Clinical Epigenetics

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Glucocorticoid exposure-induced alterations in epigenetic age from human preterm infants and human lung fibroblasts and hippocampal neuronal cells

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Abstract

Background Maternal antenatal corticosteroid treatment is standard care to accelerate fetal maturation. However, there are growing concerns that antenatal corticosteroid administration may harm fetal neurodevelopment. Quantitative assessments of the effects of antenatal corticosteroid on the neonates have not been performed and poorly understood about their complex biology.

Results We collected Methylation BeadChips-generated DNA methylation data from the Gene Expression Omnibus (GEO) database and then employed "multi-tissue predictor" to quantify the DNAm age of saliva from 36 preterm neonates, which were stratified by the absence ($n=12$) or presence ($n=24$) of antenatal corticosteroid exposure, as well as 36 full-term neonates. Next, the DNAm age of human lung fibroblast IMR90 cells and human fetal multipotent hippocampal progenitor HPC cells, with or without glucocorticoid treatment, was also determined. We observed that the DNAm age of full-term neonates was significantly higher than that of the preterm neonates, and antenatal corticosteroid exposure accelerated the DNAm age of preterm neonates, while glucocorticoid exposure accelerated the DNAm age of IMR90 cells. Conversely, dexamethasone exposure delayed the DNAm age of HPC cells during the proliferation phase. It is noteworthy that 65% of the differentially methylated probes (DMPs) linked to the multi-tissue predictor marked CpGs and corticosteroid exposure in IMR90 cells exhibited comparable methylation patterns with the DMPs associated with the antenatal corticosteroid exposure in preterm neonates. Conversely, the majority of these DMPs exhibited inverse methylation alterations in dexamethasone-induced HPC cells. Furthermore, the epigenome-wide association study (EWAS) trait enrichment analyses of the DMPs linked to the antenatal corticosteroid exposure in preterm neonates revealed significant associations with prenatal adverse environmental exposure, growth and development, and neuropsychiatric disorders.

Conclusions Our results identified the cellular and molecular evidences of epigenetic clock changes in neonatal growth and developmental trajectories with the interference of antenatal corticosteroid treatment and provided

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potential clinical guidance for the future development of noninvasive fetal assessments to identify pregnant women who could benefit from antenatal corticosteroid in a wider gestational age.

Keywords Preterm neonates, Corticosteroid, Epigenetic age, Lung maturation, Neurodevelopment

Introduction

Antenatal corticosteroid treatment is now a proven interventions to decrease perinatal mortality, especially in improving the outcomes for preterm infants [1, 2]. It promotes fetal lung maturation and reductions in risks of respiratory distress syndrome [1, 3]. However, the short- and long-term safety profile of antenatal corticosteroid treatment is still debatable. Large cohorts and epidemiology reports may overemphasize their benefits and underestimate their risks in preterm infants [4]. Their effects on child neurodevelopment are of particular concern, as corticosteroids cross the placental and blood–brain barriers and may have adverse effects on child neurodevelopment [2, 5, 6]. However, poorly understanding the biology effects of antenatal corticosteroid treatment on the fetus and the hysteresis of the clinical phenotype make it difficult to make the best decisions about who should be treated and to intervene most effectively at a preventable stage.

Stimulus and insults that occur during the critical periods of fetal growth and development can permanently alter tissue structure and function, which is termed “intrauterine programming” [7]. Epigenetic modifications, as one of the most important types of programming, could plausibly explain how long-lasting effects of prenatally administered natural or synthetic steroids could affect fetal neurodevelopment [5], such as expression of the Arp2/3 complex in the hippocampus during brain development is maintained by sustained DNA methylation modification on the *Arp2* gene promoter [8]. Prenatal bisphenol A exposure produces persistent disturbances in the postnatal changes in DNA methylation status leading to alterations in the expression of specific genes in the offspring [9]. The effects of DNA methylation as the most important epigenetic modifications on neurodevelopment persist from in utero and continue through life [10, 11]. Recent studies based on DNA methylation data have revealed clock-like patterns of epigenetic change over the human lifespan, which offers a quantitative tool for age-related progressive neurodevelopmental changes [11, 12]. It can be able to capture aberrant methylation modifications in individuals in time to quantitatively reveal deviations from their neurodevelopmental trajectories [13, 14]. The landmark epigenetic clock is the "multi-tissue predictor" developed by Horvath, who applied elastic net regression to Illumina DNAm array data from thousands of samples ($n > 8000$) from 51 different tissues and cell types and generated a predictor based on 353 CpG sites [11]. The clock has been widely used in the assessment of individual development and biological age of cells in multiple tissues [11, 13, 15–17].

