

REVIEW

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Potential role of epigenetic mechanisms in regulation of trophoblast differentiation, migration, and invasion in the human placenta

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ABSTRACT

The proper establishment and organogenesis of the placenta is crucial for intrauterine fetal growth and development. Endometrial invasion by the extravillous trophoblast cells, as well as formation of the syncytiotrophoblast (STB), are of vital importance for placental function. Trophoblast migration and invasion is often compared to tumor metastasis, which uses many of the same molecular mechanisms. However, unlike cancer cells, both initiation and the extent of trophoblast invasion are tightly regulated by feto-maternal cross-talk, which when perturbed, results in a wide range of abnormalities. Multiple factors control the trophoblast, including cytokines and hormones, which are subject to transcriptional regulatory networks. The relevance of epigenetics in transcriptional regulation of trophoblast differentiation and invasion, as well as in the onset of placenta-related pregnancy disorders, became recognized decades ago. Although, there has been tremendous progress in uncovering the molecular foundation of placental development, there is still much to be learned about the epigenetic machinery, and its role in trophoblast differentiation and invasion.

This review will provide an overview of the epigenetic control of trophoblast differentiation and invasion. It will also highlight the major epigenetic mechanisms involved in pregnancy complications related to placental deficiencies.

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Introduction

The placenta originates from the extra-embryonic structures of the conceptus, and plays a key role in fetal development and growth. This complex organ emerges through the interaction of extra-embryonic mesoderm with trophoblast stem cells (TSCs) that differentiate from the trophectoderm of the blastocyst during implantation.^{1,2} The TSCs further differentiate into cytotrophoblast (CTB) cells that give rise to the 2 major cell types of the placenta: the non-migratory villous trophoblasts (VT) and the invasive extravillous trophoblast (EVT) cells. One of the tasks of EVT cells is to invade the maternal uterine tissue and migrate toward the uterine spiral arteries where they further differentiate to become endovascular trophoblast.³ Here they degrade the tunica media and smooth muscle, and displace the endothelium in the maternal arteries, creating high volume, low resistance vessels to ensure sufficient blood flow to intervillous space of the placenta as the fetus grows and pregnancy progresses. Pregnancy complications, such as preeclampsia,⁴ miscarriage⁵ and pre-term birth,⁶ suffer from shallow or insufficient EVT invasion, whereas

conditions like placenta accreta are the consequence of excessive EVT invasion.^{7,8} The EVT cells emerge early in pregnancy from the distal portion of the anchoring villi. The VT cells form the principal maternal-fetal exchange site by fusing into an outer layer of STB, and creating a large surface area of branched villi floating in maternal blood. Defects in formation of EVT or VT cell types alter placental function, and are associated with high risk human placental pathologies.⁹ The events controlling syncytium formation and EVT differentiation are under investigation, using primary trophoblast cells, choriocarcinoma and non-tumorigenic trophoblast cell lines. The process is believed to be guided by a network of differentially expressed genes that include transcription factors,^{10,11} cell adhesion molecules, extracellular matrix components,¹² and growth factors.¹³ However, despite extensive research to decipher the molecular basis of trophoblast differentiation and invasion, the precise transcriptional mechanisms are still not fully understood.

Epigenetics is the study of heritable alterations in gene function that do not involve changes in the DNA sequence

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itself.¹⁴ Histone modification and DNA methylation are 2 major epigenetic mechanisms^{15,16} that can directly or indirectly control gene expression, either by changing the chromatin structure and influencing the accessibility of DNA, or by modifying the bindings sites of transcription regulatory subunits.¹⁷ The ultimate result is altered gene expression that produces new cellular phenotypes without changing the genotype. Non-coding RNAs (ncRNAs) are emerging as new epigenetic regulators of gene expression at both the transcriptional and post-transcriptional levels. ncRNAs represent a large part of non-protein coding transcripts, which are generally categorized into microRNAs (miRNAs) and long non-coding RNAs (lncRNAs).¹⁸ Although ncRNAs do not hold protein sequence information, they can regulate gene transcription, thus, impacting cell function, associated pathological conditions, and human diseases.¹⁹⁻²¹ In recent years, numerous studies have established a strong link between epigenetic regulation and placental development by demonstrating the critical role of epigenetic regulators, and the associated transcription factors (Table 1), in maintaining a healthy pregnancy.^{22,23}

This review summarizes recent expansions in our knowledge of the principal epigenetic machinery, including histone modifications, DNA methylation, and ncRNAs, that transcriptionally governs trophoblast differentiation, migration, and invasion during placental development. Furthermore,

we will focus on trophoblast phenotypes associated with disrupted placental development to provide mechanistic insights into the link between epigenetics and pathology.

