Placenta 35 (2014) 855–865

Contents lists available at ScienceDirect

Placenta

journal homepage: www.elsevier.com/locate/placenta

Evolutionary origins of the placental expression of chromosome 19 cluster galectins and their complex dysregulation in preeclampsia

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ARTICLE INFO

Article history:
Accepted 28 July 2014

Keywords:
ALU
AP2
Anthropoid primate
Epigenome
ESRRG
GATA
GCM1
Lectin
LINE
PP13
Pregnancy
Syncytiotrophoblast
TEF5

ABSTRACT

Introduction: The dysregulation of maternal-fetal immune tolerance is one of the proposed mechanisms leading to preeclampsia. Galectins are key regulator proteins of the immune response in vertebrates and maternal-fetal immune tolerance in eutherian mammals. Previously we found that three genes in a Chr19 cluster encoding for human placental galectin-13 (PP13), galectin-14 and galectin-16 emerged during primate evolution and may confer immune tolerance to the semi-allogeneic fetus.
Materials and Methods: This study involved various methodologies for gene and protein expression profiling, genomic DNA methylation analyses, functional assays on differentiating trophoblasts including gene silencing, luciferase reporter and methylation assays. These methods were applied on placental specimens, umbilical cord blood cells, primary trophoblasts and BeWo cells. Genomic DNA sequences were analyzed for transposable elements, transcription factor binding sites and evolutionary conservation.
Results and Discussion: The villous trophoblastic expression of Chr19 cluster galectin genes is developmentally regulated by DNA methylation and induced by key transcription factors of villous placental development during trophoblast fusion and differentiation. This latter mechanism arose via the co-option of binding sites for these transcription factors through promoter evolution and the insertion of an anthropoid-specific L1PREC2 transposable element into the 5' untranslated region of an ancestral gene followed by gene duplication events. Among placental Chr19 cluster galectin genes, the expression of *LGALS13* and *LGALS14* is down-regulated in preterm severe preeclampsia associated with SGA. We reveal that this phenomenon is partly originated from the dysregulated expression of key transcription factors controlling trophoblastic functions and galectin gene expression. In addition, the differential DNA methylation of these genes was also observed in preterm preeclampsia irrespective of SGA.
Conclusions: These findings reveal the evolutionary origins of the placental expression of Chr19 cluster galectins. The complex dysregulation of these genes in preeclampsia may alter immune tolerance mechanisms at the maternal-fetal interface.

DOI of original article: http://dx.doi.org/10.1016/j.placenta.2014.04.016.
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[1] Dedication: This manuscript is dedicated to the memory of Dr. Hans Bohn, whose scientific legacy, contribution to placentology and to this study, and inspiration will always be remembered.

http://dx.doi.org/10.1016/j.placenta.2014.07.015
0143-4004/

1. Introduction

Preeclampsia is a heterogeneous obstetrical syndrome with various etiologies and subforms, which affects 3–5% of pregnancies and is a chief cause of short- and long-term morbidity and mortality of the mother and her offspring [1–7]. Preterm or early-onset preeclampsia has often severe clinical presentation [2,6–8], and it is frequently associated with defective remodeling of the decidual and myometrial segments of maternal spiral arteries by invasive trophoblasts in early pregnancy [9–11], with oxidative stress of the placenta and the consequent placental release of toxic substances that promote the development of hypertension, proteinuria and an exaggerated systemic maternal inflammation [7,12–28], and with impaired fetal growth that often necessitates preterm delivery [6–8,28]. In this context, we found the decreased placental expression of galectin-13 (Placental Protein 13, PP13) in preterm preeclampsia [29,30] and also low first trimester maternal serum concentrations of trophoblast-secreted galectin-13, especially in cases associated with IUGR [31,32]. This phenomenon may be key in the early pathology in preterm preeclampsia, since galectin-13 and other placental galectins in a Chr19 cluster induce T cell apoptosis and may confer additional immune tolerance mechanisms in hemochorial placentation [33,34]. These observations are important from an evolutionary point of view since this galectin cluster emerged in anthropoid primates, a clade of primates consisting of New and Old World monkeys and apes, and galectin-13 evolved in Old World monkeys and apes [33,34], which have intense spiral artery remodeling by the invasive trophoblast [35–39] that is impaired in preeclampsia [10,40,41]. In fact, a recent study found that in normal pregnancies galectin-13 forms perivenous aggregates associated with leukocyte-containing zones of necrosis in early pregnancy decidua [42]. It was suggested that syncytiotrophoblast-secreted galectin-13 drains through the decidual veins, and then decidual galectin-13 aggregates attract and activate maternal immune cells, diverting them away from maternal spiral arteries and preventing them from attacking invasive semi-allogeneic trophoblasts [42].

Galectin-13 and placental Chr19 cluster galectins are predominantly expressed by the syncytiotrophoblast [29,30,32–34,43–45], a multinucleated syncytium that is in direct contact with maternal immune cells in maternal blood [29,30,33,34,42–44,46]. This cell layer is generated by the categorical reprogramming of the trophoblastic transcriptional program mainly governed by cAMP, and the consequent biochemical and morphological differentiation and fusion of the underlining villous cytotrophoblasts into this syncytium [44,46–53]. The unique transcriptomic activity of the syncytiotrophoblast [54] is responsible for the production of a large set of steroid and peptide hormones, immune proteins and other placental proteins chiefly expressed by the placenta and characteristically detectable in the maternal circulation during pregnancy [46,55]. These and non-secreted molecules enable key functions of the syncytiotrophoblast in the maintenance of pregnancy including feto-maternal gas, nutrient and waste exchange, hormonal regulation of fetal development, and the generation of an immunological barrier between the mother and the semi-allogeneic fetus [44,55]. Defects in syncytiotrophoblast formation have been implicated in the development of preeclampsia. For example, the decreased placental expression of the molecular machinery of trophoblast fusion, including GCM1 transcription factor, fusogenic retroviral proteins and their receptors have been observed in severe preeclampsia [56–62]. It was even presumed that the whole villous trophoblastic differentiation program is severely disturbed in preeclampsia [63], a hypothesis that could not be confirmed by whole-genome transcriptomic studies [49,51].

Since indirect evidence suggested that placental Chr19 cluster galectin expression is related to villous trophoblast differentiation [33,43,44], here we aimed to study how the trophoblastic expression of these galectins is related to villous trophoblast differentiation, and whether the trophoblastic expression of this galectin cluster is dysregulated in preeclampsia in relation to an altered villous trophoblast fusion or differentiation program.

2. Materials and methods

Human samples were retrieved from the Bank of Biological Specimens or were collected prospectively at the Perinatology Research Branch and Wayne State University (Detroit, MI, USA). Written informed consent was obtained from women before the collection of samples, and the research was approved by the Institutional Review Boards of the *Eunice Kennedy Shriver* National Institute of Child Health and Human Development and Wayne State University. Frozen placentas were used for RNA isolation and qRT-PCR, and for laser capture microdissection, genomic DNA isolation and bisulfite sequencing (Zymo Research Corporation, Irvine, CA, USA). Umbilical cord blood cells were used for genomic DNA isolation and bisulfite sequencing. Formalin-fixed paraffin embedded placentas were applied for tissue microarray and immunostainings with anti-PP13 (Behringwerke AG, Marburg/Lahn, Germany [64]) and anti-galectin-14 (AbD Serotec, Martinsried/Planegg, Germany) antibodies, and for *in situ* hybridization of three galectin genes. Primary trophoblasts isolated from normal term placentas and BeWo cells were used for functional experiments, including trophoblast differentiation, luciferase reporter and methylation assays, and immunostainings with anti-PP13 and anti-galectin-14 antibodies. Methylated DNA immunoprecipitation and DNA methylation arrays were performed at the University of Iowa (Iowa City, IA, USA). Genomic coordinates of genes, pseudogenes and transposable elements (TEs) in their 5' untranslated regions (5'UTRs) were retrieved from the UCSC Genome Browser (http://genome.ucsc.edu). Coding sequences were aligned and the gene tree was generated using the neighbor joining algorithm implemented in MEGA version 3.1 (www.megasoftware.net). The evolutionary conservation of transcription factor (TF) binding sites in the 5'UTRs were investigated by 'phylogenetic shadowing' using eSHADOW (http://eshadow.dcode.org). The Transcriptional Element Search System (TESS) (www.cbil.upenn.edu/tess) and the Transfac Database of the BIOBASE Biological Databases (www.biobase-international.com) were used to predict putative TF binding sites in the 5'UTRs. Demographics data were analyzed using SPSS version 12.0 (SPSS Inc., Chicago, IL), all other data were analyzed in the R statistical environment (www.r-project.org). All methods are described in detail in the Supplementary Information; additional data are shown in Supplementary Tables 1–4 and Supplementary Figs. 1–5.

