

키토산 기반 나노방출제어시스템의 세포주기진행 유전자 발현 증진 효과 및 기전

Effect and mechanism of chitosan-based nano-controlled release system on the promotion of cell cycle progression gene expression

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Purpose. In our previous studies, application of trichloroacetic acid (TCA) to gingival fibroblasts or to canine palatal soft tissue was verified to alter the expression of several genes responsible for cell cycle progression. In order to confirm this effect in a system allowing sequential release of TCA and epidermal growth factor (EGF), expression of various cell cycle genes following the application of the agents, using hydrophobically modified glycol chitosan (HGC)-based nano-controlled release system, was explored in this study. **Materials and methods.** HGC-based nano-controlled release system was developed followed by loading TCA and EGF. The groups were defined as the control (CON); TCA-loaded nano-controlled release system (EXP1); TCA- and EGF- individually loaded nano-controlled release system (EXP2). At 24- and 48 hr culture, expression of 37 cell cycle genes was analyzed in human gingival fibroblasts. Correlations and the influential genes were also analyzed. **Results.** Numerous genes such as cyclins (CCNDs), cell division cycles (CDCs), cyclin-dependent kinases (CDKs), E2F transcription factors (E2Fs), extracellular signal-regulated kinases (ERKs) and other cell cycle genes were significantly up-regulated in EXP1 and EXP2. Also, cell cycle arrest genes of E2F4, E2F5, and GADD45G were up-regulated but another cell cycle arrest gene SMAD4 was down-regulated. From the multiple regression analysis, CCNA2, CDK4, and ANAPC4 were determined as the most influential factors on the expression of ERK genes. **Conclusion.** Application of TCA and EGF, using the HGC-based nano-controlled sequential release system significantly up-regulated various cell cycle progression genes, leading to the possibility of regenerating oral soft tissue via application of the proposed system. (J Korean Acad Prosthodont 2021;59:379-94)

Keywords

Cell cycle; Epidermal growth factor; Trichloroacetic acid

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Introduction

Cells proliferate via cell cycle. The cell cycle can be divided into interphase and mitotic phase, with the interphase being further divided into G1, S, and G2 phase. In the G1 phase, enzymes and proteins necessary for DNA replication are synthesized, number of organelles is increased, and cell growth takes place. The S phase is when DNA is cloned and the amount of DNA is doubled. In the G2 phase being the final step in preparing for a split period, proteins needed for cell division such as proteins that make up the spindle fiber are synthesized, followed by the mitotic phase. In this phase that is further divided into prophase, metaphase, anaphase and telophase, two daughter nuclei are formed through fission, resulting in the formation of daughter cells via cytokinesis. Genetic damage can occur in this process, and checkpoints exist for this purpose. In G1 checkpoint, cell size, DNA database, flow factors, and nutrients are evaluated when proceeding from G1 phase to S phase, whereas, in G2 checkpoint, DNA replication and DNA damage are evaluated before mitosis begins. The spindle assembly checkpoint involves an evaluation of the attachment of the spindle fiber and the centromere in the M phase. Cell cycles are known to involve a number of factors, mainly cyclins, cyclin-dependent kinases (CDKs), and CDK inhibitors (CDKIs).^{1,2}

The mitogen-activated protein kinase (MAPK) cascade is known to play an important role in the transduction of extracellular signals into cells. MAPK families are categorized into classical MAPK (also known as ERK, the extracellular signal-regulated kinase, in human), C-Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK), and p38 kinase, and play an important role in the regulation of cell proliferation. Of these, the ERK pathway promotes proliferation, differentiation, and development by transactivating transcription factors into the nucleus to change gene expression, where ERK1 and ERK2 are instrumental. In addition, the ERK pathway can link G0/G1 mitogenic signals in the cell cycle to immediate

initial response and is known to play a central role in the regulation of cell cycle progression.³

In our preliminary pilot study, the effects of trichloroacetic acid (TCA) were evaluated in cell models.⁴ Also, the effects of TCA and epidermal growth factor (EGF) using hydrophobically modified glycol chitosan (HGC)-based nano-controlled release systems were evaluated in cell- and animal models.⁵ As a result, it was observed that TCA induces alterations of numerous gene expression in human gingival fibroblasts. In the studies, HGC-based nano controlled release systems was suggested as a solution to the risk of simple application of a strong acid, such as TCA, in the oral environment and to tightly control the its release patterns. The safety of the HGC-based nano controlled release systems was also verified, and the controlled delayed release of the EGF next to the rapid TCA release was induced. Alterations in the expression of genes in human gingival fibroblasts and in the tissue cells from canine palatal mucosa of *canis familiaris* were verified.^{4,5} From the results of the DNA microarray analysis and successive Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis in both studies, we were able to select a total of 37 cell cycle genes which would possibly alter their expression induced by the application and sequential release of TCA and EGF.

Since the application of TCA to gingival fibroblasts or to canine palatal soft tissue was verified to alter the expression of several genes responsible for cell cycle progression, the purpose of the present study was to confirm this effect in the HGC-based nano-controlled release system allowing sequential release of TCA and EGF by determining the expression of various cell cycle genes following the application of such agents. Also, to elaborate the hypothesis supporting the promoter effect of the proposed system on the cell cycle progression, correlations and influential factor genes were determined in this study as well.

Materials and methods

Fabrication of TCA- and EGF loaded nano-controlled release system

Glycol chitosan (1.0 g, 0.4×10^{-5} mol), 5 β -cholanic-acid (123.0 mg, 0.34×10^{-3} mol), N-hydroxy succinimide (NHS) (58.6 mg, 0.51×10^{-3} mol), N-(3-dimethylamino-propyl)-N'-ethylcarbodiimide hydrochloride (EDC·HCl) (97.6 mg, 0.51×10^{-3} mol), TCA (Sigma-Aldrich Co., St. Louis, MO, USA), methanol (Sigma-Aldrich Co., St. Louis, MO, USA) and EGF (Peprotech, London, UK) were purchased and used as reagents. Glycol chitosan conjugate 5 β -colanic acid particles were produced by the materials and methods described in detail in our previous study.⁵ The TCA-loaded nanoparticles and EGF-loaded nanoparticles were also produced by the materials and methods described in detail in our previous study.⁵

Cell culture

Human gingival fibroblasts (ATCC[®] PCS-201-018[™], Manassas, VA, USA) were purchased and used for cell culturing. Cells were suspended in Dulbecco's modified eagle's medium (DMEM, Invitrogen Corporation, Carlsbad, CA, USA) that consists of 10% fetal bovine serum (FBS, Sigma-Aldrich Co., St. Louis, MO, USA) and antibiotics. The suspended human gingival fibroblasts were brought to culture in humidified incubator with 5% CO₂ and 95% air at 37°C. In this study, human gingival fibroblasts cultured for 2 to 3 cell cycles were used.

Gene expression analysis

A total of 37 genes related to cell cycle progression were selected from our preliminary studies.^{4,5} These genes were analyzed by the real-time PCR for their relative mRNA expression in the groups of the control (CON); TCA-loaded nano controlled release system (EXP1); TCA- and EGF individually loaded nano controlled release system (EXP2), respectively. Human gingival fibroblasts were inoculated into 24-well CON, EXP1, EXP2 so that cell density was maintained for 1×10^4 per well for cul-

turing at 37°C, 5% CO₂ for 24 and 48 hr. The total RNA was extracted using Trizol lysis solution (TRIZOL[®] REAGENT, GIBCO BRL, Carlsbad, CA, USA) and the concentration of RNA was determined using the NanoDrop 1000 (NanoDrop Technologies, Wilmington, DE, USA). Out of total RNA, 1 μ g was reverse-transcribed with cDNA using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Berkeley, CA, USA). Based on the expression of internal control group GAPDH, mRNA of human gingival fibroblasts was finally selected through differential display PCR. The gene expression was determined using the TaqMan[®] Gene Expression Assay Kit (Applied Biosystems, Waltham, MA, USA). The genes used for real-time PCR in this study are summarized (Table 1). Chromo-4 reverse transcription-polymerase chain reactions (Bio-Rad Laboratories, Hemel Hempstead, UK) were performed using IQ Supermix (Bio-Rad, Hercules, CA, USA). The gene expression was then quantified using MJ Opticon Monitor Analysis Software (Bio-Rad, Hercules, CA, USA). The measured values were corrected by the expression level of GAPDH, and the expression levels of EXP1 and EXP2 were expressed in multiples of CON.

