Unraveling the Influence of Folic Acid on Osteogenic Differentiation : Insights from Induced Pluripotent Stem Cells

Yali MEN[1)#], Fengming YUE[1)2)#], Kensuke KAI[1)]
Chuang MA[2)3)], Daihachiro TOMOTSUNE[1)2)] and Kohei JOHKURA[1)*]

1) *Department of Histology and Embryology, Shinshu University School of Medicine*
2) *Shinshu University Interdisciplinary Cluster for Cutting Edge Research, Institute for Biomedical Sciences*
3) *Biomedical Engineering Division, Graduate School of Medicine, Science and Technology, Shinshu University*

Folic acid, a crucial B vitamin with established roles in various physiological processes, has gained recognition for its association with neural and cardiovascular health, cancer prevention, and metabolic syndrome. Despite these multifaceted roles, its specific influence on bone health, especially during fetal development, remains underexplored. This study employed induced pluripotent stem cells (iPSCs) of human to simulate in vivo osteogenic differentiation, comprehensively investigating the influence of folic acid on bone formation. As a result, a substantial increase in calcium deposition was revealed by addition of folic acid into the osteogenic differentiation of iPSCs, indicating an augmented capacity for mineralized tissue formation. The expression of key osteogenic genes (*RUNX2*, *COL1A1*, *Osteocalcin*, *PHEX*) consistently showed upregulation across various stages of osteogenic differentiation. Additionally, temporal effects of folic acid were observed, emphasizing its positive impact during the early preosteoblast stage. The role of folic acid in promoting the differentiation of preosteoblasts into osteoblasts was further confirmed using MC3T3-E1 cells. These findings provide novel insights into the molecular mechanisms underlying the influence of folic acid on bone formation, highlighting the specific stages of osteogenic differentiation where its effects are most pronounced. *Shinshu Med J 72 : 227—237, 2024*

(Received for publication March 29, 2024 ; accepted in revised form April 9, 2024)

Key words : induced pluripotent stem cells (iPSCs), folic acid, osteogenic differentiation, temporal effects

I Introduction

Folic acid, a vital member of the B-vitamin (B9), plays a crucial role in physiological processes, including nucleic acid synthesis, amino acid metabolism, and DNA methylation[1)2)]. The conversion of homocysteine to methionine is facilitated by folic acid, which is fundamental for numerous biochemical reactions[3)4)]. Traditionally known for preventing neural tube defects in pregnancy[5)6)], contemporary research has shed light on the broader implications of folic acid, including neural, reproductive, and cardiovascular health, cancer prevention, metabolic syndrome, and inflammation[7)-9)]. However, extensive studies on folic acid's specific impact on bone health and development are lacking. In adults, insufficient folic acid intake has been associated with bone-related issues, including osteoporosis and heightened osteoclast activity, which may compromise bone integrity[10)]. During pregnancy, folic acid deficiency is associated not only with neural tube defects but also with potential effects on fetal bone development[11)]. Hossein et al. reported that pregnant women receiving daily folic acid supplementation (1 mg) until childbirth exhibited significantly higher plasma levels of osteoprotegerin (OPG) concentration and lower soluble receptor activator of nuclear factor-kappa B

* Corresponding author : Kohei Johkura
Department of Histology and Embryology,
Shinshu University School of Medicine,
3-1-1 Asahi, Matsumoto, Nagano 390-8621 Japan
E-mail : kohei@shinshu-u.ac.jp
# These authors contributed equally : Yali Men & Fengming Yue

ligand (sRANKL) concentrations than those supplemented only up to the second trimester[12]. Despite these associations, the precise mechanisms by which folic acid influences bone formation, during fetal development, remain intensely investigated.

To address this issue, we utilized induced pluripotent stem cells (iPSCs) of human, a groundbreaking discovery by Yamanaka et al.[13)14)]. iPSCs possess the unique ability to be reprogrammed from adult cells, offering an ethically clear approach to studying human development, disease modeling, and regenerative medicine. This innovation allows modeling embryonic development in a dish, offering a unique vantage point to study gene and drug effects at each developmental stage[15)-17)].

The primary objective of this study was to investigate osteogenesis using iPSCs and scrutinize the effects of folic acid supplementation, elucidating the complex relationship between folic acid and bone formation, with a particular focus on understanding how folic acid influences the molecular and cellular mechanisms involved in bone development across different stages. This investigation holds the potential to provide valuable insights into therapeutic approaches for enhancing bone health and preventing associated conditions in both adults and developing fetuses.

Ⅱ Materials and Methods

A Cell culture and maintenance of iPSCs

Human iPSC line 253G1 (Riken Brc, Tsukuba, Japan) was utilized in this study. iPSC culture involved feeder layers of inactivated mouse embryonic fibroblasts (MEFs) in KnockOut™ DMEM/F-12 (Gibco), supplemented with 20 % knockout-serum replacement (Gibco), 100 mM non-essential amino acids (Wako, Japan), 1 mM sodium pyruvate (Wako), 100 mM 2-mercaptoethanol (Sigma-Aldrich, St. Louis, MO, USA), 50 U/mL penicillin, and 50 mg/mL streptomycin (Wako).

The murine calvaria-derived osteoblast-like cell line (MC3T3-E1, Riken BRC, Ibaraki, Japan) cells were cultured in α-MEM medium supplemented with 10 % FBS.

