Tubal only group; 5 CpG sites of Gene *PTPN20B*, 2 CpG sites of Gene *NDN*, and one CpG site of H19 were hypermethylated in Tubal only group (**Table 7.**, **Figure 9.**). In addition, 2 CpG sites of Gene *AQPEP* (*FLJ90650*) were suggestively hypermethylated (*P*=0.0625, 0.0637) in Tubal only group. The CpG site measured by restriction enzyme based method for *GRB10* was also significantly hypermethylated in Tubal only group (**Table 7.**, **Figure 9.**). In general, the comparison between Tubal only group or Tubal + donor egg group and Control group showed the similar results.

Table 7. Comparison of methylation levels between children conceived *in vivo* (Control n=49) and Tubal only subgroup (n=21) of candidate CpG sites in pyrosequencing or enzyme based method (*GRB10*)

		Tubal (n=21) vs. Control (n=49)	
		Pyrosequencing	
CpG ID	Gene	MD[1]	*P*[2]
cg09169283	**GRIN2C**	-0.0842	***<10^{-5}***
cg09169283-a	**GRIN2C**	-0.0502	***<10^{-4}***
cg13828758	**NDN**	0.0635	***0.0034***
cg12532169	**NDN**	0.0792	***0.0079***
cg16192575	**PTPN20B**	0.0975	***0.0067***
cg16192575-a	**PTPN20B**	0.1172	***0.0016***
cg16192575-b	**PTPN20B**	0.0897	***0.0098***
cg16192575-c	**PTPN20B**	0.0891	***0.0256***
cg16192575-d	**PTPN20B**	0.0815	***0.0441***
cg08775774	**CCDC62**	-0.0482	***0.0238***
cg08775774-a	**CCDC62**	-0.0409	***0.0443***
HpyCH4IV+realtime	**GRB10**	-0.0724	***0.0020***
cg25044651	**FLJ90650**	0.0359	0.0637
cg25044651-a	**FLJ90650**	0.0292	0.1092
cg25044651-b	**FLJ90650**	0.0405	0.0625
cg25044651-c	**FLJ90650**	0.0189	0.2328
cg07342901	**H19**	0.0339	***0.0112***
cg02829654	**LYST**	-0.0391	***0.0116***
cg26033681	**SNRPN**	0.0233	*0.0550*
cg02490034	**MEST**	-0.0303	0.0935
cg02490034-a	**MEST**	-0.0265	0.1643
cg14011639	**PCDHGB7**	0.0347	0.1091
cg12788467	**TCF2**	-0.0190	0.1482
cg12788467-a	**TCF2**	-0.0018	0.4636
cg04008843	**TTR**	-0.0120	0.1502
cg23563234	**PCDHGB7**	0.0382	0.1796
cg23563234-a	**PCDHGB7**	0.0362	0.1914
cg10977115	**CRTAM**	0.0140	0.2184
cg09059945	**MEST**	-0.0135	0.2879
cg09059945-a	**MEST**	-0.0165	0.2777
cg26081812	**IL5**	0.0061	0.3493
cg03251655	**FLJ10260**	0.0130	0.3571
cg25978208	**SNRPN**	0.0076	0.4000
cg25978208-a	**SNRPN**	-0.0412	0.0524

(1) mean difference of beta-values between groups (pyrosequencing data were also converted to represent beta-values)

(2) Student's unpaired t-Test, one-tailed , P<0.05 are in bold



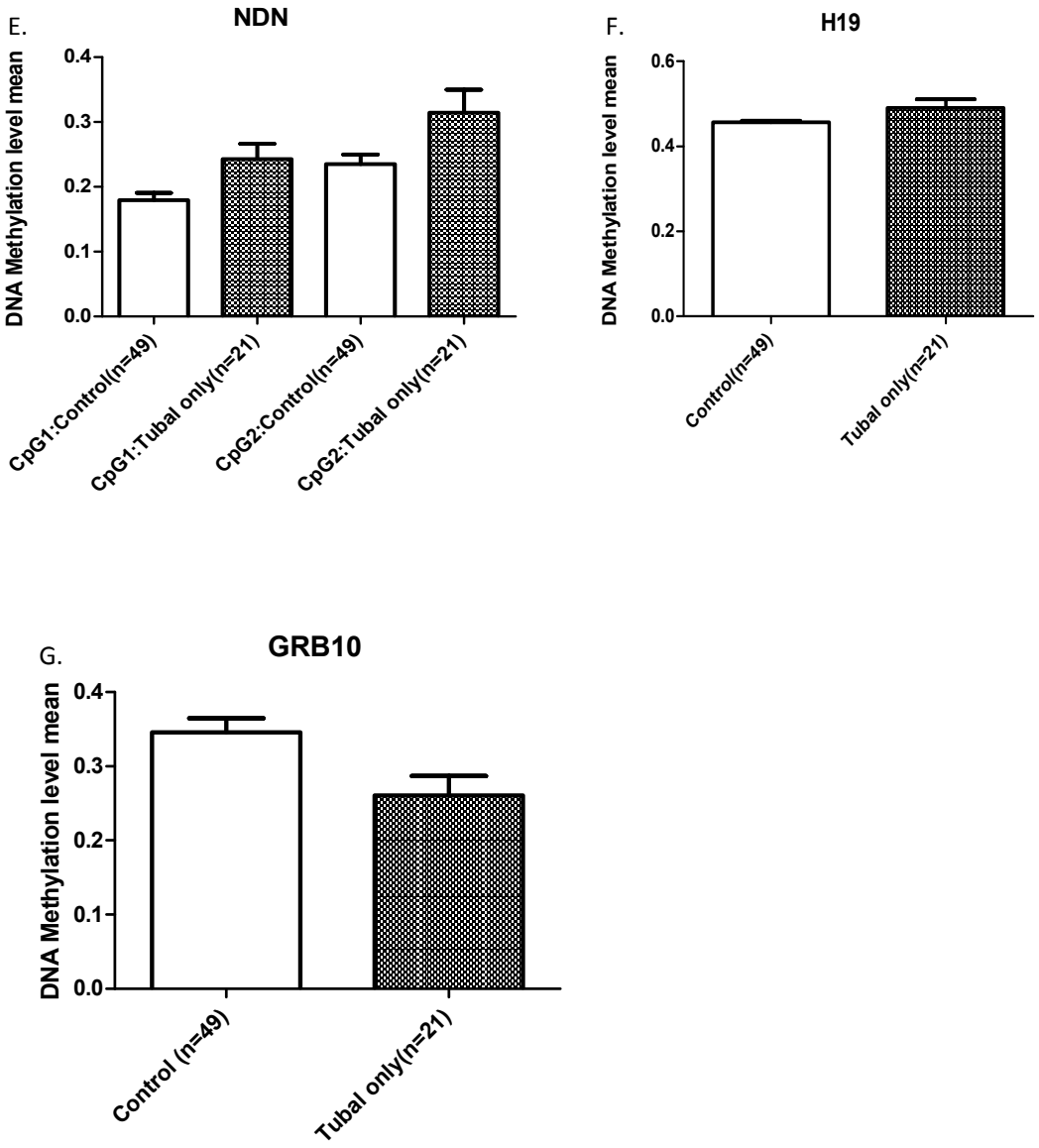


Figure 9. Bar graphs of DNA methylation level mean results represented as beta-values between Tubal only (n=21) and Control (n=49) group by pyrosequencing or restriction enzyme based method. Pyrosequencing results for A. *CCDC62*, B. *GRIN2C*, C. *PTPN20B*, D. *AQPEP*, E. *NDN* and F. *H19*. Restriction enzyme based method for G. *GRB10*. Mean difference of beta-values between groups and P values for student's unpaired t-Test are shown in Table 7.

3.7 Some Transcript Levels of Genes with Different DNA Methylation Levels of CpG Sites in Children Conceived by ART are Also Altered in Placenta.

After the validation of the DNA methylation differences between ART group or subgroups and Control in specific genes, it is interesting to examine the transcript levels of these genes, and determine whether the genes with different DNA methylation levels of CpG sites in children conceived by ART are expressed at different levels in placenta. Using the same samples as in measuring the DNA methylation levels by pyrosequencing (49 control samples, 45 tubal and donor egg samples, and 45 infertile samples), transcript levels for six of the genes in **Table 6.** (*AQPEP* (*FLJ90650*), *CCDC62*, *GRIN2C*, *MEST*, *NDN*, *PTPN20B*) were examined by Real-time RT PCR. Gene *PTPN20B* was expressed at significantly lower levels in Tubal and donor egg group (**Figure 10. A**), and *NDN* transcripts were significantly lower in the Infertility group (**Figure 10. B**). As we can see from Figure 10. A and B, there was large variance for the transcript levels of these two genes among different individuals even in the same group. We did not observe other statistically significant differences in comparisons between ART or ART subgroups and Control. One possible reason may be that the mean difference is small and the variance is large, such that we cannot distinguish such differences with this sample size. We used whole placenta as the study tissue rather than identical fetal tissues such as trophoblast. However, we did observe the differences of transcript levels for some genes in placenta between ART or ART subgroups and Control in this study or earlier study (Katari *et al.*, 2009).