Statistical analysis

The real-time PCR was performed eight times simultaneously and independently, and the mean and standard deviation were calculated. One-way ANOVA was performed in order to compare mean values, while Tukey's multiple comparison test B was used for post-hoc comparison. Pearson correlation analysis was performed to analyze the correlations between the gene expression analysis results. To determine the cell cycle genes that have statistically significant influence on the ERK1- and ERK2 expression, a stepwise method of multiple regression analysis was used (Table 2). SPSS 18.0 software program (SPSS 18.0, SPSS Inc., Chicago, IL, USA) was used for all statistical analysis.

Table 1. Gene-specific primers (Taqman® gene expression assay) used in real-time PCR

Gene	AB assay ID	NCBI reference
14-3-3σ, YWHAS, SFN, stratifin	Hs00968567_s1	NM_006142.5
ANAPC4, anaphase promoting complex subunit 4	Hs01546701_m1	NM_013367.3
BUB1, BUB1 mitotic checkpoint serine/threonine kinase	Hs01557695_m1	NM_004336.5
CDC20, cell division cycle 20	Hs00426680_mH	NM_001255.3
CDC25A, cell division cycle 25A	Hs00947994_m1	NM_001789.3
CDC25B, cell division cycle 25B	Hs01582335_m1	NM_021873.3
CDC25C, cell division cycle 25C	Hs00156411_m1	NM_022809.3
CDC7, cell division cycle7	Hs00177487_m1	NM_003503.4
CDK1, cyclin dependent kinase 1	Hs00938777_m1	NM_001786.5
CDK2, cyclin dependent kinase 2	Hs01548894_m1	NM_001798.5
CDK4, cyclin dependent kinase 4	Hs00364847_m1	NM_000075.4
CDK6, cyclin dependent kinase 6	Hs01026371_m1	NM_001259.8
CDK7, cyclin dependent kinase 7	Hs00361486_m1	NM_001799.4
CCNA1, cyclin A1	Hs00171105_m1	NM_003914.4
CCNA2, cyclin A2	Hs00996788_m1	NM_001237.5
CCNB1, cyclin B1	Hs01030099_m1	NM_031966.4
CCNB2, cyclin B2	Hs01084593_g1	NM_004701.4
CCND1, cyclin D1	Hs99999004_m1	NM_053056.2
CCND2, cyclin D2	Hs00153380_m1	NM_001759.4
CCNE1, cyclin E1	Hs01026536_m1	NM_001238.4
CCNE2, cyclin E2	Hs00180319_m1	NM_057749.2
CCNH, cyclin H	Hs00236923_m1	NM_001239.4
DBF4, DBF4 zinc finger	Hs00272696_m1	NM_006716.4
E2F1, E2F transcription factor 1	Hs00153451_m1	NM_005225.3
E2F2, E2F transcription factor 2	Hs00231667_m1	NM_004091.4
E2F3, E2F transcription factor 3	Hs00605457_m1	NM_001949.5
E2F4, E2F transcription factor 4	Hs00608097_m1	NM_001950.4
E2F5, E2F transcription factor 5	Hs00231092_m1	NM_001951.4
ERK1, extracellular signal-regulated kinase 1	Hs00385075_m1	NM_002746.3
ERK2, extracellular signal-regulated kinase 2	Hs01046830_m1	NM_002745.4
GADD45G, growth arrest and DNA damage inducible gamma	Hs02566147_s1	NM_006705.4
MAD2L1, mitotic arrest deficient 2 like 1	Hs01554513_g1	NM_002358.4
MCM6, minichromosome maintenance complex component 6	Hs00962418_m1	NM_005915.6
MPS1, TTK, TTK protein kinase	Hs01009870_m1	NM_003318.5
PLK1, polo like kinase 1	Hs00983227_m1	NM_005030.6
PTTG1, PTTG1 regulator of sister chromatid separation, securin	Hs00869689_s1	NM_004219.4
SMAD4, SMAD family member 4	Hs00929647_m1	NM_005359.5
GAPDH, glyceraldehyde-3-phosphate dehydrogenase	Hs99999905_m1	NM_002046.7

Table 2. The influential factors on the expression of ERK1 and ERK2 after respective 24- and 48 hr of culture and administration of the nano controlled release system to induce sequential release of TCA and EGF, as determined by multiple stepwise regression analysis

