Hirosaki Med. J. 66 : 135—142, 2016

ORIGINAL ARTICLE

CLOCK GENE EXPRESSIONS ARE ALTERED BY CO-CULTURE OF GINGIVAL FIBROBLASTS AND ORAL CANCER CELLS

Ken Furudate[1)], Tomoh Matsumiya[2)], Ryo Hayakari[2)], Fei Xing[2)], Kosei Kubota[1)], Hirotaka Sakaki[1)], Yoshihiro Tamura[1)], Hiroshi Kijima[3)], Tadaatsu Imaizumi[2)], Hiroto Kimura[1)] and Wataru Kobayashi[1)]

Abstract Tumor microenvironment is related to growth, survival, invasion, and metastasis of tumor cells. Several studies have proved that stromal fibroblasts play an important role in the tumor microenvironment to convert cancer-associated fibroblast (CAFs). Clock genes are known to regulate circadian rhythms, angiogenesis, and immunoreaction. In addition, clock genes play an important role in cancer development. However, little has been shown about how these clock genes function in the tumor microenvironment. In the present study, we investigated to evaluate the effect of co-culture fibroblasts with oral cancer cells on the expression of clock genes. Following the co-culture of human primary fibroblasts with human gingival carcinoma Ca9-22 cells, the expression levels of clock genes were analyzed by real-time quantitative PCR. We found that the rhythmic expression of clock genes were altered, enhanced, or disappeared by the co-culture. Such effect was observed not only in fibroblasts in the presence of Ca9-22 cells but also in Ca9-22 cells in the presence of fibroblasts. Our results suggested that clock genes might affect an important role in the tumor microenvironment.

Hirosaki Med. J. 66 : 135—142, 2016

Key words: clock genes; oral cancer cells; fibroblasts; co-culture.

Introduction

Most of the living organisms on the earth are greatly influenced by circadian rhythms. In mammals, the central system of circadian rhythm is in the suprachiasmatic nucleus (SCN) of the brain[1)]. Clock genes compose the molecular systems of circadian rhythms. Clock genes are expressed in peripheral tissues and oscillate twenty four hour cycle by forming transcriptional and translational feedback loops[2, 3)]. As positive regulators, brain and muscle ARNT-Like protein (BMAL) / circadian locomotor output cycles kaput (CLOCK) heterodimers bind to the E box, which is thought to be the key element of clock system[4)], and activate the transcription of Period (Per) 1/2/3, and cryptochrome (Cry) 1/2[5)]. On the other hand, as negative regulators, PER and CRY proteins interact with BMAL/CLOCK heterodimers and inhibit transcription of Per genes[6)]. The other negative factor differentiated embryo chondrocytes (Dec)1 and 2 suppress BMAL/CLOCK-mediated transcription[7)]. A core of 8 clock genes regulates essential time-keeping mechanisms, and organizes a variety of cell

[1)] Department of Dentistry and Oral Surgery, Hirosaki University Graduate School of Medicine, Hirosaki, Japan.
[2)] Department of Vascular Biology, Institute of Brain Science, Hirosaki University Graduate School of Medicine, Hirosaki, Japan.
[3)] Department of Pathology and Bioscience, Hirosaki University Graduate School of Medicine, Hirosaki, Japan,

Correspondence: T. Matsumiya
Received for publication, November 4, 2015
Accepted for publication, November 16, 2015

functions related to cell cycle, cell growth, and apoptosis[8]. Therefore, disruption of their function can cause cancer development[9]. Indeed, a broken circadian rhythm is associated with various cancers, including breast cancer and oral cancer[10]. For example, [11]animal studies revealed that Per2 mutant mice are likely to develop radiation-induced tumors, salivary gland hyperplasia, and lymphoma[11, 12]. In addition, the roles of clock genes on anticancer agents-induced apoptosis are reported[13, 14].

