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Genome-wide identification of enhancer elements in the placenta

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ABSTRACT

Normal placental development is essential for a healthy pregnancy, and is contingent upon tight spatiotemporal regulation of gene expression. One level of transcriptional control is via enhancer elements in the genome. Enhancers are distal *cis*-regulatory elements that can impact gene expression regardless of their position or orientation. The study of enhancers in the placenta is usually focused on one or two at a time, and the simultaneous identification of all enhancers has been limited. However, such a holistic approach is necessary if we are to gain a systems-level understanding of gene expression regulation in the placenta. Here, we review current methods for genome-scale enhancer identification, as well as studies that have applied those techniques in the placenta, with the aim of guiding future research.

1. Introduction

Enhancers are *cis*-regulatory elements that are capable of increasing gene transcription in a tissue- and time-specific manner [1]. The number of putative enhancers in the human genome has swiftly exceeded the number of protein coding genes, with some estimates nearing one million [2].

Enhancers can be kilobases (kb) or even megabases away from the genes they regulate, and multiple enhancers can act on the same gene [1], complicating genome-scale enhancer identification. In addition, enhancers, unlike promoters and protein-coding regions, do not have any apparent identifying sequences [2], hindering computational prediction. However, pinpointing enhancers is a critical step in understanding gene expression regulation, especially when considering tightly regulated processes.

Placental development is one such process. Individual enhancer elements, such as that upstream of the HLA-G gene, have been identified and characterized in the placenta. HLA-G is a Major Histocompatibility Complex (MHC) molecule that is expressed in first trimester extravillous trophoblast cells (EVTs) [3]. Ferreira et al. [4] identified a 121-bp enhancer 12-kb upstream of the HLA-G gene that, when deleted in first trimester EVTs or JEG-3 cells, lead to an abrogation of HLA-G expression in those cells. The enhancer element, dubbed Enhancer L by the authors, was bound by the CEBP and GATA transcription factors (TFs), which were found to modulate gene expression by mediating looping of the enhancer into the HLA-G promoter (Fig. 1).

Errors in placental development can have serious consequences for the mother and the baby. Preeclampsia [5], intrauterine growth restriction [6], and preterm labor [7] are among the many pregnancy disorders linked to placental defects. Furthermore, as with other complex disorders, single nucleotide polymorphisms associated with pregnancy disorders are frequently found in regions of non-coding DNA that may function as enhancers [8]. Therefore, understanding how enhancers globally contribute to gene regulation in normal and diseased placentas has great potential in furthering treatment and prevention efforts for many pregnancy complications.

Here, we briefly review computational and experimental approaches that can be used to identify enhancers on a genome-wide scale. These methods have been reviewed in more detail previously [2,9–11]. After that, we review how these methods have been utilized to understand the mechanisms of placental development (Fig. 2).

2. Methods to identify enhancers across the genome

2.1. Predicting enhancers using clusters of TF binding sites

Groups of TFs can modulate gene expression by binding to 6-12bp motifs in enhancer regions. Therefore, despite the lack of a consensus enhancer sequence, there are computational approaches to predict enhancer elements based on searching for clusters of TF binding sites within a small region [12]. Methods to predict TF binding site clusters (reviewed in Ref. [12]) involve first predicting TF binding sites, for

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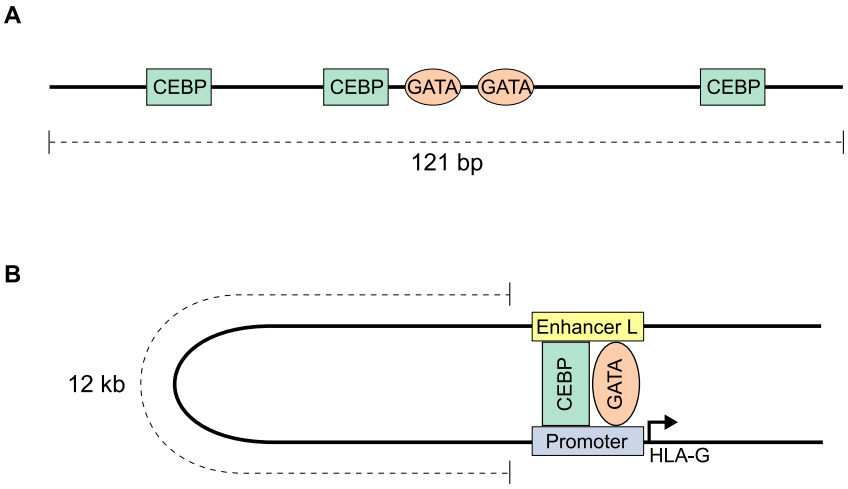

Fig. 1. Enhancer L modulates HLA-G gene expression. A) Binding sites of CEBP and GATA transcription factors within Enhancer L. B) Looping of Enhancer L to the HLA-G promoter across a 12-kb distance, mediated by CEBP and GATA transcription factors.

