Pediatric RESEARCH

BASIC SCIENCE ARTICLE

Hippocampal epigenetic and insulin-like growth factor alterations in noninvasive versus invasive mechanical ventilation in preterm lambs

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BACKGROUND: The brain of chronically ventilated preterm human infants is vulnerable to collateral damage during invasive mechanical ventilation (IMV). Damage is manifest, in part, by learning and memory impairments, which are hippocampal functions. A molecular regulator of hippocampal development is insulin-like growth factor 1 (IGF1). A gentler ventilation strategy is noninvasive respiratory support (NRS). We tested the hypotheses that NRS leads to greater levels of IGF1 messenger RNA (mRNA) variants and distinct epigenetic profile along the *IGF1* gene locus in the hippocampus compared to IMV.
METHODS: Preterm lambs were managed by NRS or IMV for 3 or 21 days. Isolated hippocampi were analyzed for IGF1 mRNA levels and splice variants for promoter 1 (P1), P2, and IGF1A and 1B, DNA methylation in P1 region, and histone covalent modifications along the gene locus.
RESULTS: NRS had significantly greater levels of IGF1 P1 (predominant transcript), and 1A and 1B mRNA variants compared to IMV at 3 or 21 days. NRS also led to more DNA methylation and greater occupancy of activating mark H3K4 trimethylation ($H3K4me^3$), repressive mark $H3K27me^3$, and elongation mark $H3K36me^3$ compared to IMV.
CONCLUSIONS: NRS leads to distinct IGF1 mRNA variant levels and epigenetic profile in the hippocampus compared to IMV.

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IMPACT:

- Our study shows that 3 or 21 days of NRS of preterm lambs leads to distinct IGF1 mRNA variant levels and epigenetic profile in the hippocampus compared to IMV.
- Preterm infant studies suggest that NRS leads to better neurodevelopmental outcomes later in life versus IMV.
- Also, duration of IMV is directly related to hippocampal damage; however, molecular players remain unknown.
- NRS, as a gentler mode of respiratory management of preterm neonates, may reduce damage to the immature hippocampus through an epigenetic mechanism.

INTRODUCTION

Prolonged invasive mechanical ventilation (IMV) of preterm human infants is associated with brain damage that may be focal or diffuse.[1–5] Brain damage is linked to poor neurodevelopmental outcomes, with worse outcomes with longer duration of IMV.[5–9] These outcomes are related to smaller size and/or volume of hippocampi of children who were born prematurely and survived.[8,10–14] Focus on the hippocampus is because it is integral for learning and memory,[15–18] which are impaired by IMV.[19,20] Noninvasive respiratory support (NRS) or early extubation to NRS is a gentler approach to respiratory management of preterm infants,[21–23] NRS is associated with less use and fewer days of intubation than IMV.[24–28] A retrospective clinical study suggested that preterm infants managed by continuous positive airway pressure at 24 h of life had better neurodevelopmental outcomes at 18 to 22 months corrected gestational age.[29] Together, these results suggest that mode of respiratory support impacts the hippocampus in preterm infants; however, molecular players remain unknown.

Insulin-like growth factor 1 (IGF1) participates in development and responses to injury.[30–32] IGF1 promotes survival and proliferation of neurons and glia.[33] Models of hypoxic ischemia[34,35] or traumatic brain damage[36,37] of immature or mature rats is associated with increased expression of IGF1 in the brain later in life. Treatment with exogenous IGF1 protein after hypoxic ischemia in neonatal or adult rats improved histological and functional outcomes later in life.[38–40] Similarly, IGF1 treatment improved neurologic outcome in 21-day-old rat pups after lipopolysaccharide-induced brain damage.[41] Thus, increasing IGF1 protein levels appears to be neuroprotective.

The *IGF1* gene is conserved among species, including sheep. The *IGF1* gene has two alternative promoters P1 and P2 that separately initiate transcription at multiple start sites that generate

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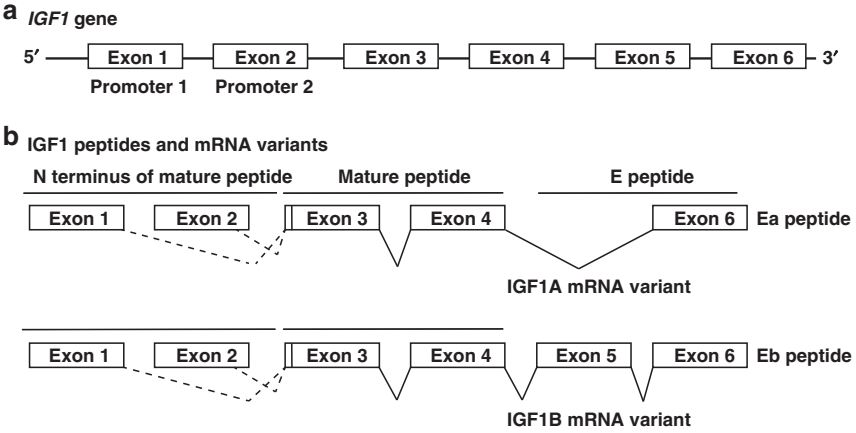

Fig. 1 Schematic of the *IGF1* gene locus. a IGF1 gene. Exons 1 and 2 serve as promoters. **b** IGF1 peptides and mRNA variants. Exon 5 exclusion or inclusion generates IGF1A or 1B mRNA variant, respectively.

Table 1. Demographic characteristics of lambs stratified by group.

	3-day-old Preterm lambs		21-day-old Preterm lambs		Unventilated controls	
	NRS	IMV	NRS	IMV	Fetus	Term
Sex (M:F)	3:1	2:2	2:2	1:3	2:3	4:5
Gestation age (days)	130 ± 1	130 ± 1	130 ± 1	130 ± 1	134 ± 1	Term
Birth weight (kg)	3.1 ± 0.7	3.0 ± 0.8	2.8 ± 0.7	3.1 ± 0.9	3.0 ± 0.8	5.6 ± 1.8*
Study-end weight (kg)	3.0 ± 0.9	2.9 ± 0.9	3.1 ± 0.9	2.9 ± 1.0	3.0 ± 0.8	5.6 ± 1.8*

IMV invasive mechanical ventilation, *NRS* noninvasive respiratory support.
No statistical differences, except as indicated: *$p < 0.05$ compared to the preterm lamb groups and unventilated control fetal group by ANOVA and Fisher's PLSD post hoc test.

