ASSOCIATION OF PLURIPOTENCY GENE PROMOTER METHYLATION WITH THE CHROMOSOMAL STATUS OF PRODUCTS OF CONCEPTION

by

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ABSTRACT OF MASTER THESIS

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Infertility affects one in six couples and often necessitates the use of assisted reproductive technology (ART). While ART is the most effective treatment, efficiency remains poor with less than 13% of transferred *in vitro* fertilization (IVF) derived embryos resulting in a live birth according to the Center for Disease Control. This has led to routine use of multiple embryo transfer to increase pregnancy rates. However, as a result of multiple embryo transfer, a significant proportion of IVF pregnancies involve multiples. Indeed, multiple gestation is the most common complication associated with ART and is now the primary focus of research and development in reproductive medicine. The ability to identify the embryo with true reproductive potential could overcome the need for multiple embryo transfer in order to achieve reasonable pregnancy rates from IVF. Differentiation and establishment of the trophectoderm lineage during preimplantation embryo development represents a potential target to identify new biomarkers of reproductive potential. Several gene promoters have already been shown to be

differentially methylated in pluripotent versus differentiated cells. These promoters include: *NANOG, PTPN6, RAB25, LYST, GBP3, MGMT, Oct4* and *Elf5*. The extent of methylation of these promoters was characterized after the development of a methodology for methylation sensitive restriction enzyme digestion followed by quantitative real-time PCR. Chromosomal aneuploidy is a well characterized marker of reproductive potential. The level of differentiation inferred from methylation status of these promoters was used to evaluate whether aneuploid and euploid conceptions possess unique levels of differentiation. Results indicate that *GBP3* promoter methylation is significantly different in aneuploid relative to euploid conceptions supporting the concept that chromosomally normal embryos may differentiate more successfully than chromosomally abnormal embryos.

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I would also like to thank Dr. Jennifer Moore from Rutgers University for letting me obtain induced pluripotent cells which was a critical factor in this work, and Dr. Manuel Krispin from Zymo Research Corporation and his team for technical assistance and discussion and for providing me with the reagents imperative for my work.

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Introduction

The first cell differentiation event in the mammalian development is the formation of two distinct cell lineages: trophectoderm (TE) and inner cell mass (ICM). The TE gives rise to the placenta, while the ICM gives rise to the three germ layers, endoderm, mesoderm and ectoderm. The TE is a vital tissue of the fetal development as it becomes the structure that provides nutrients from the mother to the developing embryo (1). The placenta is formed from three extraembryonic lineages: trophoblasts which are the epithelial cells that physically connect the embryo to the uterus, endoderm and mesoderm which are derived from ICM (2). Interestingly, the ICM differentiation does not begin until the first placental structure has formed (2). Therefore, the development of the embryo proper is dependent on proper formation of the structure that gives rise to the placenta.

One of the epigenetic modifications in mammals is gene repression accomplished by the covalent addition of methyl group to the 5-cytosine base of CpG dinucleotide (3). About 60% of promoters colocalize with CpG islands, which have approximately ten times higher CpG frequency than the genome average, and CpG sites remain mostly unmethylated in these regions. In nonisland CpG promoters, the frequency of CpG dinucleotides is the same as the genome average and includes genes whose expression is restricted to a limited number of cell types and are associated with tissue specific genes (4).

Schultz (2002) describes three functions of the maternal to zygotic transition (MZT) that are required for development. They are: destruction of oocyte-specific transcripts,

replacement of maternal transcripts with zygotic transcripts and reprogramming in the pattern of gene expression (5). It has been shown that the paternal and maternal genomes are demethylated right after fertilization and both genomes are remethylated around the time of implantation, differentially in embryonic and extraembryonic lineages (6). CpG dinucleotide methylation results in the repressive state of genes without changing the actual nucleotide sequence. When CpG dinucleotide is methylated at 5-position of the cytosine, it positions itself into the major groove of the DNA and does not interfere on the pairing of nucleotides between purines and pyrymidines (3). The maintenance of the methylation status or de novo methylation is accomplished by DNA methylatransferases Dnmt1 and Dnmt3a/3b respectively (3).

There are two developmental periods that result in reprogramming of methylation patterns: one in germ cells and the other in preimplantation embryos (6). Demethylation of sperm and egg genome occurs early in development then the germ cells enter mitotic or meiotic arrest until remethylation several days later in male germ cells and after birth in oocytes in females (6). The second major developmental period of reprogramming of methylation occurs in preimplantation embryos. Immediately after fertilization the paternal genome is actively demethylated while the maternal genome demethylation occurs by a passive mechanism (6). Remethylation of the embryo genome occurs at the time of implantation (6). Smith et al. (2012) describe a unique regulatory pattern of DNA methylation in mouse embryos through embryonic day 7.5, with methylation levels being the lowest in the ICM of the blastocyst (7).

The first differentiation pattern in embryo development occurs during the blastocyst stage and is dependent upon quantitative amount of *Oct4* for the TE/ICM differentiation (8). The ICM initially has the capacity to form all the tissues, while TE forms the extra-embryonic tissue, the placenta (9). Trophoblast from first trimester placenta shows proliferative potential, a characteristic that is not found in third trimester placenta (10).