3. Results and discussion

3.1. Villous trophoblast differentiation drives placental Chr19 cluster galectin expression

First, we characterized villous trophoblastic Chr19 cluster galectin gene expression in the placenta. *In situ* hybridization on normal term placentas showed *LGALS13*, *LGALS14* and *LGALS16* expression in the terminally differentiated syncytiotrophoblast but not in the cytotrophoblasts (Fig. 1A). In accord, the syncytiotrophoblast but not the cytotrophoblasts was immunopositive for PP13/galectin-13 and galectin-14 (Fig. 1B). Then, we examined how the expression of galectin genes are related to trophoblast syncytialization and differentiation *in vitro* by their parallel expression profiling with syncytin-1 (*ERVWE1*) and human chorionic gonadotropin (*CGB3*), markers of trophoblast syncytialization and differentiation [49,65–69] (Fig. 1C). We found increasing mRNA expression of galectin genes peaking on Days 2–3 in primary trophoblasts, following *ERVWE1* (Day 2) and preceding *CGB3* (Days 3–4) expression peaks, in parallel with the fusion of 80–90% of trophoblastic cells into syncytiotrophoblasts. The increasing mRNA expression of galectin genes peaked on Day 4 in differentiating BeWo cells, in parallel with that of *CGB3* and *ERVWE1*. When looking at the time-course of protein expression of galectins for which specific antibodies were available, cytoplasmic galectin-13 and galectin-14 immunopositivity appeared in differentiating primary trophoblasts on Day 3 and peaked on Day 7 (Fig. 1D).

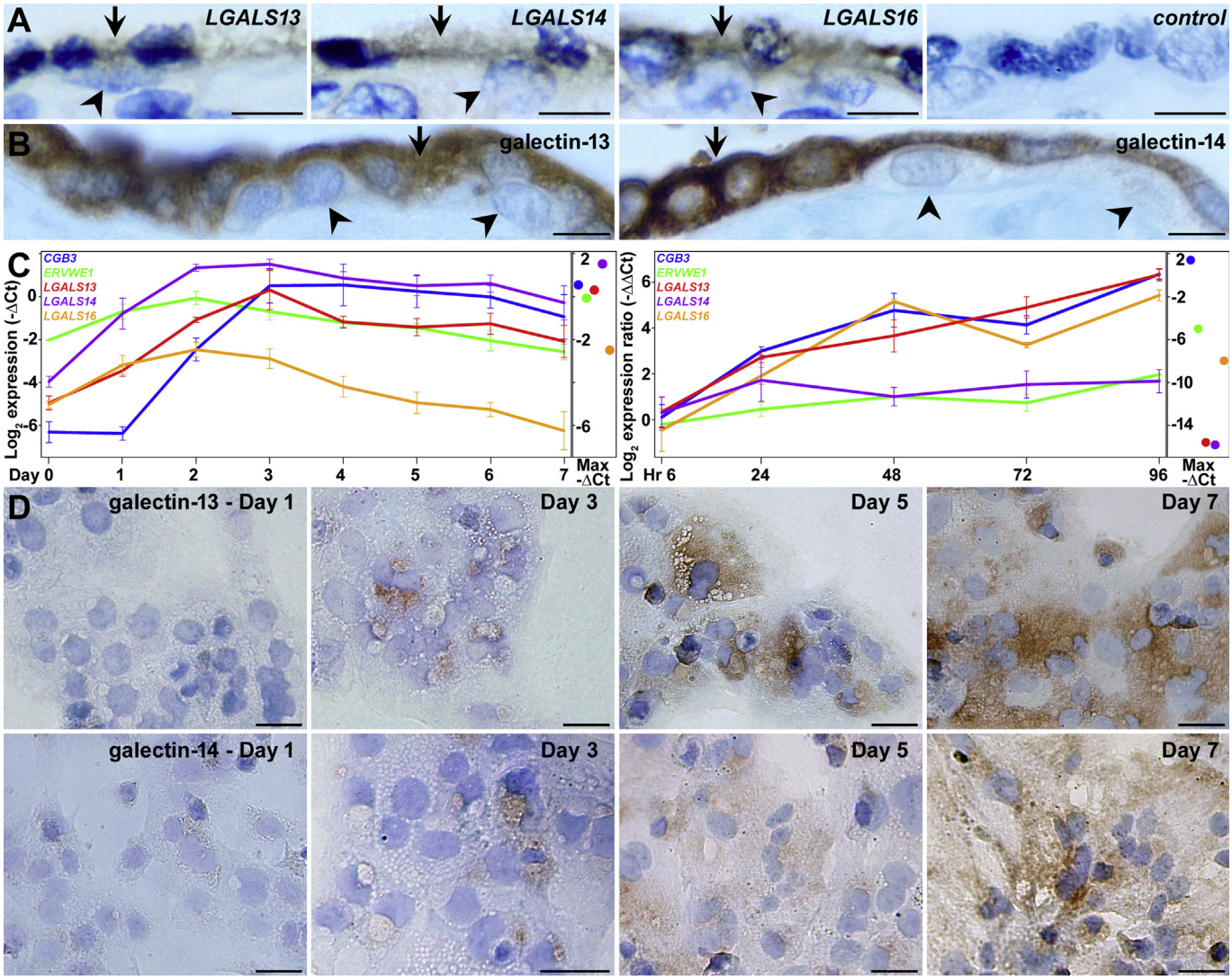

Fig. 1. Chr19 cluster galectin expression is related to villous trophoblast differentiation. (A) *In situ* hybridization of formalin-fixed paraffin embedded (FFPE) normal term placental tissue sections revealed human *LGALS13*, *LGALS14,* and *LGALS16* expression in the syncytiotrophoblast (arrows) but not in the cytotrophoblasts (arrowheads). **(B)** Immunostainings of FFPE normal term placental tissue sections showed human galectin-13 and galectin-14 expression in the syncytiotrophoblast (arrows) but not in the cytotrophoblasts (arrowheads). **(C)** qRT-PCR revealed increasing *LGALS13*, *LGALS14* and *LGALS16* expression during syncytialization and differentiation in primary trophoblast cultures (left, $n = 3$) and BeWo cells (right, $n = 3$). The expression of trophoblast syncytialization (syncytin-1, *ERVWE1*) and differentiation (human chorionic gonadotropin beta, *CGB3*) markers were also depicted. The *x* axes show the time of differentiation, the *y* axes either depict relative gene expression (left, mean ± SE) or relative gene expression ratios between Forskolin-treated and non-treated BeWo cells (right, mean ± SE). Inlets on the right of both time-scale images depict the highest relative gene expression levels (-ΔCt) during differentiation. **(D)** Galectin-13 and galectin-14 immunopositivity increased in the cytoplasm of differentiating primary trophoblasts, peaking on Day 7. Scale bars: 5 μm **(A, B)** and 10 μm **(D)**.

