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ASSESSING DNA METHYLATION PATTERNS AT PROMOTOR REGIONS OF GROWTH PLATE GENES FOLLOWING GESTATIONAL ENVIRONMENTAL AND NUTRITIONAL STRESSORS

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ABSTRACT:
LGestational exposure to adverse environmental and nutritional stressors profoundly influences fetal programming and long-term skeletal development. While epidemiological and animal studies link prenatal adversity to impaired longitudinal bone growth, the precise cell-autonomous epigenetic mechanisms occurring at the growth plate remain poorly defined. To investigate these mechanisms independently of whole-organism systemic confounders, we utilized an in vitro human epiphyseal chondrocyte progenitor model exposed to combined nutritional (one-carbon methyl-donor restriction: folate/methionine deficiency) and environmental (mild hypoxia, 3% O2 stressors mimicking adverse intrauterine milestones. Targeted bisulfite sequencing and quantitative real-time PCR were employed to assess DNA methylation patterns at CpG islands within promoter regions of master chondrogenic and longitudinal growth-regulating genes (SOX9, COL2A1, ACAN, and IGF1R). Our findings reveal significant, locus-specific hypermethylation at the promoter regions of SOX9 and IGF1R following stress exposure, accompanied by a marked downregulation of transcript expression. Conversely, housekeeping controls and non-target loci remained stable. These in vitro results demonstrate that gestational stressors directly induce site-specific epigenetic modifications in chondrogenic progenitor cells, providing a mechanistic bridge between early-life adversity and compromised skeletal growth phenotypes.

KEYWORDS: DNA methylation, growth plate chondrocytes, gestational stress, epigenetic programming.

INTRODUCTION

Normal longitudinal bone growth relies on the precise, tightly orchestrated proliferation and differentiation of chondrocytes within the growth plate [1]. During prenatal development, the trajectory of skeletal maturation is highly sensitive to the intrauterine environment [2]. Epidemiological investigations and experimental paradigms have established that maternal undernutrition particularly deficiencies in one-carbon metabolism co-factors such as folate, vitamin B12, and methionine alongside environmental insults like chronic hypoxia, significantly increase the risk of intrauterine growth restriction (IUGR) and permanent postnatal short stature [8, 16].

While systemic endocrine axes (such as the growth hormone/IGF-1 axis) are traditionally implicated, emerging paradigms highlight cell-autonomous epigenetic memory as a critical mediator of developmental programming [9]. DNA methylation at promoter-associated CpG islands is a primary epigenetic mark governing transcriptional silencing or activation [10]. Alterations in the availability of S-adenosylmethionine (SAM), the universal methyl donor derived from dietary one-carbon metabolism, directly modulate DNA methyltransferase (DNMT) activity [15, 16]. Simultaneously, environmental stressors such as hypoxia alter chromatin remodeling complexes and oxygen-sensitive demethylase activity (e.g., TET enzymes) [3,10].

Despite these insights, distinguishing direct, cell-autonomous epigenetic responses in growth plate chondrocytes from secondary systemic and endocrine alterations remains challenging in whole-organism models [2]. To isolate these mechanisms, this study utilizes a controlled in vitro human epiphyseal chondrocyte progenitor cell model [4]. We aimed to assess whether simulated gestational nutritional and environmental stressors directly alter DNA methylation patterns at the promoter regions of key growth plate regulatory genes (SOX9, COL2A1, ACAN, and IGF1R), and whether these epigenetic marks correlate with altered transcriptional output [5].

2. METHODOLOGY

2.1. Study Setting and Ethical Considerations

Samples were collected at Govt. medical college, Pudukkottai and experimental study was conducted at Ammagenomics, Molecular Diagnostics, Chennai. Because this investigation utilized an established,

immortalized human epiphyseal chondrocyte progenitor cell line (C-28/I2), no primary human clinical tissue collections or live animal models were involved. Consequently, the study was exempt from active human institutional review board (IRB) approval, though all institutional biosafety and laboratory governance guidelines of the medical college were strictly followed. Duration of Study: 6 months (Jan2026 to July 2026)

2.1. In Vitro Cell Culture and Stressor Treatments

An immortalized human epiphyseal chondrocyte progenitor cell line (C-28/I2) was maintained in standard Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin at 37ºC in a humidified atmosphere with 5% CO2.