Dependent variable	Model	Regression results	R	R ²	Sig. ¹⁾
ERK1-24h	1	ERK1-24h = 0.200+0.793 · [CCNA2-48h]	.992	.985	< .001
	2	ERK1-24 h = -0.055+0.534 · [CCNA2-48h]+0.525 · [CDK2-24h]	.998	.995	< .001
	3	ERK1-24 h = 0.242+0.532 · [CCNA2-48h]+0.803 · [CDK2-24h]-0.575 · [CDK7-48h]	.999	.997	< .001
	4	ERK1-24h = 0.486+0.448 · [CCNA2-48h]+1.162 · [CDK2-24h]-0.809 · [CDK7-48h]-0.286 · [E2F3-24h]	.999	.998	< .001
	5	ERK1-24h = 0.592+0.290 · [CCNA2-48h]+1.313 · [CDK2-24h]-0.957 · [CDK7-48h]-0.508 · [E2F3-24h]+0.270 · [CCND1-24h]	.999	.999	< .001
	6	ERK1-24h = 0.786+0.269 · [CCNA2-48h]+1.253 · [CDK2-24h]-0.812 · [CDK7-48h]-0.406 · [E2F3-24h]+0.484 · [CCND1-24h]-0.575 · [CDC25B-48h]	1.000	.999	< .001
	7	ERK1-24h = 0.736+0.105 · [CCNA2-48h]+1.276 · [CDK2-24h]-0.664 · [CDK7-48h]-0.820 · [E2F3-24h]+0.718 · [CCND1-24h]-0.577 · [CDC25B-48h]+0.226 · [E2F2-48h]	1.000	1.000	< .001
	8	ERK1-24h = 1.084-0.034 · [CCNA2-48h]+1.334 · [CDK2-24h]-1.049 · [CDK7-48h]-0.882 · [E2F3-24h]+0.792 · [CCND1-24h]-0.631 · [CDC25B-48h]+0.246 · [E2F2-48h]+0.140 · [MPS1-48h]	1.000	1.000	< .001
	9	ERK1-24h = 1.047+1.310 · [CDK2-24h]-1.014 · [CDK7-48h]-0.835 · [E2F3-24h]+0.754 · [CCND1-24h]-0.623 · [CDC25B-48h]+0.231 · [E2F2-48h]+0.128 · [MPS1-48h]	1.000	1.000	< .001
	10	ERK1-24h = 1.088+1.388 · [CDK2-24h]-1.064 · [CDK7-48h]-0.905 · [E2F3-24h]+0.750 · [CCND1-24h]-0.667 · [CDC25B-48h]+0.270 · [E2F2-48h]+0.174 · [MPS1-48h]-0.035 · [PLK1-48h]	1.000	1.000	< .001
	11	ERK1-24h = 1.156+1.392 · [CDK2-24h]-1.182 · [CDK7-48h]-0.851 · [E2F3-24h]+0.800 · [CCND1-24h]-0.736 · [CDC25B-48h]+0.300 · [E2F2-48h]+0.178 · [MPS1-48h]-0.085 · [PLK1-48h]+0.028 · [PTTG1-48h]	1.000	1.000	< .001
ERK1-48h	1	ERK1-48h = -1.493+2.493 · [CDK4-48h]	.996	.992	< .001
	2	ERK1-48h = -1.130+1.659 · [CDK4-48h]+0.466 · [CDK6-48h]	.997	.994	< .001
	3	ERK1-48h = -0.933+2.127 · [CDK4-48h]+0.686 · [CDK6-48h]-0.866 · [MPS1-24h]	.998	.996	< .001
	4	ERK1-48h = -0.861+1.863 · [CDK4-48h]+0.536 · [CDK6-48h]-1.063 · [MPS1-24h]+0.527 · [GADD45G-48h]	.999	.998	< .001
	5	ERK1-48h = -0.707+1.528 · [CDK4-48h]+1.048 · [CDK6-48h]-0.889 · [MPS1-24h]+0.571 · [GADD45G-48h]-0.552 · [DBF4-48h]	.999	.999	< .001
ERK2-24h	1	ERK2-24h = 0.644+0.318 · [ANAPC4-48h]	.987	.974	< .001
	2	ERK2-24h = 0.357+0.258 · [ANAPC4-48h]+0.362 · [14-3-3σ-24h]	.994	.988	< .001
	3	ERK2-24h = 0.627+0.309 · [ANAPC4-48h]+0.324 · [14-3-3σ-24h]-0.261 · [CCND2-48h]	.997	.994	< .001
	4	ERK2-24h = 0.494+0.270 · [ANAPC4-48h]+0.009 · [14-3-3σ-24h]-0.386 · [CCND2-48h]+0.613 · [14-3-3σ-48h]	.999	.999	< .001
	5	ERK2-24h = 0.493+0.270 · [ANAPC4-48h]-0.389 · [CCND2-48h]+0.626 · [14-3-3σ-48h]	.999	.999	< .001
	6	ERK2-24h = 0.529+0.233 · [ANAPC4-48h]-0.433 · [CCND2-48h]+0.347 · [14-3-3σ-48h]+0.324 · [CDK2-48h]	1.000	.999	< .001
	7	ERK2-24h = 0.076+0.171 · [ANAPC4-48h]+0.255 · [CCND2-48h]+0.387 · [14-3-3σ-48h]+0.430 · [CDK2-48h]-0.318 · [PLK1-24h]	1.000	1.000	< .001
	8	ERK2-24h = 0.244+0.193 · [ANAPC4-48h]+0.366 · [14-3-3σ-48h]+0.399 · [CDK2-48h]-0.202 · [PLK1-24h]	1.000	.999	< .001
	9	ERK2-24h = -0.141+0.199 · [ANAPC4-48h]+0.480 · [14-3-3σ-48h]+0.330 · [CDK2-48h]-0.482 · [PLK12-4h]+0.614 · [CCND2-24h]	1.000	1.000	< .001
	10	ERK2-24h = -0.103+0.198 · [ANAPC4-48h]+0.392 · [14-3-3σ-48h]+0.210 · [CDK2-48h]-0.540 · [PLK1-24h]+0.575 · [CCND2-24h]+0.268 · [CCNE1-48h]	1.000	1.000	< .001
	11	ERK2-24h = -0.176+0.227 · [ANAPC4-48h]+0.382 · [14-3-3σ-48h]+0.085 · [CDK2-48h]-0.560 · [PLK12-4h]+0.606 · [CCND2-24h]+0.294 · [CCNE1-48h]+0.142 · [E2F3-24h]	1.000	1.000	< .001
	12	ERK2-24h = -0.213+0.239 · [ANAPC4-48h]+0.403 · [14-3-3σ-48h]-0.587 · [PLK1-24h]+0.645 · [CCND2-24h]+0.335 · [CCNE1-48h]+0.179 · [E2F3-24h]	1.000	1.000	< .001
ERK2-48h	1	ERK2-48h = -0.433+1.397 · [CDK4-48h]	.991	.982	< .001
	2	ERK2-48h = 0.394+1.576 · [CDK4-48h]-0.973 · [CDK7-24h]	.998	.996	< .001
	3	ERK2-48 h = 0.577+0.943 · [CDK4-48h]-1.138 · [CDK7-24h]+0.616 · [CDK2-48h]	.999	.998	< .001

1) Significances of each regression model were tested by analysis of variance (n = 24).

R: coefficient of multiple correlations.

R²: coefficient of determination.

Results

Development of nano controlled release system

HGC nanoparticles were self-aggregated, encapsulated and released various chemicals and peptides in aqueous solution, and were dissolved well in a neutral pH solution.⁵ As for HGC-based nano controlled release systems manufacture, a hydrophobic cholanic acid conjugated polymer was used in chitosan where an amine group (NH₂) - a hydrophilic cationic polymer - existed. Meanwhile, self-assembling hydrophilic cholanic acid formed a core in water, while hydrophilic cationic chitosan formed a shell. Anionic TCA and EGF were loaded onto the chitosan shell by ionic bonding, and their release patterns were suggested to be controlled in sequential and sustained manner for days *in vitro* as well as *in vivo* (Fig. 1).

Gene expression

The real-time PCR analysis identified the up-regulated or down-regulated genes at 24 or 48 hr. For cyclins, the gene expression analysis results were as follows. The genes of cyclin A1 (CCNA1), cyclin A2 (CCNA2), cyclin B1 (CCNB1), cyclin B2 (CCNB2), cyclin D2 (CCND2) and cyclin E1 (CCNE1) were simultaneously up-regulated in EXP1 and EXP2 compared to CON at 24 hr, whereas the

significant up-regulations at 48 hr were in the order of CON, EXP1 and EXP2 ($P < .001$). Also, the cyclin H gene (CCNBH) was simultaneously up-regulated in EXP1 and EXP2 compared to CON at 24 hr, whereas the significant up-regulations at 48 hr were in the order of CON, EXP1 and EXP2 ($P < .01$). The genes of cyclin D1 (CCND1) and cyclin E2 (CCNE2) were significantly up-regulated at both 24- and 48 hr in the order of CON, EXP1 and EXP2 ($P < .001$) (Fig. 2).

For cell division cycles, the gene expression analysis results were as follows. The cell division cycle 7 gene (CDC7) was simultaneously up-regulated in EXP1 and EXP2 compared to CON at 24 hr, whereas the significant up-regulations at 48 hr were in the order of CON, EXP1 and EXP2 ($P < .001$). The genes of cell division cycle 20 (CDC20) and cell division cycle 25C (CDC25C) were significantly up-regulated at both 24- and 48 hr in the order of CON, EXP1 and EXP2 ($P < .001$). The cell division cycle 25A (CDC25A) was simultaneously up-regulated in EXP1 and EXP2 compared to CON at both timelines of 24- and 48 hr ($P < .001$). The cell division cycle 25B gene (CDC25B), on the other hand, showed significant up-regulation at 48 hr in the order of CON, EXP1 and EXP2 ($P < .001$), but showed no statistical differences in expression between groups at 24 hr (Fig. 3).

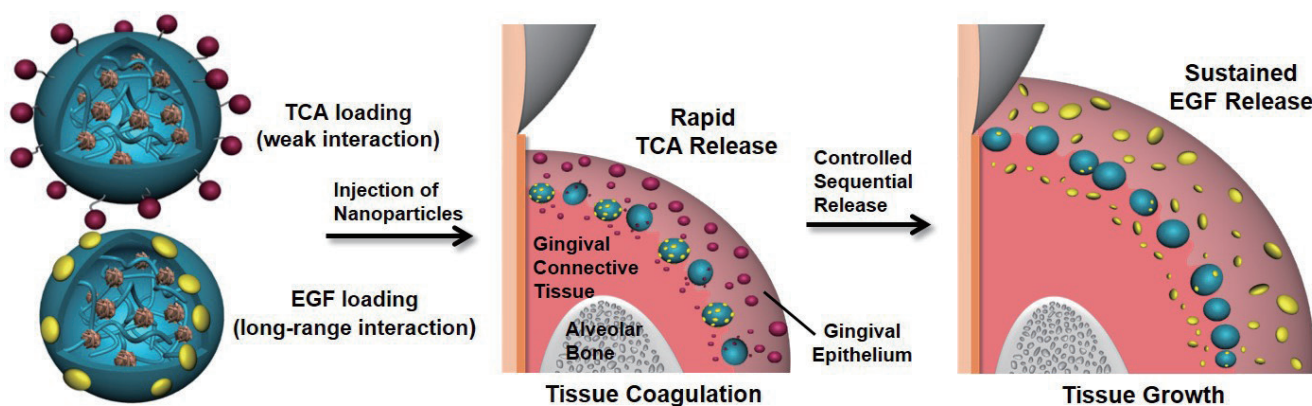


Fig. 1. After the fabrication of hydrophobically modified glycol chitosan (HGC)-based nano-controlled release system, anionic trichloroacetic acid (TCA) and epidermal growth factor (EGF) were loaded to chitosan shell by ionic bond. Note that their release patterns of TCA and EGF were suggested to be controlled in sequential and sustained manner for days *in vivo* by the proposed HGC-based nano controlled release system.