Histone modifications

Essential epigenetic regulation of gene expression in eukaryotes occurs through remodeling of higher order chromatin structure by covalent post-translational amino acid modifications.²⁴ Modifications of N-terminal tails of histones are carried out by acetylation, methylation, phosphorylation, ubiquitination, and ADP-ribosylation to modulate gene activity by controlling chromatin architecture and nucleosome positioning.²⁵ The important role of histone modifications at lineage-control gene loci in embryonic stem cells (ESCs) during early development stages has been reviewed before.²⁶ However, limited evidence is available on the crucial role of histone modifications in trophoblast differentiation, invasion, and function at advanced developmental stages.

Histone acetylation/deacetylation

Histone acetylation and deacetylation can control gene expression.^{27,28} The exposure of DNA to transcription

Table 1. Summary of epigenetic modifications related to pregnancy complications.

Epigenetic marker	Target (s)	Model(s)		Functional effect			Ref.
		Species	Type	Differentiation	Migration/invasion	Associated pathological condition	
Histone modification							
Acetylation	H2A/H2B	Ms	TS	↘	↘↘		33
	H3K9 (Maspin)	Hu	PT, CL	-	↘↘		34,35
	H3K9/14	Hu	CL	↘	-		36
Methylation	H3K27me3	Hu	Plc	↗↗	-		40
	H4K20me3	Hu	Plc	↗↗	-		40
	H3K9me3	Ms	ES	↗↗	-		42
Ribosylation	Global	Ms	ES	↗↗	↘		48-50
DNA Methylation							
	SNAI1	Hu	Plc	-	↘	PE, HELLP	69
	ERVWE1(Syncytin-1)	Hu	PT, Plc	↘	-	PE, IUGR, HELLP	72, 73
	H19	Hu	Plc, CL	↘	-	PE, IUGR, SGA	76-78
	STOX1	Hu	PT	-	↗↗	PE	112
	Leptin	Hu	Plc	-	↘↘	GD MC	81
Non-coding RNAs (ncRNAs) †							
miR-378a-5p	Nodal	Hu	Plc, CL	↘	↗↗	PE	94,96
miR-195	ActRIIA	Hu	Plc, CL	-	↗↗↗	PE	98
miR-376c	ALK7, ALK5	Hu	Plc, CL	-	↗↗↗	PE, SGA	97
miR-210	KCMF1	Hu	Plc	-	↗↗↗	PE	103
miR-125b-1-3p	S1PR1	Hu	Plc, CL	-	↗↗↗	PE	105
miR-29b	MMP2, MCL1, VEGFA, ITGB1	Hu	Plc, CL	-	↗↗↗	PE	104
miR-204	MMP9	Hu	CL	-	↗↗↗		99
miR-135b	CXCL12	Hu	CL	-	↗↗↗		100
miR-155	Cyclin D1, eNOS	Hu	Plc, CL	-	↗↗↗	PE	106,110
miR-20a	FOXA1	Hu	Plc, CL	-	↗↗↗	PE	102
miR-519d	CXCL6, NR4A2, FOXL2	Hu	Plc, CL	-	↗↗↗	IUGR	101,113
miR-424	FGFR1	Hu	PT, CL	↗	-		93

Ms, Mouse; Hu, Human; TS, Trophoblast Stem cells; ES, Embryonic Stem cells; PT, Primary Trophoblasts; CL, Cell line; Plc, Placenta explant/tissue; PE, Preeclampsia; SGA, Small-for-gestational-age; IUGR, Intrauterine Growth Restriction; GD MC, Growth-Discordant MonoChorionic twins.

