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Secreted factors from adipose tissue-derived mesenchymal stem cells suppress oxygen/glucose deprivation-induced cardiomyocyte cell death via furin/PCSK-like enzyme activity

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ABSTRACT

Clinical application of mesenchymal stem cells (MSCs) represents a potential novel therapy for currently intractable deteriorating diseases or traumatic injuries, including myocardial infarction. However, the molecular mechanisms of the therapeutic effects have not been precisely revealed. Herein, we report that conditioned media (CM) from rat adipose tissue-derived MSCs (ASCs) protected adult cardiomyocytes from oxygen/glucose deprivation (OGD)-induced cell death. We focused on furin/PCSK protease activity in ASC-CM because many therapeutic factors of MSCs and soluble cardioprotective factors include the PCSK cleavage site. We found that recombinant furin protected cardiomyocytes from OGD-induced cell death. The ASC-CM had potent furin/PCSK protease activity and the cardioprotective effect of the CM from ASCs in the OGD-assay was abolished by an inhibitor of the furin/PCSK-like enzyme. Microarray analysis and Western blot analysis showed PCSK5A, the secreted type of PCSK5, is the most abundantly secreted PCSK among 7 PCSK family members in ASC. Finally, knockdown of PCSK5A in ASCs decreased both the furin/PCSK protease activity and cardioprotective activity in the CM. These findings indicate an involvement of furin/PCSK-type protease(s) in the anti-ischemic activity of ASCs, and suggest a new mechanism of the therapeutic effect of MSCs.

1. Introduction

Recent advances in medical management have greatly improved the survival rate of patients after myocardial infarction (MI), but significant challenges remain in preventing progressive loss of cardiomyocytes and chronic heart failure [1]. Stem cell therapy or stem cell transplantation has emerged as a promising new therapeutic option to replenish cardiac cells or to improve cardiac functions in damaged hearts after MI. Both non-clinical and clinical studies showed that various types of cells applied after MI could improve cardiac function and promote regeneration of the injured heart [2–5]. Mesenchymal stem cells (MSCs) are most widely used in those studies, as they are relatively easily prepared in large amounts, and also compatible with both autologous and heterologous applications [6]. MSCs are originally isolated from bone marrow, but have also been identified in various tissues, such as adipose tissue, dental pulp, and umbilical cord blood [2,6–9]. Although beneficial effects of MSCs have been well demonstrated, the molecular mechanisms of their therapeutic effects have not been defined precisely.

In rat and porcine MI models, it has been demonstrated that the injection of MSCs increased the number of cardiomyocytes and improved heart function in parallel with myogenesis and angiogenesis [10,11]. As the protective effects were observed within a few days, it was suggested that the MSCs themselves did not differentiate into cardiomyocytes, but provided trophic effects for existing cardiomyocytes through cell–cell interactions or paracrine factors [12–14]. In fact, MSCs secrete a wide range of bioactive molecules, and administration of the conditioned media (CM) from MSC cultures to MI animal models could recapitulate therapeutic efficacy in those models [14]. Several known angiogenic or growth promoting factors, such as VEGF, IGF-1, and TSG-6 have been identified as MSC-derived cardioprotective factors, which supports the notion that well-coordinated secretion of multiple factors is the key mechanism of the MSC action [15,16].

Recently, we identified a numerous number of trophic factors secreted from three different types of MSCs, bone marrow-derived mesenchymal stem cells (BMSCs), adipose-derived mesenchymal stem cells (ASCs), and dental pulp-derived mesenchymal stem cells (DPSCs), by conducting comprehensive proteomic analysis of

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CM from these cells [17]. In the study, we also identified 124 commonly secreted factors. Because the CM of all the 3 MSCs has been known to show similar cardioprotective activities [13,17], it could be expected that those MSCs protect cardiomyocytes in the same fashion with identical trophic factors. In this context, the list of commonly secreted factors could provide promising candidates to identify factors which explain the protective activity against MI commonly observed in MSCs. In this report, we tried to identify the MI protection MSC-factor from within the list of commonly secreted factors and show evidence that furin/PCSK-like enzyme activity in the ASC-CM represents the anti-ischemic activity of ASCs, and suggests a new mechanism of the therapeutic effect of MSCs.

