Journal of Biomedical Optics

SPIEDigitalLibrary.org/jbo

Necessity of angiotensin-converting enzyme-related gene for cardiac functions and longevity of *Drosophila melanogaster* assessed by optical coherence tomography

Fang-Tsu Liao
Cheng-Yi Chang
Ming-Tsan Su
Wen-Chuan Kuo

SPIE

Necessity of angiotensin-converting enzyme-related gene for cardiac functions and longevity of *Drosophila melanogaster* assessed by optical coherence tomography

Fang-Tsu Liao,[a,b] Cheng-Yi Chang,[c] Ming-Tsan Su,[a] and Wen-Chuan Kuo[c,d]
[a]National Taiwan Normal University, Department of Life Science, Taipei 106, Taiwan
[b]Jen-Teh Junior College of Medicine, Nursing and Management, Department of Medical Technology, Miaoli 356, Taiwan
[c]National Yang-Ming University, Institute of Biophotonics, Taipei 112, Taiwan
[d]National Yang-Ming University, Biophotonics and Molecular Imaging Research Center, Taipei 112, Taiwan

Abstract. Prior studies have established the necessity of an angiotensin-converting enzyme-related (ACER) gene for heart morphogenesis of *Drosophila*. Nevertheless, the physiology of ACER has yet to be comprehensively understood. Herein, we employed RNA interference to down-regulate the expression of ACER in *Drosophila's* heart and swept source optical coherence tomography to assess whether ACER is required for cardiac functions in living adult flies. Several contractile parameters of *Drosophila* heart, including the heart rate (HR), end-diastolic diameter (EDD), end-systolic diameter (ESD), percent fractional shortening (%FS), and stress-induced cardiac performance, are shown, which are age dependent. These age-dependent cardiac functions declined significantly when ACER was down-regulated. Moreover, the lifespans of ACER knock-down flies were significantly shorter than those of wild-type control flies. Thus, we posit that ACER, the *Drosophila* ortholog of mammalian angiotensin-converting enzyme 2 (ACE2), is essential for both heart physiology and longevity of animals. Since mammalian ACE2 controls many cardiovascular physiological features and is implicated in cardiomyopathies, our findings that ACER plays conserved roles in genetically tractable animals will pave the way for uncovering the genetic pathway that controls the renin-angiotensin system. [DOI: 10.1117/1.JBO.19.1.011014]

Keywords: cardiac function; *Drosophila*; optical coherence tomography.

Paper 130200SSR received Apr. 1, 2013; revised manuscript received Jun. 27, 2013; accepted for publication Jul. 26, 2013; published online Sep. 3, 2013.

1 Background

The renin-angiotensin system (RAS) is an important regulator of blood pressure homeostasis, in which the protease renin cleaves the angiotensinogen into the angiotensin I (Ang I), followed by further cleaving of the Ang I by the angiotensin-converting enzyme (ACE) into angiotensin II (Ang II), and thereby resulting in blood vessel contraction and hypertension.[1] In hypertensive cardiovascular disease, the leading cause of death worldwide, pharmacological inhibition of the ACE and Ang II receptors has been demonstrated to be beneficial for ameliorating heart impairment.[2] The discovery of the ACE2, the ACE homologue, has introduced further complexities into the canonical RAS signal cascade, as the major biologically active product of ACE2 will hydrolyze Ang II to form Ang 1–7, and thereby counterbalancE the ACE activity.[3,4] It was thus believed that the primary function of the ACE2 is to regulate the blood pressure homeostasis (reviewed in Refs. 5–7).

Recent biochemical studies revealed that the ACE2 may modulate the RAS, and thus impact the blood pressure regulation *in vitro*.[4] Several animal models have been used to explore the biological functions of the ACE2 *in vivo*,[8,9] and genetic inactivation of the ACE2 has been shown to impair the cardiac functions in mice.[8,9] Nevertheless, mutation of angiotensin-converting enzyme-related (ACER) gene, a *Drosophila* ACE2 homolog, was proven to result in a severe defect during heart morphogenesis.[8] Other than its function in heart development, ACER also plays an important role in regulating sleeping behavior, as flies lacking ACER generally experience reduced nighttime sleep and exhibit greater sleep fragmentation.[10]

As outlined above, the mammalian ACE2 regulates cardiac contractility mainly, whereas *Drosophila* ACER regulates heart development during embryogenesis.[8] Apart from this distinction, ACER also likely regulates the heart physiology in adult flies as it is expressed in the heart of *Drosophila* during development.[11] However, the issue of whether ACER regulates the physiological functions of adult flies has not been explored in depth. Here, we took advantage of a noninvasive optical coherence tomography (OCT) imaging method to assess whether ACER modulates cardiac functions in living adult flies.