Cell adhesion is fundamental in development and guides early stages in morphogenesis by regulating epithelial differentiation and by allocating blastomeres to one of the two cell lineages of the blastocyst (11). The first epithelium of the mammalian development is the trophectoderm (11). Improper differentiation of trophectoderm has been implicated in Down syndrome, in which syncytiotrophoblast formation is defective (12). Regulated expression of certain proteins, namely ZO-1, syncytin 1 and 2, Cx43, is required for normal trophoblast development (12). Studying the methylation pattern of transcription factors that are important to the development and differentiation of the human embryo is an important undertaking that could help us understand the development of postimplantation embryo. It has been reported that fetal aneuploidy may be associated with histomorphological features like abnormalities of the trohphoblastic layer (13). However, the predictive value of chromosomally normal and abnormal abortions from chorionic villi using histology is not reliable or adequate (14).

We have chosen to study nine genes that are differentially methylated in differentiated cells versus pluripotent cells. Nishino et al. identified genes that are differentially methylated and differentially expressed in pluripotent cells. *RAB25* and *PTPN6* show hypomethylation and high expression in pluripotent cells, while *LYST* and *GBP3* show

hypermethylation and low expression in differentially methylated stem cell specific sites. *Oct4* was among the stem cell-required differentially methylated regions with hypomethylated and high expression in iPSCs (15). Ablation of *PTPN6* suggests proliferation and differentiation (16). *NANOG* and *Oct4* are genes that are critical to early embryo development as they play a role in the blastocyst proliferation and differentiation (17). Knockout of *NANOG* results in loss of pluripotency of ICM and differentiation into endoderm-like cells (17). *Oct4* is located on chromosome 6 and its mRNA levels are low in adult human tissues (18). *Oct4* knockout show loss of pluripotency in ICM and differentiation into trophoblast-like cells (17). Oct-4 and *NANOG* are tissue specific genes that are expressed during development and induced by demethylation (15). *NANOG* expression is initially detected in the interior cells of the compacted morulae and is later limited only to the ICM and eventually restricted to the epiblast (19). *Elf5* is present in the human placenta villous cytotrophoblast cells and is necessary for trophoblast stem cells self-renewal (20). *MGMT* has been shown to be hypermethylated in human embryonic stem cells and hypomethylated in normal differentiated cells (21). Gene expression analysis of *MGMT* promoter reveal that methylation status is inversely proportional with gene expression (21).

It has been described that placing a second ICM into the blastocoel cavity promotes a second zone of proliferation in the trophectoderm; whereas terminal differentiation of the trophectoderm occurs when the ICM is removed from the blastocoel (2). The developmental potential of the embryo can therefore be studied by level of differentiation of trophectoderm which eventually becomes the placenta.

Paparegious et al. studied differential methylation patterns using enrichment of free fetal DNA in maternal blood (placenta) during the pregnancy in 5 different (21, 18, 13, X, and Y) chromosomes. The group observed less methylation in first trimester placentas when compared to third trimester placentas (22). Authors also identified regions that have contrary methylation status between first and third trimesters. In chromosomes 13 and Y, most of differentially methylated regions show hypomethylation in 1st trimester but most become hypermethylated in third trimester. For chromosomes 21 and X, DMR were hypomethylated in 1st and third trimesters. For chromosome 18, DMR showed equal number of hypermethylated and hypomethylated genes (22).

The long term aim of our study is to test whether the extent of differentiation of what eventually becomes extraembrynic tissue is predictive of reproductive potential of human embryos. In the current study, we hypothesize that the level of differentiation in aneuploid embryos is not as progressive as in euploid embryos. To test this, several pluripotency and differentiation markers were selected based on the literature to study the extent of differentiation in villi cells from human conceptions.

Methods

Experimental Design

This study was divided into three phases in order to test whether the level of differentiation in aneuploid embryos is not as progressive as in euploid embryos. First, quantitative real-time PCR (qPCR) based assays of pluripotency gene promoter methylation were tested on large amounts of starting material from a fibroblast cell line

(indicative of differentiated cells) and an induced pluripotent stem cell (iPSC) line (indicative of pluripotent or undifferentiated cells) in order to confirm the assays would perform as expected. In the second phase, the same assays were evaluated on lower amounts of starting material in order to determine applicability to a trophectoderm biopsy (~5 cells). The third phase involved evaluating the methylation status of large amounts of genomic DNA from villi tissue (extraembryonic material) that have been karyotyped by SNP microarray analyses in order to determine if aneuploid and euploid conceptions had different levels of differentiation.

DNA isolation

iPSCs were obtained from Rutgers Stem Cell Research Center (Rutgers University, Piscataway, NJ). Human embryonic stem cells (hESC), cell line BG01V, were obtained from Invitrogen (Invitrogen, Carlsbad, CA). A fibroblast cell line (GM00323) was obtained from the Coriell Cell Repository (CCR, Camden, NJ). Genomic DNA (gDNA) was isolated from large numbers of cells using the Qiagen DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA). Five-cell samples were collected under a dissecting microscope in a 1 μL volume and loaded into a 0.2 mL nuclease-free polymerase chain reaction (PCR) tube (Ambion Inc., Austin, TX) using a 100 mm stripper tip and pipette (Midatlantic Diagnostics, Mount Laurel, NJ). Cells were lysed using alkaline lysis and neutralization as previously published (25). Lysates were stored at -30^{o} C until further analysis.