In this study, we collected publically available DNA methylation data detected by Illumina Infinium Methylation BeadChips from saliva of neonates with or without antenatal corticosteroid exposure during pregnancy [18], or human lung fibroblasts IMR90 cells [19] and human fetal multipotent hippocampal progenitor HPC03A/07 cells exposed to glucocorticoids [20]. We used the "multi-tissue predictor" to assess the effect of antenatal corticosteroid exposure on the epigenetic age of preterm infants and further assessed the variations in the epigenetic age of IMR90 cells and HPC cells exposed to glucocorticoids. This study quantitatively examined whether antenatal corticosteroid exposure affects ontogeny in early neonatal life and elucidates the tissue-specificity of these effects during lung maturation and neurodevelopment.

Results

Antenatal corticosteroid exposure accelerates epigenetic age in preterm infants

We first quantified the epigenetic age (DNAm age) of 36 preterm neonates and 36 full-term neonates (Table 1) using the “multi-tissue predictor,” which showed that the error value of the predicted age compared to the chronological age (uniformly treated as 0) was 0.13 years for the full-term neonate group and 0.09 years for the preterm neonate group (Fig. 1A). The small error represents the high accuracy and confidence of the prediction results obtained by the “multi-tissue predictor.” Next, we compared the DNAm age between the preterm and full-term neonates and observed that the DNAm age of the preterm neonates was significantly lower than that of the full-term neonates ($P = 0.036$, Fig. 1B, Table 2), indicating that the DNAm age can reflect the delayed developmental process of preterm neonates to a certain extent. We further evaluated the effects of corticosteroid exposure on the DNAm age in preterm neonates and observed that the DNAm age of preterm neonates with antenatal corticosteroid treatment is significantly higher that of preterm neonates without antenatal corticosteroid treatment (Fig. 1C, Table 2). Additionally, we did not observe any significant effects of antenatal magnesium sulfate exposure, maternal chorioamnionitis or late-onset sepsis, and

Table 1 Characteristics of the participants

	Full-term controls $N=36$	Preterm neonates $N=36$
Tissue	Saliva	Saliva
Gender	M/F = 18:18	M/F = 18:18
Postmenstrual age at birth/weeks	39.79 ± 1.13	28.43 ± 1.95
Postmenstrual age at scan/weeks	42.71 ± 2.07	39.71 ± 1.37
Chorioamnionitis	Yes/No/NA = 0:5:31	Yes/No = 11:25
Antenatal steroid exposure	Yes/No/NA = 0:5:31	Yes/No = 24:12
Antenatal magnesium sulfate exposure	Yes/No/NA = 0:14:22	Yes/No = 20:16
Parenteral nutrition/days	0	10.69 ± 4.79
Late-onset sepsis	Yes/No/NA = 0:5:31	Yes/No = 19:17