OCT, introduced in 1991, is a powerful tool for obtaining noncontact and noninvasive tomographic images of biological tissues.[12] In essence, OCT entails combining a broadband light source and a Michelson interferometer with a short coherence gate. The differences in the intensities of the backscattered light from the tissue are then analyzed to generate structural imaging. With the ability to image up to 3 mm in depth and to achieve better than 15 μm in axial resolution, OCT fills the

Address all correspondence to: Ming-Tsan Su, National Taiwan Normal University, Department of Life Science, Taipei 106, Taiwan. Tel: +886 2 77346244; Fax: +886 2 2931290; E-mail: mtsu@ntnu.edu.tw, and Wen-Chuan Kuo, National Yang-Ming University, Institute of Biophotonics, Taipei 112, Taiwan. Tel: +886 2 28267950; Fax: +886 2 28235460; E-mail: wckuo@ym.edu.tw

niche between ultrasound and confocal microscopies.[12] OCT had previously been successfully utilized to obtain *in vivo* images of the living heart in *Drosophila*.[13–19] Moreover, combining the frequency domain OCT system with the recently developed frequency swept source OCT (SS-OCT) further improves the detection sensitivity, and thereby makes rapid B-mode, M-mode, and Doppler OCT imaging of *Drosophila* possible.[15–19]

In this article, we applied SS-OCT and a novel algorithm[19] to evaluate the cardiac functions of *Drosophila*. Cardiac parameters, including the HR, end-diastolic diameter (EDD), end-systolic diameter (ESD), and percent fractional shortening (%FS), were automatically calculated from a large number of heartbeat M-mode OCT records. We found that the aforementioned contractile parameters declined with age in wild-type *Drosophila*. The age-dependent physiological functions of the heart were significantly reduced in ACER knock-down flies. Additionally, down-regulation of ACER increased the stress-induced heart failure rates and decreased the lifespans in flies, suggesting that ACER is essential for both the heart physiology and the longevity of *Drosophila*.

2 Methods

2.1 *Fly Stocks and Generation of Transgenic Flies*

Two *twi-24B-gal4* lines, a mesoderm and a heart driver,[20] were obtained from the Bloomington Stock Center. All fly stocks and genetic crosses were maintained on standard yeast-glucose medium at 25°C. To generate the ACER RNA interference construct, UAS-ACER-RNAi and ACER-cDNA (LD28328) were obtained from J. L Juang of the National Health Research Institutes of Taiwan. A 523-bp DNA fragment was PCR amplified using a pair of primers, 5′-CGCGTCTAGAGTGCTGGAGGCGCGTAGGTTC-3′ and 5′-CGCGTCTAGAGTCGGCATAGGAGCGGTGACC-3′ (with the *Xba*I site underlined). The amplified DNA fragment was digested with *Xba*I, and then cloned first onto the *Avr*II site. Subsequently, the same DNA fragment was subcloned onto the *Nhe*I site of a pWIZ vector as described in Ref. 21. The orientation of the DNA construct was confirmed by restriction enzyme digestion. To generate transgenic flies, the standard germ-line transformation procedure using w^{1118} as the parental line was performed.

2.2 *RT-PCR*

RT-PCR was used to determine the silencing efficiency of the transgenic flies carrying the UAS-ACER RNAi construct. Three flies carrying transgene and w^{1118} were driven by *twi-24B-gal4*. PCR was performed using the ACER-specific primers, ACER 2-1-3′ CACAAACGGCTTCTCCGGAT and ACER 2-5′ AACTGGCTTGGTATTG, as well as rps17 primers, 5′-CGAACCAAGACGGTGAAGAAG-3′ and 5′-CCATAGAGGTAGTTCAACGTCC-3′, to assess the expression of the transgene. Phenotypic analysis of all the transgenic lines exhibited similar cardiac phenotypes. Several transgenic lines were obtained for ACER-*Ri9*, which exhibited the strongest silencing efficiency and was thus used for phenotypic analysis (Fig. 1).

