Front. Med. 2019, 13(6): 639–645
https://doi.org/10.1007/s11684-019-0701-0

REVIEW

Potential functions of esophageal cancer-related gene-4 in the cardiovascular system

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Abstract **Esophageal cancer-related gene-4 (*Ecrg4*) is cloned from the normal epithelium of the esophagus. It is constitutively expressed in quiescent epithelial cells and downregulated during tumorigenesis, and *Ecrg4* expression levels are inversely correlated with the malignant phenotype of tumor cells, validating that *Ecrg4* is a real tumor suppressor gene. Unlike other tumor suppressor genes that usually encode membrane or intracellular proteins, *Ecrg4* encodes a 148-amino acid pre-pro-peptide that is tethered on the cell surface in epithelial cells, specialized epithelial cells, and human leukocytes, where it can be processed tissue dependently into several small peptides upon cell activation. Ecrg4 is expressed in a wide variety of other cells/tissues, including cardiomyocytes and conduction system of the heart, the glomus cells of the carotid body, adrenal glands, choroid plexus, and leukocytes among others, where it exerts distinct functions, such as promoting/suppressing inflammation, inducing neuron senescence, stimulating the hypothalamus–pituitary–adrenal axis, maintaining the stemness of stem cells, participating in the rhythm and rate control of the heart, and possibly gauging the responsiveness of the cardiovascular system (CVS) to hypoxia, in addition to tumor suppression. Here, we briefly review the latest discoveries on Ecrg4 and its underlying molecular mechanisms as a tumor suppressor and focus on the emerging roles of Ecrg4 in the CVS.**

Keywords tumor suppressor gene; esophageal cancer-related gene-4; cardiovascular disease, hypoxia

Introduction

Esophageal cancer-related gene-4 (*Ecrg4*) is mapped on chromosome 2 in the c2orf40 locus. It consists of four exons spanning about 14.9 kilobases [1,2]. Consistent with the definition of a typical tumor suppressor gene, *Ecrg4* is normally expressed in many tissues and downregulated in tumors, and the levels of its expression are directly correlated with the prognosis of patients with cancer [1,3,4]. However, the unique features of Ecrg4 and its much wide tissue distribution, especially in specialized epithelial-derived cells (e.g., choroid plexus epithelium, cerebral ventricular ependymal cells, and corneal epithelial cells), adrenal gland, and the heart and its conduction system where tumors rarely develop, suggest that Ecrg4 may possess important functions other than tumor suppression [2,5–8].

Unlike other known tumor suppressors that are usually intracellular or membrane proteins [9], Ecrg4 is unique because it is a 148-amino acid pre-pro-peptide that tethers budding Ecrg4 covalently with its N terminus on the cell surface [10]. This cell surface-tethered Ecrg4 plays a sentinel role in maintaining tissue homeostasis. As shown in Fig.1, the presence of Ecrg4 on the cell surface indicates sustained homeostasis, its shedding because of injury insult calls for immediate injury responses, and this cell surface Ecrg4 gradually restores and injury response dies down with wound resolution. For example, in a stab penetrating injury of the cerebral cortex in rats, Ecrg4 on the cell surface of the choroid plexus epithelium is rapidly shed, and its gene expression is downregulated in day 1 post-injury (Dpi), which is accompanied by the activation of cell proliferation and inflammation. Ecrg4 expression gradually increases and reaches the pre-injury level by 6–7 Dpi when the wound starts to heal; this observation is accompanied by inhibited cell proliferation and tissue

Received October 30, 2018; accepted May 21, 2019
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inflammation [8]. Similar results are observed in peripheral blood leukocytes after a severe burn [6], in the middle ear mucosal infection of a rat model [11], and in acute lung injury of a mouse model [12].

