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Genes, epigenetics and miRNA regulation in the placenta

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ABSTRACT

This text reviews briefly the context in which epigenetics regulate gene expression in trophoblast development and function. It is an attempt to focus on a limited number of recent papers that, according to the author, shed new light on placental development, and constitute possible trails for improving knowledge and women follow-up in pathological pregnancies.

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1. Introduction

One of the earliest events of embryo differentiation in Eutherian mammals is when two major lineages differentiate into two different types of stem cells, embryonic stem cells (ESCs), on the one hand and trophoblast stem cells (TSCs), on the other hand. ESCs will conduct to the development of the different tissues of the embryo, while TSCs are at the origin of the placenta. Clearly, early defects in the development of the placenta are susceptible to lead to functional defects, either by altering the materno-foetal immunological dialogue, the placental structure, or the placental function. These alterations will later be the cause of placental disorders, very common in humans, such as preeclampsia or Intra-Uterine Growth Restriction. Gene expression defects are considered as major culprits at the base of such diseases. Epigenetic mechanisms, that regulate gene expression without altering the DNA sequence, are now increasingly thought are important actors in the onset and severity of placental diseases in humans. This text will shortly recapitulate the major genes involved in trophoblast differentiation, then it will present briefly the different epigenetic machineries active in the cell; finally, the function of gene methylation and regulation of gene expression through the action of small RNAs will be exposed.

2. Genes of trophoblast differentiation

2.1. Embryonic versus trophoblast stem cells

The blastocyst stage is the first visible stage following fertilization where two morphologically different types of cells are evident. The periphery of the blastocyst is made of trophectoderm cells, and a cellular mass (inner cell mass, ICM) is located at one side in the blastocyste cavity. The trophectoderm cells near the ICM are the 'polar trophectoderm', while the opposite side is the 'mural trophectoderm'. In mice, the first gene associated with trophectoderm development, the transcription factor *Cdx2* (*Drosophila* caudal-type homeobox 2) was identified in 2005 [1]. In the study, the authors demonstrate in mice the existence of a balance between *Oct3/Oct4* and *Cdx2*, the former being positioned very high in the hierarchy of pluripotency, and able to block the expression and activity of the later, the reciprocal repression being also present. Besides *Cdx2*, another transcription factor belonging to the T-box family of proteins, *Eomesodermin* (Eomes) is requested for trophoblast differentiation and proliferation (as well as for mesoderm formation) [2]. In 2007, Yagi and coworkers identified the transcription factor *Tead4* (TEA-domain family member 4) as a trophectoderm determinant acting upstream of *Cdx2* [3]. When a mouse embryo develops without *Tead4*, it dies before implantation after failure to form the blastocoele (the cavity filling the blastocyst).

2.2. The network of early placental genes

Most knowledge on early development originates from the

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study of mouse embryos and depends on transcription factors, most of them being highly conserved in Eutherian mammals, suggesting that at least partly, what has been discovered in mice may be widely applicable to humans despite possible differences in the gene cascades involved [4]. In mice, mural trophectoderm differentiate into primary trophoblast giant cells (TGCs). Other trophoblast giant cells (secondary TGCs) emerge from the polar trophectoderm sometimes through a transient spongiotrophoblast stage of differentiation. TGCs will constitute an interface with the maternal deciduas and will become polyploid through endoreplication, under the control of the polyploidy-regulating gene *Geminin* [5]. Each of these placenta cell linages depends on a specific subset of genes as summarized by Maltepe and coworkers in a 2010 review [6]. Amongst those, it is important to note the transcription factor *GCM1*, a major gene of the cytotrophoblasts lineage, leading to the development of the placental syncytiotrophoblast, which allows the utero-placental exchanges in mice. The complex *HIF1-ARNT*, which is the major sensor of hypoxia in mammalian cells, plays also an essential role in spongiotrophoblast development (the cells composing the junctional zone of the mouse placenta, which is the closest to the maternal structure). Knocking-out *HIF1* in mice leads to the death of 10% of the embryos between days E6.5 and E7.5 [7]. These mice are not able to form extra-embryonic and embryonic cavities. To note as well, the importance of imprinted genes at least at later stages of development and placental growth, these genes playing a crucial role in adjusting the trade-off between mother and fetus [8]. In the recent years, in addition to the identification of crucial genes for trophoblast development, the importance of epigenetic marks has been revealed, with resistant methylated genes that prevent trans-differentiation of ES cells into TS cells [9], and the demonstration of the existence of genes able to modify the epigenetic landscape and playing a crucial role in trophoblast differentiation, such as *PRDM14* and *ARID3a*, acting respectively on DNA demethylation, and Histone acetylation [10,11].