Of importance, the peak mRNA expression level of *CGB3* was higher in BeWo cells (2-fold) than in primary trophoblasts, while there was a lower peak mRNA expression level of *ERVWE1* (29-fold), *LGALS13* (60,320-fold), *LGALS14* (162,835-fold) and *LGALS16* (43-fold) in BeWo cells than in primary trophoblasts. These results are in accord with that the regulation of *CGB3* is partly independent from syncytialization in BeWo cells [68] and that the syncytialization is less extensive in BeWo cells than in primary trophoblasts [69], suggesting that Chr19 cluster galectin gene expression is strongly related to cAMP-driven trophoblast syncytialization and differentiation. Moreover, in good accordance with earlier [33] and current data on their placental expression levels, there was a higher peak expression level of *LGALS13* (7-fold) and *LGALS14* (15-fold) than of *LGALS16* in differentiating primary trophoblasts. However, there was a lower peak expression level of *LGALS13* (234-fold) and *LGALS14* (204-fold) than of *LGALS16* in differentiating BeWo cells (Fig. 1C). All of these findings suggest that placental Chr19 cluster galectin expression is driven by villous trophoblast syncytialization and differentiation, that Chr19 cluster galectin gene expression is limited in BeWo cells due to the less extensive syncytialization, and that the expression of *LGALS13* and *LGALS14* but not of *LGALS16* is further, strongly inhibited by unknown factors in BeWo cells.

3.2. Placental expression of Chr19 cluster galectins was gained by 5'UTR evolution

Previously we found that Chr19 cluster galectin genes emerged in anthropoid primates as a result of birth-and-death evolution mainly mediated by transposable long interspersed nuclear elements (LINEs) adjacent to these genes [33]. Since the recruitment of TEs into promoters can mediate lineage- and tissue-specific

diversification, and the placenta is a frequent site of TE co-option [70], we hypothesized that the co-option of TEs might have played a role in the emergence of placental expression of Chr19 cluster galectins. Thus, we investigated the 10 kb 5'UTRs of 35 genes and non-truncated pseudogenes with 4-exon structure in the galectin cluster [33] to reveal TEs that may confer placental expression. Indeed, we found the enrichment of the anthropoid-specific L1PREC2 in the placental sub-cluster of galectins compared to those outside this sub-cluster [33] (odds ratio: 266.0, $p < 0.001$) (Fig. 2). Two copies of L1PREC2 are located adjacent to the promoters of *LGALS20* and *LGALS13* genes and pseudogenes, as well as two *LGALS14* genes and a *LGALS19* pseudogene. Three copies of L1PREC2 are adjacent to the promoters of human *LGALS14* and orangutan *LGALS14P1*, while *LGALS16* genes contain three copies of L1PREC2 in a ~7 kb distance from the ATG start codon due to the insertion of a L1PA6 TE between the promoters and L1PREC2 TEs. In general, all placental sub-cluster galectins except marmoset *LGALS19* harbor L1PREC2 in their 10 kb 5'UTR, while this TE is

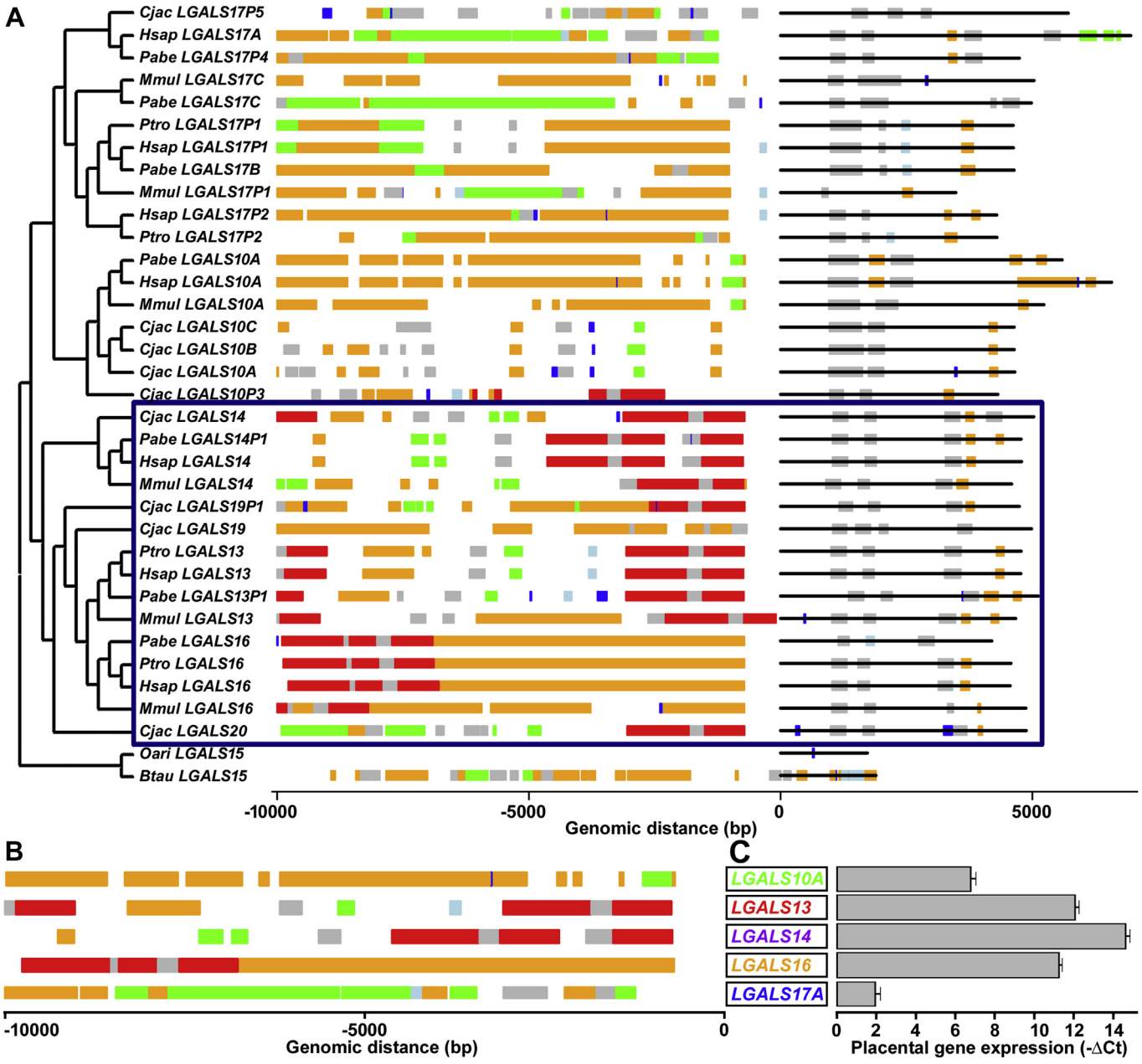

Fig. 2. Enrichment of L1PREC2 in the 10 kb 5'UTR of Chr19 placental galectin genes. (A–B) Coordinates for transposable elements (TEs) belonging to five repeat classes represented by colored lines (SINE: gray; LINE: orange; LTR: green; DNA: light blue; and Simple repeat: dark blue) were retrieved from UCSC Genome Browser. **(A)** From previously published genes and pseudogenes in the Chr19 galectin cluster [33], only genes and non-truncated pseudogenes with 4-exon structure (black lines) were included in the analysis. The L1PREC2 (red) is highly enriched (OR: 266.0, $p < 0.001$) in the 10 kb 5' untranslated region (5'UTR) of genes/pseudogenes in the placental sub-cluster (inside the blue box) compared to those outside this sub-cluster. Numbers denote genomic distance relative to the ATG start codons. The phylogenetic tree was built with MEGAv3.1 using coding sequences. *Hsap*, human; *Ptro*, chimpanzee; *Pabe*, Sumatran orangutan; *Mmul*, Rhesus macaque; *Cjac*, marmoset; *Oari*, sheep; *Btau*, cow. **(B)** TEs in the 5'UTRs of human Chr19 cluster galectin genes. Numbers denote genomic distance relative to the ATG start codons. **(C)** −ΔCt values denote mean relative *LGALS* expressions in 19 control placentas included in our study.

present in the 5'UTR of only one pseudogene (marmoset *LGALS10P3*) outside this sub-cluster (Fig. 2A).

