



HAL
open science

The early pregnancy placenta foreshadows dna methylation alterations of solid tumors

Akpéli Nordor, Djamel Nehar-Belaid, Sophie Richon, David Klatzmann, Dominique Bellet, Virginie Dangles-Marie, Thierry Fournier, Martin J. Aryee

► **To cite this version:**

Akpéli Nordor, Djamel Nehar-Belaid, Sophie Richon, David Klatzmann, Dominique Bellet, et al.. The early pregnancy placenta foreshadows dna methylation alterations of solid tumors. *Epigenetics*, 2017, 12 (9), pp.793-803. 10.1080/15592294.2017.1342912 . inserm-01617414

HAL Id: inserm-01617414

<https://inserm.hal.science/inserm-01617414>

Submitted on 21 Nov 2022

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

RESEARCH PAPER

 OPEN ACCESS



The early pregnancy placenta foreshadows DNA methylation alterations of solid tumors

Akpéli V. Nordor ^{a,b,c,d,e,f}, Djamel Nehar-Belaid^{g,h}, Sophie Richonⁱ, David Klatzmann^{g,h,j}, Dominique Bellet^{b,c,d,e,k}, Virginie Dangles-Marie^{a,l}, Thierry Fournier^{m,n,o}, and Martin J. Aryee ^{f,p}

^aInstitut Curie, PSL Research University, Département de Recherche Translationnelle, Paris, France; ^bChimie ParisTech, PSL Research University, Unité de Technologies Chimiques et Biologiques pour la Santé (UTCBS), Paris, France; ^cCNRS, UTCBS UMR 8258, Paris, France; ^dUniversité Paris Descartes, Sorbonne-Paris-Cité, UTCBS, Paris, France; ^eINSERM, UTCBS U 1022, Paris, France; ^fMassachusetts General Hospital and Harvard Medical School, Department of Pathology and Center for Cancer Research, Charlestown, Massachusetts, USA; ^gSorbonne Universités, UPMC University of Paris, Paris, France; ^hINSERM UMR_S 959, Paris, France; ⁱInstitut Curie, PSL Research University, CNRS, UMR 144, France; ^jAP-HP, Groupe Hospitalier Pitié-Salpêtrière, Department of Biotherapies, Clinical Investigation Center in Biotherapy and Inflammation-Immunopathology-Biotherapy Department (DHU I2B), Paris, France; ^kInstitut Curie, PSL Research University, Hôpital René Huguenin, Laboratoire d'Oncobiologie, Pôle Pathologie-Génétique-Immunologie-Hémobiologie, Saint-Cloud, France; ^lLaboratoire d'immunologie, Faculté de Pharmacie de Paris, Université Paris Descartes, Sorbonne-Paris-Cité, Paris, France; ^mINSERM, U1139, Faculté de Pharmacie de Paris, Paris, France; ⁿUniversité Paris Descartes, Sorbonne-Paris-Cité, UMR-S1139, Paris, France; ^oPremUp Foundation, Paris, France; ^pDepartment of Biostatistics, Harvard T.H. Chan School of Public Health, Boston, Massachusetts, USA

ABSTRACT

The placenta relies on phenotypes that are characteristic of cancer to successfully implant the embryo in the uterus during early pregnancy. Notably, it has to invade its host tissues, promote angiogenesis—while surviving hypoxia—, and escape the immune system. Similarities in DNA methylation patterns between the placenta and cancers suggest that common epigenetic mechanisms may be involved in regulating these behaviors. We show here that megabase-scale patterns of hypomethylation distinguish first from third trimester chorionic villi in the placenta, and that these patterns mirror those that distinguish many tumors from corresponding normal tissues. We confirmed these findings in villous cytotrophoblasts isolated from the placenta and identified a time window at the end of the first trimester, when these cells come into contact with maternal blood, as the likely time period for the methylome alterations. Furthermore, the large genomic regions affected by these patterns of hypomethylation encompass genes involved in pathways related to epithelial-mesenchymal transition, immune response, and inflammation. Analyses of expression profiles corresponding to genes in these hypomethylated regions in colon adenocarcinoma tumors point to networks of differentially expressed genes previously implicated in carcinogenesis and placentogenesis, where nuclear factor kappa B is a key hub. Taken together, our results suggest the existence of epigenetic switches involving large-scale changes of methylation in the placenta during pregnancy and in tumors during neoplastic transformation. The characterization of such epigenetic switches might lead to the identification of biomarkers and drug targets in oncology as well as in obstetrics and gynecology.

ARTICLE HISTORY

Received 31 March 2017
Revised 7 June 2017
Accepted 9 June 2017

KEYWORDS

Cancer; DNA methylation; epigenomics; epigenetics; hypomethylation; placenta; pregnancy

Introduction

At the beginning of pregnancy, human placental trophoblasts rely on several phenotypes that are also hallmarks of cancer to implant the blastocyst containing the embryo in the uterus. After undergoing epithelial-mesenchymal transition (EMT), placental cells invade and migrate within the endometrium. They also promote angiogenesis, while surviving hypoxia, to establish fetal/maternal exchange of nutrients, gases, and wastes. Moreover, as 50% of the placental genome has paternal origin, placental cells have to evade the maternal immune system.^{1–4}

Studies on DNA methylation in cancer cells and placental cells have highlighted similarities in their epigenetic landscapes, which are characterized by a widespread hypomethylation throughout the genome and focal hypermethylation at CpG

islands, including at promoters of tumor suppressor genes.^{5,6} A recent study also linked the observation of genes specifically expressed in both the placenta and various tumors to hypomethylation.⁷ However, to our knowledge, no study has yet directly compared genome-wide changes of DNA methylation in the placenta during pregnancy and in cancers during neoplastic transformation. We hypothesized that there may be parallels between the patterns of methylation that distinguish the early pregnancy placenta from the late pregnancy placenta and those that distinguish tumors from corresponding normal tissues, and that such similar epigenetic patterns may contribute to the regulation of shared cancer/placenta phenotypes.

Therefore, we conducted a genome-wide comparison of DNA methylation changes in placental tissues during pregnancy and

CONTACT Martin J. Aryee  aryee.martin@mgh.harvard.edu  The Jackson Laboratory for Genomic Medicine, Farmington, CT 06030, USA.

 Supplemental data for this article can be accessed on the [publisher's website](#).

© Akpéli V. Nordor, Djamel Nehar-Belaid, Sophie Richon, David Klatzmann, Dominique Bellet, Virginie Dangles-Marie, Thierry Fournier, and Martin J. Aryee. Published with license by Taylor & Francis. This is an Open Access article distributed under the terms of the Creative Commons Attribution-NonCommercial-NoDerivatives License (<http://creativecommons.org/licenses/by-nc-nd/4.0/>), which permits non-commercial re-use, distribution, and reproduction in any medium, provided the original work is properly cited, and is not altered, transformed, or built upon in any way.

in 13 types of tumor tissues during neoplastic transformation. We used publically available placenta and cancer DNA methylation data, complemented by methylome data generated from villous cytotrophoblasts isolated from placental tissues. All DNA methylation profiling was performed using the Illumina HumanMethylation450 (450K) microarray platform, thereby facilitating comparisons across data sets. We also investigated links between cancer/placenta patterns of methylation and gene expression profiles in cancer.

We report here that the early pregnancy human placenta is hypomethylated compared with later in pregnancy. This hypomethylation is organized into megabase-scale domains that frequently overlap with hypomethylated block regions observed in solid tumors.^{8,9} These hypomethylated blocks encompass genes involved in mechanisms related to hallmark cancer phenotypes.