Accumulated evidences clearly show crucial roles of microenvironment in tumor progression. Stromal fibroblasts and myofibroblasts contribute important roles in the tumor microenvironment and those stromal cells to convert cancer-associated fibroblasts (CAFs) [15, 16]. CAFs have been found in variety of human cancers. To date, the origin of CAFs has been thought to drive from normal fibroblasts[17], epithelial-mesenchymal transition[18], or mesenchymal cells[19]. CAFs are the source of cytokines such as epithelial growth factor (EGF) or transforming growth factor-β (TGF-β), which promote pro-tumorigenic signaling[16]. In addition, the pro-metastatic effects of CAFs were implied from the studies using co-culture experiment that CAFs degrade extracellar matrix (ECM), allowing the invasion cancer cells[20]. Collectively, CAFs play cardinal a role in cancer biology; however, how CAFs and cancer cells affect remain to be elucidated. In this study, we examined the effect of co-culture of gingival fibroblasts with oral cancer cells on the expression of clock genes[23]. Our results propose the important role of cancer microenvironment to alter the circadian rhythm of oral cancer cells.

Materials and Methods

Cell Culture

Human gingival fibroblasts were isolated from patients who had healthy gingival tissues and received minor oral surgery at Hirosaki university Hospital. Before treatment, the patients had given written informed consent. This study was approved by the Committee of Medical Ethics of Hirosaki University School of Medicine, Hirosaki, Japan. Gingival tissues were washed in phosphate buffered saline (PBS), (pH 7.4) and cut into small pieces, which were cultured in Alpha minimal essential medium (Sigma-Aldrich, St Louis, MO, USA) containing 10% fetal bovine serum (FBS) (Invitrogen, Carlsbad, CA, USA), penicillin (100U/mL) (Sigma-Aldrich), streptomycin (100μg/mL) (Sigma-Aldrich) and gentamicin (80μg/mL) (Sigma-Aldrich), for 2 weeks in an atmosphere of 95% air and 5% CO2 at 37 °C. When cells growing out from the explants had reached confluence, they were sub-cultured and experiments were performed on confluent cultures form third to sixth passages. A cell line of human oral squamous cell carcinoma, Ca9-22, were purchased from JCRB Cell BANK (Osaka, Japan). Sub-cultured fibroblasts and Ca9-22 cells were cultured using Dulbecco's Modified Eagle Medium (DMEM) (Sigma-Aldrich) supplemented with 10% FBS and penicillin/streptomycin.

Co-culture of fibroblast with Ca9-22

Fibroblasts were cultured on cell culture insert dishes containing a 3.0 μm pore size polyethylene terephthalate (PET) track-etched membranes (BD Falcon, Bedford, MA, USA), and Ca9-22 cells were seeded in the wells of a 6 well plate (BD Falcon) (illustrated in Figure 1). These cells were individually maintained in a 5% CO2 atmosphere at 37°C in DMEM supplemented with 10% FBS. When the cells reached approximately 80% confluence, co-culture was started for up to 7days. During the co-culture, the medium in the co-cultures was changed every 2days.

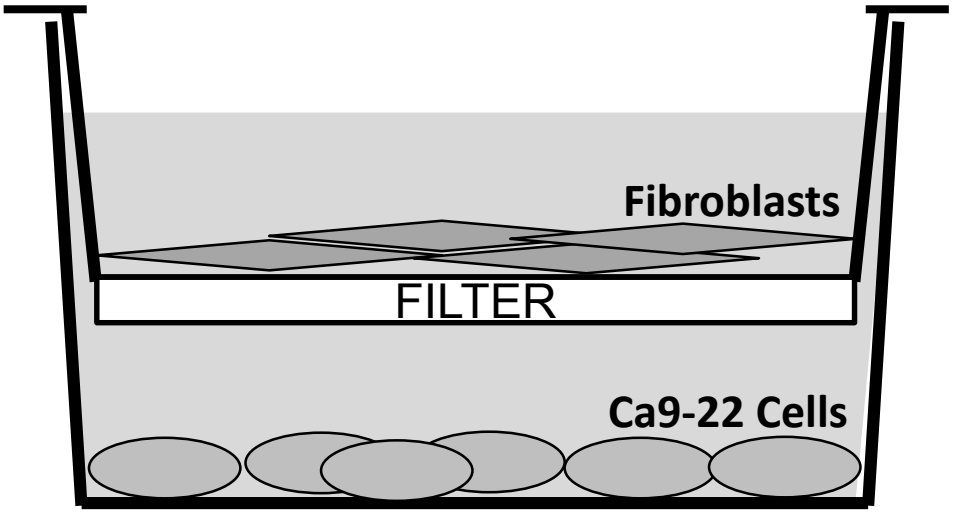

Fig.1 Illustration of co-culture system.