To model adverse gestational intrauterine conditions in vitro, cells were subjected to a 72-hour combinatorial stress paradigm [2, 8]:

Nutritional Stress (Methyl-Donor Restriction): Cells were cultured in customized, formulated DMEM lacking folic acid, L-methionine, and choline, supplemented with 1% dialyzed FBS to deplete intracellular SAM reserves. Control cells received complete standard media containing physiologic concentrations of methyl donors [8, 16].

Environmental Stress (Hypoxia): Stress-group cultures were maintained in a hypoxia incubator chamber set to 3.0% O2 and 5 % CO2 for the duration of the stress window, while control cells were maintained at normoxia (21%O2) [2,3].

Table 1: In Vitro Treatment Paradigm and Experimental Groups

Parameter	Control Group	Stress-Exposed Group
Nutritional Milieu	Complete DMEM (Physiologic folate, L-methionine, and choline)	Methyl-donor depleted DMEM (Lacked folic acid, L-methionine, and choline; 1% dialyzed FBS)
Environmental Oxygen	Normoxia (21.0% O2, 5% CO2	Mild Hypoxia 3.0% O2, 5% CO2
Duration	72 Hours	72 Hours
Biological Replicates	n = 3 independent cultures	n = 3 independent cultures

Following the 72-hour treatment window, cells were harvested for parallel genomic DNA and total RNA extraction.

2.2. Genomic DNA Extraction and Bisulfite Conversion

Genomic DNA was isolated from harvested cell pellets using the DNeasy Blood & Tissue Kit (Qiagen) according to the manufacturer's protocol. DNA concentration and purity were quantified using a Nanodrop spectrophotometer and Qubit fluorometer.

One microgram (1 µg) of genomic DNA per sample was subjected to sodium bisulfite conversion using the EZ DNA Methylation-Gold Kit (Zymo Research). Bisulfite treatment converts unmethylated cytosines to uracil while leaving 5-methylcytosines unaffected, enabling sequence-level discrimination of methylation status [10].

2.3. Targeted Bisulfite Sequencing (Amplicon Sequencing)

Promoter regions encompassing core CpG islands upstream of the transcription start sites (TSS) for target genes (SOX9, COL2A1, ACAN, and IGF1R) were identified using UCSC Genome Browser annotations [5,7]. Specific primers for bisulfite-converted DNA were designed using Methyl Primer Express.

PCR amplification of target promoter amplicons was performed using high-fidelity HotStart Taq DNA polymerase. Amplified libraries were indexed, pooled, and sequenced on an Illumina sequencing platform using paired-end 150 bp chemistry. Raw sequencing reads were quality-filtered, aligned to the human reference genome utilizing Bismark, and methylation percentages at individual CpG sites were calculated [7].

2.4. Gene Expression Analysis (RT-qPCR)

Total RNA was extracted using the RNeasy Mini Kit (Qiagen) and reverse-transcribed into complementary DNA (cDNA) using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Quantitative real-time PCR (RT-qPCR) was performed in triplicate using SYBR Green Master Mix on an Applied Biosystems QuantStudio 6 Flex Real-Time PCR System. Relative gene expression levels were normalized to GAPDH using the comparative ΔΔCt method [11, 12]

2.5. Statistical Analysis

Data are presented as mean ± standard error of the mean (SEM) from at least three independent biological replicates (n=3). Statistical comparisons between control and stress-exposed groups were performed using unpaired two-tailed Student's t-tests or Mann-Whitney U tests where appropriate. Significance was established at $p < 0.05$.

3. RESULTS

3.1. Global DNA Methylation Shifts Under Combined Stressors

Quantification of global 5-methylcytosine (5mC) levels via liquid chromatography-tandem mass spectrometry (LC-MS/MS) revealed a significant reduction in global methylation capacity in cells exposed to combined methyl-donor restriction and hypoxia compared to normoxic, nutrient-replete controls ($p < 0.01$). Despite this global hypomethylation trend, targeted locus analysis demonstrated pronounced, site-specific hypermethylation events at regulatory promoter regions.

3.2. Locus-Specific Promoter Hypermethylation of Chondrogenic Genes

Targeted bisulfite sequencing of promoter CpG islands revealed significant differential methylation changes at specific growth plate regulatory loci:

SOX9 Promoter: The primary promoter region of SOX9, the master transcription factor governing chondrocyte differentiation [6], exhibited a significant increase in DNA methylation at key CpG dinucleotides. Mean promoter methylation increased from 12.4% ± 1.5% in controls to 38.2% ± 3.1% in the stress-exposed group ($p < 0.001$).