Results

Placental methylome remodeling during pregnancy involves widespread methylation gains in CpG-poor genomic regions

To identify epigenomic features specific to the early pregnancy placenta, we first examined DNA methylation changes that occur between first and third trimester chorionic villi samples in the data set made available by the Robinson laboratory in Schroeder et al., 2013 [Gene Expression Omnibus (GEO): GSE49343] (Fig. 1A; Table S1).¹⁰ The chorionic villus represents the structural and functional unit that projects from the fetal placenta to invade the maternal uterine lining. The villous core, made of fibroblasts, mesenchymal cells, Hofbauer cells, and fetal-placental vessels, is covered by layers of villous cytotrophoblasts and syncytiotrophoblasts, which are in direct contact with maternal blood.¹¹ DNA methylation was measured on the 450K array and quantified using β values. We computed the difference in methylation between first trimester [6–10 weeks of gestation (WG), $n = 5$] and third trimester (32–39 WG, $n = 10$) chorionic villi samples. We found that while there were many alterations at CpGs distal from CpG islands with 36% of CpGs in open-sea regions affected (>4 kb from the nearest CpG island),¹² there were relatively few methylation changes at CpG islands themselves (11%) (Fig. 1B; Table S2; Figs. S1, S2 and S3). The majority (72%) of changes in open-sea regions represent hypomethylation [methylation difference < -0.05 , false discovery rate (FDR) q -value < 0.05] in first vs. third trimester chorionic villi. In contrast, methylation differences at CpG islands, in addition to being much less frequent, were equally balanced between hypermethylation (53%) and hypomethylation (47%) (Fig. 1C). We next compared placental methylomes to those of tumors profiled on the 450K array by The Cancer Genome Atlas (TCGA, <http://cancergenome.nih.gov/>) for 13 cancer types (4115 primary tumor and 460 matched normal tissue samples) (Fig. 1A; Table S1). As expected, cancer tissues displayed large changes at both CpG islands (32% altered) and at CpGs distal from islands (45% altered) as illustrated with colon adenocarcinoma (Fig. 1B; Table S2, Figs. S1, S2 and S3). Hypomethylation represents 75% of changes in open sea regions, while hypermethylation represents 78% of changes at CpG islands (Fig. 1C). To further

explore evidence of the hallmark cancer phenotype of CpG island hypermethylation in the placenta, we examined the sets of CpG island probes displaying baseline low levels of methylation in normal tissues (methylation < 0.20). In cancers, we found that, on average, 28% (5–39%) become significantly hypermethylated in the corresponding tumors (methylation difference > 0.05 and FDR q -value < 0.05). Among the subset that are unmethylated in third trimester chorionic villi, only 6% were significantly hypomethylated during the first trimester (Table S3). Taken together, these results suggest open-sea hypomethylation as a common epigenetic feature of early placental cells and neoplastic cells, and that this hypomethylation begins to resolve as pregnancy progresses.

Large hypomethylated blocks distinguish first from third trimester placenta and tumors from matched normal tissues

Given the similarities between the patterns of hypomethylation in first trimester placental chorionic villi and cancers, we sought to further characterize the genomic regions involved. Methylomes of solid tumors have been shown to display hypomethylated blocks, defined as large regions within which the average methylation is reduced compared with normal tissues.^{8,9} To search for such hypomethylated blocks, we used the block-finding procedure implemented in the R/Bioconductor *minfi* package (See Materials and Methods).^{13,14} We identified 1,240 blocks of differential methylation distinguishing first from third trimester placental chorionic villi samples, spanning 345 Mb—approximately 12% of the genome (permutation test q -value < 0.05). Among these placenta blocks, 93% are hypomethylated in the first trimester, with a median hypomethylation inside blocks of 10% (Table 1; Fig. S4). On average, each individual placenta hypomethylated block has a length of 283 kb (median 225 kb) and contains 2 genes (Fig. 2A, C, D). We investigated the similarity of placenta hypomethylated blocks to those observed in cancer by applying the same block-finding procedure to the 13 cancer types from TCGA. Among these cancers, there are on average 1,752 blocks (965–2,195) of differential methylation distinguishing tumors from their corresponding normal tissue samples (permutation test q -value < 0.05). On average, 74% (20–100%) of these are hypomethylated; most with a negative median methylation (hypomethylation) ranging from -15% in rectum adenocarcinoma to -3% in thyroid carcinoma, except in prostate adenocarcinoma with a $+3\%$ median methylation. Their coverage ranges from 212 Mb (approximately 7% of the genome) in prostate adenocarcinoma to 1,078 Mb (approximately 36% of the genome) in hepatocellular carcinoma. On average, 87% (42–100%) of these are hypomethylated (Table 1; Fig. S4). In colon adenocarcinoma, taken as an illustrative example, the average hypomethylated block has a length of 493 kb (median 376 kb) and contains 4 genes (Fig. 2B, C, D). Interestingly, using Monte Carlo simulations (See Materials and Methods), we observed that the hypomethylated blocks found in cancers overlapped on average 43% of placenta hypomethylated blocks (> 5 kb shared), with statistically significant co-localization (Bonferroni-adjusted $\alpha = 0.05$) in a subset of 5 tumor types; namely, bladder urothelial carcinoma, colon adenocarcinoma, head and

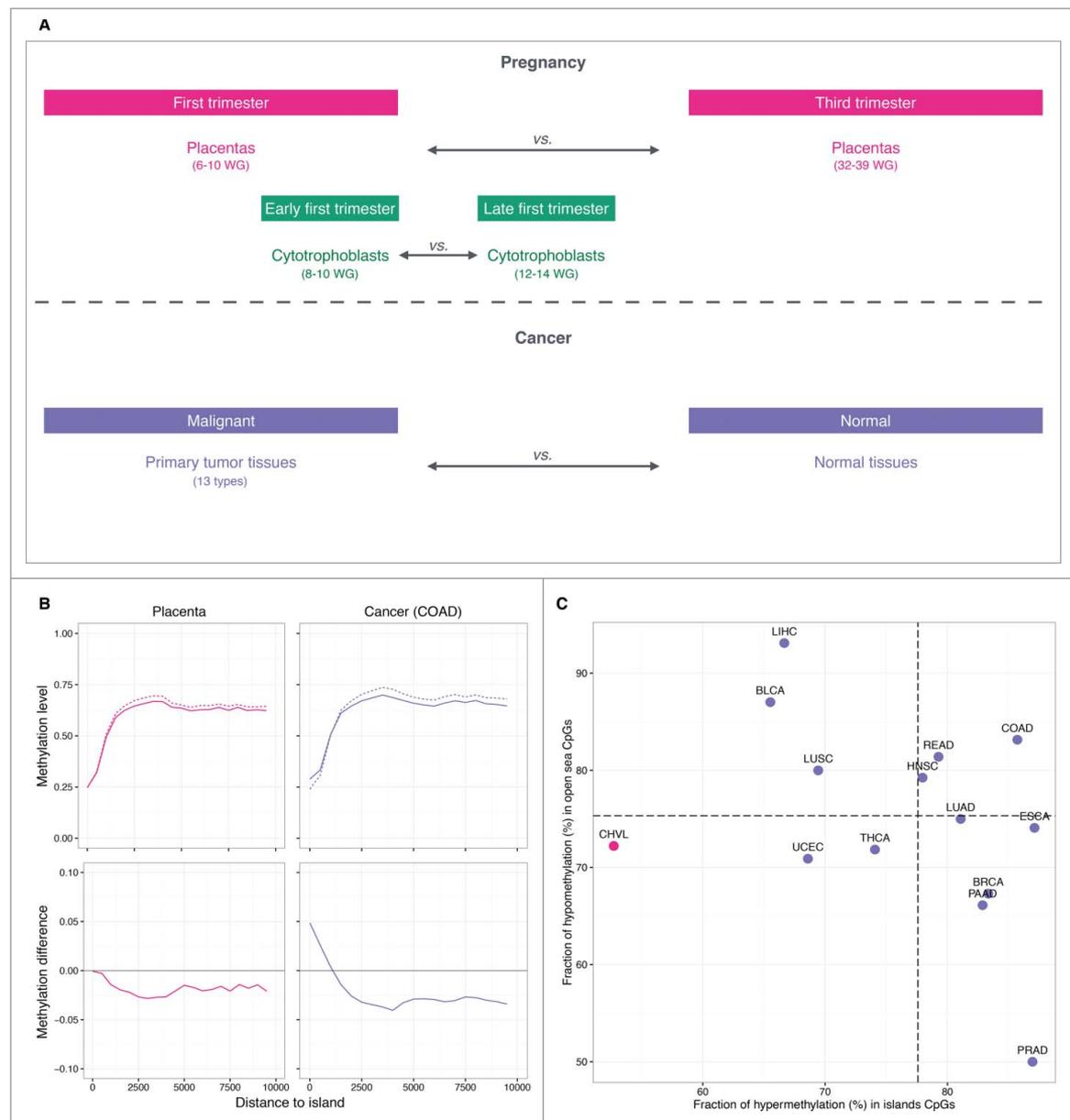


Figure 1. Comparison of DNA methylation in placenta and cancer. (A) Design of the study. (B) Top: methylation level plotted against the distance to the nearest CpG island. In pink, the solid line denotes first trimester and the dashed line denotes third trimester chorionic villi samples. In purple, the solid line denotes colon adenocarcinoma tumor samples and the dashed line denotes corresponding normal tissue samples. Bottom: methylation difference in first vs. third trimester chorionic villi samples (pink) and tumor vs. normal tissue samples (purple). (C) Fraction of hypermethylation (hypermethylation alterations / total alterations) in island CpGs (x-axis) vs. fraction of hypomethylation (hypomethylation alterations / total alterations) in open sea CpGs (y-axis) in placenta (pink) and cancer samples (purple). CpGs were classified as altered if they displayed an absolute methylation difference >0.05 with a FDR q -value <0.05 . Horizontal and vertical dashed lines represent average fractions in cancer. Abbreviations as in Table 1.

neck squamous cell carcinoma, pancreatic adenocarcinoma, and rectum adenocarcinoma (Fig. S5). Taken together, these observations reveal that the patterns of hypomethylation that distinguish first from third trimester placental tissues are similar in structure, size and location to those that distinguish tumors from matched normal tissues.