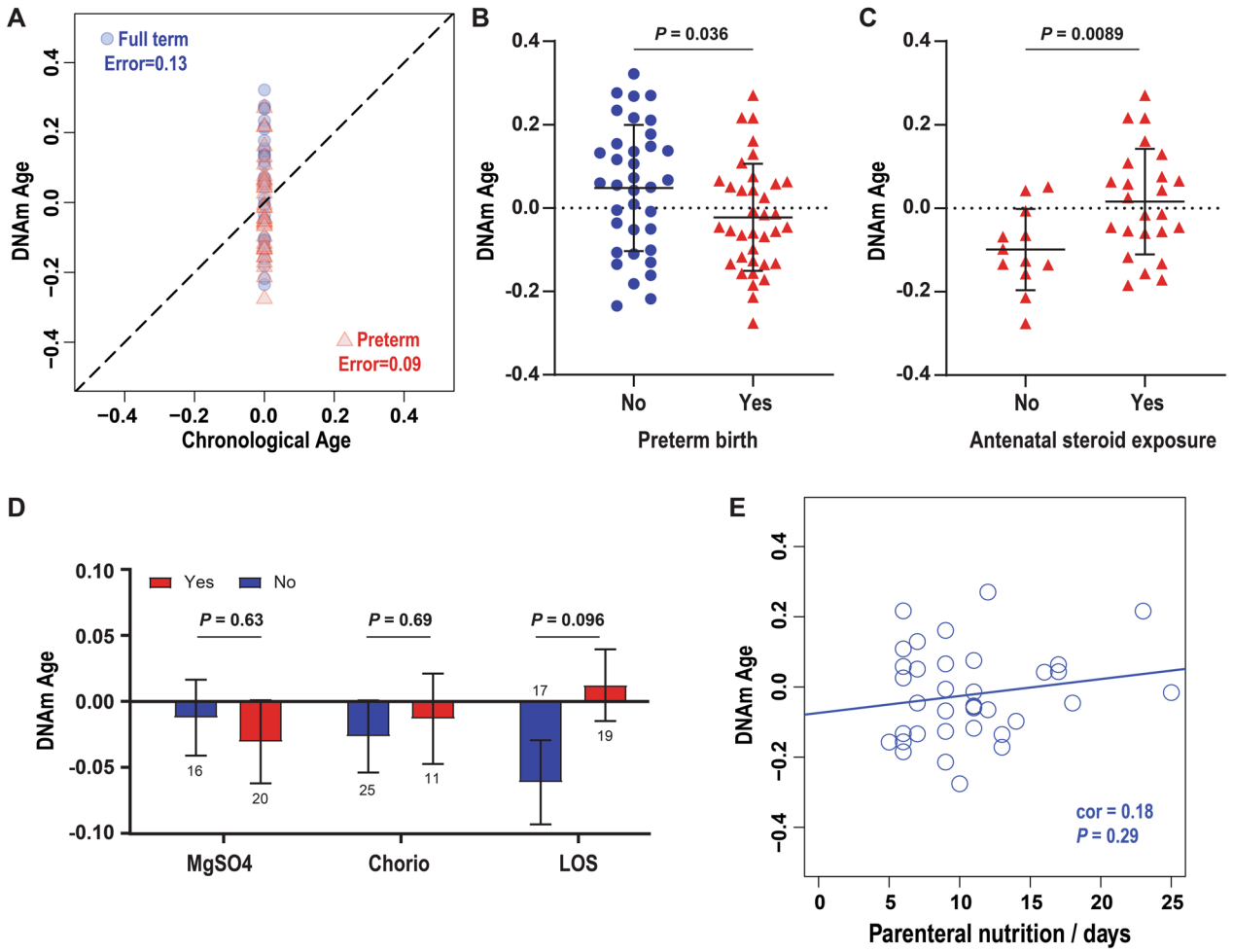

Fig. 1 Effect of antenatal corticosteroid exposure on the epigenetic age of human neonates. **A** Scatterplot of DNAm age estimated by the "multi-tissue predictor" from human neonate saliva (y-axis) against chronological age (x-axis). The black dashed line is the diagonal. The prediction error of DNAm age with chronological age from full-term neonates (blue circles) or preterm neonates (red triangles) is shown in panel. **B**, **C** Comparison of DNAm age between preterm neonates and full-term neonates (**B**) or between antenatal corticosteroid-exposed and non-exposed preterm neonates (**C**). **D** DNAm age in preterm neonates with or without exposures of antenatal magnesium sulfate (MgSO4), maternal chorioamnionitis (Chorio) or late-onset sepsis (LOS). **E** Linear regression analysis of the relationship between daily parenteral nutritional intake and DNAm age with *R* and *P* values. The *P* value derived from the Kruskal–Wallis test is shown between the indicated comparisons in the panels of **B**–**D**

Table 2 Overview of the epigenetic age of neonates with various antenatal stresses

