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Histological analysis of a Becker muscular dystrophy case, diurnal expression of dystrophin in control mice and decreased expression of dystrophin in *Bmal1* knockout mice

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Summary. Becker muscular dystrophy (BMD) is a hereditary disease characterized by dystrophin deletion that consequently induces muscle weakness, cardiac hypertrophy and cardiac failure; These conditions are similar to those in Duchenne muscular dystrophy. The circadian rhythm is a physiological phenomenon that is predominantly regulated by the transcription and translation of clock genes. *Bmal1* (*Brain and muscle Arnt-like protein 1*) is one of the core clock genes, and its deficiency disturbs the circadian rhythm, results in cardiac hypertrophy and cardiac failure. Dystrophin expression under diurnal conditions and in *Bmal1* deficiency is yet to be elucidated. In this study, we analyzed the heart and lungs sampled during a BMD autopsy. Macroscopical examination revealed a large heart and dilated cardiomyopathy. Microscopical examination revealed an undulated structure, as well as the degeneration, and necrosis of myocardial cells. We also analyzed dystrophin expression in tissues obtained from human autopsies and mice. In human autopsy cases, dystrophin expression was lower in the heart with BMD compared that in the heart with non-BMD hypertrophy. In the heart and muscle of control mice, dystrophin expression was higher at ZT0 than at ZT12. The dystrophin expression was found to be lower in heart-specific *Bmal1* knockout mice compared to that in the control mice. Hence, our study indicated that BMD was closely associated with cardiac hypertrophy and cardiac failure, while dystrophin had a diurnal expression pattern in control mice that was regulated by *Bmal1*.

Key words: Dystrophin, Becker muscular dystrophy, Bmal1, Immunohistochemistry

Introduction

Dystrophin is one of the largest genes with 79 exons-located on the X chromosome (Muntoni et al., 2003). Becker muscular dystrophy (BMD) is a hereditary disease with high/low dystrophin deletion frequency (Bushby et al., 1993). The most common deletion has been observed in exons 45-47 (Bushby et al., 1993). Reduced dystrophin expressions lead to muscle weakness and damage by mechanical stress (van den Bergen et al., 2014). BMD exhibits a milder and slower clinical progression such as cardiac involvement than that by Duchenne muscular dystrophy (DMD) (Kang et al., 2016). Cardiomyopathy and ventricular dilation occur in approximately 70% of patients with BMD, which subsequently results in heart failure (Flanigan, 2014; Chen et al., 2018). The American Pediatric Association (2005) recommends cardiac functional assessment in BMD and DMD. Microscopic analysis has revealed that BMD patients exhibit hypertrophy and necrotic myocytes (Chun et al., 2012).

Bmal1 (*Brain and muscle Arnt-like protein 1*) is one of the clock genes, and its deficiency or abnormality

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Abbreviations. BMD, Becker muscular dystrophy; DMD, Duchenne muscular dystrophy; Bmal1, Brain and muscle Arnt-like protein 1; ZT, zeitgeber time

leads to disturbances in circadian rhythm, insulin resistance, metabolic syndrome and cardiac failure (Nakao et al., 2018; Sato et al., 2018). In our previous study *Bmal1* exhibited circadian expression in mouse heart (Sato et al., 2017). *Bmal1* knockout (KO) mice showed increased expression levels of the inflammatory and cell cycle regulators p21, S100, CD206, and tumor necrosis factor-α (Sato et al., 2017). In addition, we demonstrated that a heart-specific *Bmal1* KO in mice increased heart size and weight, and resulted in cardiac failure (Kohsaka et al., 2014). Thus, *Bmal1* plays an important role in maintaining heart function and morphology. It has recently been reported that the loss of *Bmal1* induces muscle damage and dystrophic disease progression in DMD (Gao et al., 2020). This suggests that *Bmal1* deficiency may be associated with dystrophin regulation. In this study, we conducted immunohistochemical analysis of the BMD autopsy tissue. In addition, we examined diurnal variation in dystrophin expression in control mice and dystrophin expression in heart-specific *Bmal1* KO mice.