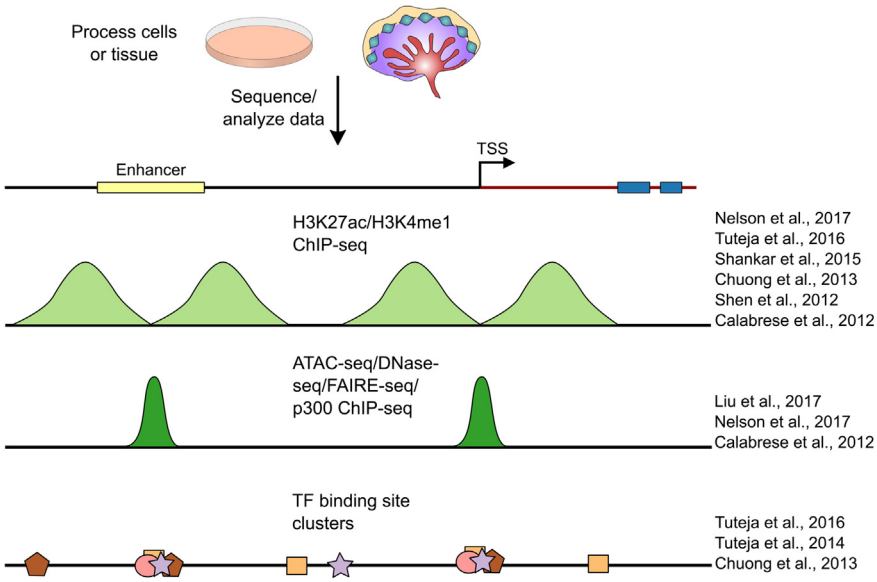

Fig. 2. Genome-wide enhancer identification methods utilized in the placenta. TSS, Transcription Start Site; H3K27ac, histone H3 lysine 27 acetylation; H3K4me1, histone H3 lysine 4 monomethylation; ChIP-seq, Chromatin Immunoprecipitation sequencing; ATAC-seq, Assay for Transposase-Accessible Chromatin sequencing; DNase-seq, DNase I hypersensitive sites sequencing; FAIRE-seq, Formaldehyde-Assisted Isolation of Regulatory Elements sequencing; TF, Transcription Factor.

example using phylogenetic footprinting, followed by searching for groups of the TF binding sites within a small region.

One disadvantage of *in silico* enhancer prediction using TF binding sites is that the binding sites are short and degenerate, resulting in many false positive predictions. In addition, certain methods assume that enhancers are conserved across species, even though many are species-specific [13,14], resulting in false negative predictions. Finally, the presence of binding site clusters is not unique to enhancers, but can occur in other genomic regions [12]. However, when combined with experimental approaches that first identify putative enhancers, binding site predictions can be used to help determine how the enhancer is regulated.

2.2. Chromatin immunoprecipitation sequencing (ChIP-seq)

ChIP-seq is a widely used technique for identifying *in vivo* protein-DNA binding events. In this experiment, regions pulled down by an antibody targeted to a specific protein are sequenced, and then sequence reads are aligned to a reference genome. Generally, if more reads align to a region in the ChIP sample compared to the input (control) sample, that region is identified as a peak and marks the binding of the target protein [15]. Since many TFs bind to enhancer regions, enhancers could be identified using TF ChIP-seq. However, TFs can also bind to other *cis*-regulatory regions, and to non-functional elements in the DNA. Furthermore, since any enhancers identified using this approach would be specific to the TF targeted in the assay, TF ChIP-seq is not used to identify all active enhancers in a particular context.

In general, active enhancers are marked by histone H3 lysine 27 acetylation (H3K27ac) and histone H3 lysine 4 monomethylation (H3K4me1), and by the binding of the CBP/p300 coactivator family [16]. H3K4me1 and p300 also mark poised enhancers when H3K27me3 is located in the same site [16]. Therefore, ChIP-seq can be used to identify regions of the genome where such marks are located, indicating the presence of enhancers.

