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Genetic recapitulation of human pre-eclampsia risk during convergent evolution of reduced placental invasiveness in eutherian mammals

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The relationship between phenotypic variation arising through individual development and phenotypic variation arising through diversification of species has long been a central question in evolutionary biology. Among humans, reduced placental invasion into endometrial tissues is associated with diseases of pregnancy, especially pre-eclampsia, and reduced placental invasiveness has also evolved, convergently, in at least 10 lineages of eutherian mammals. We tested the hypothesis that a common genetic basis underlies both reduced placental invasion arising through a developmental process in human placental disease and reduced placental invasion found as a derived trait in the diversification of Euarchontoglires (rodents, lagomorphs, tree shrews, colugos and primates). Based on whole-genome analyses across 18 taxa, we identified 1254 genes as having evolved adaptively across all three lineages exhibiting independent evolutionary transitions towards reduced placental invasion. These genes showed strong evidence of enrichment for associations with pre-eclampsia, based on genetic-association studies, gene-expression analyses and gene ontology. We further used *in silico* prediction to identify a subset of 199 genes that are likely targets of natural selection during transitions in placental invasiveness and which are predicted to also underlie human placental disorders. Our results indicate that abnormal ontogenies can recapitulate major phylogenetic shifts in mammalian evolution, identify new candidate genes for involvement in pre-eclampsia, imply that study of species with less-invasive placentation will provide useful insights into the regulation of placental invasion and pre-eclampsia, and recommend a novel comparative functional-evolutionary approach to the study of genetically based human disease and mammalian diversification.

1. Background

Phenotypic diversification between taxa may arise from the evolution of developmental programmes such that the genetic systems underlying homologous traits differ across taxa [1]. Alternatively, variation in the regulation and ontological organization of a developmental system may give rise to divergent phenotypic outcomes that have a shared underlying genetic basis. Within species, this situation is best exemplified by polyphenism [2,3], but ontogenetic shifts in complex genetic systems are also likely to be important in generating interspecific phenotypic variation [4,5]. A number of recent studies indicate that evolutionary variation of a stable underlying genetic system can give rise to repeated convergent evolution in complex traits such as *Drosophila* wing pigmentation patterns, bee eusociality and stickleback body armour [6–8]. Establishing a common genetic basis to phenotypically divergent outcomes in human and non-human species is especially important in medical research that makes use of animal models. Here, we show that three independent convergent evolutionary transitions towards reduced placental invasiveness—in rodents, primates and tree shrews—share a common

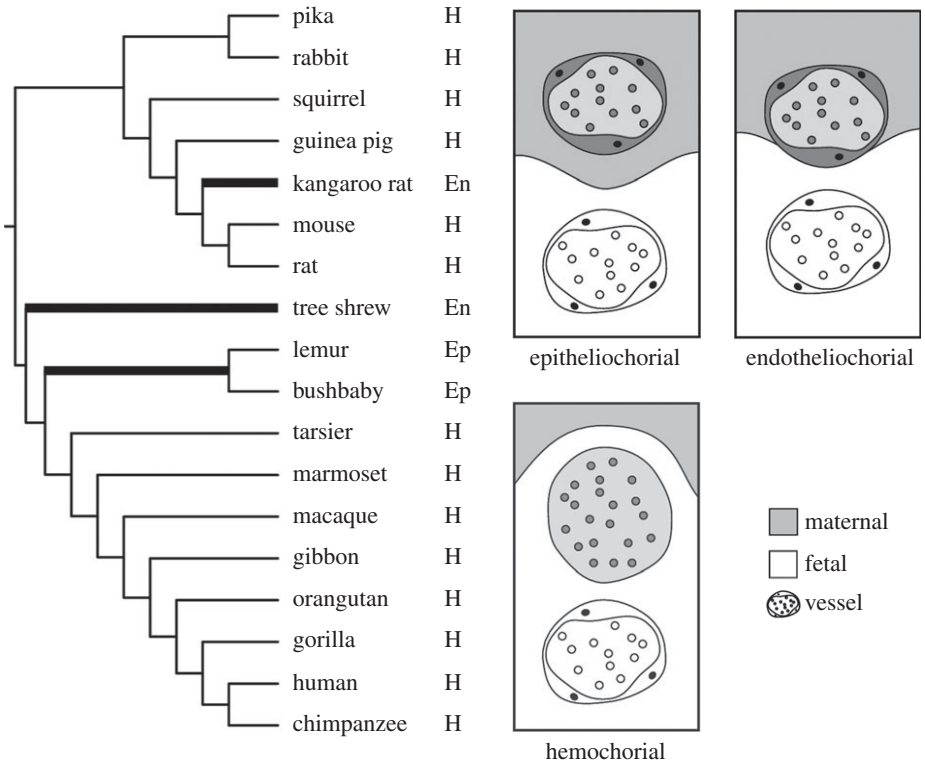

Figure 1. Left: phylogeny of species used in this study (H, hemochorial; En, endotheliochorial; Ep, epitheliochorial). Three lineages exhibiting independent transitions from hemochorial to less-invasive forms of placenta are displayed in bold. Right: schematic of hemochorial, endotheliochorial and epitheliochorial forms of placentation found in Euarchontoglires; fetal tissues are white and maternal tissues are shaded. In epitheliochorial placentation, fetal trophoblast is separated from maternal blood by maternal epithelium, connective tissue and the endothelium of maternal blood vessels. In endotheliochorial placentation, fetal tissues are separated from maternal blood only by maternal endothelium. In hemochorial placentation, maternal vascular endothelium is invaded by trophoblast such that maternal vessels are dilated and fetal tissues are bathed directly in maternal blood.

genetic basis with human diseases of placental invasion, suggesting that the ontogeny of human diseases can be recapitulated in large-scale mammalian morphological evolution.

