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ORIGINAL ARTICLE

Exploring Circadian Oscillation in Human Umbilical Cord–Derived Mesenchymal Stem Cells During Passaging

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Objective: Circadian clock genes regulate key cellular functions and regenerative medicine; however, their role in human umbilical cord–derived mesenchymal stem cells (hUC-MSCs) remains unclear. **Methods:** We analyzed the expression of core circadian genes (*CLOCK*, *BMAL1*, *PER1*, *PER2*, *PER3*, *CRY1*, and *REV-ERBα*) in hUC tissues and isolated hUC-MSCs over 48 hours at 6-hour intervals. The effects of cell confluence and passage number on gene expression, proliferation, and migration were evaluated. **Results:** Circadian clock genes were actively expressed in both hUC tissues and hUC-MSCs, indicating an intrinsic circadian regulatory mechanism. Several genes exhibited low-amplitude circadian oscillations. Notably, expression levels were significantly higher at high cell confluence, suggesting a role in cell proliferation and intercellular communication. Early-passage hUC-MSCs demonstrated superior proliferation and migration capacities compared to late-passage cells, highlighting their enhanced regenerative potential. **Conclusion:** Circadian clock genes influence hUC-MSC function, with early-passage cells demonstrating superior regenerative potential. These findings highlight the importance of passage-dependent circadian regulation in optimizing hUC-MSCs for therapeutic applications.

Keywords: Circadian clock; Oscillation; Human mesenchymal stem cells; Proliferation; Stem cell culture

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INTRODUCTION

Circadian rhythms, governed by intrinsic biological clocks, play a crucial role in regulating various cellular processes, including proliferation, differentiation, and metabolism [1-6]. Loss of clock genes (e.g., *Bmal1* or *Clock*) can lead to impaired hematopoiesis, resulting in conditions like anemia or increased susceptibility to infections. Altered expression of clock genes has been associated with neurodegenerative diseases such as Alzheimer's disease [7], where impaired circadian regulation can affect neurogenesis and contribute to cognitive decline [8]. Recent studies have highlighted the presence of circadian clock genes in stem cells, suggesting that their expression may influence stem cell maintenance and functionality [6]. Skin stem cells are regulated by circadian rhythms, influencing processes like wound healing and skin regeneration [9,10]. Disruption of circadian rhythms has been associated with various pathological conditions, indicating that maintaining these rhythms is crucial for optimal cell proliferation and function [11-13].

Human umbilical cord–derived mesenchymal stem cells (hUC-MSCs) are emerging as a valuable resource in regenerative medicine due to their unique properties, including high proliferation potential, multipotency, and low immunogenicity [14-17]. Isolated from Wharton's jelly, they provide a non-invasive alternative to bone marrow- or adipose-derived MSCs [18-20]. These cells are characterized by the expression of CD44, CD73, CD90, and CD105, while lacking CD14, CD34, CD45, and HLA-DR (human leukocyte antigen–DR isotype). Their mesenchymal identity is confirmed through differentiation into chondrogenic, osteogenic, and adipogenic lineages [18-20]. Preclinically, hUC-MSCs are utilized in tissue engineering, disease modeling, and immunomodulation studies [21], with clinical trials exploring their thera-

peutic potential in orthopedic injuries, cardiac repair, and hematopoietic transplantation [22,23].

Passage number in cell culture is another critical factor influencing stem cell properties [24]. As hUC-MSCs are expanded over multiple passages, they can undergo changes in proliferation rates and functional capabilities, often resulting in diminished stemness and increased senescence [25]. Cellular senescence is known to disrupt circadian rhythms, leading to an altered clock with a prolonged period and delayed phases. This interaction between senescence and circadian regulation has significant implications for aging and age-related diseases. In senescent cells, core circadian clock genes, including *CLOCK*, *BMAL1*, *PER*, and *CRY*, exhibit altered expression patterns [26]. Notably, the early passage hUC-MSCs possess superior migratory and proliferative abilities compared to later passages, which has significant implications for their application in tissue repair and regeneration [24,27,28].

This study aims to investigate the expression of circadian clock genes in umbilical cord tissue and hUC-MSCs across passages. Furthermore, it examines the effects of passage number on circadian clockwork, cell proliferation and migration. These insights will demonstrate the role of circadian clock genes in hUC-MSC culture during passaging.