DNA from miscarriage villi after Assisted Reproductive Technology (ART) were obtained from previously processed samples. Previous processing included isolation of

villi cells from products of conception, DNA purification, and SNP microarray karyotype analysis. SNP microarray karyotype analysis includes processing samples by whole genome amplification (WGA) on isolated genomic DNA using Genome Plex Single Cell WGA4 Kit (Sigma Aldrich Inc., St. Louis, MI). Sequentially DNA was purified using GeneElute PCR Purification Kit (Sigma Aldrich Inc., St. Louis, MI). DNA was processed on the 262K NspI SNP genotyping array per manufacturers recommendations (Affymeterix Inc., Santa Clara, CA). The Qiagen DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA) was used for isolation of genomic DNA. Concentration of DNA was determined using nanodrop 8000 spectrophotometer (Thermo Scientific, Wilmington, DE). Genomic DNA was stored at -30^{o} C until further analysis.

Methylation Sensitive Restriction Enzyme qPCR

In order to study the methylation status of genes important for differentiation or for maintenance of pluripotency, we have used methylation-sensitive restriction enzyme followed by qPCR (MSRE-qPCR). This method requires digestion of genomic DNA with methylation sensitive restriction enzyme and subsequently amplifying the digested template by PCR using gene specific primers (27). Test reaction consists of reagents and methylation sensitive enzyme that cleaves an unmethylated CpG site, and a ‘mock digestion’ reaction where enzyme is not added. The difference in Ct values between the two reactions depicts the methylation status of that particular site in the sample. High difference of delta Ct value between a test reaction and a ‘mock’ or reference reaction means the site is unmethylated as a test reaction site was digested by the enzyme.

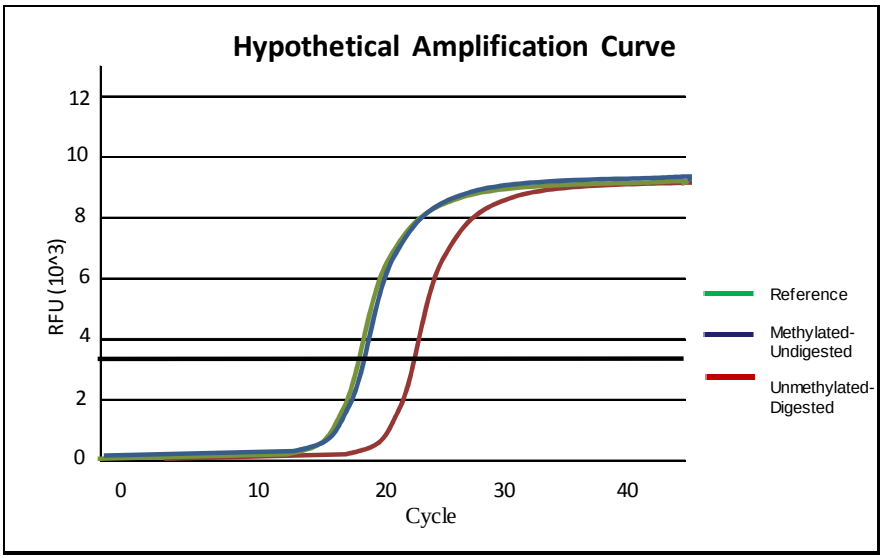

Figure 1. Possible result outcome depending on the status of methylation at the target site.

Therefore, pluripotency genes (*Oct4*, *NANOG*, *RAB25*, *PTPN6*) are expected to be hypomethylated in pluripotent cells and hypermethylated in differentiated cells. On the contrary, differentiation genes (*Elf5*, *MGMT*, *GBP3*, *LYST*) are expected to be hypermethylated in pluripotent cells and hypomethylated in differentiated cells as portrayed in figure 2.

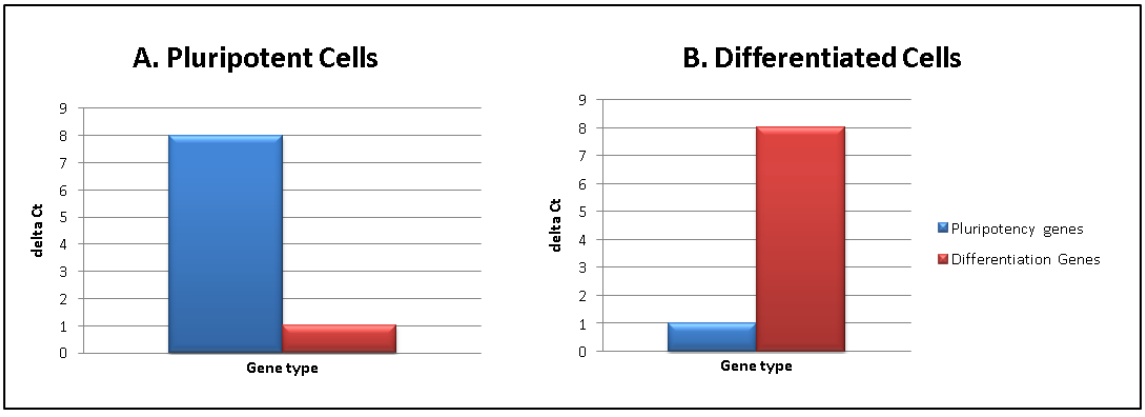

Figure 2. Hypothetical methylation status in A. Pluripotent cells B. Differentiated cells.

