Different epigenetic states define syncytiotrophoblast and cytotrophoblast nuclei in the trophoblast of the human placenta

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Highlights:

1. Epigenetic modifications can distinguish villous trophoblast cell populations
2. Syncytiotrophoblast nuclei contain non-canonical heterochromatin markers
3. Epigenetic mechanisms account for contrasting trophoblast nuclear morphologies

1. Introduction

The syncytiotrophoblast (STB) is the epithelial covering of the villous tree in the human placenta. This multinucleated syncytium displays unusual cell biology. The nuclei within the syncytiotrophoblast are terminally-differentiated and non-proliferative [1], and instead, the syncytium is sustained across gestation by continuous fusion of underlying differentiating cytotrophoblast (CTB) cells. CTB and STB nuclear populations display contrasting morphologies [2]. Undifferentiated CTB cells have a large and ovoid nucleus, with a morphologically diffuse chromatin structure. As CTB cells begin to differentiate the nuclei become more irregular in appearance. Chromatin at the periphery of the nucleus begins to condense and the nuclear volume reduces [3]. Prior to fusion, CTB nuclei become electron-dense, more irregular in outline and resemble those nuclei resident in the syncytium [4]. A range of morphologies is observed in the STB: most nuclei are small with a convoluted nuclear envelope and contain varying degrees of heterochromatin. The most highly condensed nuclei are aggregated into knots, where nuclei are closely juxtaposed and have smooth outlines with euchromatin restricted to areas near nuclear pores or to a central island [5].

Our understanding of the regulation of nuclear turnover in the trophoblast is being re-interpreted in light of new investigations [6]. It had been thought that the condensation in STB nuclei was indicative of apoptotic changes initiated during CTB differentiation [7], and that STB nuclei are held in latent states of apoptosis and transcriptionally inactive [8]. However, recent studies have shown that apoptosis is absent from the syncytium [9, 10], and that the majority of STB nuclei are transcriptionally active at all stages of gestation [11, 12]. Nonetheless, a range of transcriptional states are observed amongst nuclei within the syncytium. The most highly condensed nuclei residing in syncytial knots have been demonstrated to be transcriptionally inactive, with evidence of associated oxidative damage [13]. These recent observations suggest that the

characteristic chromatin condensation observed in STB nuclei may be a feature of the biology of the syncytiotrophoblast, serving to regulate transcription during the life-cycle of a nucleus.

Chromatin structure is epigenetically regulated by histone modifications and DNA methylation. Specific histone modifications confer active or repressive transcriptional states [14]. Trimethylation of Histone3-Lysine9 (H3K9me3), Histone3-Lysine27 (H3K27me3) and Histone4-Lysine20 (H4K20me3) are markers of tightly-packaged heterochromatin and are associated with gene repression [15]. Levels of H4K20me3 are increased in senescent fibroblasts [16]. By contrast, tri-methylated Histone3-Lysine4 (H3K4me3) residues are enriched at promoters of expressed genes and are features of open euchromatic structure. Histone modification states are reversible, which allows for dynamic regulation of chromatin structure in accordance with cellular differentiation, development, and responses to environmental signals [17].

Methylation and hydroxymethylation of cytosine residues modify DNA and are associated with its interactions with transcription factors and other DNA-associated proteins. DNA methylation occurs at cytosine bases which are converted to 5'methylcytosine (5mC) by DNA methyltransferase enzymes and is associated with local control and typically , with genomic imprints, repetitive elements and the inactive X chromosome displaying hypermethylation [18]. Methylated cytosine residues can mediate heterochromatin formation through the recruitment of methylcytosine binding proteins, linker histones and other remodelling complexes and is usually associated with gene repression [19]. DNMT1 is involved in maintaining existing methylation patterns whereas DNMT3a and 3b regulate *de novo* methylation by adding methyl groups to unmodified cytosine bases . Hydroxymethylation, generated by the oxidation

of mC by TET enzymes cytosine, has been associated with active DNA demethylation and DNA repair however, its functional roles are not well understood. In mouse and human embryonic stem cells 5hmC is detected in euchromatic areas of the nucleus, suggesting that this epigenetic modification may be associated with gene activity [21, 22] .

