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Nuclear Matrix Association: Switching to the Invasive Cytotrophoblast☆

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ABSTRACT

Abnormal trophoblast invasion is associated with the most common and most severe complications of human pregnancy. The biology of invasion, as well as the etiology of abnormal invasion remains poorly understood. The aim of this study was to characterize the transcriptome of the HTR-8/SVneo human cytotrophoblast cell line which displays well characterized invasive and non-invasive behavior, and to correlate the activity of the transcriptome with nuclear matrix attachment and cell phenotype. Comparison of the invasive to non-invasive HTR transcriptomes was unremarkable. In contrast, comparison of the MARs on chromosomes 14–18 revealed an increased number of MARs associated with the invasive phenotype. These attachment areas were more likely to be associated with silent rather than actively transcribed genes. This study supports the view that nuclear matrix attachment may play an important role in cytotrophoblast invasion by ensuring specific silencing that facilitates invasion.

1. Introduction

Placental development and invasion are vital for the successful continuation of pregnancy. Inadequate trophoblast invasion has been associated with abnormal pregnancy outcomes, including preeclampsia and intrauterine growth restriction. The initial events of placental invasion reflect a carefully choreographed sequence of cell differentiation events mediated through the production of various cell signaling pathways [1]. The cytotrophoblast invades beyond the syncytiotrophoblast and forms columns from which emanate the EVT lineage [1]. The EVT is highly invasive and migrates through the decidua basalis [3] to the superficial third of the myometrium [1]. The invasion of the EVT terminates at the distal branches of the maternal spiral arteries. This process eventually remodels the maternal spiral arteries which are converted into flaccid channels that supply the placenta and thus the fetus with oxygenated maternal blood [1,2]. Failure of the extravillous trophoblasts to invade the maternal spiral arteries has been associated with preeclampsia and growth restriction as well as other complications during pregnancy [3].

The eukaryotic genome contains two forms of stored information: the DNA sequence (genome) and the epigenetic information (epigenome) that influences gene expression without changing the DNA sequence itself [4]. Epigenetic marks are integral to differential developmental fates [5] that can be maintained and stably transmitted during mitosis [5,6]. Within the nucleus this information is indexed by the nuclear matrix, an amorphous structure comprised of DNA, RNA and more than 500 proteins [7]. Interactions of the nuclear matrix can be visualized as the base of DNA loops that extend beyond the chromosome territory, appearing in association with the induction of transcription [8,9]. When transcription is repressed, genes are often repositioned into or near nuclear lamina associated heterochromatic regions [10]. At interphase, chromosomes are specifically partitioned into territories within the nucleus [11,12], with late-replicating and gene poor regions located at the nuclear periphery, and gene rich-regions located more centrally [13,14]. MARs can provide a boundary function and/or facilitate long-range interactions of activation or repression [15–17] markedly effecting the phenotype. This is exemplified by the nuclear matrix protein SATB1, an essential

Abbreviations: aCGH, array Comparative Genomic Hybridization; CVS, Chorionic Villous Sampling; EVT, Extravillous Cytotrophoblasts; HTR, Human Trophoblast Cells Obtained From HTR-8/Svneo Cell Line; HTR-M, HTR-8/Svneo Cells Grown On Matrigel (with invasive phenotype); HTR-F, HTR-8/Svneo Cells Grown On Fibronectin (with proliferative phenotype); MAR, Matrix Attachment Region; NM, Nuclear Matrix; SKY, Spectral Karyotyping.

☆ The authors do not have any potential or actual personal, political, or financial interests in the material, information, or techniques described in this paper. Responsible for Reprint Requests: Stephen A. Krawetz, PhD. The array data reported in this publication are available at GEO as GSE17501.

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component that determines the developmental fate of T-lymphocytes [18] through its interaction with AT-rich rich-regions of the genome. The roles of many of the components of the nuclear matrix have been revealed in the pathology of various diseases typified by laminopathies. These present as a wide range of human diseases including dilated cardiomyopathy, limb girdle muscular dystrophy 1B (LGMD1B), Charcot–Marie-Tooth type 2B1(CMT2B1), Hutchinson–Gilford progeria syndrome (HGPS), and atypical early-onset Werner syndrome [19].