Digestions were performed with HaeII and HinP1I enzymes (New England Biolabs Inc., Ipswich, MA) for *Oct4* and *Elf5* genes respectively. A prealiquoted test reaction mix was used from Zymo Research (Zymo Research, Orange, CA) to study *NANOG, PTPN6, GBP3, LYST, RAB25, MGMT* genes, which included AccII, HpaII and HpyCH4IV enzymes.

Amplicons of CpG differentially methylated promoters were based on the literature. The design of primers for *Oct4* was targeting sites -175 and +42 relative to the transcription site as described in Freberg, et al. (26). Primers for *Elf5* were targeting site -152 from the transcription start site (20). Primer design was done using FileBuilder 3.1 software using a sequence based on literature for the specific gene promoter of interest that included approximately 100 base pairs around the target site. We have utilized *Taqman* assays which are known to be highly specific for the target of interest. *Oct4* and *Elf5* primers were obtained from Life Technologies (Grand Island, NY). Additional primer assay for *LYST* gene was obtained from Qiagen (Qiagen, Valencia, CA) to test for better efficiency of the protocol. Qiagen's method uses both methylation-sensitive and methylation-dependent enzyme reactions.

Table 1. Genes and amplicon sequences used.

Gene	Primer	Primer Sequence	Amplicon Sequence
Elf5	Forwad primer Reverse primer	GTGACACGGCTCCTTGGA CCTGGGCTGGGAGTGG	CTGCGCACAAAAGCA
Oct4_+42	Forwad primer Reverse primer	CTTCGCAAGCCCTCATTTC AC GGCGAGAAGGCGAAATCC	CCCATGGCGGGACACCT
Oct4_-175	Forwad primer Reverse primer	CTGCACTGAGGTCCTGGAG TGGTGGCAATGGTGTCTGT	CCGGGAGACACAACTGGCGC
NANOG	Forwad/Reverse primer	NA	CTAGAAGTATTTGTTGCTGGGTTTGTCTTCAGGTTCTGTTGCTCGGTTTTC TAGTTCCCCACCTAGTCTGGGTTACTCTGCAGCTACTTTTGCATTACAAT GGCCTTGGTGAGACTGGTAGACGGGATAACTGAGAATTCACAAGGGTGG GTCAGTAGGGGGTGTGCCCGCCAGGAGGGGTGGGTCTAAGGTGATAGAG CCTTCATTATAAATCTAGAGACTCCAGGATTTTAACGTTCTGCTGGACTG
PTPN6	Forwad/Reverse primer	NA	GTAAGTCCCGGGCACCATCGGGGTCCCAGTCTCCTGTTAGTTTTGGAGGG AGGGAGGGCTTTGTTGATGCTCACTCCGACGTGTGTGAACGTGAGTGCGA TCTGCCGCTGCCCTGC
RAB25	Forwad/Reverse primer	NA	CAGCCCTCAGTGGGCTGTCTCTGAAGGTCCTGTCCCTTTTTCGCTTCCCC CCCGCTGGAGCTGCTTCTCCCGCTTGCGGGAGCCCAGGCTGAGAGCAGA CACCCAACCTGTCGAACCTGTCTGACGTATCATCTCTCCACCCACCTGG GCCCCAGGTCTCCAGCCACCCCGCTCTTCCTGTTCTCAGCTTCCGTCCTC TCTGCTTCCTTACAGCACCCCCA
GBP3	Forwad/Reverse primer	NA	CAGCTGCAGCCTAATTTGGTCCTGGTCATTTTTAAGAAAATGAACTGAC TTATAAATTCCTTCCCATCCTTGCCACAACGTTATAGGCTCCACGTCCCT GAGCTGAGGTACTTCAG
LYST	Forwad/Reverse primer	NA	GAATACAACTTTCCCACGTAAGAATGAATAAACACTGAAAGAGGCCAA AACCCCAAACACTCTGGTATGAGGACTGCTCTTCTCAAAGCCAAAAGGT CATTGGGATGGCTTCTTAG
MGMT	Forwad/Reverse primer	NA	GCACGTGGCAGGTCGCTTGCACGCCCGCGGACTATCCCTGTGACAGGAA AAGGTACGGGCCATTTGGCAAACTAAGGCACAGAGCCTCAGGCGGAAG CTGGGAAGGCGCCGCCCGGCTTG

Results

Phase I-Validation of assays on optimal amounts of starting material

MSRE qPCR assays were tested on gDNA from fibroblast cell line, hESC and iPSCs using optimal amounts of starting material, 1 ng/ul for Zymo primers and 250 ng/ul for primers from Applied Biosystems. As expected *Oct4* is hypermethylated in differentiated cells and no digestion is observed in the fibroblast cell line. *Oct4* is hypomethylated in pluripotent cell lines as depicted by higher delta Ct values. *Elf5* is expected to be hypermethylated in pluripotent cells, however the level of methylation in our sample is not significant (Figure 3).

