scientific reports

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Extrauterine support of pre-term lambs achieves similar transcriptomic profiling to late pre-term lamb brains

Jennifer L. Cohen[1,2,3,4]✉, Felix De Bie[2], Angela N. Viaene[5,6], Nicholas O'Grady[7], Stefan Rentas[8], Barbara Coons[2], James K. Moon[2], Eric E. Monson[9], Rachel A. Myers[7], Jennifer M. Kalish[3,4] & Alan W. Flake[2]

Our group has developed an extra-uterine environment for newborn development (EXTEND) using an ovine model, that aims to mimic the womb to improve short and long-term health outcomes associated with prematurity. This study's objective was to determine the histologic and transcriptomic consequences of EXTEND on the brain. Histology and RNA-sequencing was conducted on brain tissue from three cohorts of lambs: control pre-term (106–107 days), control late pre-term (127 days), and EXTEND lambs who were born pre-term and supported on EXTEND until late pre-term age (125–128 days). Bioinformatic analysis determined differential gene expression among the three cohorts and across four different brain tissue sections: basal ganglia, cerebellum, hippocampus, and motor cortex. There were no clinically relevant histological differences between the control late pre-term and EXTEND ovine brain tissues. RNA-sequencing demonstrated that there was greater differential gene expression between the control pre-term lambs and EXTEND lambs than between the control late pre-term lambs and EXTEND lambs (Supplemental Figs. 1 and 2). Our study demonstrates that the use of EXTEND to support pre-term lambs until they reach late pre-term gestational age results in brain tissue gene expression that more closely resembles that of the lambs who reached late pre-term gestation within their maternal sheep's womb than that of the lambs who were born prematurely.

Keywords Prematurity, Brain development, RNAsequencing, Gene set enrichment analysis, Artificial womb

Preterm delivery is estimated to impact close to 15 million births globally[1]. Extreme pre-term delivery of human infants can lead to many short and long-term health sequelae that increase morbidity and mortality. While the mortality associated with pre-term birth has declined, the neurocognitive and neurodevelopmental morbidity associated with preterm birth remains a significant concern[2,3]. Studies cite approximately 25% of premature individuals having long-term neurological morbidity, with the earlier gestation pre-term survivors having more substantial involvement[4,5]. The neurodevelopmental outcomes include cerebral palsy[6], ADHD and attention difficulties[7,8], autism spectrum disorder[9–11], schizophrenia and other psychiatric diagnoses[12,13], and cognitive impairment[3].

What has been termed 'encephalopathy of prematurity' is due to a combination of periventricular leukomalacia (cerebral white matter axonal disease) and decreased volume of neuronal structures (thalamus, basal ganglia, cerebral cortex, and cerebellum)[14]. At the cellular level, pre-term birth-related brain insults impact neurons, astrocytes, microglia, and oligodendrocyte precursor cells[15]. These cell types play various roles in the excitotoxic insults, inflammation, release of cytokines, and increased metabolic demands that lead to such pre-

[1]Department of Pediatrics, Division of Medical Genetics, Duke University, 905 S. Lasalle Street, Durham, NC 27710, USA. [2]Department of Surgery, Division of General, Thoracic, and Fetal Surgery, Children's Hospital of Philadelphia, Philadelphia, PA, USA. [3]Department of Pediatrics, Division of Human Genetics, Children's Hospital of Philadelphia, Philadelphia, PA, USA. [4]Departments of Pediatrics and Genetics, The Perelman School of Medicine at the University of Pennsylvania, Philadelphia, PA, USA. [5]Department of Pathology and Laboratory Medicine, Children's Hospital of Philadelphia, Philadelphia, PA, USA. [6]Department of Pathology and Laboratory Medicine, University of Pennsylvania Perelman School of Medicine, Philadelphia, PA, USA. [7]Department of Medicine, Duke University, Durham, NC, USA. [8]Department of Pathology, Duke University, Durham, NC, USA. [9]Center for Data and Visualization Sciences, Duke University Libraries, Durham, NC, USA. ✉email: jennifer.L.Cohen@duke.edu

term birth-related insults[15]. Prior data suggest that developmental glial populations play a key role in preterm brain injury[16–19] and there are certain genes and pathways enriched in the preterm brain that may explain the observed alterations in cortical structure development[19]. Pre-term birth interrupts the precise timing of gene transcription that allows for appropriate cortical patterning during prenatal development[19].

Studies have postulated that the link between preterm birth and neurodevelopmental impairment may be due to changes in the epigenome and variation in DNA methylation[15,20]. For instance, a study looking at preterm vs. term neonates' DNA methylation signature (using buccal cells) found alterations in genes that may be important in neural development[21]. One gene, *SLC7A5*, was found to be differentially methylated in pre-term birth neonates[21]. Mutations in this gene have been associated with autism spectrum disorder and a mouse model knockout of this gene produces a behavioral phenotype[22]. Additionally, genes associated with schizophrenia, bipolar disorder (*SLC1A2* and *APOL1*, respectively) and altered social processing (*NPBWR1*) were differentially methylated[21]. Pre-term birth is often associated with maternal separation and the gene *NR3C1* has been shown to have increased methylation in pre-term infants after 4 days or more of maternal separation[23] or in the case of extreme maternal stress[24].

Prior human studies that investigated cord blood and fetal brain and lung tissue have shown that DNA methylation and unique differentially methylated regions are associated with gestational age[25]. Differentially methylated regions implicate genes involved in embryonic development, inflammation, and the innate immune system[25–27]. Another study investigated gene and pathway enrichment to understand alterations in cortical structure development occurring in preterm birth and identified regional expression of genes to highlight the genes expressed in glial cell populations during time points in development that are critical and most impacted by pre-term birth[19]. A study investigated the saliva of 258 neonates (buccal epithelium is of ectodermal origin like the brain) to test the impact of gestational age on brain development by linking differential methylation with functional analyses of white matter brain connectivity and found enrichment in gene ontology terms related to cell-cell contacts and cell-extracellular matrix contacts[20]. In that study, DNA methylation showed widespread differences in relation to gestational age at birth, which also corroborates prior DNA methylation investigations in fetal brain tissue[28]. Interestingly, another study investigating DNA methylation from blood of pre-term birth neonates found that while there are alterations in DNA methylation in these individuals, not all methylation changes appear to be permanent when re-investigated in the same individuals at 18 years of age[29]. A conclusion that can be drawn from this study is the investigation of epigenetic changes related to pre-term birth in neonates may be developmentally timed and tissue dependent. Thus, evaluating epigenetic factors that are related to neurodevelopmental outcomes in pre-term birth may be better served by direct testing of brain tissue.

To limit the negative health sequelae of prematurity, our group has developed an extra-uterine environment for newborn development (EXTEND) using an ovine model, that aims to mimic the womb in an attempt to improve short and long-term health outcomes[30]. The purpose of our study here was to determine, based on RNA sequencing (RNA-seq) analysis and histology from ovine brain tissue, if the premature lambs supported on the EXTEND system are more similar to the lambs delivered prematurely who were not supported on EXTEND (*Pre-term control*), or more similar to the lambs delivered at late pre-term gestation (*Late Pre-term control*) whose degree of brain maturation is considered comparable to brain maturation of full-term human infants. While our group has previously shown the physiologic benefits of the EXTEND model[30], this work is the first to investigate the transcriptomic sequelae of the brain when exposed to the extracorporeal support system. The transcriptomic analysis (and thus, the epigenetic changes occurring at the level of the brain) may serve as a surrogate to begin understanding what, if any, neurodevelopmental sequelae may result from supporting extreme prematurity with an artificial womb.