2.3 *SS-OCT Measurements and Quantification of Heartbeat Parameters*

To acquire the contractile parameters of adult flies, similar protocols were adopted.[13,19] Briefly, in each experiment, two-dimensional (2-D) OCT images were first obtained in the longitudinal direction to identify the dorsal midline of the A1–A3 abdominal segments of the *Drosophila*. Then, the OCT image orientation was rotated by 90 deg, such that the conical chamber (CC) of the heart was acquired in the transverse plane. Measurements were always made in the same location in the abdominal segment. A group of 20 flies was examined with an SS-OCT system (OCM1300SS, Thorlabs Inc., Newton, New Jersey). The median wavelength of the SS-OCT system was 1310 nm with an axial resolution of ~12 μm in tissue, a total power of 10 mW, and an A-scan rate of 16 kHz. Since heart function of *Drosophila* decelerates greatly between 5 and 7 weeks of age, to assess the age-dependent heartbeat parameters, 1-, 3-, and 7-week old flies were used. It has been previously reported that anesthetic treatment induces cardiac arrhythmias in mice, and that carbon dioxide or ether affects heart performances in *Drosophila*.[22–24] To sidestep the aforementioned issues, during the image assessment, the *Drosophila* were first anesthetized with triethyl amine, and then gently immobilized on wax gel with the dorsal side facing the OCT probe. The flies were subsequently allowed to return to a fully awake state on the bench for 20 min before being subjected to OCT scanning.

Figure 2 is a representative video depicting a transverse 2-D OCT image of the CC in the heart during diastole and systole in 1-week old wild-type *Drosophila*. By acquiring in-depth scans at the midline of the CC overtime without scanning, one can obtain an M-mode image of the vertical movements of the edges of the heart (y-axis) overtime (x-axis). M-mode OCT images can provide the contraction pattern which gives us an objective assessment of heart wall motion.

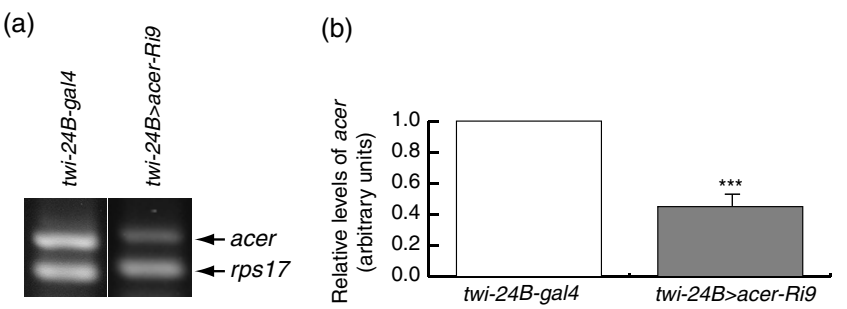

Fig. 1 (a) The expression levels of angiotensin-converting enzyme-related (ACER) mRNA in knock-down flies. (b) RT-PCR showed that the expression levels of *twi-24B*>ACER-*Ri9* were 45% of the wild-type control. Rps17 was used as a loading control. The relative expression levels were compared using Student's *t*-test; ***$P < 0.001$.

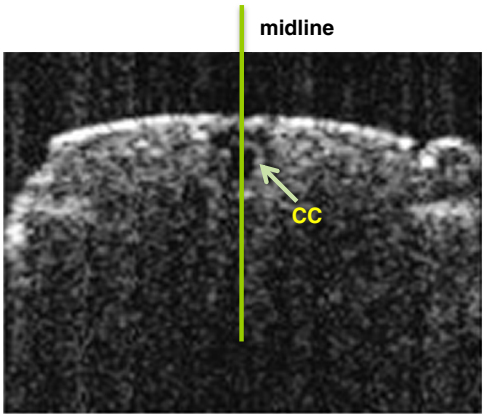

Fig. 2 A representative B-mode optical coherence tomography (OCT) in male *twi-24B-gal4/+* in 1 week of age (Video 1, MPEG, 1.97 MB) [URL: http://dx.doi.org/10.1117/1.JBO.19.1.011014.1].

We then employed a method we had previously proposed[19] for the rapid analysis of the beat-to-beat contraction–relaxation parameters of the heart in *Drosophila* by using semiautomatic cardiac chamber segmentation in B-mode OCT images based on the random walker algorithm. Random walker[25] is a semiautomatic segmentation method based on graph theory that can provide a unique solution that is robust to weak/noisy object boundaries such as the cardiac tube of *Drosophila*. Further details and proofs can be found in Refs. 19 and 26. Briefly, after contrast enhancement of the image, we created an initial set of seed points on the first frame. In the next frame, the cross-sections of the heart tube were automatically segmented in a total of 2000 frames, and the size of the inner margin was represented by the area for each *Drosophila*. Then, depending on the histogram distribution of the changing area during each heartbeat cycle, various contractile parameters, including the HR, EDD, ESD, and %FS, were determined accordingly (see Ref. 19 for details). The FS was calculated as $[(\mathrm{EDD}-\mathrm{ESD})/\mathrm{EDD}]\times 100$, which represents the extent of the changes in the cardiac diameter during systole; this can provide an estimate of the contractility of the heart tube.