ChIP-seq is a powerful technique, but not without its limitations. For example, the most well-established protocols require large amounts of starting material, restricting the tissue or cell types in which the assay can be performed [15]. In addition, activity domains identified in H3K27ac and H3K4me1 datasets are usually about 1 kb long, making it difficult to identify the exact location of the enhancer. However, this can be circumvented by combining ChIP-seq with methods used for identifying open chromatin regions, such as DNase- or ATAC-seq, which are discussed below.

Table 1
Enhancer-associated human placenta datasets available through the ENCODE or Epigenomics Roadmap consortia.

	DNase-seq	FAIRE-seq	H3K4me1 ChIP-seq	H3K27ac ChIP-seq
Fetal placenta (102 days)	X			
Fetal placenta (112 days)	X		X	X
Fetal placenta (113 days)	X		X	X
Fetal placenta (116 days)	X			
Fetal placenta (56 days)	X			
Fetal placenta (59 days)	X			
Fetal placenta (53 days)	X			
Fetal placenta (101 days)	X			
Fetal placenta (105 days)	X			
Fetal placenta (85 days)	X			
Fetal placenta (108 days)	X			
Fetal placenta (91 days)	X			
Fibroblast of villous mesenchyme	X			
Fetal placenta amnion			X	X
Fetal placenta chorion smooth			X	X
HTR8/SVneo cell line	X	X		
BMP4-treated human ESCs	X		X	X

2.3. Assaying chromatin accessibility

Enhancer elements require protein binding to exert their regulatory functions, and therefore tend to be in nucleosome-free chromatin regions. Thus, assays of chromatin accessibility, which provide an indication of how “open” a region is, can be used to identify enhancer elements. One method that can be used to identify nucleosome-free regions is Formaldehyde-Assisted Isolation of Regulatory Elements sequencing (FAIRE-seq). This method separates nucleosome-bound and nucleosome-free regions using phenol-chloroform, but has high background [17]. Therefore, more commonly used methods are based on the increased susceptibility of open regions to enzymatic cleavage, such as DNase I hypersensitive sites sequencing (DNase-seq), and the Assay for Transposase-Accessible Chromatin sequencing (ATAC-seq) [17]. The protocol for ATAC-seq is less complex, and can be carried out on fewer cells, making it the preferred method since it was developed [17]. While these methods are able to identify narrow regions of accessibility in the genome, accessibility is not always indicative of an active enhancer. Therefore, these methods are best coupled with other experiments, such as enhancer-mark ChIP-seq.

2.4. Machine learning (ML)

Many ML-based tools are available for predicting enhancers on a genome-wide scale using supervised and unsupervised algorithms on high-throughput data. Some of the popular algorithms are hidden Markov models [18,19], artificial neural networks [20], random forests [21], and support vector machines [22]. These tools build prediction models by integrating different histone-mark datasets, treating them as individual features and using them to train the classifier.

One drawback of ML-based tools is the requirement of a large number of datasets, when there are typically a limited number available in a given context. Another drawback is the specificity of the models generated to the tissue or cell type in which they were made, limiting the scope of the prediction model. However, new tools have been developed that integrate heterogeneous data, including histone modifications, motif conservation, gene expression, and DNA methylation from different cell types and tissues, broadening the scope of the predictions [23]. Overall, the exponential growth of high-throughput data and new computational methods will help make ML-based enhancer predictions more accurate across tissues.

2.5. Self-transcribing active regulatory region (STARR) sequencing

The techniques described thus far can be used to identify putative enhancer regions, but do not provide evidence of enhancer functionality. STARR-seq is a technique that combines next-generation sequencing with reporter assays, providing simultaneous high-throughput identification and functional validation of enhancer elements [24]. In a typical experiment, DNA is sheared and the resulting fragments are cloned downstream of a minimal promoter, allowing active enhancers to transcribe themselves. After transfecting the clone library into a cell line of interest, RNA is collected, converted to complimentary DNA (cDNA), and sequenced. The higher the activity of the enhancer, the more sequence reads that will align to it.

STARR-seq is ideal for Drosophila-sized genomes, but the size of the human genome is overwhelming in comparison. In general, the larger the genome, the more difficult it is to construct clone libraries and transfect them [25]. This can be circumvented by simply narrowing down the library size, as in CapStarr-seq, a variant of STARR-seq that starts with putative regulatory elements rather than the entire genome [26]. Recently, a technique that builds on CapStarr and STARR was developed, called Whole Human Genome (WHG-)STARR-seq [27], which shows great promise as a genome-wide method to identify active enhancers in highly complex genomes.