Hypomethylated blocks also distinguish placental villous cytotrophoblasts before and after they come into contact with maternal blood

Since substantial environment and cell type composition changes in the placenta occur during the 9 months of gestation,

we next sought to more narrowly define the time period and cell types associated with the observed placental methylation changes. We hypothesized that significant changes may occur concurrently with the major physiologic shifts that occur when maternal blood first comes into contact with the chorionic villi during the 10th to 12th weeks of pregnancy.¹¹ To investigate methylation changes that occur during this period, we looked for differences that arise between the early first trimester (8–10 WG, $n = 9$) and late first trimester (12–14 WG, $n = 10$) villous cytotrophoblast samples (Fig. 1A; Table S1), isolated *ex vivo* from chorionic villi samples using sequential enzymatic digestions. DNA methylation was profiled using the 450K array. At the individual probe level, we found that, as in the chorionic

Table 1. Large blocks of differential methylation in placenta and cancer tissues.

	Number of Blocks (N)	Genomic Size (Mb)	Prop. Hypo. Blocks (%)	Med. Diff. Methylation (%)
First trimester vs. Third trimester				
Chorionic villi (CHVL)	1240	346	93	-10
Malignant vs. Normal				
Bladder urothelial carcinoma (BLCA)	1865	887	99	-11
Breast invasive carcinoma (BRCA)	2195	774	52	-5
Colon adenocarcinoma (COAD)	1978	843	81	-8
Esophageal carcinoma (ESCA)	965	306	94	-9
Head and neck squamous cell carcinoma (HNSC)	2099	916	73	-7
Liver hepatocellular carcinoma (LIHC)	1856	1078	99	-12
Lung adenocarcinoma (LUAD)	1620	593	72	-6
Lung squamous cell carcinoma (LUSC)	2063	966	84	-8
Pancreatic adenocarcinoma (PAAD)	1058	212	81	-6
Prostate adenocarcinoma (PRAD)	2105	568	26	3
Rectum adenocarcinoma (READ)	1183	422	100	-15
Thyroid carcinoma (THCA)	1752	422	20	-3
Uterine corpus endometrioid carcinoma (UCEC)	2041	686	76	-8

Prop. Hypo. Blocks (%): proportion of hypomethylated blocks in percent. Med. Diff. Methylation (%): median of the average difference between first and third trimester/malignant and normal.

villi samples, CpGs distal from islands showed hypomethylation in the early first trimester villous cytotrophoblasts, with 20% of open-sea probes affected (methylation difference < -0.05 , FDR q -value < 0.05). Also, consistent with the observations in chorionic villi, only 3% of cytotrophoblast probes in island regions show a difference related to time. (Table S2; Fig. S6). Then, using the same block-finding procedure, we also identified large regions of differential methylation distinguishing early from late first trimester villous cytotrophoblast samples. There were 997 blocks spanning 223 Mb (approximately 7% of the genome, permutation test q -value < 0.05). On average, each individual cytotrophoblast hypomethylated block has a length of 261 kb (median 219 kb) and contains 2 genes (Fig. 3A; Fig. S4). Importantly, cytotrophoblast hypomethylated blocks have a highly significant 85% overlap (P -value < 0.001) with hypomethylated blocks identified in bulk placental chorionic villi samples (Fig. 3B). This finding indicates that the placenta hypomethylated blocks identified are a phenomenon observable in individual cell types, rather than a consequence of cell type composition changes during pregnancy.

Cancer and placenta hypomethylated blocks encompass genes related to hallmark cancer pathways

To gain insight into the biologic functions potentially affected by hypomethylated blocks, we performed gene set analyses using MsigDB hallmark gene sets,¹⁵ and Ingenuity Pathway Analysis (IPA) gene sets related to immune response (Fig. 4A; Fig. S7). Gene set enrichment was assessed for genes encompassed in: (i) placenta and cytotrophoblast hypomethylated blocks; (ii) cancer hypomethylated blocks (using colon adenocarcinoma as an illustrative example of cancers); (iii) cancer/placenta hypomethylated blocks, i.e., cancer hypomethylated blocks that co-localize with placenta hypomethylated blocks; and (iv) cancer unique hypomethylated blocks, i.e., cancer hypomethylated blocks that do not co-localize with placenta hypomethylated blocks. As expected, placenta and cytotrophoblast hypomethylated blocks showed similar gene set enrichment (P -value < 0.05), including “Allograft rejection,” “Inflammatory response” and “EMT.” To investigate potentially shared epigenetic mechanisms between cancer and placenta,

we focused on the subset of cancer/placenta hypomethylated blocks. This 44% subset of cancer hypomethylated blocks is enriched for 8 out of 9 gene sets significant in the full set, including “Allograft rejection,” “Angiogenesis” and “EMT.” Interestingly, we also found 2 gene sets that are significant only when looking specifically at cancer/placenta hypomethylated blocks, namely “TNF- α signaling via NF- κ B” (P -value = 0.005) and “Downregulated UV response” (P -value = 0.01). We finally investigated the expression profile of the corresponding genes located within cancer/placenta hypomethylated blocks using RNA-sequencing data from TCGA for colon adenocarcinoma. An IPA analysis revealed 2 networks of genes that are differentially expressed in tumor vs. normal tissues (FDR q -value < 0.01). The first network was based on “TNF- α signaling via NF- κ B” and included *CD83*, *CSF2*, *CXCL2*, *CXCL3*, *DUSP4*, *INHBA*, *OLR1*, and *SERPINB2*, which were significantly upregulated, and *CD69*, *CCL5*, and *KLF6*, which were significantly downregulated (Fig. 4B). The second network was based on “Downregulated UV response” and included *ANXA2*, *BDNF*, and *COL3A1*, which were significantly upregulated, and *AMPH*, *EFEMP1*, *ITGB3*, *KIT*, *MAGI2*, *NRP1*, and *SNAI2*, which were significantly downregulated (Fig. 4C). Notably, the upregulated NF- κ B complex represents a key hub in both networks. These results highlight processes with potentially shared epigenetic regulatory mechanisms during carcinogenesis and placentogenesis.

Discussion

Over the 20th century, successive technological innovations have allowed the characterization of striking similarities between placental and cancer cells. The microscope initially allowed the observation of common phenotypes;^{16,17} later, modern biochemistry and molecular biology allowed the characterization of molecular circuits underlying these similarities. Thus, we now know that placental cells, even though they do not possess the somatically altered genomes characteristic of cancer cells, rely on hallmark cancer molecular programs to establish pregnancy.^{1-4,18} More recently, independent studies in cancer biology and placenta biology have suggested similarities between epigenetic patterns in placental and cancer cells.^{5,6}