Synchronization of circadian rhythm

To synchronize circadian rhythm in cells before starting the co-culture study, we used the serum shock method as reported by Balsalobre at al[21]. Briefly, the cells were stimulated with 50% horse serum (Invitrogen) for 2 h. Then, the medium was replaced with fresh DMEM supplemented with 5% FBS (" 0 h") and incubated for up to 48 h. The cells were harvested every 4 h for RNA extraction.

RNA extraction and quantitative RT-PCR.

Total RNA was extracted from the cells using an RNeasy total RNA isolation Kit (Qiagen, Hilden, Germany) with on-column DNase I (Qiagen) digestion. One microgram of total RNA served as template for single-strand cDNA synthesis in a reaction using oligo $(dT)_{18}$ primers and M-Mulv reverse transcriptase (Invitrogen) under conditions indicated by the manufacturer. A CFX96 real-time PCR detection system (Bio-Rad) was used for quantitative assessment of mRNA for Bmal1, Clock, Cry1/2, Per1/2, Dec1/2, REV-ERBα, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The sequences of the primers were shown in Table 1.

The amplification reactions were performed with SsoAdvanced Universal SYBR Green Supermix (Bio-Rad, Hercules, CA, USA) according to the manufacturer's specifications. Amplification conditions were as follows: 2min at 50° C followed by 3min at 95° C and then 40 cycles of 15s at 95° C, 30s at 58° C, and 30s at 72° C. After amplification was completed, a melting curve was generated by heating slowly at 0.1° C per second to 95° C with continuous collection of fluorescence. Analysis of the data was performed using a CFX manager (Bio-Rad).

Result

Co-culture with Ca9-22 oral cancer cells alter expression of clock genes in fibroblasts

Following the synchronization of circadian rhythm by serum shock, the expressions of 8 clock genes in co-cultured fibroblasts with Ca9-22 oral cancer cells were analyzed. In the absence of Ca9-22 cells, rhythmic oscillations of Clock, Cry1, Cry2, Dec1, and Rev-Erbα are 20 h, 8 h, 24 h, 8 h, and 20 h, respectively (Figure 2). No rhythmic expressions of Bmal1, Per1, Per2, and Dec2 were observed in fibroblasts (data not shown). In the presence of Ca9-22 cells, the rhythmic oscillations

Table 1 Oligonucleotide primers for real-time PCR.

	Forward	Reverse
Bmal1	AAGGATGGCTGTTCAGCACATGA	CAAAAATCCATCTGCTGCCCTG
Clock	AAGTTAGGGCTGAAAGACGACG	GAACTCCGAGAAGAGGCAGAAG
Cry1	CTCCATGGGCACTGGTCTCAGTG	TCCCCACCAATTTCAGCTGCAAC
Cry2	CCAAGAGGGAAGGGCAGGGTAGA	AGGATTTGAGGCACTGTTCCGAGG
Per1	TGGCTATCCACAAGAAGATTC	GGTCAAAGGGCTGGCCCG
Per2	GGCCATCCACAAAAAGATCCTGC	GAAACCGAATGGGAGAATAGTCG
Dec1	GAAAGGATCGGCGCAATTAA	CATCATCCGAAAGCTGCATC
Dec2	CGCCCATTCAGTCCGACTT	CGGGAGAGGTATTGCAAGACTT
Rev-Erbα	AGAGCACCAGCAACATCACCAAGC	TTCTTGAAGCGACATTGCTGGCAG
GAPDH	CCACCCATGGCAAATTCCATGGCA	TCTAGACGGCAGGTCAGGTCCACC

of Clock and Rev-Erbα mRNA expression were altered from 20 h to 12 h (Figure 2A) and 14h (Figure 2E), respectively. In addition, the level of Dec1 mRNA was enhanced by the co-culture with Ca9-22 in a time-dependent manner (Figure 2D). The rhythmic expressions of Cry1/2 were disappeared in fibroblasts co-cultured with Ca9-22 cells (Figure 2B and 2C).