The role of epigenetics in early placental invasion is just beginning to be examined. Using the BeWo and JEG-3 choriocarcinoma cell lines as models of invasion, Novakovic et al. have shown that during invasion the active chromatin state as measured by tumor suppressor and proto-oncogene transcripts is correlated with methylation status [20,21]. Suppression of trophoblast methylation specifically increased the levels of E-cadherin and plakoglobulin mRNAs in conjunction with impaired trophoblast migration and wound-healing [21]. This likely reflects its release from methylation induced silencing. Recently, Kimura et al., have suggested that chromatin looping may be required to facilitate the expression of placental specific regions of the human growth hormone locus [22]. This is likely mediated by nuclear matrix attachment. However, the role of MARs and chromatin looping in trophoblast differentiation or invasion in normal or pathological pregnancies remains to be elucidated.

To fill this void we have characterized the chromosomal-wide matrix attachment binding using an invasive human cytotrophoblast model, the immortalized cell line HTR-8/SVneo [41]. These first trimester cytotrophoblast cells proliferate essentially maintaining an undifferentiated state until grown on Matrigel basement membrane, which induces their extravillous differentiation to an invasive phenotype [36]. Like primary cultures of first trimester cytotrophoblast cells [23,24], HTR-8/SVneo cells switch their expression of integrins from ITGA6 to ITGA1 and begin to produce HLA-G protein during culture on Matrigel [36], providing useful protein markers of extravillous differentiation. Accordingly, we have used the HTR-8/SVneo cytotrophoblast model to investigate changes in matrix attachment during extravillous differentiation. A significantly higher density of matrix attachment was observed in the invasive cytotrophoblast cells. This is consistent with the tenet that genomic plasticity is likely reduced during differentiation thereby directing cell fate [23,24].

2. Materials and methods

2.1. Cell culture conditions

HTR-8/SVneo cells, maintained at passage 38–45, were cultured in mass at the Cell Culture Facility of the Cleveland Clinic (Cleveland OH, USA) in 1:1 Ham's F12: DMEM medium containing 10% fetal bovine serum and antibiotics (penicillin and streptomycin). Cells were grown to confluence at 37 °C in a humidified 5%CO_2/95% air incubator. Prior to assessing invasion, the adherent HTR-8/SVneo cells were harvested following treatment with trypsin/EDTA then transferred to roller bottles coated with either 10 μg/ml fibronectin (diluted with sterile PBS), or Matrigel™, diluted 1:10 with sterile PBS. The cells were then rinsed free of serum then cultured on Matrigel™, to induce an invasive phenotype as described above or fibronectin (to allow for cell attachment to the bottle, resulting in cell proliferation in a monolayer without differentiation) for 24 h in serum free 1:1 Ham's F12: DMEM medium supplemented with 5 mg/ml BSA. Subsequent to fibronectin or Matrigel™ culture the cells were harvested with trypsin/EDTA. Western blot analysis for human leukocyte antigen-G (HLA-G) and α1 integrin (ITGA1) was used to confirm the phenotype as extravillous when cultured on Matrigel™ or non-invasive when cultured on fibronectin.

2.2. Spectral karyotyping

HTR-8/SVneo cells at passage 38 were cultured for 1 day at 37 °C in 1:1 Ham's F12: DMEM containing 10% fetal bovine serum. Mitotic cells were harvested and then treated with colcemid for 2 h. Chromosomal slides were prepared essentially as described [25,26]. After pepsin treatment and fixation with formaldehyde followed by dehydration, the chromosomal slides were denatured then hybridized to the denatured painting probes (SkyPaint, Applied Spectral Imaging: Vista, CA) for at least 48 h at 37 °C. The chromosomes were stained with DAPI and mounted with antifade subsequent to washing and hybridization detection [25,26]. Twenty mitotic figures were randomly selected for SKY image analysis providing that high-quality hybridization signals with minimal overlapping chromosomes for the mitotic spread were observed. The degree of chromosomal abnormality was not a selection criterion. Chromosomes were analyzed by the color and size using software developed by Applied Spectral Imaging (Vista, CA).