METHODS

Collection of human umbilical cord tissues and hUC-MSC isolation

The human umbilical cord (UC) samples were collected from the Tissue Bank at the University Medical Center, University of Medicine and Pharmacy, Ho Chi Minh City, Vietnam, with the approval of the Institutional Review Board (approval number: DTNN.2011.06.29). Three UC tissues were stored at 4°C until hUC-MSC isolation was performed. The collection and processing of samples followed the standard protocols established by the University Medical Center and Stem Cell Institute.

The isolation of hUC-MSCs followed the standard protocol established by the Stem Cell Institute, University of Science, Vietnam National University, Ho Chi Minh City, Vietnam, as previously described [20]. Briefly, small fragments of Wharton's jelly (1−2 mm^2) were cut and attached to the surface of T25 culture flasks (SPL Life Sciences). Primary MSC isolation medium (Regenmedlab, Stem Cell Institute) was added, and cultures were maintained at 37°C in a humidified incubator with 5% CO_2. The culture medium was refreshed every 3 days, and cells were allowed to adhere and proliferate for 7 days before further processing.

Cell culture

The hUC-MSCs were cultured in a specialized MSC medium, commercially known as MSCCult I (Regenmedlab, Stem Cell Institute), supplemented with 1% Antibiotics-Antimycotics (Gibco). All cells were cultured until around 90% and then subcultured. The cells were incubated at 37°C, 5% CO_2, and humidified conditions until reaching 80%−90% confluence for further experiments. The medium was changed every 48 hours.

Flow cytometry

The hUC-MSCs were stained with positive MSC markers, including CD44 (Miltenyi Biotech) and CD73 (BD Biosciences), as well as negative MSC markers, including CD34 (BD Biosciences) and HLA-DR (BD Biosciences), with dilution at 5 μL per 1,000,000 cells. Hoechst 33258 (Sigma) with dilution at 1:100, incubated for 30 minutes. The cells were analyzed by flow cytometry using a FACS Melody (BD Biosciences).

Cell proliferation

The hUC-MSCs were cultured in a cell culture medium including MSCCult I (Regenmedlab), 1% Antibiotics-Antimycotic (Gibco), maintained in an incubator of 5% CO_2 at 37°C, and humidified conditions. For the cell viability assay, these cells were seeded in 96-well plates with a density of 3,000 cells per 100 μL. On day 1 (24 h), day 2 (48 h), day 3 (72 h), and day 4 (96 h), hUC-MSCs were analyzed following AlamarBlue Cell Viability Reagent guidance (Invitrogen). For analysis, the results were read the fluorescence by a multimode detector (DTX 800/880, Beckman Coulter) with an excitation wavelength of 535 nm and a fluorescence emission wavelength of 595 nm after 45 minutes of incubation.

Trypan blue

The hUC-MSCs were fixed with 4% paraformaldehyde for 20 minutes. The cell viability was stained with 0.4% trypan blue (Sigma-Aldrich) for 30 minutes. The stained samples were washed with PBS, then the pictures were taken under 5×, 10×, 20×, and 40× objectives by a fluorescent microscope incorporated with Carl Zeiss™ Axiovert40C (Carl Zeiss).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

The total RNA of hUC-MSCs was collected and extracted by using TRIzol-RNA isolation reagent (Ambion, Invitrogen) following the optimized protocol. Subsequently, the total RNA was run the real-time PCR by using Luna Universal One-step RT-qPCR Kit Protocol (New England Biolabs) in an Eppendorf gradient S thermal Cycler (Eppendorf-AG) for detecting the circadian targeted genes. The primer sets are shown in Supplementary Table 1. The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was employed as a reference gene to normalize the targeted relative mRNA expression by calculating the $2^{-\Delta\Delta Ct}$, whereas $\Delta\Delta Ct=(Ct^{\text{target gene}}-Ct^{GAPDH})\text{treated}-(Ct^{\text{target gene}}-Ct^{GAPDH})\text{control}$. The Ct value is referred to as the cycle threshold.