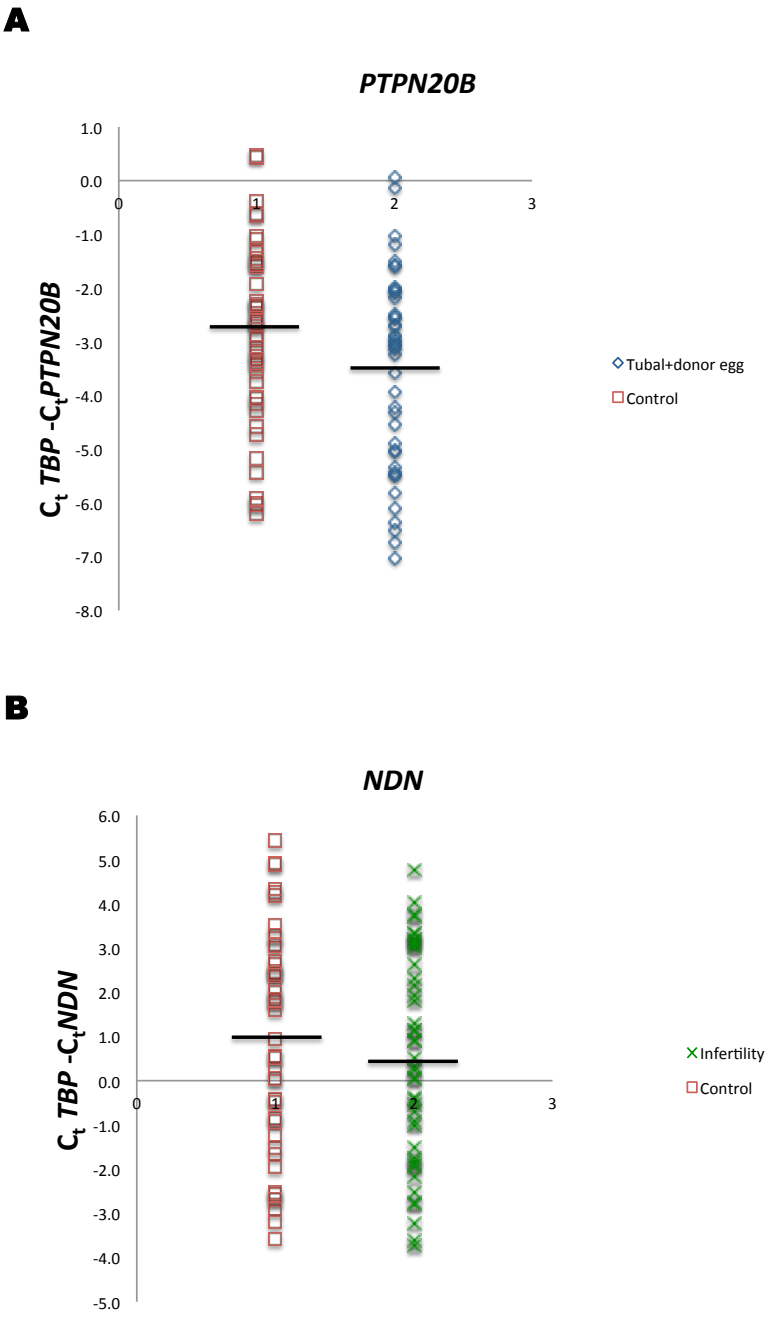


Figure 10. Real-time RT-PCR analysis of transcript level in placenta of individual children conceived by ART (Infertiltiy group or Tubal+donor egg group) and individual children conceived *in vivo* (Control) for *PTPN20B* (A; n = 48 control, 43 tubal+donor

egg; Student T-test, $P<0.05$), *NDN* (B; n = 49 control, 44 infertility; Student T-test, $P<0.005$)

CHAPTER 4

DISCUSSION

In summary, I analyzed the data from a quasi-transcriptome-wide DNA methylation profile (the Infinium Methylation Array) and selected 20 candidate genes to validate from the comparison between children conceived *in vivo* and children conceived *in vitro* (Control (n=24) *vs.* ART (n=24)). Subsequently, I used the same samples (Control (n=24) *vs.* ART (n=24)), as well as additional samples (Control (n=49) *vs.* ART (n=90)) from both groups to validate the DNA methylation differences between the ART and the Control group by pyrosequencing or the DNA methylation sensitive restriction enzyme based method. I also compared the subgroups of ART (the tubal + donor egg group (n=45) or the tubal only group (n=21)) with the Control group (n=49) to determine whether ART procedures or infertility issue of the ART children's parents are associated with the placental epigenetic alterations of ART children. Independent ART samples (n=69) from the samples used in the array were then compared with Control samples (n=49) to confirm the DNA methylation differences. The flow chart of my study is in Figure 11. I found six genes (*AQPEP*, *PTPN20B*, *CCDC62*, *GRIN2C*, *NDN* and *GRB10*) to be differentially methylated in the CpG islands of promoter regions in the placenta of ART children as compared to Controls. Moreover, mean methylation levels at these six genes were significantly different between tubal and donor egg individuals (n=45) and Controls (n=45) (**Table 6.**, **Figure 7.**, **Figure 8.**). This result is the major conclusion of

my study; i.e., the methylation differences observed between the placentas of ART children and Controls are most likely associated with some aspect of the ART procedure rather than infertility, *per se*.

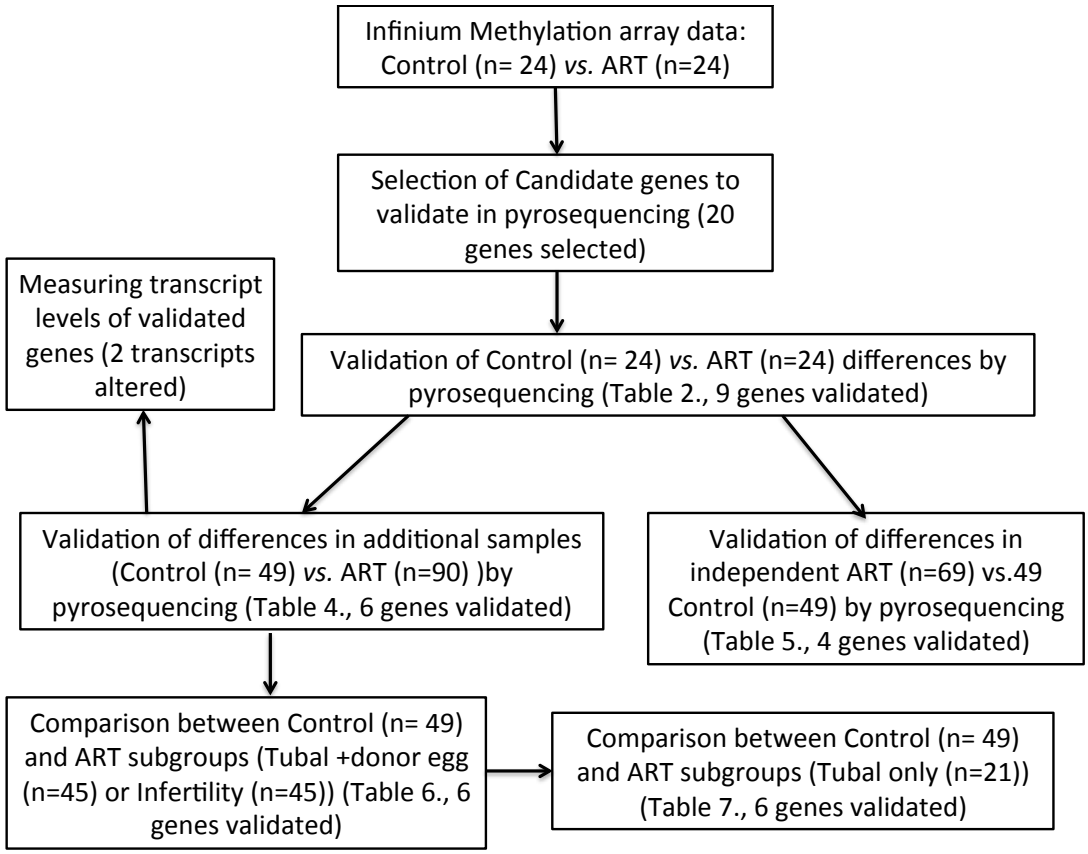


Figure 11. Flowchart in this study. Results of each step are referred

The following sections describe what is know about the genes for which I found significant differences between ART and Control individuals.

4.1 AQPEP (Laeverin/FLJ90650) – Aminopeptidase Q

It is interesting to note that the CpG sites in laeverin, a gene encoding a membrane-bound cell-surface metallopeptidase (Fujiwara *et al.*, 2004), were both hypermethylated in the Tubal + donor egg group and the infertility group. Laeverin is specifically expressed on human embryo-derived extravillous trophoblasts that invade the uterus during placentation (Maruyama *et al.*, 2007). As a specific cell-surface marker of human extravillous trophoblasts (EVT) throughout pregnancy, laeverin inactivates motility-suppressive peptides to induce EVT migration (Horie *et al.*, 2012). This enzyme plays important roles in human placentation by regulating biological activity of key peptides at the embryo-maternal interface. Furthermore, it shows homology to CD13/aminopeptidase N, which controls endothelial cell-motility through cytoskeletal rearrangement leading to filopodia formation (Petrovic *et al.*, 2007). Interestingly, the expression level of laeverin was 10-fold up-regulated in severe preeclampsia placenta (Sitras *et al.*, 2009). Preeclampsia is a clinical syndrome defined as new-onset hypertension and proteinuria after 20 weeks gestation in a previously normotensive woman (Sibai *et al.*, 2005). It affects 3–8% of all pregnancies and is a major cause of maternal and perinatal morbidity and mortality world-wide. The placenta plays a primary role in preeclampsia's etiology. Moreover, placental gene expression reflects the feto–maternal interaction and seems to control the maternal phenotype (Oudejans & van Dijk, 2008). Although we do not know the extent of the alteration in DNA methylation level can influence the expression of laeverin in placenta, the DNA methylation level alteration

of laeverin merits further investigation to clarify the potential effects of ART procedures to epigenetic reprogramming in placenta and placentation.