Changes in patterns of histone modifications and DNA methylation are associated with cellular differentiation [23]. Chromatin remodelling results in the silencing of subsets of genes while simultaneously activating other genes characteristic of the differentiated cell type. Here we investigate the hypothesis that the variations in epigenetic modifications observed between CTB and STB nuclei underlie the different chromatin structures observed cytologically in these nuclear populations.

2. Methods

2.1 Sample preparation

Blocks from paraffin-embedded placentas ranging from 11-19 weeks (n=5) and 31-39 weeks of gestation (n=4) were obtained from an archive collected in accordance with ethical protocols [2]. Samples from 11-19 weeks of gestation were obtained from terminated pregnancies which were otherwise clinically normal. Placental samples from later gestational ages were obtained from clinically normal pregnancies which underwent spontaneous onset of labour.

2.2 Knot identification

Knots were identified as previously described [24]. Briefly, serial sections were cut at 5 µm to minimize the superimposition of nuclei. Every 4th section in the series was

stained for the target antigen, whereas the adjacent sections were stained with haematoxylin and eosin. True knots were defined a sessile aggregations which protruded gently from the surface of the syncytium, and were observed to appear and disappear when moving through the series.

2.3 Immunostaining

Sections were rehydrated in Histo-clear (Sigma, Poole, UK), graded ethanol, and deionized water. Heat-induced antigen retrieval was performed by boiling sections in 0.1 mol/L Tris-EDTA buffer (pH 9.0) in a pressure cooker. Sections were blocked in non-immune serum for 30 minutes at room temperature. Endogenous peroxidases were quenched by incubating the sections in 3% H_2O_2 for 15 minutes. Primary antibodies including anti-H3K9me3, anti-H3K27me3, anti-H4K20me3 and anti-H3K4me3 (1:100; ab8898, ab6002, ab9053 and ab8580; Abcam, Cambridge, UK), anti-5'hydroxymethylcytosine (1:100; Catalogue number 39769; Active Motif, Rixensart, Belgium), anti-5'methylcytosine (1:200; sc56615; Santa Cruz, Santa Cruz, CA), DNMT1 (1:200; sc20701; Santa Cruz) and DNMT3a (1:200; sc20703; Santa Cruz) were added and incubated overnight at 4°C. Sections were washed in Tris-buffered saline with 0.1% Tween-20 (Sigma) and 0.1% Triton X-100 (Sigma). Biotin-labeled species-specific secondary antibodies were added at a concentration of 1:200 and incubated at room temperature for 1 h. Vectastain Elite ABC system (Vector Labs, Burlingame, CA) and SigmaFast DAB (Sigma) were used according to manufacturer instructions. Sections were lightly counterstained with hematoxylin, rinsed in deionized water, and dehydrated in increasing grades of alcohol and Histo-clear. Coverslips were mounted with DPX (Sigma). Images were captured and viewed using a Nanozoomer slide scanner and NDP.view2 software (Nanozoomer 2.0-RS; Hamamatsu Photonics, Hertfordshire,

UK). CTB and STB nuclei were identified on the basis of their characteristic location within the trophoblast layers.

2.4 Data analysis

We used a semi-quantitative method similar to those used in clinical assessments of tumour [25]. Briefly, 50 counting frames were applied to each section and the relative proportion of positive nuclei was determined to generate a scoring system (+, 10-20%; ++, 20-50%; +++, >50%).

3. Results

Semi-quantitative immunohistochemical analysis demonstrates that CTB and STB nuclear populations display different repertoires of histone modifications with some differences observed at different gestational ages.

CTB nuclei show a range of immunoreactivities for H4K20me3, with positive and negative nuclei observed in close proximity to each other within samples (Fig 1 A and B). The relative proportions of H4K20me3-postive CTB nuclei remain constant from 1st trimester to 3rd trimester with around 20-50% of nuclei displaying immunoreactivity. A higher proportion of STB nuclei stain intensely for this modification in the first and early second trimester placentas. However, the proportion of H4K20me3-positive STB nuclei is reduced to less than half in the third trimester (Fig 3). Syncytial knots are composed almost entirely of intensely staining nuclei (Fig 1 C).