5′-untranslated regions (UTRs) with multiple out-of-frame ATGs. Transcription from P1 splices out exon 2, while P2 starts transcription with exon 2 (66). The human and sheep *IGF1* gene has six exons (Fig. 1). Either exon 1 or exon 2 is spliced to exon 3, which encodes the N terminus of the mature peptide.[42–44] Exon 1-derived (i.e., P1) transcripts predominate in every tissue expressing the *IGF1* gene, except the liver, in which relatively high levels of exon 2-derived (i.e., P2) transcripts are expressed.[45,46] Exons 3 and 4 encode mature peptide. Exon 5 is alternatively spliced. Splicing exon 4 to exon 6 causes early termination of translation and generates the smaller isoform IGF1A. Transcripts that include exon 5 code for isoform IGF1B (80), and IGF1A variant accounts for the majority of transcripts.[47] IGF1A and 1B messenger RNA (mRNA) variants encode the Ea and Eb peptides, respectively. The predominant form, IGF1Ea, participates in physiological processes, whereas IGF1B participates in responses to other stimuli.[34] IGF1B is a potent neuroprotective factor.[48–50]

Epigenetic mechanisms participate in expression of the *IGF1* gene.[22] Epigenetic regulation of transcription includes modifications to DNA and histone proteins, without altering DNA sequence.[51] DNA methylation in promoter regions typically negatively corelates with gene expression.[52] Histone (H) modifications, including histone acetylation (ac) or methylation (me), affect gene transcription. For example, histone H3 lysine (K) 9 acetylation (ac), H3K14ac, and H3K4 trimethylation (me^3) are often associated with gene activation, while H3K36me^3 is associated with actively transcribed regions.[53,54] Moreover, H3K27me^3 is associated with gene silencing.[53] These histone marks are also vulnerable to perinatal insults.[55,56]

A gap in knowledge is whether IGF1 mRNA variant levels and epigenetic profile in the hippocampus are different depending on the mode and duration of respiratory management of preterm neonates. To address this knowledge gap, we used our preterm lamb model to compare hippocampal outcomes following NRS versus IMV, neither of which is associated with germinal matrix hemorrhage nor periventricular leukomalacia. The absence of these gross pathological lesions suggests that diffuse brain damage happens in the brain of chronically ventilated preterm lambs, and that they may develop learning and memory deficits later in life. We hypothesized that NRS leads to greater levels of IGF1 mRNA variants and distinct epigenetic profile along the *IGF1* gene locus in the hippocampus compared to IMV. Epigenetic profile, in this context, is the pattern of DNA methylation at promoters and the pattern of histone modifications along the *IGF1* gene locus. The principal results show that 3 or 21 days of NRS leads to higher levels of IGF1 mRNA variants and distinct epigenetic profile along the *IGF1* gene locus compared to IMV for the same durations.

METHODS

Preparation and management of preterm lambs

Protocols adhered to APS/NIH guidelines for humane use of animals for research and were prospectively approved by the IACUC at the University of Utah Health Sciences Center. We used the brain of preterm lambs of both sexes for which pulmonary outcomes are reported[57–59] to secondarily analyze the hippocampus.

Preterm lambs (~130 days gestation; term ~150 days) were delivered via cesarean section and divided into two groups: IMV or NRS.[57–59] Matched subgroups were managed for 3 days ($n = 4$/group) or 21 days ($n = 4$/group; Table 1; Supplementary Material Fig. 1). Block randomization was used for assignment before delivery.

Fetal lambs were exposed to antenatal steroid, perinatal surfactant, and postnatal caffeine citrate (details are provided in the Supplementary Material). Lambs were endotracheally intubated for resuscitation and continuing IMV (Bird VIP ventilator; model

Table 2. Primers for sheep IGF1 transcription start sites (TSS) in the hippocampus.

	Forward primers	Reverse primers	Probe (acc# x69472)
Real-time RT-PCR			
IGF1	TTGGTGGATGCTCTCCAGTTC	CAGCACTCATCCACGATTCCT	CTTTTATTTCAACAAGCCC
IGF1 P1	TTTGTGATTTCTTGAAGCAGGTG	GCAAGCACAGGGCCAGATA	CCTCCTCGCATCTC
IGF1 P2	AAATGGTTACACCTACACAGGTGAA	GCAAGCACAGGGCCAGATAG	CCTCGCATCTCTTC
IGF1A	CAGCGCCACACCGACAT	CCCTCTGCTTGTGTTCTTCAAA	CAAGGCTCAGAAGGAAGTA
IGF1B	CAGCGCCACACCGACAT	TTCATTTTCTTGTTGGTAGATGGGA	CAAGGCTCAGAAGTATCA
5′-RACE			
IGF1 exon 1	TGTGACATTGCTCTCAACATCTC	(outer) ACTGGCATCTTCACCTGCTT (inner) CAAGAAATCACAAAAGCAGCACTT	
IGF1 exon 3	TGTGACATTGCTCTCAACATCTC	(outer) GCAAGCACAGGGCCAGAT (inner) GAAGAGATGCGAGGAGGATGT	
Beyond the farthest TSS found in brain based on exon 1 and exon 3 results			
	AGAGAAGGCAAGCGTTCCC	(outer) AGGGATTTAGAGAAAATCCTCACATT (inner) TATCTACAAAACACAGACACTGTAGA	

15215, Palm Springs, CA). Initial ventilator settings were respiratory rate of 60 breaths/min, inspiratory time of 0.32 s, peak inspiratory pressure (PIP) of 21 cmH_2O, and positive end-expiratory pressure of 8 cmH_2O. Fractional inspired oxygen started at 0.50 and was adjusted to attain target oxygen saturation 88–92% by pulse oximetry (model SurgiVet V9200IBP/Temp, Smith Medical ASD Inc., St. Paul, MN). PIP was adjusted to attain target ventilation ($PaCO_2$) between 45 and 60 mmHg, resulting in pH between 7.25 and 7.35. Target expiratory tidal volume, measured by the ventilator, was 5–7 mL/kg. Details of monitoring, feeding, fluid homeostasis, and treatment for infection are provided in the Supplementary Material.

Management of anesthesia was different between the preterm groups. The IMV group was kept anesthetized (3–5 mg/kg pentobarbital sodium, intravenously (i.v.)) and given buprenorphine hydrochloride (5 µg/kg, i.v., every 6 h; Reckitt and Colman Pharmaceuticals, Richmond, VA). Pentobarbital sodium was given as needed to prevent breathing over the ventilator, swallowing, or chewing. The NRS group was extubated ~3 h after preterm birth. At transition, an uncuffed 3.0 Murphy tube was placed in one nostril.[57–59] Preterm lambs managed by NRS were given buprenorphine hydrochloride only to allow effective spontaneous breathing.