The notable diversity found in mammalian placental development provides outstanding opportunities to analyse the relationship between phylogeny and ontogeny in this context. Phylogenetic reconstruction indicates that the earliest members of the superorder Euarchontoglires (comprising rodents, lagomorphs, tree shrews, colugos and primates) bore an invasive hemochorial placenta in which the fetal trophoblast is bathed directly in maternal blood, a situation found in a majority of extant Euarchontogliran mammals including humans [9]. The phylogeny of this group includes three independent evolutionary transitions towards less-invasive endotheliochorial or epitheliochorial placentation—in tree shrews, strepsirhine primates and heteromyid rodents—in which maternal blood is physically separated from fetal tissues during gestation (figure 1). Reduced invasion of maternal vessels by fetal trophoblast tissue is characteristic not just of these derived forms of placentation but is also a central feature of pre-eclampsia in humans. The pre-eclamptic placenta, while remaining hemochorial, is characterized by markedly reduced invasion of the placental bed and reduced remodelling of the maternal vasculature by fetal tissues, which in healthy placentation supports nutrient supply to the fetus during pregnancy [10–14]. Although the precise causes of pre-eclampsia remain uncertain, symptoms accompanying reduced placental invasion include inappropriate expression of an immunologically pro-inflammatory and anti-angiogenic cytokine profile, misregulation of cell adhesion and apoptotic processes at the fetal–maternal interface, kidney damage and proteinuria, and maternal hypertension resulting in vascular damage, convulsions and sometimes death [15]. Hypertensive disorders of pregnancy are a leading cause of maternal and fetal morbidity and mortality, accounting for 9.1% of maternal deaths in Africa and Asia, 25.7% in Latin America and 16.1% in developed countries [16]. Pre-eclampsia is often regarded as a condition unique to humans (and perhaps great apes: [17,18]), and the apparent absence of naturally occurring analogues in non-human model species has hampered the study of its aetiology and the development of effective treatments [19].

Previous studies on the genetic basis of interspecific placental variation have attempted to identify lineage-specific genes or transcript variants that may underlie the development of placental structures unique to taxa including elephants [20], carnivores [21], cattle [21,22] and haplorhine primates [23,24]. Based on similarities in the changes to physiological systems that underlie reduced invasiveness of non-hemochorial placentation in Euarchontoglires and also reduced placental invasion in pathological, human hemochorial placentation—especially modifications to uterine vascular patterning, reduced motility of fetally derived trophoblast in the endometrium and altered immunological relations between mother and fetus—we here address the hypothesis that variation across and within species in placental invasiveness depends not merely on lineage-specific adaptation but also on variation within a core, overlapping set of genetic systems that regulate placental invasion.

To identify a set of genes undergoing adaptive evolution during the losses of hemochorial placentation in Euarchontoglires, we conducted genome-wide phylogenetic tests for statistical signals of positive selection specifically within the

three lineages associated with the origin of endotheliochorial or epitheliochorial placentation. We then tested for involvement of these genes in the ontogeny of placental invasiveness, by determining whether they show genetic or gene-expression associations with pre-eclampsia.

2. Material and methods

(a) Sequence alignments

The Ensembl Perl application programming interface [25] was used to generate, for each human gene identifier, a file containing collected unaligned one-to-one orthologous protein-coding sequences from the 18 Euarchontogliran taxa available: pika (*Ochotona princeps*), rabbit (*Oryctolagus cuniculus*), ground squirrel (*Spermophilus tridecemlineatus*), guinea pig (*Cavia porcellus*), kangaroo rat (*Dipodomys ordii*), mouse (*Mus musculus*), rat (*Rattus norvegicus*), tree shrew (*Tupaia belangeri*), lemur (*Microcebus murinus*), galago (*Otolemur garnettii*), tarsier (*Tarsius syrichta*), marmoset (*Callithrix jacchus*), macaque (*Macaca mulatta*), gibbon (*Nomascus leucogenys*), orangutan (*Pongo abelii*), gorilla (*Gorilla gorilla*), human (*Homo sapiens*) and chimpanzee (*Pan troglodytes*). Each file was scanned for the presence of non-hemochorial species (kangaroo rat, tree shrew, lemur and galago), and files were discarded from further analysis if they contained no sequence data for these species. The remaining files were each aligned using a codon model in the probabilistic alignment application PRANK [26]. Tests for positive selection are sensitive to alignment quality. A recent review [27] found PRANK's codon model (which aligns codons rather than nucleotides or amino acids, thus making use of information from both the primary nucleotide sequence and also the translation) to yield the most consistently high-quality alignments, with respect to success in inference of sitewise positive selection, of a number of methods assessed. Resultant alignments were manually checked for quality, and low-quality regions for which 50% or more species exhibited gaps were excised from the alignments prior to further analysis.

(b) Detection of selection

For each gene, a base phylogenetic tree (figure 1) was pruned according to the sequence data available. Focal branches upon which transitions from hemochorial to non-hemochorial placentation occurred (the branch leading to kangaroo rat, the branch leading to tree shrew and the branch leading to strepsirhine primates) were labelled as foreground branches for analysis in PAML (phylogenetic analysis by maximum likelihood) [28]. Owing to the genomic data and/or inference of one-to-one othologues by Ensembl being incomplete, the resultant phylogeny varied across genes in the number and identity of terminal taxa. For 2093 genes, a phylogeny bearing one transition from hemochorial to non-hemochorial placentation was available, for 4293 genes a phylogeny bearing two such transitions was available and for 10 192 genes a phylogeny bearing all three possible transitions was available.

PAML was used to fit a branch-sites positive selection model (in PAML, model = 2 and NSsites = 2). The sites component of the model allows gamma-distributed variation in evolutionary rate across sites of a gene, modelling the fact that diversifying selection is likely to be rare and arising only within portions of the entire coding sequence. The branch component of the model allows the ratio of non-synonymous to synonymous substitutions (ω) to increase above unity—signifying positive, diversifying selection—only on branches of the phylogeny exhibiting transitions from invasive hemochorial placentation to less-invasive non-hemochorial forms. A likelihood ratio test was used to identify genes for which the likelihood of the branch-sites positive selection model is significantly higher than the likelihood of its neutral counterpart in which focal branches are restricted such that $\omega \leq 1$ (in PAML, fix_omega = 1). It is expected that some genes, for example those involved in antagonistic coevolution with genes expressed by parasites or disease vectors, may be under positive selection on all branches of the phylogeny. These genes may fit the branch-sites model better than the neutral model even though selection is not specifically restricted to the branches of interest that bear evolutionary transitions in placental invasiveness. In order to exclude such genes from our list of positively selected genes, we also fitted a third model in PAML in which a single evolutionary regime of positive selection was applied to all branches of the phylogeny (in PAML, model = 0 and NSsites = 2). Genes for which this global selection model fitted better than the branch-sites model were excluded from the list. As this model is not nested within the branch-sites model as a special case, Akaike's information criterion [29] was used to assess model fit rather than the likelihood ratio test. Finally, we excluded any genes for which the branch-sites model appeared to be the best fit but which exhibited signs of failure of convergence (i.e. genes for which the inferred positively selected $\omega = 1$ or for which the proportion of sites assigned to the positive selection class = 0). All models were fitted to the data multiple times and the maximum-likelihood inferred over the course of these multiple trials was used in the likelihood tests.