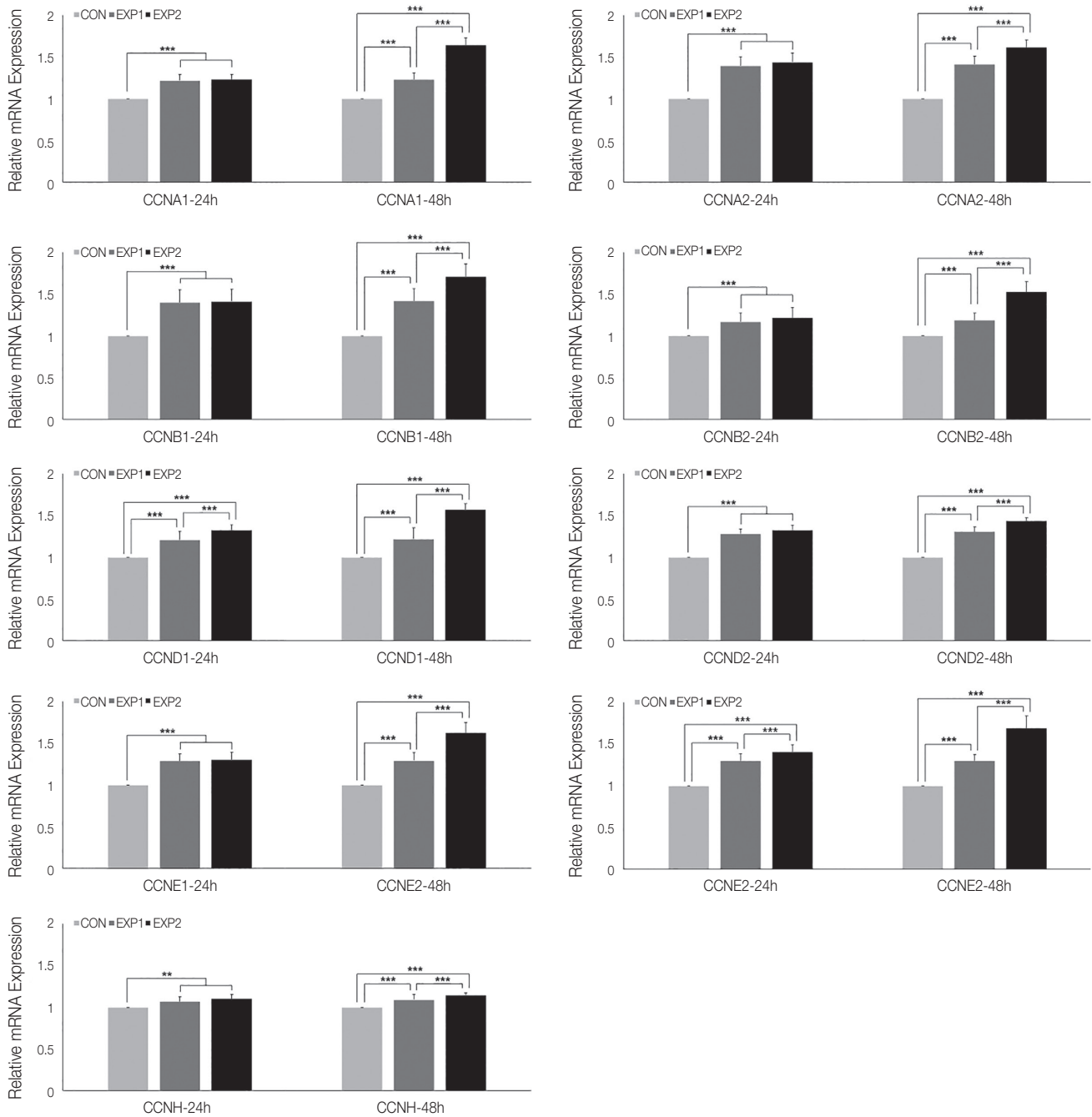


Fig. 2. Comparison of CCNA1, CCNA2, CCNB1, CCNB2, CCND1, CCND2, CCNE1, CCNE2, CCNH expression of human gingival fibroblasts cultured for 24- and 48 hr in CON, EXP1, EXP2 using the real-time PCR. The relative expression levels were analyzed by normalizing with GAPDH, and are presented as fold changes relative to the control, CON. One-way ANOVA (n = 8). ***: significant difference ($P < .001$). **: significant difference ($P < .01$).

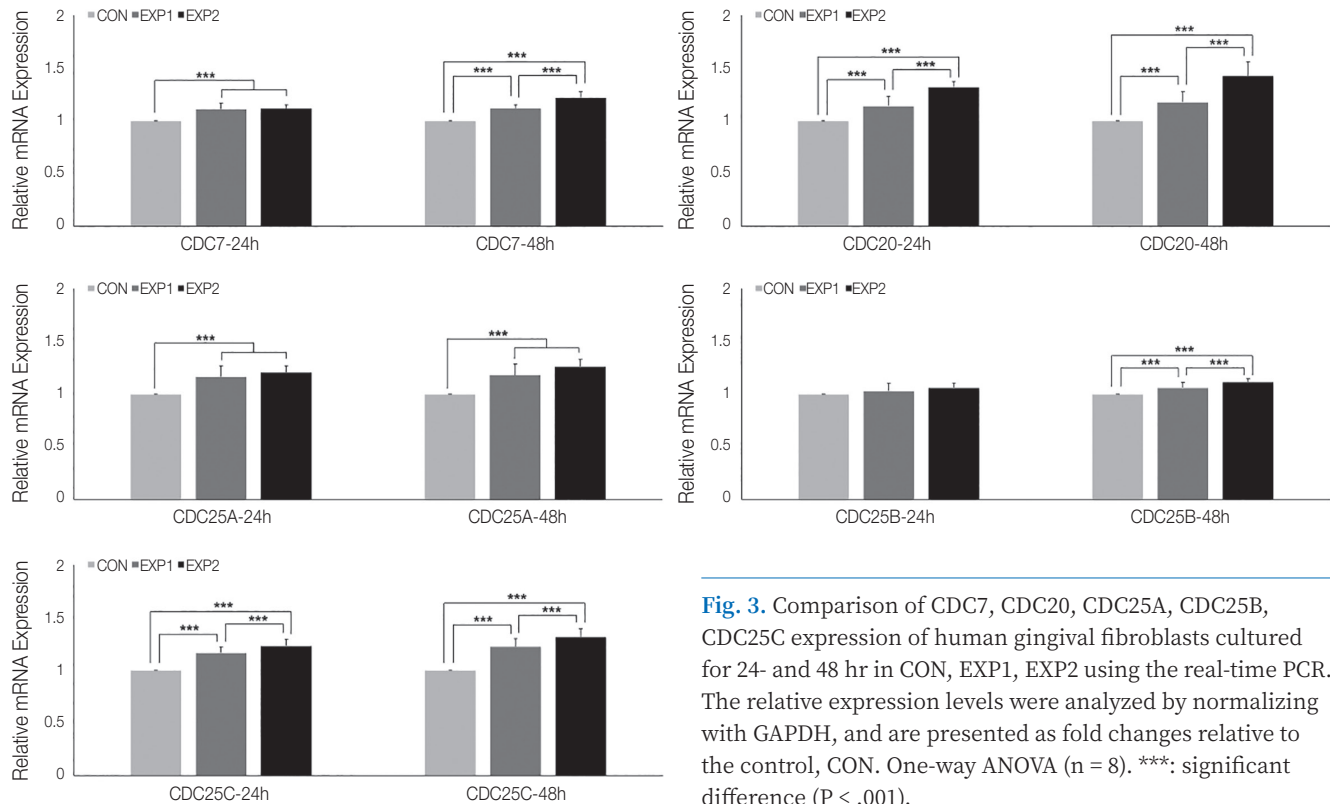


Fig. 3. Comparison of CDC7, CDC20, CDC25A, CDC25B, CDC25C expression of human gingival fibroblasts cultured for 24- and 48 hr in CON, EXP1, EXP2 using the real-time PCR. The relative expression levels were analyzed by normalizing with GAPDH, and are presented as fold changes relative to the control, CON. One-way ANOVA (n = 8). ***: significant difference ($P < .001$).