2. Materials and methods

2.1. ASC-CM preparation

Adipose tissue was isolated from lateral epididymis region in 6- to 8-week-old Wistar male rats (Charles River). All experimental procedures were performed in accordance with the in-house guidelines of the Institutional Animal Care and Use Committee of Daiichi Sankyo Co., Ltd. After visible blood vessels were removed from the adipose tissue, it was minced by scalpel and digested by 0.1% collagenase type I (Worthington) for 1 h at 37 °C. Isolated cells were filtered through a 70 μm cell strainer, washed by PBS, and suspended at the concentration of 1.5×10^6 cells/mL in αMEM (Life Technologies) with 9%FBS (Life Technologies) and 1% Antibiotic-Antimycotic (AA). Cells were cultured at 37 °C in a humidified atmosphere of 5% CO_2 and 5% O_2, and used for experiments after 3–6 passages. MSC surface markers were confirmed by using a flow cytometer. The ASCs were positive for CD44, CD29 and CD90 and negative for CD11b/c and CD45 (data not shown).

ASCs grown nearly confluent were washed three times with PBS and cultured for an additional three days in serum free αMEM with 1% AA. The collected medium was filtered by 0.22 μm filters (Millipore) and was concentrated and replaced with glucose-free DMEM (Life Technologies) containing 1% AA using Amicon Ultra 3 kDa centrifugal filter (Millipore).

2.2. Rat adult cardiomyocyte OGD assay

Rat adult cardiomyocytes were isolated as previously described [18]. Male Sprague–Dawley rats (Charles River) were anesthetized with intraperitoneal administration of Somnopentyl (Kyoritsu Seiyaku Corporation) 1 mL/kg. The heart was quickly removed from the chest, and perfused with the perfusion buffer (120 mM NaCl, 14.7 mM KCl, 0.6 mM KH_2PO_4, 0.6 mM Na_2HPO_4, 1.2 mM $MgSO_4$, 10 mM HEPES, 4.6 mM $NaHCO_3$, 5.6 mM glucose, 10 mM HEPES, 10 mM 2,3-butanedione monoxim, 30 mM taurine, pH 7.4) for 5 min. The buffer was switched to the digestion buffer containing 1 mg/mL collagenase type II (Worthington) and 0.1 mg/mL protease (Sigma), and digested for 10–15 min until the heart became swollen and turned pale. The ventricles were removed and transferred into 8 mL of the stopping buffer containing 10% FBS in the perfusion buffer, and the tissue was minced by scissors. The dispersed cardiomyocyte suspension was filtered by a 100 μm cell strainer. The isolated cardiomyocytes were washed by glucose-free DMEM, and re-suspended in the oxygen- and glucose-free DMEM with 1% AA (OGD medium), in which oxygen was eliminated by N_2 gas bubbling for 2 h. The cardiomyocytes were seeded at 90–100% confluence in a 24-well culture plate coated with laminin (Sigma) 40 μg/mL, and incubated in a low oxygen (1% O_2) condition for 2 h before adding the tested materials, ASC-CM, recombinant furin, and/or furin inhibitors. After four hours treatment with test materials, glucose was added to the media at 4.5 g/L final concentration, and the cells were incubated in the normoxia condition overnight. Cells were co-stained with 1 mM calcein-AM (Life Technologies) and 1 mM EthD-1 (Life Technologies), fixed with 2% paraformaldehyde, and washed by PBS. Images were analyzed by IN Cell Analyzer 6000 (GE Healthcare) with IN Cell Developer Toolbox image analysis software. The indices for counting living cardiomyocytes were: major axis/minor axis >3, staining area $>100\ \mu mm^2$, form factor <0.6 among calcein-AM positive cells, except for cells indicating EthD-1 signal density >800. The number of living cardiomyocytes was counted four times in 9 fields.