3. Genome-wide identification of enhancer elements in the human placenta

The NIH Roadmap Epigenomics Mapping Consortium [28] and the Human ENCODE project [29] encompass a wide range of epigenomic datasets in various human tissues, including the placenta. The placental datasets from enhancer-associated assays (DNase-seq, FAIRE-seq, H3K4me1 ChIP-seq, and H3K27ac ChIP-seq) were generated in fetal placenta (ranging from 53 days gestation to 116 days gestation), placenta chorion, placenta amnion, fibroblasts from villous placental tissue, HTR8/SVneo cells, and Bone Morphogenetic Protein 4 (BMP4)-treated human embryonic stem cells (hESCs) (Table 1). In addition to generating these data, the consortia also used a machine-learning tool, ChromHMM, to identify genomic regions likely to act as enhancers in the placenta [30]. They also identified placenta-specific enhancer modules, and predicted the transcription factors that are likely to regulate these modules [30]. These examples demonstrate the valuable insights that can be gained from the consortia data.

Utilizing a dataset from the Roadmap project, Liu et al. [31] studied the DNase-seq landscape of BMP4-treated hESCs to identify *cis*-elements regulating early placental development. Though previously debated [32], recent evidence suggests that BMP4-treated hESCs represent early invasive trophoblast cells [33–35]. By comparing the DNase hypersensitive sites (DHS) of H1 hESCs and BMP4-treated hESCs, Liu et al. identified 17,472 trophoblast-specific DHS that were enriched for placenta-associated Gene Ontology terms. They also found trophoblast-

specific DHS near genes known to be important for placental development, including *Hand1* and *Foxo1*. Candidate TF regulators were identified by searching for TF motifs that were enriched in trophoblast-specific DHS, and then determining which TFs had higher expression in BMP4-treated hESCs compared to H1 hESCs, according to RNA-seq data. This study used DNase-seq and gene expression data to identify putative enhancers and novel TFs involved in early trophoblast development, while also providing further support for the trophoblast identity of BMP4-treated hESCs.

In another study, Shankar et al. [36] integrated ChIP-seq and RNA-seq data to investigate syncytialization in BeWo cells. When treated with forskolin, BeWo cells adopt a syncytial phenotype [37]. The study found that, after 72 h of forskolin treatment, the most prominent epigenomic change was an increase in the number of active marks, including enhancers marked by H3K27ac. From RNA-seq data, *Sgk1*, *Fosb*, and *Junb* were identified as key transcriptional regulators of syncytialization, and all three had increased H3K27ac activity at their promoters after 72 h of forskolin treatment. This study highlights how combining histone mark data with RNA-seq data is a powerful way to identify enhancers and key transcription factors.

4. Genome-wide identification of enhancer elements in the mouse placenta

The mouse is the most widely used model to study placental development due to its well-characterized genome, and similarities with human placentation [38]. For example, mouse and human placentas are both classified as hemochorial, and many placental genes and pathways are conserved between them [38].

In addition to the human project, the ENCODE consortium also has a mouse project [39], which has two enhancer-associated datasets (H3K27ac and H3K4me1 ChIP-seq) generated in e14.5 placenta by Shen et al. [40]. Shen et al. utilized those datasets to define over 60,000 placenta enhancers. By comparing H3K4me1 signal in the placenta and 18 other tissues, they also defined placenta-specific enhancers, within which they saw binding site enrichment for known placental TFs, including Tcfap2, Nr2f2, Nfe2, Rxr, and Ap1. Again, these analyses demonstrate the value in consortia data, where multiple cell types are assayed using multiple techniques.

Tuteja et al. [41] utilized sequence conservation in developing an automated framework to identify tissue-specific TFs and enhancers in the placenta. After combining TF binding site predictions and target gene function annotations to define placental TFs, they identified 2,216 TF binding site clusters, or putative placenta enhancers, that were conserved in the mouse and human genomes. They then experimentally validated several of the putative enhancers in mouse placental cell lines. This framework could be especially useful when combined with histone modification ChIP-seq or chromatin accessibility data to identify functionally related enhancers and the TFs that bind them in the placenta. For example, in another study, Tuteja et al. [42] carried out H3K27ac ChIP-seq to identify enhancers involved in the process of trophoblast invasion in mouse. They compared H3K27ac-marked regions at two time points during placental development: e7.5, an early post-implantation time point when *Mmp9*, an invasion-associated gene, is highly expressed [43]; and e9.5, when blood flow has been established. They found 1,977 e7.5-specific enhancers, and then, using a framework similar to the one described above, identified clusters of Ap1, Ets, and Tcfap2 motifs enriched within a subset of those enhancers, which were predicted to regulate many invasion-associated genes. Here, the combination of histone modification ChIP-seq with binding site predictions led to the identification of potentially crucial enhancers involved in the regulation of trophoblast invasion.

