Tracing the cis-regulatory changes underlying the endometrial control of placental invasion

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Among eutherian (placental) mammals, placental embedding into the maternal endometrium exhibits great differences, from being deeply invasive (e.g., humans) to noninvasive (e.g., cattle). The degree of invasion of placental trophoblasts is positively correlated with the rate of cancer malignancy. Previously, we have shown that fibroblasts from different species offer different levels of resistance to the invading trophoblasts as well as to cancer cell invasion. Here we present a comparative genomic investigation revealing cis-regulatory elements underlying these interspecies differences in invasibility. We identify transcription factors that regulate proinvasibility and antiinvasibility genes in stromal cells. Using an in vitro invasibility assay combined with CRISPR-Cas9 gene knockout, we found that the transcription factors GATA2 and TFDP1 strongly influence the invasibility of endometrial and skin fibroblasts. This work identifies genomic mechanisms explaining species differences in stromal invasibility, paving the way to therapies targeting stromal characteristics to regulate placental invasion, wound healing, and cancer dissemination.

cancer malignancy | evolution of cancer | placenta | endometrium | stomal invasibility

Collective epithelial invasion into the stromal compartment is a recurrent phenomenon in physiology (e.g., placentation) and pathology (tumor dissemination). The stroma has long been considered as a passive barrier which must be breached by cancer cells as the first step toward the transformation of a benign tumor into a metastatic disease (1). Invasion of fetal trophoblasts into the maternal endometrium has been similarly studied as a phenomenon with trophoblast being the active participant. We have recently shown that the endometrial stromal fibroblasts (ESFs) are key regulators of trophoblast invasion, explaining species differences in placentation among mammals (2). The extent of placental invasion and the rate of cancer malignancy are correlated among mammals, suggesting that evolutionary changes in endometrial regulation of placental invasion may be mechanistically linked to stromal control of cancer dissemination (3, 4). In this paper we aim to identify some of the genetic factors that underlie species differences in the invasibility of endometrial and skin stroma.

Placentation presents a remarkable phenotypic diversity between different eutherian (placental) mammals (5). This variation is often attributed to the fact that placentation involves the interaction of genetically distinct maternal and fetal tissues (6–8). The maternal–fetal interface is classified by the depth of invasion into the endometrium as epitheliochorial (e.g., cattle and dugong), endotheliochorial (e.g., dogs, cats, and other carnivores), and hemochorial (e.g., humans and other primates, and rodents) (9–11). The rate of malignancy in melanoma is much lower in species with epitheliochorial placentation (e.g., cows and horses) vs. those with hemochorial placentation (e.g., humans and rodents) (3, 4). This interspecies diversity of invasion presents opportunities to delineate molecular mechanisms that control stromal reactions to invasion.

For long, it was posited that the higher stromal invasion during hemochorial placentation (including in humans) is due to higher invasive capability of placental trophoblasts (12, 13). Genomically informed ancestral state reconstruction showed, however, that epitheliochorial placentation has evolved more recently from the ancestral hemochorial placentation, which is present in the stem lineage of eutherian mammals (10, 14, 15). We have hypothesized and experimentally validated that the evolution of epitheliochorial placentation is in part the result of stromal fibroblasts evolving increased resistance to invasion by placental trophoblasts (2). This hypothesis, termed the evolved levels of invasibility (ELI), posits that stromal invasibility itself is an evolving phenotype, resulting in higher resistance to trophoblast invasion within bovines (and likely other species with epitheliochorial placentation). The ELI model also assumes that evolution of endometrial resistance to invasion can have pleiotropic effects on stromal cells in the rest of the body. A consequence of lower invasibility in other stromal tissues is limited cancer dissemination (Fig. 1*A*) (2, 16). Species differences in stromal invasibility, therefore, present an attractive model to explore mechanisms which influence stromal invasibility in pregnancy, as well as in cancer dissemination.

Significance

Cellular invasion into adjacent tissue is observed in pregnancy, where the fetal placenta invades the mother's endometrium, and in cancer, where tumor dissemination into stroma is a necessary step before metastasis. The behavior of the surrounding tissue, and its role in either facilitating or resisting this invasion, has not been as deeply studied as the role of the invading cells. The degree of placental invasion into the endometrium varies significantly between different eutherian mammals. Having previously shown that the endometrium of different mammals is significantly responsible for these differences, we identify cis-regulatory elements underlying these differences and show the functional effect of the corresponding transcriptional factors in regulating invasion.

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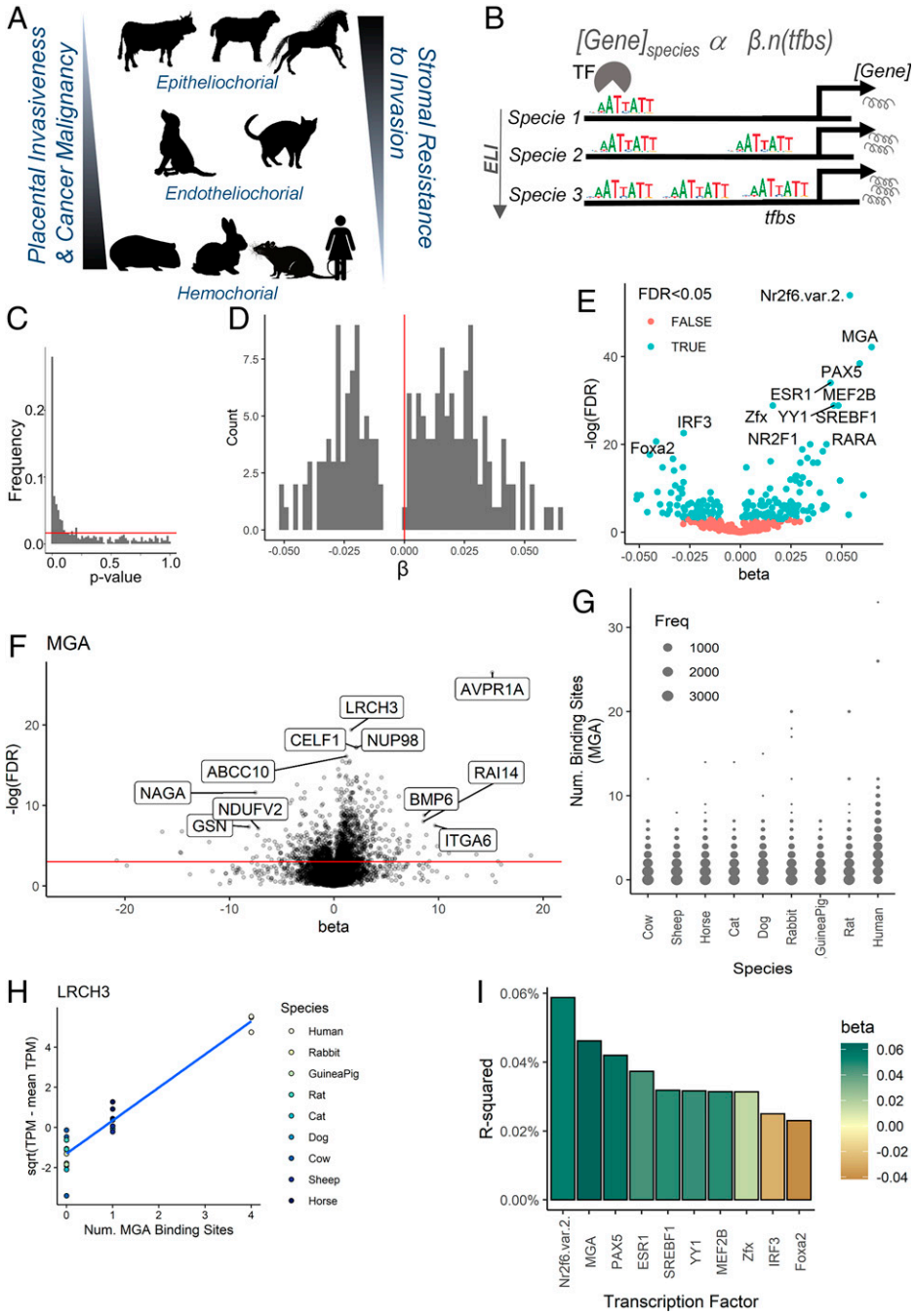