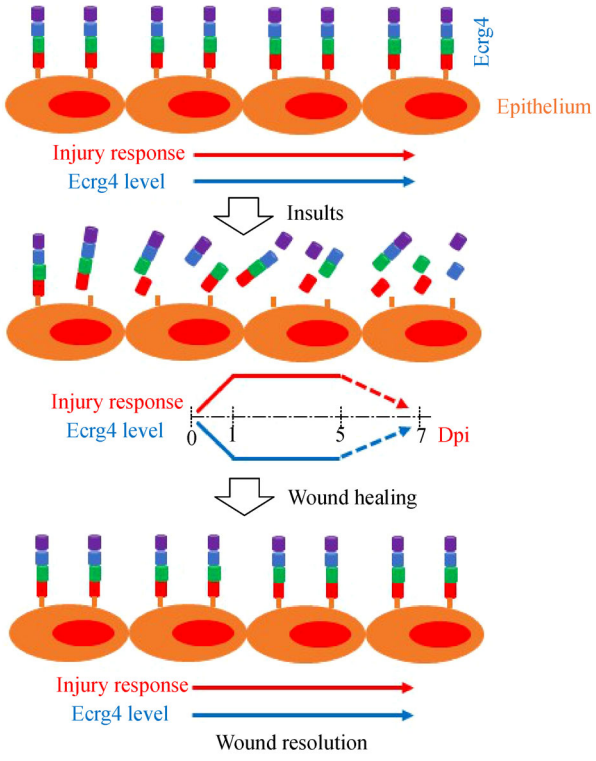

Fig. 1 Sentinel model of Ecrg4 in tissue homeostasis. Ecrg4 is covalently attached on the cell surface in quiescent state, and shed and/or proteolytically processed into various small peptides in 24 hours after insult. The loss of Ecrg4 and/or the processed Ecrg4 peptides activate injury responses including cell proliferation and inflammation, which lasts for 2–4 days. With the restoration of the cell surface Ecrg4, the inflammation dies down and wound resolves on 6–7 days post-injury (Dpi).

Ecrg4 plays a critical role in the cardiovascular system (CVS). During mouse embryonic development, the expression of *Ecrg4* in the atrial chamber continuously increases from embryonic day 10.5 onward, and its expression is significantly higher than that in ventricles at the same time. In adult rats, Ecrg4 is expressed in cardiomyocytes and the conduction system of the heart [13]. In humans, the Ecrg4 expression is constitutively expressed in atrial appendages and downregulated in the specimens of patients with atrial fibrillation (AFib). In agreement with the sentinel role of Ecrg4 in wound healing models, knocked down *Ecrg4* in atrial myocytes activates the expression of genes involved in inflammation and cardiac remodeling and modulates the functions of cardiac ion channels [5,14]. *Ecrg4* expression is also detected in the glomus cells of the carotid body of mice [15]. Here, we briefly review the latest development of Ecrg4 as a tumor suppressor and specifically focus on the potential roles of Ecrg4 in the CVS.

Ecrg4 as a tumor suppressor

Ecrg4 was cloned via the differential display of mRNA in 1998. Four mRNAs are differentially expressed between the normal and the cancerous epithelium of the esophagus, and no homologs are found in the GenBank; therefore, they are named as *Ecrg1*, *Ecrg2*, *Ecrg3*, and *Ecrg4* [3]. Further studies have shown that *Ecrg4* is constitutively expressed in the normal epithelium of the esophagus and downregulated in esophageal cancer, and the levels of its expression are directly correlated with prognosis [1]. This discovery has been supported by several laboratories, showing that the constitutively expressed Ecrg4 is down-regulated in gastric cancer, prostate cancer, hepatocellular carcinoma, nasopharyngeal carcinoma, thyroid carcinoma, glioma, colorectal carcinoma, and breast cancer among others [2,4]. This finding validates that *Ecrg4* is a pan-tumor suppressor gene. Consistently, Ecrg4 has low to non-detectable expression levels in tumor cell lines [16], and Ecrg4 restoration reverses the malignant phenotype *in vitro* and decreases tumor burden in xenograft mouse models [17–19].

Molecular mechanisms underlying the functions of Ecrg4

Ecrg4 can be processed tissue dependently into several small peptides possessing various functions, suggesting that the molecular mechanisms underlying the tumor-suppressive effect of Ecrg4 may not be simple. In 2009, the same laboratory that cloned *Ecrg4* was the first to investigate the molecular mechanisms underlying its tumor-suppressive effect and demonstrate that Ecrg4 restoration in esophageal squamous cell carcinoma (ESCC) cell line inhibits cell proliferation, colony formation, anchorage-independent growth, cell cycle progression, and tumor growth in a xenograft mouse model. These tumor-suppressive effects may be attributed to the inhibition of the expression and activity of nuclear factor κ-light-chain-enhancer of activated B cells (NF-κB) and its downstream cyclooxygenase-2 and to the interaction with Ecrg1 (transmembrane protease, serine 11A), a proteolytic enzyme that increases p27 expression through the p53 signaling pathway and blocks the cell cycle at the G_1 phase [20,21]. Since these early discoveries, others have attributed the molecular mechanisms of the anti-tumor effect of Ecrg4 to cell cycle arrest at the G_0/G_1