At the end of the 3-day study or the 21-day study, preterm lambs were given heparin (1000 U, i.v.). The NRS group was reintubated and briefly managed with the same ventilator settings during NRS. All lambs were euthanized, using Beuthanasia solution (65 mg/kg; Intervet Inc., Madison, NJ). The brain was removed and chilled during dissection to remove the hippocampus, which was snap frozen in liquid nitrogen and stored at −80 °C.

Unventilated control lambs for normal developmental reference
Hippocampi were collected from two unventilated control groups: fetal lambs ($n = 6$) and term newborn lambs ($n = 6$; Supplementary Fig. 1).[57–59] Males and females are shown in Table 1.

Molecular analyses (methodological details are provided in the Supplemental Material)
RNA isolation and real-time RT-PCR. Total RNA was extracted from hippocampi, using NucleoSpin RNA II Kit (MACHEREY-NAGEL Inc., Bethlehem, PA), and quantified using a BioTek* Epoch* Microplate Spectrophotometer (Fisher Scientific Inc., Pittsburgh, PA). Real-time reverse transcriptase-PCR was used to quantify levels of IGF1 total mRNA and IGF1 mRNA variants, with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal control. Based on high homology, we designed primer and probe sets (Table 2), using Primer Express (Applied Biosystems, Foster, CA). All primers were confirmed by PCR and sequencing. Relative quantification of PCR products was based on value differences between the target and GAPDH control, using the comparative CT method (Taqman Gold RT-PCR manual, PE Biosystems, Foster City, CA). Samples were run in quadruplicate.

5′-Rapid amplification of complementary DNA ends (5′-RACE). mRNAs were extracted from normal newborn lamb hippocampus, lung, and liver, using NucleoSpin RNA II Kit and NucleoTrap® mRNA Mini Kit (MACHEREY-NAGEL Inc. Bethlehem, PA) and quantified, using a BioTek* Epoch* Microplate Spectrophotometer (Fisher Scientific Inc., Pittsburgh, PA). Extracted mRNA was subjected to 5′ RACE, using FirstChoice® RLM-RACE Kit (Life Technologies, Carlsbad, CA) according to the manufacturer's instructions. The primers used are shown in Table 2. The PCR products were cloned, and positive clones were sequenced, using DNAMAN Version 5.2 (Lynnon Biosoft, Quebec, Canada).

DNA isolation and sodium bisulfite sequencing. Genomic DNA was extracted from hippocampi. DNA was quantified, using standard spectrophotometry, and subjected to sodium bisulfite modification according to the manufacturer's protocol (CpGenome DNA Modification Kit, Chemicon International, Temecula, CA, USA) to determine site-specific CpG methylation. Based on our 5′-RACE results, 12 CpG sites in the upstream region of the farthest transcription start sites (TSS) (about 667 bases) were selected for analysis. Two primer sets listed in Table 2 were used for PCR amplification (amplicon lengths of 260 and 350 bp, respectively).

Chromatin immunoprecipitation (ChIP) assay and real-time PCR. ChIP was carried out with commercial antibodies that cross-reacted with sheep epitopes. We used antibodies directed against H3K9ac (Cell Signaling Technologies, Beverly, MA, USA), H3K14ac (EMD Millipore Corporation, Billerica, MA, USA), H3K4me^3, H3K27me^3, and H3K36me^3 (Abcam, Cambridge, MA, USA), as described by our group.[60] Primers and probes are listed in Table 2.

Statistical analyses
Results are shown as mean ± SD. Real-time RT-PCR mRNA and ChIP data were analyzed by one-way analysis of variance (ANOVA) and post hoc comparisons (Fisher's protected least significant difference test), whereas DNA methylation data were analyzed by Fisher's exact test (for small sample size) or χ^2 test (for bigger sample size), using Statview® (SAS institute, NC) software. We assessed statistical significance at $p < 0.05$.