Results

The demographics of the three cohorts of ovine subjects are reported in Table 1. Gestational age of EXTEND lambs ranged from 105 to 110 days at birth and their time on EXTEND ranged from 20 to 22 days leading to a range of 125–128 days at necropsy. Pre-term control lambs ranged from 106 to 107 days gestational age

Category	Animal name and number	Surgery date	Breed	Sex	EGA at cannulation	Weight at cannulation	Duration on EXTEND (days)	EGA at necropsy (days)
EXTEND	2798L1	10/17/2018	Suffolk	F	110	2.38	16[a]	126
	5016L1	01/29/2019	Suffolk	F	106	1.32	22	128
	0270L1	02/27/2019	Suffolk	M	107	1.96	20[b]	127
	0319L1	03/12/2019	Suffolk	F	106	2.36	21	127
	3596L1	03/21/2019	Suffolk	F	105	1.43	20	125
Late pre-term control	0847	04/16/2019	Suffolk	M	NA	NA	NA	127
	0043	04/16/2019	Suffolk	M	NA	NA	NA	127
Preterm control	2933	03/22/2019	Suffolk	F	NA	NA	NA	107
	15053	04/04/2019	Suffolk	M	NA	NA	NA	106

Table 1. Demographic data of ovine subjects. *F* female, *M* male, *EGA* estimated gestational age, *NA* not applicable. [a]Died of sepsis (bacillus found in EXTEND biobag "amniotic fluid" culture). [b]Sildenafil dose finding study (dosed twice), cord bleed with need for transfusion followed by transfusion reaction and death.

at necropsy; and Late Pre-term control lambs were 127 days gestational age at necropsy. While 145 days is considered full-term gestation for lambs, our controls (Late Pre-term) were harvested at 127 days gestational age since the EXTEND animals required delivery following approximately 3 weeks on the circuit based on the ongoing protocol at the time of these experiments. The EXTEND model aims to bridge the crucial time between human gestational age of 24–28 weeks. For the EXTEND model, we based gestational age equivalency on lung development as follows: 102–105 day gestational age lambs are equivalent to human 24 weeks gestational age (in terms of lung development). Conversely, brain maturity largely differs between humans and sheep. Lamb brain maturity is more advanced than humans at birth[31]. 127 days gestation for lambs is closer to human full-term brain development (as compared to full-term lamb gestation at 145 days, at which time the lambs' brain development would be comparatively over-mature).

Histology results

Histologic examination of the hippocampus, motor cortex, basal ganglia, and cerebellum regions revealed no significant structural abnormalities and no histologic signs of brain injury in any of the animals (Fig. 1). Pre-term control brains had slightly thinner cortices and their cerebellums contained a thicker external granular cell layer and thinner internal granular cell layer, in keeping with gestational age. Late Pre-term control brains and EXTEND brains showed no histologically appreciable developmental differences.

RNA-sequencing results

27,054 genes filtered down to 16,332 were analyzed among the RNA sequencing data collected from the various brain tissue sections for each lamb included in the study. One sample, a control preterm cerebellum sample from #2933 was excluded from analysis due to a low number of read counts (QC criteria). The PCA plots calculated within each brain type and across the three groups of ovine subjects—Pre-term control, Late Pre-term control, and EXTEND—demonstrates clustering of Late Pre-term control and EXTEND groups, particularly in the cortex and hippocampus (Fig. 2).

Differential gene expression analysis

The number of differentially expressed genes between the three groups of ovine subjects, controlling for brain section are shown in Table 2. This analysis showed that 2050 genes were significantly downregulated in the Pre-term control cohort compared to the EXTEND lambs and 2893 genes were significantly upregulated in the Pre-term control cohort compared to the EXTEND lambs. This was in contrast to the smaller number of genes significantly downregulated in the Late Pre-term control ($n=147$) and significantly upregulated in the Late Pre-term control group ($n=184$) compared to the EXTEND lambs. Therefore, when compared to the EXTEND group and controlling for brain section, 4943 differentially expressed genes were found in the Pre-term control group, but only 331 differentially expressed genes were found in the Late Pre-term control group.

Differential gene expression analysis demonstrated that 199 genes were differentially expressed in the EXTEND group compared to both the Late Pre-term and Pre-term control group (Supplemental Table 1) and 132 genes (Supplemental Table 2) were uniquely differentially expressed in only the Late pre-term control group compared to the EXTEND group. This contrasts with the 4744 genes (Supplemental Table 6) uniquely differentially expressed in the Pre-term control group (Fig. 3; Supplemental Fig. 3).

Differential gene expression analysis was also conducted to determine the number of differentially expressed genes among the three groups of lambs and separated by brain tissue type (Table 2). These data demonstrated that when each brain tissue was examined separately, the only brain tissue type with differential expression between EXTEND and Late Pre-term control lambs was the basal ganglia ($n=65$ genes), (Supplemental Table 3). Of note, there were no significant differentially expressed genes between EXTEND and Pre-term control lambs in the basal ganglia.

Using Ensembl BioMart for those ovine genes that had orthologous human gene names, the phenotype description, Mendelian Inheritance of Man (MIM) description and accession were analyzed for the 199, 132, and 65 gene lists, respectively (Supplemental Fig. 3). Our analysis included phenotypic descriptions, as available in Ensembl BioMart, for these three smaller differentially expressed gene lists (Supplemental Tables 1–3). Of the genes that were differentially expressed in both Pre-term and Late Pre-term controls compared to EXTEND lambs, there were only a handful of genes that were differentially expressed in opposite directions (Supplemental Table 1) and among those, only one gene with an associated phenotypic description: *BRF1*, a gene associated with cerebellar-facial-dental syndrome was upregulated in Pre-term lambs and downregulated in Late pre-term lambs compared to EXTEND. The gene's associated condition is ausotomal recessive and includes cerebellar hypoplasia, thin corpus callosum, and enlarged ventricles.

Gene set enrichment analysis (GSEA)

GSEA was conducted using Gene Ontology – Cellular Components (GO-CC) and Kyoto Encyclopedia of Genes and Genomes (KEGG)[32–34] to compare the enriched pathways in the Pre-term control cohort vs. EXTEND. Figure 4 shows the twenty most significant pathways that are activated and suppressed in the Pre-term control compared to EXTEND. Regarding GO-CC pathways, mitochondrial functions (mitochondrial inner membrane, mitochondrial envelope, mitochondrial membrane, and mitochondrion) and cell junction appear to be suppressed in the pre-term lambs compared to EXTEND, while ribosomal functions (ribosome, ribosomal subunit) and collagen pathways appear to be activated in the Pre-term lambs compared to the EXTEND lambs (Supplemental Table 4). Regarding KEGG network plots, activated pathways in the Pre-term lambs include TGF-beta signaling pathway, axon guidance, cell cycle, Fanconi anemia pathway, and ECM-receptor interaction. Suppressed pathways in the Pre-term lambs include oxidative phosphorylation, cardiac muscle contraction, carbon metabolism, vascular smooth muscle contraction, glycerophospholipid metabolism,