phase; induction of apoptosis by upregulation of Bcl-2-associated X protein, cleaved-caspase-3, and cleaved-poly (ADP-ribose) polymerase, and the simultaneous inhibition of Bcl-2 [22]; and the inhibition of molecules, Polo-like kinase 1 (PLK1), cyclin-dependent kinase 4 (CDK4), procollagen-lysine, 2-oxoglutarate 5-dioxygenase (PLOD1 and PLOD2) that are associated with cell apoptosis, cell cycle, and metastasis [23]. However, the molecular mechanisms underlying Ecrg4's tumor suppression in brain tumors vary and remain controversial. In glioma, the thrombin-processed Ecrg4 (133–148) peptide reduces the glioma tumor burden by promoting monocyte recruitment and activating microglia in a T/B cell-independent mechanism [22]. However, using a glioma initiating cell (GIC) generated from neural stem cells, Moriguchi *et al*. showed that *Ecrg4*$^{-/-}$ GIC, when implanted into the brain of immunocompetent mice, forms tumors, whereas *Ecrg4*$^{+/+}$ GIC does not, suggesting that the tumor suppression function of Ecrg4 requires an intact immune system because the depletion of CD4^{+}, CD8^{+}, or NK cells restores the tumorigenicity of *Ecrg4*$^{+/+}$ GIC. They further demonstrated that type-I interferon (IFN) signaling is important for the observed anti-tumor effect [24]. The identification of Ecrg4 receptors further supports the immunomodulatory role of Ecrg4 in its tumor suppression. Using expression cloning, Moriguchi *et al*. reported that the AA71-132 of Ecrg4 binds specifically to lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1), a membrane protein, and several other scavenger receptors (Scarf1, CD36, and Stabilin-1) that facilitate internalization, leading to the activation of NF-κB in a MyD88-dependent manner in microglia [25]. MyD88 is an adaptor protein that recruits signaling molecules to Toll-like receptors (TLRs) with the consequent induction of various immune responses [26]. This Ecrg4-LOX1 signaling pathway seems consistent with the anti-inflammatory role of Ecrg4 as a tumor suppressor and sentinel molecule in wound healing models of penetrating cerebral injury, middle ear infection, and severe burn injury in which the loss of Ecrg4 activates NF-κB signaling pathway and tissue proliferation [1,6,8,11,22].

Other functions of Ecrg4

In contrast to other tumor suppressor genes that encode intracellular or membrane proteins, *Ecrg4* encodes a 148-amino acid (AA) pre-pro-peptide that contains a unique 30-AA signal peptide tethering budding Ecrg4 covalently on a cell surface [10], and several conserved proteinase restriction sites processing Ecrg4 tissue dependently into approximately a dozen of small peptides upon cell activation [27]. These processed peptides have been shown to possess unique, shared, or even opposite biological effects, although the exact peptide(s) responsible for each reported function remains to be defined [6,17,28–31]. Ecrg4 is expressed not only in the epithelium but also in the specialized epithelium, such as middle ear mucosa, epithelium of ventricular systems of the brain, choroid plexus, ependyma, oligodendrocytes, cornea, chondrocytes, endocrine tissues, and CVS [5,7,8,15,32]. This wide tissue distribution also suggests that Ecrg4 may play other important distinct functions beyond its

Table 1 Representative articles showing *Ecrg4* cloning/distinct functions and distribution