B Osteogenic differentiation of iPSCs

The iPSCs were differentiated into osteogenic lineages using a tailored protocol that mimicked the early stages of bone formation[18)19)]. This process involves the application of specific differentiation factors. Briefly, 2×10^5 iPSCs were maintained under feeder-free conditions. Differentiation was initiated in gelatin-coated 24-well plates using a mixture of 20 % mTeSR1 medium (StemCell Technologies, Vancouver, BC, CA) and 80 % osteogenic differentiation medium. The osteogenic differentiation medium comprised KnockOut DMEM (Gibco, Grand Island, NY, USA), supplemented with 20 % FBS (Gibco), 1 % non-essential amino acids (Wako), 0.1 mM 2-mercaptoethanol (Sigma), 2 mM Gluta-MAX (Wako), 10 mM glycerol-2-phosphate (Sigma), 1 nM dexamethasone (Sigma), 50 μg/mL L-ascorbic acid 2-phosphate sesquimagnesium salt hydrate (Sigma), 1 μM all-trans-retinoic acid (Sigma), and 10 μM rock inhibitor Y-27632 (Wako). On day 2, the medium was replaced with 100 % osteogenic differentiation medium, excluding Y-27632, and subsequently changed every two days. During the induction of differentiation, we supplemented the culture medium with 50 μM folic acid (Wako, Osaka, Japan) to investigate the impact of folic acid on bone development. This concentration of folic acid was determined based on our preliminary experiment of osteogenic differentiation by using a gradient of 0, 10, 25, 50, 100, 150, and 200 μM.

C Osteogenic differentiation of MC3T3-E1

Differentiation was induced using a medium containing 50 μg/ml vitamin C (Wako) and 10 mM β-glycerophosphate (Wako). To further investigate the factors influencing osteoblast differentiation, an additional component, folic acid (Wako), was added to the culture medium at the concentration of 10 μM or 50 μM.

D Alizarin staining for calcium deposition

For Alizarin Staining cells were initially fixed in a culture dish using 4 % paraformaldehyde (PFA, Wako). After fixation, 1 % Alizarin staining solution (Muto Chemicals, Tokyo, Japan) was applied and incubated at room temperature for 10 min to promote the Alizarin staining reaction. Subsequently, the cells were washed with water to remove the excess staining solution. Microscopic observations were conducted to

visualize alizarin-stained (red crystalline deposits) cells.

E Quantification of calcium deposition

iPSCs-derived osteogenic cells were exposed to 1 N hydrochloric acid (HCl) for 24 h. Calcium content in the HCl supernatant was quantified using an o-cresol phthalein complexone (OCPC) kit (N-assay L Ca ; Nittobo Medical, Tokyo, Japan) at 660 nm (reference wavelength : 800 nm) with a microplate spectrophotometer. Calcium content was normalized to the cell number.

F Real-time RT-PCR analysis

Total cellular RNA was extracted using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. Genomic DNA was removed by DNase treatment using a PrimeScript RT reagent kit with a gDNA eraser (Takara Bio, Shiga, Japan). Subsequently, 1 μg of total RNA was reverse-transcribed into single-stranded DNA using random primers and reverse transcriptase. qRT-PCR analysis was conducted as described previously[20] using a Thermal Cycler Dice Real-Time System (Takara Bio). mRNA levels were normalized to beta-actin, and the original cycle threshold values were analyzed using the $2^{-\Delta\Delta Ct}$ method.

G Immunostaining for protein expression

Cells were fixed in 4 % paraformaldehyde for 10 min, washed with 0.01 M phosphate-buffered saline. Subsequently, the cells were incubated with primary antibodies at 4 ℃ overnight. Antibodies used for immunohistochemistry were against osteocalcin (1 : 100 ; Thermo Fisher Scientific) and Collagen I (1 : 100 ; Santa Cruz Biotechnology). Antibody specificity was confirmed by validation using appropriate tissues or cells as positive controls. Antigen localization was visualized using secondary antibodies conjugated with fluorescein, specifically, anti-rabbit or anti-mouse immunoglobulin G (IgG) (Alexa Fluor 568 and 488 ; Molecular Probes Inc., Eugene, OR, USA). Imaging was performed using confocal laser scanning microscopy (Leica TCS SP8) and fluorescence microscopy (Keyence BZ-X800).

H Statistical analysis

Data from three independent experiments are expressed as mean ± standard deviation. Statistical significance for qRT-PCR and other assays was assessed using unpaired two-tailed Student's t-tests, with a p-value < 0.05 considered significant.

Ⅲ Results

A Osteogenic differentiation from iPSCs

To explore the effect of folic acid on osteogenic differentiation, iPSCs with self-renewal and pluripotent differentiation capabilities were used. Sequential iPSC differentiation mimicked the osteogenic lineage, which was induced to simulate in vivo bone formation, encompassing osteoprogenitor, pre-osteoblast, osteoblasts, and osteocytes **(Fig. 1)**.