For CDKs, the gene expression analysis results were as follows. CDK1 was simultaneously upregulated in EXP1 and EXP2 compared to CON at 24 hr, whereas the significant up-regulations at 48 hr were in the order of CON, EXP1 and EXP2 ($P < .001$). CDK2, CDK4 and CDK6 were significantly up-regulated at both 24- and 48 hr in the order of CON, EXP1 and EXP2. CDK7, on the other hand, showed significant up-regulation in EXP2 compared to both CON and EXP1 with no significant difference between CON and EXP1 at 24 hr, but showed significant up-regulations at 48 hr in the order of CON, EXP1 and EXP2 ($P < .001$) (Fig. 4).

For E2F transcription factors and ERKs, the gene expression analysis results were as follows. E2F1 and E2F5 were significantly up-regulated at both 24- and 48 hr in the order of CON, EXP1 and EXP2 ($P < .001$). E2F2 at 24 hr showed significant up-regulation in EXP2 compared to both CON and EXP1 ($P < .001$) with no significant dif-

ference between CON and EXP1. E2F4 at 48 hr showed significant up-regulation in EXP2 compared to CON ($P < .05$). All other comparisons including E2F4 at 24 hr, E2F3 at 24 hr, E2F3 at 48 hr and E2F4 at 24 hr showed no statistical differences between groups (Fig. 5). Both ERK1 and ERK2 were significantly up-regulated at both 24- and 48 hr in the order of CON, EXP1 and EXP2 ($P < .001$) (Fig. 6).

The expression results of other miscellaneous cell cycle genes were as follows. Anaphase promoting complex subunit 4 (ANAPC4), DBF4 zinc finger gene (DBF4), growth arrest and DNA damage inducible γ (GADD45G), mitotic arrest deficient 2 like 1 (MAD2L1), minichromosome maintenance complex component 6 (MCM6), TTK protein kinase (MPS1), polo like kinase 1 (PLK1) and PTTG1 regulator of sister chromatid separation (PTTG1) were significantly up-regulated at both 24- and 48 hr in the order of CON, EXP1 and EXP2 ($P < .001$). The stratifin gene (14-3-3 σ) was significantly up-regulated in EXP2

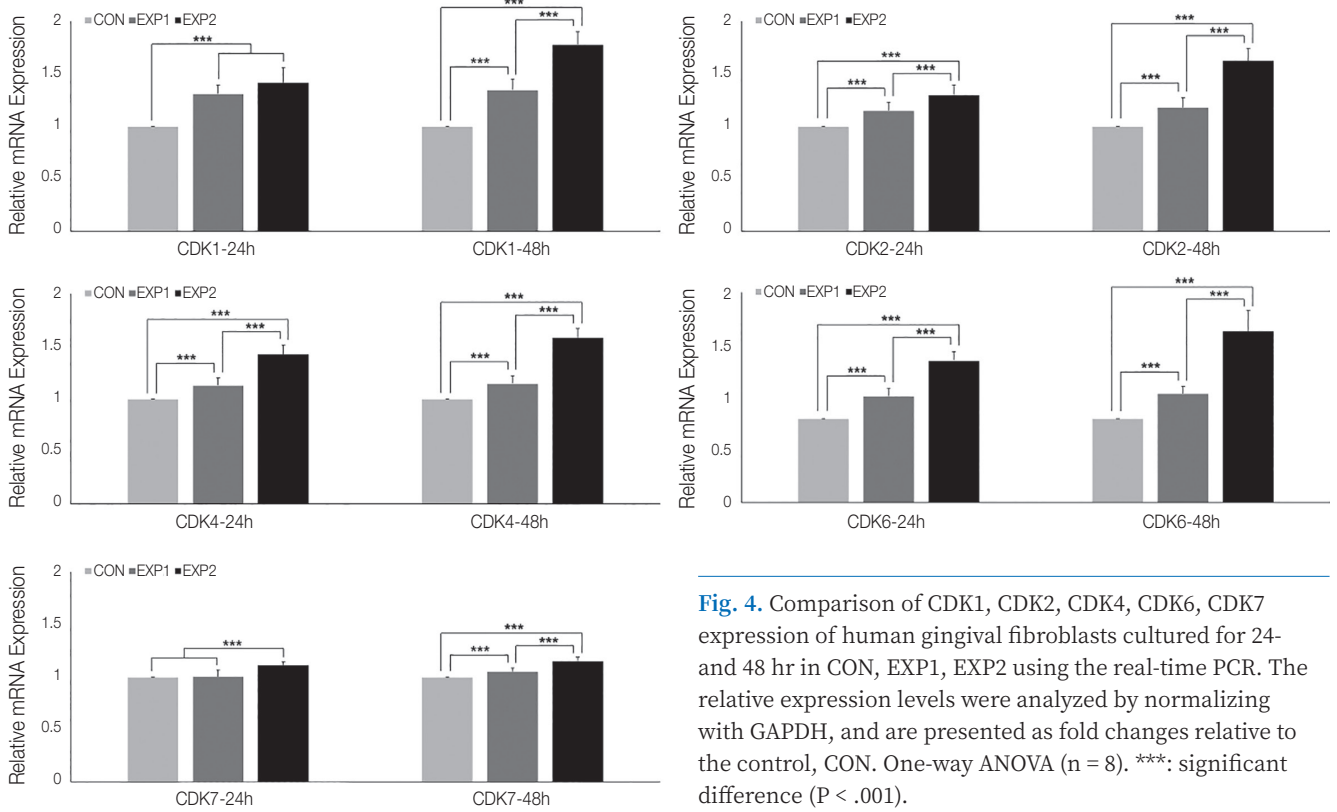


Fig. 4. Comparison of CDK1, CDK2, CDK4, CDK6, CDK7 expression of human gingival fibroblasts cultured for 24- and 48 hr in CON, EXP1, EXP2 using the real-time PCR. The relative expression levels were analyzed by normalizing with GAPDH, and are presented as fold changes relative to the control, CON. One-way ANOVA (n = 8). ***: significant difference (P < .001).