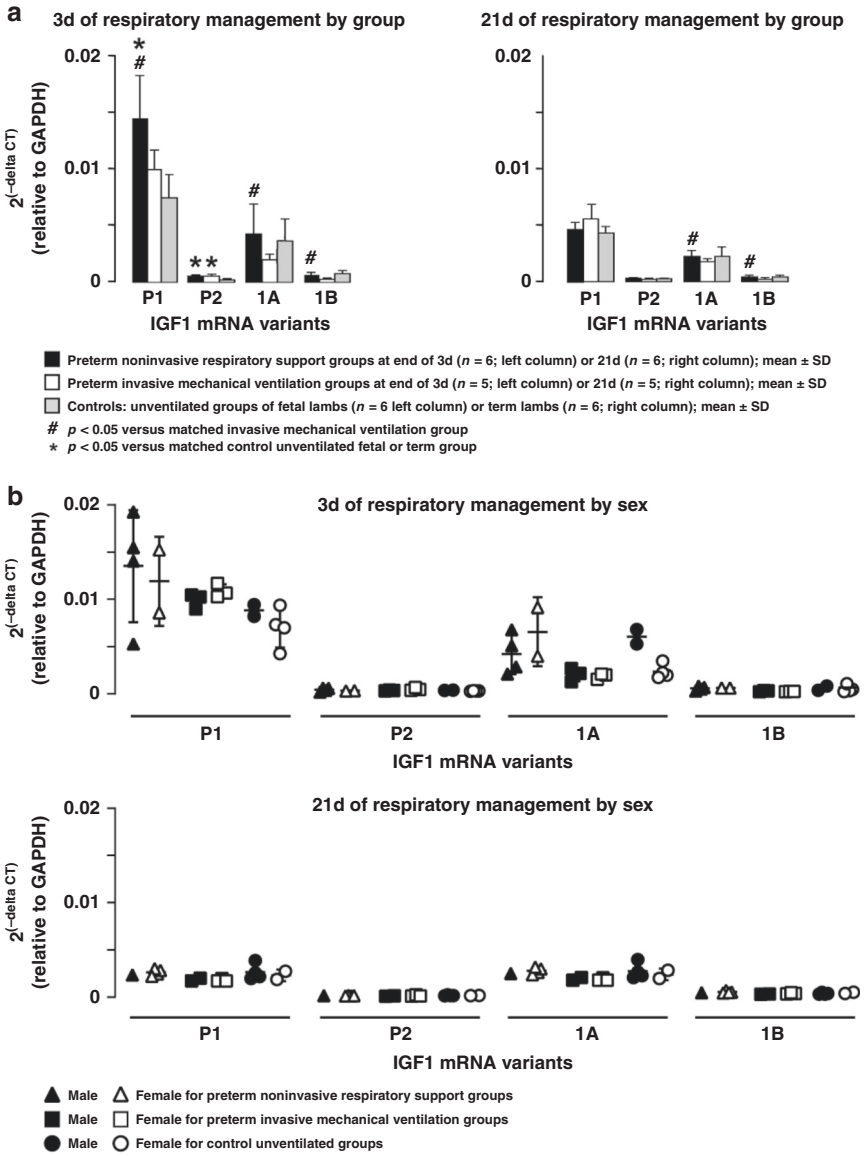

Fig. 2 Effect of mode and duration of respiratory management on expression of IGF1 mRNA variants in the hippocampus of sheep. **a** IGF1 mRNA variant results (mean ± SD) summarized for the two groups of preterm lambs that were managed for either 3 days (left graph) or 21 days (right graph) of respiratory management, as well as term control lambs that were not ventilated. **b** IGF1 mRNA variants displayed by sex for either 3 days (top graph) or 21 days (bottom graph) of respiratory management. Three days of noninvasive respiratory support (NRS; black bars; $n = 6$; 4 M:2 F) led to greater expression of IGF1 P1 and 1A mRNAs ($p < 0.05$) compared to 3 days of invasive mechanical ventilation (IMV; white bars; $n = 6$; 3 M:3 F). Twenty-one days of NRS (black bars; $n = 5$; 1 M:4 F) led to greater expression of IGF1 1A and 1B mRNAs ($p < 0.05$) compared to 21 days of IMV (white bars; $n = 5$; 2 M:3 F). Control unventilated groups are fetal-end lambs (gray bars $n = 6$; 2 M:4 F; control for the preterm 3-day groups) and term lambs (gray bars; $n = 6$; 4 M:2 F; control for the preterm 21-day groups). **b** Male and female results are individually presented as whisker dot plots for the same groups shown in **a**.

RESULTS

Demographic characteristics of the lamb groups are summarized in Table 1. Gestational age and birth weight at preterm delivery, and weight at study end, were the same among the preterm lamb groups. As expected, birth weight of preterm lambs was less than birth weight of matched term control lambs ($p < 0.05$).

IGF1 mRNA variant levels in the hippocampus of lambs

Results are shown for sets of preterm lamb groups: (a) 3-day NRS versus 3-day IMV, and (b) 21-day NRS versus 21-day IMV (Fig. 2). Results also are shown for two unventilated control groups.

For the 3-day groups (Fig. 2a), NRS had significantly greater mRNA levels of IGF1 P1, 1A, and 1B compared to IMV ($p < 0.05$). Either mode of respiratory management had significantly greater IGF1 P1 and P2 mRNA transcript levels compared to the fetal unventilated control group ($p < 0.05$).

For the 21-day groups (Fig. 2a), NRS also had significantly greater mRNA levels of IGF1 1A and 1B compared to IMV ($p < 0.05$). Neither NRS nor IMV affected IGF1 mRNA variant levels compared to the term unventilated control group.

These results also are shown as whisker plots (Fig. 2b) for males and females. Differences were not detected for sex as a biological variable.

IGF1 TSS in the hippocampus of lambs

We determined the location of TSS (Fig. 3) because IGF1 sequences for sheep are limited in GenBank. We analyzed hippocampus, lung, and liver of normal newborn lambs to determine DNA

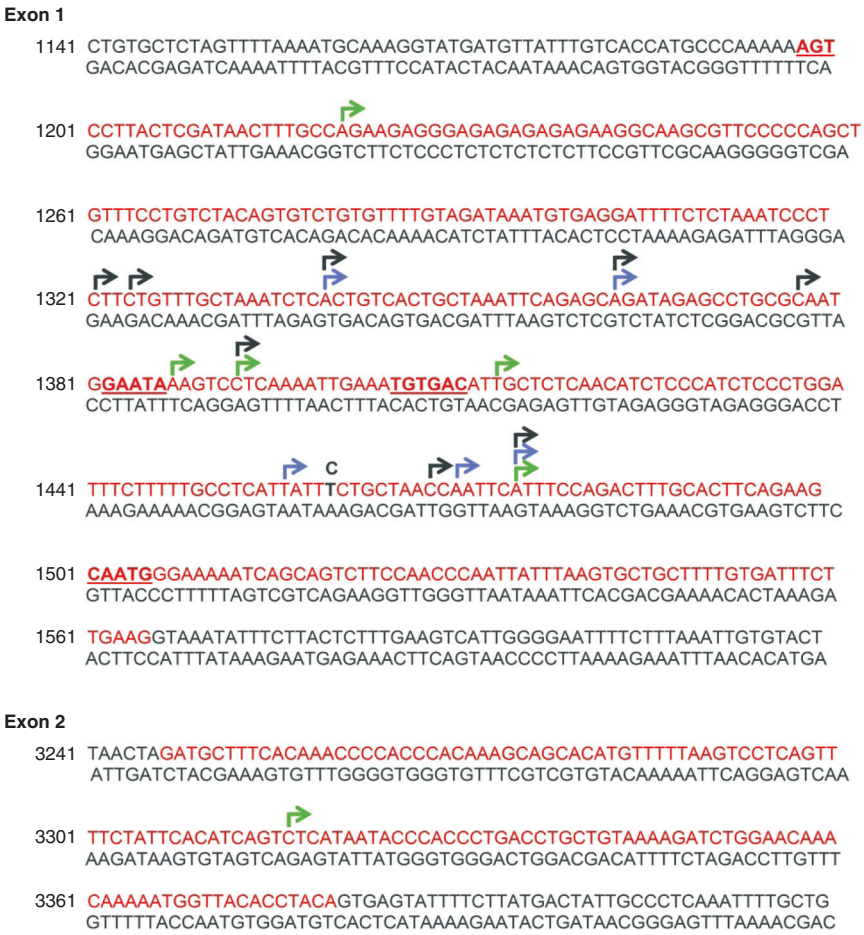

Fig. 3 RACE results for IGF1 transcription start sites in sheep. 5′-Transcription start sites are shown for the hippocampus (black arrows), lung (blue arrows), and liver (green arrows) that determined the promoter region for DNA methylation measurement. Left column of numbers indicates the location of nucleotide bases relative to the sequence in GenBank (acc# 69472). Sequences in red are known regions of exon 1 (top panel) and exon 2 (bottom panel). Underlined sequences are transcription start sites (TSS) known in sheep (GenBank, acc# x69472). A polymorphism (C/T) nucleotide is at location 1461. In exon 1 (top panel), nine TSS were in the hippocampus (black arrows), five TSS were in the lung (blue arrows), and five TSS were in the liver (green arrows). In exon 2 (bottom panel), one TSS was in the liver (green arrow) only. No sequences were in exon 2 for either the hippocampus or lung.

methylation status in the 5′-flanking region of exons 1 and 2. We found, using the 5′-RACE technique, multiple TSS in the *IGF1* gene P1 region in the hippocampus, lung, and liver. For the hippocampus, the farthest TSS was 124 bases downstream of known TSS. For the lung, the farthest TSS was 143 bases downstream of known TSS. For the liver, the farthest TSS was 24 bases downstream of known TSS. For exon 2, TSS were found in the liver only (GenBank: M31735, acc# 69472). Therefore, we determined DNA methylation status in the hippocampus in the P1 region only.