Materials and methods

Tissue preparation

Histological specimens of one BMD case were retrieved from the archives of the Shingu Municipal Medical Center according to the guidelines of the Japanese Society of Pathology. This study was approved by the Wakayama Medical University Research Committee and Shingu Municipal Medical Center Ethics Committee. Clinical and histological information of a 32-year-old man revealed deletion of dystrophin exons 45-48 at the age of 6, and he was clinically diagnosed with BMD. He had severe muscular atrophy of the femur and hip. The patient had recurrent cardiac failure since ten years of age. Although he was treated with pharmacotherapy, he did not survive. His cousin was also diagnosed with BMD but the detailed clinical information was unclear. Autopsy was performed, and the heart and lungs were sampled. We prepared the heart tissue from one myocardial infarction (MI) case and two hypertrophy cases without BMD, as previously described (Sato et al., 2017). Six-to-eight-week-old male C57BL/6 mice were housed under 12 h each of light (8AM; ZT0) and dark (8 PM; ZT12) conditions as previously described (Sato et al., 2015, 2019). We used 10 mice, and sacrificed 5 each under light and dark conditions, respectively. The mice hearts and femoral muscles were sampled at the aforementioned time points and were subjected to immunohistochemistry. Fourteen-week-old male *Bmal1*$^{flx/flx}$ and *Myh6-Cre*;*Bmal1*$^{flx/flx}$ (heart-specific *Bmal1* KO) mice were housed as previously described (Sato et al., 2017), and their heart tissues were sampled at ZT16. For analysis, 5 mice each were used.

Immunohistochemistry

Dystrophin expression in human and mouse tissues was evaluated using serially deparaffinized sections. Immunohistochemistry was performed using Discovery Auto-Stainer with automated protocols (Ventana