The majority of CTB nuclei are H3K27me3- (Fig 1 D, E and F) and H3K9me3-positive (Figs 1 G, H, and I). In contrast to CTB nuclei STB nuclei contain low levels of these modifications, with only 10-20% of nuclei displaying immunoreactivity.

Both CTB and STB nuclear populations show heterogenous staining for H3K4me3, with immunopositive and negative nuclei observed in each compartment, including syncytial knots (Figure 1 J, K and L).

Over 50% of CTB nuclei are 5mC-positive. In contrast, and unexpectedly, the majority of STB nuclei do not display appreciable levels of 5mC staining across gestation (Fig 2 A, B and C). DNMT1 is detected in almost all CTB nuclei in both early and late gestation (Fig 3A, B, C; solid arrows). DNMT1 levels are heterogeneous in the STB compartment, with approximately 20-50% of nuclei displaying immunoreactivity in 1st and 2nd trimester samples (Fig 3A, B, C; dashed arrows). This proportion is reduced to 10-20% in the third trimester. Similar staining patterns are observed with DNMT3a. CTB nuclei are mostly immunopositive for DNMT3a, with more intense staining observed in early gestation, and STB nuclei exhibit heterogeneous staining for DNMT3a. The presence of DNMTs in most CTB nuclei correlates with the high proportion of 5mC-positive nuclei in this compartment. Syncytial knots contain DNMT1 and DNMT3a-negative nuclei, which is correlative with the low levels of 5mC observed in these structures.

In contrast to their staining for 5mC, a greater proportion of STB nuclei are immunopositive for 5hmC; 20-50% of STB nuclei in 1st and 2nd trimester and greater than 50% in the third trimester are immunopositive. The 5hmC-positive STB nuclei stain intensely for this modification. Moreover syncytial knots consist entirely of 5hmC-positive nuclei (Fig 2F). This compares with 10-20% of CTB nuclei which are immunopositive for hmC at all stages investigated and which show a much higher proportion of mC positive nuclei (Fig 2 D and E).

CTB 50% mC and 20% hmC (has Dnmts) – STB show 50% hmC and no mC and v low Dnmts. Knots show 100% hmC and no mC and no Dnmts.

4. Discussion

Villous cytotrophoblasts (CTB) have the potential to differentiate into the syncytiotrophoblast (STB) of the placenta, which forms the feto-maternal interface of the placenta and also, in the first trimester, into invasive extravillous trophoblast cells. At cytological resolution, the differentiation of CTB nuclei into STB nuclei appears to be accompanied by an increase in nuclear condensation and heterochromatin formation. Here we show that there are epigenetic differences between CTB and STB nuclei, suggesting that differentiation is associated with changes in the epigenetic state as evident by changes in histone modifications and DNA methylation. However, in these trophoblast cell types, at immunocytological resolution, the patterns of immunostaining are not consistent with the functions usually attributed to these modifications.

As STB nuclei are heavily condensed in comparison to CTB nuclei we investigated canonical markers of constitutive and facultative heterochromatin, H3K9me3 and H3K27m3. [26, 27]. We found that STB nuclei contain lower levels of these histone modifications than CTB nuclei. The unexpected paucity of H3K9me3 in the STB nuclei may reflect a cell-specific effect, as it has been shown that modifications can silence genes in a cell-specific manner [28, 29]. Previous studies have demonstrated that the human growth hormone gene cluster is regulated by distinct histone modifications in the brain and placenta. Brain-specific isoforms are regulated by broad domains of histone acetylation, whereas the placental isoforms have additional discrete foci of H3K4 di- and tri- methylation [30]. Thus the absence of canonical heterochromatin markers may be further evidence of trophoblast-specific epigenetic mechanisms. Higher levels of H3K27me3 in progenitor CTB nuclei may serve to regulate genes involved in CTB to STB differentiation. In other contexts, bivalent domains containing both

repressive H3K27me3 and active H3K4me3 are found at developmentally-regulated genes, with levels of H3K27me3 decreasing upon differentiation allowing transcription to proceed [17]. In the trophoblast, H3K27me3 may allow for transient repression of genes which are required rapidly upon fusion of a CTB nucleus into the STB. If H3K27me3 is indeed regulating a transient repressive state, the apparent reduction in the proportion of H3K27me3-positive CTB across gestation suggests that they might be losing their ability to dynamically regulate bivalent genes. This is consistent with the coincident reduction in H3K4me3 as gestation proceeds. The increase in H3K9me3 in later gestation CTB cells may suggest the acquisition of a less dynamic repressive state.