DNA methylation status of IGF1 P1 in the hippocampus of lambs
Based on the 5′-RACE results, we designed two primer sets for bisulfite sequencing to detect DNA methylation status on 12 CpG sites in the P1 region that spanned 670 bp upstream of the farthest TSS in the hippocampus (Fig. 4).

For the 3-day groups (Fig. 5a, b), NRS had significantly more total CpG methylation compared to IMV ($p < 0.05$). NRS also had significantly more total CpG methylation compared to the fetal unventilated control group ($p < 0.05$). By comparison, IMV had significantly less total CpG methylation compared to the fetal unventilated control group ($p < 0.05$). Among the 12 methylation sites, NRS had significantly more CpG methylation on one site (−327) compared to the fetal unventilated control group ($p < 0.05$). IMV had significantly less CpG methylation on 7 of the 12 sites (−641, −465, −462, −436, −340, −327, and −310 relative to the farthest TSS in exon 1 set as +1) compared to the fetal unventilated control group ($p < 0.05$).

For the 21-day groups (Fig. 5a, c), both NRS and IMV had significantly more total CpG methylation compared to the term unventilated control group ($p < 0.05$). NRS also had less CpG methylation at 2 of the 12 sites (−641 and −590; $p < 0.05$) compared to the IMV group, and significantly more CpG methylation on another site (−327 site) compared to the term unventilated control group ($p < 0.05$). IMV had significantly more CpG methylation on one site (−271 site; $p < 0.05$) compared to the term unventilated control group ($p < 0.05$).

Histone modifications along IGF1 locus in the hippocampus of lambs
We assessed H3K9ac, H3K14ac, H3K4me^3, H3K27me^3, and H3K36me^3 as representative histone modifications along the *IGF1* gene locus. Neither mode nor duration of respiratory management affected H3K9ac occupancy along the IGF1 locus (data not shown). In contrast, both mode and duration of respiratory management affected H3K14ac, H3K4me^3, H3K27me^3, and H3K36me^3 occupancy along the IGF1 locus (Fig. 6).

Fig. 4 DNA methylation status of IGF1 promoter 1 in the hippocampus of sheep. Left column of numbers indicates the location of nucleotide bases relative to the sequence on GenBank (acc# 69472). The sequence in red indicates exon 1 in sheep (GenBank, acc# 69472). Underlined sequences indicate transcription start site (TSS) in sheep (GenBank, acc# x69472). Black arrow represents the TSS by 5′-RACE. Yellow highlighted CGs are the sites for DNA methylation analysis.

For H3K14ac, no differences were detected between NRS versus IMV for 3 days (Fig. 6a). NRS or IMV had significantly more H3K14ac occupancy in IGF1 P1 region compared to the fetal unventilated control group ($p < 0.05$). NRS also had significantly less H3K14ac occupancy in IGF1 exon 5 region compared to the fetal unventilated control group ($p < 0.05$). At 21 days, neither NRS nor IMV altered H3K14ac occupancy along the IGF1 locus compared to the term unventilated control group (Fig. 6a).

For H3K4me^3, NRS for 3 days had significantly more H3K4me^3 occupancy at IGF1 P2 region ($p < 0.05$) compared to IMV (Fig. 6b). NRS or IMV had significantly more H3K4me^3 occupancy in IGF1 P1 region ($p < 0.05$) compared to the fetal unventilated control group. IMV also had significantly less H3K4me^3 occupancy in IGF1 exon 6 region compared to the fetal unventilated control group ($p < 0.05$). At 21 days, NRS had significantly more H3K4me^3 occupancy in all IGF1 regions examined ($p < 0.05$), except IGF1 P1, compared to IMV (Fig. 6b). NRS also had significantly more H3K4me^3 occupancy in all IGF1 regions examined ($p < 0.05$), except IGF1 P1, compared to the term unventilated control group.

For H3K27me^3, no differences were detected between the 3-day NRS versus IMV groups (Fig. 6c). NRS or IMV had significantly more H3K27me^3 occupancy in the IGF1 P1 region ($p < 0.05$) compared to the fetal unventilated control group, indicating that the bivalent mark H3K4me^3/H3K27me^3 existed. Conversely, both IMV and NRS had significantly less H3K27me^3 occupancy in IGF1 exon 6 region ($p < 0.05$) compared to the fetal unventilated control group. At 21 days, NRS had significantly more H3K27me^3 occupancy in all IGF1 regions examined ($p < 0.05$), except P1, compared to IMV (Fig. 6c). NRS also had significantly less H3K27me^3 occupancy in IGF1 P1 region, but more H3K27me^3 occupancy in P2 region, where the bivalent mark H3K4me^3/H3K27me^3 existed ($p < 0.05$) compared to the term unventilated control group. By comparison, IMV had significantly more H3K27me^3 occupancy in IGF1 P2, but less H3K37me^3 occupancy in exon 4, 5, and 6 regions versus the term unventilated control group ($p < 0.05$).

For H3K36me^3, NRS for 3 days had significantly more H3K36me^3 occupancy in all IGF1 regions examined ($p < 0.05$) compared to IMV (Fig. 6d). NRS also had significantly more H3K36me^3 occupancy along the IGF1 locus at all regions ($p < 0.05$), except P1, compared to the fetal unventilated control group. By comparison, IMV had significantly less H3K36me^3 occupancy at the IGF1 P1 region ($p < 0.05$) compared to the fetal unventilated control group. At 21 days, NRS had significantly more H3K36me^3 occupancy along the IGF1 locus at all regions ($p < 0.05$), except P1, compared to IMV (Fig. 6d). NRS also had significantly more H3K36me^3 occupancy in IGF1 P2 region ($p < 0.05$) and exon 4 region ($p < 0.05$) compared to the term unventilated control group. By comparison, IMV had significantly more H3K36me^3 occupancy along the IGF1 P2 region only ($p < 0.05$) versus the term unventilated control group.