†Only the individually studied ncRNAs with known targets are listed.

factors depends on the chromatin compaction status.²⁴ The attachment of an acetyl group to lysine residues by a histone acetyltransferase (HAT) complex neutralizes the histone positive charge, resulting in the disruption of interactions between adjacent nucleosomes, chromatin relaxation, and initiation of transcription. In contrast, removal of acetyl groups, mediated by histone deacetylase complexes (HDAC), reverses the process, resulting in the chromatin condensation and gene repression.²⁹ A decade ago, it was established that the interaction between hypoxia-inducible factor 1 (HIF1) and HDAC is essential for proper murine trophoblast differentiation.³⁰ Mutations in ARNT, a component of the HIF1 complex, suppresses HDAC activity, which increases global histone acetylation; thus, an insufficient spongiotrophoblast population with increased numbers of giant cells leads to placental failure and fetal death.^{31,32} The role of histone acetylation in retaining multipotency of trophoblast stem (TS) cells is illustrated by acetylation of histones H2A and H2B by CREB-binding protein (CBP) acetyltransferases, which decreases the epithelial-mesenchymal transition (EMT) and reduces invasiveness of murine TS cells, while maintaining the epithelial stemness phenotype.³³ Interestingly, the negative effect of acetylation on trophoblast invasion seems to extend to later stages of placental development. Maspin, a tumor suppressor gene that has been negatively correlated to human trophoblast motility and invasion³⁴ was overexpressed in second trimester human placentas after treatment with trichostatin A, a HDAC inhibitor. This alteration was associated with increased acetylation at H3K9, accompanied by H3K4 methylation.³⁵ Pharmacological inhibition of HDACs up-regulates human pregnancy-specific glycoproteins (PSGs), which are early markers of cytotrophoblast differentiation expressed only by the STB.³⁶ More research is needed to better understand the role of acetylation/deacetylation in trophoblast differentiation.

Histone methylation

The process of histone methylation was first described in 1964 by Murray, who identified lysine and arginine residues as the only targets for methylation of histone core proteins.³⁷ Histone H3 lysines at positions 4, 9, 27 and 36, as well as lysine 20 in H4, are highly susceptible to mono-, di- or tri- methylation,³⁸ whereas arginine residues can only be mono- or dimethylated.³⁹ Histone methylation has a different function than histone acetylation in the regulation of gene expression. Depending on position and form of methylation, this post-translational modification can be associated with active or repressive states of chromatin.³⁹ The function of histone

methylation in trophoblast differentiation is controversial. In a recent study, the heterochromatin methylation marker H3K27me3 was found to be highly active in CTB. That was explained by rapid and transient repression of genes at the time of STB formation. Also STB nuclei were enriched for H4K20me3, a repressive chromatin modification.⁴⁰ However, this report was in contrast to another study reporting that the CTBs were enriched with the trimethylated H3K4, a modification associated with active promoters, and that the STBs were transcriptionally activated by the chromatin marker H3K4Me2, which was co-localized with active RNAP II in the majority of STB nuclei.⁴¹ The role of H3K9 methyltransferase G9a in transcriptional repression, and its contribution in trophoblast differentiation, has been studied in the murine model. Deletion of the G9a gene induced embryonic lethality at a very early developmental stage due to defects in chorioallantoic attachment.⁴² The differentiation of invasive murine trophoblast giant cells was also influenced by histone methylation, as demonstrated by a mutation in the Polycomb group family members⁴³ that form the Polycomb repressive complex (PRC) 2 and 3 to mediate H3K27 methylation.²² On the other hand, arginine methylation of histone mediated by protein arginine-N-methyltransferases (PRMTs) appears to have a different function in placental development. Prmt1 deficient mouse embryos failed to develop proamniotic cavities, ectoplacental cavities, or amniotic folds.⁴⁴ Thus, distinct methylation patterns could determine the destiny of trophoblast derivatives.