Group	Sample (n)	Age (years)	DNAm age (Mean ± SD)	DNAm age (ΔMean ± SD)	95%CI	Cohen's d	95%CI	*P* value	Power (%)
Human neonates									
Full-term	36	0	0.048 ± 0.025	0.070 ± 0.033	[0.004, 0.136]	0.501	[0.030, 0.969]	0.036	56.7
Preterm	36	0	−0.022 ± 0.021						
Preterm neonates with Antenatal steroid exposure									
No	12	0	−0.099 ± 0.028	−0.115 ± 0.042	[−0.199, −0.030]	−0.972	[−1.696, −0.236]	0.009	85.2
Yes	24	0	0.015 ± 0.026						
Preterm neonates with Antenatal magnesium sulfate exposure									
No	16	0	−0.012 ± 0.029	0.018 ± 0.044	[−0.070, 0.107]	0.141	[−0.518, 0.798]	0.633	7.1
Yes	20	0	−0.031 ± 0.031						
Preterm neonates born to mothers with Chorioamnionitis									
No	25	0	−0.027 ± 0.027	−0.013 ± 0.047	[−0.109, 0.082]	−0.103	[−0.812, 0.607]	0.693	6.1
Yes	11	0	−0.013 ± 0.034						
Preterm neonates born to mothers with Late-onset sepsis									
No	17	0	−0.061 ± 0.032	−0.074 ± 0.042	[−0.158, 0.011]	−0.590	[−1.255, 0.083]	0.096	42.0
Yes	19	0	0.012 ± 0.027						

daily parenteral nutritional intake on the DNAm age of neonates (Fig. 1D, E, Table 2). Antenatal steroid exposure caused a significant increase in the DNAm age of preterm neonates, indicating that corticosteroid medications do play a role in promoting accelerated fetal maturation. This result is indeed in line with the most previous perceptions that the antenatal corticosteroid exposure accelerates fetal maturation. But is this really the case? If so, whether this stimulation was tissue-specific or not during the critical periods of fetal growth and neurodevelopment? We then explored this query in different tissue cells.

Epigenetic age is accelerated in human lung fibroblasts but delayed in human hippocampal progenitor cells by glucocorticoid induction

To assess the role of corticosteroids in fetal maturation, we then evaluated the effects of glucocorticoids on the DNAm age of human lung fibroblast IMR90 cells. We observed that DNAm age displayed an increasing trend line ($P=0.26$, Fig. 2A) in the IMR-90 cells after treatment with 100 nM cortisol for 4 h; however, DNAm age displayed a significant decrease ($P=0.004$) in the cells after treatment with 500 nM relacorilant for 4 h, a selective glucocorticoid receptor modulator that competitively antagonizes the effects of cortisol activity, as compared with the untreated vehicle or the cortisol treatment. In addition, DNAm age displayed a significant increase ($P=0.037$) in the cells after treatment with a combination of these two compounds for 4 h as compared to the cells treated with relacorilant (Fig. 2A). These results suggest that glucocorticoids affect the maturation of human lung fibroblast IMR-90 cells.

We further evaluate the effects of glucocorticoid receptor activation on DNAm age of human multipotent hippocampal progenitor HPC cells during neurogenesis. We calculated the DNAm age of HPC cells after treatment with 1 μM of dexamethasone (a member of the glucocorticoid family) or vehicle (ethanol) during the 3-day proliferation phase (Pro; 3 d) or during both the 3-day proliferation and 7-day neuronal differentiation phases (Pro + Diff; 10 d) using DNA methylation data as described previously [20]. We observed that DNAm age displayed a significant decrease ($P=0.004$) in HPC cells after treatment with 1 μM of dexamethasone for 3 days during the proliferation phage (Fig. 2B). However, we did not observe significant alteration ($P=0.28$) in DNAm age of HPC cells after treatment with dexamethasone for 10 days during both the 3-day proliferation and 7-day neuronal differentiation phases (Pro + Diff; 10 d). Taken together, these results suggest that corticosteroid exposure may play a role in accelerating fetal lung maturation, but at the same time it is likely to impair neurological development.

Glucocorticoid-stimulated epigenetic regulation pattern of IMR90 is consistent with preterm neonates but opposite for HPC

To explore the function implication of DNA methylation, we performed comparative genome-wide DNA methylation analysis to identify differentially methylated probes (DMPs) associated with preterm birth or antenatal corticosteroid exposure. We identified 3,710