Genome-wide assays to identify enhancers have also been carried out to study different aspects of mouse trophoblast cells *in vitro*. In a notable study, Chuong et al. [44] investigated the role of *cis*-regulatory elements in placental evolutionary diversification. They performed ChIP-seq for H3K4me1 and H3K27ac in mouse and rat trophoblast stem cells (TSCs) and found 52,476 and 41,142 putative enhancers based on the H3K4me1 data in mouse and rat, respectively. From the H3K27ac data, they identified 25,736 mouse and 4,471 rat active enhancers. Each set of enhancers was significantly enriched near genes with placental functions. The authors also found enrichment of species-specific endogenous retroviruses (ERVs) in the enhancers, including the mouse-specific ERV family, RLTR13D5. Enhancers containing this ERV family were bound by Cdx2, Eomes, and Elf5, which are key regulators in TSCs. In this study, the combination of two enhancer marks in two species revealed valuable insight into the evolution of the placenta.

Nelson et al. [45] used the TSC ChIP-seq data generated by Chuong et al. [44] and generated ATAC-seq data in TSCs, as well as in TSCs after two days of differentiation (d2), to investigate the regulatory mechanisms behind trophoblast differentiation. The authors identified ~57,000 accessible chromatin regions in TSCs. Interestingly, TSC ATAC-seq peaks that overlapped with 8-cell stage ATAC-seq peaks were more likely to colocalize with enhancer marks (H3K4me1/H3K27ac), while those overlapping with ESC ATAC-seq peaks were more likely to colocalize with promoters (H3K4me3/H3K27ac). This indicates that some TSC enhancers may be established at the 8-cell stage. When comparing ATAC-seq data from TSCs to d2 TSCs, it was found that regions with enhanced accessibility in TSCs were enriched for RLTR13 repeats, the same ERV family identified by Chuong et al. [44]. In regions with enhanced accessibility in d2 TSCs, however, such repeat regions were significantly depleted, and instead, enrichment was found for binding sites of trophoblast TFs, such as Tcfap2, Ets, and Gata. In addition to identifying many active enhancers and predicting the TFs that bind them, this study also identified Blimp1 target genes, and provided evidence that Blimp1 silences TSC and other lineage-specific gene expression. By combining ATAC-seq data, histone modification ChIP-seq data, and binding site predictions, this study identified regulatory processes and genes that play prominent roles during trophoblast differentiation.

In another study utilizing mouse TSCs, Calabrese et al. [46] investigated the mechanisms of X inactivation. The authors proposed that X inactivation is mediated by silencing specific regulatory elements, rather than a chromosome-wide lack of binding of transcriptional machinery. While the main focus of the authors was not to define enhancer elements, they generated data in TSCs that could be used for this purpose, including ChIP-seq for H3K27ac and H3K4me1, FAIRE-seq, and DNase-seq. The data from this study, as well as from many others discussed in this review, are publicly available. This allows researchers to integrate data from the same placental cell types, and increase statistical power when identifying enhancer elements in that cell type, or integrate data from different placental cell types, to discover novel aspects of *cis*-regulation in the placenta.

5. Future directions

Tight regulation of gene expression is critical during placental development, and enhancer elements can play an important role in regulating placental gene expression. While there are multiple studies in the placenta that include TF ChIP-seq, or focus on one or two enhancer regions, there are few studies investigating active enhancers on a genome-wide scale. Now that putative human TSCs have been isolated [47], identifying enhancers in those cells would be an important step towards understanding the mechanisms of human TSC differentiation, which could then be compared to mouse TSC differentiation. In addition, placental enhancers have mainly been studied in the rat and the mouse model, but studies in other species would provide further insight into the evolution of the placenta. In general, a holistic approach to identifying enhancers and transcriptional regulatory networks in normal and diseased placenta is essential. Such studies will deepen our understanding of placental gene regulation and may have implications in the early detection or treatment of certain pregnancy disorders.

Conflicts of interest

The authors declare no conflict of interest.

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