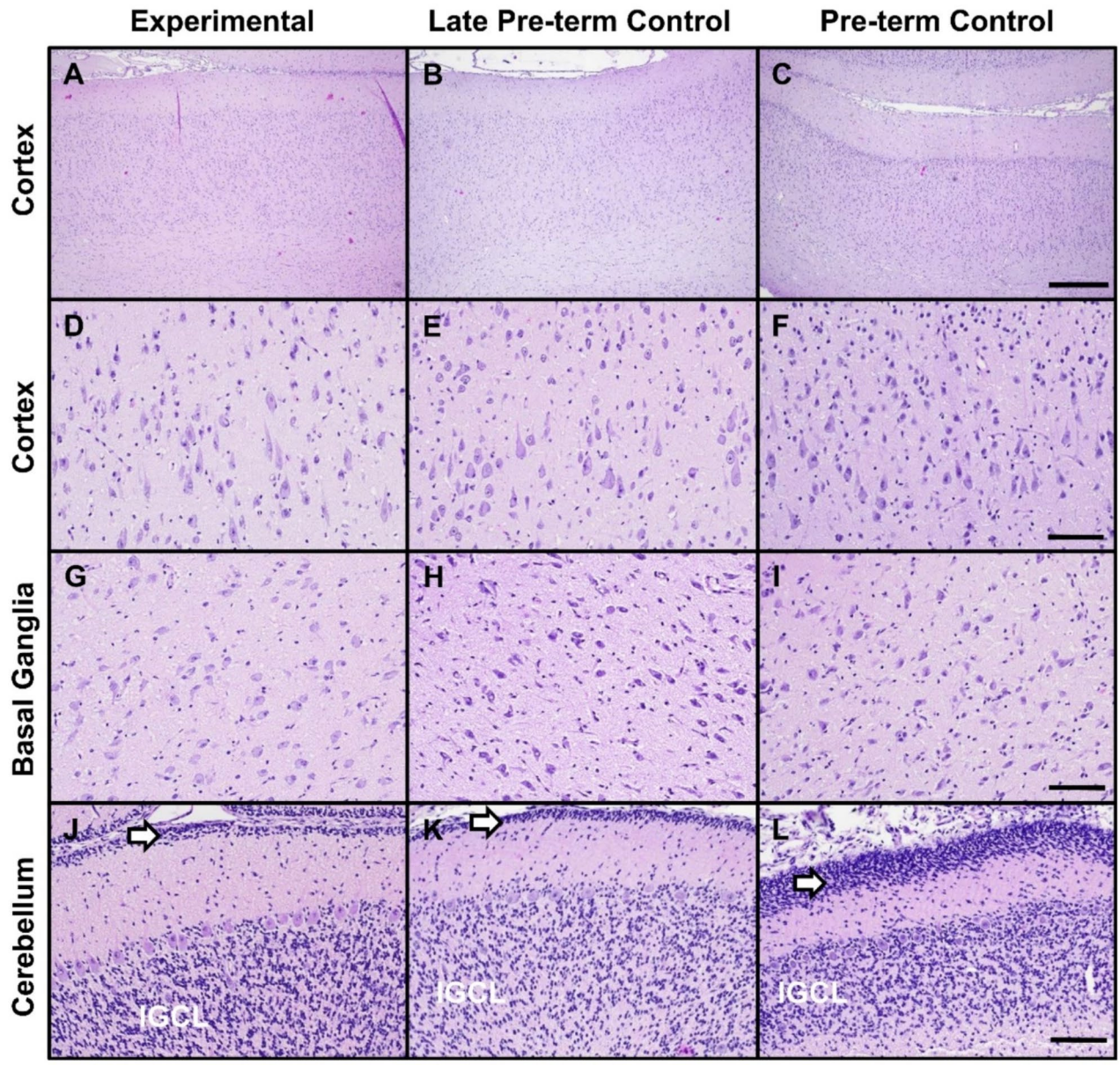

Fig. 1. Histologic features of sheep brains. Low magnification views of cortex for EXTEND, late pre-term control, and pre-term control animals (**A**–**C**, respectively) show normal cortical architecture. The cortex was slightly thinner in the pre-term controls, in keeping with gestational age. Higher magnification views of cortex (**D**–**F**) and basal ganglia (**G**–**I**) show neurons without any significant hypoxic/ischemic changes. The cerebellum (**J**–**L**) also did not show hypoxic/ischemic changes and was normally developed for age. The external granular cell layer (white arrows) was thicker in pre-term controls with a thinner internal granular cell layer (IGCL), consistent with gestational age. All images are hematoxylin and eosin stains. Scale bar in C corresponds to 0.5 mm and applies to images (**A**–**C**) (40x magnification). Scale bars in (**F**, **I**, **L**) correspond to 0.1 mm, and apply to images (**D**–**L**) (200× magnification).

diabetic cardiomyopathy, HIF-1 signaling pathway, chemical carcinogenesis – reactive oxygen species, folate biosynthesis, and numerous neurodegenerative diseases (Supplemental Table 5).

GSEA was conducted using GO-CC and KEGG to compare the enriched pathways in the Late Pre-term control cohort vs. EXTEND. Figure 5 shows the pathways that are activated and suppressed in the Late Pre-term control lambs compared to EXTEND. Regarding GO-CC pathways, cilia pathways are suppressed in the Late Pre-term control lambs (axoneme, ciliary plasm, 9 + 2 motile cilium, motile cilium, and cilium). Activated pathways in the Late Pre-term controls include complex of collagen trimers, collagen trimer, and supramolecular polymer and supramolecular complex (Supplemental Table 4). Regarding KEGG network plots, activated pathways in the Late Pre-term control lambs include linoleic acid metabolism, protein digestion and absorption, cell cycle,

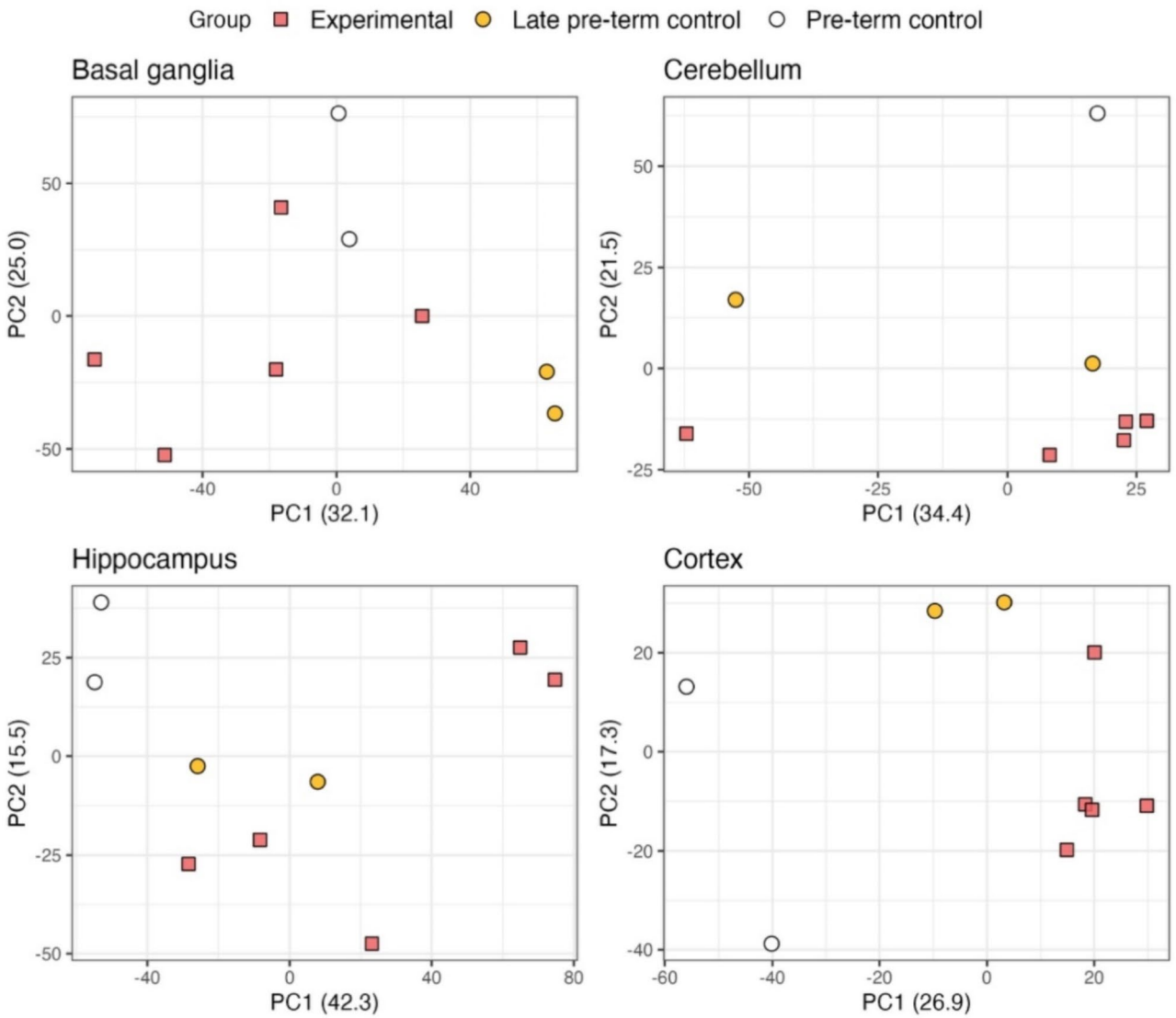

Fig. 2. Principal component analysis (PCA). PCA was performed within each brain section and stratified by experimental group. Principal components (PC) one and two are plotted on the x and y axis respectively. Legend: 'Experimental' refers to the EXTEND cohort.

ECM-receptor interaction, and chemical carcinogenesis – reactive oxygen species. Of note, neurodegenerative disease pathways are absent from this comparison (Supplemental Table 5).