2.4 *Electrical Pacing*

To monitor the stress-induced cardiac performance of adult flies, external heart pacing stress protocol was adopted and modified.[14] For each genotype, 100 flies were anesthetized with FlyNap® (Carolina Biological Supply Company, Burlington, North Carolina) for 3 min and 30 s and paced with a standard square wave stimulator at 40 V and 6 Hz for 30 s. In heart failure, we meant that either cardiac arrest or fibrillation was observed via the phase difference microscope within 2 min. The failure rate is defined as the number of flies with heart failure divided by the total number of flies.

2.5 *Survivorship Assay*

A group of 10 newly enclosed flies were collected and cultured with standard media in a vial at 25°C. Viable flies were scored and transferred to a new vial every 5 days. The log-rank test was performed to determine the differences in the lifespan of the flies.

2.6 *Statistical Analyses*

Statistical analyses were performed using SPSS (version 14.0, SPSS Inc., Chicago). P values < 0.05 were considered to be statistically significant. The relative differences between the two groups were compared using the Student's t-test; $^{***}P < 0.001$; $^{**}P < 0.01$; $^{*}P < 0.05$.

3 Results

3.1 *Down-Regulation of ACER Impaired Contractile Properties of Drosophila Heart*

Previous studies indicated that ACER is critical for the heart morphogenesis of *Drosophila*.[8] Nevertheless, the function of ACER in heart physiology has not been precisely determined. To address this question, we have utilized RNA interference to knock-down the ACER expression. The expression of the silencing construct was driven by the mesodermal- and cardial-specific *gal4* driver, *twi-24B-gal4*, using a UAS/gal4 system.[27] Quantitative RT-PCR showed that the knock-down efficiency of double-stranded ACER RNAi construct in late-fly embryos was moderate. In comparison with the case of the control *twi-24B-gal4* flies, the relative expression levels of ACER mRNA was 45% in ACER knock-down flies, *twi-24B-gal4*>ACER-*Ri9* (Fig. 1).

Having successfully knock-downed the expression of ACER, we next employed SS-OCT imaging to acquire various heartbeat parameters. As can be seen from Fig. 2, a transverse image of the conical CC of an adult *Drosophila* underneath the dorsal midline of the A1–A3 abdominal segments was obtained using SS-OCT. The cardiac chamber contracted rhythmically with occasional irregular pulsing activities (see Video 1). Since the flies did not possess red blood cells in their circulatory fluids, the heart chambers showed up as darker pixels under OCT. During heart contractions, the darker pixels, corresponding to the edges of the heart tube, crossed over to become lighter background pixels.

We found that ACER knock-down flies exhibited significantly enlarged systolic chamber dimensions with markedly impaired systolic functions as demonstrated in the B-mode images (see Fig. 3, Video 2). The representative M-mode OCT images of both wild-type control and ACER knock-down flies in 1, 3, and 7-week old flies are also shown in Fig. 4. The results for ACER knock-down *Drosophila* at

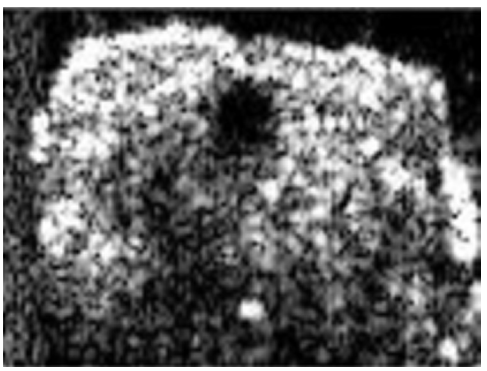

Fig. 3 A representative B-mode OCT image in ACER knock-down flies in 3 week of age (Video 2, MPEG, 1.94 MB) [URL: http://dx.doi.org/10.1117/1.JBO.19.1.011014.2].