Cloning/functions/distribution	References
Cloning of *Ecrg4*	Su, T., *et al*. Chin J Oncol. (Zhonghua Zhong Liu Za Zhi)1998; 20 (4): 254–257 [3].
In organs/tissues other than CVS	
As a tumor suppressor	Yue, C. M., *et al*. World J Gastroenterol. 2003; 9(6): 1174–1178 [45].
Chondrocyte development/differentiation	Huh, Y. H., *et al*. Gene. 2009; 448 (1): 7–15 [37].
Hypothalamus corticotrophin-releasing factor	Tadross, J. A., *et al*. Br J Pharmacol. 2010;159 (8): 1663–1671 [30].
Alzheimer's disease related	Woo, J. M., *et al*. Int J Mol Med. 2010; 25(5): 667–675 [34].
Neurosenescence factor	Kujuro, Y., *et al*. Proc Natl Acad Sci. 2010;107(18): 8259–8264 [29].
Brain homeostasis factor in wound healing	Gonzalez, A. M., *et al*. Fluids Barriers CNS 2011; 8(1): 6 [27].
Epithelium Ecrg4 is surface tethered/secreted	Dang, X., *et al*. Cell Tissue Res. 2012; 348 (3): 505–514 [10].
Apoptosis inhibitor	Matsuzaki, J., *et al*. Carcinogenesis 2012; 33(5): 996–1003 [31].
Inhibition of neuronal stem cell proliferation	Nakatani, Y., *et al*. Development 2019; 146 (4): dev168120 [38].
In CVS	
Ecrg4 in the carotid body (gene microarray)	Balbir, A., *et al*. Am J Physiol Lung Cell Mol Physiol. 2007; 292 (3): L704–L715 [15].
Ecrg4 expression in the A-V node (*in situ*)	Mirabeau, O., *et al*. Genome Res. 2007; 17(3): 320–327 [32].
Ecrg4 expression in cardiomyocytes of rat (IHC)	Porzionato, A., *et al*. Eur J Histochem. 2015; 18; 59 (2): 2458 [7].
Distribution of Ecrg4 in rat heart (IHC and qPCR), in specimens of atria in patients with AFib. (IHC), and in canine model of AFib. (qPCR). Involved in AFib.	Huang, L. *et al*. Sci Rep. 2017; 7(1): 2717 [5].

anti-tumor effect (Table 1). For example, in the central nervous system, Ecrg4 functions as a sentinel factor because an acute cortical injury causes a rapid down-regulation of Ecrg4 in the choroid plexus, which is increased gradually to the pre-injury level upon wound resolution in rats; and in zebrafish embryos, the loss of Ecrg4 causes a dose-dependent hydrocephalus-like phenotype that can be rescued by Ecrg4 overexpression [8,27]. Ecrg4 acts as a corticotrophin-releasing factor (CRF). When hypothalamus explants of rats are incubated with augurin, the mature form of Ecrg4 significantly elevates CRF; and in rats, after augurin is injected into the third ventricle or into the paraventricular nucleus of the hypothalamus, plasma adreno-cortico-tropic-hormone (ACTH) and corticosterone significantly increase, but this observation can be blocked by the peripheral injection of a CRF receptor antagonist [30]. Ecrg4 serves as a neuronal senescence inducer. Ecrg4 expression increases naturally with aging and in a serum-induced mouse oligodendrocyte precursor cell (OPC) senescence model, consequently forced expression of Ecrg4 induces senescence, whereas knocked down *Ecrg4* prevents the senescent phenotype in the OPC senescence model, respectively [29]. Ecrg4 may also be a pathogenic factor for Alzheimer's disease (AD) as shown by the increased Ecrg4 expression in neurofibrillary tangles within the cerebral cortical white matter of patients with AD, and this observation is consistent with the significantly increased expression of Ecrg4 in a transgenic mouse overexpressing Tau, which is a protein responsible for the formation of neurofibrillary tangles in AD [33–35]. In wound healing models of cortical stab injury, middle ear infection, and severe burn injury, Ecrg4 also functions as a sentinel factor because the constitutively expressed Ecrg4 rapidly decreases immediately after injury, which is accompanied by the activation of inflammation and tissue proliferative response. This decreased Ecrg4 expression is sustained for a few days and followed by a gradual restoration to the resting level upon wound resolution [6,8,11]. Consistently, forced expression of Ecrg4 inhibits injury-induced cell proliferation, cell migration, and pro-inflammatory response [6,11,36]. Ecrg4 may play important roles in other systems as well, although experimental evidence remains fragmental. For example, Ecrg4 expression is relatively high in a resting state compared with that in an activation state in lymphocytes, and Ecrg4 overexpression in Fas-sensitive Jurkat cells confers resistance to Fas-induced apoptosis [31]. In chondrocyte development, Ecrg4 expression is low in mesenchymal cells, dramatically increases during chondrogenic differentiation, and decreases again in differentiated chondrocytes and in osteoarthritis of both patients and animal models [37]. Lastly, Ecrg4 supports the proliferation or survival of hematopoietic stem cells or hematopoietic progenitor cells in embryonic development (United States Patent 7320880) and contributes to a decrease in a self-renewal activity of neural stem cells with aging [38].