The potential effect of L1PREC2 TEs on placental gene expression was substantiated by the comparison of mean placental gene expression data in our control placentas as well as the number and location of L1PREC2 TEs in the 10 kb 5'UTRs of human Chr19 cluster galectin genes in the UCSC Genome Browser data. High expression was observed for *LGALS13*, *LGALS14* and *LGALS16* that contain L1PREC2. The highest expression among these was observed for *LGALS14* which contains three L1PREC2 adjacent to the promoter followed by *LGALS13* that contains two copies of this TE in the same close location, and then *LGALS16* with three copies in a large distance (Fig. 2B–C).

3.3. Trophoblastic Chr19 cluster galectin expression is regulated by transcription factors key in trophoblast-specific functions

To reveal the functional relevance of L1PREC2, we used ~1.5 kb 5'UTR reporter constructs of human *LGALS13*, *LGALS14* and *LGALS16*, which harbor similar promoters however one copy of L1PREC2 in *LGALS13* and *LGALS14* and part of L1PA6 in *LGALS16* (Fig. 3A). BeWo cells transfected with these reporter constructs were either treated with 25 μM Forskolin to induce differentiation or kept as non-differentiating controls. Luciferase assays showed that the *LGALS13* and *LGALS14* reporters were capable of driving ~2.87-fold ($p = 1 \times 10^{-11}$) and 2.95-fold ($p = 6.1 \times 10^{-12}$) increase in luciferase activity in differentiating compared to control cells,

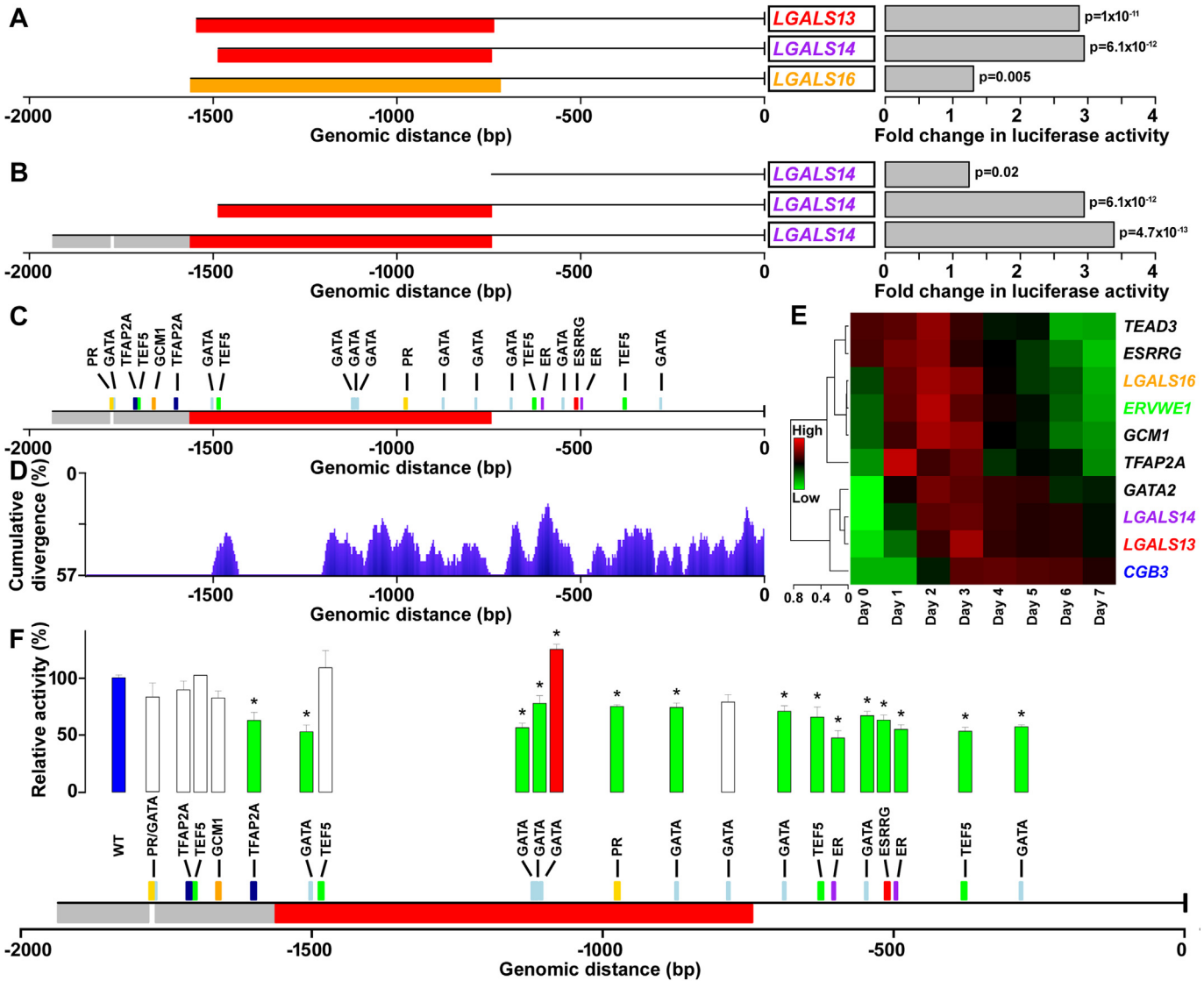

Fig. 3. Trophoblastic expression of Chr19 cluster galectins is controlled by key transcription factors in trophoblast differentiation. (A–B) Luciferase reporter assays of ~1.5 kb 5′ untranslated region (5'UTR) constructs of human *LGALS13*, *LGALS14* and *LGALS16* **(A)**, as well as 0.7 kb, 1.5 kb and 2 kb 5'UTR constructs of human *LGALS14* **(B)**. Transposable elements (TEs) in the 5'UTRs (colored as in Fig. 2) are shown on the left. Fold-changes between the mean relative luciferase activities of the various galectin 5'UTR reporter constructs compared to that of the *GAPDH* 5'UTR reporter construct in 25 μM Forskolin-induced and control BeWo cells are plotted on the right. Associated *p* values were derived from standard errors by t statistics. All luciferase reporter assays were run in triplicate. **(C)** Predicted binding sites are shown in the 2 kb 5'UTR of human *LGALS14* for transcription factors (TFs) involved in trophoblastic gene expression. **(D)** Phylogenetic shadowing revealed that most TF binding sites are positioned in conserved regions of the 5'UTRs of *LGALS13, LGALS14, LGALS19,* and *LGALS20* genes. Cumulative divergence plot of genomic sequences was generated by eSHADOW with a sliding window size of 50bp. **(E)** Heatmap shows gene expression changes in differentiating primary trophoblasts between Day 0–7. Co-expression of genes and TFs was visualized by clustering based on Pearson-correlation. **(F)** Luciferase reporter assays of 21 mutated and the wild-type 2 kb reporter constructs of human *LGALS14*. The relative luciferase activities of the mutated constructs (fold-changes between 25 μM Forskolin induced and control BeWo cells) were compared to that of the wild-type construct (100%), and were depicted as percentages with the bars. Stars denote significant differences compared to control, green color depicts down-regulation, while red color depicts up-regulation. Luciferase reporter assays were run in triplicate.

respectively, while the increase in *LGALS16* reporter activity was only mild (1.3-fold, $p = 0.005$) (Fig. 3A). We also tested different length reporter constructs of the *LGALS14* 5'UTR. The ~0.7 kb promoter drove 1.24-fold ($p = 0.02$) increase in luciferase activity in differentiating compared to control cells, similar to the 1.5 kb *LGALS16* reporter, suggesting that only the promoter but not the L1PA6 in *LGALS16* 5'UTR contributed to the change in its luciferase activity. The ~2 kb *LGALS14* reporter, which contains two ALUs in addition to the L1PREC2 and the promoter, had a slightly larger induction of luciferase activity (3.39-fold, $p = 4.7 \times 10^{-13}$) than the 1.5 kb reporter that does not contain the ALUs (2.95-fold, $p = 6.1 \times 10^{-12}$) (Fig. 3B), proving that it is mainly the L1PREC2 which drives trophoblast differentiation-related *LGALS14* expression.