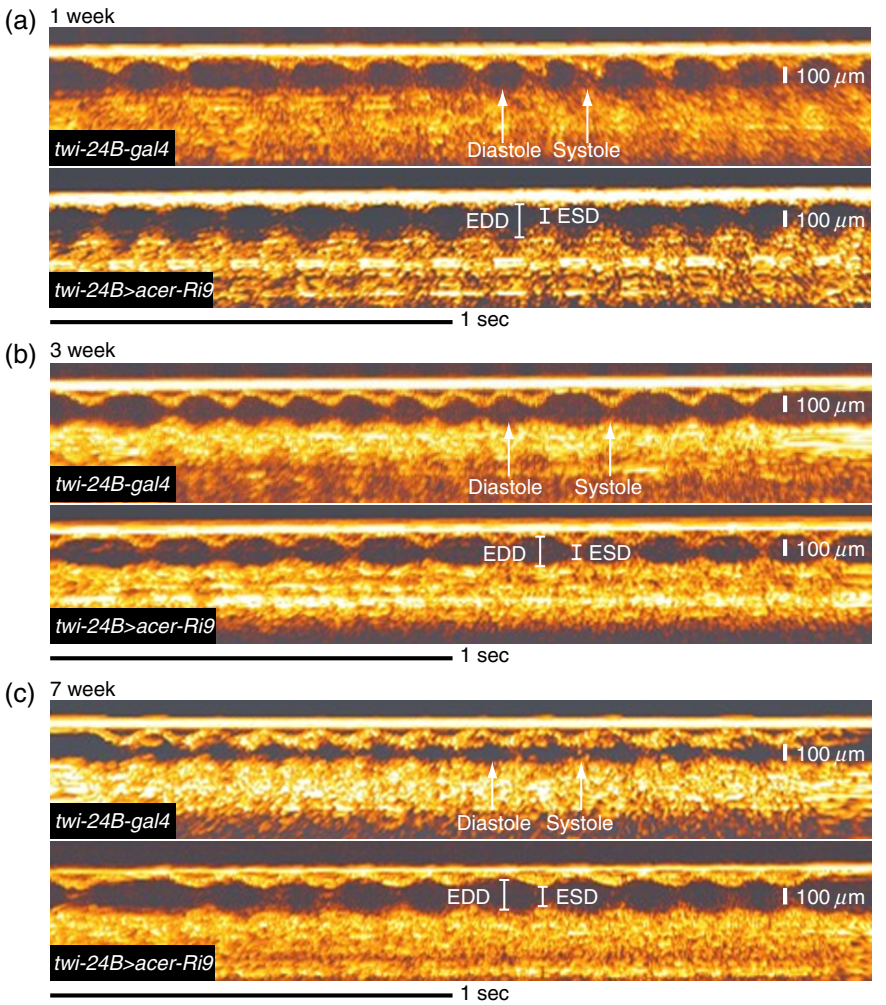

Fig. 4 Representative M-mode OCT images in wild-type control (*twi-24B-gal4/+*) and ACER silencing lines (*twi-24B*>ACER-*Ri9*) in (a) 1, (b) 3, and (c) 7 weeks of age.

different ages conclusively indicated impaired systolic functions with enlarged diastolic and systolic diameters as compared with wild-type flies.

To better assess the age-dependent contractile properties of *Drosophila*, parameters including the HR, EDD, ESD, and FS were computed from wild-type and ACER-*Ri9* flies during aging. Both wild-type and ACER-*Ri9* flies exhibited an age-dependent decline in the HR [Fig. 5(a)]. Nevertheless, the decline in HR of ACER-*Ri9* flies was consistently slower than that of wild-type flies. The differences in the HR between wild-type and ACER-*Ri9* flies were more evident than in aged animals [Fig. 5(a)]. The average HR of wild-type and ACER-*Ri9* flies were 261.86 and 242.02 bpm, respectively. In young wild-type flies, the ESD was not significantly altered. However, in 7-week old wild-type flies, ESD decreased [Fig. 5(b)]. Unlike the case of wild-type flies, the ESD of ACER knock-down flies increased with age [Fig. 5(b)]. The EDD of the wild-type flies was indistinguishable from that of the ACER-*Ri9* flies [Fig. 5(c)]. However, the EDD of both wild-type and ACER-*Ri9* flies decreased at the age of 7 weeks, and the EDD of the ACER-*Ri9* flies was significantly larger than that of the wild-type flies [Fig. 5(c)]. Moreover, we found that the FS of the wild-type flies did not decline with age [Fig. 5(d)]. In contrast, the FS of ACER-*Ri9* flies declined consistently with age and was significantly decreased compared with the wild-type flies [Fig. 5(d)]. Since the FS represents the extent of the changes in the cardiac chamber during systole and can be used as an index of cardiac contractibility, our results suggested that the down-regulation of ACER impaired the contractile properties of the *Drosophila* heart.

3.2 *Impact of Down-Regulation of ACER on Cardiac Performance of Adult Flies*

As shown above, silencing the expression of ACER reduced the contractile properties of the *Drosophila* heart (Fig. 5), and it is very likely that the deficit in ACER may also affect the heart performance in flies. To study this issue, a stress protocol was employed to test the stress-induced heart functions of the ACER knock-down flies. We found that down-regulating the ACER expression increased the likelihood of heart failure during electrical cardiac pacing in ACER knock-down flies in the ages of 1 week (chi-squared test, $P < 0.05$) and 7 weeks (chi-squared test, $P < 0.01$) (Fig. 6). Since down-regulation of ACER affected both the contractibility and the performance of the adult heart in *Drosophila*, we conclude that ACER is essential for the cardiac functions in *Drosophila* (Figs. 5 and 6).