(c) Gene set enrichment

Enrichment analysis was used to test the set of positively selected genes for functional signatures associated with placentation and placental disease. Enrichment analysis of gene ontology biological processes and Reactome canonical pathways was accomplished using ClueGo [30] (see the electronic supplementary material, tables, for parameters). Disease associations of positively selected genes were identified by extracting gene sets associated with disease MeSH terms from the Genetic Association Database [31] (which includes pre-eclampsia and other reproductive disorders within a comprehensive list of human diseases) and testing for enrichment using Fisher's exact test. Enrichment of human and mouse tissue types was tested using Enrichr [32]. All multiple tests were subject to Benjamini–Hochberg false discovery rate correction.

(d) Detection of selected substitutions of large phenotypic effect

Genes bearing positively selected amino acid substitutions of large phenotypic effect may constitute novel candidate genes for involvement in the evolution of placental invasiveness and, potentially, in the pathogenesis of pre-eclampsia and other diseases of human placental invasion. PAML can be made to output, for each site of an alignment, a Bayes Empirical Bayes probability that the given site is subject to diversifying selection. Based on the alignments, it is possible to calculate, for each site with Bayes Empirical Bayes $p > 0.95$, the amino acid(s) present in non-hemochorial species that are non-synonymous with the amino acid present in human beings. The software application PROVEAN [33] was used to predict, computationally, which of these apparently positively selected amino acid substitutions would be associated with a major phenotypic effect, were they to arise in human beings.

3. Results

(a) Identification of positively selected genes

Based on alignments of 16 578 protein-coding genes across 18 taxa (four of which have non-hemochorial placentas, figure 1), we identified a subset of 1254 genes which are inferred to have evolved adaptively in the three independent focal

lineages undergoing evolutionary transitions towards reduced placental invasion, but neutrally in the remaining lineages undergoing no such evolutionary transition. These genes are presumed to be involved, potentially, in the macroevolution of less-invasive placentation.

(b) Significant overlap of positively selected genes with genes known to be involved in human reproductive disorders

Functional overlap between genes subject to adaptive evolution during independent losses of hemochorial placentation in tree shrews, strepsirhine primates and kangaroo rats, and genes associated with human disorders of placental invasion, was assessed using tests for enrichment of disease-association gene sets. Fishers exact tests based on the Genetic Association Database [31], comprising 167 130 gene–disease associations derived from human studies, indicate that the adaptively evolving gene set is significantly enriched with genes that underlie pregnancy-related disorders (including premature birth, chorioamnionitis, pre-eclampsia and cardiovascular complications of pregnancy) and circulatory disorders involving blood clotting and atherosclerosis (table 1). Pre-eclampsia is both a placental and a vascular disorder, giving rise to maternal vascular lesions that are highly similar to those found in atherosclerosis [34] and predisposing towards hypertension, ischaemic heart disease, cerebrovascular disease and thromboembolism [35]. The enrichment results are thus consistent with positive selection on genes involved in pathology of the placenta and uterine vascular system, especially pre-eclampsia.

(c) Significant overlap between positively selected genes and genes involved in pre-eclampsia

Studies on the genetic basis of pre-eclampsia are dominated by two broad classes of approach. First, candidate gene and genome-wide association studies have been used to identify single nucleotide polymorphisms (SNPs) associated with pre-eclampsia. A comprehensive database of pre-eclampsia-associated SNPs [36] identifies within the set of 16 578 studied genes, 149 bearing putatively pre-eclampsia-associated SNPs in their coding sequence, introns or enhancer regions. These genes were significantly enriched within the set of adaptively evolving genes (22 genes, $p = 0.002$). Of the 149 SNP-bearing genes, 92 are significantly associated with pre-eclampsia with $p < 0.05$ in at least one study, and this more restricted set of loci is also enriched within the set of adaptively evolving genes (14 genes, $p = 0.009$).

Second, studies of differential gene expression in pre-eclamptic versus normal placenta and endometrium have been used to identify genes significantly upregulated or downregulated in the disease condition. A recent review of such studies [37] identifies, within the set of 16 578 studied genes, 84 genes that are found to be differentially expressed in at least one placental or endometrial tissue during pre-eclamptic pregnancies. Of these genes, 12 exhibit the statistical signal of adaptive evolution, constituting significant enrichment ($p = 0.024$). It has been noted that there is only a modest consensus in results from gene association and differential expression studies [37]. Nevertheless, of the pre-eclampsia-associated genes present in our set of 16 578 tested for adaptive evolution, 12 bear pre-eclampsia-associated SNPs and are also differentially expressed in pre-eclampsia. To account for possible non-independence of results from genetic-association and gene-expression studies, we also calculated enrichment statistics for the set of unique pre-eclampsia-associated genes combined from both approaches. Of 221 such genes, 33 exhibit the statistical signal of adaptive evolution ($p < 0.001$).

Table 1. Diseases exhibiting significant genetic overlap with the set of 1254 genes subject to adaptive evolution during evolutionary transitions towards less-invasive forms of placentation in kangaroo rats, strepsirhine primates and tree shrews; significance reported as Benjamini–Hochberg adjusted q-value.

disease MeSH term	proportion of genes	q
obstetric labour, premature	33/184	0.004
premature birth	40/250	0.004
infection of amniotic sac and membranes	31/174	0.004
chorioamnionitis	31/176	0.005
fetal membranes, premature rupture	31/179	0.005
multiple sclerosis	64/503	0.010
venous thrombosis	15/61	0.011
pre-eclampsia	35/229	0.014
thrombosis, deep-vein	8/20	0.015
fetal diseases	23/129	0.019
gastrointestinal diseases	8/23	0.029
patent ductus arteriosus	21/119	0.032
heart defects, congenital	13/58	0.039
bronchial hyperreactivity	20/114	0.039
infection	21/123	0.039
myocardial infarct	19/107	0.042
angina pectoris	9/32	0.043
myocardial ischaemia	12/53	0.044
musculoskeletal diseases	20/118	0.045
atherosclerosis, coronary	22/136	0.046