Another proof of the importance for L1PREC2 in placental gene expression is that in human *LGALS13* and *LGALS14* 5'UTRs (Fig. 3C) this TE harbors several putative binding sites for GATA and TEF5, TFs that drive trophoblast-specific gene (e.g. *CGA, CGB, CSH1, ERVWE1, HSD3B1)* expression [71–76], while the L1PA6 TE in the *LGALS16* 5'UTR is not enriched by these binding sites. In addition, the human *LGALS13*, *LGALS14* and *LGALS16* promoters contain putative binding sites for GATA, TEF5 and ESRRG, which latter is important in the regulation of trophoblast metabolism [77,78]. Of note, besides GATA and TEF5, the ALU TE adjacent to L1PREC2 harbors putative binding sites for TFAP2A and GCM1, TFs involved in trophoblast differentiation and fusion [48,50,52,59,79–82]. Evolutionary analysis showed that putative binding sites for these TFs are positioned in conserved regions in the 5'UTRs of placental Chr19 cluster genes, suggesting their functional importance (Fig. 3D).

Next, we indirectly tested whether placental Chr19 cluster galectins' trophoblastic expression is regulated by these TFs with putative binding sites in the 5'UTRs. Transcription profiling of primary trophoblasts during differentiation showed that the expression of *LGALS16* closely clustered with that of *TEAD3, ESRRG*, *GCM1*, and *ERVWE1,* peaking on Day 2. These data suggest that *LGALS16* expression is driven by TEF5 and ESRRG via binding to its promoter, and that GCM1 may have an indirect promoting effect by facilitating the expression of these TFs. The expression of *LGALS13* and *LGALS14* tightly clustered with *GATA2*, and their peak expression was on Day 3, following *GATA2* (Fig. 3E). These data suggest that in addition to the early effects of ESRRG and TEF5 on their promoters, GATA2 may drive an extended and higher *LGALS13* and *LGALS14* expression as compared to *LGALS16* by binding to several binding sites on their L1PREC2 TEs (Fig. 3E). Further supporting the role of trophoblastic GATA2 in galectin gene expression, *LGALS10,* a gene regulated by GATA TFs in myeloid cells [83], had also a marked up-regulation during trophoblast differentiation (Supplementary Fig. 1).

To reveal direct evidence on the role of these TFs in driving Chr19 cluster galectin genes' trophoblastic expression, we ran luciferase reporter assays with 21 reporter constructs containing unique mutations for the predicted TF binding sites in the ~2 kb *LGALS14* 5'UTR. The luciferase activity of these mutated reporter constructs were compared to that of the wild-type construct in Forskolin-treated and control cells (Fig. 3F). In the promoter, the mutation of all eight binding sites inhibited differentiation-induced luciferase activity. Out of these, the mutation of two estrogen receptor elements (−54% and −45%) that can also bind ESRRG [84], as well as a TEF5 (−47%), GATA (−43%) and ESRRG (−37%) binding site had the most pronounced effects. In L1PREC2, out of the eight putative binding sites the mutation of four GATA (−23 to −48%) and a progesterone receptor (PR) (−25%) element inhibited luciferase activity. In ALU, all except a TFAP2A binding site (−38%) turned to be non-functional. As an evolutionary proof of their regulatory role, the functionally active binding sites were located in the most conserved regions in the 5'UTRs of placental sub-cluster galectin genes (Figs. 2A and 3D). These results provide further evidence for GATA2, TEF5 and ESSRG in driving trophoblastic Chr19 cluster *LGALS* expression by binding to their promoter, and for GATA2 to drive a heightened expression of *LGALS13* and *LGALS14* by binding to the L1PREC2 TEs in their 5'UTRs. Since the mutation of the putative GCM1 binding site was not inhibitory, GCM1 may promote *LGALS* expression via facilitating other TF's expression.

3.4. The down-regulation of LGALS13 and LGALS14 in preterm preeclampsia is related to dysregulated trophoblastic transcription factor expression

Since previously we found down-regulated placental *LGALS13* expression in preterm preeclampsia [29], we were interested whether all placental Chr19 cluster galectins are differentially expressed in this syndrome, and if this supposed phenomenon may be related to an altered villous trophoblast fusion [56–59,62] or differentiation [63] program as shown or hypothesized before. Since our preterm preeclampsia placental microarray dataset [85] did not contain expression data for *LGALS16*, we performed a qRT-PCR study for the investigated ten genes on placentas from women with preterm severe preeclampsia with SGA and preterm controls.

Of note, *LGALS13* and *LGALS14* had the largest down-regulation in preeclampsia (−2.1-fold, $p = 0.03$ for both), while *GCM1* and *ESRRG* had less significant inhibition (−1.5-fold, $p < 0.05$, for both). There was a non-significant down-regulation of *LGALS16, ERWVE1, TEAD3*, *TFAP2A* and *GATA2* (−1.5-fold, −1.3-fold, −1.4-fold, −1-fold, and −1.4-fold, respectively) and a non-significant up-regulation of *CGB3* (+1.2-fold) in preeclampsia. A strong co-expression of *LGALS13, LGALS14* and *LGALS16* with *ESRRG* was observed, while *CGB3* clustered out from this set of genes (Fig. 4A). The down-regulation of *LGALS14* was confirmed by immunostainings of TMAs constructed from the same placentas and semi-quantitative immunoscorings (1.8-fold, $p = 4.6 \times 10^{-7}$) (Fig. 4B). In accord with earlier placental and *in vitro* trophoblastic data [50,56–59,62,68,79,82,86], our results support that cAMP-induced, GCM1-driven trophoblast fusion is negatively impacted in preterm severe preeclampsia associated with SGA, while trophoblast differentiation – characterized here by *CGB3* expression – is partially independent of trophoblast fusion and is not significantly impaired in preeclampsia. This finding confirms the results of a large set of placental microarray studies in preeclampsia [85,87] failing to detect the global alteration of the characteristic transcriptomic signature of villous trophoblast differentiation [49,51]. In addition, our results may also imply that the GCM1-driven down-regulation of *ESRRG* impairs placental Chr19 cluster galectin expression, and alterations in GATA2 expression may intensify the dysregulation of *LGALS13* and *LGALS14* compared to *LGALS16*.