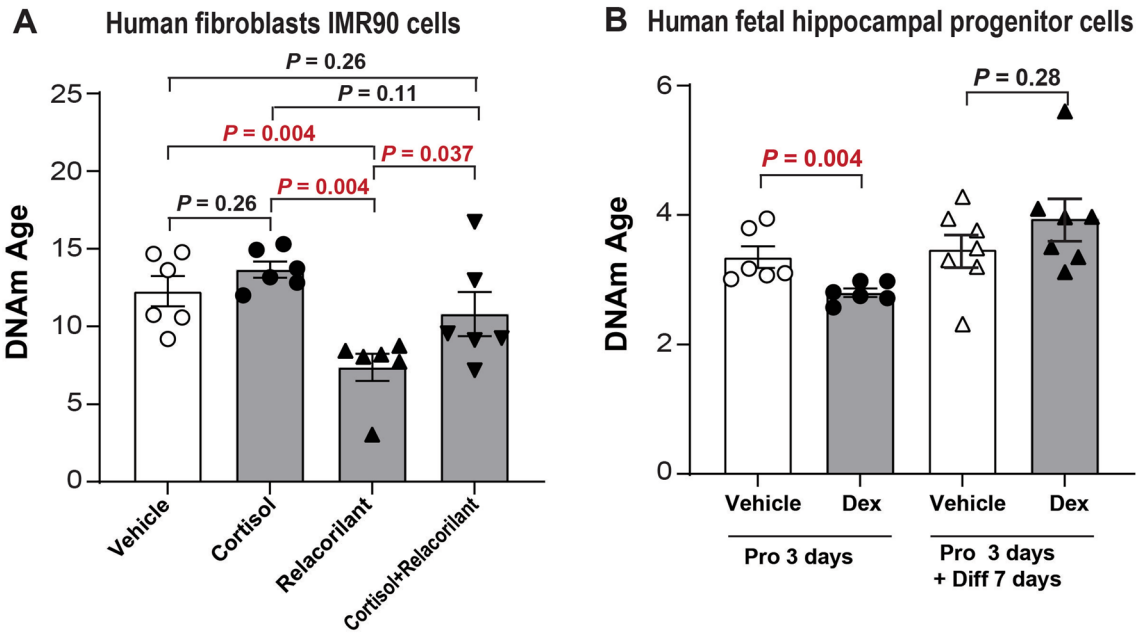

Fig. 2 Effect of glucocorticoids exposure on the epigenetic age of human lung fibroblasts IMR90 cells and human fetal multipotent hippocampal progenitor HPC cells. **A** Comparison of DNAm age of IMR-90 cells pre-treated with vehicle, cortisol, relacorilant, or cortisol + relacorilant for 4 h. **B** Comparison of DNAm age of HPC cells treated with ethanol (Vehicle) or 1 μM of dexamethasone (Dex) during 3-day proliferation phase (Pro 3 days) or during both the 3-day proliferation and 7-day neuronal differentiation phases (Pro 3 days + Diff 7 days). The *P* value from Kruskal–Wallis tests is shown between the indicated comparisons

DMPs between preterm and full-term neonates, while 2,177 DMPs between antenatal corticosteroid-exposed and non-exposed preterm neonates. Of these, 92 DMPs were found to be consistently altered in DNA methylation (Fig. 3A). The EWAS trait enrichment analyses of the 92 DMPs revealed significant associations with prenatal adverse environmental exposure, growth and development, and neuropsychiatric disorders (Fig. 3B, Supplementary File 1 Table 5). Notably, the EWAS trait enrichment analyses of the multi-tissue predictor marked 353 CpG sites also exhibited similar associations (Fig. 3C, Supplementary File 1 Table 6). This indicated that the methylation levels of multi-tissue predictor marked CpGs are likely to be associated with tissue development.

Subsequently, we found that 57 of the 353 CpG loci were significantly different in methylation levels between preterm and term neonates; next, we further conducted a differential methylation analysis of the multi-tissue predictor marked 353 CpG sites involved in the epigenetic clock between antenatal corticosteroid-exposed and non-exposed preterm neonates, cortisol-exposed and non-exposed IMR90 cells, or dexamethasone-exposed and non-exposed HPC cells. We identified that 188 DMPs showed significant methylation alterations in at least one of the aforementioned three pairwise comparisons (Fig. 3D). We next performed Gene Ontology-biological processes (GO-BP) analysis and revealed that those DMPs-annotated genes were functionally enriched in terms related to "stem cell differentiation," "neuronal differentiation," "osteoblast proliferation," "regulation of steroid hormone receptor signaling pathway" and "steroid metabolic processes" (Fig. 3E). Among those 188 DMPs, 10 of 16 DMPs (all hypoDMPs) linked to the antenatal corticosteroid exposure in preterm neonates also exhibited altered methylation in the cortisol-exposed IMR90 cells (4 hypoDMPs and 1 hyperDMPs) or the dexamethasone-exposed HPC cells (6 hyperDMPs; Figs. 3F). In particular, DMP cg19724470, annotated in *CD274* gene, was hypomethylated in both the antenatal corticosteroid-exposed preterm neonates and the cortisol-exposed IMR90 cells but hypermethylated in the dexamethasone-exposed HPC cells. After we added antenatal magnesium sulfate exposure as a covariate to the analysis, the results showed that the methylation levels of the remaining loci still showed significant alterations, except for cg26620959. These results further support that the effects of corticosteroid exposure on epigenetic modification show a tissue or developmental stage-dependent pattern.