3. Genetics versus epigenetics of trophoblast differentiation

3.1. Definition of epigenetics

Epigenetics may be defined as the study of mechanisms regulating gene expression, that are heritable through cell division, but not involving changes in the nucleotide sequence. From an operational point of view, epigenetics works through the action of a battery of cellular machineries that regulate genome function. Epigenetics is involved in various processes of development, physiological homeostasis, and adaption to external stresses (be them physical, chemical or of biochemical/biological origins). The epigenetic marks that tag the genes as expressed or repressed are inheritable through cell divisions, such as in the mitosis processes, but also in a limited number of cases, through the meiosis process. This is the basis of 'transgenerational inheritance', when without exposure, an epigenetic mark is transmitted more than three generations after the exposure. For instance, exposure of female rats to the endocrine disrupter vinclozolin, leads to anomalies of sperm number and function (apoptosis, movement) even up to the fourth generation [12]. Mechanisms for this type of transmission remain elusive, albeit it has recently been shown that some histone marks survive the multiple erasure mechanisms of male meiosis [13] and could carry an epigenetic message; another possible explanation is given by the stock of stable mi-RNA that are present in the cells and especially in the sperm [14]. These miRNAs, may be stabilized by a specific methylation by Dnmt2 (Trdmt1), since Dnmt2 invalidated mice apparently lose the ability to transmit at least some epigenetic memories in the case of paramutations [15].

3.2. The epigenetic machinery

Epigenetic marks are either apposed on the chromatin (on histones, or directly on the DNA), while epigenetic modulators may be stored in the cytoplasm of the cells such as miRNAs. It is not possible here to describe all the elements of the epigenetic machinery, and the reader can analyze numerous excellent reviews on these subjects. Among these reviews, the recent paper by Zhang and Pradhan [16], gives a comprehensive and recent vision of the various mechanisms, from DNA methylation, hydroxymethylation and demethylation to the list of enzymes able to post-translationnaly modify histones.

A highly simplified list of these different actors is presented as Fig. 1.

4. Normal epigenetic marks in the placenta

The actual profiling of histone marks in the placenta is not well known today, possibly because histone marks on chromatin may not be very stable. However, a recent paper analyzed extensively the placental methylome by an approach combining the use of the 450K Illumina methylation array and MethylC seq approaches [17]. A recent review listing the different technical approaches available for quantifying DNA methylation is given in Calicchio et al. (2013), [18].

4.1. The epigenetic status of the normal placenta

4.1.1. The placental methylome

Many papers deal with placental DNA methylation, and it is not possible to analyze exhaustively the literature in the present article. Many recent reviews can be consulted on the subject such as for instance [19]. Amongst the most recent global studies, the paper of Schroeder and co-workers appear as a landmark in the field, since it compares systematically results from the Illumina 450K methylation array and MethylC-seq, in parallel with evaluating gene expression by RNA-seq [17]. The authors first show that the Illumina and MethylC-seq approaches resulted in a very similar description of the methylation profiles along the chromosomes. Another important and innovative result was the demonstration that the methylation profile is stable in the placenta during the three trimesters of pregnancy. This was unsuspected, since from an expression point of view, placentas from first, second and third trimester are quite different [20]. Before publication of the Schroeder paper, it was known that placental methylation is lower on average than in other tissues (see for instance Table 1 in Ref. [21]). The Schroeder and coworker 2013 paper revealed that the methylation is distributed in specific domains, and some of them called 'Partially Methylated Domains' are absent from adult tissues.

4.1.2. Links between DNA methylation and gene expression

More and more studies tend to negate the idea that high methylation is associated simply with gene repression. The Schroeder study showed that high methylation in the gene body is rather associated to high expression levels, while the methylation inside gene promoters can be associated either to repression or increase of gene expression. To note, however, most studies attempt to correlate methylation analyses with transcriptome data. Classical transcriptome analysis measures the steady-state of mRNAs that are present in the cell at a specific moment. Therefore, the more stable mRNA may have a low expression and have a high concentration inside the cell. By contrast, short-lived mRNA will not accumulate inside the cell even if they have a very active transcription [22]. This could more accurately be tackled by translatome

