



MOLECULAR PATHOGENESIS OF GENETIC AND INHERITED DISEASES

Let-7c-5p Drives Collagen Gene Repression Linking Phenobarbital Teratogenicity to Cleft Palate



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The treatment of seizures during pregnancy remains challenging due to the teratogenic effects of many anticonvulsants. Phenobarbital (PB), despite its known risks, is widely used and associated with congenital malformations such as cleft palate (CP). CP is a common craniofacial defect resulting from disrupted palatogenesis, influenced by genetic and environmental factors, including maternal medication. miRNAs are key post-transcriptional regulators implicated in palate development and possibly modulated by teratogenic agents. This study investigated whether miRNA dysregulation observed in CP patients reflects epigenetic mechanisms induced by PB in human embryonic palatal mesenchymal (HEPM) cells. miRNAs dysregulated in palatal tissues from CP patients were previously identified by our group. PB-treated HEPM cells exhibited significant alterations in miRNA expression, notably down-regulation of miR-29b-3p and up-regulation of let-7c-5p, consistent with the miRNA expression patterns previously observed in CP patient tissues through multiplexed profiling. Bioinformatic analyses predicted collagen fibril organization as a common targeted pathway. Quantitative RT-PCR validation confirmed down-regulation of key collagen genes in CP patient tissues and PB-treated HEPM cells. Functional inhibition of let-7c-5p restored *COL1A2* and *COL3A1* expression, suggesting let-7c-5p mediates collagen gene repression in response to PB and potentially contributes to CP pathogenesis. The findings reveal a shared epigenetic regulatory axis involving let-7c-5p and collagen genes linking PB teratogenicity and CP, providing new insights into extracellular matrix remodeling during palatogenesis. (*Am J Pathol* 2026, 196: 1700–1707; <https://doi.org/10.1016/j.ajpath.2026.04.008>)

The treatment of seizures during pregnancy presents significant challenges, largely due to the recognized teratogenic effects of most anticonvulsants.¹ Despite advances in safer antiepileptic drugs, phenobarbital (PB) continues to be widely used globally. However, PB exposure during gestation is associated with an increased risk of congenital malformations, including cardiovascular abnormalities² and cleft palate (CP).³

CP is a common congenital craniofacial defect, accounting for approximately one-third of oral clefts, with an incidence ranging from 1 to 25 per 10,000 live births worldwide.⁴ It is a multifactorial disorder influenced by both genetic and environmental factors acting during palatogenesis, which occurs between the 6th and 10th

gestational week.⁵ Secondary palate (between the incisive foramen and the uvula) development involves the vertical growth, horizontal juxtaposition, and fusion of the two palatal shelves through a well-orchestrated apoptotic process in the medial edge epithelia. Failure at any stage can result in CP. A recent review has reported 131 candidate genes potentially implicated in CP pathogenesis.⁶ Expression of these or other specific genes may be modulated by environmental factors operating during palate development

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possibly by disruption of crucial signaling pathways, such as *BMP*, *FGF*, *WNT*, and *SHH*.⁷ Nevertheless, the precise molecular mechanisms through which environmental factors, including maternal medication, contribute to CP remain under investigation.

Recently, the research focus has shifted from the consequences of constitutive mutations on the gene product to the epigenetic mechanisms by which the functionality of a gene can be altered. miRNAs are endogenous 18- to 24-nucleotide RNAs that modulate gene expression post-transcriptionally by inducing mRNA degradation or translational repression.⁸ Multiple miRNAs have been implicated in palate development, although many remain to be characterized.^{6,9} It has been hypothesized that some teratogenic effects of antiepileptic drugs result from miRNA modulation. This hypothesis was examined in human embryonic palatal mesenchymal (HEPM) cells treated with phenytoin and PB, focusing on drug-induced dysregulation of miRNAs previously linked to CP.^{10,11}

An earlier study¹² identified a set of significantly dysregulated miRNAs in palatal tissues from 11 CP patients using the NanoString miRNA expression panel (Diatech Lab Line, Jesi, Italy). This work aims to determine whether the miRNAs found to be dysregulated in palatal tissues of CP patients may reflect a shared epigenetic mechanism underlying both the pathogenesis of the defect and the teratogenic effects induced by PB in HEPM cells.