Discussion

Antenatal corticosteroids induce fetal lung maturation and reduce neonatal respiratory morbidity and mortality [1, 21], but their widespread over-treatment has

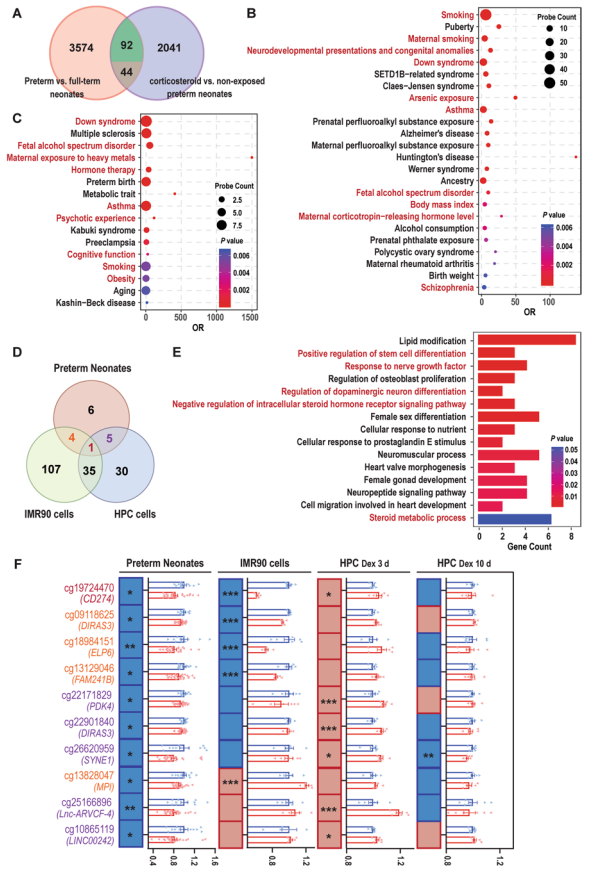

Fig. 3 Characterization of glucocorticoid-induced DMPs in epigenetic clock. **A** Venn diagram showing overlapping relationships. The numbers indicate the counts of the DMPs associated with preterm birth or prenatal corticosteroid exposure in neonates. The 92 DMPs demonstrated a consistent pattern of methylation alterations across the two comparisons. **B** EWAS trait enrichment analysis of the above 92 DMPs. **C** EWAS trait enrichment analysis of the multi-tissue predictor marked 353 CpGs. **D** The overlapped multi-tissue predictor marked DMPs associated with the prenatal corticosteroid exposure in neonates (Neonates), cortisol exposure in IMR90 cells (IMR90 cells), and dexamethasone exposure in HPC cells during the proliferation phase (HPC cells). **E** Gene Ontology-biological processes (GO-BP) analysis of 188 DMPs-annotated genes. **F** The relative DNA methylation levels of 10 overlapping DMPs in the non-treated control (blue bar) are compared to the respective corticosteroid-exposed neonates (Neonates, red bar), cortisol-exposed IMR90 cells (IMR90), dexamethasone-exposed HPC cells (HPC) during the 3-day proliferation phase (Dex 3 d), and during both the 3-day proliferation and 7-day neuronal differentiation phases (Dex 10 d). *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$ from Student *t* test