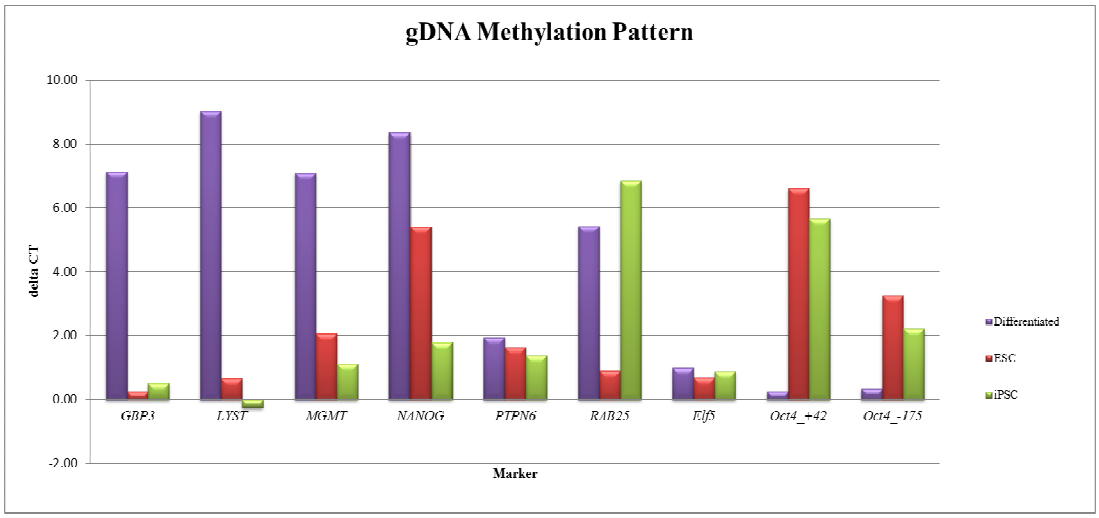

Figure 3. Methylation pattern of differentiated cells and pluripotent cells using optimum amount of starting material.

GBP3, *LYST* and *MGMT* show expected methylation patterns in differentiated and pluripotent cells as studied by Nishino and colleagues (15). *NANOG* and *PTPN6* however show contrary results as what was described by Nishino et al. (15). Contrary to Nishino's group observations, we observed hypomethylation of *NANOG* and *PTPN*, although not significant in *PTPN6*, in differentiated cells when compared to pluripotent cells.

To control for technical variance, such as pipetting, we have utilized an endogenous control assay, RNaseP. Variations in Ct values of 0.1-0.2 cycles were observed, which is within the acceptable variability range of +/-0.3 cycles (28).

We have also tested assays from different companies (Zymo and Qiagen), however results indicate that primers from Qiagen for the specific gene tested (*LYST*) show that it is hypomethylated, contrary to the expected results, while Zymo primers are hypermethylated. The reason for this is that the primers used are designed for different regions: Qiagen designed primers for the CpG island, while Zymo's primers are designed

for CpG sites in the promoter. Therefore, Zymo and Qiagen's primers study different sites of the markers. Zymo's site is at 29514651-2914650 while Qiagen's is at 234096210-234097220 for *LYST*.

Phase II-Validation of assays on limited amounts of starting material

Subsequently, we utilized the primers on 10 replicates of 5-cell lysates from the same cell line used in the previous experiment using gDNA. However, the delta Ct values for 5-cell lysates were not as significant as in gDNA experiment and did not illustrate the same pattern of methylation as in gDNA for the same cell lines (Figure 4). Increasing digestion time did not result in improvement of delta Ct values and delta Ct values were variable between replicates. To isolate the technical variability that can result in unequal distribution of DNA we have added the necessary reagents, centrifuged, vortexed and centrifuged the samples and then distributed the samples into equal volumes into new tubes. There was much less variation observed, however the delta Ct values were still not significant.

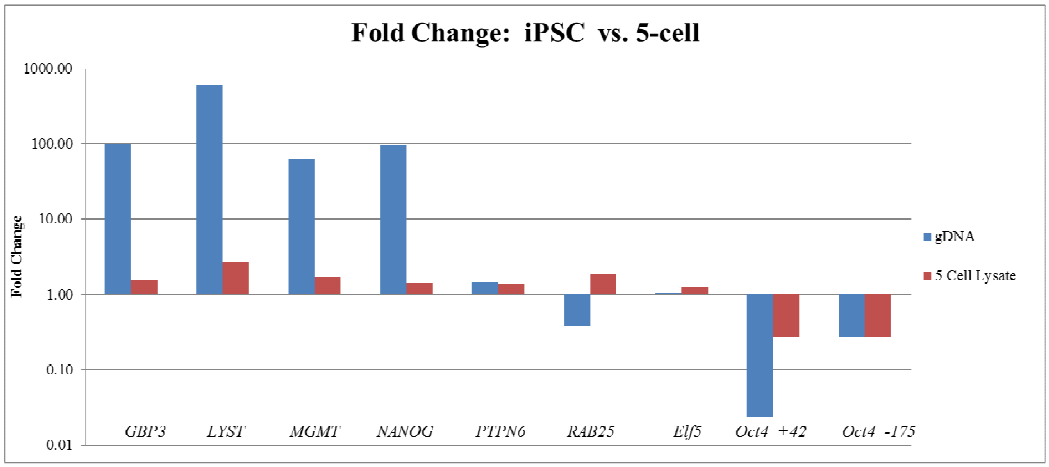

Figure 4. Methylation pattern of pluripotent cells relative to differentiated cells. 5-cell lysates do not show the same methylation pattern as the optimal amounts of genomic DNA.