Materials and Methods

Cell Culture and Cell Proliferation Assay

HEPM cells (LGC Standards, Milan, Italy; Lot/Batch No: 58798172) were cultured in Dulbecco's modified Eagle's medium/F12 medium (Sigma-Aldrich, St. Louis, MO) supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin, and 1% L-glutamine (all from Sigma-Aldrich). Cultures were maintained at 37°C in a humidified incubator with 5% CO₂. For assays, cells were seeded at 5000 cells/well in 96-well plates using serum-free medium and allowed to adhere for 6 hours. PB stock solution (0.1 mol/L) was prepared by dissolving 25.4 mg of PB powder (Sigma-Aldrich) in 1 mL of sterile ultrafiltered water. Working concentrations (0.1, 1, 3, 6, and 10 mmol/L) were obtained by serial dilution in complete Dulbecco's modified Eagle's medium/F12 medium.

Cell viability following PB treatment was quantified at 24 and 48 hours using PrestoBlue Cell Viability Reagent (Invitrogen, Carlsbad, CA), according to the manufacturer's protocol. Briefly, 10 μ L of PrestoBlue reagent was added to each well containing 90 μ L of medium, and the plates were incubated for 1 hour at 37°C in the dark. Wells containing only medium were used as background controls and subtracted from all samples. Fluorescence measurements were taken with excitation at 570 nm and emission at 620 nm on an automated microplate reader (NB-12-0035; Neo Biotech,

Nanterre, France). Each condition was performed in eight technical replicates. Relative viability (%) was calculated by normalizing fluorescence of treated wells to vehicle controls.

Clinical Tissue Samples

Seven CP patient mucosal tissue samples (mean $\pm$ SD age, 6 $\pm$ 2.08 months) were collected from surgical palate reconstruction at the San Bortolo Hospital in Vicenza, Italy, and preserved in RNAlater (Sigma-Aldrich) for subsequent RNA extraction. This study adhered to the Declaration of Helsinki and was approved by the ethics committee of reference for AULSS 8 Berica - Ospedale di Vicenza (CET ASOV protocol n. 45/23; approval date May 26, 2023), with written informed consent from parents.

Cell Treatment and Reverse Transcription

After determining the optimal concentration and duration for treatment, HEPM cells were seeded in 35-mm culture dishes at a density of 300,000 cells/well in serum-free Dulbecco's modified Eagle's medium/F12 medium and allowed to adhere for 6 hours. Cells were then treated with 6 mmol/L PB or vehicle control for 24 hours. Total RNA was extracted using the miRNeasy Mini Kit (Qiagen, Valencia, CA) in accordance with the manufacturer's instructions.

For miRNA reverse transcription, 1000 ng of total RNA was used according to the method described by Balcells et al.¹³ For cDNA synthesis of other RNA species, 500 ng of total RNA was reverse-transcribed using the PrimeScript RT Master Mix Kit (Takara Bio, Kusatsu, Japan), adhering to the instructions provided by the manufacturer.

miRNA Target Gene Analysis and Enrichment

Lists of predicted functional miRNA targets were obtained by analyzing the online miRDB database v6.0 (<https://mirdb.org>, last accessed June 5, 2025).¹⁴ miRNA-predicted target genes were evaluated using enrichment analysis against gene ontology categories from the PANTHER (Protein ANALysis THrough Evolutionary Relationships) Classification System (<https://pantherdb.org>, last accessed June 5, 2025), focusing specifically on GO: Biological Process and PANTHER: Protein Class categories. Fold enrichment of genes in the uploaded list compared with the expected background was assessed using Fisher's exact test, with false discovery rate correction applied to account for multiple testing.