2.3. Microarray analysis

Total RNA of rat ASCs was extracted using RNeasy mini kit (QIAGEN) according to the manufacturer's protocol. Biotinylated cRNA was synthesized using 3′ IVT Express Kit (Affymetrix) from 250 ng of total RNA. The biotinylated cRNA was fragmented and hybridized to the GeneChip Rat Genome 230 2.0 Array. The array was washed, stained, and scanned using the GeneChip Fluidics Station 450 and GeneChip Scanner 3000 7G according to the GeneChip Expression Analysis Technical Manual (http://www.affymetrix.com/support/downloads/manuals/expression_analysis_technical_manual.pdf).

2.4. Quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

Total RNA of ASCs and rat hearts was extracted as described above. cDNA was synthesized with random hexamer primers using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Real-time PCR was run on the ABI Prism 7900 sequence detection system with pre-designed primer sets (Applied Biosystems). The expression levels of genes were normalized to Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) used as internal control.

2.5. Antibody

Primary antibodies used were: rabbit polyclonal anti-PC1/3 (Cell signaling), goat polyclonal anti-PC2 (Santa Cruz biotechnology), rabbit anti-PCSK5 (Proteintech), rabbit anti-PCSK6, and rabbit anti-furin (Abcam). Secondary antibodies, donkey anti-rabbit IRDye 800CW and donkey anti-goat IRDye 800 CW, were obtained from Li-COR.

2.6. Western blotting

Cultured ASCs were lysed with the cell lysis buffer (50 mM Tris–HCl pH 7.5, 150 mM NaCl, 0.1% TrtionX-100) containing protease inhibitor cocktail (Roche). ASC lysate, ASC-CM (10 μg), or rat tissue protein (30 μg) were loaded onto NuPAGE Novex 4–12% Bis-Tris Gels (Life Technologies). Separated proteins were blotted onto a nitrocellulose membrane (Immobilon-FL; Millipore), which was then blocked with Odyssey Blocking Buffer (Li-COR) for 30 min at room temperature. The membrane was incubated with primary antibodies (1 μg/mL) overnight at 4 °C, washed with Tris-buffered saline containing 0.075% Tween 20 (TBST), and then incubated with an appropriate fluorescent secondary antibody (0.1 μg/mL) for 1 h. The protein bands were detected by an Odyssey Infrared Imaging System (Li-COR).

2.7. Furin/PCSK-like enzyme activity

Furin/PCSK-like enzyme activity in ASC-CM was assessed by using a fluorescent substrate t-Butyloxycarbonyl-L-arginyl-L-valyl-

L-arginyl-L-arginine 4-methylcoumaryl-7-amide (Boc-Arg-Val-Arg-Arg-MCA), which was designed to assess furin/PCSK-like activity. ASC-CM or recombinant furin (R&D Systems) and/or furin inhibitor (ENZO Life Sciences) were added to 50 μM of the synthetic substrate in assay buffer (50 mM Tris-HCl, 15 mM NaCl, 0.005% Brij35, 10 mM $CaCl_2$). The enzyme reaction was performed at 37 °C for 2 h, and liberated AMC was measured by excitation at 380 nm and emission at 460 nm.

2.8. RNA Interference

The siRNAs for PSCK5 and the control (Life Technologies) were AUAACAUGCUGUACGUCUCUCCAGG and AUACCAACGUUCUGCAUGUCUCAGG, respectively. ASCs 1.5×10^6 cells were plated in a 15 cm dish 18 h before siRNA transfection. Each siRNA at 20 nM was transfected 2 times at 24 h-intervals using Lipofectamine RNAiMAX (Life Technologies). Transfected cells were washed 3 times with PBS and cultured for 3 days in serum free αMEM containing AA.

2.9. Statistical analysis

All statistical analyses were performed using SAS System Release 8.2 (SAS Institute Inc., Cary, NC, USA), and data were expressed as means with standard deviation (SD). The statistical significance of differences between groups was tested by Student's *t*-test or Dunnett's test.

