frontiers in
GENETICS

ORIGINAL RESEARCH ARTICLE
published: 20 February 2012
doi: 10.3389/fgene.2012.00022

Naturally occurring variation in trophoblast invasion as a source of novel (epigenetic) biomarkers

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During the first trimester of pregnancy fetal trophoblasts invade the maternal decidua, thereby remodeling the maternal spiral arteries. This process of trophoblast invasion is very similar to cancer cell invasion, with multiple signaling pathways shared between the two. Pregnancy-related diseases, e.g., pre-eclampsia, and cancer metastasis start with a decrease or increase in cellular invasion, respectively. Here, we investigate if first trimester placental explants can be used to identify epigenetic factors associated with changes in cellular invasion and their potential use as biomarkers. We show that the outgrowth potential of first trimester explants significantly correlates with promoter methylation of *PRKCDBP* and *MMP2*, two genes known to be differentially methylated in both placenta and cancer. The increase in methylation percentage of placental cells coincides with an increase in invasion potential. Subsequently, as a non-invasive marker must be detectable in blood, plasma samples of pregnant and non-pregnant women were analyzed. The *MMP2* promoter showed high methylation levels in non-pregnant plasma samples, which decreased in pregnant plasma samples which also contain placental DNA. The decrease in methylated plasma DNA during pregnancy is most likely due to the fractional increase in unmethylated placental DNA. This suggests that the level of unmethylated DNA has the potential to be used as an invasion marker, where higher levels of unmethylated DNA indicate a lower invasion potential of trophoblasts. These proof of principle data provide evidence that human first trimester placental explants are an excellent *ex vivo* model system to identify (epigenetic) factors and thus potential biomarkers associated with changes in cellular invasion, e.g., to detect pregnancy-related diseases or cancer metastasis. To identify novel biomarkers the next step is to correlate naturally occurring variation in invasion potential to changes in (epigenetic) factors by genome-wide approaches such as massively parallel sequencing.

Keywords: placenta, pre-eclampsia, cancer metastasis, invasion potential, biomarker

INTRODUCTION

During the first trimester of pregnancy fetal trophoblasts invade the maternal decidua. By invading the decidua the maternal spiral arteries are remodeled by the invasive trophoblasts. This process of trophoblast invasion is very similar to cancer cell invasion, with multiple signaling pathways shared between the two (Ferretti et al., 2007; Holtan et al., 2009), giving rise to, e.g., migration and invasion of stroma and arteries, epithelial-mesenchymal transition, heterotypic interactions, and the shedding of particles into the circulation. Interestingly, multiple pregnancy complications, i.e., IUGR (intra-uterine growth restriction) and pre-eclampsia, show a reduction in trophoblast invasion during first trimester (Steegers et al., 2010).

The fact that trophoblast invasion and cancer cell invasion share many signaling pathways makes the first trimester placenta an excellent model to study factors involved in either increased or decreased invasion potential.

The aim of this study is to investigate if the naturally occurring variation in invasion potential of human first trimester placental explants correlates to changes in epigenetic factors. In the future, this set up can be used as an *ex vivo* model system to identify factors by genome-wide approaches (e.g., massively parallel sequencing), and thus potential biomarkers associated with changes in cellular invasion, e.g., to detect pregnancy-related diseases or cancer metastasis.

Previously, we identified the *STOX1 Y153H* SNP to be a susceptibility allele for pre-eclampsia in Dutch females (Van Dijk et al., 2005). When the invasion potential of first trimester placental explants was established by measuring the outgrowth of the explants a significant reduction in invasion potential was found in the placentas homozygous for the *Y153H* allele (Van Dijk et al., 2010). This finding showed that genetic differences can give rise to measurable changes in outgrowth potential. The study described here investigates if also epigenetic differences can be detected in first trimester placentas with different invasion potentials. When this is the case, not only genetic and protein or mRNA expression differences can function as potential factors, but also epigenetic differences, increasing the identification of novel biomarker panels by genome-wide approaches.