Ecrg4 expression in CVS

Since the discovery of Ecrg4 as a potential tumor suppressor in the epithelium of the esophagus [1,3], research had focused on *Ecrg4* as a tumor suppressor gene in other organs until 2008 [4,39–41]. Using Markov modeling, Mirabeau *et al*. found that Ecrg4 is a novel secreted peptide and expressed in mouse endocrine tissues, such as pituitary, adrenal gland, pancreas, and choroid plexus, and in the atrio-ventricular (A-V) node of the heart [32]. This novel finding has reignited research interests in Ecrg4. In a study on the tissue distribution of Ecrg4 in rats, Porzionato *et al*. showed that Ecrg4 is expressed heterogeneously in ventricular myocytes [7]. To further examine the region-specific distribution of Ecrg4 in the heart, Huang *et al*. reported that the expression of *Ecrg4* is higher in the atria and the sinus node than in the ventricles; its expression is also higher in the left side than in the right side of the heart. These findings have been validated by immunohistochemistry, which shows a homogeneous strong Ecrg4-positive immunostaining in the sinus node, the A-V node, and atria, and isolated cardiomyocytes with strong staining in a homogeneous light staining background in the ventricles [5]. The higher expression of Ecrg4 in the atrium than in the ventricle is further supported by microarray data sets. In mouse embryonic development, the *Ecrg4* expression is continuously increasing from embryonic day 10.5 to E18.5 in the atrial chamber; at the same time, the *Ecrg4* expression level in the ventricles is much lower and remains almost unchanged. In adult rats, the *Ecrg4* expression is higher in the atrium than in the ventricle [13]. Using human induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CMs), Lan *et al*. showed that Ecrg4 is detectable in hiPSCs. Upon differentiation, *Ecrg4* expression decreases over time until day 20 and gradually increases over time, reaching a stable level on day 50 [14]. In the vascular system, the *Ecrg4* expression is also intriguing. Its expression is higher in the glomus cells of the carotid body in DBA/2J mouse, a higher responder to hypoxia than that in A/J mouse, a lower responder to hypoxia [15].

Potential functions of Ecrg4 in CVS

The expression of Ecrg4 in cardiac tissue, especially in the conduction system of the heart argue against the tumor suppressive role of Ecrg4 in the heart. Intrigued by the expression of Ecrg4 in the conduction system of the heart and down-regulation in patients with tumor, we reasoned

that Ecrg4 might be involved in the rate/rhythm control of the heart and the long-sought molecule underlying the higher incidence of atrial fibrillation (AFib) in patients with tumor than in patients without tumor and the general population [42]. Indeed, our laboratory has shown that the *Ecrg4* expression in the atrial appendages of patients with AFib is significantly lower than that of patients with a sinus rhythm [5]. Consistently, the Ecrg4 expression in an AFib canine model is significantly decreased compared with that in the control. In neonatal cardiomyocytes, knockdown *Ecrg4* expression significantly modulates the expression of genes (Gja1, MMP3, s100a1, and s100a8) commonly implicated in atrial remodeling, activates proinflammatory genes (IL1a, IL6, and MCP1) and significantly shortens the action potential duration (APD50 and APD90) [5]. In hiPSC-CMs, knockdown *Ecrg4* modulates the expression of sodium voltage-gated channel α subunit 5 (SCN5A), potassium voltage-gated channel subfamily H member 2 ($KCNH_2$), potassium voltage-gated channel subfamily D member 3 (KCND3), intermediate conductance calcium-activated potassium channel protein 4 (KCNN4), and potassium/sodium hyperpolarization-activated cyclic nucleotide-gated channel 2 (HCN2) [14]. These results suggest that Ecrg4 plays a critical role in the pathogenesis of AFib and that Ecrg4 may be responsible for the high incidence of AFib in patients who have tumors and whose Ecrg4 levels in the heart may also be decreased with tumor tissues. Consistent with the potential role of Ecrg4 in atrial electric remodeling, previous results demonstrated that the rapid electric stimulation of HL1 cells, a mouse atrial myocyte cell line, for 24 h significantly decreases the *Ecrg4* expression [43]. Similarly, in neonatal rat cardiomyocytes, the mRNA expression of *Ecrg4* is significantly decreased after 12 h of rapid electric stimulation (unpublished observation). An *Ecrg4*-knocked out mouse has been generated and grossly characterized by the International Mouse Phenotype Consortium (IMPC). One of the significant features of the knockout mouse is the shortened QRS complex duration, supporting that Ecrg4 plays a critical role in the rate/rhythm control of the heart. In the vascular system, the expression of Ecrg4 in the glomus cells of the carotid body may suggest that Ecrg4 participates in blood pressure regulation, blood oxygenation, and changes in pH and temperature [15].