To investigate the effect of GCM1 inhibition on placental Chr19 cluster galectin expression *in vitro*, we knocked-down *GCM1* in differentiating BeWo cells and studied the changes in expression of our ten genes of interest. There was a time- and concentration-dependent down-regulation of all TFs, with *ESSRG*, *TEAD3* and *TFAP2A* expression strongly impacted on Day 3 and the least effect on *GATA2* (Fig. 4C). Similarly, the impact of *GCM1* knockdown on target gene expression was the strongest on Day 3, with substantial decrease in *LGALS16*, *ERVWE1* and *CGB3* expression. Remarkably, *LGALS13* and *LGALS14* were not down-regulated. These data confirmed that the *GCM1* down-regulation in placentas from women with preeclampsia may lead to the inhibition of *ERVWE1* expression and trophoblast fusion, and the decrease in *ESRRG* and *TEAD3* expression may lead to the consequent down-regulation of

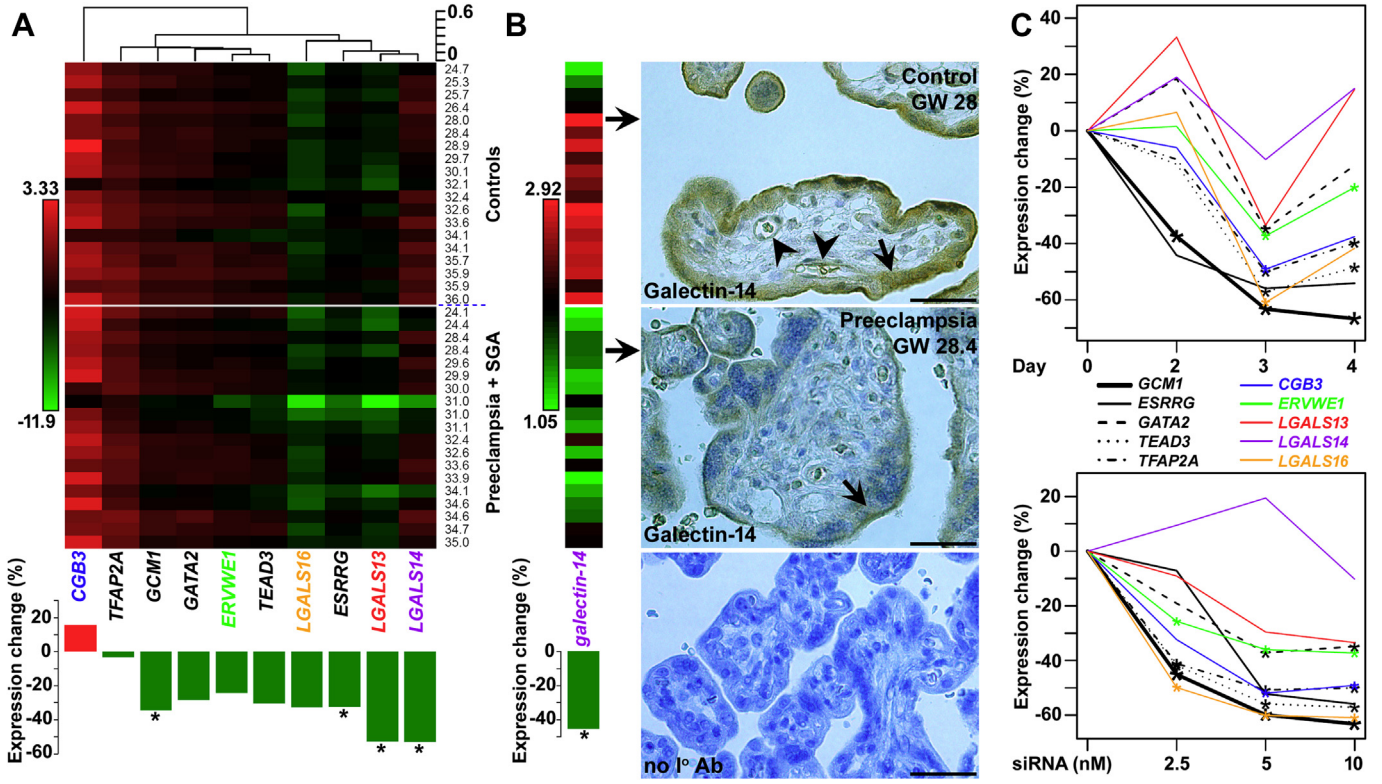

Fig. 4. Placental down-regulation of *LGALS13* and *LGALS14* expression in preeclampsia. (A) *Upper panel*: The heatmap represents the gene expressions sorted by gestational age within each group. The color code for −ΔCt values is depicted on the bar. Hierarchical clustering was applied using Pearson correlation and average linkage. *Lower panel*: Differential gene expression in preeclampsia is shown with bar charts. *, $P < 0.05$. **(B)** Galectin-14 immunoscores for the same placentas were coded according to the colors depicted on the bar. The bar chart shows the differential expression of galectin-14 in preeclampsia. The two arrows link the immunoscore and representative TMA images from formalin-fixed paraffin embedded placentas of a control woman (GW28) and a preeclampsia patient (GW28.4). Galectin-14 expression is down-regulated in the syncytiotrophoblast (arrows) in preeclampsia. Fetal endothelia (arrowheads) were also immunopositive. Scale bars: 50 μm *, $P < 0.05$. **(C)** *GCM1* knock-down in BeWo cells leads to a time- and concentration-dependent down-regulation of transcription factors and target genes. *Upper panel* shows gene expression changes in response to 10 nM GCM siRNA at different time-points. *Lower panel* depicts gene expression changes under different *GCM1* siRNA concentrations at day 3. The *y* axis shows changes in relative gene expression in *GCM1* siRNA-treated compared to 10 nM scrambled siRNA-treated BeWo cells. Associated *p* values were derived from standard errors by *t* statistics. *, $P < 0.05$. Experiments were run in triplicate.

LGALS16. The lack of effect on *LGALS13* and *LGALS14* in BeWo cells might be the result of the modest decrease in *GATA2* expression upon GCM1 knock-down, which could still support these two genes' expression. However, a more reasonable explanation for this finding is that *LGALS13* and *LGALS14* had very low absolute expression levels in BeWo cells, and their expression could not be further decreased upon *GCM1* knock-down. We hypothesized that this might be originated from the aberrant DNA methylation of BeWo cells [88] also affecting *LGALS13* and *LGALS14*, and that similar epigenetic mechanisms might also play a role in their marked down-regulation compared to *LGALS16* in placentas from women with preeclampsia.

3.5. Epigenetic mechanisms regulate trophoblastic Chr19 cluster galectin expression and their implication in preeclampsia

To investigate this hypothesis, we treated BeWo cells with 5-azacytidin, a DNA methyltransferase inhibitor, and observed the up-regulation of *LGALS13* and *LGALS14* but not *LGALS16* in a dose-dependent manner. This effect was more significant when BeWo cells were induced to differentiate by Forskolin (Fig. 5A), underlining that DNA methylation inhibits *LGALS13* and *LGALS14* expression in BeWo cells. Out of the ten genes investigated, only *ERVWE1* had a similar increase in expression upon 5-azacytidin treatment, corresponding with previous data on its epigenetic regulation in the placenta and the hypermethylation of its promoter in preeclampsia [62] (Supplementary Fig. 2). To get insight where the differential methylation occurred in placental Chr19 cluster galectin genes, we isolated genomic DNA from 5-azacytidin treated and control cells, and performed methylated DNA immunoprecipitation and methylation arrays. The mean methylation in *LGALS13* and *LGALS14* increased from the 5'UTRs towards the intragenic regions, and the intragenic methylation peaks in BeWo cells were concomitant with those in 13 non-trophoblastic cells and tissues revealed by the Human Reference Epigenome Mapping Project (Supplementary Fig. 3). Since the largest demethylation occurred in the intragenic regions upon 5-azacytidin treatment, we hypothesized that alterations in developmental methylation changes in these regions may play a role in the differential expression of *LGALS13* and *LGALS14* in the trophoblast in preeclampsia.