Scratching assays

The cells were cultured in growth media on a 12-well plate with 50−100,000 cells/mL. The cells were fully attached to the surface, cell scratches were then created by the p200 tip. The scratch is a straight vertical line on the plate. The unattached cells were washed

with 1 mL PBS. Next, 24-h and 48-h cell migration was stained with trypan blue and observed under a microscope and analyzed by ImageJ (version 1.54g; National Institutes of Health). The wound area percentage is calculated by

$$\frac{0\ h\ scratch\ width - 24\ h\ scratch\ width}{0\ h\ scratch\ width} \times 100\%.$$

Data analysis

All statistical analyses were done by using the Mann-Whitney test and Tukey's multiple comparison tests (n≥3). Differences were considered statistically significant if the p-value was <0.05. Graphs were all made with GraphPad Prism 10.0 (GraphPad Software, Inc.). The circadian oscillation is tested using Cosinor.Online Calculator (https://cosinor.online/app/cosinor.php) to determine the diurnal shift [29].

RESULTS

Detection of circadian clock genes in human umbilical cord tissues

To identify the key circadian clock genes (e.g., *CLOCK*, *BMAL1*, *PER1*, *PER2*, *PER3*, *CRY1*, *REV-ERBα*) in umbilical cord (UC) tissues, three samples were collected, and total RNA was extracted.

Analysis revealed that the heterodimer of *CLOCK* and *BMAL1* was highly expressed. Among the PER family genes, *PER1* mRNA levels were higher than those of *PER2* and *PER3*. *CRY1* mRNA was detected at low levels, while *REV-ERBα* mRNA expression was high (Figure 1).

The robust detection of *CLOCK*, *BMAL1*, *PER1*, *PER2*, *PER3*, *CRY1*, and *REV-ERBα* mRNA in human UC samples suggests that these circadian clock genes may play an important role in regulating stem cell behavior and maintaining tissue homeostasis.

Early-passage hUC-MSCs exhibited greater functional capacity than late-passage cells

The isolation of hUC-MSCs was performed according to the standard protocol established by the Stem Cell Institute, University of Science, Vietnam National University, Ho Chi Minh City, Vietnam. The identity of the isolated hUC-MSCs was shown by the stem cell morphology (Figure 2A), which was confirmed by flow cytometry, with positive expression of CD44 and CD73, and negative expression of CD34 and HLA-DR. The percentage of CD44 expression was not altered in both early and late passage; the trend of CD73 expression was not significantly decreased at late passage than early passage (Figure 2B and C).

To perform the cell proliferation and migration of hUC-MSCs during passaging, passage 5 (early passage) and 10 (late passage) were examined using the AlamarBlue test for 4 days and the scratching assay, respectively. The rate of cell growth at early passage is significantly higher than at late passage. The cell count was relative to the fluorescence intensity (Figure 2D and E). Similarly, the rate of cell closure of the scratch at early passage is significantly faster than late passage (Figure 2F).

Circadian clock gene expression in hUC-MSCs over a 48-hour period

To explore the circadian clock gene expression in hUC-MSCs, samples of hUC-MSCs were harvested at 6-hour intervals over a 48-hour period to assess circadian gene expression. However, the mRNA expression of key circadian clock genes (*CLOCK*, *BMAL1*, *PER1*, *PER2*, *PER3*, *CRY1*, and *REV-ERBα*) was detected in both early and late passages. At early passage, four genes (*CLOCK*, *PER1*, *PER2*, and *REV-ERBα*) demonstrated the significant oscillation (Figure 3A, B and Table 1). At late passage, five genes of *BMAL1*, *PER1*, *PER3*, *CRY1*, and *REV-ERBα* have indicated the circadian oscillation (Figure 3A, C and Table 2). Here, circadian clock genes are actively expressed in hUC-MSCs, with passage number influencing their oscillatory patterns.

Upregulation of circadian genes after 24 hours following culture

To investigate the regulation of circadian genes during proliferation, hUC-MSCs at early passage (P5–P6) were collected at 6-hour intervals over a 24-hour period, followed by an additional 24-hour collection during continued proliferation.

Results showed increased mRNA expression of key circadian genes after 24 hours of expansion. Specifically, at early passage, *CLOCK*, *PER2*, *PER3*, *CRY1*, and *REV-ERBα* exhibited signifi-