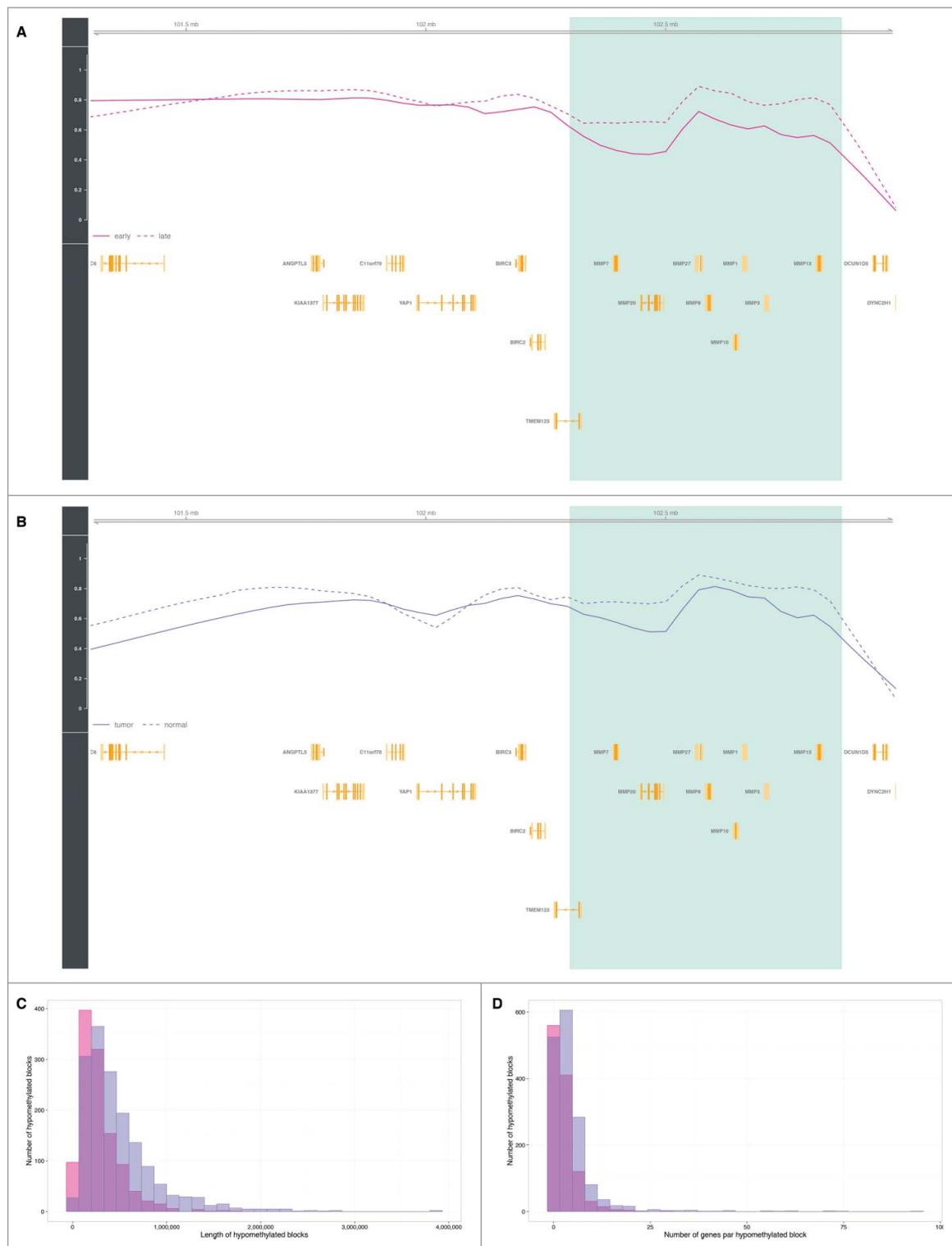


Figure 2. Hypomethylated blocks in placenta and cancer. (A) The light green zone shows an illustrative placenta hypomethylated block representing a difference in average methylation between first trimester (solid line) and third trimester (dashed line) chorionic villi samples. Gene loci are marked in yellow. (B) The light green zone shows an illustrative cancer hypomethylated block representing a difference in average methylation between colon adenocarcinoma tumor (solid line) and corresponding normal tissue (dashed line) samples. Gene loci are marked in yellow. (C) Distributions of the lengths of placenta hypomethylated blocks in pink and cancer hypomethylated blocks (colon adenocarcinoma) in purple. (D) Distributions of the number of genes per placenta hypomethylated blocks in pink, and cancer hypomethylated blocks in purple (colon adenocarcinoma). Abbreviations as in Table 1.

The study presented here focused on the identification of commonalities between methylation dynamics during pregnancy and neoplastic transformation. We conducted the first direct genome-wide comparison between DNA methylation changes in placenta throughout pregnancy, and in cancer

during neoplastic transformation. We used publicly available DNA methylation and gene expression data, and complemented this with a newly generated data set representing the first genome-wide DNA methylation profiles from isolated villous cytotrophoblasts obtained before and after the cells come

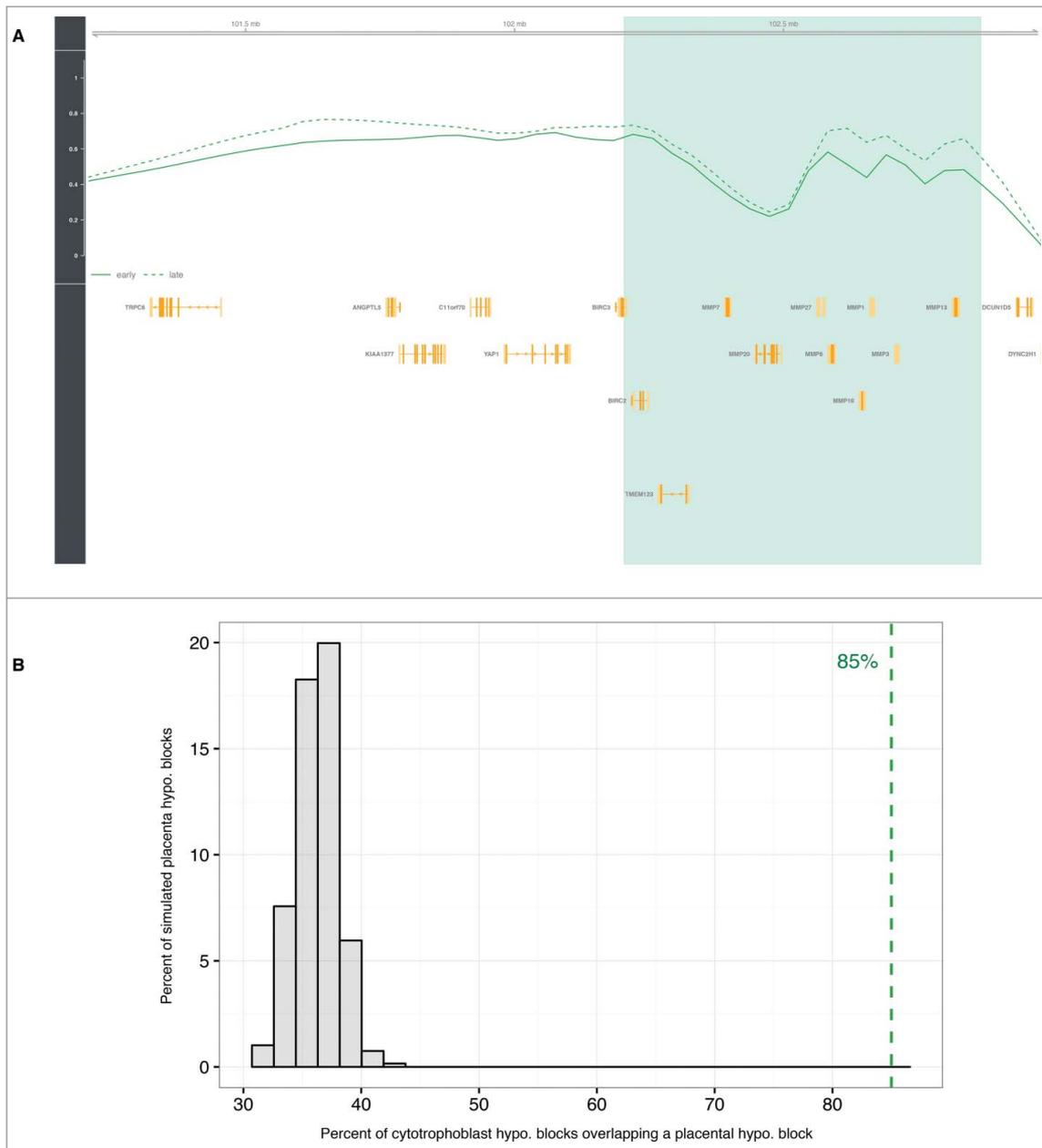


Figure 3. Hypomethylated blocks in isolated villous cytotrophoblasts. (A) The light green zone shows an illustrative cytotrophoblast hypomethylated block representing a difference in average methylation between early first trimester (solid line) and late first trimester (dashed line) villous cytotrophoblast samples. Gene loci are marked in yellow. (B) Null distribution of overlap fraction between cytotrophoblast hypomethylated blocks and randomly sampled regions equal in size to placenta hypomethylated blocks (chorionic villi). The dashed green line represents the observed overlap fraction (85%, P -value <0.001). Abbreviations as in Table 1.

into contact with maternal blood, a key step in the establishment of pregnancy. We report here that epigenetic similarities between cancer and placenta are most striking in early pregnancy placenta, and that the similarities are partially erased as pregnancy progresses. We also find that these shared epigenetic patterns relate to pivotal genes in both placentogenesis and carcinogenesis.