Expression of clock genes in Ca9-22 cells are affected by co-culture with fibroblasts

We next examined the effect of co-culture with fibroblasts on the rhythmic expressions of clock genes in Ca9-22 cells. In Ca9-22 cells, Clock, Cry1, and, Cry2 oscillated their mRNA expression (Figure 3). The rhythmic oscillation of mRNA expressions for Clock, Cry1, and Cry2 were 8h (Figure 3A, 3B, and 3C). A weak oscillation of Cry2 and Per1 mRNA expressions (Figure 3C and 3D) and little oscillation of Rev-Erbα mRNA expression (Figure 3E) were observed in Ca9-22 cells. No rhythmic expressions of Bmal1, Per2, Dec1, and Dec2 were detected in Ca9-22 cells (data not shown). In the presence of fibroblasts, the circadian interval of mRNA expressions for Clock was elongated (approximately 12h) (Figure 3A). Moreover, the co-culture with fibroblasts enhanced the rhythmic expressions of Per1 and Rev-Erbα. In contrast, the expressions of Cry1/2 were lowered and observed the very weak rhythmic oscillation in Ca9-22 cells co-cultured with fibroblasts (Figure 3B and 3C).

Discussion

In this study, we explored the effect of co-culture of cancer cells with fibroblasts originated from the oral cavity on the expressions of clock genes. Combined with our results in fibroblasts co-cultured with Ca9-22 cells and in Ca9-22 cells co-cultured with fibroblasts, we found the effect of the co-culture on the clock genes was categorized into three groups. The first group comprises clock genes whose rhythmic oscillation is altered by the co-culture. Clock, Rev-Erbα and Dec1 are categorized into this group. The rhythmic expression of Clock was influenced both in fibroblasts co-cultured with Ca-9-22 cells and Ca9-22 cells co-cultured with fibroblasts. These results suggest that oral cancer cells and fibroblasts interact and regulate the rhythmic expression of Clock. This might partly explain the essential role of tumor-stromal interactions. Expression of Rev-Erbα is controlled by the components of clock genes[22]. Co-culture with fibroblasts and Ca9-22 cells clearly shortened the circadian expression of Rev-Erbα. Furthermore, the co-culture synchronized the rhythmic expression of Rev-