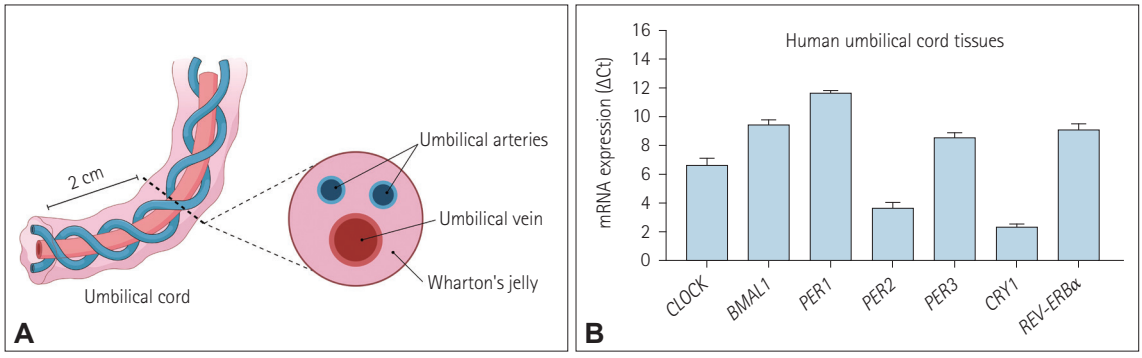

Figure 1. mRNA expression of circadian clock genes in human UC tissues. A: Human UC tissue was cut for RNA sample; the number of tissues is 3. B: Identification of key circadian clock genes (e.g., *CLOCK*, *BMAL1*, *PER1*, *PER2*, *PER3*, *CRY1*, *REV-ERBα*) in UC tissues, n=6. UC, umbilical cord.