DISCUSSION

This study compared mRNA variant levels and epigenetic profiles of IGF1 in the hippocampus of preterm lambs at the end of 3 or 21 days of continuous respiratory management by NRS or IMV. We focused on the hippocampus because of its role in learning and memory. We showed that all IGF1 mRNA variants are conserved in sheep, like humans and rats.[61,62] Our results show that NRS for 3 days had significantly greater levels of IGF1 P1, 1A, and 1B mRNA variants compared to IMV for 3 days. Greater levels of IGF1A and 1B mRNA variants persisted at 21 days of NRS compared to 21 days of IMV. Our results also show that more DNA methylation and greater occupancy of H3K4me^3, H3K27me^3, and H3K36me^3 along the *IGF1* gene locus occurs at both 3 and 21 days of NRS compared to 3 and 21 days, respectively, of IMV. We conclude that mode and duration of respiratory management lead to different IGF1 mRNA variant levels and epigenetic profile in the hippocampus of preterm lambs.

A frequent unintended consequence of prolonged IMV of preterm human infants is brain damage. Brain damage may

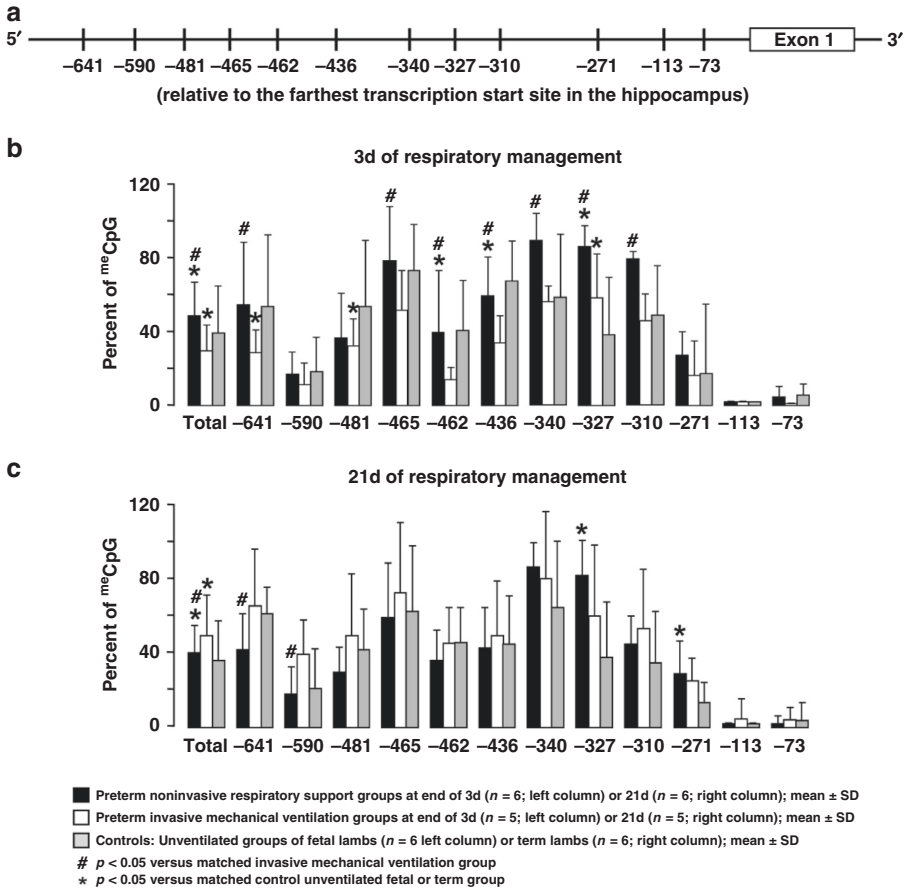

Fig. 5 Effect of mode and duration of respiratory management on CpG methylation at the IGF1 P1 promoter region in the hippocampus of sheep. Results are presented as mean ± SD. **a** Schematic of sheep proximal promoter of exon 1. Vertical lines indicate the location of the CpG sites examined relative to the farthest translation start site in exon 1 set as +1. **b** and **c** Percent of CpG sites for either 3 days (top graph) or 21 days (bottom graph) of respiratory management. Three days of noninvasive support (NRS; black bars; $n = 6$; 4 M:2 F) led to greater DNA methylation ($p < 0.05$) at nine locations compared to 3 days of invasive mechanical ventilation (IMV; white bars; $n = 6$; 3 M:3 F). Twenty-one days of NRS (black bars; $n = 5$; 1 M:4 F) led to greater DNA methylation ($p < 0.05$) at three locations compared to 21 days of IMV (white bars; $n = 5$; 2 M:4 F). Control unventilated groups are fetal-end lambs (gray bars $n = 6$; 2 M:4 F; control for the preterm 3-day groups) and term lambs (gray bars; $n = 6$; 4 M:2 F; control for the preterm 21-day groups).

include gross lesions, such as germinal matrix hemorrhage-intraventricular hemorrhage or periventricular leukomalacia,[63] which are associated with cerebral palsies. Alternatively, brain damage may be diffuse yet still be associated with suboptimal neurodevelopmental outcome, such as learning and memory deficits.[64,65] However, the molecular basis of these outcomes in chronically ventilated preterm human infants remains incompletely understood.

NRS is a strategy for respiratory management of preterm human infants. Early use of NRS, such as nasal continuous positive airway pressure, is associated with better lung outcomes than IMV.[24–28] Mechanistic insights by which lung outcomes are better stem in part from experimental animal studies. Functional studies using preterm lambs indicate that expression of acute-phase response genes and levels of inflammatory markers are lower compared to IMV.[66]

Studies using preterm baboons also show that early use of NRS is associated with less overall cerebral damage[67,68] and cerebellar damage.[69] However, molecular players remain to be identified. Results of our study show that IGF1 P1 mRNA levels are persistently higher when NRS is used compared to IMV. A caveat is that management of preterm lambs by IMV for 3 days also led to greater IGF1 P1 mRNA levels compared to fetal unventilated control lambs. We suggest that this caveat reflects the impact of the abrupt environmental consequences of preterm birth and stresses of prolonged respiratory support and neonatal intensive care. These environmental consequences were not experienced by the control unventilated fetal or term lambs. We suggest that the mode of respiratory management may affect epigenetic determinants of IGF1 transcriptional regulation in the hippocampus by altering the activities and/or levels of epigenetic enzymes, such as DNA methyltransferases or ten-eleven translocation methylcytosine dioxygenases, as well as activities and/or levels of histone acetyltransferases, deacetylases, and nucleosome remodeling complexes.