Fig. 1. Modeling the genomic basis of interspecies expression differences using cis-regulatory elements. (*A*) Illustration showing the observation that placental invasibility and cancer malignancy are inversely related, along the epitheliochorial to hemochorial axis. (*B*) Schematic showing the gain and loss of binding sites in the promoter regions of a given gene in different species and its effect on gene expression. β is a coefficient estimating the extent of effect gain of a binding site has on gene expression. (*C*) The distribution of *P* values for individual TFBS motifs, when fit using the model described in *B* where occurrence is tested for an effect on interspecies gene expression differences. The overrepresentation of low *P* values beyond the distribution expected by chance (red line) is evidence of a real effect. (*D*) Histogram for the effect size (beta) found for different TFBS motifs that were found to be statistically significant (FDR < 0.05). Beta represents the amount the scaled (square root of TPM) expression changes with one binding site, on average. Positive beta implies an enhancer effect, while negative beta implies a repressor effect on gene expression of downstream genes. (*E*) TFs whose putative binding sites affect the variation in expression across selected eutherian species. Each point represents a single TF binding motif. The statistical significance is plotted in terms of the FDR on the *y* axis, while the effect size beta (increase or decrease in expression) for additional binding sites is plotted on the *x* axis. Statistically significant motifs are labeled in green. (*F*) Illustration of the effect of downstream effect of MGA binding sites on the expression of individual genes. Here each point represents a gene whose expression was independently tested for a linear relationship to the number of binding MGA binding sites. The gene-specific effect size (beta) and significance (FDR) are plotted on the *x* and *y* axes, respectively. (*G*) Distribution of the number of binding site motifs matched in the promoter regions of genes in different species, shown here for the MGA binding site motif. (*H*) Illustration of the effect of MGA binding sites on the expression of LRCH3, across different species listed in the order of placental invasiveness. (*I*) The R-squared (fraction of variance explained) for the most statistically significant TFs affecting global ESF gene expression across species. The colors correspond to the effect size, with blue for an overall increase of gene expression with the particular TF binding in its promoter region and orange for an overall decreasing effect for additional binding.

In this paper, we identify genes whose expression in endometrial fibroblasts is correlated with placenta phenotype (ELI genes). We then characterize the gain or loss of transcription factor (TF) binding sites (TFBSs) in the promoter region of ELI genes to explain the variation in gene expression between these species. We found that differences in TFBS number could explain a many times higher fraction of variance in gene expression for ELI genes compared to other genes. We also found candidate TFs which antagonistically act as potential enhancers of proinvasibility genes and repressors of antiinvasibility genes across eutherians and vice versa. Finally, we experimentally show that GATA2 and TFDP1 control the capacity of stromal fibroblasts to resist invasion. CRISPR-based knockout (KO) of these TFs in stromal cells not only results in decreased invasibility of endometrial and skin fibroblasts by trophoblast or melanoma cells, but also changes the migratory characteristics of the invading cells, without any manipulation to the invading trophoblasts or melanoma cells themselves. Our work highlights the importance of stromal biology in regulating tissue invasion in placentation and cancer and presents genes driving the evolution of stromal resistance to invasion, with implications for personalized cancer medicine, wound healing, and pregnancy.

Results

TFBS Abundance Is Correlated with Gene Expression. The ELI hypothesis states that bovine (and other epitheliochorial) stromal fibroblasts have evolved to be more resistant to trophoblast invasion and that endometrial invasibility also is correlated with susceptibility to cancer malignancy between species (2) (Fig. 1*A*). In this study, we use ESFs from nine species with invasive and noninvasive placentation (Fig. 1*A*). We use a linear regression model to estimate the extent to which cis-regulatory TFBS numbers could statistically explain gene expression differences across species (Fig. 1*B*). Using gene expression quantified as transcripts per million (TPM), of 8,639 genes with 1–1 orthology among our species sample, we first calculated their square root TPM values to decrease the influence of extremely high expression values. We then subtracted the gene-wise mean sqrt(TPM) across all species from the square root of the species specific sqrt(TPM). This scaled expression measure was fit to a linear model with the number of TFBSs in promoter regions as the predictor variable.

Regressing the TFBS number for 572 TFs for which consensus binding site motifs are available in the JASPAR database (17), we identified 187 TFs at a false discovery rate (FDR) less than 0.05, which could explain a significant portion of the interspecies differences in gene expression in endometrial stromal cells (Fig. 1*C*). A large fraction of TFBSs statistically influence interspecies gene expression variation, indicating that the gene expression profile of endometrial stromal cells has evolved, in part, through changes in cis-regulatory elements (Fig. 1*C*).

We found that β, the regression coefficient describing the extent to which TFBS abundance could explain variation in gene expression, was positive for most TFs (Fig. 1*D*). A positive β indicates that the gain of TFBS is correlated with increased expression of the target gene, suggesting that most TFs acted as positive regulators of gene expression (i.e., activating TFs) in ESFs. The range of β values was similar for positive and negative regression coefficients (Fig. 1*D*). We found that for many TFs, $|\beta|$ was above 0.025 for the whole transcriptome, with high significance (FDR < 10^{-20}) (Fig. 1*E* and Dataset S1). Notably, averaged β over all genes did not reflect the large effect a single TF may have on the expression of individual genes (Fig. 1 *E* and *F*). For example, the regression coefficient $|\beta|$ for the TF MGA could be 20 times higher for some genes than the average β over all genes, demonstrating specificity for particular target genes (Fig. 1*F*). The interspecies distribution of the number of binding sites for the same TF for all genes showed that enrichment for specific TFBS was high for a small number of genes among hemochorial species (Fig. 1*G*). As an example, Fig. 1*H* shows the expression of the target gene LRCH3, as a function of the number of binding sites for MGA.

Line_NoWe then identified the top TFs with the highest R^2 values (Fig. 1*I*). These TFs consisted of various nuclear receptors including NR2F6, a steroid receptor which is also a repressor of IL17, the Estrogen Receptor ESR1, as well as SREBF1, the sterol regulatory element binding TF 1 (18). NR2F6 coregulates and inhibits thyroid receptor (19) and oxytocin receptor (OXTR) (20, 21), while ESR1 directly regulates OXTR expression (22) and is considered essential for uterine receptivity and endometrial decidualization (23, 24). In addition, among the top candidates an important factor with a negative β value was FOXA2, the Forkhead Box A2, an essential TF for fertility and uterine function (25, 26). In addition, other top TFs with high β values were Max Dimerization Protein MGA, a negative regulator of MYC pathway and implicated in endometrial cancers (27, 28), and PAX5 is involved in gene regulation in many tissues. Overall, we found that differences in TFBS numbers could explain variation in gene expression to a modest extent overall, while there are also many TFs explaining a large extent of interspecies variation for specific genes. Next, we sought to identify stromal genes whose expression correlates with the invasive phenotype.