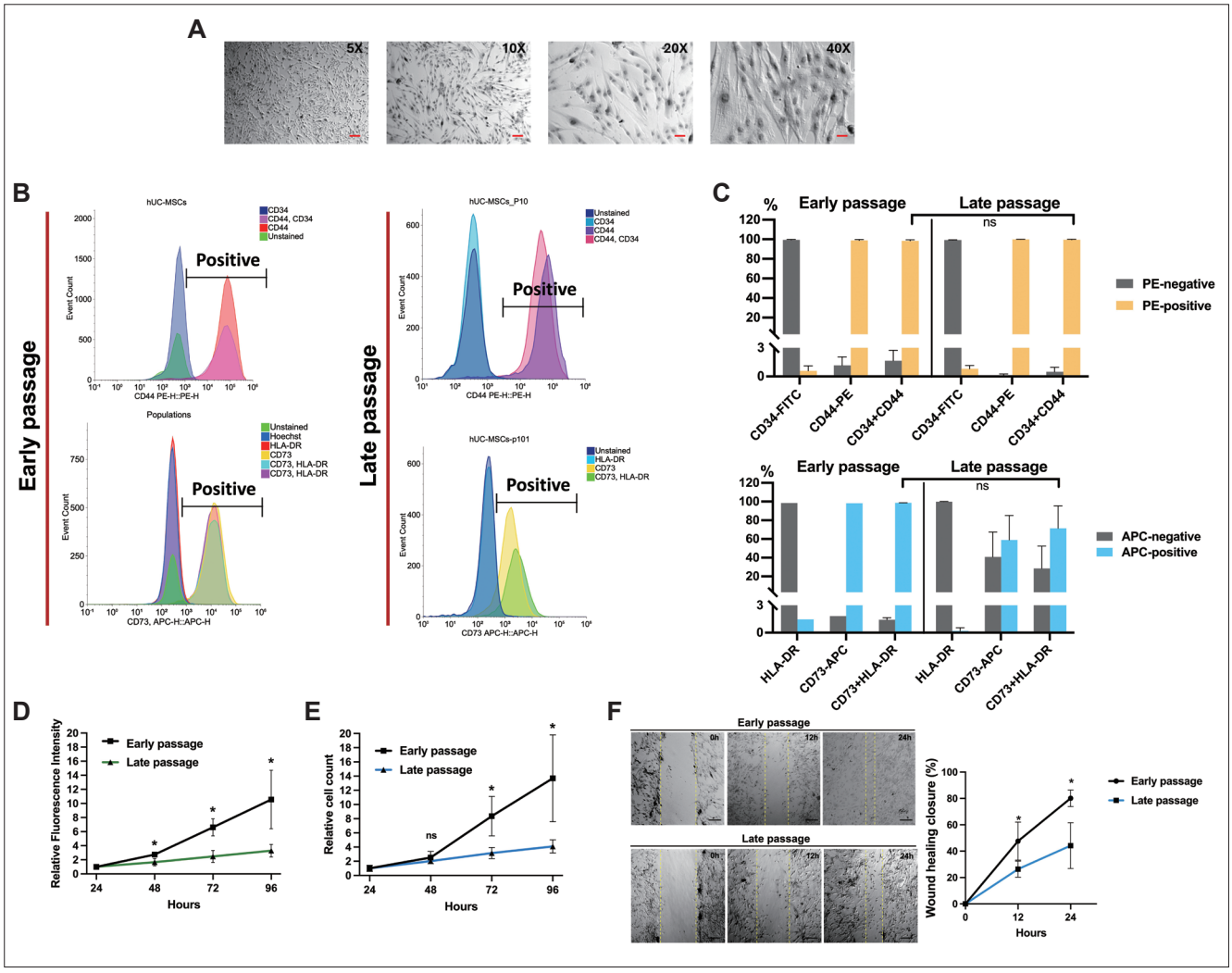

Figure 2. Early passage of hUC-MSCs showed the capacity more than late passage. A: Morphology of post-isolation of hUC-MSCs. B: CD44 and CD73 markers showed the positive cells with PE and APC, respectively. C: Percentage of cells indicated the positive of CD44-PE and CD73-APC in early and late passage (n=3). D and E: Relative of flourencense and relative of cell number of hUC-MSCs were detected at early and late passage (n≥32). F: The migration capacity of early passage of hUC-MSCs was faster than late passage (n≥24). *significant difference. hUC-MSCs, human umbilical cord–derived mesenchymal stem cells; PE, phycoerythrin; APC, allophycocyanin.

cantly higher expression (Figure 4A). At late passage, *PER1* and *CRY1* showed elevated mRNA levels after 24 hours (Figure 4B). These findings suggest that circadian genes may contribute to regulating cell proliferation, particularly during active cell division.

DISCUSSION

The characterization and functional dynamics of hUC-MSCs provide essential insights for their use in regenerative medicine [14-17]. Our study shows that key circadian clock genes, including *CLOCK*, *BMAL1*, *PER1*, *PER2*, *PER3*, *CRY1*, and *REV-ERBα*, are actively expressed both in hUC tissues and in isolated hUC-MSCs, indicating that these cells possess an intrinsic circadian regulatory mechanism. When monitored over a 48-hour period with sampling every 6 hours, most genes exhibit mRNA expression patterns with low amplitude, suggesting that similar patterns with some other isolated cell types may lack strong circadian oscillations in circadian gene expression. In human embryonic stem cells (ESCs), both undifferentiated and differentiated states exhibit a cyclical expression pattern of the core circadian genes *CLOCK*, *BMAL1*, *PER*, and *CRY*, with distinct peaks observed at 8, 18, and 22 hours, respectively [30]. Knockout studies of *Bmal1* in mouse ESCs reveal its essential role in sustaining pluripotency and influencing differentiation pathways. Furthermore, loss-of-function of *Bmal1* is associated with altered metabolic gene expression, reduced glycolytic flux, and elevated mitochondrial reactive oxygen species (mtROS) production [31]. In hMSCs, CLOCK and BMAL1 proteins predominantly localize within the cytoplasm, while PER1 and PER2 are distributed across both the nuclear and cytoplasmic compartments. Silencing of *CLOCK* or *PER* genes impairs adipogenic differentiation and leads to reduced hMSC adherence and migratory capacity, indicating a critical role for these circadian regulators in stem cell fate and functionality [6].

The expression of circadian clock mRNA in hUC-MSCs is notably higher at high cell confluence compared to low confluence in both early passage and later passage. This observation suggests