For testing the use of first trimester epigenetic differences in relation to invasion potential the methylation levels of 10 genes were chosen to study. Eight well-known tumor suppressor

genes (*TP73*, *RASSF5*, *RASSF1*, *APC*, *DAB2IP*, *PRKCDBP*, *WT1*, *MORF4L1*) were chosen which all were described by Grigoriu et al. (2011) to be differentially methylated in first trimester placenta. From the extensive list of proteins and pathways shared by cancer cell invasion and trophoblast invasion, e.g., the WNT signaling pathway, integrins, enzymes, growth factors, and their receptors (see for overviews Ferretti et al., 2007; Holtan et al., 2009), two matrix metalloproteinases were chosen to study, *MMP9* and *MMP2*. Both are found to be expressed in the invasive trophoblast as well as malignant tumors (Ferretti et al., 2007; Holtan et al., 2009).

MATERIALS AND METHODS

PLACENTAL DNA SAMPLES

Placental DNA used ($n = 32$) originates from placental samples with established outgrowth potential as described previously (Van Dijk et al., 2010). In short, small fragments of placental villi from 6 to 9 week gestation were dissected from placenta, placed on Matrigel and cultured at 3% O_2 and 37˚C in serum free DMEM/F12 media. Simultaneously, small fragments of the same placentas were kept for DNA isolation. At day 2 and day 4 pictures were taken to measure changes in outgrowth. The explant outgrowth potentials were measured by taking the difference of the distance the extravillous trophoblasts migrated between day 2 and day 4. From each placenta at least three explants were grown, explants that did not show any growth were left out of analyses because they were either not viable or were not properly dissected. For the current study only DNA of placentas were used that had at least two explants with growth.

PLASMA DNA SAMPLES

Simultaneous isolation of DNA and RNA from plasma samples was performed as described previously (Oudejans et al., 2003) with modifications (Go et al., 2007). Pregnant sample collection was approved by the ethics committee of the VU University Medical Center. Informed consent was obtained from all women. Isolated DNA samples ($n = 26$) were precipitated and concentrated to be able to use the DNA amount isolated from 1 ml plasma (∼20 ng) in each reaction. Characteristics of the plasma samples analyzed can be found in **Table 1**.

DNA METHYLATION ASSESSMENT

The level of DNA methylation was measured using the OneStep qMethyl kit (Zymo Research). For use with this assay, 10 primer pairs were designed within CpG rich (promoter) regions according to the OneStep qMethyl kit guidelines. Primer sequences can be found in **Table 2**. In brief, to measure methylation percentages 20 ng DNA (or DNA isolated from 1 ml plasma) in duplicate is placed in a test and a reference reaction mix including primers. The samples are placed in an ABI7300 with the following protocol: 2 h 37˚C; 10 min 95˚C; 50 cycles 30 s 95˚C, 1 min 54˚C, 1 min 72˚C followed by an dissociation stage to check specificity of the PCR products. The Ct values obtained are used to calculate ΔCt values [Ct(test) – Ct(reference)]. From this methylation percentages are calculated using Methylation% $= 100 \times 2^{-\Delta Ct}$. This calculation is similar as calculating fold differences in a standard quantitative PCR assay, except that the fold differences are now expressed as percentages by multiplying the fold difference by 100. For example, a "fold difference" of 0.125 (which represents a ΔCt value of 3) using this method gives a methylation percentage of 12.5%, while a ΔCt of 0 (no difference between reference and test reaction) gives a fold difference of 1 indicating 100% methylation. Each primer set used was tested for reaction efficiency by measuring dilutions of fully methylated and unmethylated DNA samples provided with the kit (**Table 3**). Secondly, calibrator samples consisting of 0, 35, 65, and 100% methylated DNA were run in each experiment. From these the inter-assay and intra-assay reproducibility was investigated, shown in **Tables 4** and **5**, respectively. From this it can be concluded that at low percentages the errors are smaller, and the assay therefore more sensitive, than at higher levels of methylation.