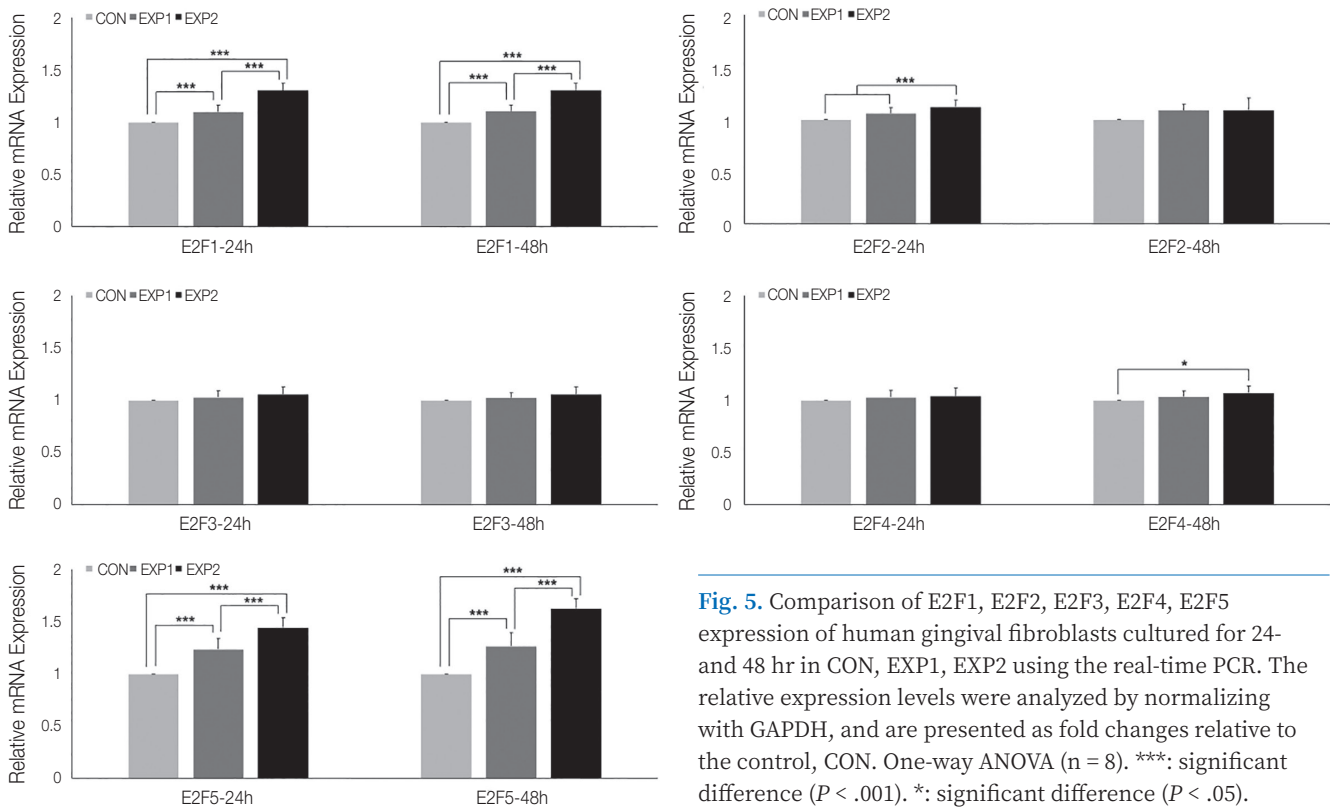


Fig. 5. Comparison of E2F1, E2F2, E2F3, E2F4, E2F5 expression of human gingival fibroblasts cultured for 24- and 48 hr in CON, EXP1, EXP2 using the real-time PCR. The relative expression levels were analyzed by normalizing with GAPDH, and are presented as fold changes relative to the control, CON. One-way ANOVA (n = 8). ***: significant difference (P < .001). *: significant difference (P < .05).

compared to CON or EXP1 at 24 hr, whereas, at 48 hr, significant up-regulations were shown in the order of CON, EXP1 and EXP2 ($P < .001$). The BUB1 mitotic checkpoint serine/threonine kinase 1 gene (BUB1) was significantly up-regulated at both 24- and 48 hr in the order of CON, EXP1 and EXP2 ($P < .001$). The SMAD family member 4 gene (SMAD4) at 24 hr showed significant down-regulation of EXP1 compared to CON, whereas at 48 hr, both EXP1 and EXP2 were significantly down-regulated compared to CON ($P < .01$) with no statistical differences between EXP1 and EXP2 (Fig. 7).

Correlation and influence

The bivariate Pearson correlation analysis was performed using all the results of the cell cycle related gene expression determined in this study. The results of the analysis have shown a significant correlation in all comparisons ($P < .01$) (data not shown). In order to identify the gene that affects the ERK in the most significant manner, multiple regression analyses were performed using ERK1-24h, ERK1-48h, ERK2-24h, and ERK2-48h as the dependent variables and the expression results in all other genes at 24- and 48 hr in this study as independent variables. As a result, 14-3-3 σ -24h, 14-3-3 σ -48h, ANAPC4-48h, CCNA2-48h, CDK2-24h, CDK2-48h, CDK4-48h, CDK6-48h, CDK7-48h, E2F2-48h, E2F3-24h, CCND1-24h, CCND2-24h, CCND2-48h, CCNE1-48h, CDC7-24h, CDC25B-48h, DBF4-48h, GADD45G-48h, MPS1-24h, MPS1-48h, PLK1-24h,

PLK1-48h, and PTTG1-48h were selected as influential factors on the expression of ERK1-24h, ERK1-48h, ERK2-24h or ERK2-48h ($P < .001$). The most significantly influential factors were determined as; CCNA2-48h for ERK1-24h; CDK4-48h for ERK1-48h; ANAPC4-48h for ERK2-24h; CDK4-48h for ERK2-48h ($P < .001$). The representative correlation results between ERK1-24h and CCNA2-48h; ERK1-48h and CDK4-48h; ERK2-24h and ANAPC4-48h; ERK2-48h and CDK4-48h are presented (Fig. 8).

Discussion

Cyclin levels change based on specific phases of the cell cycle and determine the timing of CDK activity and their substrate specificity.⁶ Cyclin A-CDK2 complex level begins to rise early in the S phase and is essential for the S phase progression of the cell cycle.⁷ Cyclin A-CDK1 complex is involved in the transition from G2 to the M phase.² In this study, both cyclin A1- and cyclin A2 genes (CCNA1 and CCNA2) were significantly up-regulated in EXP1 and EXP2. Along with the upregulation of CDK1 in EXP1 and EXP2, this suggests that the controlled release of TCA with or without the sequential release of EGF could induce the activation of the S phase and the transition from G2 to the M phase. Cyclin B-CDK1 complex, also called mitosis promoting factor, is a precursor to the onset of mitosis when this complex is translocated from the cytoplasm to the nucleus.⁸ In addition, the de-

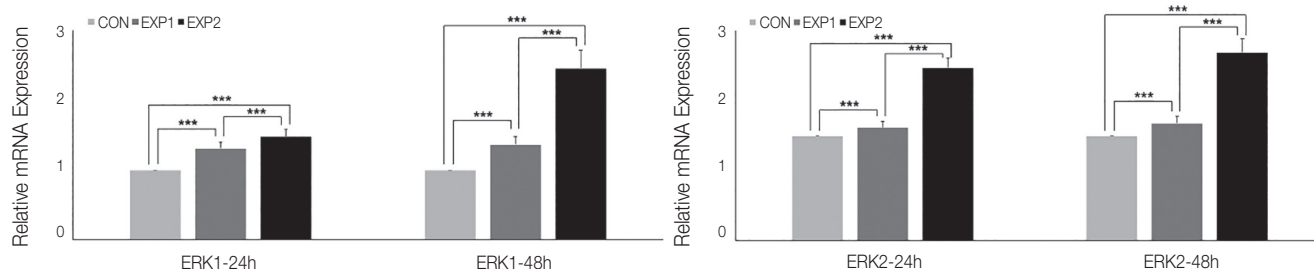


Fig. 6. Comparison of ERK1, ERK2 expression of human gingival fibroblasts cultured for 24- and 48 hr in CON, EXP1, EXP2 using the real-time PCR. The relative expression levels were analyzed by normalizing with GAPDH, and are presented as fold changes relative to the control, CON. One-way ANOVA (n = 8). ***: significant difference ($P < .001$).