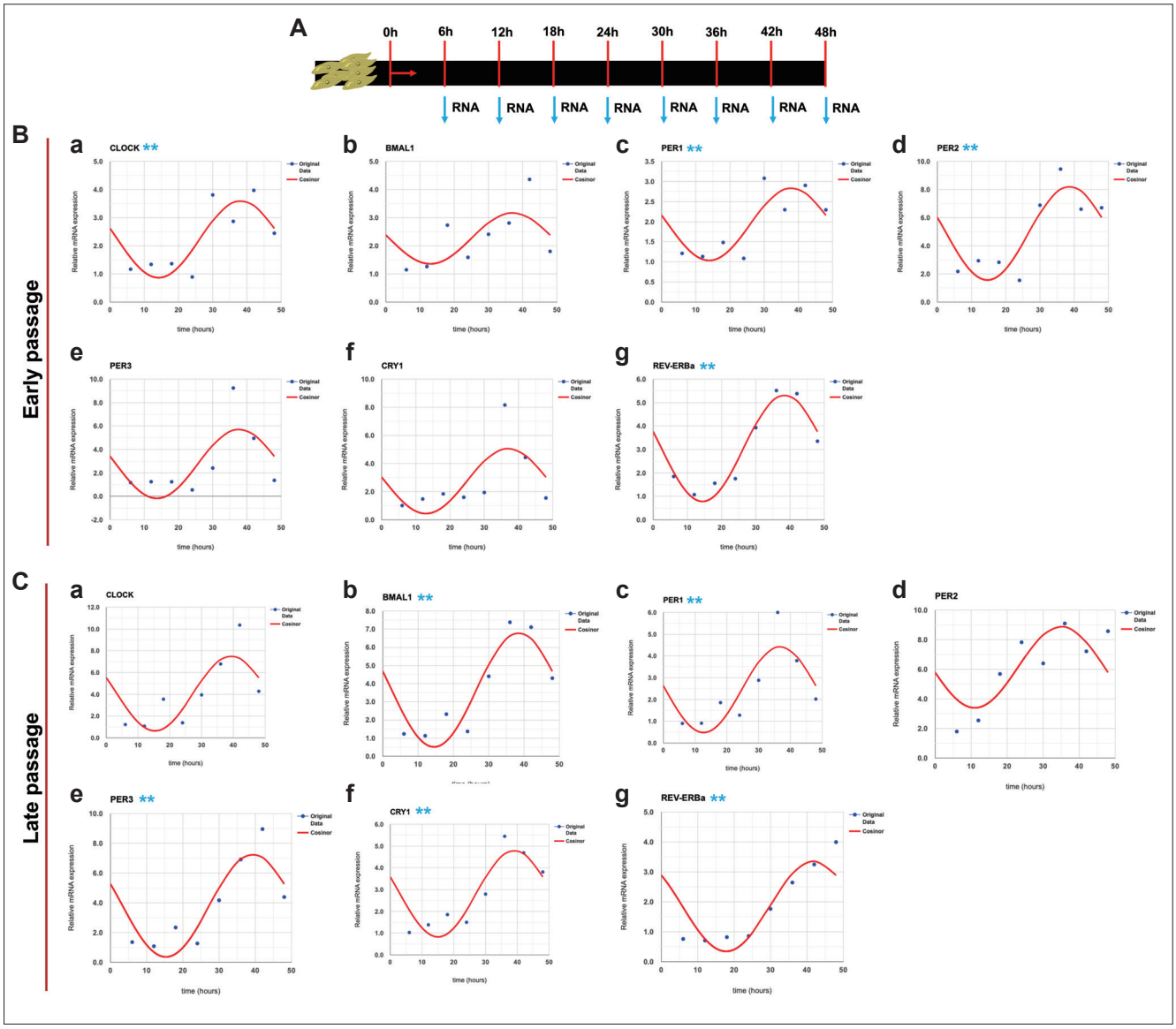

Figure 3. Circadian clock gene indication in hUC-MSCs. A: Timeline of RNA collection for checking the circadian genes. B and C: Analysis of circadian clock genes (e.g., *CLOCK*, *BMAL1*, *PER1*, *PER2*, *PER3*, *CRY1*, and *REV-ERBα*) expression in hUC-MSCs with early (B) and late (C) passage. The circadian oscillation is tested using Cosinor.Online Calculator (https://cosinor.online/app/cosinor.php) to determine the diurnal shift. The pictures were taken by Objective at 5x, 10x, 20x, and 40x. Each gene was quantified in six independent replicates (n=6). **The gene shows circadian oscillation. hUC-MSCs, human umbilical cord–derived mesenchymal stem cells.

that circadian genes may play a role in cell doubling, division, or intercellular interactions. Moreover, the robust circadian gene expression observed during hUC-MSC passaging suggests the maintenance of a functionally undifferentiated state, which may contribute to their high regenerative potential. Importantly, our results highlight the role of passage number in preserving the inherent properties of hUC-MSCs. As cells undergo extended passaging, shifts in gene expression profiles, cellular morphology, and functional capacity may occur [32].