Identifying Gene Expression Signatures Correlated with Placental Invasibility. To explore the evolutionary history of genes that have changed expression along with the advent of noninvasive epitheliochorial placentation, we expanded the number of eutherian species in our transcriptomic analysis (2). We isolated and cultured the stromal fibroblasts from endometrial tissues of rabbit, guinea pig, rat, cat, horse, humans, sheep, dog, and cow. This selection of species comes from two eutherian clades, the Euarchontoglires (primates, rodents, and others) and Laurasiatheria (ungulates, carnivores, and others), together forming the clade of Boroeutheria (9). This balanced taxon sample contained species with invasive placentation (human, rabbit, rat, and guinea pig) and less-invasive endotheliochorial or epitheliochorial placentation (cat, dog, horse, sheep, and cow) (Fig. 2*A*). Our aim was to uncover genes that render hemochorial species to be more invasible (ELI^{up} genes) or endow the stroma of epitheliochorial species with the resistance to trophoblast invasion (ELI^{dn} genes) (Fig. 2*B*). We created a rough invasibility index based on the anatomical presentation of the fetal–maternal interface in these species (9, 29). Specifically, species with epitheliochorial placentation were given a rank of 0, i.e., no invasion (cow, sheep, and horse); carnivores with endotheliochorial placentation were ranked 1 (cat and dog); and rodents with hemochorial placentation ranked as 2 (rabbit, guinea pig and rats), while humans, which along with primates have a particularly invasive hemochorial placentation, were ranked 3 (Fig. 2*A*). In order to identify genes that correlate with the placental invasibility phenotype, we calculated the correlation between gene expression and the placental invasion phenotype with both a naıve linear model and a phylogenetically informed linear model (*Methods*) (Fig. 2*C* and Dataset S2). The phylogenetic linear model corrects for correlations that arise due to shared descent (30). As expected, the phylogenetic linear model showed less significant *P* values than the naıve model since it allows for fewer degrees of freedom. Nevertheless, the same genes show high correlations in either model showing that confounding effect of phylogenetic relatedness is minimal (Fig. 2*C*). Therefore, either model can be used to prioritize candidate genes correlated with invasibility.

Fig. 2*D* shows an example of a gene with higher expression correlated with increased placental invasiveness (defined as

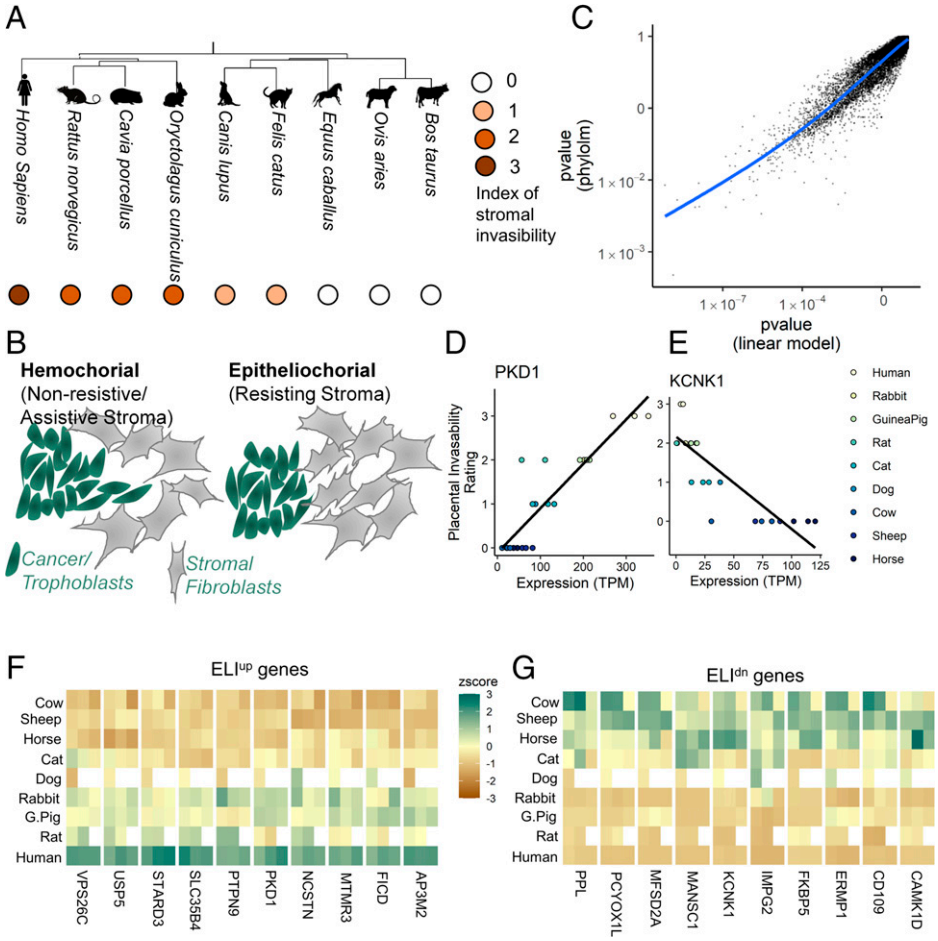

Fig. 2. Finding genes correlated with the ELI of the endometrial stroma across eutherian mammals. (*A*) Phylogenetic hierarchical representation of selected eutherian species whose ESFs were analyzed in the manuscript. The tree is balanced for invasive and less invasive placental species. Color legend denotes the extent of placental invasion ranked from 0 to 3. (*B*) Schematic explaining the ELI principle, showing that among the epitheliochorial placental mammals, the stroma has evolved to resist trophoblast invasion with secondary manifestation in other tissues resulting in decreased cancer malignancy. (*C*) Statistical significance of the expression of individual genes for correlation with invasibility among different species, evaluated using a simple linear model (*x* axis) and a phylogenetic linear model (*y* axis). *P* values are larger under the phylogenetic linear model, but similar ordering of the genes under the two methods is visible. (*D*) Illustration of the linear fit test used to probe whether the gene expression of a particular gene (in this case, PKD1) in ESFs across species is correlated with the invasibility. (*E*) Illustration of a gene whose expression is anticorrelated with invasibility. (*F* and *G*) Heat maps of expression across species for the genes most correlated with the invasibility phenotype across species, either positively (ELI^{up} genes) or negatively (ELI^{dn} genes).

ELI^{up} gene), while Fig. 2*E* shows an example of a gene whose expression is anticorrelated to invasibility (defined as ELI^{dn} gene). The ELI^{up} genes are purportedly proinvasible as their stromal expression increases along with increase in placental invasion, while ELI^{dn} genes are purportedly antiinvasible as they increase in expression with reduced stromal invasion. Fig. 2 *F* and *G* show the top 10 genes ranked in order of their positive and negative influence on invasibility, respectively. We selected a balanced set of 200 statistically most significant genes (all had FDR < 0.05), with 100 of them from the ELI^{up} set and 100 from ELI^{dn}. This gene set, termed "ELI gene set," is a rank ordered set of genes which show consistent differences across species from the least invasive to the most invasive placentation. This gene set was used to identify candidate TFs that regulate stromal invasibility.

TFBS Numbers Statistically Explain Expression of Genes Correlated with Stromal Invasibility. Modeling specifically the ELI-specific set of 200 genes, we found that TFBS could statistically explain a much higher fraction of gene expression differences than for all 1–1 orthologous genes (Fig. 3 *A* and *B*). For many TFs, both the absolute β values and the fraction of variance explained (Figs. 1*I* and 3*C*) were substantially higher than for the whole gene set, with most TFs exhibiting a positive β value. Many of these TFs were related to the myosin enhancer family of TFs, with all major MEF TFs, e.g., MEF2A-D, showing significant effects. In addition, we found that GATA TFs were highly enriched among the TFs showing significant effects. These included GATA1 and GATA2, which are classically referred to as hemotopoeitic TFs (31), and GATA5 and GATA6 (32), which are classically referred to as cardiac specific TFs. Crucially, GATA1 and GATA2 are key TFs up-regulated in cancer associated fibroblasts and induce increased cancer dissemination (33). All four GATA factors are also important in trophoblast development (34), and GATA2 acts as a key TF regulating decidualization (16, 35). These data highlight genomically encoded transcriptional regulation of decidual genes in species with increased endometrium–trophoblast interaction. Notably, we found relatively few TFs with significant negative β values, but they included key developmental TFs like PAX6, SOX10, and SIX2.