4.2 PTPN20B (Protein Tyrosine Phosphatase, Non-receptor Type 20B/A)

There are 38 classical tyrosine-specific PTPs (protein tyrosine phosphatases) encoded by the human genome. Many PTPs have been shown to regulate fundamental cellular processes and several are mutated in human diseases. The product of the *PTPN20* locus at chromosome 10q11.2 is alternatively spliced to generate 16 possible variants of the classical human non-transmembrane PTP20 (hPTPN20). One of these variants, hPTPN20a, was expressed in a wide range of both normal and transformed cell lines. hPTPN20a was identified as a novel and widely expressed phosphatase with a dynamic subcellular distribution that is targeted to sites of actin polymerization in response to varied extracellular stimuli (Fodero-Tavoletti *et al.*, 2005). *PTPN20B* or *PTPN20A* is the gene which is transcribed into hPTPN20a. *PTPN20A* is the aliase of *PTPN20B*.

Early vertebrate embryonic development is characterized by three processes, cell proliferation, differentiation and migration. The protein product of *PTPN20B* is important for normal gastrulation cell movements. In vertebrates the earliest two processes conducted by cell migration are the formation of the three germ layers during gastrulation by epiboly and internalization (or ingression/emboly, depending on the organism), and

the formation of the mediolateral body axis by convergence and extension (C/E) cell movements (Keller, 2002; Warga & Kimmel, 1990). Ptpn20 is a structural paralogue of PTP-BL and both phosphatases inhibit RhoA activity and were required for proper cell polarization and normal C/E cell movements (van Eekelen *et al.*, 2012). It is possible that the alterations of DNA methylation levels of CpG sites in *PTPN20B* and other genes involved in cell proliferation, differentiation and migration in ART children have influence on their embryonic development, because of ARTs as an essential factor in the increased risk of preterm birth and low birth weight.

4.3 CCDC62 (Coiled-coil Domain Containing 62, ERAP75, Estrogen Receptor Alpha 75)

Estrogens regulate the growth, differentiation, and development functions in a broad range of human target tissues, including both male and female repro- ductive systems (Couse & Korach, 1999). The estrogens actions are mediated by the ERs, members of the nuclear receptor (NR) superfamily (Green *et al.*, 1986; Greene *et al.*, 1986; Kuiper *et al.*, 1996; Mosselman *et al.*, 1996), which are encoded by two distinct genes, estrogen receptor 1 (ER alpha) and estrogen receptor 2 (ER beta). ERs share a common structural architecture with other NR superfamily members and consist of independent but functionally interactive domains: an amino terminal A/B domain containing a ligand-independent transactivation function (AF1), a DNA binding domain (DBD), and a C-terminal ligand binding domain (LBD) containing a ligand-dependent transactivation function (AF2) (Giguere *et al.*, 1988; Tsai & O'Malley, 1994;

Mangelsdorf *et al.*, 1995; Evans, 1988). Recent studies have shown that a wide range of ER coregulators can modulate ERs at target promoters (Lonard *et al.*, 2007). *CCDC62*/*ERAP75* functions as a nuclear receptor coactivator that can modulate ERα function in the prostate stromal cells (Chen *et al.*, 2008). ERα can interact with the C terminus of *CCDC62* via its ligand binding domain both *in vivo* and *in vitro* in prostate stromal cells. And *CCDC62* can enhance ERα transactivation in a dose-dependent manner and up-regulate the expression of the endogenous ERα target gene, stromal-derived factor-1 (SDF-1), in the prostate stromal cells (Chen *et al.*, 2008). In addition, *CCDC62*/*ERAP75* also functions as an ERβ coactivator to enhance ERβ -mediated transactivation, target genes expression and cell growth in the prostate cancer epithelial cells (Chen *et al.*, 2009). We can identify an important role of CCDC62 as a coactivator in ER signaling pathway, and the alterations of DNA methylation levels of this gene might potentially influence its protein function in reproductive system.

4.4 GRIN2C (Glutamate Receptor, Ionotropic, N-methyl D-aspartate 2C)

Glutamate is the major excitatory neurotransmitter in the mammalian central nervous system and serves as the neurotransmitter of the pyramidal cells. These are the source of efferent and interconnecting pathways of the cerebral cortex and limbic system (Tsai & Coyle, 2002). A key factor in glutamatergic neurotransmission is the N-methyl-D-aspartate (NMDA) receptors, which are involved in brain development, excitatory neurotransmission, synaptic plasticity, and memory formation (Bliss & Collingridge, 1993; Yang *et al.*, 1999; Akashi *et al.*, 2009; Zhuo *et al.*, 2009). The NMDA receptors

are composed of multiple subunits including at least one NR1 subunit (encoded by the *GRIN1* gene) and one or more NR2 subunits (encoded by the *GRIN2A-D* genes) (McBain & Mayer, 1994), and less commonly, a NR3 subunit (encoded by the *GRIN3A-B* genes) (Das *et al.*, 1998; Chatterton *et al.*, 2002).

NMDA receptors are important for normal brain development due to their involvement in the regulation of synapse maturation (Cohen & Greenberg, 2008). NR1 and NR2B knockout mice die shortly after birth, which indicates a requirement for NMDA receptor function in nerve circuit development (Forrest *et al.*, 1994; Kutsuwada *et al.*, 1996). It has also been shown that NMDA receptor blockade in rodents during postnatal early brain development leads to alterations in NMDA receptor binding still evident at adulthood (du Boris *et al.*, 2009), and maternal stress in rodents had influenced brain development and NMDA function of the offspring for life (Fumagalli *et al.*, 2009). These investigations suggest that disturbance of NMDA receptor function early in life has an impact on brain development. Moreover, because of the critical role of NMDA receptors in excitatory synaptic transmission and plasticity in the central nervous system, they govern a range of physiological conditions including neurological disorder caused by excitotoxic neuronal injury, psychiatric disorders and neuropathic pain syndromes (Cull-Candy *et al.*, 2001). *GRIN2C* is also expressed in placenta, I did not find significant different expression levels between ART children and children conceived *in vivo*. The differences of DNA methylation levels of NMDA receptors subunit-*GRIN2C* gene in ART children might potentially influence their brain development and the incidence of other physiological conditions.

4.5 NDN (Mouse Necdin Homolog)

The human *necdin* gene (*NDN*) is maternally imprinted and maps to 15q11–q12, a region known to be deleted in patients suffering from the Prader–Willi syndrome (PWS) (Jay *et al.*, 1997; MacDonald & Wevrick, 1997; Sutcliffe *et al.*, 1997). Murine Ndn was originally identified as a gene whose expression is upregulated in retinoic-acid-treated P19 embryonal carcinoma cells (Maruyama *et al.*, 1991) and is strongly expressed during development, including nervous (Aizawa *et al.*, 1992), muscle (Gerard *et al.*, 1999; Kuwajima *et al.*, 2004), and somitic tissues (Andrieu *et al.*, 2003; Brunelli *et al.*, 2004). Necdin null mice that manage to survive to adulthood have reduced numbers of oxytocin- and luteinizing hormone-releasing hormone-producing hypothalamic neurons, consistent with hypothalamic insufficiency observed in PWS, and display behavioral and cognitive disruptions that bear some resemblance to characteristics observed in humans with PWS (Muscatelli *et al.*, 2000). Together, these studies indicate that Necdin has an important role in development and/or maintenance of discrete cells in the nervous system, and disruption of its function almost certainly plays a crucial role in the pathogenesis of PWS.

Necdin is a growth suppressor that facilitates the entry of the cell into cell cycle arrest. Necdin has been shown to physically interact with cell-cycle-promoting proteins such as SV40 large T, adenovirus E1A, and the transcription factor E2F1 (Taniura *et al.*, 1998). The E2F proteins act as important components of the cell cycle machinery, and E2F1 specifically binds and trans-activates genes that are involved in cell cycle

progression. This pathway is regulated by retinoblastoma protein (Rb), which represses E2F1 activation by binding to the transactivating domain of E2F1 during the G1 phase. When cells move toward the S phase, Rb repression is released due to the phosphorylation of Rb, and the E2F1 complex becomes free to transactivate cell cycle progression genes (Yoshikawa, 2000). Necdin acts in a way similar to that of Rb; by binding to E2F1. Necdin represses E2F1 function which leads to cell growth arrest (Taniura *et al.*, 1998). Necdin also interacts with p53, a well-known tumor suppressor that transactivates genes involved in growth arrest and apoptosis. Necdin binds to the transactivating domain of p53 and in this way inhibits p53- induced apoptosis and represses p53-dependent activation of the p21/WAF. Even though Necdin represses the activation of the p21/WAF promoter, it does not repress p53-induced growth arrest in SAOS-2 cells. In these cells p53 and Necdin work in an additive manner to inhibit cell growth (Taniura *et al.*, 1999). Thus, it is likely that Necdin acts as a specific growth suppressor and anti-apoptotic protein in early neurons through interaction with p53 and E2F proteins. In addition, Necdin is a transcriptional modulator that inhibits apoptosis and promotes differentiation in neurons and muscle (Barker & Salehi, 2002; Bush & Wevrick, 2008; Deponti *et al.*, 2007). It is not surprising to find out the alterations of DNA methylation levels of this kind of imprinted genes in ART children, it suggests a potential molecular mechanism for how the development and growth of the cells in the nervous system are affected during the epigenetic reprogramming. The details of the mechanism require further study.