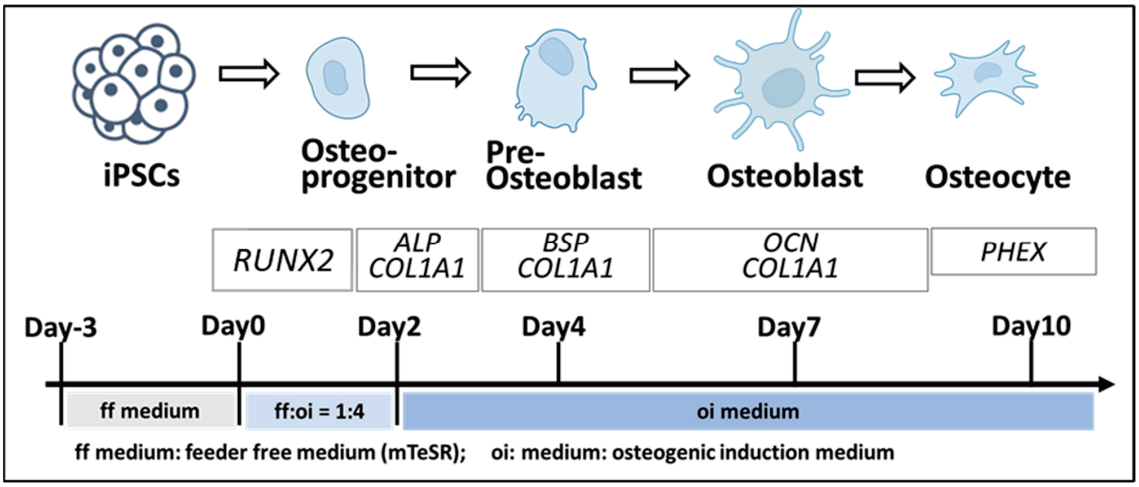

Fig. 1 Osteogenic differentiation from iPSCs. The iPSCs were differentiated into osteogenic lineages using a tailored protocol that mimicked the early stages of bone formation, including osteoprogenitor, pre-osteoblast, osteoblasts, and osteocytes.

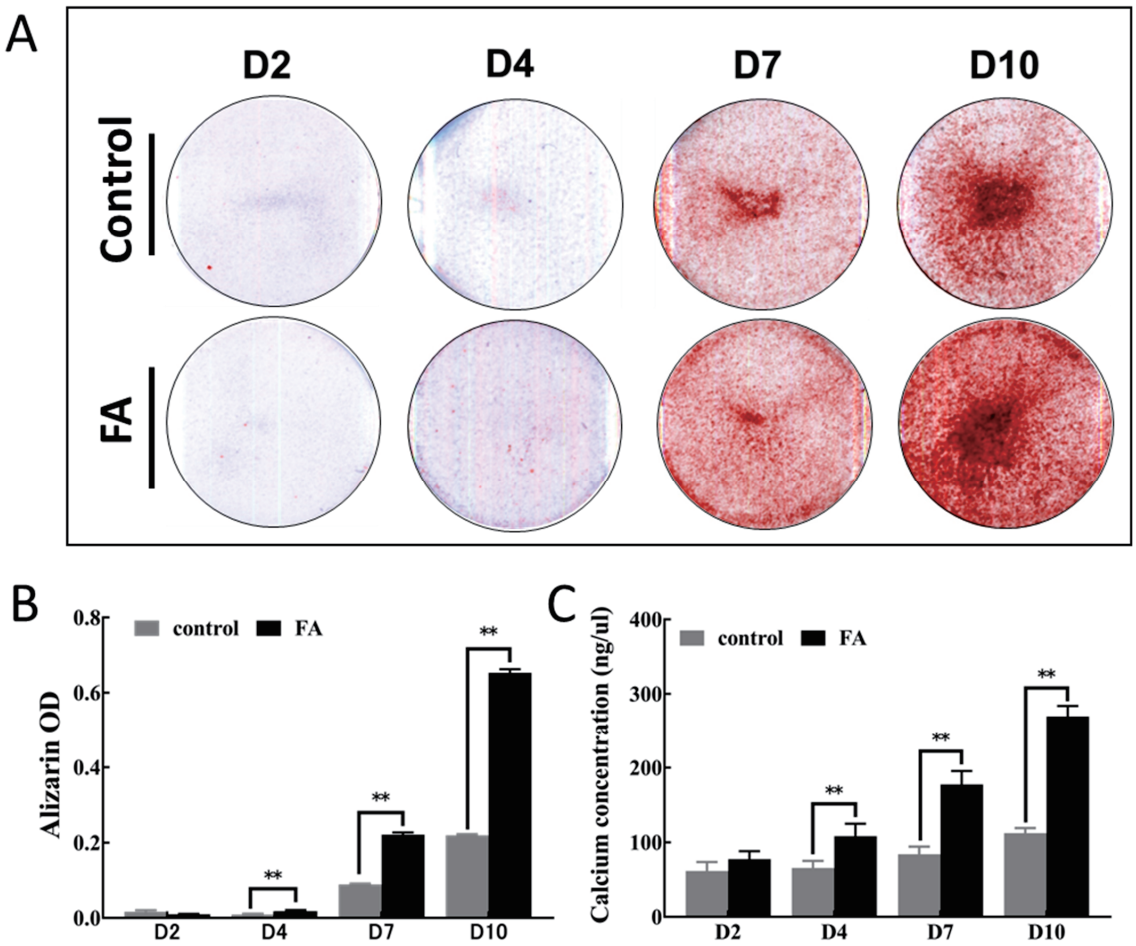

Fig. 2 Impact of folic acid on osteogenic differentiation and calcium deposition in iPSCs. (A) Representative images of Alizarin Red staining (red granules) during osteogenic differentiation in the folic acid-added group and the untreated group (control). (B, C) The evaluation of bone formation on the indicated day was quantified with a dissolved alizarin solution (B) and the deposition of calcium (C). FA : folic acid. The results were normalized by cell number. Data expressed as the mean ± SD (n = 3) ; $^{*}p < 0.05$, $^{**}p < 0.01$.

B Calcium deposition in bone-like nodules

Alizarin Red staining was used to visualize and quantify calcium deposition in the bone-like nodules, which is a crucial indicator of osteoblast activity. Alizarin Red staining on days 2, 4, 7, and 10 revealed a noticeable trend of darker staining in the folic acid-supplemented experimental group compared to the control group, particularly on days 7 and 10 **(Fig. 2A)**. In addition, a bone-like nodule dissolution experiment was conducted by adding acetic acid to dissolve pigments. Absorbance measurements of the dissolved solution were conducted to assess calcium phosphate content, indicative of osteoblast and osteocyte differentiation. The higher Alizarin OD in the folic acid-supplemented group exhibited greater osteoblast differentiation on days 4, 7, and 10 than the control group **(Fig. 2B)**, and calcium quantification using OCPC showed a significant increase in calcium deposition in the folic acid-supplemented group on days 4, 7, and 10 **(Fig. 2C)**. These results suggested that folic acid supplementation promoted calcium deposition during osteogenic differentiation.