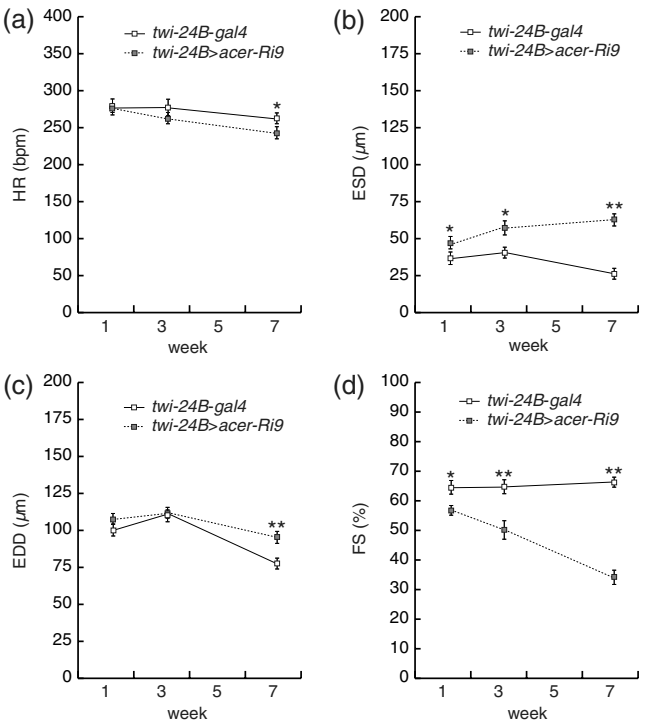

Fig. 5 Cardiac parameters in male wild-type control (*twi-24B-gal4*/+) and ACER silencing lines (*twi-24B*>ACER-*Ri9*) at 1, 3, and 7 weeks of age showing (a) heart rate (HR), (b) end-systolic diameter (ESD), (c) end-diastolic diameter (EDD), and (d) percent fractional shortening (%FS). Data points represent the mean (±SEM) for 20 flies per data point; ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$.

3.3 *Necessity of ACER for the Longevity of Drosophila*

It has been shown that modulation of the gene expression in the heart can alter the longevity of *Drosophila*.[28] As shown above, cardiac-specific down-regulation of ACER did indeed exacerbate the age-dependent cardiac functions of *Drosophila*. We posit that the down-regulation of ACER directly impacts the longevity of the flies. In a survivorship assay, we found that the

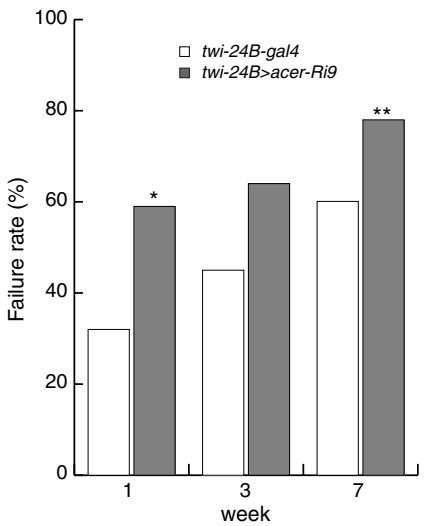

Fig. 6 Heart failure rate during pacing in both wild-type control (*twi-24B-gal4*/+) and ACER silencing lines (*twi-24B*>ACER-*Ri9*) at 1, 3, and 7 weeks of age; ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$.

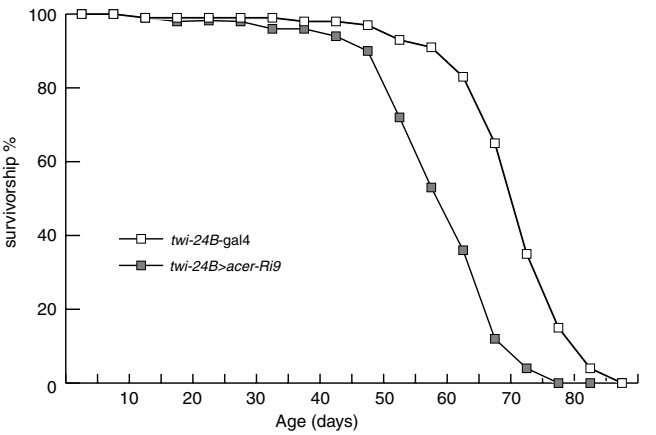

Fig. 7 Down-regulation of ACER reduced the lifespan of *Drosophila*. Survivorship curve of both wild-type control (*twi-24B-gal4*/+) and ACER silencing lines (*twi-24B*>ACER-*Ri9*) log-rank analysis.

mean lifespans of control *twi-24B-gal4* and *twi-24B-gal4*>ACER flies were 66.85 and 54.90 days, respectively (Fig. 7). Statistical analyses revealed that the differences in the survivorships of the wild-type control and ACER knock-down flies were significant (log-rank test, $P < 0.001$) indicating that the activity of ACER is essential for the survival of the flies.