To identify miRNAs potentially involved in gene set regulation, such as collagen genes, Gene Set Enrichment Analysis (<https://www.gsea-msigdb.org>, last accessed June 5, 2025) was used; all 29 collagen isoform genes were compared against gene sets predicted to be targets of miRNAs from the miRBase database v22, as implemented

via miRDB v6.0 with MirTarget v4 prediction scores >85 to ensure high-confidence target selection.

Quantitative Real-Time RT-PCR

Oligonucleotide sequences for the miRNA expression analysis were designed using miRprimer2 version 2.0,¹⁵ and primer sequences used for quantification of target gene expression were designed using Primer-BLAST (<https://www.ncbi.nlm.nih.gov/tools/primer-blast>).¹⁶ *RNU44* and *RPL13* served as reference genes for miRNA and mRNA normalization, respectively (Table 1).

Quantitative real-time RT-PCR (qRT-PCR) was performed on the QuantStudio 5 Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA). Each 20-μL reaction contained 10 μL of Power SYBR Green Master Mix (Life Technologies, Foster City, CA), 100 nmol/L of each forward and reverse primer, and 300 nmol/L of cDNA. The cycling conditions consisted of an initial denaturation step at 95°C for 2 minutes, followed by 40 cycles of 15 seconds at 95°C and 60 seconds at 60°C. A melt curve analysis was conducted at the end of each run to confirm amplification specificity.

Cell Transfection

HEPM cells were seeded in 6-well cell plates and grown to approximately 60% confluence at the time of transfection. Cells were simultaneously treated with PB 6 mmol/L and transfected with Lipofectamine RNAiMAX (Thermo Fisher Scientific) plus *mirVana* miRNA inhibitor let-7c (Thermo Fisher Scientific) for 24 hours at 37°C. A

mirVana miRNA inhibitor Negative Control #1 (Thermo Fisher Scientific) was included as a baseline control for comparison of target gene expression in PB-treated HEPM cells. Successful inhibition of let-7c-5p was validated measuring the up-regulation of its target gene, *HMGA2*, by qRT-PCR.

Statistical Analysis

Statistical analysis of all experiments was performed using SPSS Statistics software version 29.0 (IBM Corp., Armonk, NY). For cell viability assays involving multiple PB concentrations and time points, one-way analysis of variance was applied, followed by Tukey’s post hoc pairwise comparisons. Relative gene and miRNA expression levels were analyzed with the ΔΔC_T method.¹⁷ Independent-samples *t*-test was performed to compare the ΔC_T values of cases and controls. Relative expression levels were calculated by raising 2 to the power of the negative ΔΔC_T values, which represents the fold change in expression levels. The 95% CIs of fold changes were calculated on ΔΔC_T values using the *t*-test distribution and subsequently transformed to the fold-change scale.

Results

Considering the established teratogenic effects of PB, the aim of this study was to investigate how PB influences cellular epigenetic mechanisms that may promote the onset of CP. As expected, PB treatment reduced HEPM viability in a dose- and time-dependent manner. One-way analysis of variance revealed a statistically significant reduction in

Table 1 Primer Sequences (5′ → 3′) Used for Quantitative Real-Time RT-PCR Expression Analysis