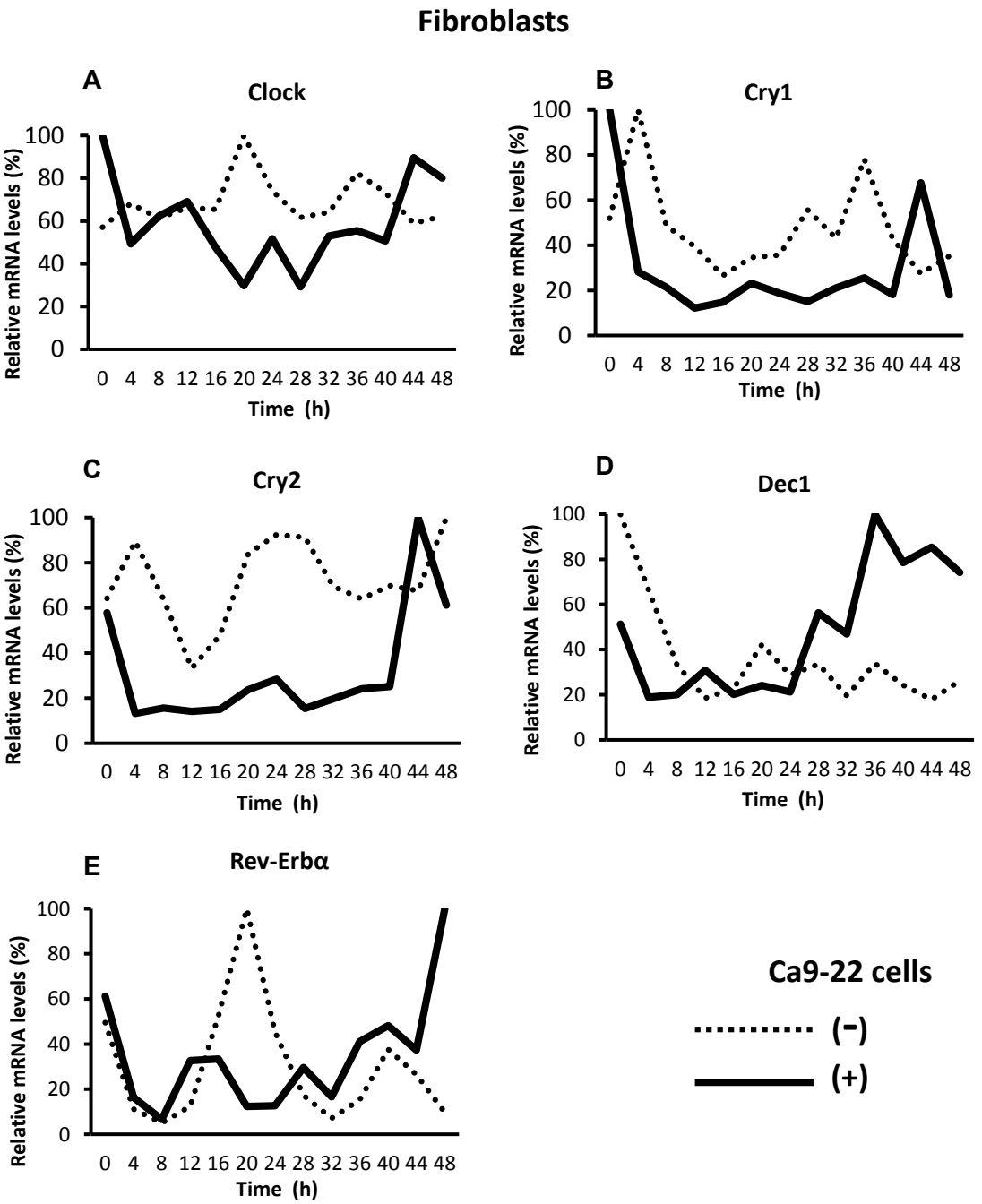

Fig.2 Effect of co-culture with Ca9-22 cells on the expression of clock genes in fibroblasts. Fibroblasts were cultured in semipermeable membrane dish in the absence (dot line) or the presence (solid line) of Ca9-22 cells. The expressions of mRNA for clock genes and GAPDH are analyzed by PCR. Peak expression during each time-course set to 100%. Data represent the average of three measurements.

Erbα in both fibroblasts and Ca9-22 cells. These suggested the importance of stromal fibroblasts to regulate circadian rhythm in tumor biology. The rhythmic expression of Dec1 was also altered in fibroblasts in the presence of Ca9-22 cells. Overexpression of Dec1 in tumor tissues was found in various tumors[23-25]. Since Dec1 is a regulator of the mammalian molecular clock, alternation of Dec expression may modify the expressions of clock-regulated genes.