TFs displaying the highest statistical significance showed R^2 values 50 times higher than for similar calculations aggregated

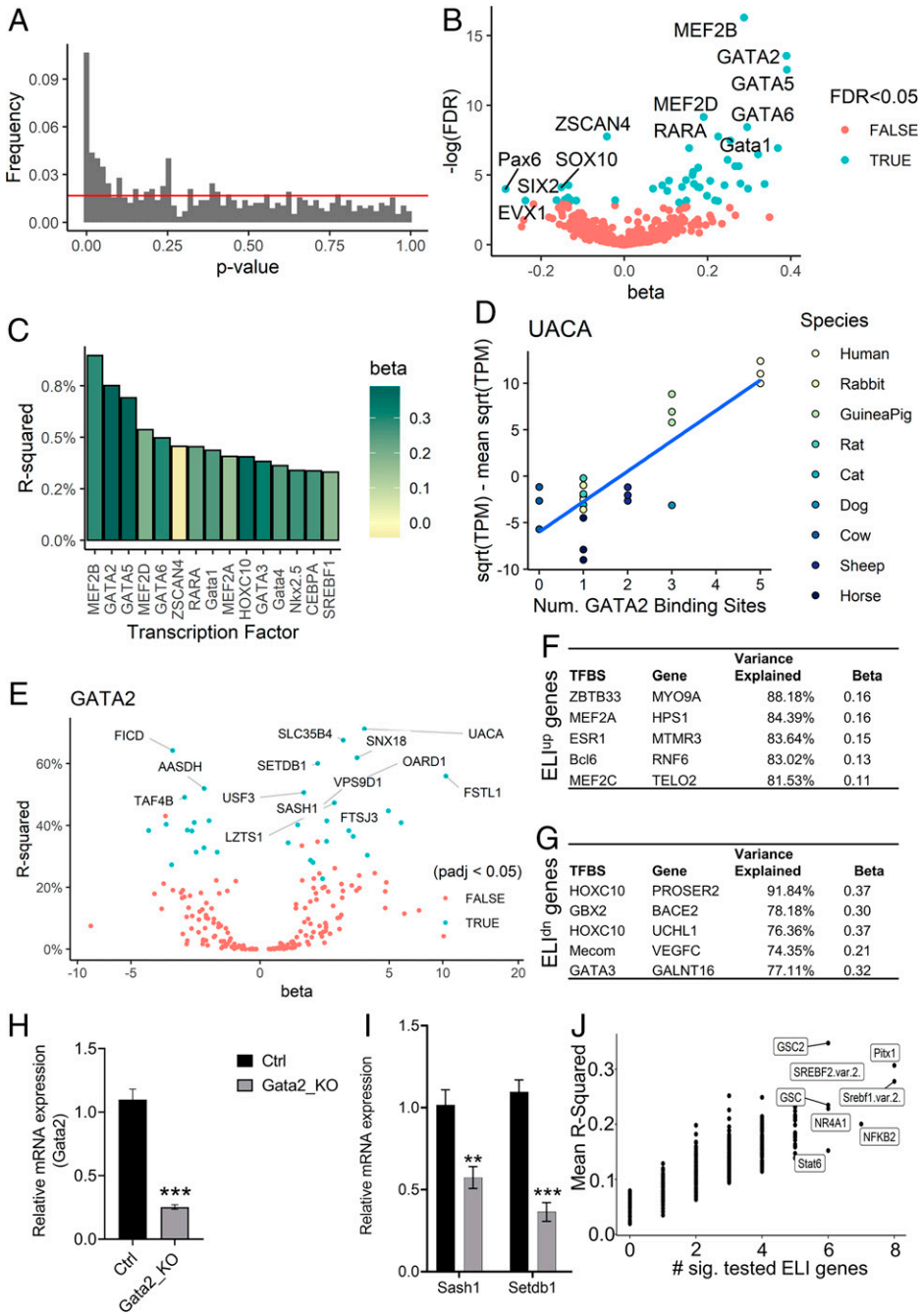

Fig. 3. Genomic basis for the regulation of genes correlated with stromal invasibility among eutherian species. (*A*) The distribution of *P* values for the effect of individual TFBS motifs, tested for the effect on expression of ELI-related genes, with the red line representing the distribution expected by chance. (*B*) The TFs whose binding sites affect the expression of genes purportedly involved in the invasibility of the endometrium (the ELI-related genes). Statistical significance (*y* axis, FDR) with the effect size (*x* axis, beta). (*C*) The fraction of variance (R-squared) of the expression across genes explained only by the occurrence of a particular binding motif aggregated over a balanced set of the most significant 100 ELI^{up} and 100 ELI^{dn} genes, shown here for the most significant TF binding motifs. The colors of the bars represent the effect size, green being higher. (*D*) Illustration of the linear fit used to calculate the statistical significance and effect size for an affected gene, UACA. Shown are the changes in gene expression across species with increasing binding sites for the TF GATA2. Species are ordered on the basis of their extent of placental invasion. (*E*) Specific genes likely to be regulated by GATA2. The fraction of variance explained (*y* axis) and the effect size (*x* axis, beta) are calculated for a linear model relating the (scaled) gene expression across species for a single gene to the number of GATA2 binding site motifs in the promoter regions among the different species. (*F*) ELI-correlated (ELI^{up}) and (*G*) ELI-anticorrelated (ELI^{dn}) genes whose variance is most explained by the number of specific binding site sequences. Beta is the common effect size found for the binding site sequence found for all ELI genes. For individual TF–gene pairs, a large extent of interspecies variance in the direction (or against the direction) of placental invasiveness could be explained by the cis-regulatory TFBS elements. (*H*) qPCR analysis showing reduction in expression of GATA2 72 h after Cas9/sgRNA transfection, (*I*) resulting in reduced expression of two genes with the highest R2 values, Sash1, Stdb1. $n = 3$ samples; error bars represent SEM. Statistical significance for *H* and *I* established at $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. (*J*) TFBSs whose occurrence is related to the expression of genes experimentally tested (2) for their role in modulating invasibility. Each point represents a binding site sequence motif, with the mean variance explained on the *y* axis and the number of genes with $P < 0.05$ for the correlation on the *x* axis.

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over all genes (Figs. 1*I* and 3 *B* and *C*). Individual genes likely to be involved in regulating placental invasibility (such as UACA) can be seen to be affected by differences in the number of binding sites of particular TFs (such as GATA2) (Fig. 3*D*). Further, as for the whole transcriptome, an aggregated R^2 value for a TF over all ELI genes masked the substantial effect of TFBS in explaining the variance in gene expression for individual target genes (Fig. 3*E*). As an example, binding sites for GATA2 could explain more than 50% of the interspecies variance for many ELI genes. Interestingly, GATA2 is acting as an enhancer for some genes and a repressor of gene expression for others (Fig. 3*E*). For many ELI genes, a substantial extent of the interspecies transcriptional difference is explained by changes in the number of cis-regulatory binding sites (Fig. 3 *F* and *G* and Dataset S3). We then sought to experimentally test whether these TFs could affect the downstream gene expression. As predicted, qPCR confirmed that gene KO of TF GATA2 by CRISPR/Cas9 gRNAs significantly reduced the expression of Sash1 and Setdb1, both implicated in preeclampsia (Fig. 3 *H* and *I*).