miRNA or gene	Forward primer	Reverse primer
miRNA		
miR-100-5p	5′-CAGAACCCGTAGATCCGA-3′	5′-GTCCAGTTTTTTTTTTTTTTTTCACAAG-3′
miR-29b-3p	5′-CAGTAGCACCATTGAAATCAG-3′	5′-GGTCCAGTTTTTTTTTTTTTTTAACAC-3′
miR-205-5p	5′-CCTTCATTCCACCGGAGT-3′	5′-GGTCCAGTTTTTTTTTTTTTTTCAGA-3′
miR-200c-3p	5′-AGTAATACTGCCGGGTAATGA-3′	5′-GGTCCAGTTTTTTTTTTTTTTTCCA-3′
miR-125a-5p	5′-CCCTGAGACCCTTTAACCT-3′	5′-GGTCCAGTTTTTTTTTTTTTTTCACA-3′
miR-5682	5′-GGTAGCACCTTGCAGGA-3′	5′-GGTCCAGTTTTTTTTTTTTTTTACCT-3′
miR-6871-3p	5′-CAGCACCTGTGGCT-3′	5′-GTCCAGTTTTTTTTTTTTTTCTGTG-3′
let-7c-5p	5′-GCAGTGAGGTAGTAGGTTGTA-3′	5′-GGTCCAGTTTTTTTTTTTTTTTAACCA-3′
<i>RNU44</i>	5′-CCTGGATGATGATAGCAAATGC-3′	5′-GTCCAGTTTTTTTTTTTTTTTAGTCAGT-3′
Gene		
<i>COL1A1</i>	5′-TAGGGTCTAGACATGTTTCAGCTTTGT-3′	5′-GTGATTGGTGGGATGCTCTTCGT-3′
<i>COL1A2</i>	5′-GTGGTTACTACTGGATTGAC-3′	5′-CTGCCAGCATTGATAGTTTC-3′
<i>COL2A1</i>	5′-GCGACGACATAATCTGTGA-3′	5′-GTCCTTTGGGTCCTACAATA-3′
<i>COL3A1</i>	5′-ATTACCTACACAGTTCTGG-3′	5′-TGCGTGTTTCGATATTCAAAG-3′
<i>COL4A1</i>	5′-AAAGGGAGATCAAGGGATAG-3′	5′-TCACCTTTTCTCCAGGTAG-3′
<i>COL5A1</i>	5′-TTGACGAGAACTACTACGAC-3′	5′-ATCCCTTCATAGATGGTATCC-3′
<i>COL11A1</i>	5′-AGATGAGGCAAACATCGTTGA-3′	5′-ATCAGAATCCCTGCCGTCTA-3′
<i>RPL13</i>	5′-ATTCACAAGAAGGGAGACAG-3′	5′-GAAATCTCTCTCTTCCTCAGTG-3′
<i>HMGA2</i>	5′-GAAAAACGCCAAGAGGCAG-3′	5′-GATCCAACCTGCTGCTGAGGT-3′

RNU44 and *RPL13* were used as reference genes for normalization; *HMGA2* was the target gene used to monitor let-7c-5p inhibition efficiency.

HEPM viability at both 24 hours ($F = 103$, 5 Df, $P < 0.001$) and 48 hours ($F = 80$, 5 Df, $P < 0.001$) following PB exposure (Figure 1). Based on post hoc comparisons, a 24-hour treatment with 6 mmol/L PB was selected for further experiments, as it induced a significant decrease in viability while preserving more than 50% cell survival.

Initially, the impact of PB treatment on the expression levels of five miRNAs, which the research group had previously identified as among the most deregulated in palatal tissues of patients with CP, was assessed.

Two miRNAs up-regulated in patient tissues, miR-205-5p and miR-200c-3p, also showed fold changes greater than 1 in PB-treated HEPM cells; however, these expression increases were not statistically significant (Table 2). Among the miRNAs down-regulated in patient tissues, miR-29b-3p exhibited a significant twofold decrease in expression following PB treatment ($P = 0.02$).

miRDB predicted 360 high-confident target genes (with score $\geq 85\%$) for miR-29b-3p. Ontology analysis of these predicted targets using the PANTHER Classification System revealed a strong enrichment linked to extracellular matrix structural protein (false discovery rate = 9.9×10^{-17} , fold enrichment = 15.9) (Supplemental Tables S1 and S2). Several collagen-coding genes fell into this category. Consequently, the expression of selected collagen genes in PB-treated HEPM cells was evaluated (Table 3). Expression analysis revealed that PB consistently down-regulated collagen genes; however, this effect appeared independent of miR-29b-3p, which was also down-regulated.

Table 2 Expression Profile in HEPM + PB of the miRNAs Found to Be Most Deregulated in Cleft Palate Patients by the NanoString Platform (NanoString, Seattle, WA)

miRNA	$\Delta\Delta C_T$	t -Test	Df	P value	Fold change	95% CI
mir100-5p	-0.01	-0.08	14	0.94	1.01	0.79–1.28
mir29b-3p	0.98	2.53	14	0.02	0.51	0.29–0.90
mir205-5p	-0.42	-0.77	14	0.46	1.34	0.59–3.02
mir200c-3p	-0.09	-0.27	14	0.79	1.07	0.64–1.78
mir125a-5p	0.01	0.05	14	0.96	0.99	0.78–1.27

Bold indicates statistically significant P values.