(d) Functional enrichment

Genes subject to adaptive evolution were also significantly enriched with biological processes involved in placental function (the electronic supplementary material, table S2). A cluster of genes involved in angiogenesis and blood vessel development was the most strongly enriched (especially involving interleukin 6, an angiogenic cytokine possibly involved in trophoblast invasion into the endometrium [38,39]), consistent with selection on vascular patterning during the evolution of placental invasiveness and also consistent with the centrality of misregulated angiogenesis to the pathogenesis of pre-eclampsia [40,41]. Significant enrichment was also identified in a cluster of genes involving cytokine and chemokine regulation of the immune system and inflammatory response, and in clusters of genes related to cell migration and apoptosis, all central to fetal–maternal interactions *in utero* [42–44].

Analysis of enriched canonical pathways (electronic supplementary material, table S3) indicates especially strong selection on G-protein-coupled receptors, integrin and NOTCH receptors as well as some of their downstream intracellular signalling cascades PI3K and AKT, all known for involvement in promoting trophoblast motility [45], as well as numerous other signalling pathways associated with placentation and placental disease including fibroblast growth factor and interferon gamma [46,47]. Statistical identification of gene clusters enriched in common between adaptively evolving genes and combined pre-eclampsia-associated genes yielded similar results (electronic supplementary material, tables S6 and S8) confirming the pre-eclampsia associations of these processes and pathways.

(e) Identification of novel evolutionarily informed candidate genes for involvement in pre-eclampsia

One implication of the enrichment of genes associated with placental pathology within the set of adaptively evolving genes is that the latter may contain novel candidate genes and pathways involved in human placental disorders. To formally prioritize the set of genes subject to adaptive evolution for possible involvement in pre-eclampsia and related conditions, we used PROVEAN, a software tool for predicting the phenotypic impact of nucleotide substitutions in humans based on evolutionary conservation of amino acid sites [33]. We identified 289 genes that evolved adaptively during loss of hemochorial placentation in Euarchontoglires, for which positive selection can be ascribed to specific amino acid sites with high probability (Bayes Empirical Bayes $p > 0.95$) and which also exhibit, in non-hemochorial Euarchontoglirans, substitutions at those sites that are predicted to be of major phenotypic effect relative to the normal human allele (PROVEAN score < -2.5). These genes are significantly enriched with putatively pre-eclampsia-associated SNPs (eight genes, $p = 0.005$, table 2; electronic supplementary material, tables S4 and S5) though not with transcripts differentially expressed in pre-eclampsia. Genes already known to be associated with pre-eclampsia were excluded and, through comparison with the combined set of pre-eclampsia-associated genes, those remaining were categorized into three subsets. First, we identified members of pathways and biological processes enriched in common between pre-eclampsia-associated and adaptively evolving genes (58 genes with robust functional support for involvement in pre-eclampsia; electronic supplementary material, tables S10 and S12). Second, we identified members of pathways and biological processes enriched uniquely in the adaptively evolving gene set (48 genes that may be involved in pre-eclampsia or may be involved in adaptations allowing low levels of placental invasion to be compatible with healthy mothers and offspring in kangaroo rats, tree shrews and strepsirhine primates; electronic supplementary material, tables S11 and S13). Finally, we identified a subset of 111 adaptively evolving genes with large predicted phenotypic effects involved in direct physical or genetic interactions with known pre-eclampsia-associated genes [48]. The combination of these subsets constitutes a list of 199 novel, evolutionarily informed candidate genes for involvement in the evolution of placental invasiveness and in the pathogenesis of pre-eclampsia and other diseases of placental invasion, listed in full in the electronic supplementary material, table S14; those genes with known placental functions are described in table 3.

Gene Ontology biological processes, Reactome pathways, KEGG pathways and Wikipathways enriched among the 199 candidate genes are illustrated in figure 2. Overlapping gene sets are clustered and identified using ClueGo [30] under default parameters. A number of disease-associated modules are identified—including tuberculosis, leishmaniasis, cholera infection and amyotrophic lateral sclerosis. We suspect that the enrichment of such categories is a consequence of high levels of research effort into identification of disease-associated genes and arises from enrichment of genes generally involved in immunity and pathological processes. This view is supported by the fact that highly specific disease-associated gene sets are tightly clustered with more generic immune and cytokine processes. Hence, the enriched tuberculosis and amyotrophic lateral sclerosis (ALS) gene sets are tightly clustered with a number of pathways and processes involved in tumour necrosis factor signalling and inflammation. Tumour necrosis factor plays a major role in the response of the human body to tuberculosis infection [49] but additionally regulates trophoblast migration, invasion and apoptosis in early pregnancy [50–52], and proteins associated with the tumour necrosis factor pathway are specifically involved in remodelling of spiral arteries [53]. Similarly, the leishmaniasis gene set is tightly clustered with biological functions and processes involved in angiogenesis and endothelial cell migration. Progressive angiogenesis and tissue remodelling in the spleen is characteristic of visceral leishmaniasis in mammals [54] and angiogenesis inhibitors are a potential treatment for the parasite [55]. As noted above, pathological angiogenesis is a fundamental outcome in pre-eclampsia [40,41]. Several other enriched clusters broadly overlap with aspects of pre-eclampsia, including genes involved in NOTCH signalling, known to be involved in early angiogenesis [56–58] and the nitric oxide pathway, involved in the control of vasoconstriction and misregulated in pre-eclampsia [59–61].

4. Discussion

The enrichment among the set of genes targeted by natural selection of distinct but overlapping human placental disorders ranging from premature membrane rupture to pre-eclampsia (table 1), along with significant overlap with the set of genes known to be differentially expressed in pre-eclampsia or bearing pre-eclampsia-asssociated SNPs (table 2), constitute robust support from multiple, independent approaches for the existence of a core set of genes underlying both pre-eclampsia in humans and the adaptive evolution of reduced placental invasion in Euarchontogliran mammals, and evidence against the notion that the pathogenesis of pre-eclampsia involves placental processes unique to humans. We have used a novel evolutionary computational approach to identify 199 genes (table 3), not currently known to underlie the pathogenesis of pre-eclampsia, as candidates for involvement with pre-eclampsia and other diseases of human pregnancy.