resulted in unnecessary and inappropriately timed corticosteroid exposure in 50% of fetus [21]. For some, the benefits of preventing transient morbidity may not outweigh the unknown long-term risks of exposing the fetus to antenatal corticosteroids [22]. Our study found that antenatal steroid exposure caused a significant increase in the DNAm age of preterm neonates, indicating that corticosteroid medications do play a role in promoting accelerated fetal maturation. The epigenetic clock is capable of providing estimates of internal physiological condition and other measures of development and senescence. Deviations of predicted age from actual age can be considered biomarkers of development (aging). The DNAm age older than one's chronological age has been associated with accelerated senescence (which in neonates indicates accelerated maturation); conversely, it is associated with developmental delay. This idea has been widely used in studies related to assessing abnormalities in the early development of individuals or abnormalities in the rate of aging [13, 15, 23]. However, there was a non-significant increasing trend level in the DNAm age of human lung fibroblasts IMR90 cells under cortisol-treated, but a significant decrease in DNAm age of dexamethasone-exposed human hippocampal progenitor HPC cells only during the proliferation phase. In fact, there are also studies that multiple antenatal steroid doses are no marked effective in preventing neonatal chronic lung disease, whereas the risk of causing neurodevelopmental defects is extremely high [2, 24]. We also found that the DNAm age of the HPC cells under a 10-day dexamethasone treatment during both the proliferation and neuronal differentiation phases rebounded to a non-difference state from that of vehicle, suggesting that the effect of dexamethasone on DNAm age displayed developmental specific pattern. It is also shown that DNAm age is dynamic changes in both directions, i.e., it increased rapidly in response to environmental stress and reversed after recovery from stress. The elevation of biological age by stress may be a quantifiable and actionable target for future interventions [16]. This quantitative tracking approach can be a potent mediators of lifelong health captured by the concepts of the Developmental Origins of Health and Diseases, providing valuable guidance for clinical decision-making about when, how long, and who should be treated with antenatal corticosteroid treatment, as well as prognostic protocols. We should also pay more attention to the potential effects of maternal health, environmental factors, and prenatal care on the DNAm age in neonates, such as gestational hypertension, gestational diabetes, prenatal depression, adverse environmental exposures during pregnancy, and poor maternal lifestyle habits.

Deviations from the epigenetic clock provide an interface between the organism and its environment [25, 26], so we analyzed the molecular mechanisms by which a brief exposure to environmental insults such as corticosteroid exposure drives changes in DNAm age through clock-marked DMPs. Surprisingly, the corticosteroid exposure-induced DMPs identified in human IMR90

cells overlapped 65% isotropically with the preterm infants, while there was up to 93% anisotropic overlap in human HPC cells. This overlapping variation illustrates the cross-tissue commonality and specificity of corticosteroid-induced DNAm changes and is highly consistent with the trend of DNAm age. It also suggests that the findings of the two in vitro models may translate to human pregnancy and that DMPs with cross-tissue effects could serve as biomarkers for conditions associated with antenatal corticosteroids exposure. For example, the high expression of the overlapping gene *CD274* promotes migration and invasion of lung fibroblasts [27], while its hypermethylation is associated with the PD-1/PD-L1 pathway in which it resides affects neurodevelopment and leads to cognitive deficits associated with the hippocampus [28].

Strengths and limitation

Saliva was chosen for this study because of its high accessibility and significant correlation with free cortisol levels in blood [29]. Currently, published studies about saliva methylation largely focused on cancer, psychiatry, environmental, and lifestyle-related disease [29]. More importantly, venous blood collection would cause the fluctuation of cortisol levels, which decrease the accuracy of evaluation. Saliva collection is more convenient and accurate, making it more suitable for clinical diagnosis. The "multi-tissue predictor" was chosen to calculate DNAm age because it was developed by 51 different tissues and cell types and is more accurate in calculating DNAm age from non-blood samples than other models developed using only whole blood data [11]. The ability of "multi-tissue predictor" to predict biological age has been verified in studies concerning individual development [15, 30], respiratory disease [26], and neurodevelopmental disorders [13, 31]. The main limitation of this study is lacked in vivo confirmation and we should be designed animal experiments to control the dosage of corticosteroids, interval, approach of administration, and at what stage to medicine during pregnancy to assess under what conditions the neurological effects of corticosteroid exposure on newborn individuals can be minimized. Another minor limitation is that this study did not adjust for differences in probe counts. Because our study only focused on alterations in methylation in different groups for the 353 probes involved in the multi-tissue predictor, the methylation levels of these probes change with age. However, our subjects were neonates, and antenatal corticosteroid exposure has not been able to show an extremely significant effect on the methylation levels of those locus, so we used *P* values for screening. We used $P < 0.05$ as an indicator of significance to screen for probes with potential effects on the biological age of individuals. Methylation differences in this fraction of probes are likely to accumulate with age. Due to the small number of probes screened, it is difficult to have multiple genes present the same GO function item at the same time during the GO analysis of their regulatory genes, so the function of related genes was discussed only by *P* values.