DATA ANALYSIS

Statistical analysis of the obtained data was carried out with the GraphPad Prism program. In here, linear regression analysis was performed for **Figures 1A,B** and **2B** and **Table 3**. The p values of the linear regression lines represent the testing of the null hypothesis that the slope is zero, where the p value is calculated from an F test. Data in **Tables 1**, **4**, and **5** and **Figure 2A** were obtained calculating the mean, median, SD, and SE of the mean of the measurements.

RESULTS

OUTGROWTH CORRELATES WITH DNA METHYLATION

DNA samples of first trimester placentas with known outgrowth potential were used to study the epigenetic status of the promoters of two matrix metalloproteinases (*MMP9*, *MMP2*) known to be involved in both placentation and cancer (Ferretti et al., 2007), and eight tumor suppressor genes (*TP73*, *RASSF5*, *RASSF1*, *APC*, *DAB2IP*, *PRKCDBP*, *WT1*, *MORF4L1*) known to show partial DNA methylation in first trimester placentas (Grigoriu et al., 2011). From the 10 genes chosen to study, *PRKCDBP* and *MMP2* showed a significant linear regression, $p = 0.017$ and

Table 1 | Characteristics of plasma samples analyzed.

	Non-pregnant ($n = 12$)	Pregnant		
		Total ($n = 14$)	1st Trimester (≤90 days gestation; $n = 10$)	2nd Trimester (>90 days gestation; $n = 4$)
Age women in years (range)	32.3 (27–40)	34.6 (28–38)	34.4 (29–38)	35.3 (28–38)
Gestational age in days (range)	NA	91.6 (42–182)	71.7 (42–86)	141.3 (102–182)
Methylation % (range)	62.5 (0.0–100.0)	33.0 (8.1–53.7)	36.4 (12.5–53.7)	24.5 (8.1–44.3)

Table 2 | Primer sequences used in OneStep qMethyl kit.

Gene	Forward primer (5′–3′)	Reverse primer (5′–3′)
TPA73	AAGCGAAAATGCCAACAAAC	CACCGACGTACAGCATGGTA
RASSF5	GGTTCTCTTGGGTCGTCCTT	TCCAATAGTAGCGGGTACGG
RASSF1	ACCTAGTCCTCGGGAGCTGT	ACCTCTGTGGCGACTTCATC
APC	AAGCCAGCAACACCTCTCAC	AGTACCTGGGAACAGCATCG
DAB2IP	TCCGGGGTTAGGTGAGTAGA	GCCGAAAATGCCTTTTGTAT
PRKCDBP	CGGAGGCTCTGTACCTTCTG	GTAAGGAGCTGCCAGGATCA
WT1	ATCGGACACGGGTTTGATTA	CTTGGCCACTCGATTCTCTC
MORF4L1	CGGAGAGAGCAGCCTATTGT	GGCCATTTTACAACGCACTT
MMP2	CTACGATGGAGGCGCTAATG	CGGGGAACTTGATGATGG
MMP9	CATCGTCATCCAGTTTGGTG	GAAATAAGTGCGGGCTGAAA

Table 3 | Reaction efficiencies of the primer sets used.

Primer set	Slope	r^2
TP73	1.0040 ± 0.008801	0.9998
RASSF5	0.9948 ± 0.016310	0.9995
RASSF1	0.9864 ± 0.023650	0.9989
APC	0.9736 ± 0.062900	0.9917
DAB2IP	0.9458 ± 0.113500	0.9720
PRKCDBP	0.9981 ± 0.003968	1.0000
WT1	1.0000 ± 0.008441	0.9999
MORF4L1	1.0020 ± 0.008762	0.9998
MMP2	0.9929 ± 0.018690	0.9993
MMP9	0.9980 ± 0.003860	1.0000

The slope value is obtained by analyzing the linear regression of the percentage of methylated DNA input plotted against the percentage of methylation measured. The r^2 value indicates the goodness of fit of the different percentages of methylated DNA input on the linear regression line where 1 indicates a perfect fit.