Although Ecrg4 seems to play critical roles in CVS, the subcellular localization of Ecrg4 in the cardiomyocytes and glomus cells of the carotid body remains to be characterized. Given the homogeneous immunohistochemistry staining of Ecrg4 in cardiomyocytes, the molecular mechanisms of how Ecrg4 relays signals may not be the same as that characterized in other cell types in which proteolytically processed cell surface Ecrg4 binds to the Ecrg4 receptor, leading to the activation of downstream signaling pathways.

Regulation of Ecrg4 expression

Ecrg4 (aka c2orf40) gene is about 14.9 kilobases and mapped to 2q14.1-3 in humans. Transcription produces a 772-base pair (bp) mRNA that contains a 447 bp open reading frame (GenBank: AF325503.1). *Ecrg4* core promoter is mapped to the -780 to $+420$ region where the transcription initiation site is at -11 relative to the A in the start codon ATG. Bioinformatics analysis has shown that the sequence is rich in GC and does not contain TATA and CAAT boxes, but a CpG island, a canonical hypoxia response element, and tandem-conserved Sp1 binding sites immediately upstream of ATG that are typical features of a housekeeping gene. *In vitro* methylation of the core promoter significantly inhibits the *Ecrg4* promoter activity [44]. Likewise, the application of 5-azicytidine, a DNA methylation inhibitor, to tumor cell lines that express a negligible level of Ecrg4 significantly restores Ecrg4 expression and thus reverses the malignant phenotypes of tumor cell lines [1,44,45]. Furthermore, tissue biopsy shows that CpG islands are highly methylated in tumor tissues, and the degree of methylation is inversely correlated with Ecrg4 expression levels and thus its prognosis [40,46–48]. Therefore, the degree of methylation/demethylation is one of the main mechanisms gauging the Ecrg4 expression in tumors, which is in sharp contrast with other known tumor suppressor genes where mutations or DNA polymorphisms are usually responsible for the decreased or loss of tumor suppressor function in tumorigenesis [49,50]. To confirm that the observed downregulation of the Ecrg4 expression in AFib is correlated with promoter methylation, we compared the methylation status of the CpG islands in atrial appendages between patients in the sinus rhythm and those in Afib through bisulfite sequencing, and the results showed that the percentage of CpG methylation in the predicted CpG islands was significantly higher in patients with AFib than that in patients with a sinus rhythm (unpublished observation). In addition to promoter methylation, the Ecrg4 expression is positively regulated by Sp1 [49,50] but is negatively regulated by hypoxia (manuscript in preparation).

Perspectives

Ecrg4 is a hormone-like peptide expressed in cardiomyocytes and the conduction system of the heart, where it is implicated in rate/rhythm control and possibly in cardiac ischemia. In contrast to the cell surface localization in epithelial cells, Ecrg4 expression in cardiomyocytes seems homogeneously in the cytoplasm and the nucleus, suggesting that the processing of Ecrg4 and the molecular mechanisms responsible for its cardiac effects may differ.

The subcellular distribution and processing of Ecrg4 should be characterized, and the molecules interacting with or downstream of Ecrg4 in cardiomyocytes should be identified.

Acknowledgements

This study was funded by the Collaborative Innovation Center for Prevention and Treatment of Cardiovascular Disease of Sichuan Province, Southwest Medical University (No. xtcx-2016-16), the National Natural Science Foundation of China (No. 81770336), and a seeding grant from Southwest Medical University.

Compliance with ethics guidelines

Rui Zhou, Yuanshu Liu, Wenjun Huang, and Xitong Dang declared that they have no conflict of interest to disclose. This manuscript is a review article and does not involve a research protocol requiring approval by a relevant institutional review board or ethics committee.

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