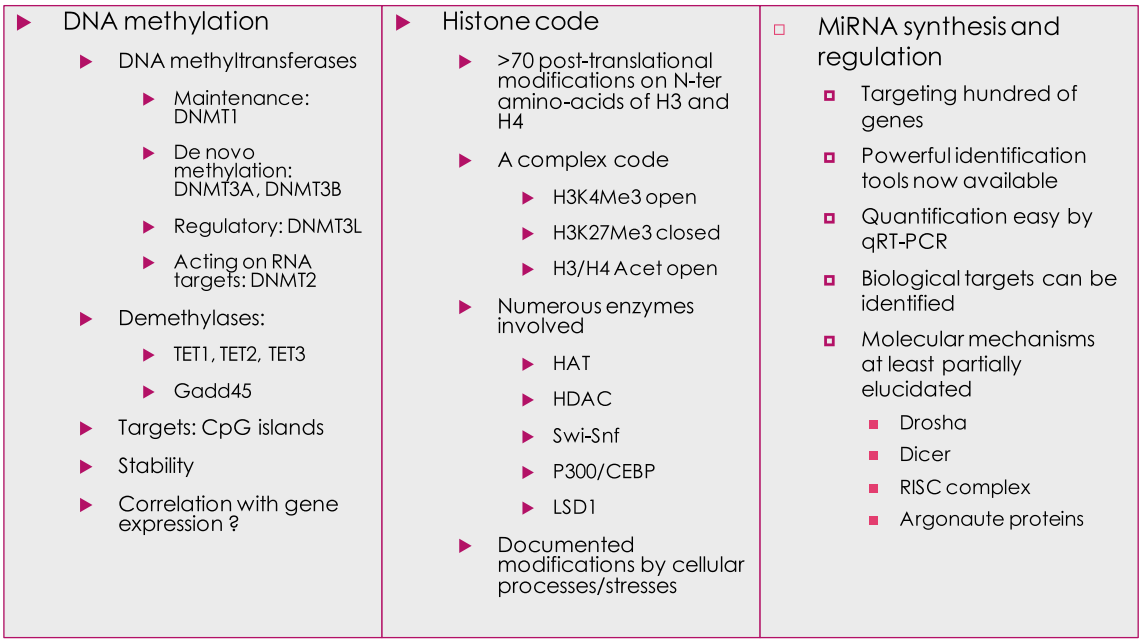

Fig. 1. A short and non-exhaustive list of the mechanisms and genes involved in modulating the epigenetic anlagen in mammalian cells. To this table could be added genes involved in hydroxymethylation, an issue that has not been studied in the context of trophoblast cells.

approaches that prior to microarray analysis, purifies polysomes with their associated mRNA being translated, and thus be indicative of the genes that are translated in proteins at a given moment [23]. To have an instant vision of the expression level of all the mRNAs is much more challenging, and may be approached rather by ChIP-seq approaches using antibodies against RNA polymerase II. For the moment such methods have not been applied to the placenta. Nevertheless, there is a clear association of the expression of several developmental genes (such as DLX5/DLX6) and specific methylation patterns in the placenta [17]. The mechanistic link between expression and methylation at the gene level is not very frequent in the scientific literature.

Abnormal environmental conditions during development may alter gene methylation. Among examples, prenatal stress imposed to mothers has been shown to modulate the methylation of the *Hmox1* promoter [24]. Preeclampsia (cause or consequence) is associated with alterations of the methylation of various genes, such as *TIMP3* [25], *RassF1* and *SERPINB5* [26], *SERPINA3* [27,28] *Cullin7* and *Cullin 4b* [29], *11βHSD* [30], *HLA*-G [31], and several imprinted genes [32]. In 2012, it has been shown that *SERPINA3* expression is regulated by the transcription factor *ZBTB7B*, depending upon the state of methylation of the *SERPINA3* promoter. *ZBTB7B* is also involved in regulation extracellular matrix genes [33]. The ZBTB family of genes has been associated to highly expressed/highly methylated gene promoters [34,35]. Overall, high-methylation in a gene is classically associated with a low level of transcription. However, many recent results show that exceptions to this paradigm are numerous.

5. Regulation of gene expression by microRNA

5.1. Definition of miRNAs, number in the mammalian genome

MiRNAs are small RNA molecules (~22 nucleotides) synthesized from the genome by the regular cell machinery either from bona fide genes (and transcribed in pri-mi-RNA), or from introns of host genes. Drosha and Pasha (DGCR8) proteins are involved in the handling of the pri-miRNA and its transformation into pre-miRNA. These are exported towards the cytoplasm through the action of Exportin 5, then the Dicer complex will eliminate the loop, and the dimer will be associated to the RISC complex (RNA-induced Silencing Complex), composed of proteins of the Argonaut family, and of a helicase able to dissociate the two RNA strands. The one that is kept in the RISC complex will hybridize to target mRNA inducing degradation or blocking protein synthesis. The processes are summarized in Treiber (2012) [36].