C Gene and protein expression profiling at different stages of osteogenic differentiation

Real-time RT-PCR analysis was employed to assess the expression levels of genes related to osteogenic differentiation, including *RUNX2*, *COL1A1*, *Osteocalcin* (*OCN*), and *PHEX* **(Fig. 3A)**. This molecular approach provided valuable data on the influence of folic acid on the genetic regulation of bone formation.

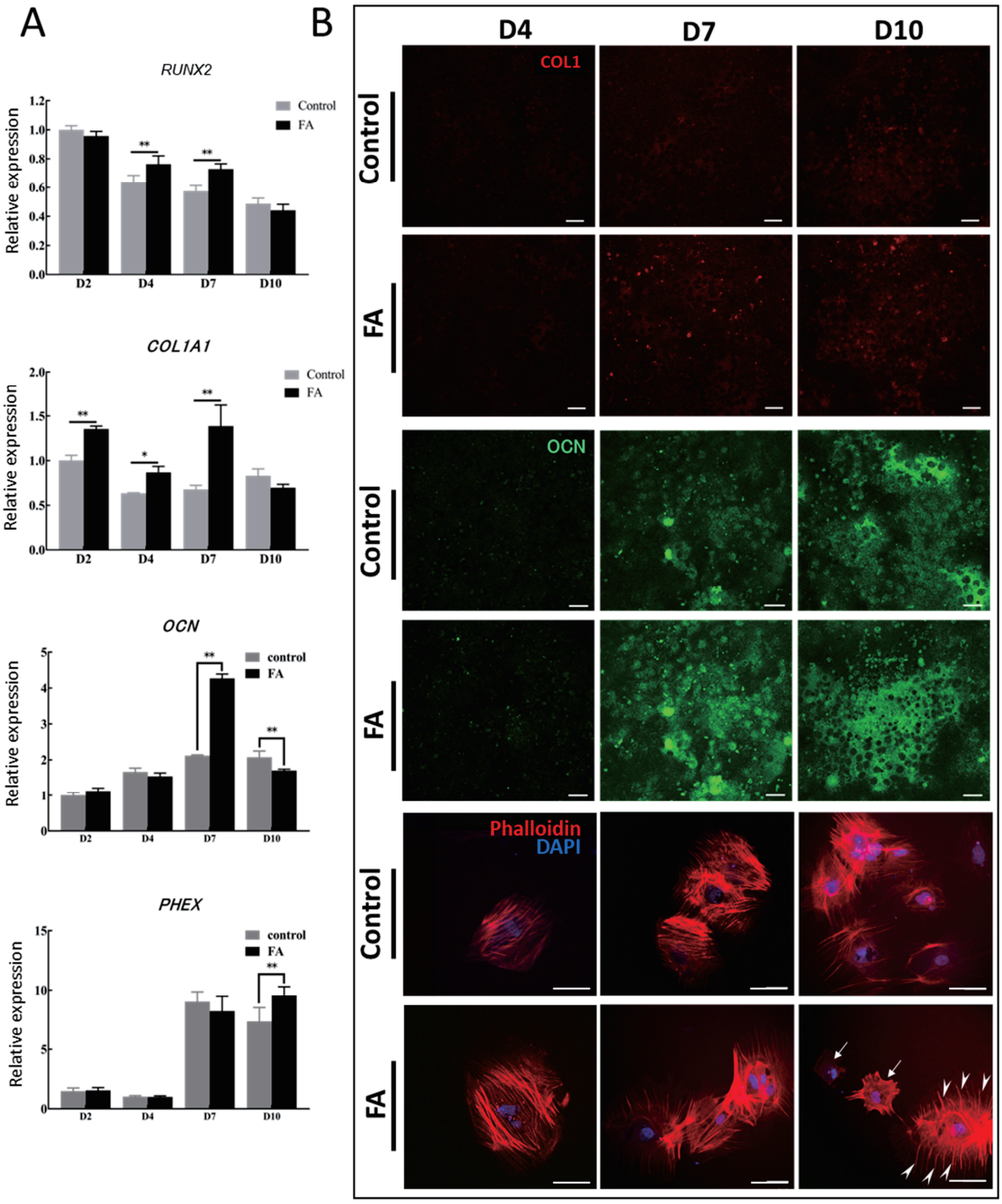

Fig. 3 Gene and protein expression profiling during osteogenic differentiation. (A) Quantification of the expression of osteogenic differentiation stage-related genes *RUNX2*, *COL1A1*, *OCN*, and *PHEX*. Data expressed as the mean ± SD (n = 3) ; *p < 0.05, **p < 0.01. (B) Immunostaining of type I collagen (COL1, red), osteoblast-specific protein Osteocalcin (OCN, green), and Phalloidin (red). The presence of the cells was confirmed by staining nuclei with DAPI (blue). Scale bars : 30 μm. FA : folic acid

RUNX2, a scaffold for nucleic acids and regulatory factors involved in skeletal gene expression, exhibited significant differences on days 4 and 7, indicating that folic acid promotes bone formation. *COL1A1*, which encodes the principal component of type I collagen, the fibrillar collagen present in various connective tissues, including bone and cartilage, exhibited elevated expression in the folic acid-supplemented group on days 2, 4, and 7. *OCN*, which is among the most abundant proteins in bone and is produced ex-

clusively by osteoblasts, showed maximum expression in the folic acid-supplemented group on day 7, with a significant difference compared to the control. *PHEX,* a marker of osteocytes, was highly expressed in the folic acid-supplemented group on day 10. These findings suggested that folic acid influences gene expression at various stages of osteogenic differentiation, supporting its potential role in promoting bone formation.