Table 4 | Inter-assay reproducibility based on three independent assays.

% Methylated DNA input	% Methylation measured			
	Mean (SD)	Median	Minimum	Maximum
0	5.889 (8.095)	2.547	0.000	15.12
35	35.52 (6.276)	36.14	27.35	42.48
65	53.58 (12.65)	56.35	36.17	65.45
100	107.1 (15.42)	100.5	88.66	123.8

$p = 0.028$, respectively, where the increase in methylation percentage coincided with an increase in invasion potential (**Figure 1**). The remaining eight genes did not give significant changes in methylation level upon changes in outgrowth potential (data not shown).

NON-INVASIVE BIOMARKER DETECTION IN BLOOD

To function as a non-invasive marker an (epigenetic) factor must be detectable in blood. Therefore, methylation of *PRKCDBP* and *MMP2* was tested in pregnant plasma and compared to non-pregnant plasma samples. *PRKCDBP* showed low levels of methylation in both pregnant and non-pregnant samples (data not shown). The *MMP2* promoter showed high methylation levels in non-pregnant plasma samples, which decreased in first trimester samples and further decreased in second trimester (**Figure 2A**;

Table 5 | Intra-assay reproducibility showing the positive and negative errors of percentage methylation measured for the four methylated DNA input controls in two independent assays.

% Methylation input	% Methylation error	Mean (SD)
0	Positive	4.426 (2.423)
	Negative	3.663 (1.618)
35	Positive	2.851 (0.9235)
	Negative	2.621 (0.8695)
65	Positive	8.404 (8.145)
	Negative	6.924 (6.308)
100	Positive	12.67 (2.874)
	Negative	11.00 (1.805)

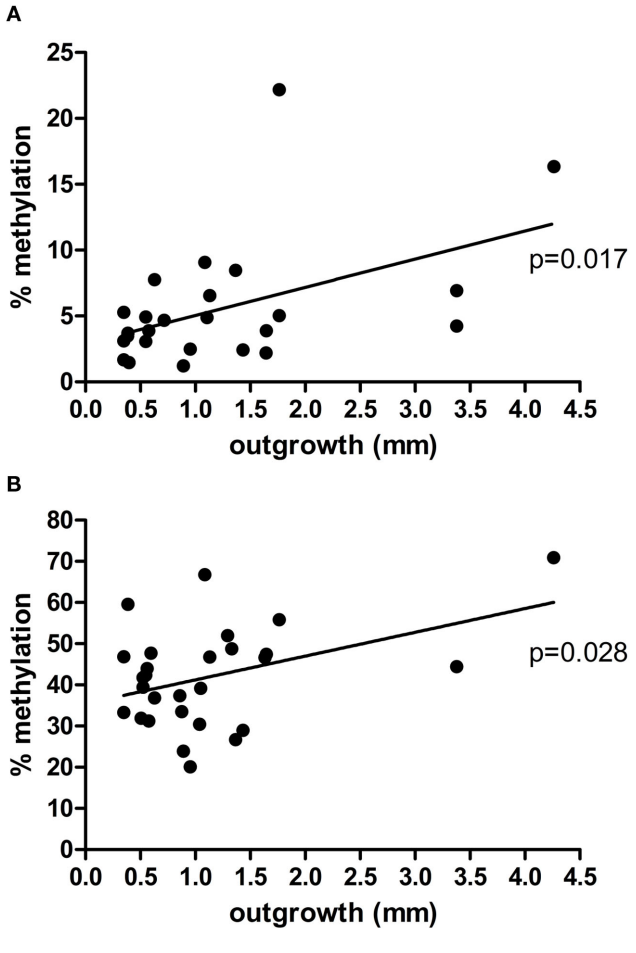

FIGURE 1 | Correlation between placental explant outgrowth and *PRKCDBP* (A) and *MMP2* (B) promoter methylation in first trimester placenta.