H4K20me3 has been shown to be a conserved marker of pericentric heterochromatin, and co-localises with DAPI-dense condensed regions of nuclei [31]. Levels of H4K20me3 have also been observed to be increased in ageing cells [32]. We see constant proportions of H4K20me3-positive CTB nuclei across gestation. In contrast the majority of dispersed first trimester STB nuclei are H4K20me-positive with this proportion reduced in later stages. Despite this reduction, syncytial knots, which are more abundant in the third trimester, are almost entirely composed of H4K20me3-positive nuclei. We therefore speculate that the patterns of H4K20me3 staining in the STB may reflect the heterogeneity of nuclear age within the syncytium: STB nuclei may contain low amounts of H4K20me3 at the time of incorporation into the syncytium, and begin to accumulate the modification as the syncytium ages, until the oldest nuclei with the highest levels of this modification are aggregated into syncytial knots. The movement of nuclei into knots would result in reduced proportions of immunopositive nuclei within the dispersed compartment. Tracer studies using a marker for recent incorporation could test this hypothesis [11].

We also investigated the distributions of H3K4me3, which is associated with active euchromatin. Intermediate proportions of both nuclear populations were determined to

be H3K4me3-positive across gestation. This is in contrast to the findings of a previous study which suggested that H3K4me3 immunoreactivity was mainly confined to CTB nuclei [12]. The conflicting results may be due to differences in gestational ages of the sampled tissues. Ellery et al. performed immunohistochemistry for H3K4me3 only on first trimester samples from 5 – 17 weeks of gestation, whereas this present study included samples from 13-19 weeks gestation and the third trimester. The presence of both H3K4me3-positive and –negative STB nuclei may reflect the range of transcriptional states in this tissue. It may reflect activation of genes bivalently marked in the CTB but also H3K4me3 has been shown to persist in nuclei as a marker of recent transcription [33]. The STB produces and secretes very large quantities of hormones throughout pregnancy. Towards the end of pregnancy the STB secretes 1-4g of human placental lactogen per day, revealing the high transcriptional and translational capacities of this tissue [34]. It is likely that the high H3K4me3 in the STB across gestation reflects the transcriptional activity of that tissue.

We investigated states of methylation as DNA methylation is associated with gene repression, inversely correlated with transcription factor binding and it can also influence chromatin organisation by interacting with linker histones and other chromatin associated proteins [35]. Unexpectedly, we observed higher numbers of 5mC-immunoreactive CTB nuclei in comparison to STB nuclei at all stages investigated. The relative proportions of 5mC-positive nuclei remains similar in the two compartments across gestation, perhaps suggesting that chromatin and gene regulation by methylation is not subject to major fluctuations. Indeed, genome-wide sequencing has revealed the presence of partially demethylated domains in the placenta. These large domains of DNA containing lower levels of DNA methylation than the rest of the genome are thought to be a unique feature of the placenta, perhaps reflecting its differential epigenetic control and divergence from the inner cell mass and all somatic

cells in early embryogenesis [36]. Thus the lower levels of methylation in placental cells suggests that regulation by methylation may be involve much smaller regions that can not be detected by our assay. Furthermore, non-canonical mechanisms of regulation by methylation may act in these cell types.

We also investigated the tissue distribution of selected DNMTs in the trophoblast. DNMT1 is considered to be the major enzyme regulating the maintenance of methylation patterns. [37]. While DNMT3a and 3b both regulate *de novo* methylation DNMT3a methylates at a higher rate than DNMT3b [38]. As expected, we found levels of DNMTs to correlate with the levels of 5mC in the nuclear populations: nearly all CTB nuclei contain DNMT1 and 3a and also are 5mC-positive whereas STB nuclei, which contain low levels of 5mC, have lower levels of DNMTs.