Aging MSCs often show a decline in the expression of key surface markers, which are associated with their stemness and functional capabilities [33-35]. During passaging, surface markers such as CD73 of hUC-MSCs at late passage are reduced compared with early passage. Our findings demonstrate that hUC-MSCs at early passage exhibit greater proliferative and migratory capacities than those at later passage. These capacities are key attributes for effective cell-based therapies, as rapid proliferation and directed migration to injury sites are essential for successful tissue regeneration. This decline in performance could be attributed to senescence or changes in the microenvironment that affect stem cell behavior. Therefore, minimizing the number of passages before application in regenerative therapies may be beneficial for preserving their therapeutic potential.

In conclusion, this study demonstrates the conservation of circadian clock gene expression from human umbilical cord tissues to hUC-MSCs, with expression levels influenced by cell confluence. Early passage hUC-MSCs exhibit enhanced circadian gene expression, proliferation, and migration, underscoring their superior functional capacity for regenerative applications.

Table 1. Oscillation analysis of early-passage hUC-MSCs using Cosinor analysis software

	CLOCK	*BMAL1*	*PER1*	*PER2*	*PER3*	*CRY1*	*REV-EBRα*
Cosinor analysis							
Mesor	2.23259	2.26445	1.93605	4.89228	2.7732	2.75274	3.05085
Amplitude	1.36481202	0.90559663	0.90040249	3.32351491	2.94382155	2.30937966	2.26703009
Acrophase (hours, decimal)	38.17240399	36.9935037	37.9089857	38.6380246	37.6758811	36.93371324	38.42328119
Bathyphase (hours, decimal)	14.17240399 or 62.17240399	12.99350373 or 60.99350373	13.9089857 or 61.9089857	14.6380246 or 62.6380246	13.67588107 or 61.67588107	12.93371324 or 60.93371324	14.42328119 or 62.42328119
Zero-amplitude test							
Df1 (numerator degrees of freedom)	2	2	2	2	2	2	2
Df2 (denominator degrees of freedom)	5	5	5	5	5	5	5
F-value	6.23269	1.79373	5.98723	8.22083	3.31148	2.71735	48.20207
p-value	0.04385*	0.25868	0.04709*	0.02626*	0.12138	0.15894	0.00054*

*p-values were equal to or smaller than 0.05. hUC-MSCs, human umbilical cord–derived mesenchymal stem cells.

Table 2. Oscillation analysis of late-passage hUC-MSCs using Cosinor analysis software

	CLOCK	*BMAL1*	*PER1*	*PER2*	*PER3*	*CRY1*	*REV-EBRα*
Cosinor analysis							
Mesor	4.07488	3.65411	2.45334	6.13962	3.80983	2.81177	1.84909
Amplitude	3.43227796	3.14177999	1.97052632	2.7456074	3.44486492	1.98281917	1.50440651
Acrophase (hours, decimal)	39.31968702	38.5244815	36.6810761	35.0063973	39.3245903	39.0339768	41.80727587
Bathyphase (hours, decimal)	15.31968702000 or 63.31968702000	14.52448150000 or 62.52448150000	12.68107607000 or 60.68107607000	11.00639729000 or 59.00639729000	15.32459030000 or 63.32459030000	15.03397680000 or 63.03397680000	17.80727587000 or 65.80727587000
Zero-amplitude test							
Df1 (numerator degrees of freedom)	2	2	2	2	2	2	2
Df2 (denominator degrees of freedom)	5	5	5	5	5	5	5
F-value	4.81812	14.92898	6.81974	3.66054	10.62957	10.9659	8.4438
p-value	0.06821	0.00779*	0.03727*	0.10491	0.01582*	0.01485*	0.02494*

*p-values were equal to or smaller than 0.05. hUC-MSCs, human umbilical cord–derived mesenchymal stem cells.