4.6 Transcript Levels of Genes with Different DNA Methylation Levels in ART Children in Placenta

In my study, I also examined the transcript levels for six genes (*AQPEP* (*FLJ90650*), *CCDC62*, *GRIN2C*, *MEST*, *NDN*, *PTPN20B*) by Real-time RT PCR in the same samples as measuring the DNA methylation levels by pyrosequencing (49 control samples, 45 tubal and donor egg samples, and 45 infertile samples). Only gene *PTPN20B* was expressed at significantly lower levels in Tubal and donor egg group (**Figure 10. A**), and *NDN* transcripts were significantly lower in the Infertility group (**Figure 10. B**). Methylation levels of CpG sites in promoter region of *PTPN20B* were also significantly higher in Tubal and donor egg group by both pyrosequencing and Infinium methylation array. This phenomenon can be explained as the DNA methylation of the promoter region represses the transcription of gene *PTPN20B*. However, there were not statistically significant differences of DNA methylation levels between the Infertility group and Control group for NDN. Although I did not find other statistically significant differences in comparisons between ART or ART subgroups and Control, all the genes I measured were expressed in placenta. It is easier to show a difference between groups for DNA methylation than for transcription, this is because of the small differences in means and large differences in variance of beta values, which constrained between "0" and "1". However, CT values which reprensents the transcript levels differ by orders of magnitude.

I also observed a large range of variation of the transcript levels compared to the methylation levels for all genes I measured. This is not unusual, one of the most

surprising observations from human transcriptome profile is the very high level of inter-individual variability found in steady state mRNA levels of many genes (Turan *et al.,* 2010a). The similar large inter-individual differences from many genes observed in the arrays have been validated by quantitative RT-PCR in my laboratory and other groups (Turan *et al.,* 2010a; Cheung & Spielman, 2009; Peters *et al.*, 2007; Deutsch *et al.*, 2005; Whitney *et al.*, 2003). Another possible reason may be that instead of the identical fetal tissues such as trophoblast, the whole placenta was used in my study which could potentially influence the results of gene expression levels.

4.7 Limitations and Strengths of the Study

Although we used a moderate sample size in validation, more than 40 placenta samples for each group (Tubal + donor egg group, Infertility group and Control group), there were only 21 Tubal samples available and three of them were derived from frozen embryo transfer (FET) cycles. The Tubal only samples were limited and our results suggest that it would be better if we could use Tubal samples as a specific internal control group when we study the influence of ART procedures to ART children instead of using samples from children whose parents had underlying infertility issues. Furthermore, as we used a single placental sample from a standardized location, we cannot be certain that the DNA methylation profile or DNA methylation levels will be the same in all parts of placenta. However, we believe that this is a representative site to study placenta DNA methylation and gene expression as it includes central villous parenchyma. And the early study showed that there is less intraplacental variation than interplacental variation (Turan *et al.*, 2010a).

Our study is the first study using the Tubal group as an internal control to minimize the infertility factors which may affect the DNA methylation levels of ART children, and confirm the role of ART procedures in affecting the epigenetic reprogramming and the potentially the fetal development and growth.

4.8 Placenta and Epigenetics

The placeta is of paramount importance to ensure proper growth and in utero development of the fetus from its earliest stages till the end of pregnancy. Called by some a hallmark of mammalian development (Jaenisch, 1997), the placenta is responsible for the establishment of a tight contact between mother and fetus, providing the fetus with nutrients, allowing for waste to be transferred and ultimately excreted by the mother, enabling the exchanging of gas and protecting the fetus from maternal immune system attacks (Rossant & Cross, 2001). In addition, the placenta has a degree of metabolic and endocrine activity, is involved in secreting hormones responsible for maintaining and regulation various stages of pregnancy, and performs biochemical reactions to protect wnenever possible the fetus from exposure to toxicants or other harmful chemicals (Sood *et al.*, 2006). All of these functions of the placenta together with placental gene expression thus respond to and are marked by environmental insults (Sood *et al.*, 2006; Guo *et al.*, 2008) including ART, and in many ways, the placenta could serve as a record of in utero exposure and pathology. Various compounds and drugs, including but not limited to alcohol (Beattie, 1986), nicotine (Bush *et al.*, 2000), cocaine (Bailey,1987; Pastrakuljic *et al.*, 1999), lead(Rastogi *et al.*, 2007), and phthalates (Mose *et al.*, 2000) have been shown to cross the placenta and alter placental gene expression; some even accumulate in the placental tissue.

Several animal studies have suggested that placental tissues are more sensitive to preimplantation epigenetic disturbance than embryonic tissues. Superovulation followed by *in vivo* development resulted in biallelic expression of imprinted gene Snrpn and H19 in placenta, but loss of imprinting (LOI) did not happened in the embryos (Fortier *et al.*, 2008). And the expression of IGF-II which linked to H19 was increased in the placenta but not in the embryos following superovulation with or without embryo transfer (Fortier *et al.*, 2008). After *in vitro* preimplantation culture, proper imprinted expression for the most part was preserved in the embryo while placental tissues displayed activation of the normally silent allele for H19, Ascl2, Snrpn, Peg3 and Xist (Mann *et al.*, 2004). Moreover, embryo transfer, with or without embryo culture, also led to LOI in placental tissue but not in the embryo (*Rivera et al.*, 2008). This indicates that appropriate imprinting is not restored during post-implantation development of the placenta. In Haycock and Ramsay study, although ethanol-exposed placenta and embryos were severely growth retarded in comparison with saline-treated controls, DNA methylation at paternal and maternal alleles was unaffected in embryos (Haycock & Ramsay, 2009). While paternal alleles were significantly less methylated in ethanol-treated placenta in comparison with saline-treated controls (Haycock & Ramsay, 2009). These studies suggest that imprinting control mechanisms in the embryo are more robust to environmental perturbations, and tissue of trophectoderm origin are more sensitive to preplantation epigenetic ditubance than embryonic tissue.

Deregulation of placentation can lead to adverse outcomes for both mother and child; derailed growth can lead to invasive, sometimes malignant trophoblast disease in the mother (Hui *et al.*, 2005), whereas hypoplastic development is associated with maternal hypertension (preeclampsia; Cross, 2003; Fisher, 2004) and fetal growth retardation (Chaddha *et al.*, 2004; Gluckman *et al.*, 2004; Monk *et al.*, 2004). Intrauterine growth retardation and low birthweight (LBW) are major negative health predictors for

newborns and children, and are associated with chronic diseases expressed later in life, e.g. cardiovascular disease, hypertension and type 2 diabetes (Barker *et al.*, 1993; Curhan *et al.*, 1996a, b; Boyko, 2000; Eriksson *et al.*, 2003; Ross and Beall, 2008). A significant factor in placental development and function is epigenetic regulation. Imprinted genes for instance, which are epigenetically regulated, are abundantly expressed in the placenta and usually lacking in non-placental organisms (Reik and Walter, 2001). As I mentioned in the introduction part, ARTs in humans have been associated with LBWs, small for gestational age, preterm deliveries, birth defects and rare imprinting disorder. Recently, studies from my lab (Katari *et al.*, 2009; Turan *et al.*, 2010) and other researchers (Gomes *et al.*, 2009; Katagiri *et al.*, 2010; Kobayshi *et al.*, 2009; Zechner *et al.*, 2010) showed a aberrant epigenotype in children conceived *in vitro*. Taken together, it seems that environmental effects (e.g. ART) can disturb placental epigenetics and herewith placental development and function, with possible downstream consequences for maternal morbidity, fetal development and disease susceptibility in later life. Especially since several animal studies have suggested that placental tissues are more sensitive to preimplantation epigenetic disturbance than embryonic tissues and ART are increasingly used worldwide, this emphasizes the need for further investigations in human placentas.

4.9 Significance

The causes of the increases in risks of pregnancy complications and adverse perinatal outcomes have been the subject of much controversy—is the reproductive technology to blame or could the adverse outcomes be attributed to factors related to the infertile couple? However, separation of the reproductive technology from those of factors leading to infertility is difficult. When we study the influence of ART procedures to the children by this technology at molecular level, DNA methylation, gene expression

or other quantitive characteristics, researchers are confounded by the same problem. If we can not separate these two tangled factors, we can never know the real cause of the observed adverse outcomes, not to mention the mechnisms behind them. My study is the first human study to separate these two factors (ART procedures and underlying infertility of the parent(s)) at the molecular level by using tubal and donor egg samples as a internal control to ART procedures. By using these two subgroups of ART children, we have show that the DNA methylation differences we observe in the placentas of ART children are associated with some procedure used in ART. We may now begin dissecting the role od individual ART procedures in the adverse outcomes of the children conceived by ART.

CHAPTER 5

CONCLUSIONS AND FUTURE DIRECTIONS

5.1 Conclusions

The quasi-transcriptome-wide DNA methylation profile is altered in the placentas of ART children. We validated six differentially methylated genes in ART children compared to children conceived *in vivo*. This validation may help in developing novel epigenetic biomarkers of ART procedures and improving the procedures in the future. We used Tubal +donor eggs samples or Tubal samples to exclude one confounding factor – underlying infertility – as the only cause of these differences and our results suggest that one or more steps of the ART procedures, not simply infertility of the ART children's parents are associated with the differences of DNA methylation levels of specific genes. The Tubal samples, especially the Tubal samples whose parent(s) were without any underlying infertility issue, are better ART samples to study the influence of ART procedures to children conceived *in vitro*.