Previously, we experimentally demonstrated that human stromal fibroblasts could be rendered more resistant to trophoblast invasion by modifying gene expression to match that of bovine stromal fibroblasts (2). Here we tested whether interspecies variation in these experimentally validated ELI genes could be explained by cis-regulatory evolutionary changes (Fig. 3*J*). Indeed we found that among 10 experimentally validated ELI genes (ACVR1, BMP4, CD44, DDR2, LPIN1, MGAT5, NCOA3, STARD7, TGFB1, and WNT5B), many TFBSs correlated with their interspecies expression differences. These included homeobox TFs like PITX1 and GSC; sterol binding factors like SREBF1, SREBF2, and NR4A1; and nuclear signal transducers like NFKB2 and STAT6. Notably, the experimentally validated genes were positively correlated with the invasibility phenotype (i.e., the validated genes are ELI^{up} genes), and the regulating TFs also had positive β values.

Overall, these data strongly suggest that cis-regulatory genomic sequences explain variation in gene expression associated with increased stromal invasibility or stromal resistance to placental invasion.

Evolution of Increased Resistance to Placental Invasion Is Associated with Distinct Patterns of Genomic Regulation. Phylogenetic analysis has settled the debate over the evolution of noninvasive epitheliochorial placentation in eutherians, which is now considered to have evolved after the ancestral invasive hemochorial placentation (10, 14, 15). However, anatomical reports suggest that among primates, placentation has continued to evolve into a very aggressively invading form (36, 37). We asked whether cis-regulatory genomic changes in the stroma could specifically explain gain or loss of the phenotype of placental invasibility. Toward this objective, we tested if the ELI genes that correlate with increased (or decreased) placental invasion are differently influenced by TFBS.

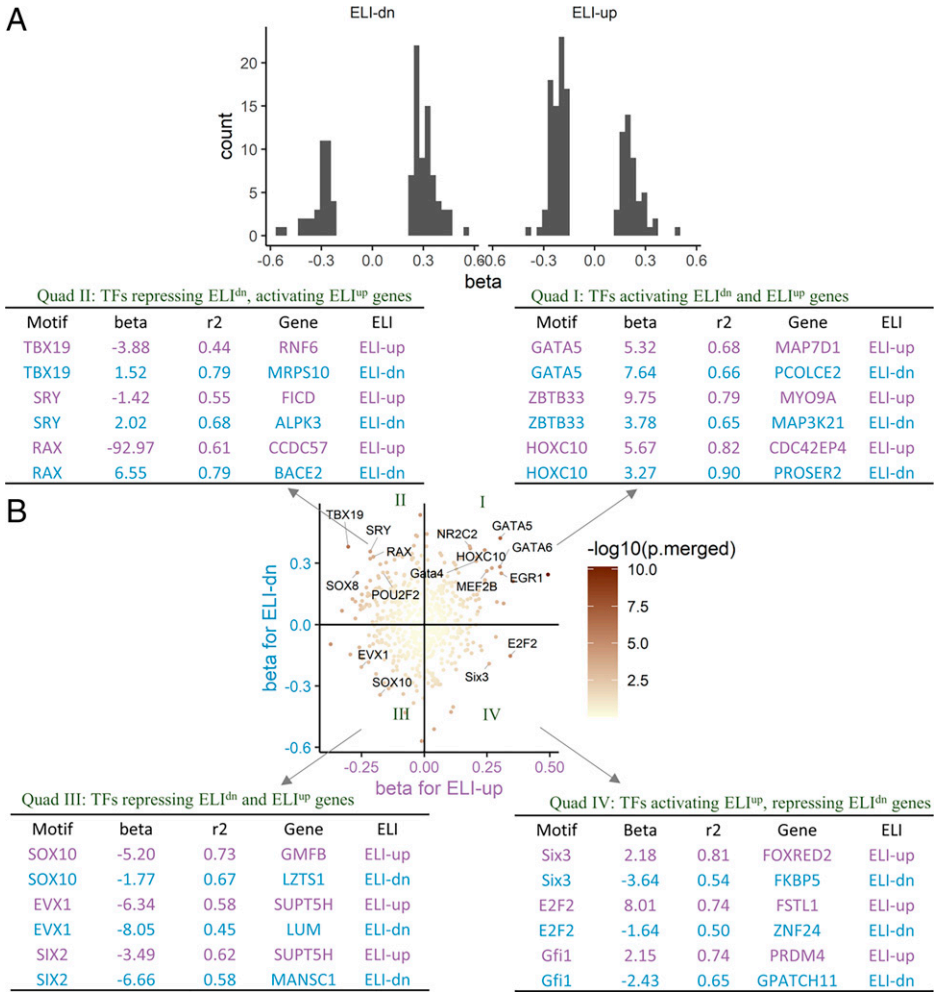
We used the top 100 genes which were the most positively correlated (ELI^{up} genes) and the top 100 genes most anticorrelated (ELI^{dn} genes) with the placental phenotype (FDR < 0.05 for all selected genes). Separate treatment of either gene set in our model could be influenced by systematic interspecies differences in overall TFBS frequencies. To avoid this artifact, we normalized the frequencies of each TFBS across the species by subtracting the TFBS abundance in the genome from the TFBS number at the promoter of each ELI gene. Overall, we found that a higher number of TFs showed enhancing effects on the expression of ELI^{dn} genes, while relatively more TFs showed repressive effects on expression of ELI^{up} genes (Fig. 4*A*). According to our model, this suggests that the majority of TFs positively regulate stromal resistance to invasion.

For each TF, we analyzed the effect size of TFBS number changes for either gene set (ELI^{up} and ELI^{dn}) (Fig. 4*B*). We found that while few TFs act as either enhancers or repressors for both ELI^{up} and ELI^{dn} genes (quadrants I and III), there were also many TFs which acted as antagonistic regulators for ELI^{up} and ELI^{dn} genes (quadrants II and IV). The tables in Fig. 4*B* show examples of TFs which regulate a given ELI^{up} and ELI^{dn} gene either as an activator or a repressor. Indeed, for many genes, a large fraction of their interspecies variation is explained by a single TF. The statistical significance of the regulation by each TF for individual ELI genes and for the two sets of 100 ELI^{up} and ELI^{dn} genes are listed in Dataset S3.

Quadrant I lists TFs activating both ELI^{up} and ELI^{dn} genes and includes many GATA factors and MEF2 factors. All GATA factors and MEF2 factors are key TFs regulating human and mouse ESF decidualization. We speculate that these TFs may regulate resistive genes in ESFs of epitheliochorial placentation and also genes which are present in the more resistive decidualized fibroblasts in hemochorial species. Other TFs also suggest a link to decidualization and preparation for pregnancy including estrogen responsive factor, HOXC10 (38), and early growth response gene 1 (EGR1) (39). EGR1 is induced by placenta growth factor and is an inducer of hypoxia inducible factor 1 (HIF1α) necessary for early implantation (40). Quadrant III shows TFs which acted as repressors for both ELI^{up} and ELI^{dn} genes.