The *IGF1* gene is regulated epigenetically. One of the epigenetic mechanisms is DNA covalent modification that leads to DNA methylation. CpG methylation is generally considered to repress transcription, either directly by blocking transcription factor binding or indirectly by recruiting methylated DNA binding proteins that occupy critical site(s) to prevent transcription factor binding.[70–72] Little is known, however, about transcription factors that upregulate IGF1 in the brain. Transgene studies reveal that growth hormone upregulates IGF1 in the brain, but predicted growth hormone receptor binding sites have not been identified in the promoter region.[73] We screened the promoter for potential transcription factor binding sites known to be methylation sensitive, but none were detected. Also, DNA methylation within CpG sites in promoters is not always associated with gene silencing.[74,75] For example, DNA methylation is an inconsistent epigenetic mechanism in pig embryonic day 10 epiblast, hypoblast, trophectoderm,

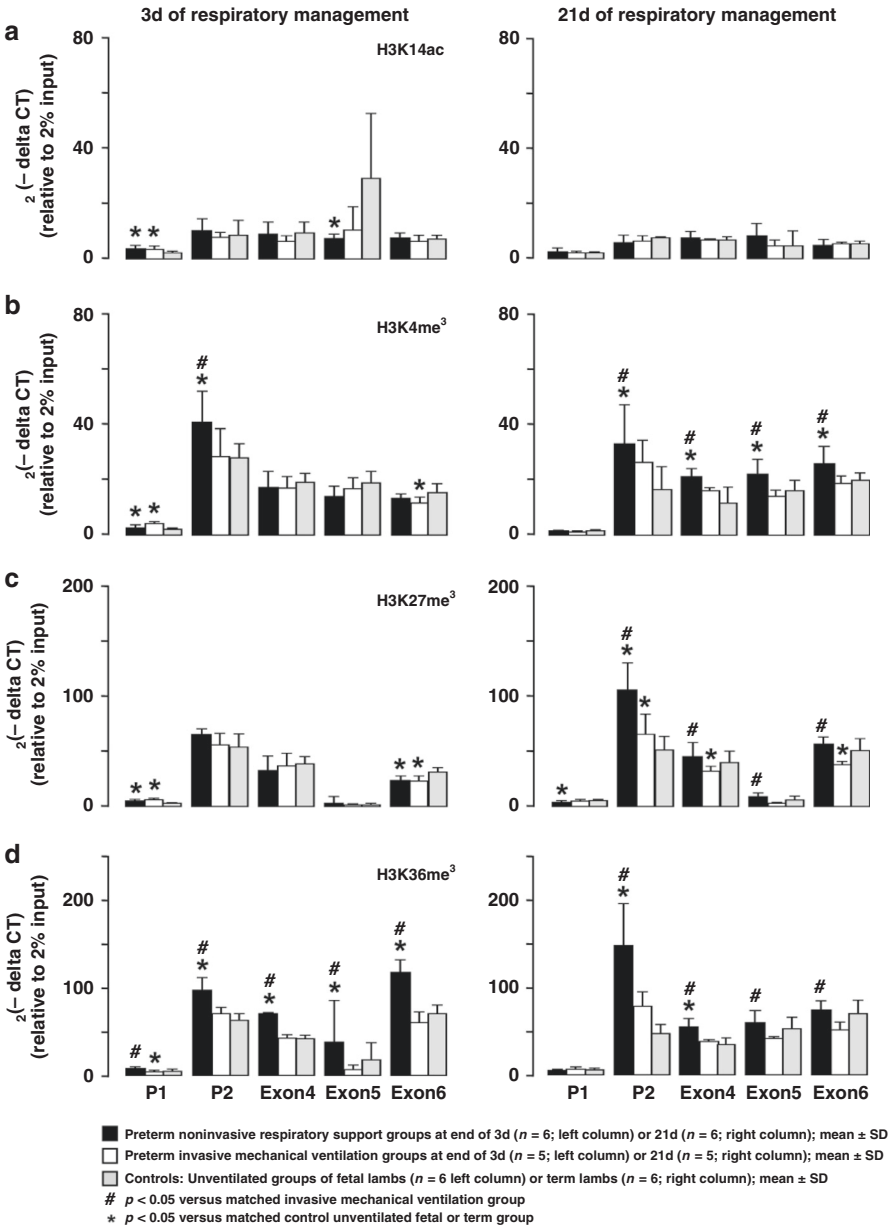

Fig. 6 Effect of mode and duration of respiratory management on histone covalent modifications along the *IGF1* gene locus in the hippocampus of sheep. Results are presented as mean ± SD for H3K14ac (**a**), H3K4me^3 (**b**), H3K27me^3 (**c**), and H3K36me^3 (**d**) at 3 days (left column) or 21 days (right column) of respiratory management. For comparison between noninvasive support (NRS) and invasive mechanical ventilation (IMV) outcomes, 3 days of NRS (black bars; $n = 6$; 4 M:2 F) led to greater H3K36me^3 occupancy ($p < 0.05$) at all locations of mRNA variants ($p < 0.05$) compared to 3 days of IMV (white bars; $n = 6$; 3 M:3 F). For the same comparison, 21 days of NRS (black bars; $n = 5$; 1 M:4 F) led to greater H3K4me^3 occupancy ($p < 0.05$) at four locations of mRNA variants ($p < 0.05$) compared to 21 days of IMV (white bars; $n = 5$; 2 M:3 F). Control unventilated groups are fetal-end lambs (gray bars $n = 6$; 2 M:4 F; control for the preterm 3-day groups) and term lambs (gray bars; $n = 6$; 4 M:2 F; control for the preterm 21-day groups).

and epiblast-derived neural progenitor cells.[76,77] Our results also have the same inconsistency because 3 days of NRS had more DNA methylation and greater levels of IGF1 mRNA variants compared to 3 days of IMV. We interpret these results to mean that other epigenetic mechanisms may be involved in the regulation of IGF1 transcription, such DNA hydroxymethylation (5-hmC). 5-hmC is a biologically important epigenetic marker and effects transcriptional activation.[78,79] This recently identified type of DNA modification occurs when the hydrogen atom at the C5 position in cytosine is replaced by a hydroxymethyl group. Levels of 5-hmC are the highest in the brain.[80] However, the technique for sodium bisulfite sequencing, which we used, does not distinguish 5-hmC from 5-methylcytosine (5-mC) because 5-hmC behaves like its precursor, 5-mC.[81] Future studies will focus on 5-hmc as a potential epigenetic player.