5.2 Future Directions

We notice that the DNA methylation level for most of genes we studied have a relatively large range. It will be interesting to study clinically the individual samples with extreme DNA methylation levels of one or more genes and follow them up in the future. As the technology progresses and more and more people benefit from it, it is worth investigating different subgroups of ART samples and discover more genes whose DNA methylation levels, expression levels or both are different in ART children and obtain the whole picture of the affected pathway. Because the ART procedure is probably influencing the fetal development and growth by affecting epigenetic reprogramming, the progress of epigenetics definitely can help us understand the mechanism of epimutations in ART children. Placental tissue sampled after placental-related pathologies and form pregnancies resulting from ART, can be used to screen for epigenetic disturances, instead of being discarded as a meaningless byproduct. Early screening using placenta may lead to earlier intervention or better surveillance of children who may be at risk later in life. With epigenetic marks being reversible, epigenetic therapy may eventually provide promising new challenges. The need for more fundamental research to investigate the involvement of histone modifications other epigenetic factors in placental regulation is increasing too. Although the epigenetic reprogramming in the embryo is more robust to environmental perturbations, it is also interesting to studying the influenced genes or pathways which probably lead to long-term results such as audulthood diseases.

REFERENCES CITED

Aizawa, T., Maruyama, K., Kondo, H., Yoshikawa, K. (1992). Expression of necdin, an embryonal carcinoma-derived nuclear protein, in developing mouse brain. *Brain Res Dev Brain Res.* 68, 265–274.

Akashi, K., Kakizaki, T., Kamiya, H., Fukaya, M., Yamasaki, M., Abe, M., Natsume, R., Watanabe, M., Sakimura, K. (2009) NMDA receptor GluN2B (GluRe2/NR2B) subunit is crucial for channel function; postsynaptic macromolecular organization; and actin cytoskeleton at hippocampal CA3 synapses. *J Neurosci,* 29, 10869–10882.

Andrieu, D., Watrin, F., Niinobe, M., Yoshikawa, K., Muscatelli, F., Fernandez, P.A. (2003) Expression of the Prader-Willi gene Necdin during mouse nervous system development correlates with neuronal differentiation and p75NTR expression. *Gene Expr Patterns.* 3, 761–765.

Barker, D. J., Hales, C. N., Fall, C. H., Osmond, C., Phipps, K., Clark, P.M. (1993) Type 2 (non-insulin-dependent) diabetes mellitus, hypertension and hyperlipidaemia (syndrome X): relation to reduced fetal growth. *Diabetologia.* 36, 62–67.

Bailey, D. N. (1987) Cocaine detection during toxicology screening of a university medical center patient population. *J Toxicol Clin Toxicol.* 25, 71–79.

Barker, P.A., Salehi, A. (2002) The MAGE proteins: Emerging roles in cell cycle progression, apoptosis, and neurogenetic disease. *J Neurosci Res.* 67, 705–712.

Basso, O., Baird, D. D. (2009) Infertility and preterm delivery, birthweight, and Caesarean section: a study within the Danish National Birth Cohort. *Hum Reprod*. 18, 2478–2484.

Beattie, J. O. (1986) Transplacental alcohol intoxication. *Alcohol Alcohol*. 21, 163–166.

Berger, S. L. (2007) The complex language of chromatin regulation during transcription. *Nature*. 447, 407–12.

Bliss, T.V., Collingridge, G. L. (1993) A synaptic model of memory: Long-term potentiation in the hippocampus. *Nature.* 361, 31–39.

Borghol, N., Lornage, J., Blachere, T., Sophie Garret A., Lefevre A. (2006) Epigenetic status of the H19 locus in human oocytes following *in vitro* maturation. *Genomics.* 87,417–26.

Bowdin, S., Allen, C., Kirby, G., Brueton, L., Afnan, M., Barratt, C., Kirkman-Brown, J., Harrison, R., Maher, E. R., Reardon, W. (2007) A survey of assisted reproductive technology births and imprinting disorders. *Hum Reprod*.22, 3237–3240.

Boyko, E. J. (2000) Proportion of type 2 diabetes cases resulting from impaired fetal growth. *Diabetes Care.* 23, 1260–1264.

Brunelli, S., Tagliafico, E., De Angelis, F.G., Tonlorenzi, R., Baesso, S., Ferrari, S., Niinobe, M., Yoshikawa, K., Schwartz, RJ., Bozzoni, I., Cossu, G. (2004) Msx2 and necdin combined activities are required for smooth muscle differentiation in mesoangioblast stem cells. *Circ Res.* 94, 1571–1578.

Buitendijk, S. E. (1999) Children after in vitro fertilization. An overview of the literature. *Int J Technol Assess Health Care*.15, 52–65.

Bush, J.R., Wevrick, R. (2008) The Prader-Willi syndrome protein necdin interacts with the E1A-like inhibitor of differentiation EID-1 and promotes myoblast differentiation. *Differentiation.* 76, 994–1005.

Bush, P. G., Mayhew, T. M., Abramovich, D. R., Aggett, P. J., Burke, M. D., Page, K. R. (2000) Maternal cigarette smoking and oxygen diffusion across the placenta. *Placenta.* 21, 824–833.

Carrell, D.T., Hammoud, S. S. (2010) The human sperm epigenome and its potential role in embryonic development. *Mol Hum Reprod.* 16, 37–47.

Centers for Disease Control and Prevention. 2009 assisted reproductive technology success rates: national summary and fertility clinic reports.

Chaddha, V., Viero, S., Huppertz, B., Kingdom, J. (2004) Developmental biology of the placenta and the origins of placental insufficiency. *Semin Fetal Neonatal Med.* 9, 357 – 369.

Chang, A.S., Moley, K.H., Wangler, M., Feinberg, A.P., DeBaun, M.R., (2005) Association between Beckwith-Wiedemann syndrome and assisted reproductive technology: a case series of 19 patients. *Fertil Steril*. 83, 349–354.

Chatterton, J.E., Awobuluyi, M., Premkumar, L. S., Takahashi, H., Talantova, M., Shin Y., Cui, J., Tu, S., Sevarino, K. A., Nakanishi, N., et al. (2002) Excitatory glycine receptors containing the NR3 family of NMDA receptor subunits. *Nature.* 415, 793–798.

Chen, M., Ni, J., Chang, H. C., Lin, C. Y., Muyan, M.,Yeh, S. (2009) CCDC62/ERAP75 function as a coactivator to enhance estrogen receptor beta-mediated transactivation and target gene expression in prostate cancer cells. *Carcinogenesis.* 30, 841-850.

Chen, M., Ni, J., Zhang, Y., Muyan, M., Yeh, S. (2008). ERAP75 functions as a coactivator to enhance estrogen receptor α transactivation in prostate stromal cells. *Prostate.* 68, 1273-1282.

Cheung, V.G., Spielman, R.S. (2009) Genetics of human gene expression: mapping DNA variants that influence gene expression. *Nat Rev Genet.* 10, 595-604.

Choufani, S., Shuman, C., Weksberg, R. Beckwith–Wiedemann syndrome. (2010) *Am J Med Genet C Semin Med Genet*.154C, 343–354.

Cohen, S., Greenberg, M. E. (2008) Communication between the synapse and the nucleus in neuronal development; plasticity and disease. *Annu Rev Cell Dev Biol.* 24, 183–209.

Couse, J. F., Korach, K. S. (1999) Estrogen receptor null mice: What have we learned and where will they lead us? *Endocr Rev.* 20, 358 – 417.

Cox, G. F., Burger, J., Lip, V., Mau U. A., Sperling, K., Wu, B. L., Horsthemke, B. (2002) Intracytoplasmic sperm injection may increase the risk of imprinting defects. *Am J Hum Genet.* 71, 162–164.

Cross, J.C. (2003) The genetics of pre-eclampsia: a feto-placental or maternal problem? *Clin Genet.* 64, 96–103.

Cull-Candy, S., Brickley, S. & Farrant, M. (2001) NMDA receptor subunits: diversity, development and disease. *Curr. Opin. Neurobiol.* 11, 327–335.

Curhan, G. C., Chertow, G. M., Willett, W. C., Spiegelman, D., Colditz, G. A., Manson, J. E., Speizer, F. E., Stampfer, M. J. (1996a) Birth weight and adult hypertension and obesity in women. *Circulation.* 94, 1310–1315.

Curhan, G. C., Willett, W. C., Rimm, E.B., Spiegelman, D., Ascherio, A.L., Stampfer, M.J. (1996b) Birth weight and adult hypertension, diabetes mellitus, and obesity in US men. *Circulation.* 94, 3246–3250.

Das, S., Sasaki, Y. F., Rothe, T., Premkumar, L. S., Takasu, M., Crandall JE, Dikkes P, Conner DA, Rayudu PV, Cheung W., *et al.* (1998) Increased NMDA current and spine density in mice lacking the NMDA receptor subunit NR3A. *Nature.* 393, 377–381.

de Waal, E., Yamazaki, Y., Ingale, P., Bartolomei, M., Yanagimachi, R., McCarrey, J. R. (2012) Primary epimutations introduced during intracytoplasmic sperm injection (ICSI) are corrected by germline-specific epigenetic reprogramming. *PNAS.* 109, 4163-4168.

DeBaun, M.R., Niemitz, E.L., Feinberg, A. P. (2003) Association of in vitro fertilization with Beckwith–Wiedemann syndrome and epigenetic alterations of LIT1 and H19. *Am J Hum Genet.*72, 156–160.

Deponti, D., Francois, S., Baesso, S., Sciorati, C., Innocenzi, A., Broccoli, V., Muscatelli, F, Meneveri, R., Clementi, E., Cossu, G., Brunelli, S. (2007) Necdin mediates skeletal muscle regeneration by promoting myoblast survival and differentiation. *J Cell Biol.* 179, 305–319.