GSEA was conducted using GO-CC and KEGG to compare the enriched pathways in the Late pre-term control cohort vs. EXTEND within the basal ganglia. Figure 6 shows the pathways that are activated and suppressed in the Late Pre-term control basal ganglia compared to EXTEND. Regarding GO-CC pathways, suppressed pathways include cilia pathways (9 + 2 motile cilium, sperm flagellum, axoneme, ciliary plasm, motile cilium, cilium). Activated pathways in the Late Pre-term control basal ganglia include mitochondrial respirasome, respiratory chain complex, inner mitochondrial membrane protein complex, mitochondrial protein-containing complex, and collagen trimer (Supplemental Table 4). Regarding KEGG network plots, activated pathways in the Late Pre-term control basal ganglia include the citrate cycle, linoleic acid metabolism, oxidative phosphorylation, diabetic cardiomyopathy, protein digestion and absorption and melanogenesis. Interestingly, the neurodegenerative pathways (Alzheimer disease, Prion disease, Parkinson disease) are activated in the Late Pre-term control group basal ganglia compared to EXTEND (Supplemental Table 5).

Utilizing a review of the available literature[15,19,20,35,36] to specifically investigate genes and pathways that have been implicated in the neurologic development of pre-term infants and pre-term birth in general, we noted statistically significant differential gene expression and differential gene pathway expression among these genes and pathways between the EXTEND and control groups, with the majority of differential expression seen between Pre-term control and EXTEND (Supplemental Figs. 4 and 5).

	Comparison	Down	Up	NotSig
All brain sections	EXTEND vs. Late Pre-term control	147	184	16,001
	EXTEND vs. Pre-term control	2050	2893	11,389
Within basal ganglia	EXTEND vs. Late Pre-term control	41	24	16,267
	EXTEND vs. Pre-term control	0	0	16,332
Within cerebellum	EXTEND vs. Late Pre-term control	0	0	16,332
	EXTEND vs. Pre-term control	122	66	16,144
Within hippocampus	EXTEND vs. Late Pre-term control	0	0	16,332
	EXTEND vs. Pre-term control	117	66	16,149
Within motor cortex	EXTEND vs. Late Pre-term control	0	0	16,332
	EXTEND vs. Pre-term control	656	735	14,941

Table 2. Number of differentially expressed genes when compared to EXTEND group. The first row, "all brain sections" displays the number of up or down regulated significantly expressed genes, evaluating experimental groupings, while controlling for brain sections. The remaining rows display the number of up or down regulated significantly expressed genes, within each brain section. Significance defined as adjusted p. value ≤ 0.05.

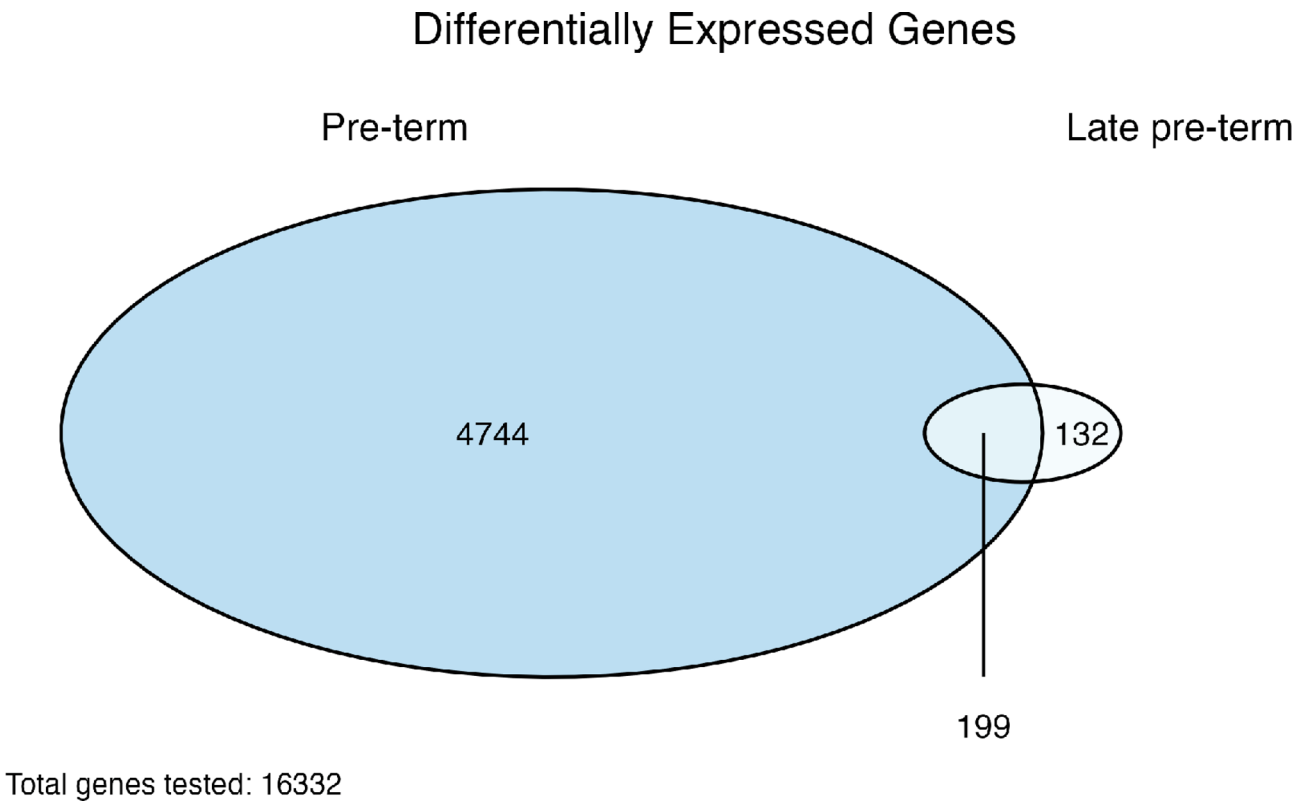

Fig. 3. Venn diagram of differentially expressed genes. The Venn diagram displays that the Late Pre-term control cohort has fewer genes differentially expressed than the Pre-term control cohort when compared to the EXTEND group. The plot displays the number of differentially expressed genes found in each cohort. Each group (Pre-term and Late Pre-term control) was compared to EXTEND and this plot displays the total number of differentially expressed genes, and which of those genes are unique to each group ($n = 4744$) and ($n = 132$) and which are present in both ($n = 199$).

Discussion

Our study used an ovine model of prematurity to investigate histological and transcriptional differences in four sections of brain tissue among three cohorts of infant lambs—Pre-term control, Late Pre-term control, and Pre-term EXTEND lambs who had been supported on EXTEND until reaching equivalent late pre-term gestational age. Histological features of the lambs' brains demonstrated that there are no concerning differences among the EXTEND lambs when compared to the Late Pre-term control lambs. Overall, there are no histologic signs of brain injury (including hypoxic-ischemic changes) or structural abnormalities noted in any of the investigated