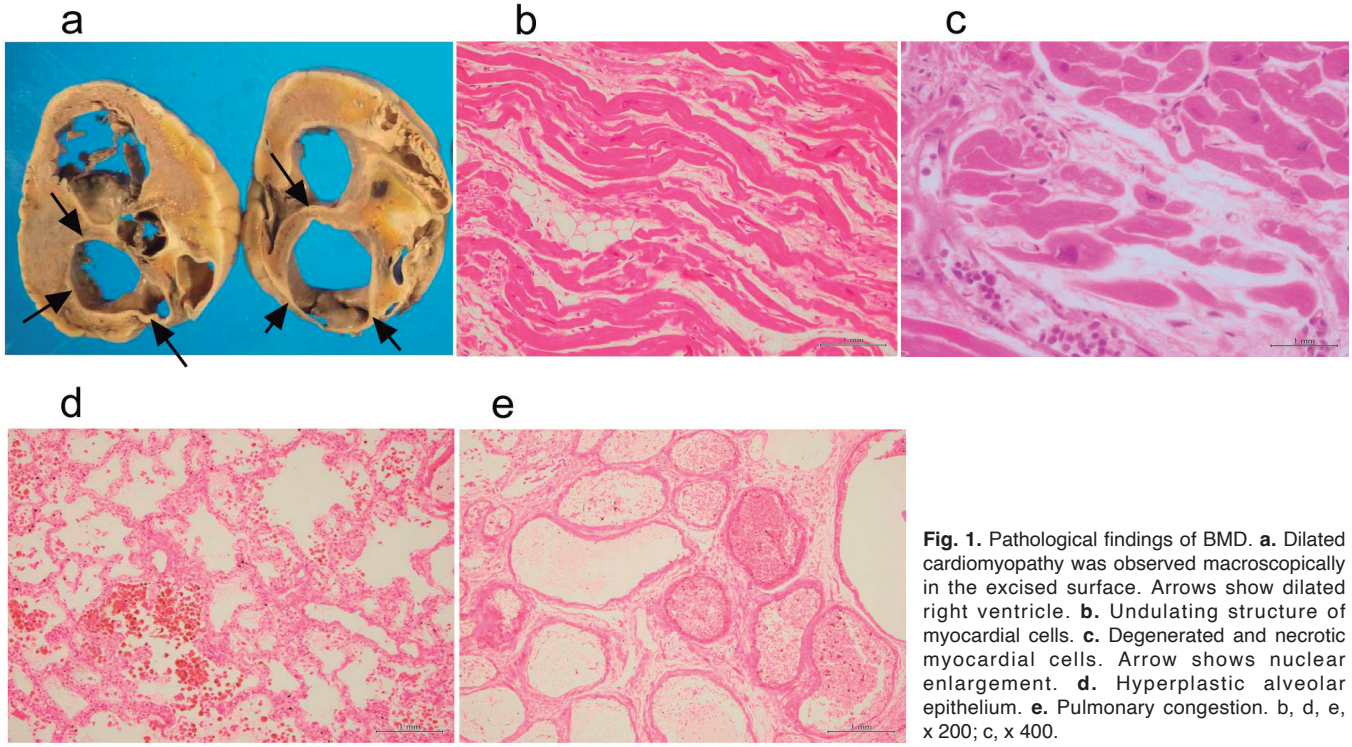

Fig. 1. Pathological findings of BMD. **a.** Dilated cardiomyopathy was observed macroscopically in the excised surface. Arrows show dilated right ventricle. **b.** Undulating structure of myocardial cells. **c.** Degenerated and necrotic myocardial cells. Arrow shows nuclear enlargement. **d.** Hyperplastic alveolar epithelium. **e.** Pulmonary congestion. b, d, e, x 200; c, x 400.

Medical Systems, Inc., Tucson, AZ, USA; Roche, Mannheim, Germany), as described previously (Le et al., 2019; Sato et al., 2019a,b). Dystrophin antibody (mouse monoclonal, sc-365954, 1:100) was purchased from Santa Cruz Biotechnology (Dallas, TX, USA).

Results

Pathological findings of the heart and lungs in BMD

The hearts and lungs of one patient with BMD were sampled via autopsy. The weights of the heart, right lung, and left lung were 590 g, 695 g, and 540 g, respectively. The heart was very large and showed dilated cardiomyopathy in macroscopic examination (Fig. 1a). Microscopic analysis revealed a marked undulated structure, degeneration and necrosis of the myocardial cells (Fig. 1b and c). Although no significant pathological findings of lungs were observed macroscopically in the lungs, focal hyperplastic alveolar epithelium and pulmonary congestion were observed on microscopic examination (Fig. 1d and e).