Deutsch. S., Lyle. R., Dermitzakis. E.T., Attar. H., Subrahmanyan. L., Gehrig. C., *et al.* (2005) Gene expression variation and expression quantitative trait mapping of human chromosome 21 genes. *Hum Mol Genet*. 14, 3741-9.

Doherty, A. S. , Mann, M. R., Tremblay, K. D., Bartolomei, M. S., Schultz, R. M. (2000) Differential effects of culture on imprinted H19 expression in the preim-plantation mouse embryo. *Biol Reprod.* 62, 1526–35.

Doornbo, M. E., Maas, S. M., McDonnell, J., Vermeiden, J. P. W., Hennekam, R. C. M. (2007) Infertility, assisted reproduction technologies and imprinting disturbances: a Dutch study. *Hum Reprod*. 22, 2476–2480.

Draper, E. S., Kurinczuk, J. J., Abrams, K. R., Clarke, M. (1999) Assessment of separate contributions to perinatal mortality of infertility history and treatment: a case-control analysis. *Lancet.* 353, 1746–1749.

du Boris, T.M., Deng, C., Han, M., Newell, K. A., Huang, X. F. (2009) Excitatory and inhibitory neurotransmission is chronically altered following perinatal NMDA receptor blocade. *Eur Neuropsychopharmacol.* 19, 256– 265.

Ericson, A., Nygren, K. G., Olausson, P. O., Ka¨lle´n, B. (2002) Hospital care utilization of infants born after IVF. *Hum Reprod.* 17, 929–932.

Eriksson, J. G., Forsen, T. J., Osmond, C., Barker, D. J. (2003) Pathways of infant and childhood growth that lead to type 2 diabetes. *Diabetes Care.* 26, 3006–3010.

Evans, R. M. (1988) The steroid and thyroid hormone receptor super-family. *Science.* 240, 889–895.

Fajardy. I., Moitrot, E., Vambergue, A., Vandersippe-Millot, M., Deruelle, P., Rousseaux, J. (2009) Time course analysis of RNA stability in human placenta. *BMC Mol Biol,*10, 21.

Fauque, P., Jouannet, P., Lesaffre, C., Ripoche, M. A., Dandolo, L., Vaiman, D., et al. (2007) Assisted reproductive technology affects developmental kinetics, H19 imprinting control region methylation and H19 gene expression in individual mouse embryos. *BMC Dev Biol*. 7, 116.

Fisher, S. J. (2004) The placental problem: linking abnormal cytotrophoblast differentiation to the maternal symptoms of preeclampsia. *Reprod Biol Endocrinol.* 2, 53.

Fodero-Tavoletti, M. T., Hardy, M. P., Cornell, B., Katsis, F., Sadek, C.M., Mitchell, C. A., Kemp, B. E. & Tiganis, T. (2005) Protein tyrosine phosphatase hPTPN20a is targeted to sites of actin polymerization. *Biochem J.* 389, 343-354.

Forrest, D., Yuzaki, M., Soares, H. D., Ng, L., Luk, D. C., Sheng, M., Stewart, C. L., Morgan, J. I., Connor, J. A., Curran, T. (1994) Targeted disruption of NMDAR 1 gene abolishes NMDA response and results in neonatal death. *Neuron.* 13, 325–338.

Fortier, A. L., Lopes, F. L., Darricarrere, N., Martel, J., Trasler, J.M. (2008) Superovulation alters the expression of imprinted genes in the midgestation mouse placenta. *Hum Mol Genet.* 17, 1653–65.

Fujiwara, H., Higuchi, T., Yamada, S., Hirano, T., Sato, Y., Nishioka, Y., et al. (2004) Human extravillous trophoblasts express laeverin, a novel protein that belongs to membrane-bound gluzincin metallopeptidases. *Biochem Biophys Res Commun.* 313, 962–8.

Fumagalli, F., Pasini, M., Frasca, A., Drago, F., Racagni, G., Riva, M.A. (2009) Prenatal stress alters glutamatergic system responsiveness in adult rat prefrontal cortex. *J Neurochem.* 109, 1733–1744.

Gardiner-Garden, M., Frommer, M.(1987) CpG islands in vertebrate genomes. *Journal of Molecular Biology.* 196, 261–82.

Gelbaya, T., Tsoumpou, I., Nardo, L. (2010) The likelihood of live birth and multiple birth after single versus double embryo transfer at the cleavage stage: a systematic review and meta-analysis. *Fertil Steril.* 94, 936–945.

Gerard, M., Hernandez. L., Wevrick, R., Stewart, C.L. (1999) Disruption of the mouse necdin gene results in early post-natal lethality. *Nat Genet.* 23, 199–202.

Gicquel, C , Gaston, V., Mandelbaum, J., Siffroi, J. P., Flahault, A., Le Bouc, Y. (2003) *In vitro* fertilization may increase the risk of Beckwith–Wiedemann syndrome related to the abnormal imprinting of the KCN1OT gene. *Am J Hum Genet.* 72, 1338–1341.

Giguere, V., Yang, N., Segui, P., Evans, R. M. (1988) Identification of a new class of steroid hormone receptors. *Nature,* 331, 91– 94.

Gluckman, P. D., Hanson, M.A. (2004) Maternal constraint of fetal growth and its consequences. *Semin Fetal Neonatal Med.* 9, 419–425.

Gomes, M. V., Huber, J., Ferriani, R. A., Amaral, Neto, A. M., Ramos, E. S. (2009) Abnormal methylation at the KvDMR1 imprinting control region in clinically normal children conceived by assisted reproductive technologies. *Mol Hum Reprod.* 15, 471–477.

Green, M. P., Mouat, F., Miles, H. L., Hopkins, S. A., Hofman, P. L., Peek, J., Cutfield, W. S. (2010) Anthropometric and endocrine differences exist between children conceived after the transfer of a fresh or thawed embryo compared to naturally conceived controls. *Aust N Z JObstet Gynaecol*. 50, 25–27

Green, S., Walter, P., Kumar, V., Krust, A., Bornert, J. M., Argos, P., Chambon, P. (1986) Human oestrogen receptor cDNA: Sequence, expression and homology to v-erb-A. *Nature.* 320, 134 – 139.

Greene, G.L., Gilna, P., Waterfield, M., Baker, A., Hort, Y., Shine, J. (1986) Sequence and expression of human estrogen receptor complementary DNA. *Science.* 231, 1150 – 1154.

Guo, L., Choufani, S., Ferreira, J., Smith, A., Chitayat, D., Shuman, C., Uxa, R., Keating, S., Kingdom, J., Weksberg, R. (2008) Altered gene expression and methylation of the human chromosome 11 imprinted region in small for gestational age (SGA) placentae. *Dev Biol*. 320, 79–91.

Halliday, J., Oke, K., Breheny, S., Algar, E., Amor, D. J. (2004) Beckwith – Wiedemann syndrome and IVF: a case-control study. *Am J Hum Gene.* 75, 526 – 528.

Hammoud, S.S., Purwar, J., Pflueger, C., Caims, B.R., Carrell, D.T. (2010)Alterations in sperm DNA methylation patterns at imprinted loci in two classes of infertility. *Fertil Steril.* 94, 1728–33.

Haycock, P.C., Ramsay, M. (2009) Exposure of mouse embryos to ethanol during preimplantation development: effect on DNA methylation in the h19 imprinting control region. *Biol Reprod.* 81, 618–627.

Hiendleder, S., Wirtz, M., Mund, C., Klempt, M., Reichenbach, H. D., Stojkovic, M., et al. (2006) Tissue-specific effects of *in vitro* fertilization procedures on genomic cytosine methylation levels in overgrown and normal sized bovine fetuses. *Biol Reprod.* 75, 17–23.

Horie, A., *et al.* (2012) Laeverin/aminopeptidase Q induces trophoblast invasion during human early placentation. *Hum. Reprod.* 27, 1267-1276.

Hui, P., Martel, M., Parkash, V. (2005) Gestational trophoblastic diseases: recent advances in histopathologic diagnosis and related genetic aspects. *Adv Anat Pathol.* 12, 116–125.

Hvidtjørn, D., Grove, J., Schendel, D. E., Vaeth, M., Ernst, E., Nielsen, L. F., Thorsen, P. (2006) Cerebral palsy among children born after in vitro fertilization: the role of preterm delivery—a population-based, cohort study. *Pediatrics*. 118, 475–482.

Hvidtjørn, D., Grove, J., Schendel, D., Sværke, C., Schieve, L. A., Uldall, P., Ernst, E., Jacobsson, B., Thorsen, P. (2010) Multiplicity and early gestational age contribute to an increased risk of cerebral palsy from assisted conception: a population-based cohort study. *Hum Reprod*. 25, 2115–2123.

Iliadou, A. N., Janson, P. C. J., Cnattingius, S, (Karo- linska Institutet, Stockholm; Sweden). (2011) Epigenetics and assisted reproductive technology (Review). *J Intern Med.* 270, 414–420.

Jaenisch, R. (1997) DNA methylation and imprinting: why bother? *Trends Genet.* 13, 323–329.

Jay, P., Rougeulle, C., Massacrier, A., Moncla, A., Mattei, M. G., Malzac, P., Roeckel, N., Taviaux, S., Lefranc, J. L., Cau, P., Berta, P., Lalande, M., and Muscatelli, F. (1997) The human necdin gene, NDN, is maternally imprinted and located in the Prader–Willi syndrome chromosomal region. *Nat. Genet.* 17, 357–361.

Jenkins, T. G., Carrell, D. T. (2011) The paternal epigenome and embryogenesis: poising mechanisms for development. *Asian J Androl*. 13, 76–80.