Next, we investigated the intragenic regions of placental Chr19 cluster galectin genes for differential methylation between primary trophoblasts and cord blood cells to reveal the impact of trophoblastic development on DNA methylation and potential sites for differential methylation in preeclampsia. We designed amplicons for the most methylated intragenic regions of the placental Chr19 cluster galectin genes, and developed sensitive and robust bisulfite sequencing assays, which yielded a median sequencing read of 749 (range: 79–2246) per CpG. Remarkably, the regions surrounding the transcription start sites and the 3′ ends of *LGALS13*, *LGALS14* and *LGALS16* were strongly or moderately hypomethylated in the trophoblast compared to cord blood cells, and the absolute differences in DNA methylation ratios between these cells were mostly

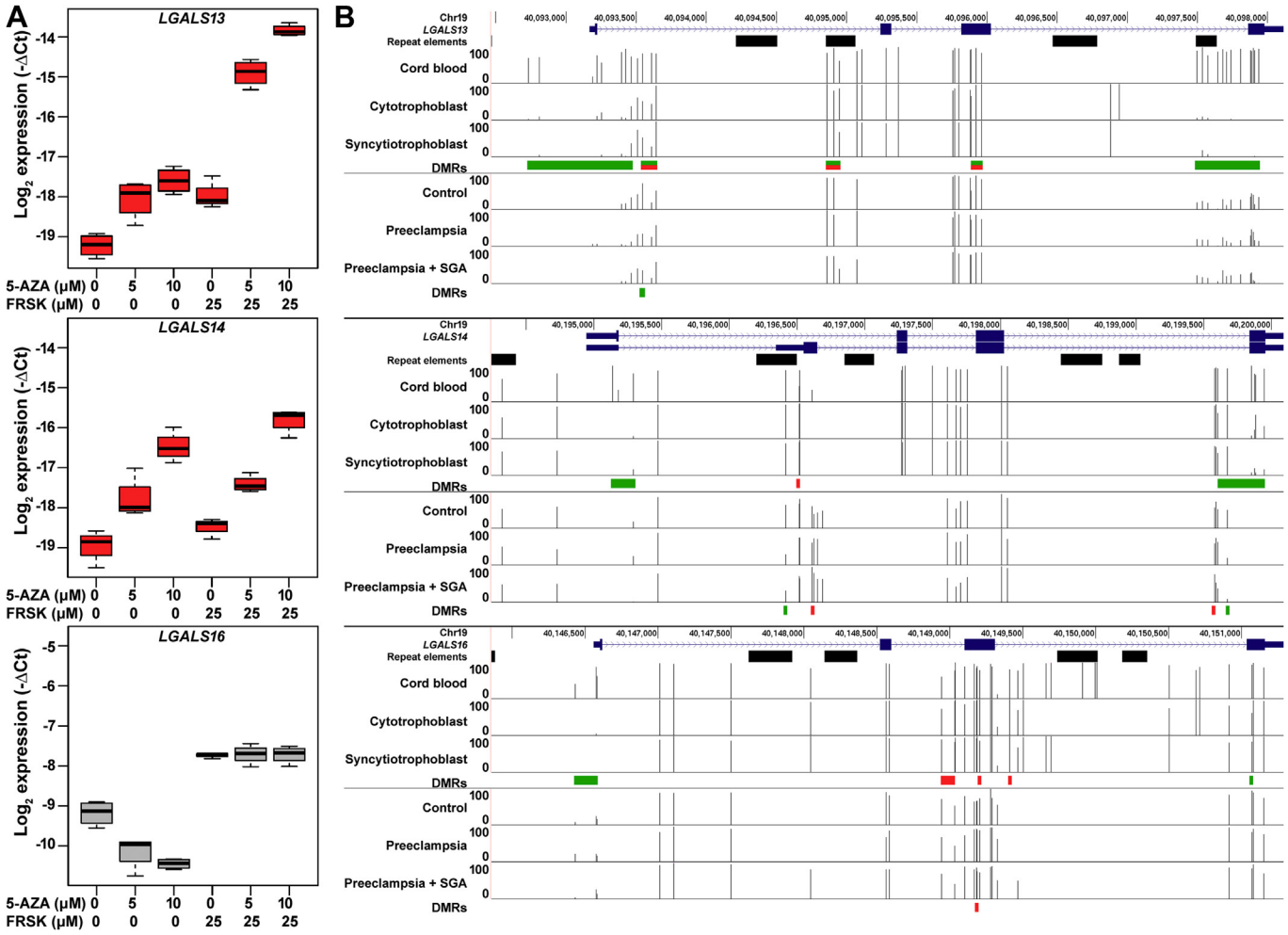

Fig. 5. The effect of DNA methylation on placental Chr19 cluster galectin gene expression. (A) BeWo cells were treated with 0, 5 or 10 μM 5-azacytidin (5-AZA), with or without co-treatment with 25 μM Forskolin (FRSK). Treatment with 5-azacytidin led to a concentration-dependent up-regulation of *LGALS13* and *LGALS14* but not *LGALS16* after 24 h, whose effect was more prominent after 25 μM Forskolin treatment. The *y*-axis depicts −ΔCt values. Experiments were run in quadruplicate. **(B)** Mean DNA methylation levels (0–100%) of individual CpGs in Chr19 cluster galectin genes are depicted in umbilical cord blood cells, cytotrophoblasts and syncytiotrophoblasts differentiated for 7 day. Cord blood cells ($n = 3$) and cytotrophoblasts ($n = 3$) were obtained from the same fetuses. Trophoblastic mean DNA methylation data corresponds to RNA expression data on Day 1 and Day 7 of differentiation as depicted in Fig. 1. Mean DNA methylation levels of individual CpGs in the clinical groups are also shown. Among differentially methylated regions (DMRs), green bars show hypomethylation, red bars depict hypermethylation, while red and green bars depict mixed DMR pattern in the trophoblast compared to cord blood cells or in the trophoblast of patients with preeclampsia compared to controls. Genomic coordinates are shown above the graphs. Barplots in Supplementary Fig. 4 and 5 provide detailed statistics for sample numbers and methylation levels at individual CpGs.

≥0.5 (Fig. 5B, Supplementary Fig. 4). Of note, there was mainly mild difference in DNA methylation ratios in these genes between cytotrophoblasts and the syncytiotrophoblast (Fig. 5B, Supplementary Fig. 4). The results suggest that the hypomethylation of differentially methylated regions (DMRs) around the transcription start sites in these genes may allow active transcription. This permissive status is already present in the cytotrophoblast, and the increase in the expression of key TFs of trophoblast differentiation drives the expression of placental Chr19 cluster galectin genes.

Finally, we tested whether the developmental differences in galectin gene DNA methylation may play a role in their dysregulated expression in preterm preeclampsia. We laser-captured villous trophoblasts from the same placentas that were examined by qRT-PCR, isolated genomic DNA and subjected to bisulfite sequencing. To look for differential methylation unique for preterm preeclampsia associated with SGA compared to preterm preeclampsia without SGA, we also subjected trophoblastic genomic DNA isolated from women in the latter group. In spite of the quantity of the obtained DNA was low (median: 394 ng, range: 137–1048 ng), bisulfite sequencing assays yielded a median sequencing read of 57 (range: 3-1112) per CpG. We found a hypomethylated CpG in *LGALS13* (chr19:40093547) in preeclampsia, with a larger absolute methylation ratio difference in cases without SGA (−0.35). Similarly, the hypermethylation of two CpGs in *LGALS16* (chr19:40149169, chr19:40149184) in preeclampsia were larger in cases without SGA (methylation ratio differences: 0.25 for both). On the other hand, the differential methylation of three CpGs in *LGALS14* (chr19:40196416, chr19:40196625, chr19:40199675) in preeclampsia was larger in cases with than without SGA (methylation ratio differences: −0.28, 0.42 and −0.37, respectively). Of note, two CpGs in *LGALS14* (chr19:40196610, chr19:40199582) were hypermethylated only in preeclampsia associated with SGA (methylation ratio differences: 0.37 and 0.22, respectively) (Fig. 5B, Supplementary Fig. 5). Our findings suggest that the differential DNA methylation in these genes is mainly associated with preeclampsia itself rather than SGA associated with preeclampsia, and these alterations may interfere trophoblastic transcription of placental Chr19 cluster galectin genes in

other ways than interfering with the permissive function of the hypomethylated DMRs.