In contrast, quadrant II shows TFs which act as repressors for ELI^{up} genes and activators for ELI^{dn} genes and thus are positive regulators of resistance to invasion. Since ELI^{dn} genes are more highly expressed in species with epitheliochorial placentation, these TFs may act as important regulators promoting stromal resistance, by both activating resistive genes and downregulating genes which promote invasion. These TFs include POU2F2/3, SRY, Nkx3.2, and TBX19. Quadrant IV lists TFs, including SIX3 and E2F2, which act as antagonistic repressors of proinvasibility ELI^{up} and activators of antiinvasibility ELI^{dn} genes and thus could be positive regulators of invasibility. The presence of antagonistic transcriptional regulators suggests that the different phenotypic outcomes are achieved partially by shared mechanisms. These proinvasibility TFs present attractive targets to inhibit the invasive processes by gene knockdowns. This would, for instance, be desirable in tumor-associated fibroblasts as the KD of such a gene is expected to increase the resistance of these cells to invasive cancer cells. Furthermore, the KD of any one of these TF genes will affect the expression a number of effector genes that influence stromal invasibility.

Silencing of GATA2 and TFPF1 Enhances Stromal Resistance to Invasion. Our model has identified candidate stromally expressed TFs regulating genes correlated with placental invasion among mammals. We therefore asked if modulating the expression of these TFs could influence stromal invasibility, the key phenotype posited to have evolved according to the ELI framework. Unlike collective migration of cells, stromal invasion is composed of complex interactions between the stromal cells and the invading cells, as well as with the extracellular matrix (1). Invasion into a stromal monolayer is also a kinetically slow process requiring many days of observation to quantitatively resolve small phenotypic differences. We have therefore previously described a quantitative stromal invasion platform composed of juxtaposed populations of invasive cells and stromal fibroblasts on a nanofabricated substrate mimicking the topography of collagen fibers in connective tissues (2, 41). The anisotropic arrangement of the submicron features not only provides a physiological context to observe stromal invasion but also aligns the actomyosin machinery within the cells in one direction, thereby increasing the speed of invasion and sensitivity of observation (Fig. 5*A*).



Quad II: TFs repressing ELI^{dn}, activating ELI^{up} genes

Motif	beta	r2	Gene	ELI
TBX19	-3.88	0.44	RNF6	ELI-up
TBX19	1.52	0.79	MRPS10	ELI-dn
SRY	-1.42	0.55	FICD	ELI-up
SRY	2.02	0.68	ALPK3	ELI-dn
RAX	-92.97	0.61	CCDC57	ELI-up
RAX	6.55	0.79	BACE2	ELI-dn

Quad I: TFs activating ELI^{dn} and ELI^{up} genes

Motif	beta	r2	Gene	ELI
GATA5	5.32	0.68	MAP7D1	ELI-up
GATA5	7.64	0.66	PCOLCE2	ELI-dn
ZBTB33	9.75	0.79	MYO9A	ELI-up
ZBTB33	3.78	0.65	MAP3K21	ELI-dn
HOXC10	5.67	0.82	CDC42EP4	ELI-up
HOXC10	3.27	0.90	PROSER2	ELI-dn

Quad III: TFs repressing ELI^{dn} and ELI^{up} genes

Motif	beta	r2	Gene	ELI
SOX10	-5.20	0.73	GMFB	ELI-up
SOX10	-1.77	0.67	LZTS1	ELI-dn
EVX1	-6.34	0.58	SUPT5H	ELI-up
EVX1	-8.05	0.45	LUM	ELI-dn
SIX2	-3.49	0.62	SUPT5H	ELI-up
SIX2	-6.66	0.58	MANSC1	ELI-dn

Quad IV: TFs activating ELI^{up}, repressing ELI^{dn} genes

Motif	Beta	r2	Gene	ELI
Six3	2.18	0.81	FOXRED2	ELI-up
Six3	-3.64	0.54	FKBP5	ELI-dn
E2F2	8.01	0.74	FSTL1	ELI-up
E2F2	-1.64	0.50	ZNF24	ELI-dn
Gfi1	2.15	0.74	PRDM4	ELI-up
Gfi1	-2.43	0.65	GPATCH11	ELI-dn

Fig. 4. Differences in the TFBS explained regulation of genes correlated (ELI^{up}) or anticorrelated (ELI^{dn}) with placental invasiveness in ESFs. (*A*) Histograms of the effect sizes (beta) of different binding sites, when tested on only ELI^{dn} and ELI^{up} genes. Only effect sizes for TFBSs that are statistically significant ($P > 0$) in the respective genes (ELI^{dn} or ELI^{up}). (*B*) Scatterplot shows the effect sizes (beta) of individual TFs for ELI^{dn} (*y* axis) and ELI^{up} (*x* axis) genes. Names of some selected TFBSs are labeled. Selected genes with a high fraction of variance explained by TFBS motif occurrences are shown as examples in the four tables next to each quadrant.

We choose two TFs to investigate: GATA2 and TFDP1, a dimerization partner of E2F, which were predicted to significantly enhance expression of ELI^{up} genes in ESFs (*P* values for ELI^{up} genes were 7.987×10^{-11} and 1.086×10^{-3}). We hypothesized that since human ESFs (hESFs) have an overall higher constitutive expression of ELI^{up} genes compared to their expression in other species, knockdown of these TFs should increase stromal resistance to invasion. Using CRISPR/Cas9-based gene KO, we targeted these TFs in hESFs, as well as in BJ skin fibroblasts. Unperturbed trophoblasts (HTR8), breast epithelial cells (MCF10A1), or malignant melanoma cells (A375), all of human origin, were patterned along with either the endometrial fibroblasts or skin fibroblasts (BJ) (Fig. 5*B*). Morphological changes in the interface were observed using live cell fluorescence and phase-contrast microscopy for 24 h to identify the extent of aerial invasion into the stroma. It is possible for the invasive fronts to deeply invade the stromal monolayer without a homogeneous aerial movement. Invasive fronts involve specification of a leader cell, which forges its way through the stromal monolayer, followed by other cells maintaining cell–cell connection through adherens junctions, and are commonly described in cancer dissemination and other invasive processes (42, 43) (Fig. 5 *C* and *D*).

Using the stromal invasion assay, described earlier (2), we performed experiments to measure the effect of GATA2 and TFDP1 gene silencing in hESFs and human skin fibroblasts BJ cells and its effect on the invasion of trophoblasts, breast epithelial cells, and melanoma cells for 24 h (Fig. 5*E* and Dataset S3).

We found that both GATA2 and TFDP1 gene KO in the hESFs resulted in significant reduction in the invasion by HTR8 trophoblasts (Fig. 5*F*). Furthermore, the morphological nature of invasion by HTR8 cells was also influenced by the gene KO. To focus on the depth of stromal invasion by the invasive forks (Fig. 5 *C* and *E*), we calculated the percentage of the vertical segment occupied by the invading monolayer at every location in the direction of invasion, showing stark differences in the profile along the depth of invasion by TFDP1 silencing (Fig. 5*G*). We also identified the tip for each invading fork and calculated the depth forged by each fork from its initial location (Fig. 5*H*), finding that TFDP1 silencing in hESFs significantly reduced the depth of invasion by HTR8 cells (Fig. 5*H*).