4 Discussions and Conclusion

The present study was the first investigation of *in vivo* functional changes in the heart of ACER knock-down flies. It has been reported that the disruption of the ACER results in a severe and a lethal defect during heart morphogenesis in *Drosophila*.[8] Nevertheless, genetic analysis of the same ACER-mutant allele revealed that there existed a second mutation on the secondary chromosome, in which case it was likely to contribute to the embryonic lethality of the ACER-mutant allele (unpublished observation by M.T. Su). To sidestep this complicated issue and to better ascertain whether ACER plays a physiological role in adult animals, we adopted the RNA-interference approach. Our data indicated that ACER knock-down flies exhibited age-dependent changes in both the heart beating and the contractility of the heart tube, and lifespan were reduced when ACER was down-regulated suggesting that ACER is essential for both adult heart physiology and longevity of *Drosophila*.

Although we have shown that the HR of wild-type control flies is reduced in an age-dependent manner [Fig. 5(a)], our results were less significant when compared with other studies.[28] Since the heartbeat of *Drosophila* was both myogenic and neurogenic, it was affected by temperature, aging, and genetic background, as well as anesthetic agent.[23,29] To reduce the variability of heartbeat, decapitated flies were used as previously reported.[29,30] As we have used OCT to obtain the heartbeat parameters from live flies in this study, it was expected that variability of heartbeat was increased, which may reduce the statistical significance. However, these age-dependent cardiac functions declined significantly when ACER was down-regulated. On the other hand, due to differences in size between flies, large variations existed in both EDD and ESD within the tested groups; however, use of %FS can eliminate the effect of body size.

Although we have shown that down-regulation of ACER affects the age-dependent heart functions and the longevity,

the mechanism by which ACER deficiency leads to the above phenotypes was not clear. It has been reported that the expression levels of NADPH oxidase and reactive oxygen species (ROS) formation were significantly higher in ACE2 KO mice.[31,32] Since oxidative stress was elevated in congestive heart failure patients and correlates well with the development and progression of cardiovascular diseases (reviewed in Ref. 33), it will be interesting to study in the future whether ROS levels also increase in the heart of ACER knockdown flies.

Acknowledgments

This work was supported by grants from the National Science Council (Grant No. NSC 96-2628-M-003-001-MY3, NSC 100-2325-B-003-002, and NSC 101-2311-B-003-002) and National Health Research Institute (NHRI-EX91-9109SC) of Taiwan.