Efficiency of digestion was controlled for by adding an internal positive control to each sample. Low delta Ct values in the positive control indicate inefficient digestion on the 5-cell lysates (Table 2).

Table 2. Delta Ct values of samples with an internal positive control.

Cell Line	CF2	Elf5	Oct4_+42	Oct4_-175
GM00323	0.4	-1.1	-1.0	-0.6
iPSC	0.2	0.0	1.6	1.4

To investigate what could be the cause of the inefficient digestion in the 5-cell lysates we have set up an experiment comparing 5-cell equivalent samples and 5-cell equivalent amount (30pg) from gDNA. 5-cell equivalent samples were prepared by pooling lysates together and redistributing them into 5-cell equivalents. 5-cell equivalent amount was prepared by diluting purified gDNA to 30pg. Results indicate that digestion is more efficient on 5-cell equivalent amount on gDNA than on 5-cell equivalent lysates. Since the DNA in the two sample types was obtained differently (DNA sample lysates were obtained by lysis protocol, while gDNA from 5-cell lysates was obtained by isolation using the Qiagen DNeasy Blood and Tissue Kit), we conclude that reagents in the lysis reaction is inhibiting the digestion reaction on the 5-cell preAmp leftover products. Purifying the DNA from 5-cell preAmp leftover products could result in loss of the already limited material. As a consequence we decided to study the level of differentiation on gDNA obtained from villi samples rather than from preAmp leftover products.

Phase III-Characterization of methylation status of genomic DNA from extraembryonic material

Since digestion is inefficient of 5-cell preAmp product leftovers, we utilized the gene promoter methylation assays on gDNA samples isolated by Qiagen DNeasy Blood and Tissue Kit. Our sample size consisted of 15 gDNA samples isolated from villi tissue from spontaneous abortions that have been karyotyped by microarray. Characterizing the villi gDNA would allow us to describe the methylation pattern of euploid and aneuploid products of conception after assisted reproductive technology (ART). We hypothesize that villi from aneuploid samples do not have the same differentiation level as euploid samples.

Of the 15 samples characterized by microarray, 9 had a normal karyotype, 6 abnormal karyotypes consisted of two trisomies 22, one trisomy 4 one trisomy 17 one trisomy 13, and one insertional mutation at chromosome 8. All of the samples analyzed did not go beyond 40 days of pregnancy. Most of the spontaneous abortions (95 %) occurred in the 21-30 day timeslot after start of pregnancy. For the euploid cohort, 78 % samples aborted in the 21-30 day mark window, where as 83% were aborted in the 31-40 day mark window for aneuploid samples.

Scatter plots were used to study possible relationships between DNA methylation and the gestational age of the fetus at which it was aborted. T-test method was used to measure the statistical significance of DNA methylation of the nine genes between euploid and aneuploid villi samples. P values indicate no difference of euploid versus aneuploid samples in regards to methylation status for all genes studied except for *GBP3* (Table 3).

Table 3. Statistical significance between euploid and aneuploid samples as measured by t-test.

Elf5	*Oct4_+42*	*Oct4_-175*	*GBP3*	*LYST*	*MGMT*	*NANOG*	*PTPN6*	*RAB25*
0.058	0.490	0.534	0.045	0.484	0.542	0.380	0.774	0.955

Lack of statistical significance may be due to low number of samples analyzed. *Elf5* is one of the genes studied that could potentially have a higher statistical significance if sample size was increased. There is no high variation between cohorts, except for *MGMT* and *NANOG*, as indicated in the box plots in figure 5.