2.3. Expression analysis

The expression profiles of the HTR-8/SVneo cells grown on Matrigel™ or fibronectin were determined and compared using the Illumina Sentrix Human-8 v2 Expression BeadChip arrays [27]. Total RNA was isolated using the RNeasy kit (Qiagen Inc., Valencia, CA, USA). The resulting RNA was then amplified and labeled by *in vitro* transcription using the Illumina RNA amplification system (Ambion, Austin, TX, USA). A 750 ng aliquot of the *in vitro* transcribed probe was used for hybridization. The array analyses were carried out in duplicate for HTR-8/SVneo cells grown on either Matrigel™ or fibronectin, for a total of four independent isolations. The data was analyzed using Illumina Bead Studio. The average signal for each reporter across bead replicates was cubic spline normalized to determine a standardized expression value. Expressed genes were identified by signal values higher than the internal spike-in controls for expression ($S_{min} > 3000$). The biological replicates exhibited a correlation coefficient (r) of ~0.99 for both the HTR-F and the HTR-M replicates.

2.4. Nuclear matrix and loop extractions

To determine the optimal time to prepare nuclear matrices from HTR-8/SVneo cells, nuclei from cells grown on either Matrigel™ or fibronectin were exposed to 2 M NaCl for varying amounts of time essentially as described [28]. Both HTR-F and HTR-M, matrix and loop DNA were isolated after optimal extraction as described [29,30]. Briefly, the halo structures were gently washed with REact® 3 restriction buffer (Invitrogen, Carlsbad, CA, USA) for 20 min at room temperature then centrifuged at 1000*g* at 4 °C. This was repeated a total of three times to remove any residual non-nuclear matrix proteins. After the third wash, the DNA loops were separated from the matrix-bound DNA by restriction digestion with 400 U of *Eco*RI (Invitrogen) at 37 °C for 3 h. Subsequent to restriction digestion, the DNA was centrifuged at 16,000*g* for 5 min at 4 °C to pellet the nuclear matrix-bound portion of the DNA. The supernatant, containing the loop-associated DNA, was then transferred to a separate tube. The nuclear matrix-bound DNA was washed 2 additional times with React® 3 (Invitrogen) buffer to minimize loop contamination of the matrix-bound DNA. The remaining residual proteins were removed from both the loop and matrix associated DNA by overnight digestion with 50 μg/ml proteinase K buffered with 50 mM Tris–HCl buffer, pH 8.0, with 50 mM NaCl, 25 mM EDTA and 0.5% SDS. DNA from each fraction was then purified using the Quantum-Prep Matrix kit (BioRad, Hercules, CA, USA), and then resuspended in nuclease-free deionized water and stored at −20 °C.

2.5. Verification of fractionation and aCGH hybridization

The fractionation of loop and matrix associated DNA was confirmed by real-time PCR as previously described [17,27,31]. Regions that were previously described as loop or matrix associated including the human growth hormone locus (unpublished data) were initially amplified in triplicate to verify fractionation. Purified DNA from each nuclear matrix and loop-associated fraction was then analyzed using array 7 to interrogate chromosomes 14–18. (NimbleGen Systems CGAR0150-WHG8 CGH isothermal oligonucleotide 8 array system, NimbleGen Systems Inc., Madison, WI, USA). The with a median probe spacing was 713 bp. This includes the human growth hormone locus that is known to be under epigenetic control in human placenta [22]. Two biological replicates were completed for the cells grown under either culture condition. All hybridizations were performed by NimbleGen (Reykjavik, Iceland). The loop and matrix array signals exhibited a correlation coefficient of 0.976 for the cells grown on fibronectin and 0.966 for the cells grown on Matrigel™.

2.6. Identification of MARs by aCGH

To assess similarity between replicates, CGH data was initially evaluated using SignalMap (v. 1.9) (Roche NimbleGen Inc., Madison, WI, USA). Methods previously detailed [27,30] were used to evaluate the dataset and to identify a parsimonious set of MARs found in both cell types and unique to either cell type. Briefly, the sites of significance were identified if the putative MAR fulfilled three criteria. First, the probe must exhibit a $\log_2$ signal ratio in the lower 2.5% of the ranked signal on both replicates. Second, two additional probes with concordant signal within a 3 kb region on each side of the site were required. Third, the average signal across the restriction fragment that was interrogated was required to be concordant. Restriction fragments meeting these requirements but with inconsistent signal across the length of these fragments were excluded. Regions meeting these criteria in both replicates were then selected for further analysis. The significance of associations