Dystrophin expression in the BMD heart

Dystrophin expression, when examined in BMD,

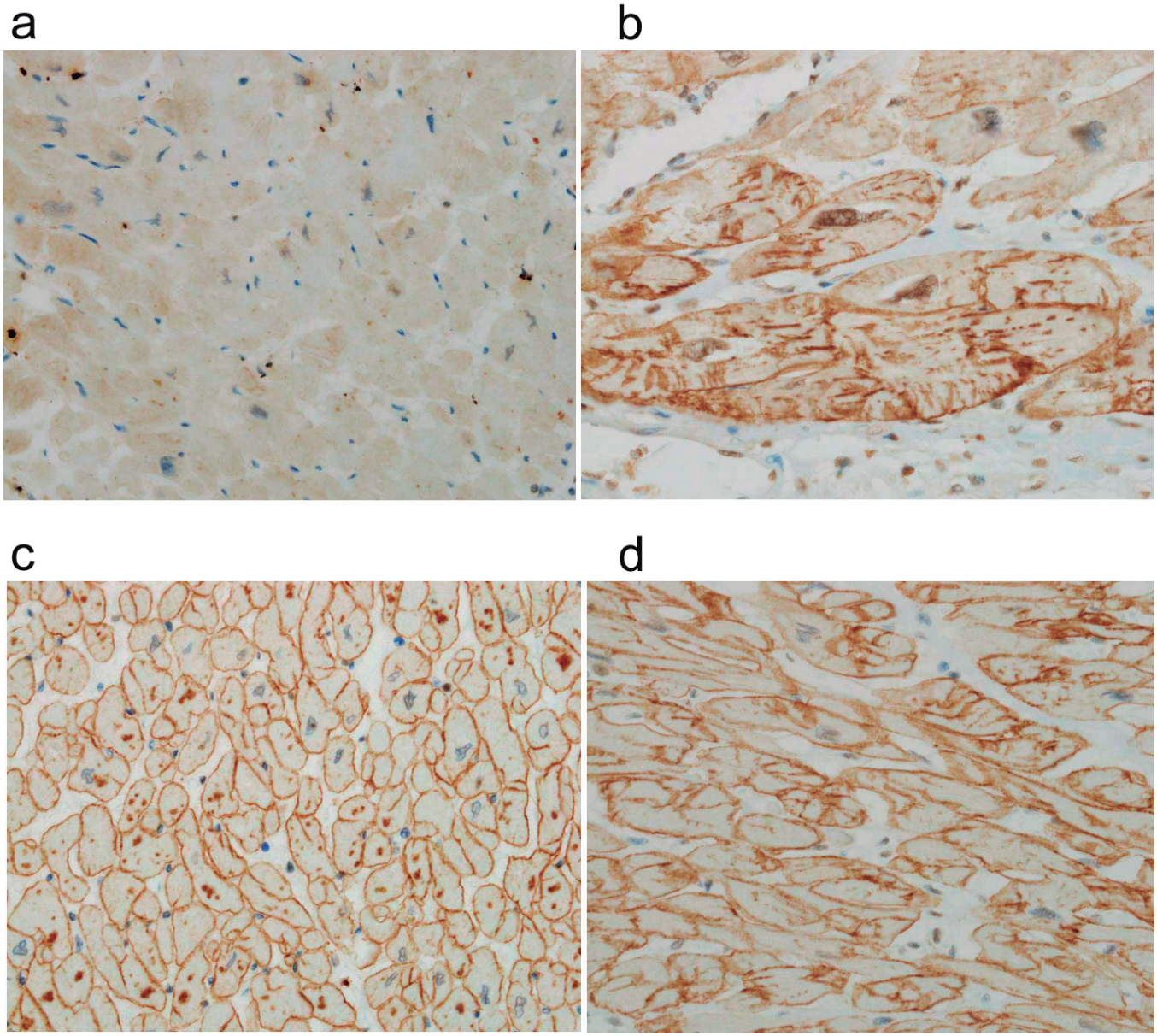

Fig. 2. Dystrophin expression decreased in heart of BMD. Dystrophin immunoreactivity in the heart of **(a)** BMD. **b.** MI. **c.** hypertrophy case 1. **d.** hypertrophy case 2. x 400.