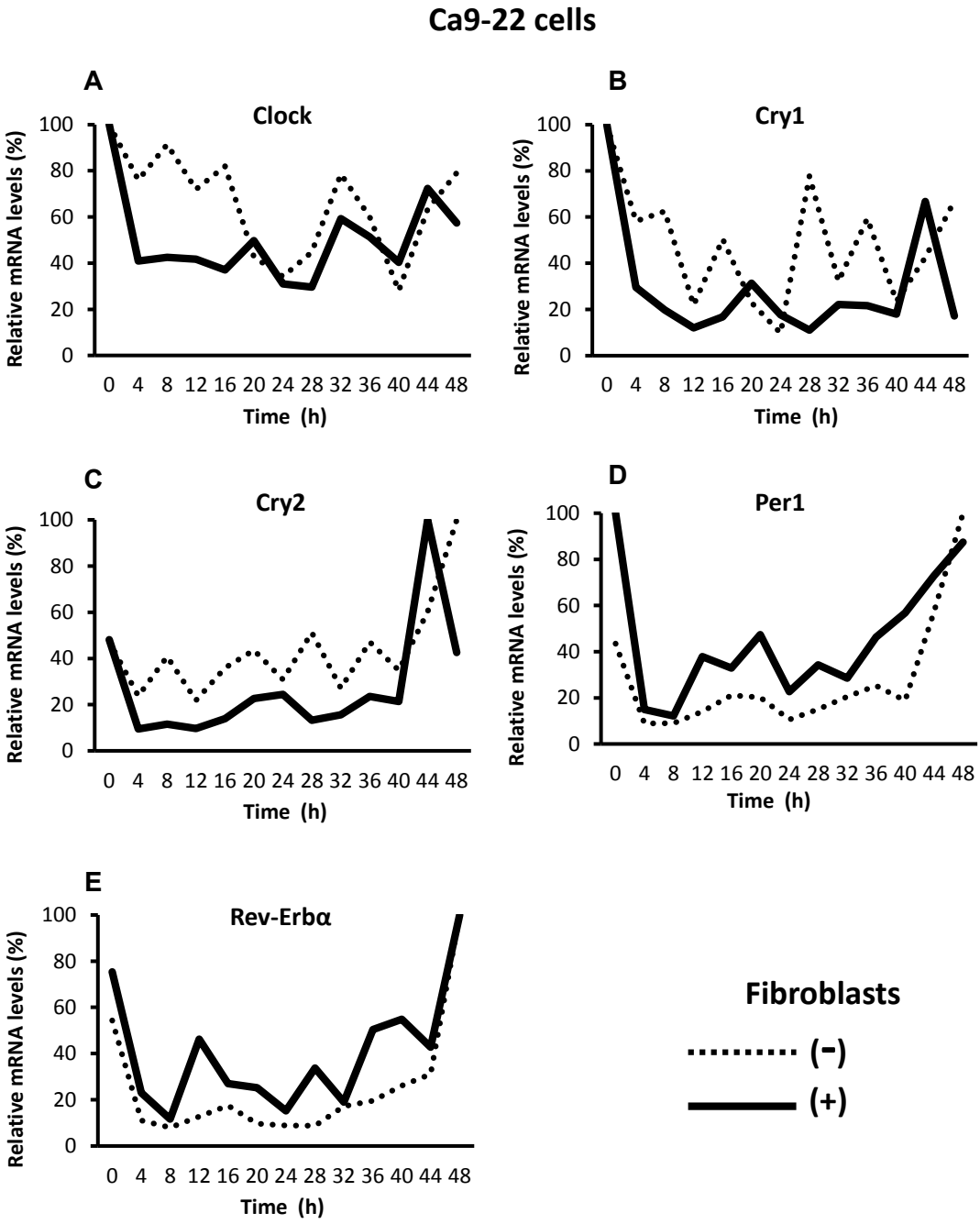

Fig.3 Effect of co-culture with fibroblasts on the expression of clock genes in Ca9-22 cells. Cell culture condition is described in Figure 1. The expressions of mRNA for clock genes and GAPDH are analyzed by PCR. Peak expression during each time-course set to 100%. Data represent the average of three measurements.