Immunostaining was employed to detect and visualize specific proteins linked to osteoblasts and osteocytes, including Type I collagen (COL1) and osteocalcin (OCN) **(Fig. 3B)**. Osteoblasts synthesize bone matrix via collagen secretion and hydroxyapatite crystal deposition. COL1 expression in bone-like nodules induced from iPSCs showed a uniform distribution in the control and folic acid-supplemented group. Notably, a more pronounced positive signal was discerned in the folic acid group on day 7. OCN, exclusively produced by osteoblasts, serves as a standard osteoblast marker. On the fourth day of induction, both the control and folic acid-supplemented groups exhibited OCN-positive cells within the bone-like nodules, with a stronger positive signal observed in the folic acid-added group on days 7 and 10. Following the isolation of cells from bone-like nodules through collagenase treatment, staining with phalloidin was conducted to visualize the actin filaments. Most cells isolated on days 4 and 7 exhibited a columnar morphology consistent with osteoblasts. As mineralization progresses, they transform into dendritic-shaped cells with spines on day 10 **(**arrowhead in **Fig. 3B)**. As osteogenic differentiation, osteoblasts gradually transform into mature osteocytes, characterized by morphological changes such as a reduction in cell volume and the adoption of a star-shaped appearance **(**arrow in **Fig. 3B)**.

D Folic acid effects at different stages of osteogenic development

To investigate the role of folic acid at various stages of bone development, folic acid was added on days 0, 2, 4, and 7 during the induction of differentiation. Visual observation of alizarin staining revealed that the group treated with folic acid on day 2 exhibited larger and darker bone-like nodules compared to the other groups **(Fig. 4A)**. Key genes associated with different stages of bone development were selected, and their expression was analyzed **(Fig. 4B)**. On day 1, the expression of the osteoprogenitor-related gene *RUNX2* showed a significant increase in the folic acid-supplemented group. On day 2, an increase in the expression of the pre-osteoblast-related gene *ALP* was observed although statistical analysis did not reveal a significant difference. On day 4, the expression of the osteoblast-related gene Bone sialoprotein (*BSP*) was significantly increased in the group that received folic acid on the 2nd day of differentiation. On day 7, the expression of the osteoblast-related genes *OCN* and *COL1A1* showed a significant increase in the group that received folic acid on day0 and day2. On day 10, the expression of the osteocyte-related gene *PHEX* significantly increased in all folic acid-supplemented groups, except for that supplemented on day 7, with the most notable increase observed in the group that received folic acid on the 2nd day. These results indicate that the addition of folic acid has affected different stages of bone development, with the most significant promotion of osteoblast differentiation observed when folic acid was added during pre-osteoblast differentiation.

E Confirmation of folic acid role in osteoblast differentiation using MC3T3-E1 cell line

To further validate the role of folic acid in osteoblast differentiation, the MC3T3-E1 cell line was employed, and cells were treated with 10 μM and 50 μM folic acid. On days 5th and 10th day of differentiation, Alizarin Red staining revealed a deeper red color in the two folic acid-added groups **(Fig. 5A)**. Gene expression analysis of *COL1A2* and *OCN* showed significant enhancement in the folic acid supplemented groups **(Fig. 5B)**. Additionally, on day 10 of differentiation induction, cells were passage into single cells and subjected to immunostaining. The results showed an increased number of OCN-positive cells and enhanced expression of COL1 protein in the folic acid-supplemented groups. Phalloidin staining further demonstrated that a greater proportion of cells in the folic acid-supplemented group underwent a transition