HEPM, human embryonic palatal mesenchymal cells; PB, phenobarbital.

cDNA samples from palate tissue of CP patients remaining from the previous investigation¹² were used to evaluate collagen gene expression compared with HEPM cells. Several collagen genes were strongly down-regulated in patient tissues (Table 4).

The concurrent down-regulation of collagen genes in both patient tissues and PB-treated HEPM cells was considered biologically relevant and potentially linked to CP pathogenesis. Therefore, a bioinformatics approach was used to identify additional miRNAs potentially driving this effect. Using the Gene Set Enrichment Analysis tool, the list of 26 collagen genes was queried against miRDB to identify miRNAs predicted to target these genes with high confidence scores (≥ 85). The analysis revealed several miRNAs likely regulating different collagen genes: miR-29b emerged as the principal regulator, predicted to bind 21

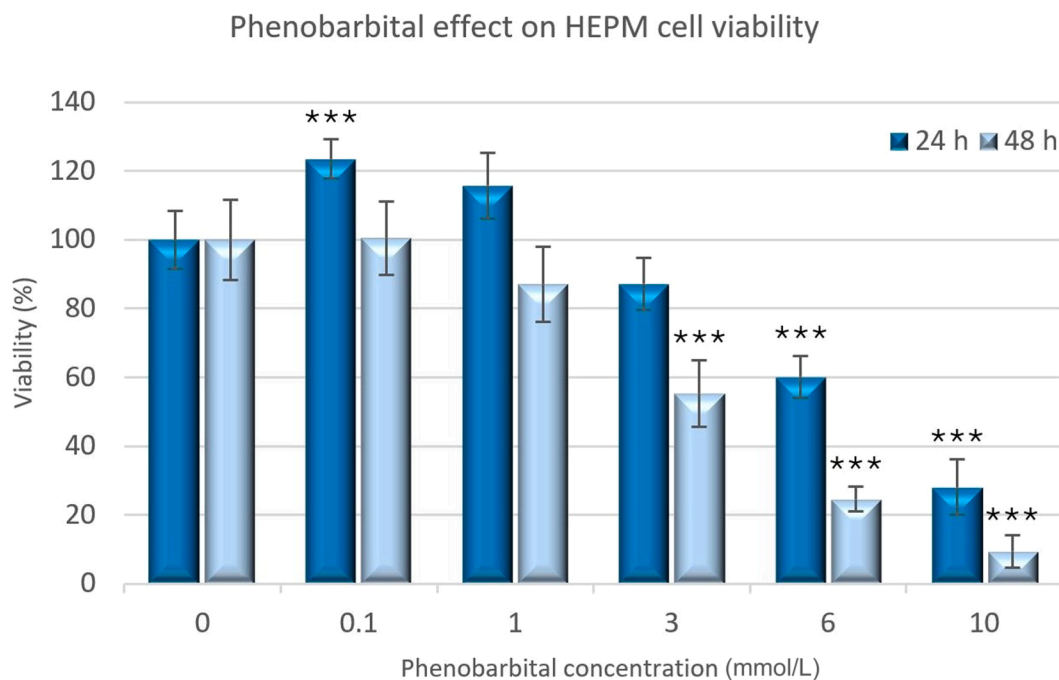


Figure 1 Viability of human embryonic palatal mesenchymal (HEPM) cells treated with varying concentrations of phenobarbital after 24 and 48 hours. Error bars represent 95% CIs. *** $P < 0.001$ versus untreated control.