Katagiri, Y., Aoki, C., Tamaki-Ishihara, Y., Fukuda, Y., Kitamura, M., Matsue, Y., *et al.* (2010) Effects of assisted reproduction technology on placental imprinted gene expression. *Obstet Gynecol Int.* 2010.

Katari, S., Turan, N., Bibikova. M., Erinle, O., Chalian, R., Foster, M., et al. (2009) DNA methylation and gene expression differences in children conceived *in vitro* or *in vivo*. *Hum Mol Genet.* 18, 3769–78.

Keller, R.(2002) Shaping the vertebrate body plan by polarized embryonic cell movements. *Science.* 298, 1950–1954.

Khosla, S., Dean, W., Brown, D., Reik, W., Feil, R. (2001) Culture of preimplantation mouse embryos affects fetal development and the expression of imprinted genes. *Biol Reprod 2001*. 64, 918–26.

Kobayashi, H., Hiura, H., John, R. M., Sato, A., Otsu, E., Kobayashi, N., *et al.* (2009) DNA methylation errors at imprinted loci after assisted conception originate in the parental sperm. *Eur J Hum Genet.*17, 1582–91.

Kuiper, G. G., Enmark, E., Pelto-Huikko, M., Nilsson, S., Gustafsson, J. A. (1996) Cloning of a novel receptor expressed in rat prostate and ovary. *Proc Natl Acad Sci USA.* 93, 5925– 5930.

Kutsuwada, T., Sakimura, K., Manabe, T., Takayama, C., Katakura, N., Kushiya, E., Natsume, R., Watanabe, M., Inoue, Y., Yagi, T., et al. (1996) Impairment of suckling response, trigeminal neuronal pattern formation, and hippo-campal LTD in NMDAR epsilon 2 subunit mutant mice. *Neuron.* 16, 333–334.

Kuwajima, T., Taniura, H., Nishimura, I., Yoshikawa, K. (2004). Necdin interacts with the Msx2 homeodomain protein via MAGE-D1 to promote myogenic differentiation of C2C12 cells. *J Biol Chem.* 279, 40484–40493.

Lewis, A., Mitsuya, K., Umlauf, D., Smith, P., Dean, W., Walter, J., et al. (2004) Imprinting on distal chromosome 7 in the placenta involves repressive histone methylation independent of DNA methylation. *Nat Genet*. 36, 1291–5.

Li, T., Vu, T. H., Ulaner, G. A., Littman, E., Ling, J. Q., Chen, H. L., et al. (2005) IVF results in de novo DNA methylation and histone methylation at an Igf2-H19 imprinting epigenetic switch. *Mol Hum Reprod.* 11, 631–40.

Lidegaard, O., Pinborg, A., Andersen, A. N. (2005) Imprinting diseases and IVF: Danish National IVF cohort study. *Hum Reprod.* 20, 950–954.

Lim, D., Bowdin, S. C., Tee, L., Kirby, G. A., Blair, E., Fryer, A., Lam. W., Oley, C., Cole, T., Brueton, L. A. et al. (2009) Clinical and molecular genetic features of Beckwith-Wiedemann syndrome associated with assisted reproductive technologies. *Hum Reprod.* 24, 741–747.

Lonard, D. M., *et al.* (2007) Nuclear receptor coregulators: judges, juries, and executioners of cellular regulation. *Mol. Cell*. 27, 691–700.

Ludwig, M. (2009) Are adverse outcomes associated with assisted reproduction related to the technology or couples' subfertility?. *Nat Clin Pract Urol.* 6, 8 – 9.

MacDonald, H. R., Wevrick, R. (1997). The necdin gene is deleted in Prader–Willi syndrome and is imprinted in human and mouse. *Hum. Mol. Genet.* 6, 1873–1878.

Maher, E. R., Brueton, L. A., Bowdin, S. C., Luharia, A., Cooper, W., Cole, T. R., Macdonald, F., Sampson, J.R., Barratt, C. L., Reik, W. et al. (2003) Beckwith–Wiedemann syndrome and assisted reproduction technology (ART). *J Med Genet*. 40, 62–64.

Makhoul, I. R., Tamir, A., Bader, D., Rotschild, A., Weintraub, Z., Yurman, S., Reich, D., Bental, Y., Jammalieh, J., Smolkin, T. et al. (2009) *In vitro* fertilisation and use of

ovulation enhancers may both influence childhood height in very low birthweight infants. *Arch Dis Child Fetal Neonatal Ed.* 94, 355 –359.

Mangelsdorf, D.J., Thummel, C., Beato, M., Herrlich, P., Schutz, G., Umesono, K., Blumberg, B., Kastner, P., Mark, M., Chambon, P., Evans, R. M. (1995) The nuclear receptor superfamily: The second decade. *Cell.* 83, 835 – 839.

Manipalviratn, S., DeCherney, A., Segars, J. (2009) Imprinting disorders and assisted reproductive technology. *Fertil Steril.* 91, 305-31.

Mann, M. R., Lee, S. S., Doherty, A. S., Verona, R. I., Nolen, L.D., Schultz, R. M., et al. (2004) Selective loss of imprinting in the placenta following preimplantation development in culture. *Development.*131, 3727–35.

Marques, C. J., Francisco, T., Sousa, S., Carvalho, F., Barros, A., Sousa, M. (2010) Methylation defects of imprinted genes in human testicular spermatozoa. *Fertil Steril*. 94, 585–94.

Maruyama, K., Usami, M., Aizawa, T., Yoshikawa, K. (1991) A novel brain-specific mRNA encoding nuclear protein (necdin) expressed in neurally differentiated embryonal carcinoma cells. *Biochem Biophys Res Commun,* 178, 291–296.

Maruyama, M., *et al.* (2007) Laeverin/aminopeptidase Q, a novel bestatin-sensitive leucine aminopeptidase belonging to M1family of aminopeptidases. *J. Biol. Chem.* 282, 20088-20096.

McBain, C. J., Mayer, M. L. (1994) N-methyl-D-aspartic acid receptor structure and function. *Physiol Rev.* 74, 723–760.

Middelburg, K. J., Heineman, M. J., Bos, A. F., Pereboom. M., Fidler. V., Hadders-Algra M. (2009) The Groningen ART cohort study: ovarian hyperstimulation and the *in vitro* procedure do not affect neurological outcome in infancy. *Hum Reprod*. 24, 3119–3126.

Middelburg, K.J., Haadsma, M.L., Heineman, M.J., Bos, A.F., Hadders-Algra, M. (2010) Ovarian hyperstimulation and the *in vitro* fertilization procedure do not influence early neuromotor development; a history of subfertility does. *Fertil Steril*. 93, 544–553.

Miles, H. L., Hofman, P. L., Peek, J., Harris, M., Wilson, D., Robinson, E. M., Gluckman, P. D., Cutfield, W.S. (2007) *In vitro* fertilization improves childhood growth and metabolism. *J Clin Endocrinol Metab.* 92, 3441–3445.

Monk, D., Arnaud, P., Frost, J., Hills, F. A., Stanier, P., Feil, R., *et al.* (2009) Reciprocal imprinting of human *GRB10* in placental trophoblast and brain: evolutionary conservation of reversed allelic expression. *Hum Mol Genet*. 18, 3066–74.

Monk, D., Moore, G. E. (2004) Intrauterine growth restriction—genetic causes and consequences. *Semin Fetal Neonatal Med,* 9, 371–378.

Moore, T., Haig, D. (1991) Genomic imprinting in mammalian development: a parental tug-of-war. *Trends Genet.* 7, 45–49.

Morison, I. M., Ramsay, J. P., Spencer, H. G. (2005) A census of mammalian imprinting. *Trends Genet.* 21, 457–65.

Mose, T., Knudsen, L. E., Hedegaard, M., Mortensen, G.K. (2007) Transplacental transfer of monomethyl phthalate and mono(2-ethylhexyl) phthalate in a human placenta perfusion system. *Int J Toxicol*. 26, 221–229.

Mosselman, S., Polman, J., Dijkema, R. (1996) ER beta: Identification and characterization of a novel human estrogen receptor. *FEBS Lett.* 392, 49 – 53.

Muscatelli, F., Abrous, D. N., Massacrier, A., Boccaccio, I., Le Moal, M., Cau, P., Cremer, H. (2000) Disruption of the mouse Necdin gene results in hypothalamic and behavioral alterations reminiscent of the human Prader-Willi syndrome. *Hum Mol Genet.* 9, 3101–3110.

Nanassy, L., Carrell. D. T. (2011a) Abnormal methylation of the promoter of CREM is broadly associated with male factor infertility and poor sperm quality but is improved in sperm selected by density gradient centrifugation. *Fertil Steril.* 95, 2310–4.

Nanassy, L., Carrell, D. T. (2011b) Analysis of the methylation pattern of six gene promoters in sperm of men with abnormal protamination. *Asian J Androl.* 13, 342–6.

Ombelet W, De Sutter P, Van der Elst J, Martens G. (2005) Multiple gestation and infertility treatment: registration, reflection and reaction–the Belgian project. *Hum Reprod Update*. 11, 3–14.

Orstavik, K. H., Eiklid, K., van der Hagen, C. B., Spetalen, S., Kierulf, K., Skjeldal, O., Buiting, K. (2003) Another case of imprinting defect in a girl with Angelman syndrome who was conceived by intracytoplasmic semen injection. *Am J Hum Genet.* 72, 218–219.

Oudejans, C. B. M., van Dijk, M. (2008) Placental gene expression and pre-eclampsia. *Placenta.* 29, 78–82.