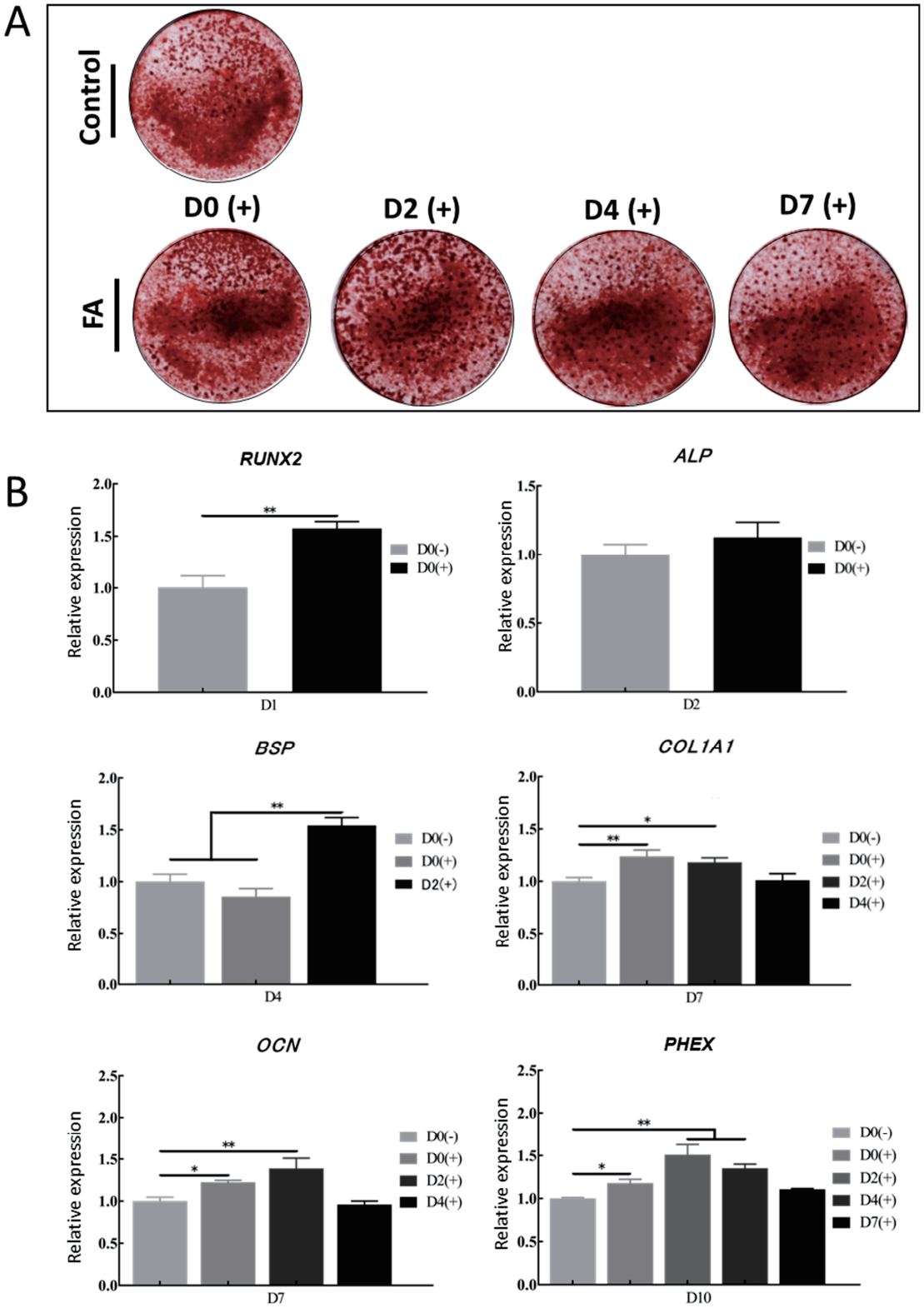

Fig. 4 Folic acid effects at various stages of bone development. (A) Images of Alizarin Red staining on Day 10 in the control and folic acid-added group. Folic acid was added on Day 0, 2, 4, and 7. Note the larger bone-like nodules with deep red staining at the upper left part of the image of addition on Day 2 (D2(+)). FA : folic acid. (B) Key gene expression at different stages of osteogenic differentiation, *RUNX2*, *ALP*, *BSP*, *COL1A1*, *OCN*, and *PHEX*, expressed as a value relative to that of the control group on Day 0. Data expressed as the mean ± SD (n = 3) ; $^{*}p<0.05$, $^{**}p<0.01$.