4. Summary

The trophoblastic expression of placental Chr19 cluster galectins is regulated by TFs that drive villous trophoblast differentiation and trophoblast-specific gene expression [50,59,71–75,77–82]. Luciferase reporter assays showed that ESRRG, GATA2, and TEF5 regulate trophoblastic expression of these genes by binding to the promoters, while the L1PREC2 TE further enhances trophoblastic expression via binding GATA2 at several binding sites. Functional assays uncovered that GCM1 also plays a role in the regulation of placental Chr19 cluster galectin genes by facilitating the expression of their regulatory TFs during trophoblast fusion and differentiation. Based on the evolutionary conservation of TF binding sites in the promoters and L1PREC2 TEs in the 5'UTRs of placental Chr19 cluster galectins genes, and the adjacent positions of the L1PREC2 and ALU TEs to the promoters and gene duplication boundaries [33] in most of these genes, the following evolutionary scenario can be best envisioned for the emergence of placental expression of these galectins: 1) an ancestral gene in the cluster underwent promoter evolution and acquired binding sites for TFs, which supported placental expression; 2) the primate-specific L1PREC2 TE was inserted adjacent to the promoter of this ancestral gene, which enhanced placental expression; 3) during the birth-and-death evolution of cluster galectins [33], this ancestral gene was duplicated several times, and the gene duplication units contained the promoter as well as L1PREC2 and ALU TEs adjacent to the boundaries; 4) these TEs were then duplicated or deleted in certain genes and lineages; and 5) the insertion of L1PA6 between the promoter and L1PREC2 in *LGALS16* 5'UTR considerably decreased the regulation of *LGALS16* by GATA2 and the extent of trophoblastic *LGALS16* expression compared to *LGALS13* and *LGALS14*.

This latter data also suggest a key role for GATA2 in enhancing trophoblastic gene expression of placental Chr19 cluster galectin genes. In this context it is important that *LGALS10*, a Chr19 cluster galectin gene chiefly expressed by eosinophils and Treg cells [89,90] is considerably induced during trophoblast differentiation, although its absolute expression levels in the placenta and differentiating trophoblast are much lower than that of *LGALS13*, *LGALS14* or *LGALS16*. The *LGALS10* promoter does not contain TF binding sites conserved in placental Chr19 cluster galectin genes; however, it contains a GATA binding site functional in eosinophils [83], suggesting that this is also key in its trophoblastic expression. In spite of the presumably shared regulation of *LGALS10* and placental Chr19 cluster galectin genes by GATA factors in the trophoblast, this is not the case in myeloid cells where placental galectins are not expressed [33,34]. Two assumptions can be made according to these observations: 1) ESRRG, GATA2 and TEF5 may be necessary to act in concert to define placenta-specific expression by binding to Chr19 cluster galectin genes' promoters; and 2) epigenetic factors may need to be operational to restrict these genes' expression to the placenta.

Regarding point 1, the finding that site-directed mutagenesis of single TF binding sites led only to a 23%–54% decrease in luciferase activity as well as the close vicinity of these TF binding sites suggest that these may form *cis*-regulatory modules where complexes of TFs may bind to. Indeed, this would be a similar scenario to the regulation of other placenta-specific genes like *CGA*, *CSH1*, *ERVWE1*, or *HSD3B1* [48,50,52,73,74,80]. Regarding point 2, a key observation of this study showed that DMRs in placental Chr19 cluster galectin genes may confer their placenta-specific expression beyond the transcriptional machinery. In cord blood cells which do not express placental Chr19 cluster galectins [33,34], these DMRs are strongly methylated especially around the transcription start site, while they are hypomethylated in the cyto- and syncytiotrophoblast. This is in accord with open chromatin and active gene expression [91], suggesting that the hypomethylation of these DMRs presets the active transcription of *LGALS13, LGALS14* and *LGALS16* already in the undifferentiated cytotrophoblast, and their hypermethylation restricts gene expression in other tissues, tightly controlling developmental processes.

Interestingly, we did not find the hypermethylation of these DMRs and developmentally restricted *LGALS13* and *LGALS14* expression in preeclampsia, although this may be the case in BeWo cells. However, we found DNA methylation marks that were mainly associated with preeclampsia irrespective of the presence of SGA, although their exact relation to the pathogenesis of preeclampsia could not be established from the acquired data. In addition, we were able to detect the down-regulation of *GCM1* and *ESRRG* expression in preterm preeclampsia associated with SGA, while there was no difference in *CGB3* expression. These results suggest that alterations in GCM1-mediated trophoblast fusion may be impaired while the trophoblastic differentiation program is not altered in this severe, preterm form of preeclampsia. Of note, *GATA2* was also – non-significantly – down-regulated, which may explain why *LGALS13* and *LGALS14* had a more pronounced decrease in expression compared to *LGALS16* in these cases.

5. Conclusions

The villous trophoblastic expression of placental Chr19 cluster galectin genes is the result of promoter evolution and repeat element insertion mediated TF binding site co-option into the 5'UTR of an ancestral gene in the cluster followed by gene duplication events. The trophoblastic expression of these genes is also regulated by the methylation of DMRs in their transcription start site regions. The placental expression of *LGALS13* and *LGALS14* is impaired in preterm preeclampsia associated with SGA due to the dysregulated expression of key TFs controlling trophoblast functions, and the differential methylation of these genes is also associated with preterm preeclampsia irrespective of SGA. The complex dysregulation of placental Chr19 cluster galectin genes in preeclampsia suggests a multifaceted placental etiology of this syndrome. Future studies are warranted to examine how the dysregulation of these genes may affect deep placentation in preeclampsia.

Author contributions

N.G.T., Y.X., S.L., M.K. and H.B. designed research; N.G.T., Y.X., R.L., H.E-A., C.L., B.W., Z.D, and M.K. performed research; N.G.T., R.R., S.L., S.S.H., T.C., M.K., C.J.K. and H.B. contributed new reagents/analytic tools or clinical specimens; N.G.T., R.R., Y.X., O.E., Z.X., G.B., T.H.C., A.B., G. Sz., M.K., A.L.T., and Z.P. analyzed and interpreted data; and N.G.T., R.R., Y.X., O.E., Z.X., G.B., R.L., T.H.C., H.E-A., C.L., B.W., A.B., G. Sz., S.L., Z.D., S.S.H., T.C., M.K., C.J.K., A.L.T., and Z.P. wrote/approved the paper.

Conflicts of interest

The authors have no conflicts of interest.

Acknowledgments

We thank for Sandy Field, Hong Meng, Tara Reinholz, Sivasakthy Sivalogan, Vanessa Topping, Anita Vasudevan, Brad Baker, Po Jen Chiang, Gerardo Rodriguez and Dr. Theodore Price for technical assistance, Dr. Douglas Ruden for helpful discussions, and Sara Tipton for critical reading of the manuscript (all Wayne State

University). We thank Professor Andras Falus for inspiring the study design on transcriptional regulation by non-coding genomic regions, Dr. Katalin Eder for assistance in microarray experiments (both Semmelweis University), and Dr. Valerie Reeb (University of Iowa) and Nazeena Alvi (Roche NimbleGen, Inc.) for assistance with MEDIP chip experiments. This research was supported, in part, by the Perinatology Research Branch, Division of Intramural Research, *Eunice Kennedy Shriver* National Institute of Child Health and Human Development (NICHD), National Institutes of Health (NIH), Department of Health and Human Services (DHHS); and, in part, with Federal funds from the NICHD under Contract No. HHSN275201300006C. Microarray and evolutionary analyses were supported by the European Union FP6 Grant "Pregenesys 037244" (to N.G.T.). Manuscript writing was partly supported by the Hungarian OTKA-PD grant "104398" (to A.B.) and the Hungarian Academy of Sciences Momentum Grant "LP2014-7/2014" (to N.G.T.).

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.placenta.2014.07.015.

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