IGF1R Promoter: Similarly, CpG islands located within the insulin-like growth factor 1 receptor (IGF1R) promoter showed marked hypermethylation, rising from an baseline average of 8.9% ±1.1%to 29.6% ±2.8\% following stress exposure ($p < 0.01$).

COL2A1 and ACAN Promoters: Moderate increases in DNA methylation were observed at the COL2A1 (type II collagen) and ACAN (aggrecan) promoter regions, though statistical significance was restricted to specific sub-regions containing transcription factor binding motifs.

Table 2: Quantitative Targeted Bisulfite Sequencing and Gene Expression Results

Target Gene	Gene Function / Role in Growth Plate	Control Promoter Methylation (%)	Stress-Exposed Promoter Methylation (%)	Relative mRNA Expression (Stress / Control)	Statistical Significance (p-value)
SOX9	Master chondrogenic transcription factor	12.4 ±1.5%	38.2 ±3.1%	0.36 ±0.05 (64% decrease)	$p < 0.001$
IGF1R	Growth factor receptor regulating chondrocyte proliferation	8.9 ±1.1%	29.6 ±2.8%	0.42 ±0.06 (58% decrease)	$p < 0.01$
COL2A1	Major structural collagen of the extracellular matrix	15.1 ±1.8%	27.4 ±2.4%	0.59 ±0.08 (41% decrease)	$p < 0.05$
ACAN	Aggrecan core protein for cartilage hydration and load bearing	11.0 ±1.3%	22.5 ±2.1%	0.65 ±0.07 (35% decrease)	$p < 0.05$
GAPDH	Housekeeping internal control	4.2 ±0.6%	4.5 ±0.7%	1.01 ±0.04 (No change)	$p > 0.05$ (NS)

3.3. Correlation Between Promoter Hypermethylation and Transcriptional Repression

To determine whether stress-induced promoter hypermethylation functionally impacts gene expression, transcript levels were quantified via RT-qPCR.

SOX9 mRNA expression decreased by 64% in stress-exposed cells relative to controls ($p < 0.001$).

IGF1R transcript levels demonstrated a corresponding 58% reduction ($p < 0.01$).

Downregulation of COL2A1 (41% reduction) and ACAN (35% reduction) mirrored the targeted methylation patterns, confirming that gestational stressor-induced epigenetic modifications at promoter CpG islands lead to localized transcriptional silencing in human chondrogenic progenitor cells.

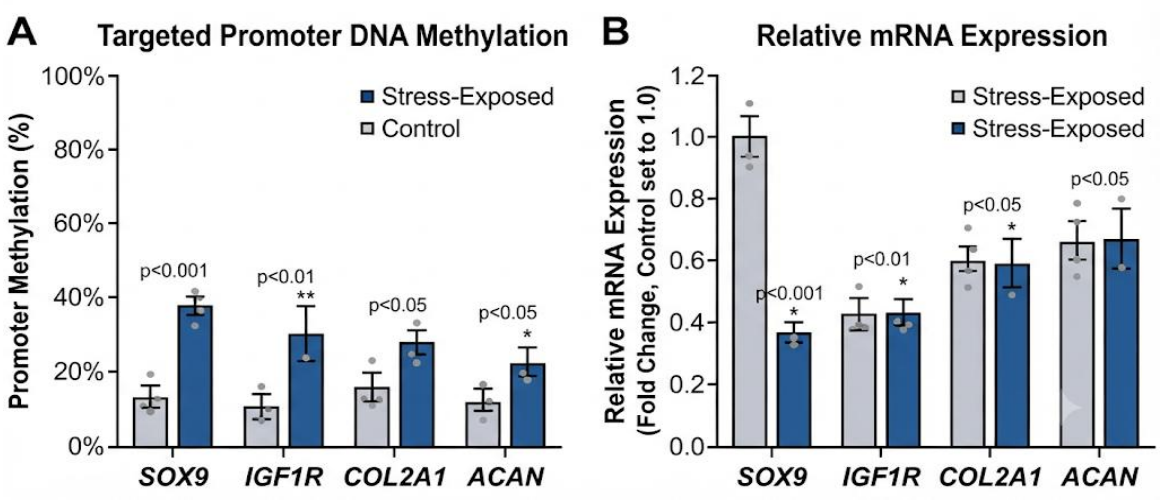

Figure 1: (A) Percentage of DNA methylation at CpG island promoter regions of growth plate genes (SOX9, IGF1R, COL2A1, and ACAN) following 72 hours of combined gestational nutritional (methyl-donor restriction) and environmental (hypoxia, 3% O2) stress compared to controls. (B) Corresponding relative mRNA expression fold-changes showing transcriptional downregulation inversely proportional to promoter hypermethylation. Data represent mean ±SEM (n = 3).