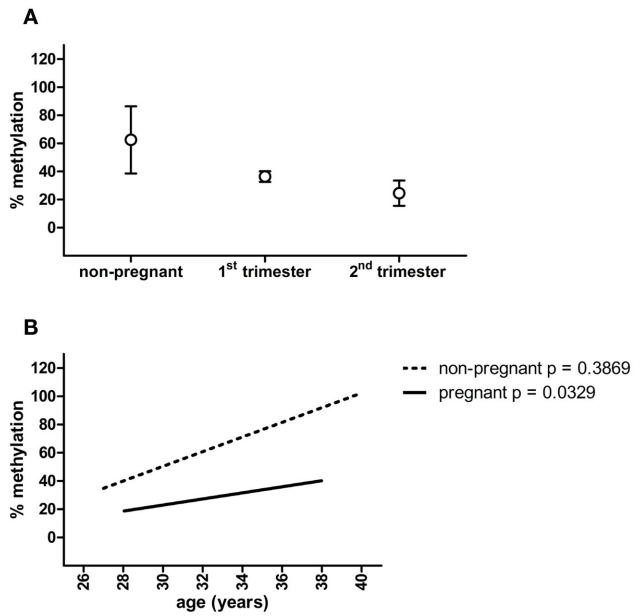

FIGURE 2 | MMP2 promoter methylation in non-pregnant, first trimester and second trimester plasma samples (A), and linear regression of *MMP2* methylation percentages plotted against the age of the women (B). Circles represent mean ± SEM.

Table 1). Plotting the methylation percentages of the same samples versus the age of the women showed a significant linear regression in pregnant women ($p = 0.0329$), while the linear regression was not significant in non-pregnant women (**Figure 2B**). In this graph first and second trimester women were combined as the age range of the second trimester women was not evenly distributed, see also **Table 1**.

DISCUSSION

The aim of this study was to investigate if the naturally occurring variation in invasion potential of human first trimester placental explants correlates to changes in epigenetic factors. Previously, we showed that genetic differences can give rise to changes in outgrowth potential, as shown for the *STOX1 Y153H* SNP (Van Dijk et al., 2010). In this study, we describe the use of first trimester placental explants to identify epigenetic differences associated with changes in cellular invasion. Increase in methylation of *PRKCDBP* and *MMP2* in placental cells showed a significant linear regression with increased invasion potential of first trimester placental explants. Increased *PRKCDBP* methylation levels have frequently been found in several human malignancies, including colorectal, lung, breast, and gastric cancers (Xu et al., 2001; Zöchbauer-Müller et al., 2005; Lee et al., 2008, 2011). Hypermethylation of the promoter region of this gene causes downregulated expression correlating with tumor progression (Lee et al., 2011). This is in accordance with our findings showing increased methylation upon increased outgrowth potential of trophoblasts. Except for the study by Grigoriu et al. (2011) identifying differential methylation of tumor suppressor genes in first trimester placenta by microarray, no other studies have been done on *PRKCDBP* or *MMP2* methylation in the placenta. MMP2 expression and activation levels, however, have been repeatedly found to be increased upon increased trophoblast invasion (Godbole et al., 2011; Onogi et al., 2011; Williams et al., 2011). The literature on increased levels and activation of MMP2 upon tumor progression in various cancer types are numerous, see for a selection of reviews (Levicar et al., 2003; Overall and Dean, 2006; Qian et al., 2011). Furthermore, a decreased methylation status of *MMP2* in different cancers types has also been found to be associated with increased tumor invasion (Chernov et al., 2009; Morán et al., 2012). Therefore, although it would have been expected to find a decrease in methylation of the *MMP2* promoter, this was not seen in this study. This inconsistency might be a good example of the main difference between trophoblasts and tumorous cells, i.e., the invasion of trophoblasts is tightly regulated in a time and place restricted manner. Methylation of *MMP2* upon increased invasion potential can be one of these regulatory mechanisms.