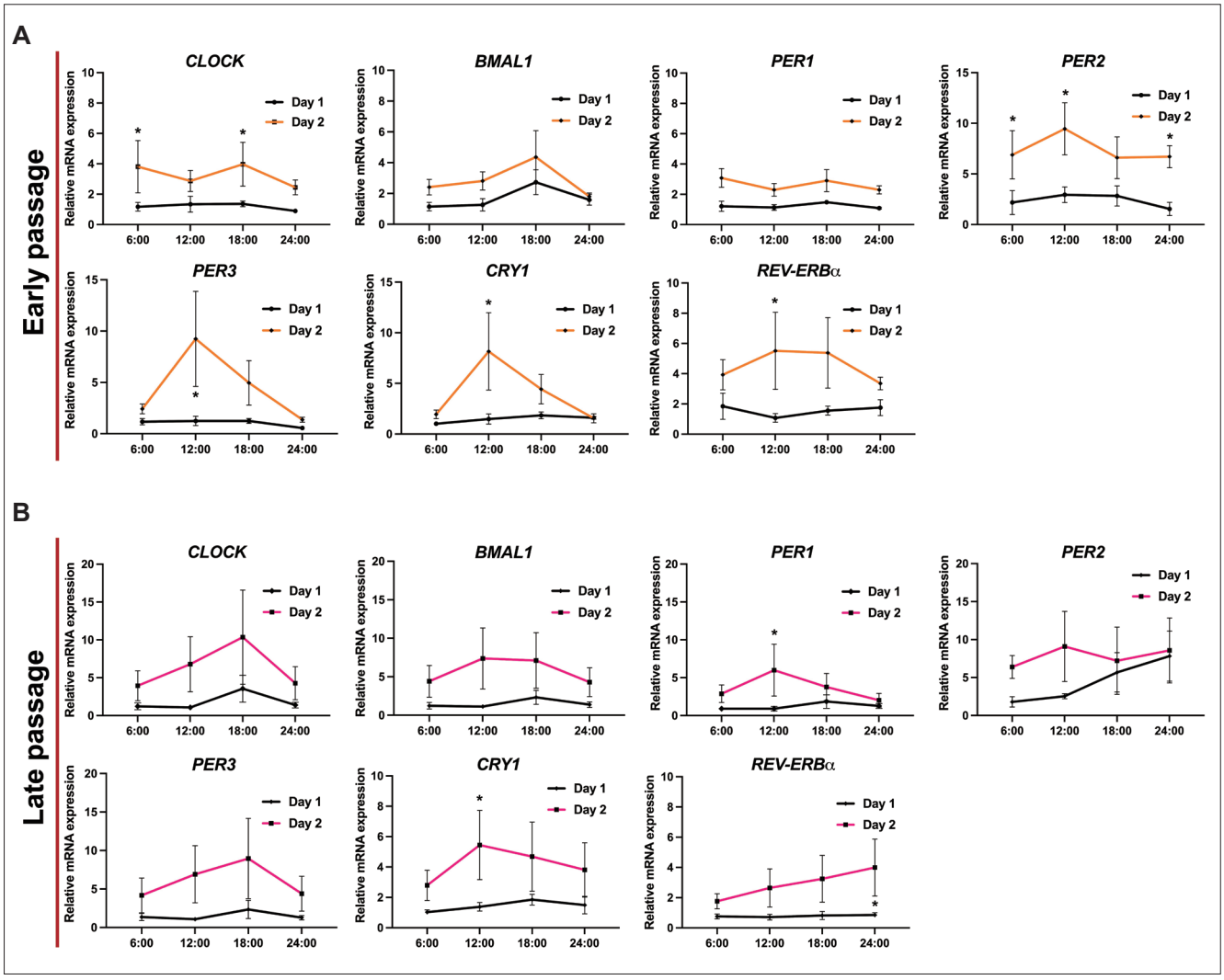

Figure 4. Increased levels of circadian genes after 24-hour proliferation. After 24 hours of culture, early passage (A) and late passage (B) of hUC-MSCs exhibit increased expression of circadian genes, suggesting an adaptive response to the culture environment (n=6). *significant difference; ns, not significant difference. hUC-MSCs, human umbilical cord–derived mesenchymal stem cells.

Supplementary Materials

The Supplement is available with this article at https://doi.org/10.33069/cim.2025.0008.

Conflicts of Interest

The authors have no potential conflicts of interest to disclose.

Availability of Data and Material

The datasets generated or analyzed during the study are available from the corresponding author on reasonable request.

Author Contributions

Conceptualization: Hong Thuan Tran. Data curation: Hong Thuan Tran. Formal analysis: all authors. Funding acquisition: Hong Thuan Tran. Investigation: Hong Thuan Tran. Methodology: all authors. Project administration: Hong Thuan Tran. Resources: Hong Thuan Tran. Software: all authors. Supervision: Hong Thuan Tran. Validation: Hong Thuan Tran. Visualization: all authors. Writing—original draft: Hong Thuan Tran. Writing—review & editing: Hong Thuan Tran.

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