Histone ribosylation

Ribosylation is a post-transcriptional modification catalyzed by poly(ADP-ribose) polymerases (PARPs) that transfer ADP-ribose from nicotinamide adenine dinucleotide to protein substrates.⁴⁵ In humans, histone H2AK13, H2BK30, H3K27, H3K37 and H4K16 are known ADP-ribose acceptor sites.⁴⁶ The process reverses the positive charge of amino acid side chains to induce chromatin relaxation, resulting in recruitment of DNA repair enzymes, cell cycle progression, replication enhancement, and regulation of gene expression.⁴⁷ The positive influence of Parp1 on trophoblast differentiation is frequently accompanied by negative regulation of invasion. An increase in the invasive trophoblast giant cell population, suppression of differentiation, and a decrease in spongiotrophoblast number are general characteristics of Parp-1^{-/-} mouse placentas and ESCs.⁴⁸⁻⁵⁰ Nude mice injected subcutaneously with Parp-1^{-/-} teratocarcinomas containing giant cell-like cells developed lighter, but highly metastatic, tumors when compared to wild-type (Parp-1^{+/+}) controls, revealing the strong effect of Parp-

1 loss on induction of invasiveness and inhibition of cell proliferation.⁵⁰ The *Parp1*^{-/-} tumors, however, contained a significantly lower spongiotrophoblast population, as verified by specific marker genes.⁴⁸ Although the reduction of *Parp-1* correlates with early trophoblast lineage formation, the causal role of histone ADP-ribosylation on placental development still remains unclear.

DNA methylation

DNA methylation was the first epigenetic mark to be discovered.⁵¹ It converts cytosine to methyl cytosine by addition of a methyl (-CH₃) group at the 5' carbon of cytosine. Methylated cytosine residues often occur adjacent to a guanine nucleotide (CpG dinucleotide), resulting in 2 altered cytosine residues sitting diagonally to each other on opposing DNA strands. Methylation of CpG sequences in a promoter generally silences a gene, but instances of transcriptional activation are also known.^{52,53} However, in certain genes, a secondary site adjacent to the promoter region is targeted by methylation, leading to differential expression. For example, in *STOX1*, methylation of a CpG island located in intron1, but not in the promoter, reduces expression.⁵⁴ The methylation marks generally cluster, and lock genes in either an "on" or "off" mode. While gene silencing can occur by inhibiting the binding of transcription factors,⁵⁵ altering chromatin packing,⁵⁶ or recruitment of methyl-binding domain proteins,⁵⁷ no mechanism has been proposed for transcriptional activation by methylation.

There are 2 classes of enzymes that regulate DNA methylation. DNA methyl transferases (DNMTs) add methyl groups on CpG sites, while 10 11 transferases (TETs) actively de-methylate the DNA through a series of intermediates.⁵⁸ Among the DNMTs, DNMT 3A and 3B are *de novo* methylation enzymes that work in a site-specific manner, whereas DNMT1 is a maintenance enzyme.⁵⁹⁻⁶¹

DNA methylation plays an important role in embryo development. This is evident from the observation that the ESC and TSC lineages have distinct DNA methylation profiles.⁶² Cells of TSC lineage are globally hypomethylated compared to those of the ESC lineage.⁶³ Disruption of the methylation profile in mouse ESC by knocking down the methylation enzymes (DNMT1, 3A, 3B) or depletion of demethylation enzymes (TET1, 2) resulted in either cell death or transformation to the TSC lineage.^{64,65} On the other hand, knocking out DNMT's in extra-embryonic tissue did not alter their survival or proliferation.⁶⁶ These observations emphasize the importance of DNA methylation and its regulation in trophoblast cell fate restriction. EVT invasion of the uterus

requires transformation of epithelial CTB cells into EVT cells through the EMT process.⁶⁷ Both EMT and invasion are tightly regulated by methylation. The zinc finger transcription factor, Snail, encoded by the *SNAI1* gene, initiates EMT.⁶⁸ Altered levels of both Snail and E-cadherin have been observed in PE and HELLP placentas.⁶⁹ Studies with villous trophoblast and EVT cell lines, BeWo and HTR-8/SVneo, showed that hypomethylation of the Snail promoter increased expression and activity, suggesting a contribution to the disease mechanism by down-regulation of E-cadherin to induce EMT.⁷⁰ Formation of STB is regulated by endogenous retrovirus group W member 1 (ERVWE1) or syncytin-1.⁷¹ During normal placental development, downregulation of DNMT1, 3A and 3B in CTB leads to hypomethylation of the syncytin-1 promoter, resulting in expression of syncytin-1, and differentiation of the CTBs into multi-nucleated STB.⁷¹ CTB cells from placentas of pathologic pregnancies suffer from reduced levels of syncytin-1, with increased methylation of its promoter.^{72,73} Further, over-expression of DNMT3A in cultured CTB cells down regulates syncytin-1, demonstrating that defects in the methylation machinery could contribute to defective trophoblast differentiation.⁷²

