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授与した学位	博　士
専攻分野の名称	歯　学
学位授与番号	博甲第６４８３号
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学位授与の要件	医歯薬学総合研究科機能再生・再建科学専攻 （学位規則第４条第１項該当）
学位論文の題目	Circadian production of melatonin in cartilage modifies rhythmic gene expression. （軟骨組織におけるメラトニンの概日リズムは、軟骨細胞の遺伝子発現リズムと代謝に影響する）
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学位論文内容の要旨

Introduction The Circadian system is an intrinsic clock regulating multiple physiological processes in mammals. The circadian system is orchestrated by a master clock in the suprachiamatic nucleus (SCN) of the hypothalamus, which in turn controls secondary self-sustained autonomous circadian clocks present in most peripheral tissues. Accumulating evidence suggested a critical role of circadian rhythm in controlling cartilage biology. Melatonin is the key regulator of circadian rhythms and is known as a sleep hormone synthesized mainly in pineal gland. Melatonin levels tend to decrease significantly with advancing age with disruptions of circadian rhythm, while increasing number of patients with cartilage destruction such as osteoarthritis observed with aging. Given that cartilage is an avascular and aneural tissue, the role of melatonin in regulating the cartilage circadian clock and maintaining cartilage homeostasis remains largely unknown and thus is worth investigating.

Methods and Results In our study, we explored the effects of melatonin on metabolism and rhythmic gene expression in chondrocytes in mice firstly. Using mouse epiphyseal cartilage and primary cultured chondrocytes, expression of melatonin receptors *Mt1* (MTNR1A, melatonin receptor 1A) and *Mt2* (MTNR1B, melatonin receptor 1B) as well as melatonin-synthesizing enzyme *Aanat* (arylalkylamine N-acetyltransferase) and *Hiomt* (hydroxyindole O-methyltransferase) mRNA was detected. Production of melatonin in chondrocytes was confirmed by mass spectrometry analysis. Addition of melatonin to cultured chondrocytes enhanced cell proliferation and increased expression of *Col2a1* (collagen type II alpha 1 chain), a maker of chondrocytes, *Acan* (aggrecan), another maker of chondrocytes and *Sox9* (SRY, sex determining region Y-box 9), an inhibitor of hypertrophy, but repressed *Col10a1* (collagen type X alpha 1 chain), a marker of hypertrophy. Addition of luzindole, an MT1 and MT2 antagonist, abolished these effects. Kinetic analysis showed that melatonin caused rapid upregulation of *Aanat, Mt1, Mt2,* followed by *Sox9* and *Ihh* (Indian hedgehog). Furthermore, expression of the clock gene *Bmal1* (aryl hydrocarbon receptor nuclear translocator-like) was induced, while that of *Per1* (period circadian clock 1) was downregulated. Chronobiological analysis of gene expression using RNA collected from cultured chondrocytes every 4 hours for approximately 2 days revealed addition of melatonin induced cyclic expression of *Aanat* with a period around 24 hours and adjusted the cyclic rhythm of *Bmal1* close to 24h period from 17h. Rhythmic expression of *Mt1* and *Mt2* was also modified by melatonin with periods around 17h, which were different from those of *Bmal1* and *Aanat*.

Furthermore, human articular chondrocytes were collected from 7 to 82 years old patients who underwent joint replacement or osteosarcoma surgery. Negative correlation between age and the expression level of *AANAT* and *BMAL1* was observed. Expression of *AANAT* and *BMAL1* mRNA was rapidly enhanced by the addition of melatonin to human articular chondrocytes from relative younger donors, however, samples from aged donors (above 80) showed no significant induction in the expression of these genes. Chronobiological analysis of gene expression confirmed that *BMAL1* and *PER1* showed rhythmic gene expression in all the samples of articular chondrocytes, but melatonin

addition did not affect the period of rhythmic cycle. However, period of the rhythmic expression of *AANAT* was modified, and appeared close to 24 hours after the addition of melatonin in all ages examined.