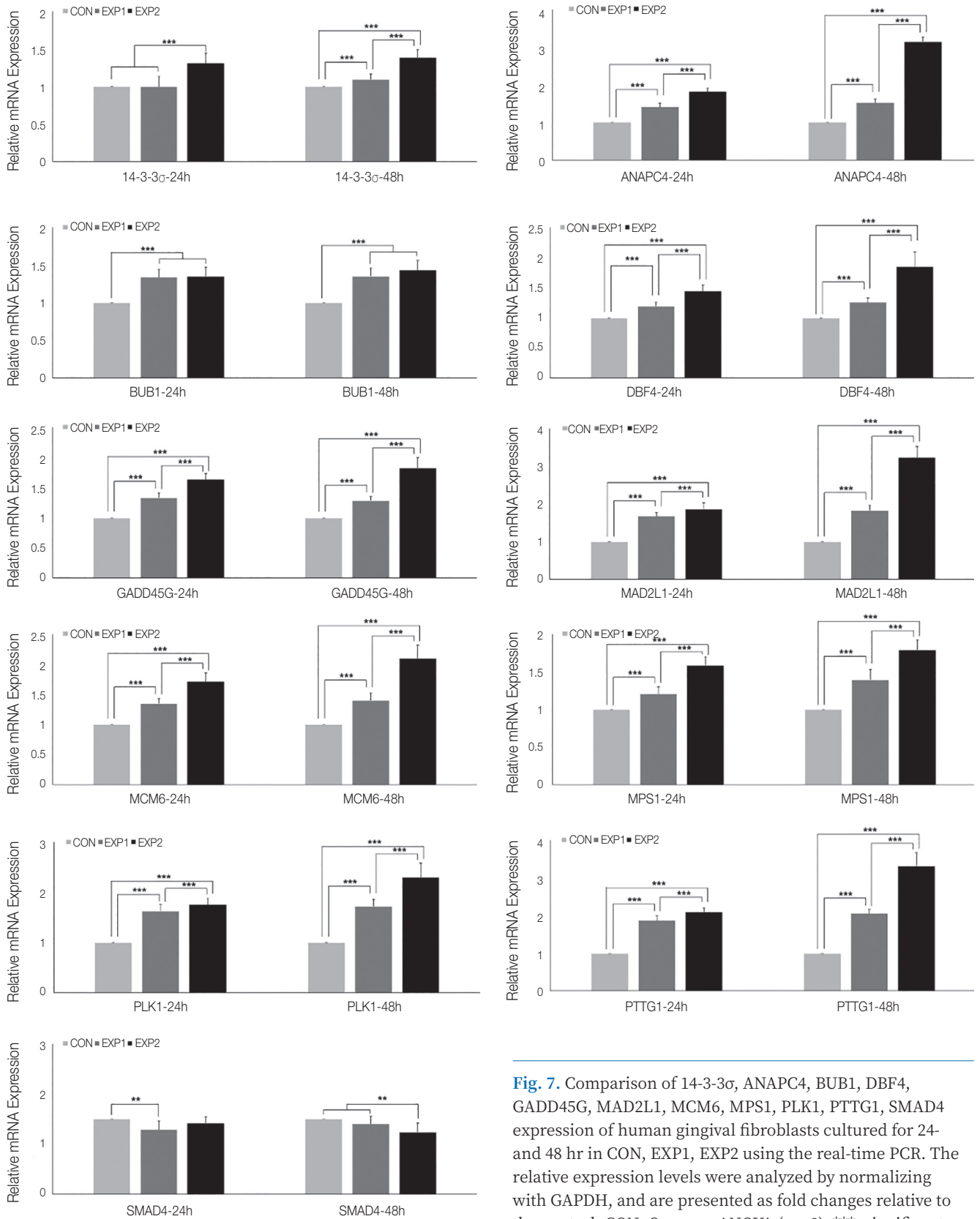


Fig. 7. Comparison of 14-3-3σ, ANAPC4, BUB1, DBF4, GADD45G, MAD2L1, MCM6, MPS1, PLK1, PTTG1, SMAD4 expression of human gingival fibroblasts cultured for 24- and 48 hr in CON, EXP1, EXP2 using the real-time PCR. The relative expression levels were analyzed by normalizing with GAPDH, and are presented as fold changes relative to the control, CON. One-way ANOVA (n = 8). ***: significant difference (P < .001). **: significant difference (P < .01).

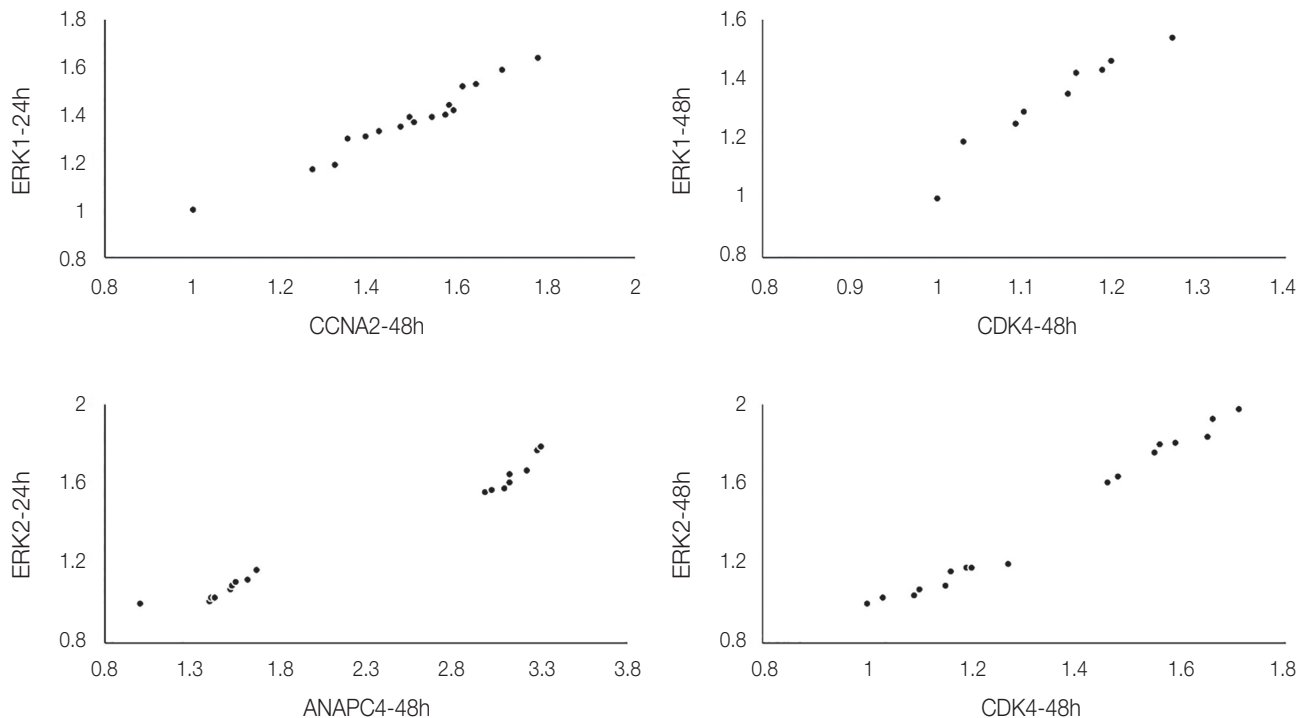


Fig. 8. Scatter-plot results from the Pearson’s correlation analysis. Significant correlations were noted between ERK1-24h and CCNA2-48h; ERK1-48h and CDK4-48h; ERK2-24h and ANAPC4-48h; ERK2-48h and CDK4-48h ($P < .01$) ($n = 24$). Note that only the correlation results of the expressed genes determined as most influential factors for ERK1, ERK2 expression at 24- and 48 hr (Table 1) are presented.

struction of cyclin B is a requirement for the completion of the cell cycle from the M phase.¹ In this study, both cyclin B1- and cyclin B2 genes (CCNB1 and CCNB2) were significantly up-regulated in EXP1 and EXP2. Along with the up-regulation of CDK1 in EXP1 and EXP2, this also suggests that the controlled sequential release of TCA and EGF activates the mitosis, M phase. Phosphorylation of pRb by sequential activation of the Cyclin D-CDK4/CDK6 complex enables cell cycle progression from the G1 phase to the S phase.^{9,10} The simultaneous significant up-regulation of CCND1 and CCND2, CDK4 and CDK6 in this study suggests that the controlled sequential release of TCA and EGF also activates the G1/S cell cycle progression. Cyclin E-CDK2 level rises and then decreases with the start of the S phase and is important for the start of DNA synthesis.¹¹ Cyclin HCDK7 complex acts as a