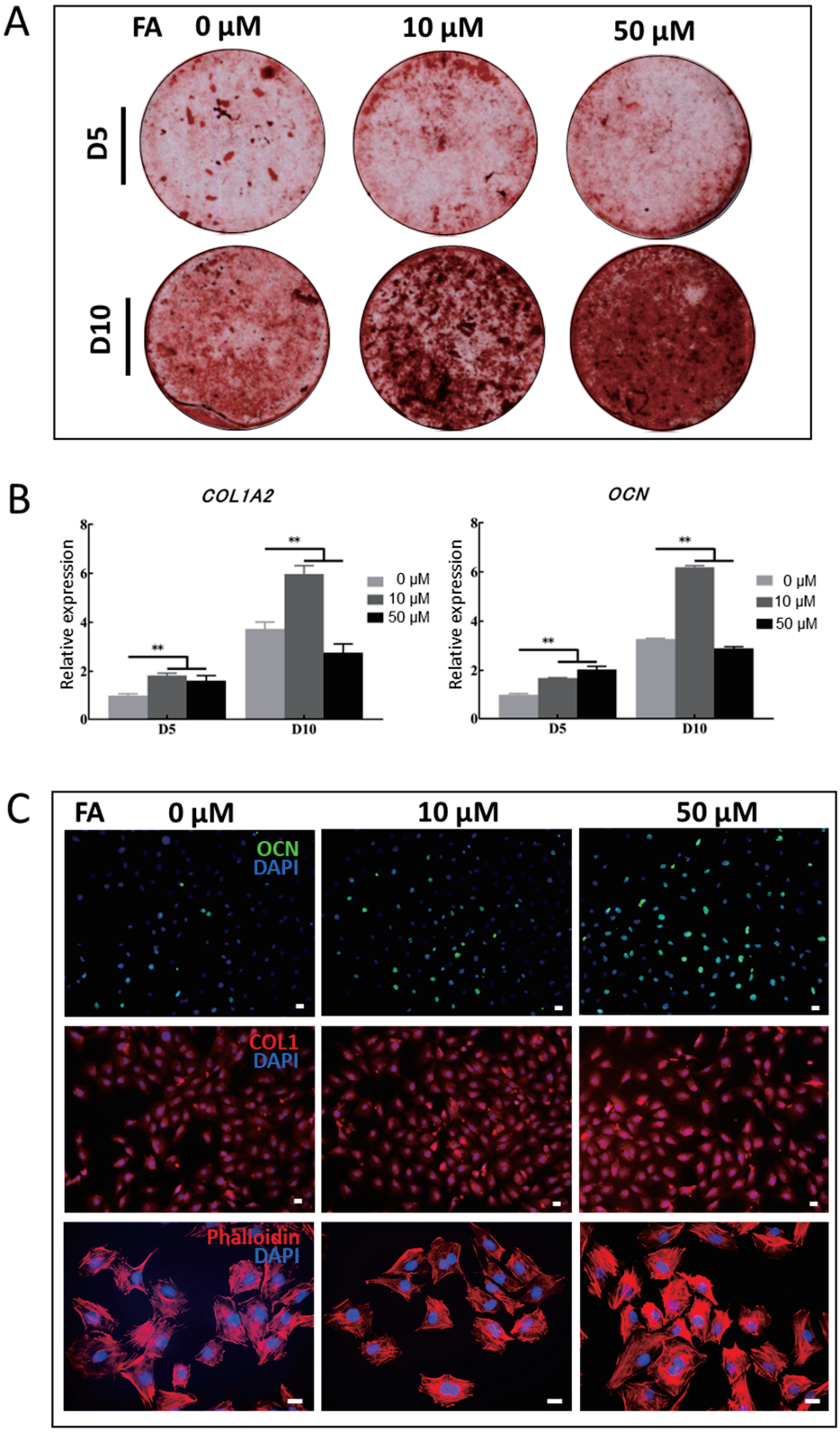

Fig. 5 Role of folic acid in osteoblast differentiation using the MC3T3-E1 cell line. (A) Alizarin Red staining on the 5th and 10th days of differentiation for groups treated with varying concentrations of folic acid. (B) Gene expression levels of crucial markers, *COL1A2* and *OCN*. Data expressed as the mean ± SD (n = 3) ; $^{**}p < 0.01$. (C) Immunostaining of osteoblast-specific protein Osteocalcin (OCN, green), type I collagen (COL1, red) and Phalloidin (red) on Day 10. Nuclei were counterstained with DAPI (blue). Scale bars : 30 μm. FA : folic acid

from a rectangular to a polygonal shape **(Fig. 5C)**.

Collectively, these results support the notion that folic acid plays an important role in enhancing osteogenic differentiation and calcium deposition, particularly during pre-osteoblast differentiation. These findings provide valuable insight into the complex relationship between folic acid and bone formation.

Ⅳ Discussion

Our study utilized iPSCs to mimic the in vivo osteogenic differentiation process and conducted a comprehensive investigation into the role of folic acid at various stages of bone formation. Folic acid supplementation significantly enhanced calcium deposition in bone-like nodules, indicating increased osteogenic activity. Crucial osteogenic differentiation genes, such as *RUNX2*, *COL1A1*, *OCN*, and *PHEX*, consistently exhibited upregulated in the presence of folic acid. Additionally, temporal variations in the effects of folic acid at distinct stages of osteogenic differentiation were observed, indicating a time-dependent manner of the responses of osteogenic cells to folic acid during bone development. Furthermore, the inclusion of the MC3T3-E1 cell line results not only validated our findings with iPSCs but also strengthen the folic acid's positive influence on osteoblast differentiation. This study represents the first in-depth exploration of the specific effect of folic acid on the early stages of bone development.

Adequate nutrient intake, including folic acid, has been associated with improved bone mineralization, emphasizing the multifaceted role of nutrition in supporting skeletal integrity, especially in aging populations. A recent study by Zheng et al.[21] demonstrated a positive correlation between folic acid intake and bone mineral density in postmenopausal women. In this study, the observed increase in Alizarin Red staining and calcium quantification in the folic acid-supplemented group suggested a stimulatory effect on osteoblast activity. The dissolution experiment further substantiated these findings, indicating increased calcium phosphate content in the folic acid-supplemented group. These results collectively point towards a promoting role of folic acid in calcium deposition, a crucial aspect of bone mineralization.

Nutrition plays a pivotal role in influencing the molecular processes underlying bone health. Multiple studies[22][23] have investigated the regulatory influence of folic acid on DNA methylation patterns within osteoblasts, suggesting potential epigenetic mechanisms capable of modulating bone formation. Our study demonstrated the upregulation of crucial osteogenic differentiation genes (*RUNX2*, *COL1A1*, *OCN*, and *PHEX*) in the presence of folic acid. Immunostaining for Type I collagen and osteocalcin further provided visual evidence of enhanced protein expression in the folic acid-supplemented group. Especially, the morphological changes observed during osteogenic differentiation revealed the dynamic role of folic acid in driving the progression from osteoblasts to mature osteocytes.

Considering the age-related changes in bone physiology, temporal variations in folic acid effects at distinct stages of osteogenic differentiation were observed. The darker bone-like nodules and upregulated expression of osteogenic genes in the group that received folic acid during pre-osteoblast differentiation suggested the influence on osteoblast differentiation. Moon et al.[24] reported that early treatment of mesenchymal stem cells with folic acid significantly promotes chondrogenic differentiation, emphasizing the importance of timing in the supplementation. Through additional validation experiments on the osteoblast-like cell line (MC3T3-E1), we confirmed the crucial role of folic acid in osteoblast differentiation, particularly during the transition from pre-osteoblasts to mature osteoblasts. This temporal dimension offers a dynamic understanding of the effects of folic acid on osteogenic differentiation at different stages. Our study hints at the time-dependent and complex responses of osteogenic cells to folic acid supplementation during different phases of bone development.

In the context of nutrition and aging, our study prompts a discussion on the potential significance of folic acid supplementation for pregnant women and older individuals. Future studies could explore the optimal dosage and duration of folic acid supplementation for bone health maintenance. Moreover, it is

essential to note that bone development is a complex process involving various nutrients. Previous studies[25)26)] have emphasized how folic acid, in conjunction with vitamins D, B6, and B12, can synergistically promote bone health. Understanding these interactions is essential for developing straightforward dietary strategies to enhance overall bone formation.

In conclusion, utilization of iPSCs for in vivo osteogenic differentiation provides comprehensive evidence that folic acid plays a pivotal role in enhancing osteogenic differentiation and calcium deposition, particularly during pre-osteoblast differentiation. These findings contribute valuable insights into the molecular and cellular mechanisms underlying the relationship between folic acid and bone formation, highlighting that supplementation with folic acid during pregnancy not only crucially prevents neural tube defects in the developing fetus but also holds potential relevance for the skeletal system development. Further research is needed to elucidate the complexities of nutrient interactions and the precise molecular pathways through which folic acid exerts its effects on osteogenic differentiation, as well as to explore its therapeutic applications in bone health.