Conclusion Our results indicate that in mouse cartilage, melatonin stimulates chondrocyte proliferation but inhibits its maturation via the MT1 and MT2 receptors. Melatonin addition induced the circadian production of melatonin in chondrocytes. Melatonin changed clock the expression of clock genes, indicating that clock gene can be a key regulator of melatonin-induced rhythmic gene expression. *Bmal1* performed peripheral rhythm in the presence of endogenous melatonin, and exogenous melatonin could adjust it to the central rhythm in mice. *Mt1* and *Mt2* showed different rhythmic pattern by melatonin addition, indicating that their rhythmic expression is regulated by different regulators other than clock genes. In human articular chondrocytes melatonin increased expression of *AANAT* and *BMAL1* in relatively young human articular chondrocytes, but not in the elder. However, unlike the observation in mice, melatonin is uncapable of affecting rhythmic expression pattern of *BMAL1* and PER1 in human cells; however, it adjusts *AANAT* rhythmic expression to a cycle of 24hours both in relatively young and elder cells, indicating that melatonin maintains the tissue function even in elder articular cartilage.

論文審査結果の要旨

Objective The circadian system is an intrinsic clock regulating multiple physiological processes in mammals. Melatonin levels tend to decrease significantly with advancing age with disruptions of circadian rhythm, while increasing number of patients with cartilage destruction such as osteoarthritis observed with aging. Given that cartilage is an avascular and aneural tissue, the role of melatonin in regulating the cartilage circadian clock and maintaining cartilage homeostasis remains largely unknown and thus is worth investigating.

Methods and Results In our study, we firstly explored the effects of melatonin on metabolism and rhythmic gene expression in chondrocytes in mice. Using mouse epiphyseal cartilage and primary cultured chondrocytes, expression of melatonin receptors *Mt1* (MTNR1A, melatonin receptor 1A) and *Mt2* (MTNR1B, melatonin receptor 1B) as well as melatonin-synthesizing enzyme *Aanat* (arylalkylamine N-acetyltransferase) and *Hiomt* (hydroxyindole O-methyltransferase) mRNA was detected. Production of melatonin in chondrocytes was confirmed by mass spectrometry analysis. Addition of melatonin to cultured chondrocytes enhanced cell proliferation and increased expression of *Col2a1* (collagen type II alpha 1 chain), a maker of chondrocytes, *Acan* (aggrecan), another maker of chondrocytes and *Sox9* (SRY, sex determining region Y-box 9), an inhibitor of hypertrophy, but repressed *Col10a1* (collagen type X alpha 1 chain), a marker of hypertrophy. Addition of luzindole, an MT1 and MT2 antagonist, abolished these effects. Kinetic analysis showed that melatonin caused rapid upregulation of *Aanat*, *Mt1*, *Mt2*, followed by *Sox9* and *Ihh* (Indian hedgehog). Furthermore, expression of the clock gene *Bmal1* (aryl hydrocarbon receptor nuclear translocator-like) was induced, while that of *Per1* (period circadian clock 1) was downregulated. Chronobiological analysis of gene expression using RNA collected from cultured chondrocytes every 4 hours for approximately 2 days revealed addition of melatonin induced cyclic expression of *Aanat* with a period around 24 hours and adjusted the cyclic rhythm of Bmal1 close to 24h period from 17h. Rhythmic expression of *Mt1* and *Mt2* was also modified by melatonin with periods around 17h, which were different from those of *Bmal1* and *Aanat*.

Conclusion In mouse cartilage, melatonin stimulates chondrocyte proliferation but inhibits its maturation. Chondrocytes could produce melatonin with their own rhythm and exogenous melatonin could adjust it to the central rhythm. In relatively younger human articular chondrocyte, melatonin increased expression of *Aanat* and *Bmal1*. Melatonin could not affect rhythmic expression pattern of *Bmal1* and *Per1*, however it could adjust Aanat pattern to the central rhythm both in relatively younger and aged cells which indicates melatonin may be able to keep the cartilage active even in aged articular cartilage.

The content of this doctoral dissertation covered the article, “Circadian production of melatonin in cartilage modifies rhythmic gene expression.” (https://doi.org/10.1530/JOE-19-0022) which was published in the Journal of endocrinology after the international peer-review. These findings are scientifically significant, providing useful knowledge that will promote the advance in cartilage science. Therefore, the thesis defense committee hereby accept this article as a doctoral dissertation in dentistry.