CDK-activating kinase and regulates the activity of CDKs in all cell cycle phases.¹² Since CCNE-CDK2 and CCNH-CDK7 were simultaneously up-regulated in EXP1 and EXP2, we suggest that the controlled sequential release of TCA and EGF by the nano controlled system not only activates the start of the S phase but also up-regulates CDKs in every phase of the cell cycle. However, 14-3-3 σ and GADD45G, known to inhibit the activity of Cyclin B1-CDK1 complex, were both significantly up-regulated in this study. Since the inhibitory function of 14-3-3 σ was reported to be upon DNA damage^{13,14} resulting in G2/M cell cycle inhibition, we suggest that this controversial result was obtained due to DNA damage of the cells caused by the cytotoxic nature of TCA. However, from the results of our study, it is suggested that a group of cells were rescued by up-regulated PLK1 because PLK1 is known for

its essential role in cell cycle re-entry by contributing to cyclin B1-CDK1 activation when G2 phase arrest occurs due to DNA damage.¹⁵

CDCs encode cell division cycle proteins that have the kinase activity. CDC7 is a key switch for phosphorylation and initiation of the DNA replication of minichromosome maintenance (MCM) DNA helicase, and plays an important role in the G1/S cell cycle progression in eukaryotic cells.¹⁶ CDC20 activates the anaphase-promoting complex (ANAPC) to regulate mitotic exit through proteasomal degradation of proteins and improves cell proliferation upon overexpression.¹⁷ CDC25 phosphatase families, CDC25A, CDC25B and CDC25C, promote cell cycle progression through dephosphorylation of CDKs. CDC25A is active and present at all stages of the cell cycle, from the S phase to mitosis, and plays an important role in the G1/S phase transition,¹⁸ while CDC25B and CDC25C promote G2/M phase transitions.¹⁹ DBF4 is a regulatory subunit of CDC7 serine/threonine-specific protein kinase and is involved in the initiation of DNA replication and the G1/S cell cycle progression.²⁰ DBF4 is also essential for DNA synthesis and initiation of replication necessary for S/G2 cell cycle progression. MCM proteins, involving MCM6 in our study, are phosphorylated by CDC7-DBF4, followed by binding to replication origins and activating the replication initiation complex.²¹ On the other hand, E2Fs can be categorized into activators (E2F1, E2F2, E2F3) and repressors (E2F4, E2F5. E2F1, E2F2) of cell cycle progression.²² E2F1, E2F2 and E2F3 positively regulate transactivation of genes essential for the G1/S phase transition of the cell cycle.²³ While E2F4 and E2F5 does not alter cell proliferation, they cause arrest in the G1 phase.²⁴ Budding uninhibited by benomyl (BUB) proteins and mitotic arrest deficient (MAD) constitute the spindle assembly checkpoint (SAC) complex and are activated when defects occur in the microtubule attachment.²⁵ This delays metaphase-anaphase transitions until the microtubule attachment becomes appropriate.²⁶ MPS1 is a dual specificity protein kinase that also regulates spindle assembly checkpoints and mediates proper microtubule

attachment to chromosomes during mitosis.²⁷ PTTG1, suppressed in most normal tissues, is an important mitotic checkpoint protein that helps keep sister chromatids together before proceeding to anaphase.²⁸ It also acts as a protooncogene, promotes cell cycle progression, maintains chromosomal stability, regulates transformation, and plays an important role in tumorigenesis.²⁹ In this study, potent activators of G1/S-, S/G2- and G2/M cell cycle progressions such as CDC7, DBFA4, MCM6, CDC20 and the family members of CDC25 were significantly up-regulated. E2F1 and E2F2, with complex functions of both activator and repressor were also up-regulated along with the G1/S cell cycle inhibitors E2F4 and E2F5. Also in this study, important regulators of spindle assembly check points such as BUB1, MAD2L1, MPS1 and PTTG1 were significantly up-regulated in EXP1 and EXP2. Taken together, we suggest that the HGC-based nano controlled release of TCA and EGF not only simultaneously up-regulated both activators and repressors of G1/S-, S/G2- and G2/M cell cycle progressions but also activated the regulators of spindle assembly check points in the later phase of metaphase to anaphase transition. Together with the down-regulated SMAD4, known to be responsible for G1/S phase cell cycle arrest,³⁰ the HGC-based nano controlled release system used in this study can be elucidated as both inducer and regulator of the cell cycle progression in all phases.

Comparison results of the expression levels and roles of the 37 genes related to cell cycle progression encouraged us to determine which gene expression profoundly affected the ultimate cellular event of proliferation and differentiation. However, these cellular events could not be determined in this study due to the cytotoxic nature of TCA *in vitro*. The up-regulated MAPKs, ERK1 and ERK2, in this study, play important roles in cell proliferation, differentiation and development.³¹ The ERK pathway also links the G0/G1 mitogenic signal with immediate initial response and the G1/S phase process requires continued activity of ERK1/ERK2.³² For this reason, we selected the expression results of ERK1 and ERK2 in different time-

lines of culture (ERK1-24h, ERK1-48h, ERK2-24h and ERK2-48h) as dependent variables for determination using the multiple regression analysis. We first performed Pearson's correlation analysis and verified that all the result concerning expression of 37 genes in different time-lines of culture were significantly correlated, which verifies that our experimental design in this study was highly valid. Further multiple regression analysis revealed the most significant influencing factors to be: CCNA2-48h for ERK1-24h; CDK4-48h for ERK1-48h; ANAPC4-48h for ERK2-24h; and CDK4-48h for ERK2-48h. Important cell cycle progression conductors and promoters such as CCNA2, CDK4 and ANAPC4 were determined as the most influential genes on the expression of cell proliferation/differentiation representative genes ERK1/ERK2.

Conclusion

We conclude by the results from various analyses in this study that the application of TCA and EGF using the HGC-based nano-controlled release systems promotes cell cycle progression in human gingival fibroblasts, confirming the possible effect of regenerating human gingival tissue under the central role of CCNA2, CDK4 and ANAPC4. Also, in the future, a full clinical investigation study in various animal models can confirm the possibility of regenerating oral soft tissue via application of the proposed system.

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키토산 기반 나노방출제어시스템의 세포주기진행 유전자 발현 증진 효과 및 기전

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목적: 이전 연구에서 치은섬유아세포 혹은 성견 구개 연조직에 trichloroacetic acid (TCA)를 적용하는 것이 세포주기진행 유전자 발현의 변화를 일으키는 것으로 밝혀졌다. 이에 따라 본 연구에서는, hydrophobically modified glycol chitosan (HGC)기반의 나노방출제어시스템을 이용한 TCA 및 상피세포성장인자(EGF)의 순차적 방출시스템에서 이 효과를 검증하기 위하여 다양한 세포주기진행 유전자들의 발현을 규명하였다. **재료 및 방법:** TCA와 EGF를 담지하는 HGC기반 나노방출제어시스템을 제작하였다. 실험군은 대조군(CON); TCA-담지형 나노방출제어시스템 투여군(EXP1); TCA- 및 EGF-담지형 나노방출제어시스템 투여군(EXP2)으로 정의되었다. 24시간 및 48시간 배양 시 37개 세포주기 유전자들의 발현을 분석하였다. 영향인자로서의 유전자 및 상관관계에 대해서도 분석하였다. **결과:** Cyclins (CCNDs), cell division cycles (CDCs), cyclin-dependent kinases (CDKs), E2F transcription factors (E2Fs), extracellular signal-regulated kinases (ERKs)와 같은 다수의 유전자들과 기타 다른 세포주기 유전자들의 발현이 EXP1과 EXP2에서 상향조절되었다. E2F4, E2F5, GADD45G와 같은 세포주기차단 유전자들의 발현도 상향조절되었으나, 또다른 세포주기차단 유전자인 SMAD4의 발현은 하향조절되었다. 다중회귀분석에서 CCNA2, CDK4 그리고 ANAPC4가 ERK 유전자 발현에 가장 영향력 있는 유전자로 선정되었다. **결론:** HGC기반 순차적 나노방출제어시스템을 이용한 TCA 및 EGF의 적용은 다양한 세포주기진행 유전자들의 발현을 상향조절함이 밝혀졌고, 이를 토대로 한 구강연조직증대 시스템 개발의 가능성이 확보되었다. (대한치과보철학회지 2021;59:379-94)

주요단어

세포주기; 상피세포성장인자; 트리클로로아세트산

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