Conclusion

In conclusion, our study identified the cellular and molecular evidences of epigenetic clock changes in neonatal growth and developmental trajectories with the interference of antenatal corticosteroid treatment and provided potential clinical guidance for the future development of noninvasive fetal assessments to identify pregnant women who could benefit from antenatal corticosteroid in a wider gestational age.

Materials and methods

Description of the datasets

Three genome-wide DNA methylation datasets from human neonate saliva (GSE72120), human multipotent fetal HPC line HPC03A/07 (GSE119846) or human lung fibroblasts IMR90 cells (GSE210301) were obtained from the Gene Expression Omnibus (GEO) database. DNA extracted from saliva of 36 preterm neonates (< 32 weeks of gestation at birth; Table 1) and 36 full-term neonates (postmenstrual age > 37 weeks) [18] or from HPC cells [20] was employed for DNA methylation assay with Illumina Infinium 450 k Human Methylation BeadChips. The HPC cells were treated with 1 μM of dexamethasone or vehicle (ethanol) during 3-day proliferation phase (Pro; 3 d) or during both the 3-day proliferation and 7-day neuronal differentiation phases (Pro + Diff; 10 d) as previously described [20]. DNA extracted from IMR90 cells, which were treated with cortisol, relacorilant, or a combination of cortisol and relacorilant [19], was employed for DNA methylation assay with Illumina Infinium MethylationEPIC BeadChips.

Determination of the DNAm age and statistical analysis

The DNAm age was measured using the "multi-tissue predictor" [11]. The chronological age of all neonates is uniformly recorded as 0; cell samples from the same dataset have the same initial age by default. The Illumina genome-wide methylation detection platforms quantify DNA methylation levels by the β value from 0 (completely unmethylated) to 1 (completely methylated). The missing values of data were imputed with the k-Nearest Neighbor approach (10 nearest markers) using the R "impute" package [32]. Data normalization was performed using the BMIQ R function, which rescales the roughly 21,368 type II probes in each array that overlap with those used

to train the "multi-tissue predictor" [11], so that their distribution matched the gold standard of the "multi-tissue predictor" [11]. This pre-processing step improved the accuracy of the age predictor and was particularly beneficial in ensuring accuracy across tissues and cells (lung fibroblasts and hippocampal progenitor cells) since the "multi-tissue predictor" covers 51 healthy tissue and cell types. See the Epigenetic Clock website (https://dnamage.genetics.ucla.edu) for a more detailed description. The Kruskal–Wallis test was applied to determine the significant difference in the DNAm ages between antenatal corticosteroid-exposed and non-exposed preterm neonates, cortisol-exposed and non-exposed IMR90 cells, or dexamethasone-exposed and non-exposed HPC cells. Statistical analyses were performed using R Studio, and *P* values of less than 0.05 were considered to be significant. Differences in methylation levels at 353 age-related methylation sites between the correspondent groups were examined using the limma package in R Studio. Entrez gene IDs of CpG sites were provided in the HumanMethylation450 annotation file, which were used to identify genes. Gene Ontology (GO) analyses were conducted in R using the "clusterProfiler" package from Bioconductor. EWAS trait enrichment analyses were conducted by the EWAS Toolkit, a web toolkit for epigenome-wide association study (https://ngdc.cncb.ac.cn/ewas/toolkit).

Supplementary Information

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Additional file 1

Additional file 2

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Author contributions
Xiaohui Wu and Cunyou Zhao wrote the main manuscript text and prepared Figs. 1–3. Chenglin Lu analyzed and interpreted the data. Zhiying Deng, Wenbo Xiao and Hongyu Ni were responsible for data acquisition and collation. All authors reviewed the manuscript.

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Availability of data and materials
The 450 k (GES72120, GSE119846) and 850 k (GSE210301) methylation datasets analyzed during this study are available in the NCBI GEO (Gene Expression Omnibus) repository, https://www.ncbi.nlm.nih.gov/geo/. The detailed description of the raw data and the statistical analysis code were provided in Supplementary file 2.

Declarations

Ethics approval and consent to participate
All datasets of this manuscript are publicly obtained from the Gene Expression Omnibus (GEO) database and other online resources. Thus, all study participants provided informed consent, and the study design was approved by the appropriate ethics review board.

Competing interests
The authors declare no competing interests.

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