Evolutionary analyses have the potential to shed light on outstanding controversies in the study of placental disease. In particular, we find selection on genes involved in hypertension and blood pressure regulation as well as placental tissue remodelling and angiogenesis, supporting the controversial view that gestational hypertension and pre-eclampsia may actually overlap to some extent in their pathogenesis beyond mere superimposition in at-risk pregnancies [62]. More generally,

Table 2. Genes associated with pre-eclampsia in human beings and also exhibiting the statistical signal of adaptive evolution during loss of invasive hemochorial placentation in kangaroo rat, strepsirhine primates and tree shrew. PV, PROVEAN score: in genes for which positive selection can be ascribed to specific amino acid sites (BEB $>$ 0.95) lower scores indicate stronger predicted phenotypic effects of observed substitutions. ω: dN/dS ratio reported by PAML, with values greater than one indicating positive selection, and 'high' indicating an estimate of ω reaching an upper limit in PAML, caused for example by an absence of synonymous substitutions; $p(\omega) > 1$: significance of the likelihood ratio test supporting positive selection; genetic-association codes: DE, differentially expressed in pre-eclampsia; SNP, gene bearing putatively pre-eclampsia-associated SNP; SNP*, gene bearing SNP associated with pre-eclampsia with $p < 0.05$ in at least one genetic-association study.

gene	association	PV	ω	$p(\omega > 1)$	biological process
S100A8	DE		high	$<$0.001	cytokine production, inflammation, apoptosis, wound healing
F5	SNP*		high	$<$0.001	cell adhesion, blood coagulation
DRD4	SNP*	−16.00	92.75	$<$0.001	MAPK activation, dopamine signalling
C1QTNF6	SNP*	−7.39	high	$<$0.001	protein oligomerization
EGLN3	DE		32.41	$<$0.001	response to hypoxia, apoptosis, cell proliferation
CD97	DE		high	$<$0.001	inflammation, cell adhesion, cell–cell signalling
SLC30A8	SNP*		high	0.002	insulin secretion, regulation of vesicle-mediated transport
CYBA	SNP	−13.00	42.01	0.003	endothelial cell proliferation, regulation of blood pressure
IL6	SNP	−6.42	2.63	0.004	apoptosis, cell proliferation, immune response, cytokine signalling
IL12RB1	SNP*		9.2	0.005	immune response, cytokine signalling
EDN2	DE		1.05	0.005	vasoconstriction, chemotaxis, prostaglandin synthesis
APOE	SNP	−7.02	5.07	0.005	endothelial cell migration, lipid transport, vasodilation
ACE	SNP*	−3.02	8.76	0.006	vessel remodelling, regulation of blood pressure, vasoconstriction
ACVR1	SNP	−16.54	5.65	0.006	patterning of blood vessels, embryonic development
F2	SNP*		11.66	0.007	blood coagulation
CYP4V2	SNP*		3.6	0.007	fatty acid omega-oxidization
CBS	SNP	−3.16	4.35	0.009	regulation of blood pressure, process in pregnancy, hypoxia
VGLL1	DE		7.51	0.011	regulation of transcription, DNA-dependent
SOD3	SNP		high	0.012	response to hypoxia
COL4A2	SNP*		22.22	0.012	response to TGF-β stimulus
BDKRB1	DE		high	0.013	inflammation, cell migration, regulation of blood pressure
APOH	SNP*		29.71	0.016	endothelial cell proliferation, blood coagulation, angiogenesis
CXCR6	DE		7.33	0.019	cell–cell signalling
SLC9A3	SNP*		19.75	0.02	ion transport, regulation of pH
SIAE	DE		1.31	0.024	sialate O-acetylesterase activity
IL4R	SNP*		22.72	0.025	inflammation, cytokine signaling, cell proliferation
SERPINI2	DE		14.97	0.029	cellular component movement, regulation of endopeptidase activity
ADRB3	SNP		high	0.031	vasodilation, energy reserve metabolism, endocytosis
FLT1	DE/SNP*		10.87	0.034	sprouting angiogenesis, patterning of blood vessels
MTR	SNP		4.06	0.04	xenobiotic metabolic process, nervous system development
IDO1	DE		3.23	0.042	inflammation, cytokine signalling, T cell proliferation
COL18A1	DE		1.86	0.047	angiogenesis, cell adhesion, cell proliferation, apoptosis
PLAUR	SNP*		14.19	0.05	cellular component movement, chemotaxis, blood coagulation

the overlap between the set of genes targeted by natural selection and genes involved in multiple placental pathologies supports the view that placental disorders are highly multifactorial and that there may exist different genetic routes to common symptomatic states [63]. Controversy has also been raised over the relative contribution to pre-eclampsia of the maternal and fetal components of the placenta, especially the role of the maternal immune system in the disease [64]. This is especially pertinent given the apparent association of disease risk with maternal exposure to partner-specific paternal alloantigens, with nulliparous women more than twice as likely to develop pre-eclampsia as multiparous women [65], and the risk of pre-eclampsia and/or pregnancy-induced hypertension inversely correlated with the duration of sexual cohabitation prior to conception [66–69] presumably as a result of tolerance arising from maternal exposure to paternal seminal antigens [70–72]. Mapping of the 199 genes bearing positively selected amino acid substitutions of large phenotypic effect to tissue-specific expression data [73] using Enrichr [32] indicates weak enrichment of proteins localized to CD14+

Table 3. Novel, evolutionarily informed candidate genes for involvement in pre-eclampsia. Associations: C, member of biological process or pathway enriched in common between positively selected genes bearing substitutions of large predicted phenotypic effect and known pre-eclampsia-associated genes; U, member of process or pathway uniquely enriched in adaptively evolving genes; I, member of physical or genetic interaction network with known pre-eclampsia-associated genes. An expanded and fully referenced version of this table is available in the electronic supplementary material, table S14.