3. Results

3.1. The ASC-CM protects against OGD-induced cell death of adult cardiomyocytes

Various regulatory functions of MSCs have been reported, including anti-inflammation, immunosuppression, angiogenesis, and tissue repair [6,16]. One of the most validated clinical beneficial effects of MSCs is protection against MI. Although the precise mechanisms of the protective function of MSCs against MI have not yet been elucidated, increasing evidence supports the notion that the injected MSCs are recruited to the damaged tissues and then produce various trophic factors to protect damaged cells from cell death. We previously identified common trophic factors secreted from three different types of MSCs, BMSCs, ASCs, and DPSCs [17]. We presumed that the protein list could provide candidate proteins which could explain the trophic action of the MSCs and also clues which would explain their various effects.

In order to identify such protective proteins from the list, we first prepared an in vitro assay method of oxygen and glucose deprivation (OGD)-induced cell death that mimics the

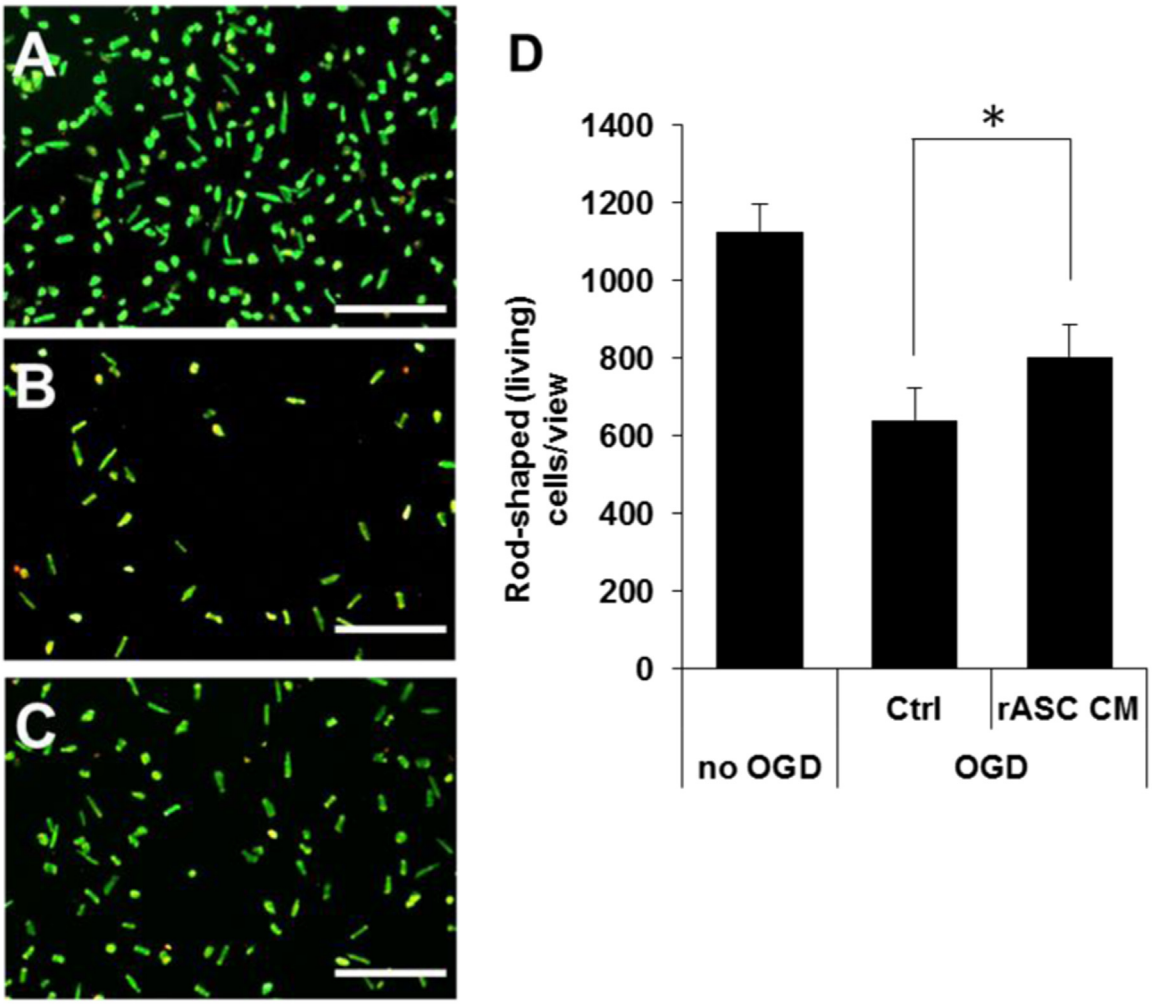

Fig. 1. Rat ASC-CM protected against adult cardiomyocyte cell death induced by OGD. Rat adult cardiomyocytes were stained with calcein-AM and EthD-1 (A, B, C). Without OGD, many living cardiomyocytes were positive for calcein-AM and negative for EthD-1 (A). OGD/reperfusion induced cell death (B), and ASC-CM treatment increased the number of living cells (C). The number of living cardiomyocytes was counted four times in 9 fields (D). Data are shown as the means ± SD (N=4); Student's *t*-test, $^*P < 0.05$ versus control. Bar=500 μm.

reperfusion injury after MI by using non-proliferating rat adult primary cardiomyocytes. Cardiomyocyte-like cell lines such as H9c2 or primary cultured neonatal cardiomyocytes are normally used for reperfusion injury assays. However, these cell lines or neonatal cardiomyocytes proliferate actively in culture and are not suitable for the analysis of ischemic cell death since adult cardiomyocytes do not proliferate. To overcome this issue, cardiomyocytes from adult rats were used in our assay. Cardiomyocytes isolated from adult rats were incubated in glucose-free DMEM under 1% O_2 (OGD condition) for 6 h, then replenished