Another potential epigenetic mechanism is histone covalent modifications. Histone covalent modifications are generally associated with gene activation or repression, depending on location and type of modification. For example, H3K9ac, H3K14ac, and H3K4me^3 are associated with gene activation[82,83] and H3K36me^3 is associated with actively transcribed regions.[84] H3K27me^3, in contrast, is associated with gene repression.[84] Therefore, we assessed multiple histone marks because epigenetic modifications in vivo likely affect multiple histone marks in multiple cells types simultaneously.[85] A principal finding for the 3-day groups is that H3K36me^3 occupancy is greater along the *IGF1*

gene locus in the NRS group compared to the IMV group. A principal finding for the 21-day groups is that occupancy of $H3K4me^3$, $H3K27me^3$, and $H3K36me^3$ also is greater in the NRS group compared to the IMV group. However, a limitation is that we did not use double immunoprecipitation and therefore our study could not identify multiple marks on the same site.

NRS for 3 days led to more IGF1 P1 mRNA transcript and distinct epigenetic profile than fetal unventilated control lambs. We suggest that the immature hippocampus has the capacity to respond to sudden environmental stresses, such as preterm birth, respiratory resuscitation, and ongoing neonatal intensive care. Also, a nearly 2-fold greater level of IGF1 P1 mRNA transcripts, as well as higher occupancy of $H3K4me^3$ and $H3K36me^3$ along the *IGF1* gene locus, were detected in the NRS group compared to the fetal unventilated control group. We suggest that these increases during NRS may be beneficial early to the immature, developing hippocampus, once removed from the in utero environment.

For the 21-day groups of preterm lambs, less DNA methylation in IGF1 P1 was detected compared to the 21-day IMV group. These differences suggest that longer-term transcription of exon 1-containing mRNA may be greater for the NRS group compared to the IMV group. We also found greater occupancy of the activation marks $H3K4me^3$ and $H3K36me^3$ along the *IGF1* gene locus compared to the 21-day IMV group. $H3K4me^3$ at the 5′ end of a gene is commonly associated with gene activation and initiation of elongation.[86–88] Importantly, $H3K4me^3$ at the body of an actively transcribed gene may also play a role in splicing. For instance, $H3K4me^3$ specifically interacts with human CHD, a protein involved in chromatin modification.[89] Depletion of CHD1 in extracts reduced splicing efficiency in vitro, indicating a functional link between CHD1 and the spliceosome.[90] Knockdown of CHD1 and H3K4me3 levels by short interfering RNA reduced association of U2 small nuclear ribonucleoprotein components with chromatin, and more importantly, altered the efficiency of pre-mRNA splicing on active genes in vivo.[90] In addition, $H3K36me^3$ at the 5′ end of a gene is typically associated with gene transcriptional elongation.[83,91] Therefore, we speculate that at 21 days of NRS, the epigenetic profile of the *IGF1* gene in the hippocampus may lead to more appropriate gene activation, transcript elongation, transcription termination, and alternative splicing than at 21 days of IMV.

Results for preterm lambs managed by IMV are like those reported by our group for rat pups that had intrauterine growth retardation.[92] Rat pups had lower levels of hepatic IGF1 mRNA variants in general, and lower $H3K36me^3$ occupancy specifically, along the *IGF1* gene locus. We speculate that lower $H3K36me^3$ occupancy in the 3-day IMV group of preterm lambs may play a role in the lower levels of IGF1 mRNA variants in the hippocampus.

An interesting observation is the presence of bivalent domains,[93] which are opposing histone marks on the same promoter of a gene. We found that occupancy of the activation mark $H3K4me^3$ coincides with occupancy of the repression mark $H3K27me^3$ in both the promoter region and along the *IGF1* gene at 3 and 21 days. In both mouse and human embryonic stem cells, bivalent genes are highly enriched for transcription factors and other developmental genes. Gene activation when embryonic stem cells differentiate is associated with loss of $H3K27me^3$.[94–96] Subsequent observations detected bivalent domains in cell types of restricted potency.[97] Genes with bivalent domains are interpreted to be in a poised state, enabling rapid activation upon suitable cues and/or environmental stimuli.[97,98] For example, the pluripotency-associated genes *SOX2*, *POU5F1*, and *NANOG* shifted from $H3K4me^3$ alone to colocalization of both $H3K4me^3$ and $H3K27me^3$ as these genes became repressed during differentiation.[95] Whether the coincident modifications occurred in the hippocampus of preterm lambs is unknown.

Our study has limitations. One limitation is that our results pertain to genome-wide assessments. We did not assess subregions or specific cell types of the hippocampus for spatial distribution of the IGF1 mRNA variants or IGF1 protein, which is the focus of a separate structural study. Also, our analysis of histone covalent modifications is limited to candidates for which reagents that recognize epitopes in sheep were available. Furthermore, our study was not powered to assess the impact of sex as a biological variable, although both sexes were included. In addition, we do not know whether our results have causal effects on hippocampal structure and/or function, topics that are the subjects of separate reports. We used term lambs as developmental control for the 21-day groups. A potential limitation is that term lambs were not ventilated and therefore may not be the optimal control. However, the term lambs breathed spontaneously for 12–24 h before terminal tissue collection so their lungs were recruited and aerated, as were the lungs of both groups of preterm lambs that were managed for 21 days. Those groups were delivered at the saccular stage of lung development and do not survive without exogenous surfactant replacement therapy and respiratory support. A potential different control would be to noninvasively resuscitate preterm lambs and continue noninvasive respiratory management; however, such an approach will require new study design. Lastly, potential effects of anesthetic agents on IGF1 transcripts and IGF1 variant distribution pattern are possible,[59,99,100] which will require separate investigation.

The results advance the field by providing the first report of IGF1 mRNA variant levels and epigenetic profiles in the hippocampus of preterm neonates that were managed by NRS versus IMV for up to 21 days. These results are foundation for ongoing histopathological analyses and long-term neuroimaging and neurobehavioral assessments, using our new model of former preterm lambs that had been managed by either NRS or IMV.[101] While we did not test IGF1 signaling in this study, we speculate that IGF1 signaling may be more active after NRS compared to IMV. A possibility of neuroprotection associated with NRS of preterm lambs has basis in other experimental models of brain injury in which elevated expression of IGF1 and/or greater IGF1 signaling are neuroprotective,[102–105] whereas IMV suppresses IGF1 expression and/or signaling.[106] Moreover, these results may provide biological basis for selecting mode of respiratory management for preterm infants.[107]

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AUTHOR CONTRIBUTIONS

All authors meet at least one of the required aspects of: substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; drafting the article or revising it critically for important intellectual content; and final approval of the version to be published.

ADDITIONAL INFORMATION

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