We first found that placental methylome remodeling during pregnancy involves methylation gains distal from CpG islands. Our findings are consistent with results from a high performance liquid chromatography (HPLC) assay that shows an increase in methylation levels during pregnancy.¹⁹ In contrast to the changes in open-sea regions, CpG islands remain largely unaltered during pregnancy, suggesting that the

hypermethylated state of tumor suppressor promoters, including *RASSF1A*, *APC*, *SFRP2*, *WIF1*, and *EN1*, described by previous studies on DNA methylation in the placenta, can remain largely stable throughout pregnancy.^{5,20-22} Intriguingly, this suggests that the two characteristic classes of cancer methylome alterations—namely, hypomethylation of CpG-sparsely regions and hypermethylation of CpG islands—can occur independently, as only the former set of regions are affected during pregnancy progression.

We found that hypomethylation in first relative to third trimester chorionic villi is organized into large blocks with an average length of 283 kb. In 2013, Schroeder et al. first described large-scale hypomethylated regions in chorionic villi,²³ which thus represents the first known normal tissue showing clear evidence of large-scale

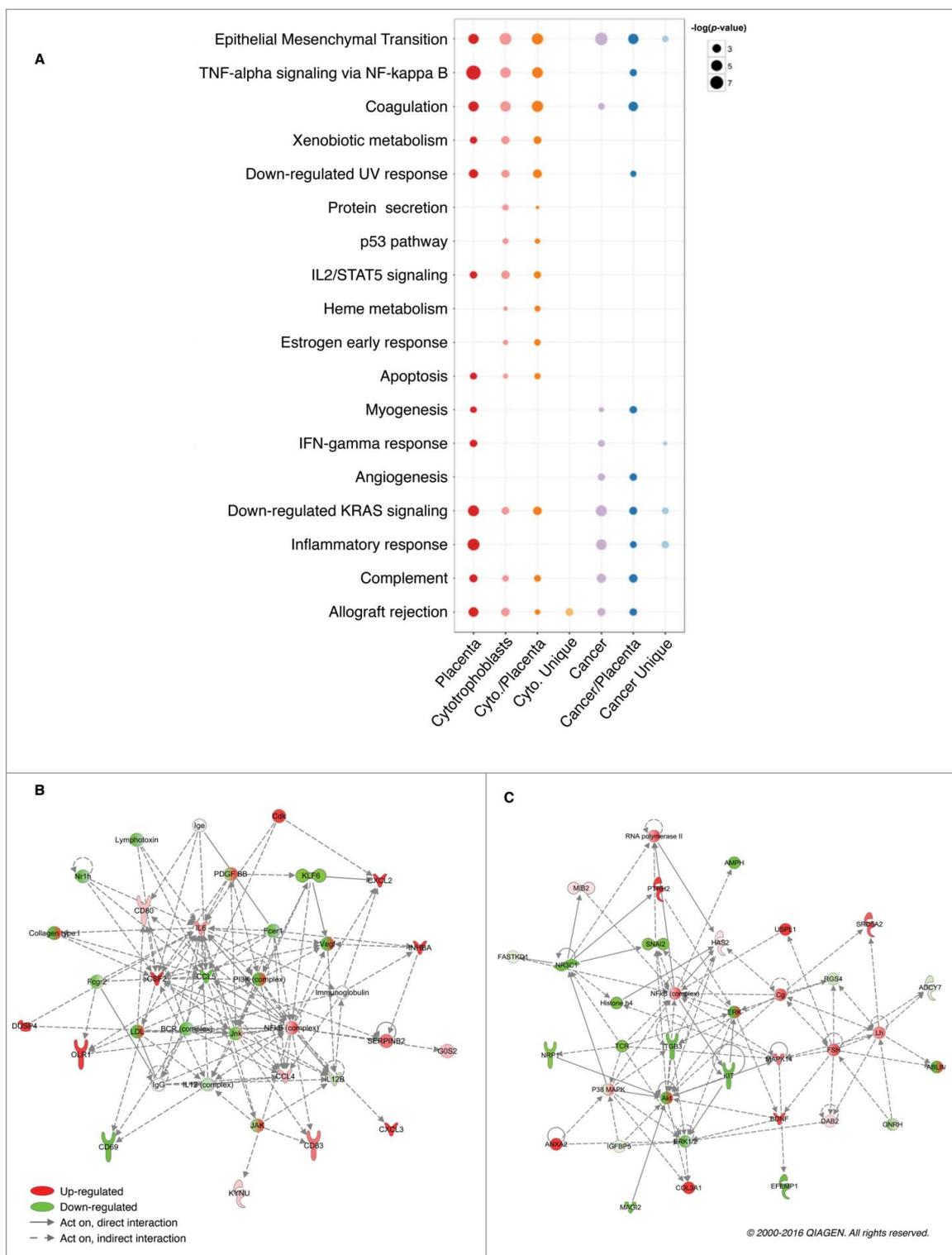


Figure 4. Gene set enrichment for genes encompassed in placenta and cancer hypomethylated blocks. (A) Bubble chart representing the enrichment of MsigDB hallmark gene sets in placenta and cancer hypomethylated blocks (colon adenocarcinoma). “Cancer/Placenta” (“Cancer Unique”) represents genes in cancer hypomethylated blocks that co-localize (do not co-localize) with a placenta hypomethylated block. Only the significant enrichments (P -value < 0.05) are presented. Networks of genes belonging to cancer/placenta blocks and relating to the (B) “TNF- α signaling via NF- κ B” gene set and the (C) “UV Response - Down” gene set were identified using IPA software. Abbreviations as in Table 1.

contiguous blocks of hypomethylation of the kind previously been described in cancer. This observation was in line with the historic observation of a global hypomethylation characteristic of placenta and cancers compared with other tissues.^{24,25} Hypomethylated blocks have initially been described in cancers using whole-genome

bisulfite sequencing. First, in colon tumors,^{8,26} then, in breast cancer cell line,²⁷ medulloblastoma tumors,²⁸ and in EBV immortalized B-cells.²⁹ In 2014, Timp et al. reported the first integrated genome-wide analysis of DNA methylation of 6 different tumor types (breast, colon, lung, pancreas adenocarcinoma, pancreas

neuroendocrine tumors, thyroid cancer samples) using the 450K array.⁹ These studies have thus established hypomethylated blocks as a universal defining epigenetic alteration in human solid tumors. Using TCGA data for 13 cancer types, we also observed hypomethylated blocks in breast, colon, lung, pancreas and thyroid cancers, as well as described such hypomethylation patterns for the first time in liver, head and neck, bladder, uterus, esophageal and prostate cancers. Strikingly, in most cases, the number of blocks, size, and overall hypomethylation we found in cancers were similar to the ones we found in early pregnancy chorionic villi. Cancer and chorionic villi hypomethylated blocks also display frequent colocalization, which was significant in several cancer types. Taken together, our observations provide the first evidence of dynamic change of these large-scale hypomethylated regions throughout pregnancy, and highlight an intriguing parallel with hypomethylation patterns in cancers.

Moreover, we also observed that hypomethylated blocks distinguish villous cytotrophoblasts before and after they first come into contact with maternal blood (weeks 10–12), providing the first non-cancer evidence of large-scale hypomethylated blocks in a purified cell type. Furthermore, this result indicates that our finding of hypomethylated blocks in the placenta is not an artifact of cell-type confounding related to changes in cellular composition during pregnancy. It also suggests that many of the methylation differences between early and late pregnancy placenta may be associated with changes in the cytotrophoblast microenvironment such as exposure to components of maternal blood and higher oxygen tension. This time point has been also shown to be associated with changes in proliferative, invasive and fusion abilities of the cytotrophoblasts.^{11,30}

Finally, our data suggest that cancer and placenta hypomethylated blocks might be involved in the regulation of hallmark cancer pathways. The regulatory implications of megabase-scale hypomethylation is poorly understood, but the finding that it is associated with increased gene expression variability has led to the suggestion that it may provide a mechanism for a phenotypic plasticity that allows adaptation and rapid growth in the setting of a host tissue.³¹ Supporting this hypothesis, we found that cancer and placenta hypomethylated blocks are enriched for pathways including EMT, allograft rejection, and inflammatory response. Interestingly, we also identified two enriched gene sets that are detectable only when focusing on the subset of cancer/placenta hypomethylated blocks rather than the full set of cancer hypomethylated blocks. Differentially expressed genes in these sets are involved in predicted networks centered on NF- κ B, a transcription factor complex playing a critical role in inflammation.³² This observation is in line with the hypothesis that the establishment of pregnancy and cancer are both pro-inflammatory states.^{33,34} These networks include *BDNF*, *SERPINB2*, and *CXCL3*, genes described previously as instrumental in both placentogenesis and cancerogenesis. The brain-derived neurotrophic factor (BDNF) has been linked to placental development³⁵ and it has also been found to regulate cell motility in colon cancer.³⁶ Plasminogen Activator Inhibitor Type 2 (PAI-2), encoded by *SERPINB2*, was initially identified in the placenta and low levels of PAI-2 has been linked to preeclampsia.^{37,38} Since then, numerous studies have attempted to elucidate its role in invasion and metastasis.³⁹ *CXCL3* has been

implicated in placental invasion and is aberrantly expressed both in severe preeclampsia⁴⁰ and in cancer during esophageal carcinogenesis.⁴¹ Our analyses also pointed to immune-related genes that were down-modulated in colon tumors, including *CD69* and *CCL5*. This last observation could be explained by the presence within the immune infiltrate of suppressive subpopulations such as regulatory T cells (Tregs), M2 macrophages, or myeloid-derived suppressive cells (MDSC), that have previously been linked to tolerance induction both in cancer and pregnancy.^{2,4,42,43} Our approach thus suggests a means for identifying genes potentially regulated by similar epigenetic mechanisms during pregnancy and neoplastic transformation.