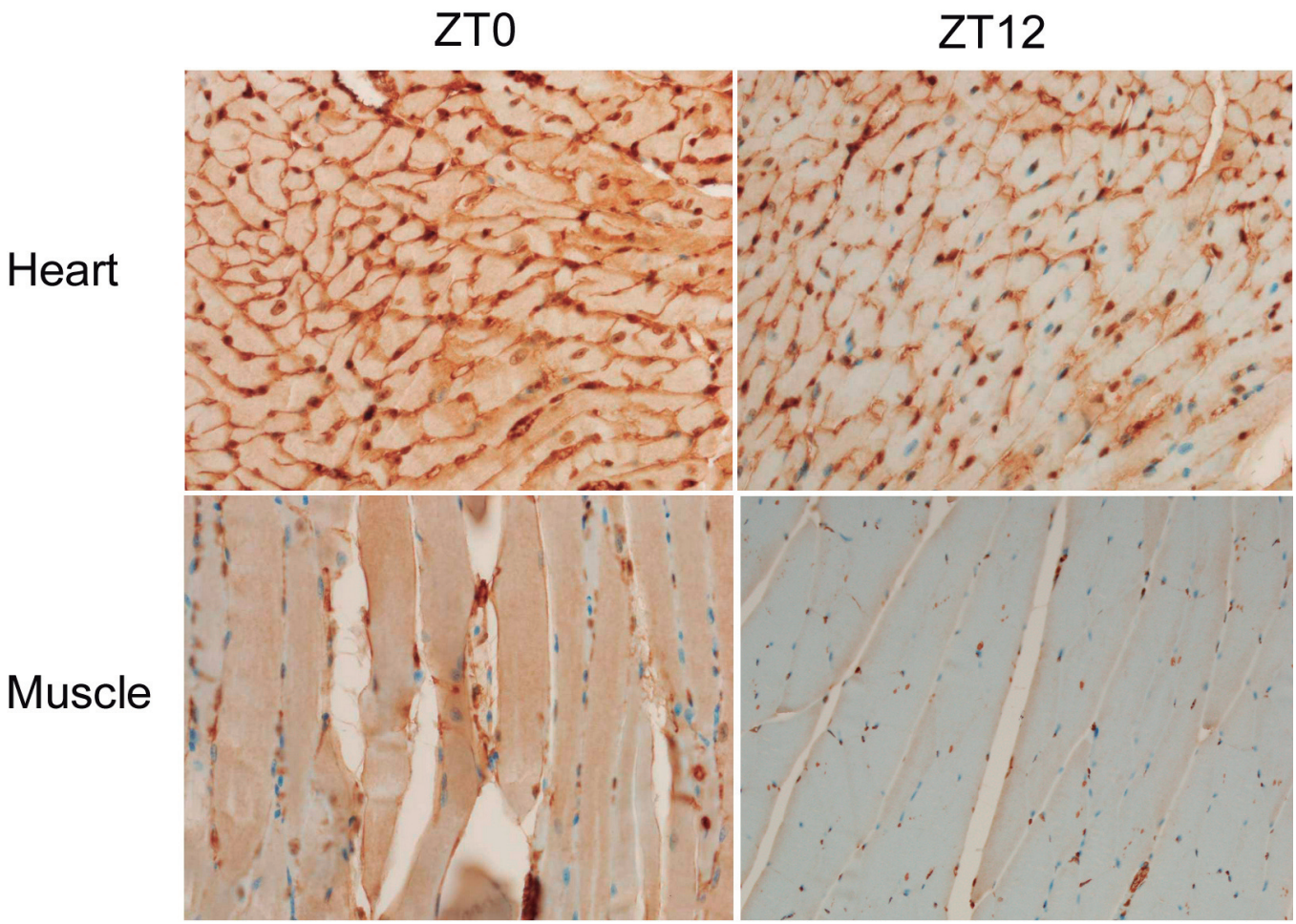

Fig. 3. Diurnal expression of dystrophin in mice heart and muscle. Dystrophin immunoreactivity in mice heart and muscle at ZT0 and at ZT12. x 400.

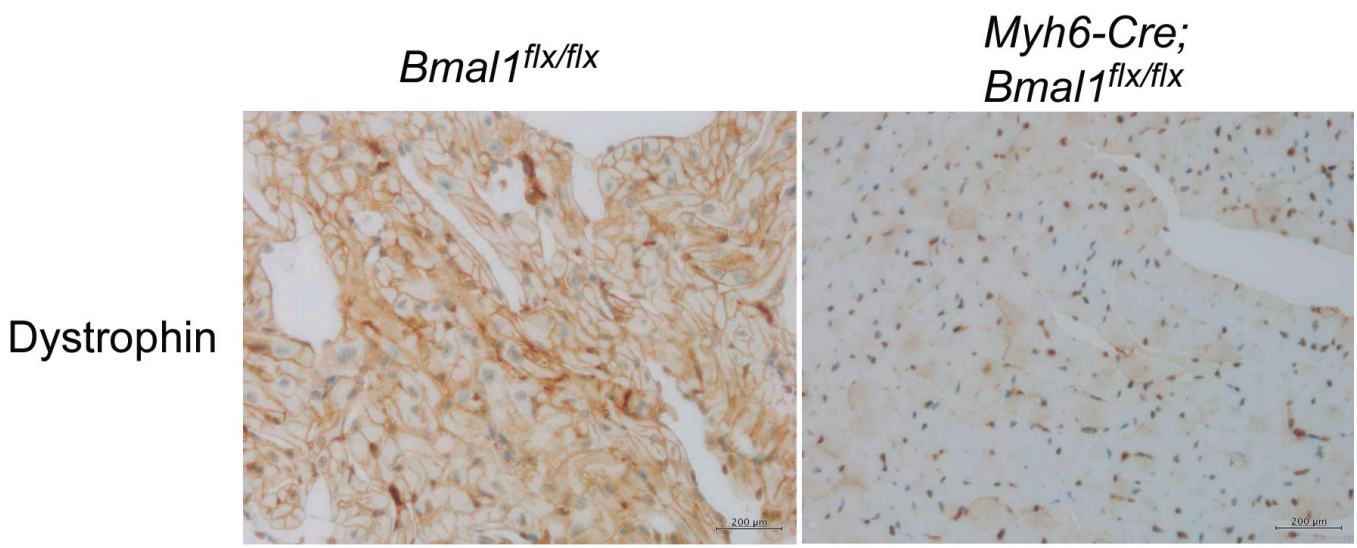

Fig. 4. Dystrophin expression decreased in the heart of *Bmal1* knockout mice. Dystrophin expression in the heart of *Bmal1*$^{flx/flx}$ and *Myh6-Cre;Bmal1* $^{flx/flx}$. x 400.