We also tested the effect of modulating stromal gene expression on invading breast epithelial MCF10A1 cells. Again, silencing of both TFs in BJ fibroblasts showed a significant effect in enhancing stromal resistance to MCF10A1 invasion (Fig. 5*I*). Notably, TFDP1 silencing in BJ also significantly reduced not only the overall aerial invasion of MCF10A1 but

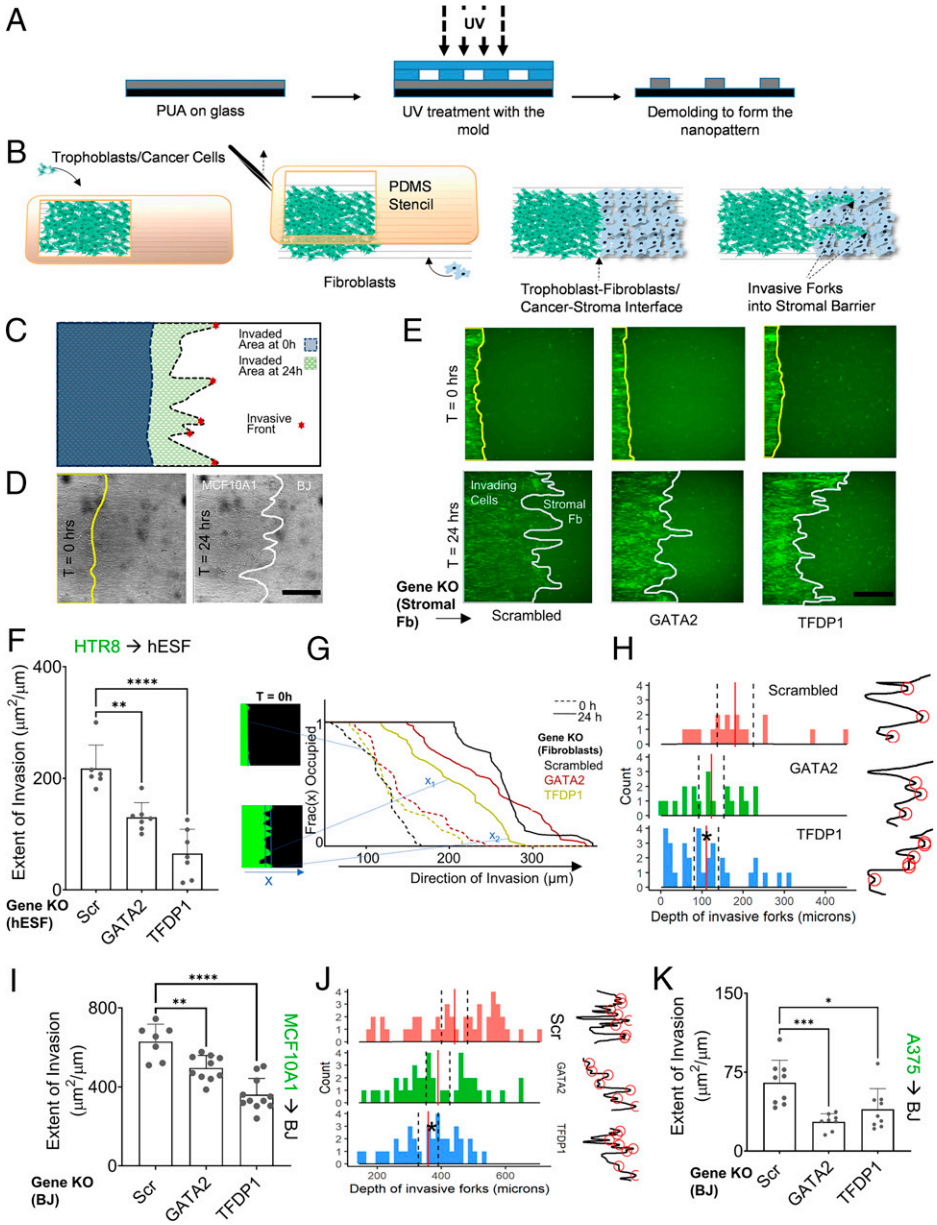


Fig. 5. TFs predicted to enhance invasibility of stromal fibroblasts functionally control invasion in multiple cellular contexts. (*A*) Method to transfer nanopatterns mimicking collagen fibers using polyurethane (PUA) replica mold with capillary force lithography. (*B*) Nanofabricated scaffolds are used as substrates to pattern invading cells and fibroblasts using poly(dimethylsiloxane) stencils, creating an interface between two cell populations. (*C*) Schematic showing a directional movement of invading fronts, accompanied by formation of invasive forks, also shown in (*D*) phase contrast images for breast epithelial cells (MCF10A1) invading into BJ fibroblasts within 24 h. Scale bars in *D* and *E* correspond to 400 μm. (*E*) Live cell microscopy snapshots of invading MCF10A1 cells into the BJ monolayer in 24 h, with BJ cells subjected to CRISPR/Cas9-based gene KOs for GATA2, and TFDP1 TFs. Scr refers to the untargeted scrambled control. (*F*) Quantification for the extent of aerial invasion, normalized to the initial interface length. (*G*) Cumulative measurement of invading fronts calculated as the fraction of pixels occupied at every location in the direction of invasion. (*Left*) Schematic explains the calculation of fraction occupied at two different locations. (*H*) Quantification for the depth of invasion by each fork within 24 h invading into the stromal barrier, here BJ fibroblasts with the given gene KOs. Shown is automated identification of the tip of the invading forks (red circles); solid red bar refers to the mean length of invasion, and black dashed lines refer to the 95% confidence intervals. (*I*) Normalized extent of invasion of HTR8s into hESF monolayers with gene KOs. (*J*) Depth of invasion by each HTR8 fork within 24 h invading into hESF monolayers. (*Right*) Representative tips of invading forks (red circles). (*K*) Normalized extent of aerial invasion of A375 melanoma cells in BJ fibroblasts with gene KOs. In *G*–*K*, error bars represent SDs, statistical significance by *t* tests established at $P < 0.05$, and denoted by $*P < 0.05$, $**P < 0.01$; $***P < 0.001$, $***P < 0.0001$. Each dot represents an independent imaged interface.

also the depth forged by the invading forks (Fig. 5*J*). Finally, we also showed that silencing both TFs in BJ cells significantly enhanced their resistance to invasion by malignant melanoma cells, A375 (Fig. 5*K*).

We previously have proposed that stromal fibroblasts in other tissues may have coevolved with endometrial fibroblasts, resulting in a more resistive stroma in species with epitheliochorial placentation (2). These experiments demonstrate that the TFs predicted by our comparative placentation analysis to regulate endometrial vulnerability to placental invasion also regulate the invasibility of skin fibroblasts by cancer cells.

Discussion

The maternal–fetal interface in placental mammals represents an apposite model to study the broader phenomenon of epithelial invasion into stroma, providing avenues to delineate and study the role of stromal reactions to invasion (44). Phylogenetic analysis has led to the rejection of the long-held hypothesis that the hemochorial placentation, also present in humans, had evolved recently. Instead, hemochorial placenta is ancestral for eutherian mammals, and the noninvasive placenta of hoofed animals is derived (10, 15, 45). Our recently advanced hypothesis, termed ELI, proposes that evolution of noninvasive epitheliochorial placentation was driven, in part, by changes in the endometrial stromal cells; i.e., less invasive placentation is caused by higher stromal resistance to invasion. We further propose that this resistance was secondarily conferred, as a pleiotropic effect, onto other tissues leading to correlated resistance to cancer malignancy (2).

We used an ordinal metric of placental invasiveness to identify candidate genes which potentially regulate invasibility among eutherian mammals using transcriptomic data of ESFs from nine species. Both a linear regression model and a phylogenetically informed model suggested a set of 200 genes, called ELI genes, strongly correlated with placental phenotype. Among these are genes that are positively correlated with invasive placenta, called ELI^{up}, where higher expression in the stromal cells is correlated with higher invasibility. The complement of these is ELI^{dn} genes, where higher expression is correlated with lower invasibility, i.e., stronger resistance to invasion. We hypothesized that species differences in ELI gene expression might be caused, in part, by cis-regulatory changes, i.e., changes in the number of TFBSs. We tested this hypothesis by regressing gene expression levels onto the number of TFBSs in the genomic neighborhood of the genes.