5.2. MiRNAs functions in trophoblast

In trophoblast cells, as in other context, there is an increasing corpus of data showing that miRNAs play important physiological and developmental roles, consistently with the estimation that they potentially regulate 60% of the genes in eukaryotic genomes [37]. Actually, for several physiological characteristics of trophoblast biology (immunity, invasion, proliferation, vasodilation), it has been shown that specific miRNAs are effective actors. An important issue for trophoblast tissue and especially in humans, is the existence of placental miRNAs that are specific of primates and mainly organized into two clusters, one on chromosome 19 (54 miRNAs) and one on chromosome 14 (34 miRNAs) [38,39].

5.2.1. Immunity

Trophoblasts express specific HLA molecules that differ from the ones of other cells of the body. Instead of HLA A and B, which are highly polymorphic, they express HLA-C and HLA-G (the former being moderately polymorphic, while the later is quasi-monomorphic [40]). It has recently been shown that HLAG expression is down-regulated by miR-148a and miR-152 [41,42]. A very interesting recent study suggests a miRNA basis for the communication between trophoblast cells and uterine Natural Killer (uNK), the major actors of the early materno-foetal immunological dialogue. The authors showed that miR-517a-3p down-regulate PRKG1 (Protein Kinase, CGMP-Dependent, Regulatory, Type I) in Bewo cells (a choriocarcinoma cell line similar to villous

trophoblast and able to fuse in culture under AMPc induction by forskolin). Exosomes from the Bewo cells were able to fuse with maternal NK cells, and the content in miRNAs induced a decrease of PRKG1 in the NK cells [43]. PRKG1 is considered as an important modulator of nitric oxide (NO) metabolism, NO being a major molecule in the regulation of the vascular system function [44]. Therefore trophoblast cells appear able to communicate with the maternal immune cells through miRNAs production, and possibly to modulate their function.

5.2.2. Invasion

Trophoblast invasion is also affected by several miRNAs, often through the alteration of the Nodal pathway. Nodal is a member of the TGFβ family that interacts with the ALK7 receptor (Activin receptor-like kinase 7). Both are expressed in villous and extravillous trophoblast, and their overexpression decreases cell migration and invasion, while silencing these genes has the opposite effect [45]. Several miRNAs target genes of the Nodal pathway (miR-373a-5p, miR-675, miR-195, miR-376c), and may have a specific impact on trophoblast physiology [46–49]. Other regulators of invasion are targeted by miRNAs (*SMAD2* by miR-18a, *FOXA1* by miR-20a, *S1PR* by miR125b-1-3p, *CXCL12* by miR135b, *cMYC* by miR-34a, *MMP9* by miR-204 [50–56]). In a recent study using a BAC encompassing the C19MC cluster, the team of Yoel Sadovsky showed that miRNAs encoded by this cluster target genes involved in trophoblast movements, while miR-519d, located inside this cluster, regulates invasion of Extra Villous Trophoblasts by targeting CXCL6, NR4A2 and FOXL2 [57].

5.2.3. Proliferation

Trophoblast proliferate massively at the first trimester, and in humans, extravillous trophoblast will plug the maternal spiral arteries of the endometrium [58], the local hypoxia being probably important for the proliferation [59]. Again, several genes involved in proliferation are regulated by mi-RNA; this is the case for *LIF*, *CCND1*, *TTN*, *MYC*/*ERK*, *IRAKM*/*NKIRAS*, respectively targeted by miR-141, miR-155, miR-144, MiR-145, 377, let7a, and finally miR-155* [60–64].

5.2.4. Vasodilation

Placentation in humans, especially the specific invasion of maternal arteries aims at providing the growing fetus with a stable hemosphere especially in terms of oxygen pressure. Indeed, while trophoblasts are well adapted to low oxygen pressure, they are very sensitive to steep variations in oxygen pressure, leading many researchers to mimic preeclampsia through a hypoxia-reoxygenation protocols, rather than a mere exposure to hypoxia [65–68]. These variations are associated to an increase in oxidative and nitrosative stress that are major actors in preeclampsia [69,70]. In eukaryotic cells, it is clear that miR-210 plays the role of an important sensor of hypoxia, and its deregulation has been recurrently reported, see for instance [71–76]. Recently in a mouse model of preeclampsia, we also reported abnormal miR-210 regulation [77]. Amongst the various molecules important for vasodilation, NO and H_2S play prominent roles. It has recently been shown that miR-21 down-regulate CSE (Cystathione lyase), a protein involved in H_2S synthesis [78].