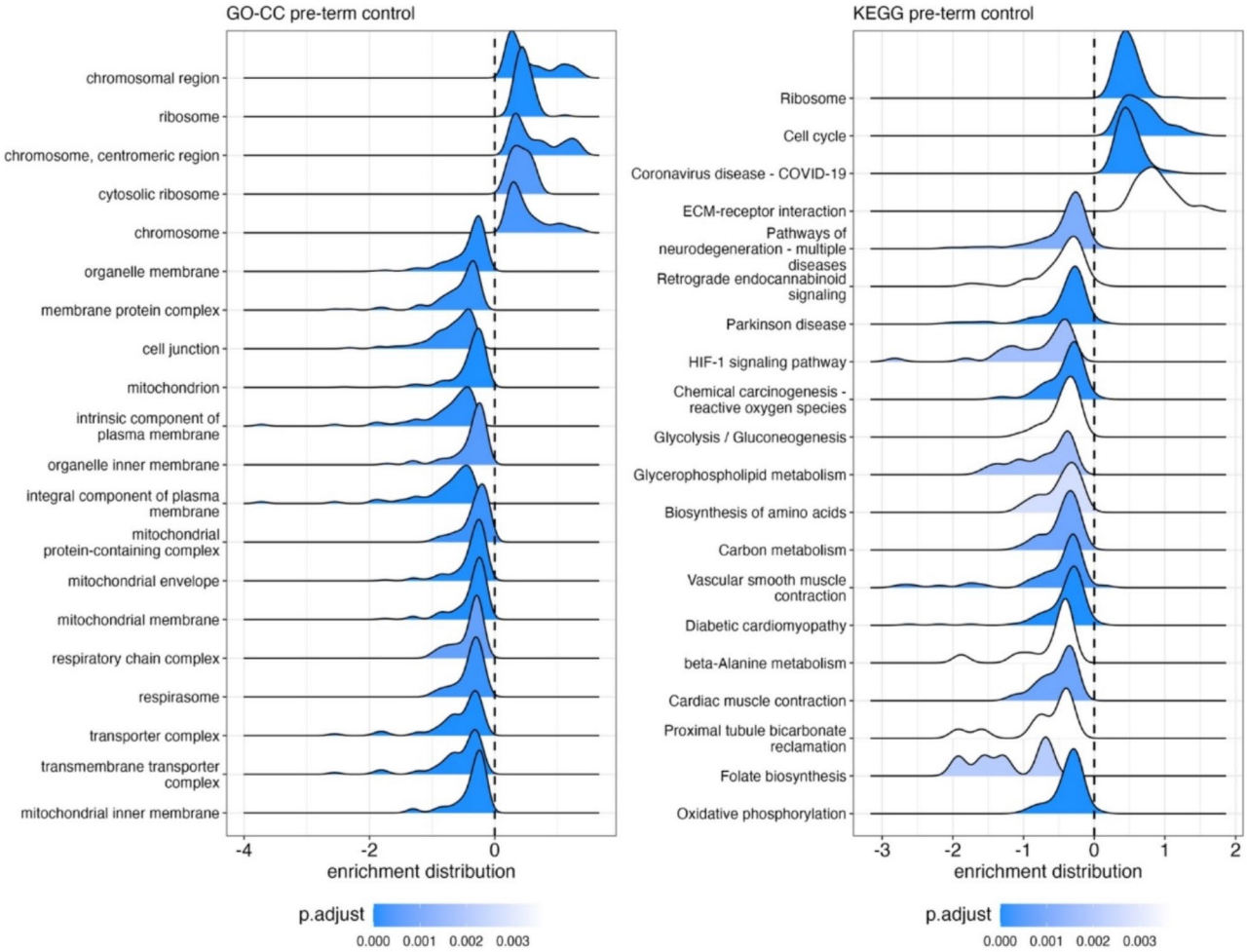

Fig. 4. GO-CC and KEGG[32–34] Pre-term control vs. EXTEND gene set enrichment analysis. We performed gene set enrichment analysis on the Pre-term control vs. EXTEND groups. Ridge plots of results from the gene ontology cellular components and KEGG databases show either suppressed or activated pathways. P-values are FDR adjusted.

brain sections. There are some subtle differences between the Pre-term animals and the EXTEND and Late Pre-term controls that are expected for the differences in gestational age (thinner cortex as well as thicker external granular cell layer and thinner internal granular cell layer). All the lambs' brains were normally developed for their gestational age and of note, the Late Pre-term controls and EXTEND animals look similar, histologically. RNA sequencing of the three cohorts of lambs' brains demonstrated that overall, based on the number of differentially expressed genes, a greater difference was noted between Pre-term control and EXTEND lambs, compared to the difference between the Late Pre-term control and EXTEND lambs (Table 2). Since the difference between Pre-term control and EXTEND lambs may be a function of gestational age itself, it is important to note that the absolute number of differentially expressed genes between the EXTEND and Late Pre-term control groups was a small proportion of the total number of genes investigated: 331/16,332 genes, (2%), which is an encouraging sign that EXTEND is allowing for appropriate brain maturation and development on a transcriptional level.

From the small subset of genes found to be uniquely differentially expressed between the Late Pre-term control and EXTEND lambs, the genes with known human disease phenotypic descriptions are reported in Supplemental Table 2 and Supplemental Fig. 3. These genes appear to represent the "gap" that EXTEND lambs may still need to fill in order to be most similar to age-matched Late Pre-term control lambs. When analysis was conducted within each brain section, three of the four brain sections studied only showed differentially expressed genes between Pre-term control lambs and EXTEND lambs (Table 2). The basal ganglia, however, had differentially expressed genes only in the Late Pre-term control lambs (compared to EXTEND). This observation may be driving the remaining transcriptional "gap" between EXTEND lambs and Late Pre-term control lambs that we observed among the overall data analysis. The basal ganglia references a group of subcortical nuclei that play a role in motor control and motor learning but also have a role in executive functioning and emotions[37]. Some of the genes differentially expressed between Late Pre-term control basal ganglia and EXTEND are associated with a neurologic human phenotype (Supplemental Table 3).

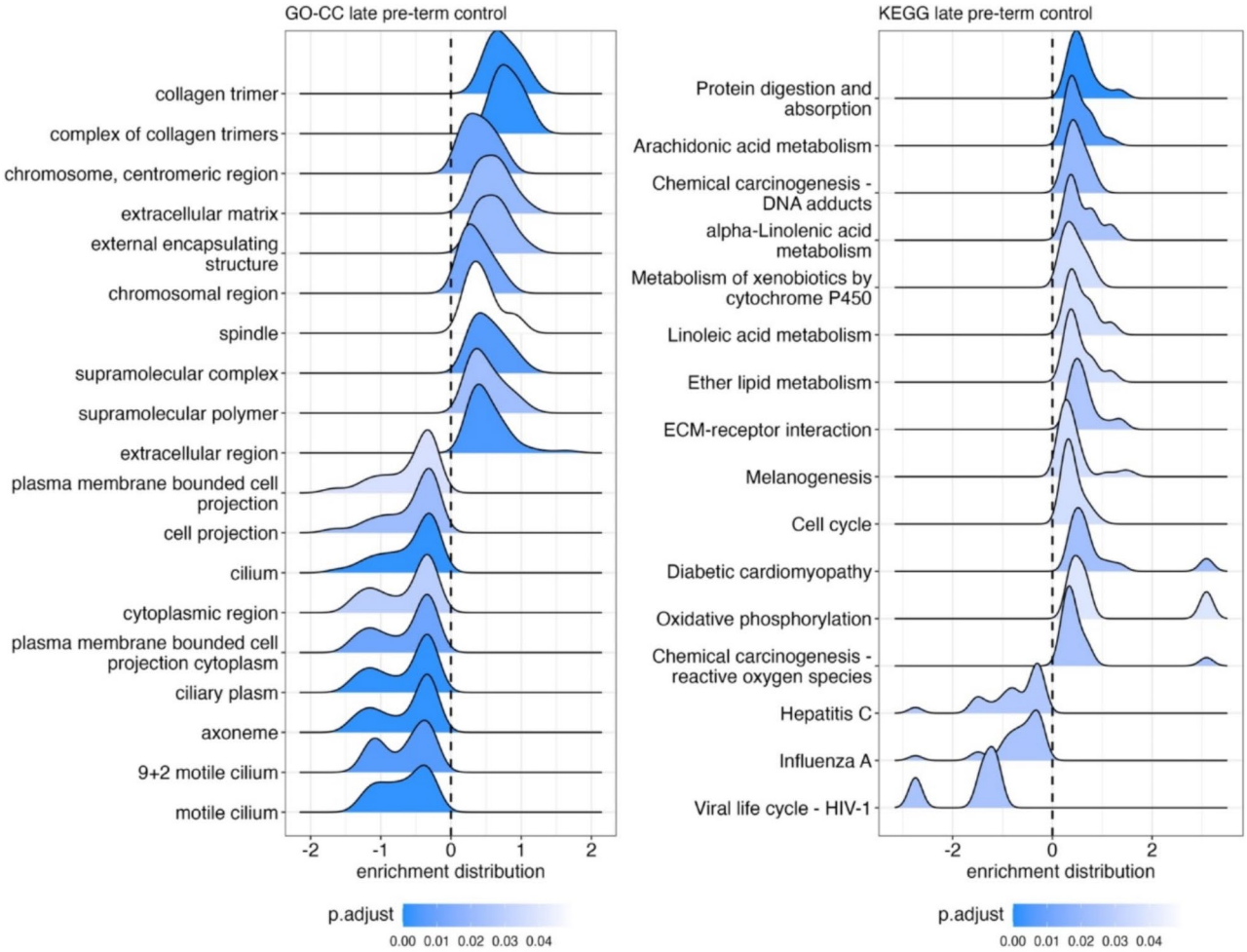

Fig. 5. GO-CC and KEGG[32–34] Late Pre-term control vs. EXTEND gene set enrichment analysis. We performed gene set enrichment analysis on the Late Pre-term control vs. EXTEND groups. Ridge plots of results from the gene ontology cellular components and KEGG databases show either suppressed or activated pathways. P-values are FDR adjusted.