The second group consists of clock genes which are rhythmically oscillated their expression by co-culture. Per1 is categorized in this group. In addition, Rev-Erbα also belongs to this group as well as to the first group. Disruption of circadian rhythm is known to associate cancer [26]. A part of this may be explained by the dysrhythmic expression of a clock gene. For example, dysregulation of Per2, which has tumor suppressor properties, is involved in human

colorectal and breast cancers[27]. In our results, the expressions of Per1 and Rev-Erbα did not exhibit the circadian rhythms in Ca9-22 cells. However, co-culture with fibroblasts markedly enhanced the rhythmic expression of Per1 and Rev-Erbα. On the other hand, the expression levels of Rev-Erbα and Per1 (data not shown) were unchanged in fibroblasts co-cultured with Ca9-22 cells. These suggest that there is the direction to regulate clock genes between tumor cells and stromal cells.

The rhythmic expression of clock genes is disappeared by co-culture in the third group. Cry1/2 are such clock genes. Co-culture with fibroblasts and Ca9-22 cells dissipated the rhythmic expressions of Cry1/2 in both fibroblasts and Ca9-22 cells. Although how such effect is exerted is unclear, co-culture of fibroblasts with Ca9-22 cells interact the expression of Cry1 and Cry2 each other. Note that the fibroblasts and cancer cells do not contact directly in the co-culture system used in the present study. Therefore, some humoral factors secreted from one type of cell may affect the circadian expression of the clock genes in another cells type. Although we have unidentified the factors in this study, it should be clarified in future studies.

In conclusion, we found that co-culture of fibroblasts with oral cancer cells alter the circadian expressions of clock genes in an individual gene-specific manner. Molecular mechanisms of which co-culture affects such alternation of the rhythmic expressions of clock genes remain unclear. In addition, we did not performed co-culture of oral cancer cells with other constituent cells in tumor microenvironment such as smooth muscle cells or vascular endothelial cells. Nevertheless our results may provide a certain role of interaction within tumor microenvironment in the circadian expression of the clock genes in oral cancer.

Acknowledgement

This work was supported by JSPS KAKENHI Grant Numbers 25861767 (K.F) and 26460567 (T.M.); a Hirosaki University Institutional Research Grant (T.I.); and a Hirosaki University Institutional Research Grant for Young Scientists (T.M.).

Reference

1) Moore R, editor Organization and function of a central nervous system circadian oscillator: the suprachiasmatic hypothalamic nucleus. Federation proceedings; 1983.

2) Reppert SM, Weaver DR. Molecular analysis of mammalian circadian rhythms. Annual review of physiology. 2001;63:647-76.

3) Gómez - Santos C, Gómez - Abellán P, Madrid JA, Hernández - Morante JJ, Lujan JA, Ordovas JM, et al. Circadian rhythm of clock genes in human adipose explants. Obesity. 2009;17:1481-5.

4) Jin X, Shearman LP, Weaver DR, Zylka MJ, De Vries GJ, Reppert SM. A molecular mechanism regulating rhythmic output from the suprachiasmatic circadian clock. Cell. 1999;96:57-68.

5) Van Der Horst GT, Muijtjens M, Kobayashi K, Takano R, Kanno S-i, Takao M, et al. Mammalian Cry1 and Cry2 are essential for maintenance of circadian rhythms. Nature. 1999;398:627-30.

6) Shearman LP, Sriram S, Weaver DR, Maywood ES, Chaves I, Zheng B, et al. Interacting molecular loops in the mammalian circadian clock. Science. 2000;288:1013-9.

7) Honma S, Kawamoto T, Takagi Y, Fujimoto K, Sato F, Noshiro M, et al. Dec1 and Dec2 are regulators of the mammalian molecular clock. Nature. 2002;419:841-4.

8) Krugluger W, Brandstaetter A, Kállay E, Schueller J, Krexner E, Kriwanek S, et al. Regulation of genes of the circadian clock in human colon cancer: reduced period-1 and dihydropyrimidine dehydrogenase transcription correlates in high-grade tumors. Cancer research. 2007;67:7917-22.

9) Sahar S, Sassone-Corsi P. Metabolism and cancer: the circadian clock connection. Nature Reviews Cancer. 2009;9:886-96.

10) Stevens RG. Circadian disruption and breast cancer: from melatonin to clock genes. Epidemiology. 2005;16:254-8.