This pilot study demonstrates that the early pregnancy placenta differs from that in later pregnancy by the existence of additional hypomethylation in the form of megabase-scale blocks. These blocks are similar in size and number to those that characterize tumors, and may contribute to the regulation of molecular programs allowing cellular growth advantage at the expense of the host. Further analyses of these patterns could eventually lead to the identification of critical epigenetic switches that prevent healthy placentas from degenerating into tumors, and whose failure allows tumor development. The study demonstrates the relevance of placental research as a platform for innovative approaches in oncology as well as in obstetrics and gynecology.

Materials and methods

DNA methylation data

Villous cytotrophoblast data was generated on the 450K platform and normalized using the *preprocessFunnorm* function implemented in the *minfi* package of the R/Bioconductor software environment,^{13,14} it is available in GEO under accession number GSE93208. Public 450K data for chorionic villi and cancers was obtained from GEO (GSE49343) and TCGA (<http://cancergenome.nih.gov/>), respectively (Table S1).¹⁰ The calculated β values from GEO were downloaded using the *getGenomicRatioSetFromGEO* function of *minfi*, and the calculated β values from TCGA (level 2 data, Feb 24th 2015 version) were downloaded via the UCSC Cancer Genomics Browser website (<https://genome-cancer.ucsc.edu/>).^{13,14} The solid tumor vs. normal tissue samples spanned 13 cancer types: bladder urothelial carcinoma (BLCA); breast invasive carcinoma (BRCA); colon adenocarcinoma (COAD); esophageal carcinoma (ESCA); head and neck squamous cell carcinoma (HNSC); liver hepatocellular carcinoma (LIHC); lung adenocarcinoma (LUAD); lung squamous cell carcinoma (LUSC); pancreatic adenocarcinoma (PAAD); prostate adenocarcinoma (PRAD); rectum adenocarcinoma (READ); thyroid carcinoma (THCA); and uterine corpus endometrioid carcinoma (UCEC).

Isolation and purification of human villous cytotrophoblasts

The *ex vivo* villous cytotrophoblast samples were isolated from placental tissues (chorionic villi) and extemporaneously frozen. They were obtained following legal voluntary interruption of 318 pregnancies in patients at the Department of Obstetrics

and Gynecology at Cochin Port-Royal 319 Hospital (Paris, France). Patients gave their informed written consent and the local ethics committee 320 (CCP IDF1, N° 13909, Paris, France) approved the study. Sequential enzymatic digestion (Trypsine and DNase) based on methods described previously by Kliman et al. were used with modifications to isolate villous cytotrophoblast cells.^{11,44} The isolation step was followed by a purification step based on Percoll gradient fractionation. A subset of the purified villous cytotrophoblasts were characterized by immunolabeling using the cytokeratin 7 (CK7) and their ability to aggregate after 48 h and to form syncytiotrophoblasts at 72 h of culture. DNA was extracted using the GenElute™ Mammalian Genomic DNA Miniprep Kit (Sigma-Aldrich).

Probe level analysis

For each probe, we averaged the methylation level (β value between 0 and 1) across individuals in each case group (e.g., chorionic villi first trimester or colon adenocarcinoma tumor) and control group (e.g., chorionic villi third trimester or colon adenocarcinoma normal). We then computed the difference at the probe level between these case and control groups in placenta and cancer. Student's t-test was used to assess statistical significance of differences, and the false discovery rate procedure implemented in the R/Bioconductor *qvalue* package was used to correct for multiple testing.^{45,46} Methylation differences were termed biologically meaningful when above 0.05 and statistically significant for an FDR q -value below 0.05.

Block-finding procedure

To investigate blocks (megabase-scale changes in methylation) in Illumina 450K data, we used the block-finding procedure implemented in the R/Bioconductor *minfi* package.^{13,14} This procedure uses open-sea CpGs, i.e., those distal from CpG islands, to identify large-scale methylation patterns. The 485,512 probes on this array target regions including CpG islands (31% of the array), CpG island shores (23%) and shelves (10%) located adjacent to CpG islands, while the remaining probes represent open-sea CpGs located at least 4 kb away from CpG islands (36%).¹² Briefly, the procedure groups adjacent open sea CpGs into clusters with a maximum gap width of 500 bp. Clusters with a width >1,500 bp are subdivided. Methylation values at CpGs within each cluster are averaged, resulting in a single mean estimate per cluster. Student's t-statistics comparing cases (e.g., chorionic villi first trimester or colon adenocarcinoma tumor) and controls (e.g., chorionic villi third trimester or colon adenocarcinoma normal) were calculated for each cluster. Then, clusters within 250 kb from each other were grouped together. Finally, the Bumhunter algorithm was applied within each group to detect blocks exhibiting differences in average methylation between cases and controls.⁴⁷ The main steps of this algorithm are the following: (i) fitting a loess curve with a 250 kb smoothing window through the t-statistics; (ii) identifying regions with an absolute smoothed t-statistic in the top 2.5% as putative blocks; and (iii) determining block P -values based on the likelihood of such dimensions occurring by chance through a sample permutation test (1,000 permutations) using *minfi* default parameters. The

P -value is calculated as the fraction of permutations that generate a block of equal or larger dimensions. Blocks containing at least 5 probe clusters with a q -value below 0.05 were declared statistically significant. Also, as we used microarray data, and not whole-genome bisulfite sequencing data, we could only identify blocks located in regions included in the design of the 450K assay; we could not tell if regions excluded from the array contain blocks.

Block overlap P -values

For each tissue, tables gathering all characteristics of the methylation blocks we detected were converted into GRanges objects of the R/Bioconductor software environment to allow convenient manipulation of information corresponding to genomic intervals.⁴⁸ Overlaps between each set of hypomethylated blocks found in cancers (the queries) and the set of placenta hypomethylated blocks (the subject) were assessed as the fraction of cancer hypomethylated blocks that overlapped a placenta hypomethylated block by at least 5 kb. Significance of the overlap rate was determined using Monte Carlo simulations. We generated 1,000 random lists of simulated placenta hypomethylated blocks. We constrained the simulation such that, compared with observed placenta hypomethylated blocks, the simulated placenta hypomethylated blocks had the same genomic coverage (within 10%) and the same number of collapsed open sea probes. We further required that hypomethylated blocks within a simulated set were non-overlapping by resampling until non-overlapping lists were obtained. Then, we counted overlaps between each of list of simulated subject (placenta) hypomethylated blocks and the observed query lists (each cancer). The 1,000 overlap fractions formed the null distribution of overlaps obtained by chance. We then compared these null distributions with the observed overlap fractions. To account for multiple testing, we declared significant those overlaps with a P -value less than the Bonferroni-adjusted threshold, i.e., P -value/number of comparisons = 0.05/13. Notably, this approach avoids biases due to the design of the 450K microarray platform.