gene	PV	ω	p	association	possible reproductive role
TNFRSF1B	−30	16.58	<0.001	C,I	inflammation and vascular cell migration
PKD1	−27	4.93	0.003	C,U	placental labyrinth formation
FLG2	−22.02	55.61	<0.001	I	possible involvement in inhibition of trophoblast differentiation through interaction with caspase-14
LOXL2	−20.61	53.6	0.005	C,U	cell matrix interactions in cytotrophoblast
ATP6AP1	−20	489.32	<0.001	U	determinant of birthweight
SH2D2A	−19	14.96	0.009	C,U,I	pathological angiogenesis, endothelial cell migration
QRICH2	−18.74	3.94	<0.001	I	physically interacts with SNAI1, a repressor of trophoblast giant cell differentiation
MKI67IP	−18.53	2.33	0.024	I	a trophoblast stem-associated gene
PTGER1	−18.19	6.1	0.026	I	regulation of placental blood pressure, extravillous trophoblast migration, parturition
IL20RA	−17	16.95	<0.001	I	inhibition of tumour necrosis factor alpha in fetal membrane
NTRK3	−16.03	3.27	0.045	C,U	receptor for NT3, a placentally expressed growth factor
TCOF1	−15	3.63	0.007	I	a trophoblast stem-associated gene
CD74	−15	12.41	0.009	C,U	expressed at the fetal–maternal interface, also associated with atherosclerotic plaques
MPP4	−12.69	2.53	0.045	I	a trophoblast stem-associated gene; in general, MMPs are involved in breakdown of maternal ECM during placental invasion
ZNF214	−12.28	17.22	<0.001	I	associated with fetal overgrowth
PRKCB	−12	7.69	0.007	C,U,I	regulates constriction of uterine arteries in sheep; a trophoblast stem-associated gene
MAML3	−11.75	3.67	0.045	C,U	NOTCH signalling
S1PR5	−11.4	82.81	<0.001	I	receptor for sphingosine 1-phosphate, a regulator of angiogenesis during pregnancy in sheep
GPC1	−11	670.08	0.013	C,U,I	a trophoblast differentiation marker
TLL1	−11	6.84	0.013	U	fetal heart development, placental ECM interactions; potentiates BMP1 which is involved in placental angiogenesis
PTGS2	−11	8.1	0.026	C,U,I	parturition
MX1	−10.74	13.09	0.003	I	an interferon-induced antiviral protein upregulated in chorion
GATAD1	−10.73	6.17	0.016	I	possibly involved in transcriptional interference with a neighbouring methylated retroviral element enriched in villous trophoblast
CREB3L1	−10.47	19.64	0.008	I	regulates the expression of GCM1, required for chorioallantoic branching and syncytiotrophoblast formation
SP5	−10.34	6.82	<0.001	I	regulator of embryogenesis
CCL16	−10.27	5.74	0.001	I	inflammatory chemokine associated with various placental disorders and arteriogenesis
NANOS1	−10	999	<0.001	C,U	embryogenesis
EIF2B2	−9.78	4.32	0.048	C	a trophoblast stem-associated gene
CRABP2	−9.73	22.95	0.003	I	involved in endometrium–trophoblast interaction during implantation
SOD1	−9.41	2.99	0.024	C,U	reaction to oxidative stress in pregnancy
F7	−9	998.96	0.004	C	coagulation at fetal–maternal interface
EPHA8	−9	4.97	0.008	C	ovulation, adhesion to fibronectin

(*Continued*.)

Table 3. (*Continued.*)

gene	PV	ω	p	association	possible reproductive role
ZNF416	−8.42	4.3	0.005	I	suppresses MAPK signalling, a pathway involved in regulation of villous trophoblast differentiation
BCS1L	−8	10.86	0.048	U	a trophoblast stem-associated gene also involved in fetal brain development
SS18	−7.46	7.04	0.049	I	transcriptional regulation of genes involved in placental vascularization and/or chorioallantoic fusion
HSPG2	−7.45	4.85	<0.001	C,U,I	involved in cell adhesion, growth factor binding and modulation of apoptosis in various placental tissues; a clinical biomarker for early rupture of fetal membranes and gestational diabetes
TNFRSF1A	−7	25.71	0.004	C,I	inflammation and vascular cell migration
MYCBP2	−6.99	6.09	0.001	U	differentially expressed in resorbing versus healthy rat embryos
FGF2	−6.77	25.68	0.009	C,U,I	regulates trophoblast differentiation
SLC25A17	−6.22	8.88	0.027	U	upregulated in the placenta during maternal food deprivation
SMTNL2	−5.77	999	<0.001	I	a marker of highly differentiated contractile smooth muscle cells in placental vasculature
IL2RB	−5.57	8.15	0.009	C	trophoblast-specific gene expression arising from endogenous retroelement promoter, promoting apoptosis
KIF1B	−5	7.72	0.026	U	a trophoblast stem-associated gene
CCNI	−4.95	10.9	0.021	I	a trophoblast stem-associated gene
ADCYAP1	−4.82	998.97	0.002	I	regulation of placental hormone milieu
HDAC1	−4.69	117.85	<0.001	C,U,I	regulates transcriptional profile of embryonic and trophoblast stem cells
PSTPIP1	−4.62	39.74	0.049	I	autoinflammatory gene
GALR1	−4.51	999	0.029	I	possible placental regulatory role
FIGLA	−4.36	999	0.012	I	oocyte-specific transcription factor essential for folliculogenesis and regulating zona pellucida proteins
NUP35	−4.3	9.4	0.028	I	a trophoblast stem-associated gene
SCARB1	−4	37.14	0.003	C,U	trophoblast giant cell immune behaviour
TAOK2	−3.99	2.91	0.006	C	possible involvement in pig conceptus development
SELPLG	−3.92	9.8	<0.001	I	hemostasis in the placental bed
PLAU	−3.76	11.7	0.011	C,I	regulates trophoblast invasion in response to hGH
NPM2	−3.73	7.26	0.007	C	a maternal-effect gene involved in implantation and early embryonic development
PRND	−3.7	32.41	0.029	C	required for early placental development
BTNL2	−3.68	998.9	0.037	I	differentially regulated in implanted blastocysts obtained after co-culture in human endometrial cells versus the sequential system
EMR2	−3.58	14.15	<0.001	I	promotes cell–cell adhesion and pro-inflammatory cytokine response
NCAM2	−3.44	154.06	0.01	I	involved in a possible rescue mechanism against placental hyperplasia associated with NCAM1 deficiency
TBCA	−3.4	999	<0.001	I	a trophoblast stem-associated gene
UBR4	−3.16	4.23	0.046	I	gene knockouts in mice exhibit unusually dilated placental blood vessels and thin labyrinth layer
VIM	−3	4.13	0.034	I	a characteristic factor of vascular trophoblast giant cells in the mouse placenta
CD5	−3	212.75	<0.001	C	maternal CD5-positive cells associated with fetal growth delay and spontaneous abortion