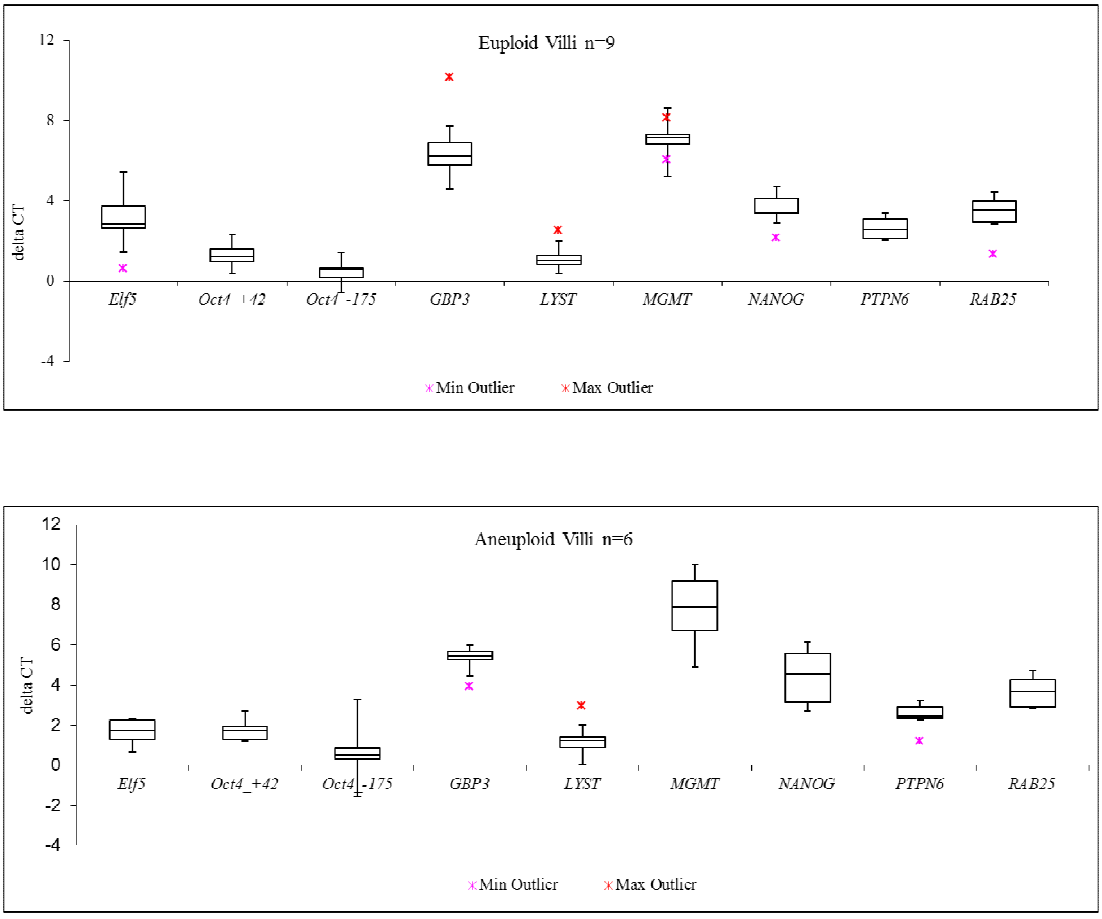

Figure 5. Methylation value of eight genes in euploid and aneuploid karyotyped gDNA villi samples.

GBP3, LYST, MGMT and Elf5 gene promoters are expected to be hypermethylated in pluripotenet cells and hypomethylated in differentiated cells. Gene promoters of *NANOG, PTPN6, RAB25* and *Oct4* are expected to be hypomethylated in pluripotent cells. *Elf5* was reported to be more strongly expressed in the first trimester and was down-regulated towards term (20). Consistent with this observation, our results indicate hypomethylation in villi samples from first trimester in both cohorts (Figure 6). As expected *Oct4*_-175 shows hypermethylation in villi samples (Figure 6). *LYST*, however, although insignificant, unexpectedly shows a tendency toward hypermethylation, in the samples analyzed (Figure 6).