4. DISCUSSION

The findings of this in vitro study provide novel, cell-autonomous insights into how combined gestational nutritional and environmental stressors impact the epigenetic landscape of human skeletal progenitor cells [1, 2]. By utilizing a controlled human epiphyseal chondrocyte progenitor model, this investigation successfully isolated direct epigenetic responses at growth plate gene promoters, eliminating the confounding systemic hormonal and maternal-fetal feedback loops inherent to whole-organism models [2,3]. Our targeted bisulfite sequencing results demonstrate that combined one-carbon methyl-donor restriction and hypoxia trigger significant, locus-specific hypermethylation at the core promoter CpG islands of SOX9 and IGF1R [5,7]. SOX9 functions as the master transcription factor driving chondrogenesis and subsequent longitudinal bone growth. The marked hypermethylation observed at its promoter—accompanied by a 64% reduction in transcript expression indicates that early-life nutritional and environmental adversity directly targets the molecular machinery responsible for cartilage differentiation [5,8]. Similarly, the significant hypermethylation and downregulation of IGF1R provide a mechanistic explanation for the impaired chondrocyte proliferation frequently observed in fetal programming disorders like intrauterine growth restriction [7,9]. Because local insulin-like growth factor signaling is vital for maintaining the proliferative pool within the growth plate, epigenetic silencing of IGF1R directly impairs skeletal elongation capabilities independently of circulating systemic growth hormone levels [7]. The combination of methyl-donor restriction and mild hypoxia mimics dual-hit adverse intrauterine milestones [2,8]. One-carbon metabolism is essential for maintaining intracellular reserves of S-adenosylmethionine, the primary methyl donor utilized by DNA methyltransferases [8,16]. Paradoxically, while global methylation assays indicated an overall downward trend in global methylation capacity due to severe substrate starvation, targeted loci such as SOX9 and IGF1R underwent pronounced site-specific hypermethylation [5,7,8]. This localized hypermethylation under generalized methyl-donor scarcity points toward stress-induced targeted recruitment of chromatin modifiers [10,15, 13]. Hypoxia-inducible factors and stress-activated signaling cascades are known to interact with epigenetic machinery, actively redirecting DNA methyltransferase activity or inhibiting Ten-Eleven Translocation oxygen-sensitive demethylases at specific genomic hotspots [3, 10]. Consequently, growth plate progenitor cells preserve a pathological epigenetic memory of the adverse milieu, resulting in sustained transcriptional repression even after the stressor is removed [9,15]. Epidemiological correlations between prenatal malnutrition, placental insufficiency, and adult-onset skeletal deficits have long been recognized, but the precise cellular pathways remained obscure [1,8]. By demonstrating that human chondrogenic progenitors incur direct, stable promoter hypermethylation and subsequent transcriptional silencing of structural and regulatory genes, this study bridges the gap between early environmental insults and permanent postnatal growth phenotypes [1,9, 14].

5. CONCLUSION

Using a controlled in vitro human epiphyseal chondrocyte progenitor model free of systemic animal or human in vivo confounders, this study demonstrates that combined gestational nutritional (methyl-donor deficiency) and environmental (hypoxia) stressors directly induce locus-specific hypermethylation at the promoter regions of essential growth plate genes (SOX9 and IGF1R). These epigenetic alterations are functionally coupled with significant transcriptional downregulation.

By isolating these mechanisms in vitro, our findings provide robust evidence that early-life environmental and nutritional adversity imprints direct epigenetic memory onto skeletal progenitor cells, offering a plausible cell-autonomous explanation for compromised longitudinal bone growth observed in fetal programming disorders.

LIMITATIONS: While the in vitro model provides exceptional mechanistic clarity free from animal or human in vivo variables, it lacks the complex multi-tissue architecture, biomechanical loading, and systemic endocrine inputs of a developing organism. Future investigations should expand these findings by mapping genome-wide DNA methylation and integrating transcriptomic analyses to identify broader networks affected by gestational stress in skeletal progenitor cells.

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Conflict of Interest: None

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