(*Continued.*)

Table 3. (*Continued.*)

gene	PV	ω	p	association	possible reproductive role
JAK2	−2.97	28.15	<0.001	C,U,I	JAK2 signalling regulates extravillous trophoblast invasiveness and is involved in decidual response to IL11
SDHD	−2.91	23.76	0.001	I	a trophoblast stem-associated gene
SSTR3	−2.88	60.34	0.015	C,I	receptor for somatostatin, a regulator of first-trimester human trophoblast function
KLRB1	−2.81	9.25	<0.001	I	activates natural killer cell cytotoxicity, involved in regulating Th1/Th2 balance at the feto-maternal interface; increased expression in uterine natural killer cells in pregnancies with implantation failures
VPS53	−2.75	8.68	0.042	I	abnormal expression associated with increased trophoblast giant cell number and abnormal placental labyrinth architecture in mice
GSG1	−2.72	2.77	0.027	I	tissue-specific methylation in macrophages of the fetal–maternal barrier
FLG	−2.71	41.88	<0.001	I	possible involvement in inhibition of trophoblast differentiation through interaction with caspase-14
NFYA	−2.69	3.96	0.035	U	a transcription factor with enriched targets in the pre-eclamptic placenta
KLB	−2.69	10.45	0.003	U,I	an aging-suppressor gene, candidate factor for vascular disease, pre-eclampsia; deficiency leads to impaired vasodilation and angiogenesis
LZTFL1	−2.65	999	0.036	I	a target of selection for low uterine capacity in rabbits
FOLR1	2.63	9.38	0.02	I	a high-affinity isoform of the folate receptor selectively expressed in syncytiotrophoblast and choriocarcinoma; a trophoblast stem-associated gene
C14orf133	−2.59	9.13	0.035	I	transcriptional regulation of e-cadherin, which in turn is involved in regulation of trophoblast differentiation
F2RL1	−2.58	2.77	0.047	C,U,I	mediates extravillous trophoblast invasion

monocytes (enrichment score = 4.15; FMNL1, BID, MANBA, TNFRSF1B, POU2F2, PSTPIP1 and JAK2). These immune cells are characteristic of maternal uterine tissues during pregnancy [74] and are differentially expressed in the decidua in pre-eclampsia [75]; in the context of maternal immunity, they are known to be differentially regulated in the placenta depending on maternal lifestyle and possibly allergen exposure [76]. A similar analysis using mouse gene atlas data [73,77] indicates enrichment for genes localized to IgE-bound mast cells (enrichment score = 4.42), which have a central role in allergy and inflammation and which are also active in the maternal reproductive tract throughout pregnancy [78] and are involved in the defective vascular remodelling of pre-eclampsia [79]. Hence, components of the maternal immune response involved in pregnancy and pre-eclampsia are targets of natural selection during evolutionary transitions in placental invasiveness, supporting a role for genetic modules underlying maternal immunity, inflammation and allergy response in evolutionary transitions between placental types and in the pathogenesis of disorders of placental invasion.

The importance of Darwinian evolution of protein-coding genes in generating phenotypic diversity remains much debated, and it has long been argued that selection on transcriptional regulation may be of equal or greater importance than selection on protein-coding sequences [80,81]. Furthermore, both protein sequence and regulatory evolution appear to be dominated by neutral drift and purifying selection rather than diversifying selection [82–84]. A strong association with placentation and diseases of pregnancy in the gene lists described above provides strong support for the role of natural selection on protein-coding sequences in placental evolution. This is not inconsistent, however, with a major role for regulatory evolution including drift. Ten of the 74 genes listed in table 3 are themselves placentally expressed transcription factors (PKD1, PRKCB, GATAD1, CREB3L1, SP5, HDAC1, FIGLA, JAK2, NFYA and LZTFL1). But, more pertinently, an *ad hoc* test using Enrichr [32] indicates that 41—more than half—of the genes listed in table 3 are collectively regulated by eight transcription factors (SNAI1, SNAI2, TCF3, ZNF148, USF2, EGR1, E2F1 and JUN). At least six of these eight transcription factors whose targets are significantly enriched in table 3 ($q < 0.005$) have known roles in regulating blastocyst implantation, vascular inflammation or trophoblast differentiation and proliferation during pregnancy [85–90]. Hence, while it is possible that positive selection on the gene lists described above is directly involved in the generation of phenotypic diversity in mammalian placentation, it is also possible that the signal of adaptive evolution identified in this study represents the accumulation of mutations in protein-coding sequences that are compensatory to drift or adaptive evolution of the broader regulatory network in which protein-coding genes are embedded.

One such regulatory evolutionary process that has been implicated in the evolution of reproductive mode in reptiles [91] and at the time of the division between eutherian and