MsigDB and IPA gene set enrichment analyses

The gene universe was defined as unique gene symbols in the R/Bioconductor *Homo sapiens* package with a transcription start site (TSS) within a region probed by block-finding procedure (i.e., regions defined by the *cpgCollapse* function of *minfi*). Using the R/Bioconductor *GeneOverlap* package, gene set enrichment P -values for MsigDB hallmark gene sets¹⁵ and IPA (<http://www.ingenuity.com/>) gene sets related to immune response were computed for genes encompassed in: (i) placenta and cytotrophoblast hypomethylated blocks; (ii) cancer hypomethylated blocks (using colon adenocarcinoma as an illustrative example of cancers); (iii) cancer/placenta hypomethylated blocks, i.e., cancer hypomethylated blocks that co-localize with placenta hypomethylated blocks; and (iv) cancer unique hypomethylated blocks, i.e., cancer hypomethylated blocks that do not co-localize with placenta hypomethylated blocks. Rows of Fig. 4A were ordered using the *ward.2* method for hierarchical clustering built in the R software environment. Gene expression

profiles of colon adenocarcinoma primary tumors (n = 241) and normal tissues (n = 39) measured by TCGA using Illumina HiSeq 2000 RNA-Seq platform were downloaded via the UCSC Cancer Genomics Browser website (level 3 RNA-seq profiles, RNAseqV2 normalized RSEM, Feb 24, 2015 version). The differentially expressed gene list was produced using the R/Bioconductor *limma* package.⁴⁹

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

Acknowledgments

We are grateful to Raphaël Bilgraer for his advice as well as to Justin Guinney for his feedback. We also thank Sophie Gil and Françoise Vibert for their help in isolating villous cytotrophoblasts.

Funding

This work was supported by the Association pour la recherche en cancérologie de Saint-Cloud (ARCS); ADEBIOPHARM; Genevieve and Jean-Paul Driot Transformative Research Grant; Ouidad Hachem Foundation Grant; Philippe, Laurent and Stéphanie Bloch Cancer Research Grant; Sally Paget-Brown Translational Research Grant; and French state funds within the Investissements d'Avenir program under Grant ANR-11-IDEX-0004-02.

Availability of data and material

DNA methylation profiles (Illumina 450K data) of the first trimester villous cytotrophoblasts are available in GEO under accession number GSE93208.

ORCID

Akpèli V. Nordor  <http://orcid.org/0000-0003-3541-0221>

Martin J. Aryee  <http://orcid.org/0000-0002-6848-1344>

References

1. Ferretti C, Bruni L, Dangles-Marie V, Pecking AP, Bellet D. Molecular circuits shared by placental and cancer cells, and their implications in the proliferative, invasive and migratory capacities of trophoblasts. *Hum Reprod Update* 2007; 13:121-41; PMID:17068222; <https://doi.org/10.1093/humupd/dml048>
2. Holtan SG, Creedon DJ, Haluska P, Markovic SN. Cancer and Pregnancy: Parallels in growth, invasion, and immune modulation and implications for cancer therapeutic agents. *Mayo Clin Proc* 2009; 84:985-1000; PMID:19880689; [https://doi.org/10.1016/S0025-6196\(11\)60669-1](https://doi.org/10.1016/S0025-6196(11)60669-1)
3. Louwen F, Muschol-Steinmetz C, Reinhard J, Reitter A, Yuan J. A lesson for cancer research: placental microarray gene analysis in pre-eclampsia. *Oncotarget* 2012; 3:759-73; PMID:22929622; <https://doi.org/10.18632/oncotarget.595>
4. Nehar-Belaid D, Courau T, Dérian N, Florez L, Ruocco MG, Klatzmann D. Regulatory T Cells orchestrate similar immune evasion of fetuses and tumors in mice. *J Immunol* 2016; 196:678-90; PMID:26643476; <https://doi.org/10.4049/jimmunol.1501834>
5. Novakovic B, Saffery R. Placental pseudo-malignancy from a DNA methylation perspective: unanswered questions and future directions. *Front Genet* 2013; 4:285; PMID:24368911; <https://doi.org/10.3389/fgene.2013.00285>
6. Allis CD, Jenuwein T. The molecular hallmarks of epigenetic control. *Nat Rev Genet* 2016; 17:487-500; PMID:27346641; <https://doi.org/10.1038/nrg.2016.59>
7. Rousseaux S, Debernardi A, Jacquiau B, Vitte A-L, Vesin A, Nagy-Mignotte H, Moro-Sibilot D, Brichon P-Y, Lantuejoul S, Hainaut P, et al. Ectopic activation of germline and placental genes identifies aggressive metastasis-prone lung cancers. *Sci Transl Med* 2013; 5:186ra66; PMID:23698379; <https://doi.org/10.1126/scitranslmed.3005723>
8. Hansen KD, Timp W, Bravo HC, Sabunciyan S, Langmead B, McDonald OG, Wen B, Wu H, Liu Y, Diep D, et al. Increased methylation variation in epigenetic domains across cancer types. *Nat Genet* 2011; 43:768-75; PMID:21706001; <https://doi.org/10.1038/ng.865>
9. Timp W, Bravo HC, McDonald OG, Goggins M, Umbrecht C, Zeiger M, Feinberg AP, Irizarry RA. Large hypomethylated blocks as a universal defining epigenetic alteration in human solid tumors. *Genome Med* 2014; 6:61; PMID:25191524; <https://doi.org/10.1186/s13073-014-0061-y>
10. Blair JD, Langlois S, McFadden DE, Robinson WP. Overlapping DNA methylation profile between placentas with trisomy 16 and early-onset preeclampsia. *Placenta* 2014; 35:216-22; PMID:24462402; <https://doi.org/10.1016/j.placenta.2014.01.001>
11. Cocquebert M, Berndt S, Segond N, Guibourdenche J, Murthi P, Aldaz-Carroll L, Evain-Brion D, Fournier T. Comparative expression of hCG β -genes in human trophoblast from early and late first-trimester placentas. *Am J Physiol Endocrinol Metab* 2012; 303:E950-8; PMID:22811468; <https://doi.org/10.1152/ajpendo.00087.2012>
12. Sandoval J, Heyn HA, Moran S, Serra-Musach J, Pujana MA, Bibikova M, Esteller M. Validation of a DNA methylation microarray for 450,000 CpG sites in the human genome. *Epigenetics* 2011; 6:692-702; PMID:21593595; <https://doi.org/10.4161/epi.6.6.16196>
13. Huber W, Carey VJ, Gentleman R, Anders S, Carlson M, Carvalho BS, Bravo HC, Davis S, Gatto L, Girke T, et al. Orchestrating high-throughput genomic analysis with Bioconductor. *Nat Methods* 2015; 12:115-21; PMID:25633503; <https://doi.org/10.1038/nmeth.3252>
14. Aryee MJ, Jaffe AE, Corrada-Bravo H, Ladd-Acosta C, Feinberg AP, Hansen KD, Irizarry RA. Minfi: A flexible and comprehensive Bioconductor package for the analysis of Infinium DNA methylation microarrays. *Bioinformatics* 2014; 30:1363-9; PMID:24478339; <https://doi.org/10.1093/bioinformatics/btu049>
15. Subramanian A, Tamayo P, Mootha VK, Mukherjee S, Ebert BL. Gene set enrichment analysis: A knowledge-based approach for interpreting genome-wide. *Proc Natl Acad Sci U S A* 2005; 102:15545-50; PMID:16199517; <https://doi.org/10.1073/pnas.0506580102>
16. Beard J. Embryological Aspects and Etiology of Carcinoma. *Lancet* 1902; 159:1758-61; [https://doi.org/10.1016/S0140-6736\(01\)89936-0](https://doi.org/10.1016/S0140-6736(01)89936-0)
17. Beard J. The cancer problem. *Lancet* 1905; 90:283
18. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. *Cell* 2011; 144:646-74; PMID:21376230; <https://doi.org/10.1016/j.cell.2011.02.013>
19. Fuke C, Shimabukuro M, Petronis A, Sugimoto J, Oda T, Miura K, Miyazaki T, Ogura C, Okazaki Y, Jinno Y. Age related changes in 5-methylcytosine content in human peripheral leukocytes and placentas: An HPLC-based study. *Ann Hum Genet* 2004; 68:196-204; PMID:15180700; <https://doi.org/10.1046/j.1529-8817.2004.00081.x>
20. Novakovic B, Rakyan V, Ng HK, Manuelpillai U, Dewi C, Wong NC, Morley R, Down T, Beck S, Craig JM, et al. Specific tumour-associated methylation in normal human term placenta and first-trimester cytotrophoblasts. *Mol Hum Reprod* 2008; 14:547-54; PMID:18708652; <https://doi.org/10.1093/molehr/gan046>
21. Guilleret I, Osterheld M-C, Braunschweig R, Gastineau V, Tailens S, Benhattar J. Imprinting of tumor-suppressor genes in human placenta. *Epigenetics* 2009; 4:62-8; PMID:19106645; <https://doi.org/10.4161/epi.4.1.7471>
22. Chiu RWK, Chim SSC, Wong IHN, Wong CSC, Lee W-S, To KF, Tong JHM, Yuen RKC, Shum ASW, Chan JKC, et al. Hypermethylation of RASSF1A in human and rhesus placentas. *Am J Pathol* 2007; 170:941-50; PMID:17322379; <https://doi.org/10.2353/ajpath.2007.060641>
23. Schroeder DJ, Blair JD, Lott P, Yu HOK, Hong D, Crary F, Ashwood P, Walker C, Korf I, Robinson WP, et al. The human placenta methylome. *Proc Natl Acad Sci U S A* 2013; 110:6037-42; PMID:23530188; <https://doi.org/10.1073/pnas.1215145110>