11) Hua H, Wang Y, Wan C, Liu Y, Zhu B, Yang C, et al. Circadian gene mPer2 overexpression induces cancer cell apoptosis. Cancer science. 2006;97:589-96.

12) Wood PA, Yang X, Hrushesky WJ. Clock genes and cancer. Integrative cancer therapies. 2009;8:303-8.

13) Sato F, Wu Y, Bhawal UK, Liu Y, Imaizumi T, Morohashi S, et al. PERIOD1 (PER1) has anti-apoptotic effects, and PER3 has pro-apoptotic effects during cisplatin (CDDP) treatment in human gingival cancer CA9-22 cells. Eur J Cancer. 2011 Jul;47:1747-58..

14) Wu Y, Sato F, Bhawal UK, Kawamoto T, Fujimoto K, Noshiro M, et al. Basic helix-loop-helix transcription factors DEC1 and DEC2 regulate the paclitaxel-induced apoptotic pathway of MCF-7 human breast cancer cells. Int J Mol Med. 2011 Apr;27:491-5.

15) Olumi AF, Grossfeld GD, Hayward SW, Carroll PR, Tlsty TD, Cunha GR. Carcinoma-associated fibroblasts direct tumor progression of initiated human prostatic epithelium. Cancer research. 1999;59:5002-11.

16) Kalluri R, Zeisberg M. Fibroblasts in cancer. Nature Reviews Cancer. 2006;6:392-401.

17) Xing F, Saidou J, Watabe K. Cancer associated fibroblasts (CAFs) in tumor microenvironment. Frontiers in bioscience: a journal and virtual library. 2010;15:166.

18) Zeisberg EM, Potenta S, Xie L, Zeisberg M, Kalluri R. Discovery of endothelial to mesenchymal transition as a source for carcinoma-associated fibroblasts. Cancer research. 2007;67:10123-8.

19) Zeisberg EM, Tarnavski O, Zeisberg M, Dorfman AL, McMullen JR, Gustafsson E, et al. Endothelial-to-mesenchymal transition contributes to cardiac fibrosis. Nature medicine. 2007;13:952-61.

20) Gaggioli C, Hooper S, Hidalgo-Carcedo C, Grosse R, Marshall JF, Harrington K, et al. Fibroblast-led collective invasion of carcinoma cells with differing roles for RhoGTPases in leading and following cells. Nature cell biology. 2007;9:1392-400.

21) Balsalobre A, Damiola F, Schibler U. A serum shock induces circadian gene expression in mammalian tissue culture cells. Cell. 1998;93:929-37.

22) Preitner N, Damiola F, Lopez-Molina L, Zakany J, Duboule D, Albrecht U, et al. The orphan nuclear receptor REV-ERBalpha controls circadian transcription within the positive limb of the mammalian circadian oscillator. Cell. 2002;110:251-60.

23) Bhawal UK, Sato F, Arakawa Y, Fujimoto K, Kawamoto T, Tanimoto K, et al. Basic helix-loop-helix transcription factor DEC1 negatively regulates cyclin D1. J Pathol. 2011;224:420-9.

24) Chakrabarti J, Turley H, Campo L, Han C, Harris AL, Gatter KC, et al. The transcription factor DEC1 (stra13, SHARP2) is associated with the hypoxic response and high tumour grade in human breast cancers. Br J Cancer. 2004;91:954-8.

25) Li Y, Zhang H, Xie M, Hu M, Ge S, Yang D, et al. Abundant expression of Dec1/stra13/sharp2 in colon carcinoma: its antagonizing role in serum deprivation-induced apoptosis and selective inhibition of procaspase activation. Biochem J. 2002;367:413-22.

26) Viswanathan AN, Hankinson SE, Schernhammer ES. Night shift work and the risk of endometrial cancer. Cancer Res. 2007;67:10618-22.

27) Sjoblom T, Jones S, Wood LD, Parsons DW, Lin J, Barber TD, et al. The consensus coding sequences of human breast and colorectal cancers. Science. 2006 13;314:268-74.