MI, and hypertrophic hearts by immunohistochemistry, was found to be markedly lower in BMD than that in the membrane of myocardial cells in MI and hypertrophy (Fig. 2).

Diurnal variation in dystrophin expression in mouse heart and muscle

Subsequent immunohistochemical examination of the diurnal variation of dystrophin expression in the mouse tissues revealed higher dystrophin expression at ZT0 than that at ZT12 (Fig. 3).

Dystrophin expression in the heart of Bmal1 KO mice

Additionally, we assessed the dystrophin expression in *Myh6-Cre*;*Bmal1*$^{flx/flx}$ heart, which revealed reduced expression in *Myh6-Cre*;*Bmal1*$^{flx/flx}$ mice than that in control *Bmal1*$^{flx/flx}$ mice (Fig. 4).

Discussion

Macroscopic examination revealed right ventricular dilation and left ventricular hypertrophy. In addition, microscopic examination revealed the undulating structure of myocardial cells, degenerated necrotic myocardial cells, and decreased dystrophin protein expression. These abnormalities may induce ventricular fibrillation and cardiac failure. These cardiac phenotypes have also been observed in BMD and DMD (Flanigan, 2014; Kang et al., 2016; Chen et al., 2018). Our pathological findings were compatible with BMD. The lung weight was within the normal range, and no obvious thrombus or severe inflammation was observed. Therefore, we speculate that the histological finding of hyperplastic alveolar epithelium and pulmonary congestion is a non-specific phenotype.

Interestingly, dystrophin expression in both the heart and muscle was higher at ZT0 than at ZT12. We previously reported that *Bmal1* expression in the heart was higher at ZT0 than at ZT12 and revealed circadian expression (Sato et al., 2017). Additionally, dystrophin expression was decreased in the heart-specific *Bmal1* KO mice. These results suggest that dystrophin expression may be regulated by *Bmal1* under circadian rhythm. Circadian rhythms are predominantly regulated by the transcription and translation of clock genes (Honma et al., 2002; Sato et al., 2018). Briefly, CLOCK and BMAL1 heterodimer binds to the E-boxes in the promoter of *DEC*, *PER*, and *CRY*, and the activated protein products of DEC, PER and CRY suppresses CLOCK and BMAL1 transactivation (Honma et al., 2002; Sato et al., 2004, 2018). The E-boxes are core elements for the regulation of the circadian rhythm (Honma et al., 2002; Sato et al., 2004, 2018). To our knowledge, *dystrophin* appears to have an E-box in its promoter. We speculated that dystrophin exhibits diurnal and circadian expression through the E-box. The current study is limited and shows circadian expression of dystrophin. Further studies are needed to clarify whether CLOCK and BMAl1 induce dystrophin transcription.

Since *Bmal1* KO mice showed cardiac hypertrophy and cardiac failure (Kohsaka et al., 2014), a decrease in the BMD-associated dystrophin expression may be due to *Bmal1* deficiency. Further studies are needed to clarify the detailed molecular mechanism how *Bmal1* deficiency decreases dystrophin expression.

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Competing interests. The authors declare no conflicts of interest.

References

American Academy of Pediatrics Section on Cardiology and Cardiac Surgery (2005). Cardiovascular health supervision for individuals affected by Duchenne or Becker muscular dystrophy. Pediatrics 116, 1569-1573.

Bushby K.M., Gardner-Medwin D., Nicholson L.V., Johnson M.A., Haggerty I.D., Cleghorn N.J., Harris J.B. and Bhattacharya S.S. (1993). The clinical, genetic and dystrophin characteristics of Becker muscular dystrophy. II. Correlation of phenotype with genetic and protein abnormalities. J. Neurol. 240, 105-112.

Chen L., Ren J., Chen X., Chen K., Rao M., Zhang N., Yu W. and Song J. (2018). A novel mutation of dystrophin in a Becker muscular dystrophy family with severe cardiac involvement: From genetics to clinicopathology. Cardiovasc. Pathol. 36, 64-70.