Table 3 Expression Analysis in HEPM + PB of miR-29b-3p Target Genes from Panther Pathway “Collagen Fibril Organization”

Gene	Target score (miRDB)	$\Delta\Delta C_T$	<i>t</i> -Test	Df	<i>P</i> value	Fold change	95% CI
<i>COL1A1</i>	98	2.86	10.09	12	3.26×10^{-7}	0.14	0.09–0.21
<i>COL1A2</i>	90	1.34	4.18	12	9.20×10^{-4}	0.39	0.24–0.64
<i>COL2A1</i>	97	2.08	5.00	6	2.45×10^{-3}	0.24	0.12–0.48
<i>COL3A1</i>	100	3.56	16.55	6	3.10×10^{-6}	0.08	0.06–0.12
<i>COL5A1</i>	100	3.03	6.37	6	7.04×10^{-4}	0.12	0.05–0.27
<i>COL11A1</i>	99	3.02	5.50	6	1.51×10^{-3}	0.12	0.05–0.31

Bold indicates statistically significant *P* values.
HEPM, human embryonic palatal mesenchymal cells; PB, phenobarbital.

collagen mRNAs, along with miR-5682, miR-6871-3p, miR-767-5p, and members of the let-7 family (Supplemental Table S3). Next, it was tested whether these candidate miRNAs were involved in collagen gene down-regulation following PB treatment in HEPM cells. Candidate miRNA expression analysis (Table 5) showed that PB treatment significantly up-regulated miR-6871-3p and let-7c-5p, approximately fourfold, suggesting their role in suppressing collagen gene expression. Conversely, miR-5682 expression was unchanged, and no reliable data were obtained for miR-767-5p due to assay quality control failure.

To demonstrate a functional link between let-7c-5p and collagen gene modulation, PB-treated HEPM cells were transfected with a let-7c-5p inhibitor. Successful knock-down of the miRNA was validated by the up-regulation of its known target gene, *HMGA2* (predicted to have the highest target score) via qRT-PCR. Inhibition of let-7c-5p in PB-treated HEPM cells markedly increased the expression of key collagen genes *COL1A2* and *COL3A1*, as detailed in Table 6, thereby providing direct functional evidence that let-7c-5p actively represses collagen expression.

Discussion

miRNAs have emerged as critical post-transcriptional regulators in diverse developmental processes, including craniofacial morphogenesis. While the involvement of miRNAs in CP pathogenesis is increasingly recognized,

this study provides novel functional insights by identifying let-7c-5p as a key epigenetic regulator repressing collagen gene expression during palatal development. Specifically, it is demonstrated, for the first time to our knowledge, that let-7c-5p modulates expression of crucial collagen genes, such as *COL1A2* and *COL3A1*, in HEPM cells exposed to PB. This finding offers further insights into the molecular mechanisms linking a pharmacologic teratogen to extracellular matrix remodeling.

Building on previous observations of multiple dysregulated miRNAs in 11 CP patient tissues,¹² the impact of periconceptional exposure to PB, a known teratogen associated with increased risk of CP formation, on miRNA profiles in HEPM cells was explored. PB treatment induced a dose-dependent reduction in HEPM cell viability, confirming its cytotoxicity.¹⁰ Comparative analysis of miRNA and target gene expression in CP patient tissues and HEPM + PB cells enabled the identification of at least one shared epigenetic regulatory pathway. Among the miRNAs analyzed, miR-29b-3p was significantly down-regulated in HEPM + PB cells. However, despite bioinformatic predictions indicating collagen fibril organization as a major target pathway, qRT-PCR assays of six collagen genes revealed no direct up-regulation attributable to miR-29b-3p dysregulation, suggesting its limited direct role in collagen regulation in this model.

Given collagen’s well-established importance in tissue remodeling, cell proliferation, and palatal shelf fusion,¹⁸ the regulatory network underlying collagen gene expression

Table 4 Expression Analysis of Collagen Genes in *ex Vivo* Palatal Tissue Samples from Cleft Palate Patients

Gene	$\Delta\Delta C_T$	<i>t</i> -Test	Df	<i>P</i> value	Fold change	95% CI
<i>COL1A1</i>	3.61	10.41	12	2.3×10^{-7}	0.08	0.05–0.14
<i>COL1A2</i>	5.31	12.64	12	2.7×10^{-8}	0.03	0.01–0.05
<i>COL2A1</i>	1.90	1.00	12	0.34	0.27	0.02–4.75
<i>COL3A1</i>	1.29	3.71	12	3.0×10^{-3}	0.41	0.24–0.69
<i>COL4A1</i>	3.17	4.29	12	1.0×10^{-3}	0.11	0.04–0.34
<i>COL5A1</i>	3.04	4.96	12	3.3×10^{-4}	0.12	0.05–0.31
<i>COL11A1</i>	0.52	0.47	12	0.65	0.70	0.13–3.71

Bold indicates statistically significant *P* values.