Studies by Hu et al, focusing on global methylation levels in normal and pathologic placentas identified caludin4 and fucosyltransferase IV in the regulation of trophoblast invasion.⁷⁴ The altered methylation of other candidates, including the imprinted gene *H19*, that regulate the differentiation of placental cytotrophoblasts⁷⁵ has also been correlated with intrauterine growth restriction (IUGR), PE and small for gestational age cases.⁷⁶⁻⁷⁸ Similarly, parental-specific hypermethylation of another imprinted gene, *STOX1*, which negatively modulates trophoblast invasion,⁷⁹ was reported in placentas carrying the Y153H preeclampsia susceptibility allele.⁵⁴ The promoter of *leptin*, a gene involved in regulation of EVT invasion,⁸⁰ is hypermethylated in the under-developed portion of placentas from growth-discordant monozygotic twin pregnancies.⁸¹ In addition to methylation, a role for hydroxymethylation of cytosine residues in trophoblast was also described recently.⁴⁰ Oxidation of the 5' methylcytosine by TET enzymes gives rise to the 5'-hydroxymethylcytosine.⁸² Unlike methylation, 5'-hydroxymethylation was found to be associated with euchromatin in embryonic stem cells and was associated with promoters having high transcriptional activity.⁸³ While the exact role of hydroxymethylation in trophoblast is not known, higher levels were detected in syncytiotrophoblast where it is stated that cytotrophoblast and syncytiotrophoblast may have distinct epigenetic profiles.⁴⁰ These studies combined with the several in-vivo and in-vitro studies in cell lines and mouse models

respectively, indicate a sensitive balance between the different methylation states and trophoblast function.^{70,84}

Non-coding RNAs (ncRNAs)

Evidence suggests that ncRNAs, and specifically miRNAs, are a key components of epigenetic regulation. miRNAs are small ncRNAs of about 20-24 nucleotides in length that target the 3' untranslated region (UTR), or the 5'UTR, of mRNA to regulate translation.⁸⁵ miRNAs are transcribed as pri-miRNA by RNA polymerase II, and subsequently processed by nuclear proteins Drosha and DGCR8 into shorter pre-miRNAs, which are exported to the cytoplasm and further processed into mature miRNAs.⁸⁶ miRNAs either inhibit translation by imperfect base pairing, or induce degradation of the mRNA.⁸⁷ Studies have shown that a multitude of diverse miRNAs are produced in the human placenta, and that they can directly target effectors of the epigenetic machinery, such as HDAC and PRC genes, to indirectly affect gene expression.⁸⁸

miRNAs are expressed during placental development, regulating trophoblast differentiation, migration and invasion. Using a miRNA microarray, numerous miRNAs were identified in placental tissues collected at different gestational ages.⁸⁹ miRNAs in the C14MC, miR-371-3 and C19MC clusters were significantly upregulated in the first trimester.⁸⁹ In contrast, miRNAs of the let-7, miR-34, miR-29a, miR-195, and miR-181c clusters were upregulated in third trimester placentas.⁸⁹ This expression pattern indicates a key role for miRNAs in placental development. Clusters C14MC and C19MC are located on chromosome 14 and chromosome 19, respectively, and are exclusively expressed in the placenta.^{90,91} Both C14MC and C19MC miRNAs are thought to regulate cellular differentiation during pregnancy.⁸⁹ Lan et al.⁹² showed that upon stable transfection of the entire C19MC cluster in HTR-8/SVneo trophoblast cells, migration is attenuated without affecting cell proliferation or apoptosis, suggesting that miRNAs within the C19MC cluster specifically regulate trophoblast migration.