When we look more closely at the specific gene pathways and individual genes where differential expression is noted among the three lamb cohorts, the following pathways and gene families are worth highlighting: inflammatory and immunologic pathways, collagen pathways, metabolic and biosynthetic pathways, folate biosynthesis, oxidative phosphorylation, HIF-1 signaling, cell-cell contacts, cilia pathways, and neurologic disease (Figs. 4, 5 and 6, Supplemental Fig. 4 and Supplemental Fig. 5).

In cross-referencing genes found to be implicated in pre-term birth in the literature[35], our study found overlap with genes uniquely differentially expressed among the Pre-term control cohort compared to EXTEND (Supplemental Fig. 5, Supplemental Table 6). Some of those genes fell into the category of inflammatory and immunologic pathway, such as toll-like receptor genes (*TLR7*), interleukins (*IL1A)*, and tumor necrosis factor genes (i.e. *TNFAIP3*)[35]. Since prior studies have shown both pro- and anti-inflammatory cytokine pathways to be differentially expressed in pre-term birth, it is interesting to note that the TGF-beta signaling pathway (a type of cytokine) was statistically significant among the KEGG pathway gene set enrichment analysis between Pre-term control and EXTEND. Anti-inflammatory cytokine genes like *IL13*[35] that have been shown to have SNPs associated with pre-term birth seemed to be relevant in our study as well, with *IL13RA1* showing unique differential expression between Pre-term and EXTEND lambs (Supplemental Table 6). Other genes fell into the category of metabolic and biosynthetic pathways, critical to embryonic development (i.e. *IGF1R*, *IGF2BP2*), or prostaglandin synthesis and function (*PTGS1*, *PTGES*, *PTGES2*, *PTGS2*)[35]. Single nucleotide polymorphisms in collagen biosynthesis genes have also been found to have association with pre-term birth[35,38,39]. In our study, multiple collagen genes were differentially methylated between the Pre-term control and EXTEND cohorts (Supplemental Table 6) and interestingly, other collagen genes were differentially methylated in both Pre-term control and Late Pre-term control when compared to EXTEND (Supplemental Table 1). GO-CC gene set enrichment showed three collagen pathways to be enriched in the Pre-term control vs. EXTEND cohorts and two collagen pathways to be enriched in the Late Pre-term control vs. EXTEND. Lastly, genes discovered to be genome-wide significant on genome-wide association studies (GWAS) investigating pre-term birth phenotypes

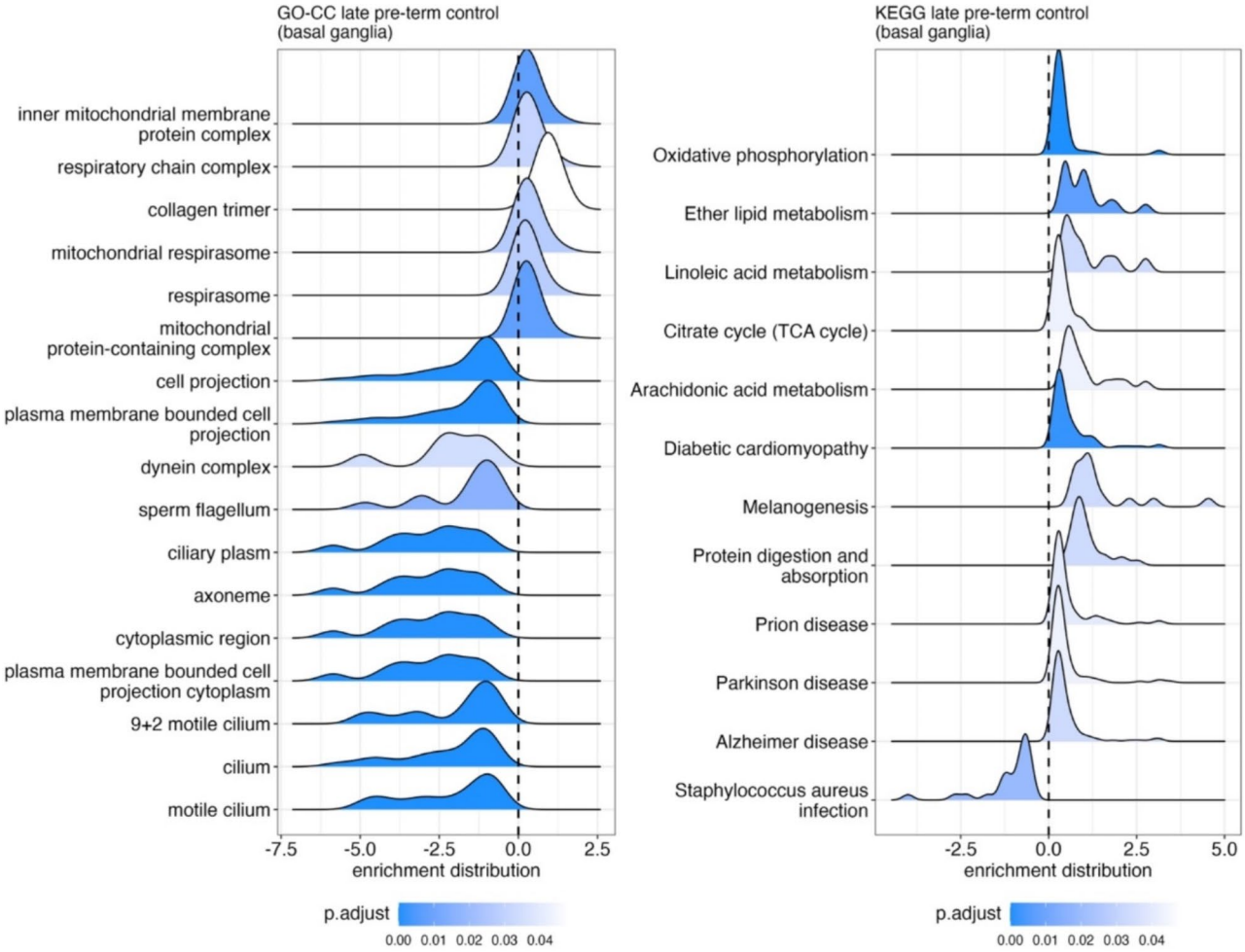

Fig. 6. GO-CC and KEGG[32–34] Late Pre-term control (basal ganglia) vs. EXTEND gene set enrichment analysis. We performed gene set enrichment analysis on the Late Pre-term control (basal ganglia) vs. EXTEND groups. Ridge plots of results from the gene ontology cellular components and KEGG databases show either suppressed or activated pathways. P-values are FDR adjusted.

were found to overlap with differentially expressed genes in our Pre-term cohort compared to EXTEND and not found in the Late Pre-term cohort: *FAF1*, *CKAP2L*, *RUVBL1*, *TLR7*, *HIVEP3*, *PMP22*, *LNPEP*, *IL1A*, *COL27A1*[35,40–42]. While these gene families and pathways are more associated with pre-term birth itself, rather than specific neurologic morbidity, these findings provide verification that our Pre-term control lambs appear to have a transcriptomic profile that aligns with premature human genomic studies.

The literature on pre-term birth and genetics notes single nucelotide polymorphisms in genes of folate and methionine synthesis to be associated with spontaneous pre-term birth[35,43]. Folate is a known critical component in prenatal/fetal neurologic development[44]. It is an important cofactor in C1 metabolism and indirectly important for DNA methylation, which is believed to be one of the epigenetic mechanisms contributing to fetal programming and brain development[44]. Given its importance, folate supplementation was adminstered in the total parenteral nutrition provided through the extracorpeal system, EXTEND. In our study, folate biosynthesis was noted to be suppressed in the Pre-term control lambs compared to EXTEND lambs and importantly, the folate biosynthesis pathway was not found to be significantly activated or suppressed in either the Late Pre-term lambs in general, or the Late Pre-term lambs' basal ganglia when compared to EXTEND lambs. It appears therefore, that regarding folate biosynthesis, the EXTEND lambs' brains behave similarly to the Late Pre-term lambs, which is an encouraging observation.