Table 5 Expression Analysis in HEPM + PB of miRNAs Other Than miR-29b-3p Targeting Collagen Genes

miRNA	$\Delta\Delta C_T$	<i>t</i> -Test	Df	<i>P</i> value	Fold change	95% CI
miR-5682	−0.09	−0.25	14	0.81	1.07	0.6–11.85
miR-6871-3p	−2.13	−4.72	14	3.30×10^{-4}	4.38	2.24–8.57
let-7c-5p	−1.77	−4.25	12	1.12×10^{-3}	3.42	1.82–6.41

Bold indicates statistically significant *P* values.
HEPM, human embryonic palatal mesenchymal cells; PB, phenobarbital.

Table 6 Expression Analysis of Collagen Genes in HEPM + PB After Let-7c-5p Inhibition

Gene	Target score (miRDB)	$\Delta\Delta C_T$	<i>t</i> -Test	Df	<i>P</i> value	Fold change	95% CI
<i>COL1A1</i>	57	0.03	0.12	6	0.90	0.98	0.65–1.48
<i>COL1A2</i>	92	−0.52	−2.69	6	0.04	1.43	1.03–1.99
<i>COL2A1</i>		−0.13	−0.80	6	0.46	1.10	0.83–1.45
<i>COL3A1</i>	95	−0.42	−3.25	6	0.02	1.34	1.07–1.67
<i>COL4A1</i>	88	0.03	0.12	6	0.91	0.98	0.67–1.45
<i>COL5A1</i>		0.14	0.59	6	0.57	0.90	0.60–1.37
<i>COL11A1</i>		0.03	0.16	6	0.88	0.98	0.69–1.39

Bold indicates statistically significant *P* values.

HEPM, human embryonic palatal mesenchymal cells; PB, phenobarbital.

changes was further investigated. Two candidate miRNAs were found to be overexpressed: miR-6871-3p and let-7c-5p.

The miR-6871-3p overexpression, showing a notable fold change of 4.38 in HEPM + PB cells, is interesting. It was not possible to evaluate its expression level in human palate tissues because of limited clinical sample availability; however, miR-6871-3p emerges as a promising target for future studies of both post-transcriptional regulation under pharmacologic stress and CP.

A pivotal finding in the present study is the significant up-regulation of let-7c-5p observed both in NanoString analysis of CP patient tissues and in the HEPM + PB model, consistent with recent reports of PB-induced modulation of let-7c-5p expression.¹⁰ Computational predictions identified multiple collagen genes as putative let-7c-5p targets, a hypothesis strongly supported by qRT-PCR results demonstrating marked down-regulation of *COL1A1*, *COL1A2*, *COL2A1*, *COL3A1*, *COL5A1*, and *COL11A1* following PB treatment. Furthermore, functional inhibition of let-7c-5p in HEPM cells confirmed its regulatory influence, inducing significant up-regulation of *COL1A2* and *COL3A1*. This reinforces let-7c-5p's role as a key molecular mediator of collagen repression under teratogenic stress and suggests it may contribute to impaired extracellular matrix remodeling observed in the absence of PB exposure, as evidenced by altered collagen expression patterns in CP tissues from patients without antiepileptic drug exposure.