In contrast to 5mC, the proportion of 5hmC-immunopositive nuclei in the STB is higher than that observed in the CTB compartment, Hence there appears to be a reciprocal relationship between 5mC and 5hmC in the two cell types, with high 5mC and low 5hmC a feature of CTB and vice versa in the STB. Although the low levels of 5mC do not change in the STB across gestation, the proportion of 5hmC-positive STB nuclei increases over time suggesting accumulation of hmC at previously mC residues. While the function of this residue has not been fully elucidated, it has been proposed that oxidative stress may result in the formation of 5hmC [39]. Increasing accumulation of 5hmC in STB nuclei as gestation proceeds in this non-replicative cell population may reflect the continuous fusion of mC-associated CTB cells into a syncytiotrophoblast that loses mC as it accumulates hmC. Consistent with this theory is our observation that syncytial knots consist almost entirely of 5hmC-positive nuclei, perhaps associated with the

integration of the older hmC positive STB nuclei. We have previously shown that nuclei in syncytial knots contain oxidatively damaged nuclei [24]; the presence of 5hmC in these nuclei may be further evidence of the role of oxidative damage in knot formation and perhaps implicates 5hmC in DNA repair processes [40].

We observe variation in histone modifications within both trophoblast compartments. STB nuclei harbour heterogeneities in their timing of incorporation within the syncytium, which is might be reflected in the variations in chromatin morphologies observed in this compartment. Although we assessed the CTB as a uniform population, asymmetric division of CTB nuclei to produce one daughter cell to fuse with the syncytium and one remaining in the progenitor pool results in variation in this compartment. It would be interesting to determine whether this asymmetry yields daughter cells which are epigenetically different from each other. GCM-1 is thought to be upregulated in the cell destined to fuse [41], and could perhaps be used as a marker to distinguish the two CTB types in epigenetic co-localisation studies.

This study describes for the first time broad epigenetic signatures of the two main populations of trophoblast nuclei in normal pregnancy. It would be intriguing to investigate patterns of epigenetic modifications in the trophoblast of placentas from pathological pregnancies. Aberrant trophoblast turnover is associated with preeclampsia and IUGR, with these placentas demonstrating reduced trophoblast volume and surface area as well as decreased the total number of trophoblast nuclei. consideration of epigenetic patterns in abnormal placentas might provide insights into the role of epigenetic states in trophoblast development and homeostasis and may contribute to our understanding of the pathophysiology of these conditions. Futhermore, increased syncytial knot formation (Tenney-Parker changes) is observed in preeclampsia and is used as a biomarker to assess placental well-being. We show here that knots can be identified by an epigenetic signature of high 5hmC and

300 H4K20me3, and low H3K27me3, H3K9me3 and 5mC. This panel of markers could be
301 employed in clinical assessments of knotting index.

302 It has been reported that preeclampsia is associated with global hypermethylation, as
303 shown by both 5mC immunohistochemistry and pyrosequencing of repeat elements [42,
304 43]. However when specific promoters were investigated it was found that some
305 exhibited hyper- and others hypomethylation relative to normotensive placentas. This
306 is likely to be due to dysregulated gene expression as some are known to be
307 upregulated and others down regulated in the pathophysiology of the disease; for
308 example, Kisspeptin is increased in preeclampsia whereas MMPs Superoxide dismutase
309 are reduced [44, 45].

310

311 This semi-quantitative investigation of global levels of histone modifications reveals
312 that there are differences between the epigenetic signatures of chromatin in STB and
313 CTB nuclei, with may contribute to the observed differences in chromatin conformation
314 and nuclear morphology between the two populations. These nuclear populations have
315 been previously demonstrated to utilize different repertoires of transcription factors to
316 promote differentiation-dependent gene expression [46]. Epigenetic mechanisms may
317 similarly confer differential gene regulation in CTB and STB nuclei. Differences in the
318 epigenetic profiles of STB nuclei across gestation may be considered in the context of
319 the syncytium as a terminally-differentiated, non-proliferative but transcriptionally
320 active tissue.

321

322 As histone modifications often interact with each other, further investigations into
323 combinations of modifications in nuclei would increase our understanding of the
324 regulatory processes. In conclusion, these qualitative observations and the presence of

transcriptionally active nuclei in the STB support the hypothesis that epigenetic factors, and not apoptosis as previously suggested, results in the observed chromatin condensation.

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