Acknowledgments

We are grateful to the Division of Instrumental Research, Shinshu University Research Center for Advanced Science and Technology.

References

1) Crider KS, Yang TP, Berry RJ, Bailey LB : Folate and DNA methylation : a review of molecular mechanisms and the evidence for folate's role. Adv Nutr 3 : 21-38, 2012
2) Lucock M : Folic acid : nutritional biochemistry, molecular biology, and role in disease processes. Mol Genet Metab 71 : 121-138, 2000
3) Ducker GS, Chen L, Morscher RJ, et al : Reversal of cytosolic one-carbon flux compensates for loss of the mitochondrial folate pathway. Cell Metab 23 : 1140-1153, 2016
4) Lan X, Field MS, Stover PJ : Cell cycle regulation of folate-mediated one-carbon metabolism. Wiley Interdiscip Rev Syst Biol Med 10 : e1426, 2018
5) Blom HJ, Smulders Y : Overview of homocysteine and folate metabolism. With special references to cardiovascular disease and neural tube defects. J Inherit Metab Dis 34 : 75-81, 2011
6) Ebara S : Nutritional role of folate. Congenit Anom 57 : 138-141, 2017
7) Zhou Y, Tang J, Wu M, et al : Effect of folic acid supplementation on cardiovascular outcomes : a systematic review and meta-analysis. PLoS One 6 : e25142, 2011
8) Cheng Z, Gu R, Lian Z, Gu H : Evaluation of the association between maternal folic acid supplementation and the risk of congenital heart disease : a systematic review and meta-analysis. J Orthop Surg Res 18 : 684, 2022
9) Ströhle A, Wolters M, Hahn A : Folic acid and colorectal cancer prevention : molecular mechanisms and epidemiological evidence. Int J Oncol 26 : 1449-1464, 2005
10) He H, Zhang Y, Sun Y, et al : Folic Acid Attenuates High-Fat Diet-Induced Osteoporosis Through the AMPK Signaling Pathway. Front Cell Dev Biol 39 : 791880, 2022
11) Hossein-Nezhad A, Mirzaei K, Maghbooli Z, Najmafshar A, Larijani B : The influence of folic acid supplementation on maternal and fetal bone turnover. J Bone Miner Metab 29 : 186-192, 2011
12) Weitzmann MN : The role of inflammatory cytokines, the RANKL/OPG Axis, and the immunoskeletal interface in physiological bone turnover and osteoporosis. Scientifica (Cairo) 2013 : 125705, 2013
13) Takahashi K, Tanabe K, Ohnuki M, et al : Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell 131 : 861-872, 2007
14) Takahashi K, Yamanaka S : Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell 126 : 663-676, 2006

15) Duan X, Tu Q, Zhang J, et al : Application of induced pluripotent stem (iPS) cells in periodontal tissue regeneration. J Cell Physiol 226 : 150–157, 2011
16) Rowe RG, Daley GQ : Induced pluripotent stem cells in disease modelling and drug discovery. Nat Rev Genet 20 : 377–388, 2019
17) Robinton DA, Daley GQ : The promise of induced pluripotent stem cells in research and therapy. Nature 481 : 295–305, 2012
18) Yue F, Era T, Yamaguchi T, Kosho T : Pathophysiological investigation of skeletal deformities of musculocontractural Ehlers–Danlos syndrome using induced pluripotent stem cells. Genes (Basel) 14 : 730, 2023
19) Kawai S, Yoshitomi H, Sunaga J, et al : In vitro bone–like nodules generated from patient–derived iPSCs recapitulate pathological bone phenotypes. Nat Biomed Eng 3 : 558–570, 2019
20) Takizawa S, Yoshie S, Yue F, et al : FGF7 and cell density are required for final differentiation of pancreatic amylase–positive cells from human ES cells. Cell Tissue Res 354 : 751–759, 2013
21) Zheng Z, Luo H, Xu W, Xue Q : Association between dietary folate intake and bone mineral density in a diverse population : a cross–sectional study. J Orthop Surg Res 18 : 684, 2023
22) Chen JR, Lazarenko OP, Blackburn ML, Mercer KE, Badger TM, Ronis MJ : p47phox–Nox2–dependent ROS Signaling Inhibits Early Bone Development in Mice but Protects against Skeletal Aging. J Biol Chem 290 : 14692–14704, 2015
23) Kaludjerovic J, Ward WE : Bone–specific gene expression patterns and whole bone tissue of female mice are programmed by early life exposure to soy isoflavones and folic acid. J Nutr Biochem 10 : 1068–1076, 2015
24) Moon Y, Patel M, Um S, et al : Folic acid pretreatment and its sustained delivery for chondrogenic differentiation of MSCs. J Control Release 343 : 118–130, 2022
25) Lewicki S, Lewicka A, Kalicki B, Klos A, Bertrandt J, Zdanowski R : The influence of vitamin B12 supplementation on the level of white blood cells and lymphocytes phenotype in rats fed a low–protein diet. Cent Eur J Immunol 39 : 419–425, 2014
26) Fernández–Villa D, Jiménez Gómez–Lavín M, Abradelo C, San Román J, Rojo L : Tissue Engineering Therapies Based on Folic Acid and Other Vitamin B Derivatives. Functional Mechanisms and Current Applications in Regenerative Medicine. Int J Mol Sci 19 : 4068, 2018

(2024. 3. 29 received ; 2024. 4. 9 accepted)