24. Feinberg AP, Vogelstein B. Hypomethylation distinguishes genes of some human cancers from their normal counterparts. *Nature* 1983; 301:89-92; PMID:6185846; <https://doi.org/10.1038/301089a0>
25. Ehrlich M, Gama-Sosa MA, Huang LH, Midgett RM, Kuo KC, McCune RA, Gehrke C. Amount and distribution of 5-methylcytosine in human DNA from different types of tissues of cells. *Nucleic Acids Res* 1982; 10:2709-21; PMID:7079182; <https://doi.org/10.1093/nar/10.8.2709>
26. Berman BP, Weisenberger DJ, Aman JF, Hinoue T, Ramjan Z, Liu Y, Noushmehr H, Lange CPE, van Dijk CM, Tollenaar RAEM, et al. Regions of focal DNA hypermethylation and long-range hypomethylation in colorectal cancer coincide with nuclear lamina-associated domains. *Nat Genet* 2011; 44:40-6; PMID:22120008; <https://doi.org/10.1038/ng.969>
27. Hon GC, Hawkins RD, Caballero OL, Lo C, Lister R, Pelizzola M, Valsesia A, Ye Z, Kuan S, Edsall LE, et al. Global DNA hypomethylation coupled to repressive chromatin domain formation and gene silencing in breast cancer. *Genome Res* 2012; 22:246-58; PMID:22156296; <https://doi.org/10.1101/gr.125872.111>
28. Hovestadt V, Jones DTW, Picelli S, Wang W, Kool M, Northcott P a, Sultan M, Stachurski K, Ryzhova M, Warnatz HJ, et al. Decoding the regulatory landscape of medulloblastoma using DNA methylation sequencing. *Nature* 2014; 510:537-41; PMID:24847876; <https://doi.org/10.1038/nature13268>
29. Hans KD, Sabuncuyan S, Langmead B, Nagy N, Curley R, Klein G, Klein E, Salamon D, Feinberg AP. Large-scale hypomethylated blocks associated with Epstein-Barr virus-induced B-cell immortalization. *Genome Res* 2014; 24:177-84; PMID:24068705; <https://doi.org/10.1101/gr.157743.113>
30. Genbacev O, Zhou Y, Ludlow JW, Fisher SJ. Regulation of human placental development by oxygen tension. *Science* 1997; 277:1669-72; PMID:9287221; <https://doi.org/10.1126/science.277.5332.1669>
31. Timp W, Feinberg AP. Cancer as a dysregulated epigenome allowing cellular growth advantage at the expense of the host. *Nat Rev Cancer* 2013; 13:497-510; PMID:23760024; <https://doi.org/10.1038/nrc3486>
32. Baltimore D. NF- κ B is 25. *Nat Immunol* 2011; 12:683-5; PMID:21772275; <https://doi.org/10.1038/ni.2072>
33. Mor G, Cardenas I, Abrahams V, Guller S. Inflammation and pregnancy: the role of the immune system at the implantation site. *Ann N Y Acad Sci* 2011; 1221:80-7; PMID:21401634; <https://doi.org/10.1111/j.1749-6632.2010.05938.x>
34. Shalapour S, Karin M. Immunity, inflammation, and cancer: an eternal fight between good and evil. *J Clin Invest* 2015; 125:3347-55; PMID:26325032; <https://doi.org/10.1172/JCI80007>
35. Kawamura K, Kawamura N, Sato W, Fukuda J, Kumagai J, Tanaka T. Brain-derived neurotrophic factor promotes implantation and subsequent placental development by stimulating trophoblast cell growth and survival. *Endocrinology* 2009; 150:3774-82.
36. Huang S-M, Lin C, Lin H-Y, Chiu C-M, Fang C-W, Liao K-F, Chen D-R, Yeh W-L. Brain-derived neurotrophic factor regulates cell motility in human colon cancer. *Endocr Relat Cancer* 2015; 22:455-64; PMID:25876647; <https://doi.org/10.1530/ERC-15-0007>
37. Kawano T, Morimoto K, Uemura Y. Partial purification and properties of urokinase inhibitor from human placenta. *J Biochem* 1970; 67:333-42; PMID:5424370; <https://doi.org/10.1093/oxfordjournals.jbchem.a129257>
38. Roes EM, Sweep CGF, Thomas CM, Zusterzeel PL, Geurts-Moespot A, Peters WH, Steegers EA. Levels of plasminogen activators and their inhibitors in maternal and umbilical cord plasma in severe preeclampsia. *Am J Obstet Gynecol* 2002; 187:1019-25; PMID:12388999; <https://doi.org/10.1067/mob.2002.126280>
39. Croucher DR, Saunders DN, Lobov S, Ranson M. Revisiting the biological roles of PAI2 (SERPINB2) in cancer. *Nat Rev Cancer* 2008; 8:535-45; PMID:18548086; <https://doi.org/10.1038/nrc2400>
40. Gui S, Ni S, Jia J, Gong Y, Gao L, Zhang L, Zhou R. Inconformity of CXCL3 plasma level and placenta expression in preeclampsia and its effect on trophoblast viability and invasion. *PLoS One* 2014; 9:e114408; PMID:25485631; <https://doi.org/10.1371/journal.pone.0114408>
41. Alvarez H, Opalinska J, Zhou L, Sohal D, Fazzari MJ, Yu Y, Montagna C, Montgomery EA, Canto M, Dunbar KB, et al. Widespread hypomethylation occurs early and synergizes with gene amplification during esophageal carcinogenesis. *PLoS Genet* 2011; 7:e1001356; PMID:21483804; <https://doi.org/10.1371/journal.pgen.1001356>
42. De Simone M, Arrigoni A, Rossetti G, Gruarin P, Ranzani V, Politano C, Bonnal RJP, Provasi E, Sarnicola ML, Panzeri I, et al. Transcriptional landscape of human tissue lymphocytes unveils uniqueness of tumor-infiltrating T regulatory cells. *Immunity* 2016; 45:1135-47; PMID:27851914; <https://doi.org/10.1016/j.immuni.2016.10.021>
43. Samstein RM, Josefowicz SZ, Arvey A, Treuting PM, Rudensky AY. Extrathymic generation of regulatory T cells in placental mammals mitigates maternal-fetal conflict. *Cell* 2012; 150:29-38; PMID:22770213; <https://doi.org/10.1016/j.cell.2012.05.031>
44. Kliman HJ, Nestlert JE, Sermasi E, Sanger JM, Strauss JF. Purification, characterization, and *in vitro* differentiation of cytotrophoblasts from human term placentae. *Endocrinology* 1986; 118:1567-82; PMID:3512258; <https://doi.org/10.1210/endo-118-4-1567>
45. Noble WS. How does multiple testing correction work? *Nat Biotechnol* 2009; 27:1135-7; PMID:20010596; <https://doi.org/10.1038/nbt1209-1135>
46. Benjamini Y, Hochberg Y, Benjamini Y, Hochberg Y. Controlling the false discovery rate: a practical and powerful approach to multiple testing. *J R Stat Soc B* 1995; 57:289-300
47. Jaffe AE, Murakami P, Lee H, Leek JT, Fallin MD, Feinberg AP, Irizarry RA. Bump hunting to identify differentially methylated regions in epigenetic epidemiology studies. *Int J Epidemiol* 2012; 41:200-9; PMID:22422453; <https://doi.org/10.1093/ije/dyr238>
48. Lawrence M, Huber W, Pages H, Aboyoun P, Carlson M, Gentleman R, Morgan MT, Carey VJ. Software for computing and annotating genomic ranges. *PLoS Comput Biol* 2013; 9:e1003118; PMID:23950696; <https://doi.org/10.1371/journal.pcbi.1003118>
49. Law CW, Chen Y, Shi W, Smyth GK. voom: Precision weights unlock linear model analysis tools for RNA-seq read counts. *Genome Biol* 2014; 15:R29; PMID:24485249; <https://doi.org/10.1186/gb-2014-15-2-r29>