Next, we show that one of the two factors identified can be detected in plasma of both pregnant and non-pregnant women. High *MMP2* methylation levels were detected in non-pregnant plasma DNA samples, which decreased in first trimester samples which also contain placental DNA. A further decrease was seen in second trimester samples. The increased amount of unmethylated DNA in pregnant plasma compared to non-pregnant plasma is most likely placental in origin, due to the additional fractional contribution of unmethylated placenta DNA to plasma during pregnancy. The additional fractional increase of unmethylated DNA during the second trimester of pregnancy is consistent with the knowledge that the invasion capacity of the placental trophoblasts also decreases (Zhou et al., 1993). This current methylation pattern seen for *MMP2* has been shown before in the *SERPINB5* promoter (Chim et al., 2005). *SERPINB5* is a well-known tumor suppressor gene in which the promoter furthermore showed downregulated methylation in pre-eclamptic plasma samples. To conclude, the increase in unmethylated DNA during pregnancy suggests that the level of unmethylated DNA has the potential to be used as an invasion marker, where higher levels of unmethylated DNA indicate a lower invasion potential of trophoblasts.

This proof-of-principle study therefore provides evidence that it is possible to use human placental explants as an *ex vivo* model to identify factors associated with changes in cellular invasion, e.g., to detect pregnancy-related diseases or cancer metastasis. This study shows that not only genetic and protein or mRNA expression differences can function as potential factors, but also epigenetic differences, increasing the identification of novel biomarker panels by genome-wide approaches.

It must be noted that within the current setting *MMP2* methylation itself is not usable as a cellular invasion marker. This is due to the high variance of methylation found in non-pregnant samples compared to the pregnant plasma samples, as was shown by the linear regression plots of the methylation percentages versus the age of the women. A linear regression in methylation versus age can be expected when measuring epigenetic effects, however, this was only the case in

pregnant samples. The high variance in non-pregnant women might be caused by the observation that plasma samples derived from parous non-pregnant women overall had lower methylation percentages as nulliparous women. However, the amount of parous samples in the non-pregnant group is small ($n = 4$) and it is not known if the nulliparous women never have been pregnant.

Another issue that needs to be addressed is the method used to measure differences in methylation percentage. The current assay used shows, although within acceptable range at low methylation levels (mean errors around 3%), at higher methylation percentages mean errors up to almost 13%. Therefore, when by genome-wide approaches epigenetic factors are being identified, the method used to measure methylation differences as potential biomarkers should be highly sensitive and accurate at all levels of methylation, which might be achieved by using pyrosequencing or methylation-specific single nucleotide primer extension (MS-SNuPE).

Although *MMP2* promoter methylation in the current setting is not usable as a biomarker, it can be envisaged that it does have the potential to be used in combination with other biomarkers yet to be identified. This proof-of-principle study shows that the described method can be used to identify novel biomarkers by correlating naturally occurring variation in invasion potential to changes in (epigenetic) factors by genome-wide approaches such as massively parallel sequencing. Using genome-wide approaches on naturally occurring variation in invasion potential of first trimester placental explants increases the success of identifying primary factors involved in cellular invasion changes. Especially the first trimester placenta is highly informative as this is the period in which trophoblast invasion starts and dysfunction in this process will give complications later in pregnancy. The same holds for cancer cell invasion; when the first signs of metastasis are recognized early, the success of treatment highly increases. As non-invasive early detection markers are still lacking in both pregnancy-related disorders and cancer metastasis new approaches on the identification of novel biomarkers are still urgently needed.

ACKNOWLEDGMENTS

The authors thank all women to participate in donating blood for this study.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 29 November 2011; accepted: 28 January 2012; published online: 20 February 2012.
*Citation: van Dijk M, Visser A, Posthuma J, Poutsma A and Oudejans CBM (2012) Naturally occurring variation in trophoblast invasion as a source of novel (epigenetic) biomarkers. Front. Gene. **3**:22. doi: 10.3389/fgene.2012.00022*
This article was submitted to Frontiers in Epigenomics, a specialty of Frontiers in Genetics.