In addition to the C19MC cluster, recent studies identify other miRNAs that regulate trophoblast differentiation, migration and invasion. miR-424, which is downregulated in human trophoblasts under hypoxic conditions, promotes cell differentiation through direct regulation of fibroblast growth factor receptor 1 (FGFR1).⁹³ Luo et al.⁹⁴ demonstrated that miR-378a-5p promotes trophoblast cell migration and invasion in first trimester placental explants by suppressing Nodal.⁹⁴ Nodal is a member of the transforming growth factor factor- β superfamily⁹⁵ that inhibits trophoblast cell migration

and invasion through the actin receptor like kinase 7 (ALK7) pathway. However, miR-378a-5p suppresses Nodal expression potentially through its binding site on Nodal 3'UTR, thus, promoting trophoblast cell migration and invasion. Most recently, the inhibitory role of miR-378a-5p on STB differentiation by down-regulation of CCNG2 expression was reported in BeWo cells.⁹⁶ miR-376c is another microRNA that targets ALK7 and ALK5 to induce trophoblast migration and invasion.⁹⁷ Similarly, miR-195 promotes trophoblast invasion by repressing ActRIIA, which is the type II receptor for ActivinA and Nodal.⁹⁸ Notably, miR-195 is downregulated in preeclamptic pregnancies.⁹⁸

Several studies report that miRNAs, such as miR-204,⁹⁹ miR-135b,¹⁰⁰ miR-519d,¹⁰¹ miR-20a,¹⁰² miR-210,¹⁰³ miR-29b,¹⁰⁴ miR-125-1-3p,¹⁰⁵ and miR-155,¹⁰⁶ inhibit trophoblast cellular activities. There is compelling evidence linking each of these inhibitory miRNAs to pregnancy complications, including PE and IUGR, in which EVT invasion of the uterine arteries is reduced¹⁰⁷ and trophoblast apoptosis is elevated¹⁰⁸ (Table 1). miRNA-210 is upregulated in PE placentas, compared to healthy placentas,¹⁰³ and its expression is inversely correlated to expression levels of potassium channel modulatory factor 1 (KCMF1), suggesting KCMF1 as a direct target.¹⁰³ Transfection of the HTR-8/SVneo cell line with miRNA-210 mimics repressed trophoblast invasion, which can be rescued by overexpression of KCMF1,¹⁰³ supporting the putative contribution of miRNA-210 to PE. miRNA microarray data indicates miRNA-210 as the most upregulated miRNA in preeclamptic placentas.¹⁰⁹ miRNA-125b-1-3p inhibits trophoblast cell invasion by targeting sphingosine-1-phosphate receptor 1 (S1PR1) in preeclampsia.¹⁰⁵ It was also documented that miRNA-29b induces apoptosis and inhibits invasion by directly targeting the 3'UTRs of myeloid cell leukemia sequence 1, matrix metalloproteinase 2, vascular endothelial growth factor A and integrin β 1.¹⁰⁴ An inverse correlation between the expression of miRNA-29b and its target genes was identified in preeclamptic placentas.¹⁰⁴ Dai et al. demonstrated a negative regulatory role of miRNA-155 in trophoblast cell migration by targeting the eNOS and cyclin D1/p27 pathway.^{106,110}

The role of miRNAs in epigenetic modifications that regulate placental development is still in the early phase of investigation, yet there is compelling evidence of a clear epigenetic miRNA interaction that regulates trophoblast cell differentiation, migration and invasion. Several studies have shown a close correlation between miRNA expression and trophoblast cell activity, and its implications for placental disease (Table 1). Epigenetics is an emerging field that has revealed a complex regulatory network involved in gene regulation that is

responsible for placental development. A recent study has shown that another group of non-coding RNAs, lncRNAs, play a putative role in epigenetic modulation of trophoblast proliferation and migration through miRNA sequestration.¹¹¹ Continued research on epigenetic regulation by miRNAs shows great promise for rapidly elucidating novel mechanisms that control human placental development.

Conclusion

Although knowledge of epigenetic regulatory mechanisms of gene expression essential for placental development has improved substantially in recent years, much remains to be learned about its contribution to the pathogenesis of abnormal placentation associated with inadequate trophoblast differentiation and invasion. The ability to analyze the entire epigenome, using innovative technologies such as next-generation sequencing, will provide a more detailed and precise perspective of the transcriptional regulatory networks. With further research focusing on gestational changes in the various placental cell types in relationship to disease, epigenetics will help to understand human development, and contribute significantly toward efforts to devise preventive and therapeutic interventions for pregnancy complications.

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