We found that the gain or loss of TFBSs in promoter regions could, to a limited extent, statistically explain interspecies variation in gene expression. Previous work has speculated that changes in cis-regulatory elements may be occurring up to 10 times faster than protein evolution following speciation and about 3 times faster following gene duplication in the same species (46). Therefore, cis-regulatory elements are an appropriate signal to start exploring interspecies differences in gene expression. TFBS abundance explained a much larger fraction of interspecies variation in ELI genes compared to other stromally expressed genes. Notably, for individual TF/target gene pairs, the number of TFBSs could explain a large portion of expression changes along the trajectory toward the evolution of reduced placental invasion, also confirmed experimentally for a key TF, GATA2, and the predicted downstream genes. Many TFs explaining large ELI-correlated transcriptomic differences belonged to the myocyte enhancer binding factor 2 (MEF2) family and the GATA-binding factor family. MEF2 proteins, in particular, MEF2B and MEF2D, are central players in muscle cell differentiation (47, 48), cardiac hypertrophy, and fibrosis (49). Similarly, GATA2 is a central factor in endothelial cell differentiation (50) and decidual differentiation in humans and mice (16, 35). The stroma of hemochorial species may be more prepared to interact with invasive trophoblasts, and indeed, GATA2 has been reported to regulate genes associated with decidualization, and decidua–trophoblast interactions (51), including placental lactogen synthesis (52, 53), proliferin (51), and prolactin-like protein-A (54). Epitheliochorial placentation among ruminants is a more recent derivation than the ancestral hemochorial placentation. Therefore, it is worth noting that ruminants lost binding sites for GATA2 and MEF2D.

In addition to the TFs which acted as positive regulators for proinvasibility genes, we found many TFs which acted antagonistically on ELI^{up} and ELI^{dn} genes. Many of these antagonistic TFs (Fig. 4*B*, quadrant II) acted as enhancers for antiinvasibility (ELI^{dn}) genes, while also acting as repressors for the proinvasibility (ELI^{up}) genes. What do these TFs tell us about the evolution of epitheliochorial placentation? Since evolutionarily, epitheliochorial placentation is more recent, our analysis suggests that both ELI^{dn} and ELI^{up} genes have recruited these TFBSs, but their effects on ELI^{dn} and ELI^{up} genes are opposite. Indeed, we also found examples of antagonism wherein certain TFs (e.g., E2F2 in Fig. 4*B*, quadrant IV) act as repressors of ELI^{dn} genes, while activating ELI^{up} genes. We underscore that while the effect of these antagonistic TFs is opposite upon the expression of ELI^{up} and ELI^{dn} genes, the effect on the phenotype of invasibility is consistent. This is because a TF with repressing effects on ELI^{up} genes and activating effects on ELI^{dn} genes is going to be have an antiinvasive effect; i.e., these TFs are consistently increasing the stromal resistance to invasion. The opposite is the case with TFs that have an activating effect on ELI^{up} genes and a repressive effect on ELI^{dn} genes. These TFs have a consistent proinvasibility effect.

Our analysis identifies attractive candidates for exploring the mechanisms of stromal invasibility. We sought to experimentally test the capability of TFs, predicted to enhance invasibility, to regulate stromal invasibility. Targeting of GATA2 and TFDP1 for KO resulted in increased resistance to invasion by trophoblasts and cancer cells. Crucially, we demonstrated that stromal gene expression state influenced the characteristics of collective invasion, even though the invading cells were unperturbed, highlighting that cancer dissemination, or placental invasion, may be more directly influenced by the stromal environment than is currently appreciated.

We speculate that these effects are caused by decreased expression of ELI^{up} genes which these TFs are expected to positively regulate. Understanding the molecular functions of these TFs in stromal cell–trophoblast interaction will reveal the mechanisms that enable stromal cells to resist invasion by both placental and cancer cells. The presented model is relatively straightforward and highly interpretable, suggesting a mechanistic link between the evolution of cis-regulatory elements and stromal invasibility in various cellular contexts: trophoblast invasion during pregnancy, epithelial invasion in wound healing, and cancer dissemination. Although the experimental identification of downstream genes of the KO TF (e.g., GATA2) was beyond the scope of this study, future studies could harness these TF-target gene pairs to specifically modulate stromal invasibility and treat diseases of pregnancy or cancer dissemination. Our work provides a roadmap inspired by evolutionary medicine to target human diseases, including cancer and pregnancy-related pathologies, through a better understanding of species differences.

Methods

Cell Sourcing. hESFs were immortalized by the Charles Lockwood laboratory and obtained from the Gil Mor group. Human SFs (BJ) were purchased from American Type Culture Collection (ATCC) (CRL-2522). ESFs from cow (*Bos taurus*), dog (*Canis lupis*), guinea pig (*Cavia porcellus*), horse (*Equus caballus*), cat (*Felis catus*), rabbit (*Oryctolagus cuniculus*), sheep (*Ovis aries*), and rat (*Rattus norvegicus*) were obtained by enzymatic digestion of uterine tissues and attachment-based positive selection.

RNA Isolation and Sequencing. RNA was isolated using RNeasy micro kit (QIAGEN) and resuspended in 15 μL of water. RNA integrity number of over 8 was observed in the samples measured by Agilent Bioanalyzer 2100. mRNA libraries prepared using TruSeq RNA Library (Illumina) were sequenced on Illumina HiSeq to generate 30 to 40 million reads per sample (single-end 75 base pair reads), and a high Q score was observed ($Q > 30$) for the sequenced data. A transcript-based quantification approach was used to analyze RNA sequencing (RNA-seq) data using the program kallisto (55). The Ensembl release 99 (56) gene annotation model was used to align raw reads. In order to facilitate comparative gene expression across species, a one-to-one ortholog dataset

consisting of 8,639 species was formulated (composed of human, rat, rabbit, horse, guinea pig, cat, sheep, cow, horse, dog, and opossum) such that the sum of TPMs across these 8,639 genes for each species totals to 10^6.

Finding Genes Related to the Modulation of Placental Invasibility. We obtained the gene expression $e_{g,s}$ of gene g in species s in TPM. For each species, we also rated the degree of placental invasibility r_s, with a value from 0 to 3. In order to find the stromal genes likely involved in modulating the degree of invasibility, we fit the linear model

$$r_s = \gamma_g e_{g,s} + \epsilon_{g,s},$$

where $\epsilon_{g,s}$ is the residual. This is fitted independently for each gene, and positive γ_g represent genes that are up-regulated in species with more invasive placenta, while negative γ_g indicates the opposite. The statistical significance is calculated for testing against the null hypothesis of $\gamma_g = 0$. The *P* values for all genes are corrected for multiple testing using the false discovery rate method. In addition, this linear model was also fitted using a phylogenetic linear model, with an assumption of Brownian motion processes producing both the traits (gene expression and species invasibility). The phylogenetic linear model was fitted using the phylolm package (30).

Stromal Invasion Assay. For the cell patterning, a PDMS (polydimethylsiloxane) mold, which was fabricated by a stereolithographic plastic mold, was used. To fabricate a PDMS stencil, the monomer and cross-linker (with a ratio of 10:1) of PDMS were mixed and cured at 80 °C for 4 h and then cast in the predesigned mold. The stencil was placed on the substrate after washing with isopropanol and drying with N_2 steam. The device was kept in a vacuum to remove air bubbles under the stencil. The Invasive cells were labeled by cell tracker green and were seeded at a density of 5×10^5 cells and attached to the surface overnight. The stencil was removed carefully using blunt-end tweezers. The unlabeled stromal cells were seeded at a density of 5×10^5 to fill and attach that area covered by the stencil before. The unattached cells were washed off after 5 h of incubation. The plate was mounted on the live microscopy stage.

Data Availability. RNA-seq data have been deposited in the National Center for Biotechnology Information BioProject (PRJNA564062). All other study data are included in the article and/or supporting information.

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