Extensive literature highlights collagen's essential contribution to palatal development, with collagen types I and III particularly enriched in palatal mesenchyme.¹⁸ Both genetic and experimental models have linked reduced collagen expression to impaired palatal shelf elevation and fusion, key events in CP pathogenesis in a mouse model^{19,20} and in humans.²¹ Supporting this, clinical case reports correlate *COL3A1* haploinsufficiency with syndromic CP, reinforcing the physiological significance of collagen regulation.²²

Findings align with studies in idiopathic pulmonary fibrosis where *COL1A2* and *COL3A1*, together with the let-7 miRNA family, form a regulatory network controlling extracellular matrix remodeling. This parallel suggests a conserved mechanism by which let-7c-5p modulates collagen gene expression across diverse

biological settings, highlighting its broader functional relevance.

The work bridges clinical observations with mechanistic understanding by integrating patient-derived tissue expression profiles with an *in vitro* HEPM model exposed to PB and combining bioinformatic predictions with experimental validations. This multidisciplinary approach supports the identification of let-7c-5p as a functionally validated post-transcriptional regulator of collagen genes and highlights its potential relevance during palatogenesis. This regulatory effect was substantiated by functional inhibition experiments that restored expression of major collagen genes (*COL1A2* and *COL3A1*), underscoring let-7c-5p's significant role in modulating extracellular matrix dynamics during palate formation.

In addition to the let-7c/*COL1A2*-*COL3A1* axis, consistent down-regulation of *COL1A1* and *COL5A1* was observed in both experimental models considered here, though this effect appears let-7c independent, implicating additional miRNAs in complex collagen regulation. Characterization of these miRNAs remains an important future direction to fully elucidate extracellular matrix dynamics in palate development. Moreover, the involvement of other miRNAs, such as miR-6871-3p, hints at a complex post-transcriptional network influenced by pharmacologic agents.

Despite these advances, limitations remain. The HEPM cell line, although useful for dissecting molecular pathways, cannot fully capture the multicellular and tissue-level complexity of palatal morphogenesis *in vivo*. Moreover, Italian law (art. 13 L. 40/2004) restricts the derivation of embryonic cell lines within the country but does not explicitly forbid the use of human embryonic cell lines that were cultivated and established abroad. Consequently, although the availability of multiple well-characterized human embryonic palate cell lines is limited, HEPM remains the only commercially available and internationally accepted reference line for human embryonic palatal mesenchyme. Although other models like dental pulp-derived mesenchymal stem cells and induced pluripotent stem cell-derived craniofacial cells exist, they are not direct substitutes. Furthermore, obtaining palatal

tissue samples from healthy newborns was not possible due to the unavailability of parental consent for tissue collection. Considering these legal and practical challenges, HEPM cells were exclusively used in this study, while acknowledging the importance of additional models for future research. Finally, beyond let-7c-5p, candidate miRNAs such as miR-6871-3p require further functional studies to define their roles.

Conclusions

The study identifies let-7c-5p as a key miRNA involved in repressing collagen genes in response to PB-induced stress in embryonic palatal mesenchymal cells. This regulatory mechanism likely contributes to extracellular matrix remodeling, which is crucial for palatal development, and supports a model where disrupted miRNA–collagen dynamics are important in CP pathogenesis. By combining patient-derived data with mechanistic insights from cell models, a strong foundation for exploring miRNA–collagen interactions in the context of CP was established. Despite some limitations, such as the simplified cellular environment and the partial characterization of all candidate miRNAs, these results expand understanding of miRNA-driven collagen regulation and enhance knowledge of CP development.

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Author Contributions

A.Pa. conceptualized the study, developed methods, performed investigations, wrote the manuscript, and acquired funding; L.S. performed bioinformatic analyses, interpreted data, wrote the manuscript, and acquired funding; A.Pe. developed methods and performed investigations; R.S. and D.L. interpreted data and reviewed and edited the manuscript; F.C. curated data and supervised the study; F.A. enrolled patients and curated data; M.M. conceptualized the study, interpreted data, wrote, reviewed, and edited the manuscript, supervised the study, and acquired funding; and all authors have read and agreed to the published version of the manuscript.

Disclosure Statement

None declared.

Supplemental Data

Supplemental material for this article can be found at <https://doi.org/10.1016/j.ajpath.2026.04.008>.

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