References

1. P. C. Santos, J. E. Krieger, and A. C. Pereira, "Renin-angiotensin system, hypertension, and chronic kidney disease: pharmacogenetic implications," *J. Pharmacol. Sci.* **120**(2), 77–88 (2012).
2. M. Volpe, "Preventing cardiovascular events with angiotensin II receptor blockers: a closer look at telmisartan and valsartan," *Expert Rev. Cardiovasc. Ther.* **10**(8), 1061–1072 (2012).
3. S. R. Tipnis et al., "A human homolog of angiotensin-converting enzyme. Cloning and functional expression as a captopril-insensitive carboxypeptidase," *J. Biol. Chem.* **275**(43), 33238–33243 (2000).
4. M. Donoghue et al., "A novel angiotensin-converting enzyme-related carboxypeptidase (ACE2) converts angiotensin I to angiotensin 1–9," *Circ. Res.* **87**(5), E1–E9 (2000).
5. S. Keidar, M. Kaplan, and A. Gamliel-Lazarovich, "ACE2 of the heart: from angiotensin I to angiotensin (1–7)," *Cardiovasc. Res.* **73**(3), 463–469 (2007).
6. L. M. Burrell et al., "The ACE2 gene: its potential as a functional candidate for cardiovascular disease," *Clin. Sci. (London)* **124**(2), 65–76 (2013).
7. W. Wang et al., "Role of ACE2 in diastolic and systolic heart failure," *Heart Fail. Rev.* **17**(4–5), 683–691 (2012).
8. M. A. Crackower et al., "Angiotensin-converting enzyme 2 is an essential regulator of heart function," *Nature* **417**(6891), 822–828 (2002).
9. S. B. Gurley et al., "Altered blood pressure responses and normal cardiac phenotype in ACE2-null mice," *J. Clin. Invest.* **116**(8), 2218–2225 (2006).
10. A. Carhan et al., "Loss of angiotensin-converting enzyme-related (ACER) peptidase disrupts night-time sleep in adult Drosophila melanogaster," *J. Exp. Biol.* **214**(4), 680–686 (2011).
11. X. Houard et al., "The Drosophila melanogaster-related angiotensin-I-converting enzymes ACER and ANCE—distinct enzymic characteristics and alternative expression during pupal development," *Eur. J. Biochem.* **257**(3), 599–606 (1998).
12. D. Huang et al., "Optical coherence tomography," *Science* **254**(5035), 1178–1181 (1991).
13. M. J. Wolf et al., "Drosophila as a model for the identification of genes causing adult human heart disease," *Proc. Natl. Acad. Sci. U. S. A.* **103**(5), 1394–1399 (2006).
14. M. A. Choma et al., "Images in cardiovascular medicine: *in vivo* imaging of the adult Drosophila melanogaster heart with real-time optical coherence tomography," *Circulation* **114**(2), e35–e36 (2006).
15. M. A. Choma et al., "Heart wall velocimetry and exogenous contrast-based cardiac flow imaging in Drosophila melanogaster using Doppler optical coherence tomography," *J. Biomed. Opt.* **15**(5), 056020 (2010).
16. A. Bradu et al., "Dual optical coherence tomography/fluorescence microscopy for monitoring of Drosophila melanogaster larval heart," *J. Biophotonics* **2**(6–7), 380–388 (2009).
17. M. T. Tsai et al., "Observations of cardiac beating behaviors of wild-type and mutant Drosophilae with optical coherence tomography," *J. Biophotonics* **4**(9), 610–618 (2011).
18. L. Ma et al., "Arrhythmia caused by a Drosophila tropomyosin mutation is revealed using a novel optical coherence tomography instrument," *PLoS ONE* **5**(12), e14348 (2010).
19. S. Y. Guo et al., "Semiautomatic and rapid quantification of heartbeat parameters in Drosophila using optical coherence tomography imaging," *J. Biomed. Opt.* **18**(2), 026004 (2013).
20. W. K. Lockwood and R. Bodmer, "The patterns of wingless, decapentaplegic, and tinman position the Drosophila heart," *Mech. Dev.* **114**(1–2), 13–26 (2002).
21. Y. S. Lee and R. W. Carthew, "Making a better RNAi vector for Drosophila: use of intron spacers," *Methods (San Diego, California)* **30**(4), 322–329 (2003).
22. D. M. Roth et al., "Impact of anesthesia on cardiac function during echocardiography in mice," *Am. J. Physiol. Heart Circ. Physiol.* **282**(6), H2134–H2140 (2002).
23. R. J. Wessells and R. Bodmer, "Screening assays for heart function mutants in Drosophila," *Biotechniques* **37**(1), 58–66 (2004).
24. G. Paternostro et al., "Age-associated cardiac dysfunction in Drosophila melanogaster," *Circ. Res.* **88**(10), 1053–1058 (2001).
25. L. Grady and G. Funka-Lea, "Multi-label image segmentation for medical applications based on graph-theoretic electrical potentials," in *Proc. 8th ECCV04 Workshop on Computer Vision Approaches to Medical Image Analysis and Mathematical Methods in Biomedical Image Analysis*, pp. 230–245, Springer-Verlag (2004).
26. L. Grady, "Random walks for image segmentation," *IEEE Trans. Pattern Anal. Mach. Intell.* **28**(11), 1768–83 (2006).
27. A. H. Brand and N. Perrimon, "Targeted gene expression as a means of altering cell fates and generating dominant phenotypes," *Development* **118**(2), 401–415 (1993).
28. R. J. Wessells et al., "Insulin regulation of heart function in aging fruit flies," *Nat. Genet.* **36**(12), 1275–1281 (2004).
29. M. Fink et al., "A new method for detection and quantification of heartbeat parameters in Drosophila, zebrafish, and embryonic mouse hearts," *Biotechniques* **46**(2), 101–113 (2009).
30. K. Ocorr et al., "KCNQ potassium channel mutations cause cardiac arrhythmias in Drosophila that mimic the effects of aging," *Proc. Natl. Acad. Sci. U. S. A.* **104**(10), 3943–3948 (2007).
31. H. Xia et al., "ACE2-mediated reduction of oxidative stress in the central nervous system is associated with improvement of autonomic function," *PLoS ONE* **6**(7), e22682 (2011).
32. R. A. Pena Silva et al., "Impact of ACE2 deficiency and oxidative stress on cerebrovascular function with aging," *Stroke* **43**(12), 3358–3363 (2012).
33. A. Whaley-Connell and J. R. Sowers, "Oxidative stress in the cardiorenal metabolic syndrome," *Curr. hypertens. Rep.* **14**(4), 360–365 (2012).