Chun J.L., O'Brien R. and Berry S.E. (2012). Cardiac dysfunction and pathology in the dystrophin and utrophin-deficient mouse during development of dilated cardiomyopathy. Neuromuscul. Disord. 22, 368-379.

Flanigan K.M. (2014). Duchenne and Becker muscular dystrophies. Neurol. Clin. 32, 671-688.

Gao H., Xiong X., Lin Y., Chatterjee S. and Ma K. (2020). The clock regulator Bmal1 protects against muscular dystrophy. Exp. Cell Res. 397, 112348.

Honma S., Kawamoto T., Takagi Y., Fujimoto K., Sato F., Noshiro M., Kato Y. and Honma K. (2002). Dec1 and Dec2 are regulators of the mammalian molecular clock. Nature 419, 841-844.

Kang Y., Chen Y.C. and Zhang Q. (2016). Left ventricular hypertrophy: A rare cardiac involvement of Becker muscular dystrophy. Kaohsiung J. Med. Sci. 32, 533-534.

Kohsaka A., Das P., Hashimoto I., Nakao T., Deguchi Y., Gouraud S.S., Waki H., Muragaki Y. and Maeda M. (2014). The circadian clock maintains cardiac function by regulating mitochondrial metabolism in mice. PLoS One. 9, e112811.

Le H.T., Sato F., Kohsaka A., Bhawal U.K., Nakao T., Muragaki Y. and Nakata M. (2019). Dec1 deficiency suppresses cardiac perivascular fibrosis induced by transverse aortic constriction. Int. J. Mol. Sci. 20, 4967.

Muntoni F., Torelli S., and Ferlini A. (2003). Dystrophin and mutations: One gene, several proteins, multiple phenotypes. Lancet Neurol. 2, 731-740.

Nakao T., Kohsaka A., Otsuka T., Thein Z.L., Le H.T., Waki H., Gouraud S.S., Ihara H., Nakanishi M., Sato F., Muragaki Y. and Maeda M. (2018). Impact of heart-specific disruption of the circadian clock on systemic glucose metabolism in mice. Chronobiol. Int. 35, 499-

510.

Sato F., Kawamoto T., Fujimoto K., Noshiro M., Honda K.K., Honma S., Honma K. and Kato Y. (2004). Functional analysis of the basic helix-loop-helix transcription factor DEC1 in circadian regulation. Interaction with BMAL1. Eur. J. Biochem. 271, 4409-4419.

Sato F., Kohsaka A., Bhawal U.K. and Muragaki Y. (2018). Potential roles of dec and bmal1 genes in interconnecting circadian clock and energy metabolism. Int. J. Mol. Sci. 19, 781.

Sato F., Muragaki Y. and Zhang Y. (2015). DEC1 negatively regulates AMPK activity via LKB1. Biochem. Biophys. Res. Commun. 467, 711-716.

Sato F., Kohsaka A., Takahashi K., Otao S., Kitada Y., Iwasaki Y. and Muragaki Y. (2017). Smad3 and bmal1 regulate p21 and s100a4 expression in myocardial stromal fibroblasts via TNF-α. Histochem. Cell Biol. 148, 617-624.

Sato F., Otsuka T., Kohsaka A., Le H.T., Bhawal U.K. and Muragaki Y. (2019a). Smad3 suppresses epithelial cell migration and proliferation via the clock gene Dec1, which negatively regulates the expression of clock genes Dec2 and Per1. Am. J. Pathol. 189, 773-783.

Sato F., Bhawal U.K., Tojyo I., Fujita S., Murata S.I. and Muragaki Y. (2019b). Differential expression of claudin 4, occludin, SOX2 and Proliferating cell nuclear antigen between basaloid squamous cell carcinoma and squamous cell carcinoma. Mol. Med. Rep. 20, 1977-1985.

van den Bergen J.C., Wokke B.H., Janson A.A., van Duinen S.G., Hulsker M.A., Ginjaar H.B., van Deutekom J.C., Aartsma-Rus A., Kan H.E. and Verschuuren J.J. (2014). Dystrophin levels and clinical severity in Becker muscular dystrophy patients. J. Neurol. Neurosurg. Psychiatry 85, 747-753.

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