α-ketoglutarate and other components of the TCA cycle are required for appropriate activity of the α-ketoglutarate dependent dioxygenases, which are thought to be critical in pre-term birth-related brain injury[15]. It is therefore notable that the Pre-term control lambs have differential expression of the TCA cycle pathway and *OGDH* (the gene that encodes for α-ketoglutarate) when compared to the EXTEND lambs. Additionally, the HIF-1 signaling pathway is downregulated in Pre-term control lambs compared to EXTEND. Since HIF-1 helps respond to low oxygen levels with compensatory mechanisms[15], it is an encouraging sign that the EXTEND (vs. Late Pre-term) lambs are not experiencing this downregulation seen in the (EXTEND vs.) Pre-term control

lambs, even if the primary reason for this is that the increased gestational age of the EXTEND lambs (compared to the Pre-term lambs) leads to more similarity between the EXTEND and the Late Pre-term lambs.

Gene ontology (GO) analysis in our study found that 'cell junction' was significant in the gene set enrichment between Pre-term control lambs and EXTEND lambs. Previous literature demonstrated that cell junction was one of 14 enriched gene ontology terms relating to cell-cell contacts in a study investigating how DNA methylation differentiation in pre-term human neonates relates to functional metrics of white matter brain disease (diffusion MRI)[20].

Cilia are critical in disease processes relevant to prematurity including bronchopulmonary dysplasia, retinopathy of prematurity, and intraventricular hemorrhage[45,46], and cilia play a role in kidney development, eye development, hearing, and sperm function. The cilia pathways were seen to be suppressed in the Late Pre-term control lambs compared to the EXTEND lambs; this finding was recapitulated when looking specifically at the basal ganglia in the Late Pre-term control lambs compared to EXTEND lambs' basal ganglia. *CFAP298* (OMIM #615500) and *RSPH1* (OMIM #615481), two genes associated with primary ciliary dyskinesia were uniquely downregulated in Late Pre-term lambs compared to EXTEND lambs. This finding will be important to investigate particularly with future studies in tissues such as the lungs.

Utilizing a prior study that identified genes expressed by oligodendrocytes and microglia associated with neuroimaging differences in the human pre-term cortex[19], our study identified overlapping genes that were differentially expressed in the Pre-term control cohort when compared to the EXTEND cohort, yet were not differentially expressed in the Late Pre-term control cohort compared to the EXTEND cohort (Supplemental Fig. 5). Some highlighted genes and their function are: *CASP3*, *CDKN1A*, *GADD45B* (apoptotic mechanisms with correlation to imaging differences in the human pre-term cortex), *S100B* (MyD88 and Toll-like receptor signaling cascades), *CIT*, *RHOB* (Rho-GTPase signaling pathway)[19]. Another study investigated DNA methylation in relation to brain dysmaturation in pre-term infants and investigated human saliva to conduct an epigenome-wide associate study[20]. Two genes noted to be among the most significant differentially methylated probes in relation to gestational age at birth were also noted to be differentially expressed in the Pre-term control cohort when compared to the EXTEND cohort; of note these genes were not differentially expressed in the Late Pre-term control cohort compared to the EXTEND cohort: *LOXL4*, *INTS1*[20]. *INTS1* (OMIM #611345) is associated with a human phenotype of neurodevelopment disorder with cataracts, poor growth, and craniofacial differences. Additional enriched pathways comparing Pre-term control to EXTEND that may be of interest given their relation to neurons and neurologic function include: Axon guidance, serotonergic synapse, pathways of neurodegeneration, and postsynapse (Supplemental Fig. 4).

When our data were cross referenced with genes reported in literature to be hypomethylated or hypermethylated in autism spectrum disorder[36], certain genes were differentially expressed in Pre-term controls compared to EXTEND in a concordant direction, while other genes were differentially expressed in a discordant direction from the directionality seen in autism spectrum disorder (Supplemental Fig. 5). These findings that demonstrate unexpected or inconsistent directionality are potential areas for continued investigation as the extracorporeal support model is further developed.

A strength of our study is that the initial agnostic approach to analyzing any statistically significant differential gene and pathway expression eliminates the bias that candidate gene studies experience. A targeted approach, which we also pursued (Supplemental Figs. 4 and 5) is influenced by prior research and therefore unable to identify novel findings. Weaknesses of our study include the small sample size, particularly having only two lambs per control cohort, and the lack of comparator data sets from pre-term brain tissue of other species; however, due to the expense of raising lambs on the EXTEND system, this was deemed a reasonable tradeoff in order to gain insight into gene expression signatures and brain development while on EXTEND. Studies such as ours that examine post-mortem tissue also have the drawback of not being able to examine causality and changes that would otherwise be experienced by the epigenome through continuous environmental interactions throughout life[15]. Longitudinal behavioral studies comparing our three study cohorts would be beneficial to explore these effects. An additional limitation of our study is the lack of a fourth group: a control group that was born pre-term and supported on conventional ventilation for a time equivalent to time supported on EXTEND. Due to the feasibility and resources needed to include this type of control, our laboratory is not currently pursuing this as a study cohort for preclinical experiments of EXTEND. The main goal of our current study was to ensure that no extensive harm was occurring in the EXTEND animals' brains when compared to late pre-term gestation (lambs that continued their gestation within the maternal ewe womb). We conducted RNA sequencing as a systematic molecular confirmation of the histology-based observations, and the results thereof are considered exploratory and descriptive.

Although the differential gene expression observed between the Pre-term control and EXTEND lambs could be a factor of their differing gestational age at necropsy, it is still encouraging to observe that the gene expression between the two groups is indeed different, perhaps reflecting the EXTEND animals' continued appropriate developmental progression expected for age.

In summary, we have shown that overall, lambs supported by EXTEND have more transcriptional similarity to their age-matched Late Pre-term control counterpart and less similarity to a Pre-term control counterpart. Many of the genes associated with neuroimaging differences in the human pre-term brain were also uniquely differentially methylated among our Pre-term control cohort compared to EXTEND, demonstrating that there is likely some correlation between our animal model and human prematurity. In summary, we observed encouraging results regarding differential expression and methylation of genes known to be associated with pre-term birth and neurodevelopment in prematurity based on human studies. The lack of difference between the EXTEND lambs to the Late Pre-term lambs suggests that EXTEND support allows for natural development of these pathways equivocal to a fetus of the same gestational age.

Methods

Animal procedures

The sheep utilized in our study were sourced from May Family Enterprises, Buffalo Mills, PA. All aspects of the cannulation procedure as well as the support and care of lambs on EXTEND have been described in detail elsewhere[30,47,48].

Sex as a biological variable

Four of the five EXTEND lambs were female; the two Late Pre-term control lambs were both male; one of the Pre-term control lambs was female. Sex was not considered as a biological variable in this study due to the limited sample size of subjects studied.

Cannulation procedure

In brief, time-dated pregnant Suffolk ewes, were used at gestational ages (GA) of 105 to 110 days (term gestation is 145 days). At the time of cannulation, fetuses are a neurological development equivalent to a 28- to 30-week pre-term human infant[48–50] and a pulmonary equivalent to 24 weeks gestation pre-term human infant[51]. Under general anesthesia (using isoflurane) and under sterile conditions, five ewes underwent a lower midline laparotomy to expose the gravid uterus and a hysterotomy was made to expose one fetus. Umbilical vein and arteries were cannulated sequentially using cutdown, custom-made cannulas connecting the fetus to the circuit[47]. The pumpless circuit consisted of a low-resistance hollow fiber oxygenator (Quadrox-ID Neonatal Oxygenator, Maquet) with BIOLINE-coated tubing (Maquet). Upon achieving stable hemodynamics, the fetal lambs were weighed and transferred to the EXTEND biobag: a warmed (temperature 38.5–40.5 °C), insulated, sterile, fluid-filled environment with continuously exchanged synthetic amniotic fluid composed of a balanced salt solution[30]. At the conclusion of the cannulation procedure, anaesthetized ewes were euthanized by pentobarbital sodium injection.