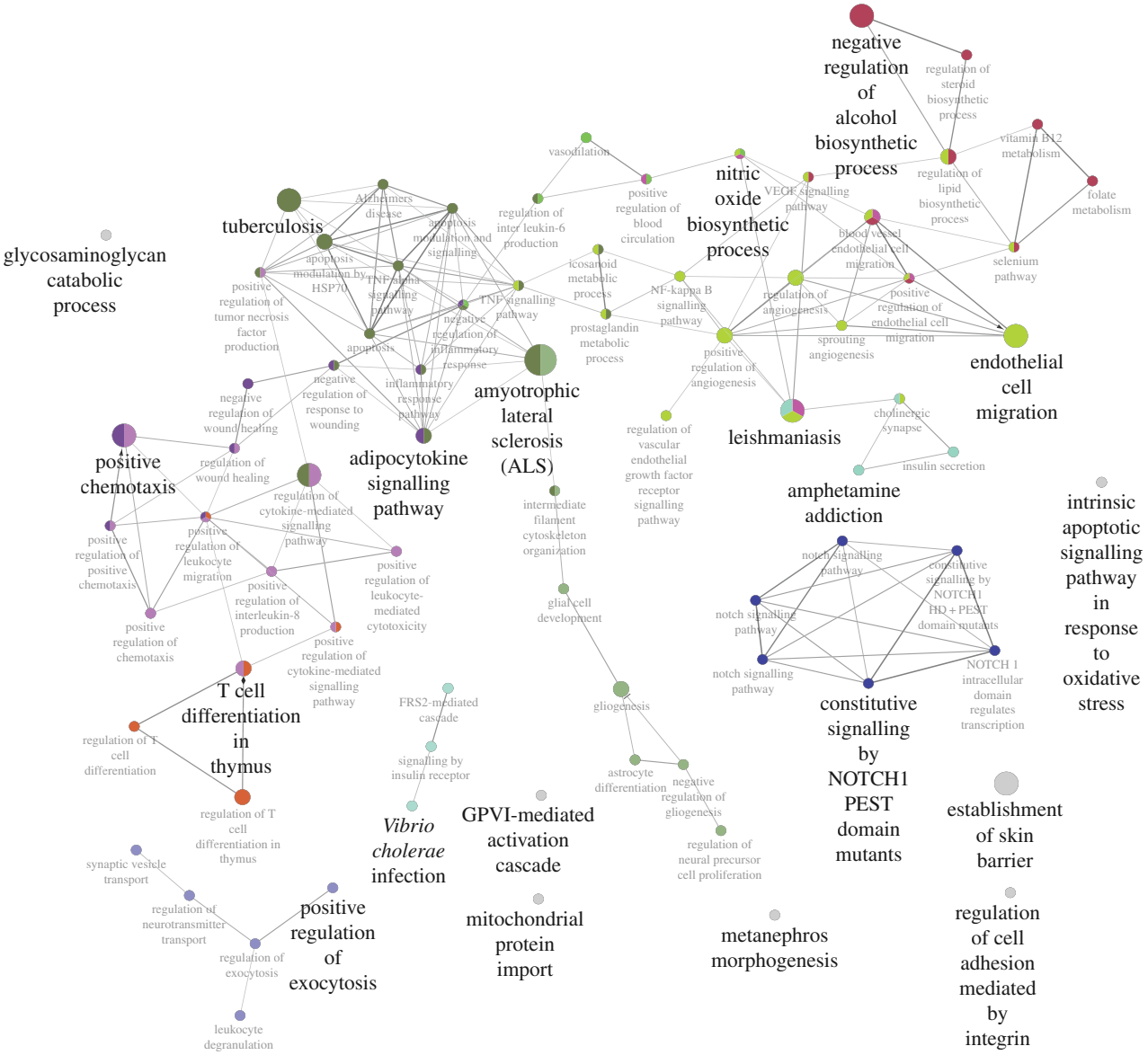

Figure 2. Enriched Gene Ontology biological processes, Reactome pathways, KEGG pathways and Wikipathways among 199 novel, evolutionarily informed candidate genes for involvement in pre-eclampsia. Overlapping gene sets are clustered and identified using ClueGo [30] under default parameters. Large text is associated with the most enriched gene set within each cluster. (Online version in colour.)

metatherian mammals [92–94] is heterochrony. Deep placental invasion in species with hemochorial placentation naturally progresses through a process of increased invasiveness throughout gestation: at the moment prior to implantation, the uterus is of course not invaded at all, and the first trimester of human pregnancy is supported primarily by maternal uterine secretions and yolk sac placentation until at least the tenth week of pregnancy, when the vascularization of the chorionic villi supports establishment of true hemochorial placentation with direct fetal access to maternal blood [95]. It is notable that species with derived, less-invasive forms of placentation exhibit prolonged yolk sac placentation and histotrophic nutrition, a phenomenon most pronounced in the epitheliochorial horses [96] but also observed in strepsirhine primates [97]. In haplorhine primates, early placental phenotypes appear to be more conserved than phenotypes arising later in gestation [98], and in farm animals, gene-expression patterns of early pregnancy are more conserved than those in late pregnancy, which are characterized by species-specific divergence [99]. These observations suggest a heterochronic model of placental evolution in which early stages of placentation are prolonged in order to accomplish reduced placental invasion, along with taxon-specific terminal addition of novel adaptations to support late pregnancy, such as the unique hemophagic regions of carnivores [100] and the areaolae or chorionic vesicles of strepsirhines [97]. King [96] has argued that the degree of placental invasiveness is primarily dependent upon maternal endometrial reactions to trophoblast, a view supported by the fact that placental tissue from species with minimal invasiveness, such as pigs, expresses a highly invasive phenotype when transplanted into an ectopic site [101,102]. A third possible interpretation of our findings, then, is that the signal of adaptive evolution identified in this study reflects compensatory mutations in proteins expressed by the fetus and/or mother in response to maternal regulatory evolution, as part of a process of parent–offspring conflict over the degree of placental invasion [103–106]. We anticipate that combining the results presented above with future data on maternal and fetal transcriptomic evolution during transitions in placental invasiveness will help to tease apart the role of adaptively evolving proteins in generating phenotypic variation, adapting to regulatory evolution of the transcriptome and participating in parent–offspring conflict.

These findings support the hypothesis that a core set of genes and pathways underlying eutherian placental invasiveness are associated with both the pathogenesis of human pre-eclampsia and the convergent evolution of less-invasive (endotheliochorial and epitheliochorial) placentation. These results, derived from the study of three independent phylogenetic replicates of evolutionary transitions towards reduced placental invasion, support and complement previous work that examines a less (taxonomically and placentally) diverse set of taxa in which a single branch of the phylogenetic tree is associated with increased invasiveness through the evolution of spiral arteries [107]. Establishing that the raw genetic basis of human placental disorders is of ancient lineage provides a fundamental empirical grounding for evolutionary theories of human placentation that are based on notions of parent–offspring or intragenomic conflict in mammals and viviparous vertebrates in general [103–106]. Furthermore, the view that endotheliochorial and epitheliochorial placentation represent extreme points along a common underlying genetic axis of variation suggests that studies of species bearing less-invasive forms of placentation, such as kangaroo rats, tree shrews and some insectivores, may provide useful models of the molecular control of maternal–fetal interactions and could yield important insights into the mechanisms underlying pre-eclampsia and other human disorders of placentation. More generally, these results suggest that disease-related ontogenetic changes can genetically recapitulate large-scale phylogenetic shifts in mammalian morphology, such that development and diversification share a common genetic basis.

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