Pandian, Z., Bhattacharya. S., Ozturk, O., Serour, G., Templeton, A. (2009) Number of embryos for transfer following *in-vitro* fertilisation or intra-cytoplasmic sperm injection. *Cochrane Database Syst Rev.* 15, Art. No.: CD003416.

Paoloni-Giacobino, A. (2007) Epigenetics in reproductive medicine. *Pediatr Res.* 61, 51R – 57R.

Pastrakuljic, A., Derewlany, L. O., Koren, G. (1999) Maternal cocaine use and cigarette

smoking in pregnancy in relation to amino acid transport and fetal growth. *Placenta*. 20, 499–512.

Peters, E.H., Rojas-Caro, S., Brigell, M.G., Zahorchak, R.J., des Etages, S.A., Ruppel, P.L., *et al.* (2007) Quality-controlled measurement methods for quantification of variations in transcript abundance in whole blood samples from healthy volunteers. *Clin Chem.* 53, 1030-7.

Petrovic, N., Schacke, W., Gahagan, J. R., O'Conor, C. A., Winnicka, B., Conway, R. E., *et al.* (2007) CD13/APN regulates endothelial invasion and filopodia formation. *Blood.* 110, 142–50.

Pinborg, A., Loft, A., Schmidt, L., Greisen, G., Rasmussen, S, Andersen, A. N. (2004) Neurological sequelae in twins born after assisted conception: Controlled National Cohort Study. *BMJ* 329, 311–314.

Powell, K. (2003) Fertility treatments: seeds of doubt. *Nature.* 422, 656–8.
Rivera, R. M., Stein, P., Weaver, J.R., Mager, J., Schultz, R. M., Bartolomei, M. S. (2008) Manipulations of mouse embryos prior to implantation result in aberrant expression of imprinted genes on day 9.5 of development. *Hum Mol Genet*. 17, 1–14.

Rastogi, S., Nandlike, K., Fenster, W. (2007) Elevated blood lead levels in pregnant women: identification of a high- risk population and interventions. *J Perinat Med.* 35, 492–496.

Rivera, R. M., Stein, P., Weaver, J. R., Mager, J., Schultz, R. M., Bartolomei, M.S. (2008) Manipulations of mouse embryos prior to implantation result in aberrant expression of imprinted genes on day 9.5 of development. *Hum Mol Genet.* 17, 1–14.

Ross, M.G., Beall, M. H. (2008) Adult sequelae of intrauterine growth restriction. *Semin Perinatol.* 32, 213–218.

Rossant, J., Cross, J. C. (2001) Placental development: lessons from mouse mutants. *Nat Rev Genet.* 2, 538–548.

Santos, F., Hendrich, B., Reik, W., Dean, W. (2002) Dynamic reprogramming of DNA methylation in the early mouse embryo. *Dev Biol*. 241, 172–82.

Sato, A., Otsu, E., Negishi, H., Utsunomiya, T., Arima, T. (2007) Aberrant DNA methylation of imprinted loci in superovulated oocytes. *Hum Reprod.* 22, 26–35.

Shi, W., Haaf, T. (2002) Aberrant methylation patterns at the two-cell stage as an indicator of early developmental failure. *Mol Reprod Dev.* 63, 329–34.

Sibai, B., Dekker, G., Kupferminc, M. (2005) Pre-eclampsia. *Lancet* 26, 785–99.
Sitras, V., *et al.* (2009) Differential Placental Gene Expression in Severe Preeclampsia. *Placenta*. 30, 424-433.

Sood, R., Zehnder, J. L., Druzin, M. L., Brown, P. O. (2006) Gene expression patterns in human placenta. *Proc Natl Acad Sci USA*. 103, 5478–5483.

Steffenburg, S., Gillberg, C. L., Steffenburg, U., Kyllerman, M. (1996) Autism in Angelman syndrome: a population-based study. *Pediatr Neurol*. 14, 131–136.

Strömberg, B., Dahlquist, G., Ericson, A., Finnström, O., Köster, M., Stjernqvist. K. (2002) Neurological sequelae in children born after *in-vitro* fertilisation: a population-based study. *The Lancet.* 359, 461–465.

Sunderam, S., Chang, J., Flowers, L., Kulkarni. A., Sentelle, G., Jeng, G., Macaluso, M. (2009) Assisted reproductive technology surveillance—United States, 2006. *Morb Mortal Wkly Rep.* 58, 1–25.

Sutcliffe, J. S., Han, M., Christian, S. L., and Ledbetter, D. H. (1997) Neuronally-expressed necdin gene: An imprinted candidate gene in Prader–Willi syndrome. *Lancet* 350, 1520–1521.

Taniura, H., Matsumoto, K., Yoshikawa, K. (1999) Physical and functional interactions of neuronal growth suppressor necdin with p53. *J. Biol. Chem.* 274, 16242–16248.

Taniura, H., Taniguchi, N., Hara, M., and Yoshikawa, K. (1998) Necdin, a postmitotic neuron-specific growth suppressor, interacts with viral transforming proteins and cellular transcription factor E2F1. *J. Biol. Chem.* 273, 720–728.

Tsai, G., Coyle, J.T. (2002) Glutamatergic mechanisms in schizophrenia. *Annu Rev Pharmacol Toxicol*. 42, 165–179.

Tsai, M. J., O'Malley, B. W. (1994) Molecular mechanisms of action of steroid/thyroid receptor superfamily members. *Annu Rev Biochem*. 63, 451–486.

Turan, N., Katari, S., Coutifaris, C., Sapienza, C. (2010a) Explaining inter-individual variability in phenotype: Is epigenetics up to the challenge? *Epigenetics*. 5, 1–4

Turan, N., Katari, S., Gerson, L., Chalian, R, Foster, M., Gaughan, J., Coutifaris, C., Sapienza, C. (2010b) Inter- and Intra- Individual Variation in Allele-Specific DNA Methylation and Gene Expression in Children Conceived using Assisted Reproductive Technology. *PLoS Genetics*. 6, e1001033

Turan, N., Ghalwash, M. F., Katari, S., Coutifaris, C., Obradovic, Z., Sapienza, C. (2012) DNA methylation differences at growth related genes correlate with birth weight: a molecular signature linked to developmental origins of adult disease? *BMC Medical Genomics*. 5, 10

van Eekelen, M., Runtuwenw, V., Masselink, W., den Hertog, J. (2012) Pair-Wise regulation of Convergence and Extension Cell Movements by Four Phosphatases via RhoA. *PLoS ONE.* 7, e35913.

Waddington, C. H. (1942) The epigenotype. *Endeavour*. 1, 18–20.

Wang, Z., Xu, L., He, F.. (2010) Embryo vitrification affects the methylation of the H19/Igf2 differentially methylated domain and the expression of H19 and Igf2. *Fertil Steril.* 93, 2729–33.

Warga, R. M., Kimmel, C. B. (1990) Cell movements during epiboly and gastrulation in zebrafish. *Development.* 108, 569–580.

Whitney. A.R., Diehn. M., Popper. S.J., Alizadeh. A.A., Boldrick. J.C., Relman. D.A., Brown. P.O. (2003) Individuality and variation in gene expression patterns in human blood. *Proc Natl Acad Sci USA.* 100, 1896-901.

Williams, M. A., Goldman, M. B., Mittendorf, R., Monson, R. R. (1991) Subfertility and the risk of low-birth-weight. *Fertil Steril*. 56, 668–671.

Wu, Ct., Morris, J. R. (2001) Genes, genetics, and epigenetics: a correspondence. *Science.* 293, 1103–1105.

Yang, S.N., Tang, Y.G., Zucker, R. S. (1999) Selective induction of LTP and LTD by postsynaptic [Ca2+]i elevation. *J Neurophysiol.* 81, 781– 787.

Yoshikawa, K. (2000) Cell cycle regulators in neural stem cells and postmitotic neurons. *Neurosci. Res.* 37, 1–14.

Young, L. E., Fernandes, K., McEvoy, T. G., Butterwith, S. C., Gutierrez, C. G., Carolan, C., Broadbent, P. J., Robinson, J. J., Wilmut, I., Sinclair, K.D. (2001) Epigenetic change in IGF2R is associated with fetal overgrowth after sheep embryo culture. *Nat Genet.* 27, 153–154.

Young, L., Sinclair, K., Wilmut, I. (1998) Large offspring syndrome in cattle and sheep. *Rev Reprod.* 3, 155–163.

Zaitseva, I., Zaitsev, S., Alenina, N., Bader, M., Krivokharchenko, A. (2007) Dynamics of DNA demethylation in early mouse and rat embryos developed *in vivo* and *in vitro*. *Mol Reprod Dev.*74, 1255–61.

Zechner, U., Pliushch, G., Schneider, E., El Hajj, N., Tresch, A., Shufaro, Y., *et al.* Quantitative methylation analysis of developmentally important genes in human pregnancy losses after ART and spontaneous conception. *Mol Hum Reprod.*16, 704–13.

Zegers-Hochschild, F. *et al.*. (2009) International Committee for Monitoring Assisted Reproductive Technology (ICMART) and the World Health Organization (WHO) revised glossary of ART terminology, 2009. *Fertil. Steril*. 92, 1520–1524

Zhu. J. L., Hvidtjørn, D., Basso, O., Obel, C., Thorsen, P., Uldall, P., Olsen, J. (2010) Parental infertility and cerebral palsy in children. *Hum Reprod*. 25, 3142–3145.

Zhuo, M. (2009) Plasticity of NMDA receptor NR2B subunit in memory and chronic pain. *Mol Brain,* 2, 4.