Fetal lamb maintenance on EXTEND

As described previously, lambs on circuit were infused with a continuous infusion of heparin (10–400 USP/hour) titrated to an activated clotting time of 160–200 s, and prostaglandin E1 (0.1 µg/kg/minute) to assure ductal patency[30]. Arterial and venous blood were analyzed every 1–8 h for blood gas, electrolyte, and coagulation values (i-Stat System, Abbott Point of Care Inc., Princeton, NJ, USA) and oxygen saturation (Avoximeter 1000E, Accriva Diagnostics, San Diego, CA, USA)[30]. Sweep gas supplied to the oxygenator was a blended mix of medical air, nitrogen and oxygen, titrated to maintain PaO_2 20–30 mmHg, $PaCO_2$ 35–45 mmHg and post-membrane oxygen delivery 20–25 mL/kg/min[47]. Circuit blood flow and pre-/post-oxygenator pressures were continuously recorded (HT110 Bypass Meter and HXL Tubing Flowsensor; Transonic Systems Inc.; LabChart 7, ADInstruments Inc.)[30]. Total parenteral nutrition was administered throughout the duration of fetal incubation as described: amino acids (TrophAmine 10%, titrated to blood urea nitrogen target level 30 mg/dL), lipids (Intralipid 20%, 0.1–0.2 g/kg/day), dextrose (titrated to blood glucose target 30 mg/dL) and iron (1 mg/kg/day)[30]. Lambs were maintained on EXTEND for up to 22 days, with studies terminated before this end-point if circuit flows or physiological parameters were considered incompatible with survival[47].

Necropsy

At GA 127 ± 2 days, fetal lambs were euthanized by a pentobarbital injection. Immediately following, brains were harvested in their entirety, snap-frozen in liquid nitrogen, and stored at -80 °C awaiting analysis. Harvesting brain tissue at 125–128 days gestation is a neurologic development equivalent of 32–34 weeks human pre-term infant[50] and a pulmonary equivalent of 32–35 weeks human pre-term infant[51].

Experimental design

Our study investigated four brain sections per lamb from nine individual lambs (Table 1). Two lambs were delivered pre-term and underwent immediate necropsy (*Pre-term control*); two lambs were delivered at 127 days and underwent immediate necropsy (*Late Pre-term control*); five lambs were delivered pre-term, underwent immediate cannulation and were supported by the EXTEND model and underwent necropsy after reaching a gestational age close to 127 days (gestational age similar to the Late Pre-term control cohort). The four brain sections investigated through both histology and RNA-sequencing were: hippocampus, motor cortex, basal ganglia, and cerebellum. In choosing these four sections we considered those that could be consistently and accurately sampled among immature brains of varying ages, while simultaneously working to survey a broad range of brain functions.

RNA-sequencing

Flash frozen tissue was procured from ovine brain at the time of necropsy and sent to GeneWiz for RNA extraction and RNA-sequencing. Total RNA was extracted from frozen cell pellet and fresh frozen tissue samples using Qiagen RNAeasy Plus Universal mini kit following Manufacturer's instructions (Qiagen, Hilden, Germany). GeneWiz conducted RNA-seq library preparation as follows: A) mRNA enrichment, mRNA fragmentation, and random priming; B) first and second strand cDNA synthesis; C) end repair, 5' phosphorylation, and dA-Tailing; D) adaptor ligation, PCR enrichment, and sequencing.

Histology

After frozen tissue was procured for RNA-sequencing, if residual tissue from the region(s) of interest remained, the tissue was placed in 4% paraformaldehyde for histologic processing. Sampled regions included primary motor and somatosensory cortices, basal ganglia, hippocampus, and cerebellum. After thawing and 24 h of

fixation, tissue was processed, paraffin-embedded, and then cut into 5 µm unstained slides by the Children's Hospital of Philadelphia Pathology Core Laboratory. Slides were subsequently stained with hematoxylin and eosin and examined by a pediatric neuropathologist (A.N.V.).

Bioinformatics analysis methodology and statistics

Sequence processing and alignment

Paired end sequences were assessed for quality using SeqKit[52]. Adapter and poly G/X tails were removed using fastp[53]. Post processing quality assessment is then repeated on the paired-end reads using SeqKit. Sequences were aligned to the ovis aries genome, version 107, using STAR[54]. Reference files are available from Ensembl (https://ftp.ensembl.org/pub/release-107/fasta/ovis_aries/). Gene expression was quantified against the gene transfer format (GTF), v107 annotation file using STAR's '--quantMode GeneCounts' parameter. The reference GTF annotation file is also available from Ensembl (https://ftp.ensembl.org/pub/release-107/gtf/ovis_aries/). Sample-level quantifications from the resulting 'ReadsPerGene.out.tab' file, column 2 for inward unstranded (IU) reads, were combined across all samples to generate the gene count matrix.

Count QC & normalization

Lowly expressed genes were filtered using EdgeR's 'filterByExpr' filtering tool[55]. Spearman correlations across paired samples are generated for the removal of outliers. Normalization factors were generated using EdgeR's 'calcNormFactors', with trimmed mean of M-values (TMM)[55], and log2 counts per million (cpm) transformed. Density plots across raw, filtered, and log cpm normalized data, and principal component analysis (PCA) on log cpm data are generated for study design considerations and removal of outliers. Samples over 3 standard deviations away from the spearman correlation mean, on filtered data, are removed from downstream analysis.

Differential analysis method

Two experimental designs were used for the discovery of differentially expressed genes (DEG). Both designs used EXTEND group values as the reference, and then were set up as follows: (1) Expression values as a function of experimental groups (Pre-term control and Late Pre-term control) while controlling for brain section (e.g. ~experimental group + brain section), and (2) Expression values as a function of experimental groups (Pre-term control and Late Pre-term control), stratified by brain subsection, (i.e. separate analyses for each the basal ganglia, cerebellum, hippocampus or motor cortex). For differential expression analysis, we utilize Limma's linear modeling, and empirical Bayes[56]. Significantly DEGs are defined as having a false discovery corrected p-value ≤ 0.05, using the Benjamini-Hochberg procedure[57].

Secondary analysis

GSEA was performed on the Pre-term control and Late Pre-term control, and Late Pre-term within basal ganglia groups. For GSEA, genes were ranked by log fold change and passed into R's clusterProfiler[58]. GSEA was performed on the KEGG database, via the 'gseKegg' tool from clusterProfiler. We set a minimum gene set size for analyzing to five, and cap at a max of 800. Results from gseKegg are restricted to a false discovery corrected p-value ≤ 0.05.

All statistical analyses were completed using R Statistical Software v. 4.1.1 (2021)[59]. Code available upon request.

Phenotypic analysis

Ensembl's Biomart (version accessed on May 19, 2023) was used to determine which of the differentially expressed ovine genes identified in the study have orthologous human genes and determine their corresponding known phenotype descriptions and mendelian inheritance of man (MIM) descriptions. Those that had a human phenotype description(s) available were extracted and analyzed in Supplemental Tables 1–3 and reported in Supplemental Fig. 3.

Following a literature review of genes and gene pathways reported to be implicated in pre-term birth and/or pre-term birth-related brain injury, a list of genes and gene pathways were generated and specifically examined for statistically significant differential expression between the EXTEND group and the Pre-term and Late pre-term control groups. For pathways, search terms looking for all variations of TCA/tricarboxylic/Kreb/and citric were used. These results are reported in Supplemental Figs. 4 and 5.

Data availability

Raw data generated and analyzed during the current study are available through the gene expression omnibus (GEO) under accession number GSE275228.

Received: 7 August 2024; Accepted: 6 November 2024
Published online: 21 November 2024

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Acknowledgements
The authors would like to thank Haiying Li, Philip Zoltick, and Whitney Frederic for their guidance and assistance with RNA extraction.

Author contributions
JLC, JMK, and AWF designed the study. JLC, FDB, BC, JM procured the brain samples. ANV conducted dissections of brain and histology staining. NO'G and RAM conducted bioinformatic analyses. EM assisted in the preparation of figures. JLC prepared the initial manuscript; SR provided content expertise and contributed to the writing of the manuscript. All authors critically reviewed and edited the manuscript in its final form.

Declarations

Competing interests
Alan W. Flake holds multiple patents related to the EXTEND technology, and holds option rights and is a Paid Medical Consultant for Vitara Biomedical Inc. All other authors have declared that no conflicts of interest exist.

Study approval
These experiments were approved by the Institutional Animal Care and Use Committee of Children's Hospital of Philadelphia Research Institute (N°:19–000984) and followed the 'Animal Research: Reporting of In Vivo Experiments' (ARRIVE) guidelines[60]. The animal vendors and facilities operated under the United States Department of Agriculture (USDA) national guidelines.

Additional information
Supplementary Information The online version contains supplementary material available at https://doi.org/10.1038/s41598-024-79095-7.

Correspondence and requests for materials should be addressed to J.L.C.