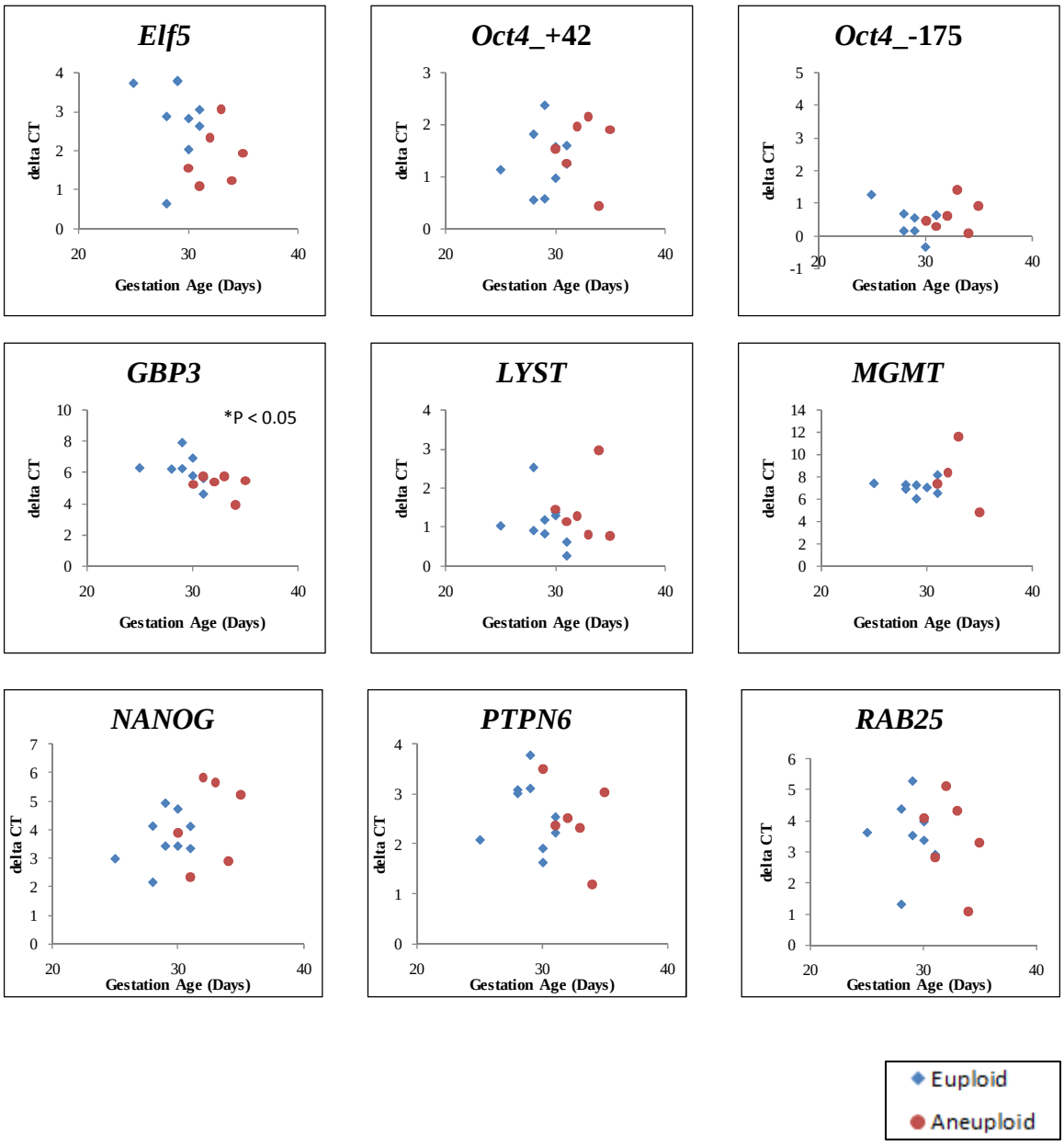

Figure 6. Relationship of euploid (blue diamond) or aneuploid (red circles) villi samples to gestational age measured in days. Euploid samples show a statistically differential methylation status within the *GBP3* promoter compared to aneuploid samples.

LYST is hypomethylated in differentiated cells when compared to iPSCs (15).Our results indicate hypomethylation of *LYST* in fibroblasts which represent differentiated samples (Figure 3). However, in villi samples *LYST* shows a tendency toward hypermethylation (Figure 6). *LYST* is a lysosomal trafficking regulator gene and was implicated to function as an adaptor protein that affects proteins involved in intracellular membrane fusion reactions (29).

Of the nine gene promoters studied, *GBP3* shows differential methylation status between euploid and aneuploid villi samples. As expected *GBP3* gene promoter is hypomethylated in differentiated cells. Results indicate hypomethylation in euploid samples versus aneuploid samples.

Discussion

In this study, we have begun to develop a method to screen preimplantation embryos for their reproductive potential using several candidate gene promoter methylation markers of pluripotency. We characterized the methylation patterns in products of conception using a set of gene promoter methylation sites. We found that euploid samples have a statistically different methylation status within the *GBP3* promoter compared to aneuploid villi samples. The guanylate binding proteins (GBPs) belong to a family of cytokine-induced GTPases and are located on chromosome 1 (30). Moon et al. (2011) identified *GBP3* as one of the genes that is upregulated in early stages of induced abortion in mice (31). Methylation regulates gene expression and can be used as a molecular marker for such. Our results indicate hypomethylation of the *GBP3* gene promoter in the euploid compared to aneuploid products of conception. In addition,

aneuploid samples in our study aborted at a later time point than euploid samples and hypothetically they should have been more differentiated. *GBP3* is a marker of differentiation and should therefore be less methylated in tissue that is more differentiated. Our outcomes indicate that aneuploid tissue possesses significantly more methylation of the *GBP3* gene promoter indicating that euploid tissue is better differentiated. It is therefore possible to utilize *GBP3* as a diagnosis marker of differentiation level in embryos. However, we have only studied *GBP3* in tissues from spontaneous abortions making further experimentation necessary to determine the applicability to preimplantation embryo reproductive potential.

Indeed, one of the original aims of this study was to characterize the methylation status of products of conception on small amounts of starting material (i.e. trophectoderm biopsy) from DNA left over from clinical genetic testing. We have noted that very low amounts of DNA (30pg) do not behave in the expected manner as optimal amounts of DNA do. This observation could be due to non-optimal ratio of enzyme to DNA concentration. The observed outcome could also be due to the fact that cell line samples were at different passage numbers (P25 and P32) when tested at optimal versus limited amounts of starting material . It has been acknowledged previously that passage number does play a role in the methylation status, where the number of differentially methylated regions dramatically decreased from an early passage (P10 to P20) to late passage (P30 to P40). (15). We have identified that using available excess DNA from leftover samples was not possible and conclude that this is likely due to the composition of the lysis reagents/protocol used rather than a limitation of the small amounts of starting material. Unfortunately, modifying the lysis protocol was not feasible within the present study as it

will require considerable amounts of testing and validation, and acquisition of new research materials. Alternatively, we utilized samples with large amounts of starting material that have been prepared in a way that enzyme digestion is not inhibited. This allowed us to study the methylation status in products of conception to identify possible associations between the methylation status and the chromosomal normalcy status of post-implantation samples.

Future studies will aim to characterize the methylation status of discarded embryos, rather than from excess DNA from embryos used in clinical practice. This will provide an opportunity to investigate alternative methods of lysis and represents an area of active investigation in collaboration with Zymo Research Inc. Another area of investigation stemming from the present study involves characterizing the genome wide methylation status of the human preimplantation embryo. This project, unlike the present study, will not be limited to evaluating a subset of targeted gene promoters (i.e. pluripotency gene promoters). In addition, it may allow for the identification of differential methylation in gene promoters which may have otherwise not been hypothesized to be of importance to differentiation or reproductive potential in the human embryo.

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