5.3. Defining miRNA targets, some examples

Compared to other epigenetic regulations, the mode of action of miRNAs is much more understood in molecular terms. In addition, it is possible to define specifically and experimentally if a given gene is a direct target for a miRNA. The idea is to clone the presumed miRNA target of the gene (often contained into the 3′ Untranslated Region) in front of the luciferase coding sequence in a reporter vector. Then cells are transfected with this construction and either siRNA mimicking the miRNA or with a control siRNA. If the miRNA targets the gene, then it will lead to a reduction of the luciferase activity, thus validating the target. Following are some examples in the context of placenta and placental diseases.

5.3.1. miR-1324 in preeclampsia

In 2013, Oudejans and coworkers identified polymorphisms in the gene *INO80B* as associated with an increased risk of preeclampsia. Interestingly the susceptible allele (rs34174194) is located in the 3′UTR of the gene and modifies a binding site for the miRNA miR-1324. The common variant is highly conserved throughout mammalian species. By luciferase assays, the authors demonstrated that the repression of the gene is only marginal in the mutant, thus leading to an increased risk of preeclampsia [79].

5.3.2. miR-675 and placental development

Another example is given by miR-675, a micro-RNA which is encoded by a highly conserved sequence present in the imprinted non-coding gene *H19* [80]. In mice miR-675 synthesis is prevented during the first 11 days of gestation by binding with the HuR protein, which decreases during pregnancy. Then, miR-675 is released, and targets the *Igf1R* mRNA, encoding the active receptor of Igf2, as well as NOMO1 (Nodal Modulator 1).

5.3.3. miR-34a and placental diseases

MiR-34 is frequently associated with cancer [81]. It is also strongly expressed in placental cells, especially at the end of pregnancy [82], while the pri-miR appears strongly expressed in early placentas [83]. We could show that its expression is correlated to methylation of its promoter, that this methylation is considerably increased by hypoxia. Functionally, it has been recently shown by Sun and coworkers that miR-34a inhibits cell migration and invasion by directly targeting the c-MYC proto-oncogene [51].

5.4. Sets of preeclampsia-associated deregulated miRNAs

One of the major interests of miRNAs is that they have a double hat. They regulate directly genes by repressing their expression, and are thus important in physiology and development, but they are also very stable in the plasma (at least several days) [84,85], and constitute exciting potential biomarkers for a large number of human diseases; among those there are more than 20 articles exploring preeclampsia and circulating miRNAs, for instance [86–92]. For the moment, however, no consensual list of miRNAs emerges from these screening experiments; possibly, a better classification of the patients in term of preeclampsia severity, combination with IUGR, HELLP syndrome, eclampsia, or considering the ethnical background, could be very helpful to classify the various miRNA profiles and sort them more efficiently.

6. Conclusion

This short review aims at presenting the role of the epigenetic machinery, in the context of trophoblast differentiation and normal/pathological placental development. The histone code is accessible through technically challenging and costly methods, such as in particular ChIP-seq experiments, using antibodies directed against the different histone variants. While these approaches have a great potential for the better understanding of placental development, they appear difficult to set up in the context of placental diseases. DNA methylation is now very easily implemented in humans using high-throughput microarrays that interrogate simultaneously and with a high quantitative precision more

than 450,000 CpG in the genome. However, besides the use of demethylated maspin (SERPINB5) in the maternal blood [93], such epigenetic marks are not envisaged for a systematic use in the context of placental diseases. An additional problem is that the link between DNA methylation and gene expression down-regulation is far from obvious [35]. Micro-RNA are probably the most promising biomarkers, cumulating the advantage of a good knowledge of their mode of action, high stability in the plasma, and the advantage of being easily detectable in the women plasma. For the moment, no clear subset of miRNAs appears as characteristic of preeclampsia; this may be due to the heterogeneity of the disease, and possibly a better clinical classification of the samples could drive significant progress in the field. This would imply analyzing a much higher number of human samples thoroughly characterized, which has not been done today. A more distant additional use of small RNA could be therapeutic, since siRNA could in principle be used to correct specifically anomalies of gene expression. Attempts to address small vesicles encompassing siRNA or therapeutic proteins towards the syncytiotrophoblast could be an interesting approach in the future.

Conflict of interest

No conflict of interest declared.

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