with glucose (25 mM) and 20% O_2 for overnight. The OGD treatment induced a morphological change of isolated cardiomyocytes from rod shape into round shape, subsequently causing cell death, and mimicking the condition in MI (Fig. 1A, B). The rat ASC-CM protected adult cardiomyocytes from OGD-induced cell death (Fig. 1C). This result suggests that ASC secreted protein(s) could suppress ischemic cell death of cardiomyocytes.

3.2. *Furin/PCSK-like enzyme activity is protective against OGD-induced cardiomyocyte cell death*

Then, we screened the trophic factors which exert a protective effect in the OGD assay. Among 124 proteins commonly produced from the three MSCs [17], we first focused on growth factors and cytokines. Recombinant proteins of Activin A (consisting homodimer of inhibinβA), C1q and tumor necrosis factor related protein 3 (C1qTNF3), serpin peptidase inhibitor, clade E (SERPINE1), follistatin like 1 (FSTL1), and nephroblastoma overexpressed (NOV) were evaluated. However, the single treatment of these recombinant proteins did not suppress OGD-induced cell death in our assay (data not shown). We assumed that not a sole protein, but proteins which modulate functions of various proteins could play roles in this process. According to this idea, we focused on proteases. Furin is known to cleave various secreted proteins in cells and regulates the function and secretion of those proteins. Several cardioprotective factors such as EGF, IGF, and GDF-15 possess furin cleavage sites [19,20]. We noticed that the list of commonly secreted factors contains PCSK5 and PCSK6 and decided to validate the possibility of PCSKs playing important roles in the cardioprotective activity of the ASC-CM. In order to evaluate whether furin/PCSK-like activity is important to the cardioprotective activity of ASC-CM, recombinant furin protein was tested in the OGD-assay. Recombinant furin protein was used since there were no commercially available PCSK5 proteins and we were unable to produce the recombinant secreted form of PCSK5 protein. Recombinant furin dose-dependently suppressed myocardiocyte cell death to the same extent as ASC-CM in the OGD-assay (Fig. 2A). The protective activity of recombinant furin was completely canceled by a furin inhibitor (Furin inhibitor II) (Fig. 2B). Furthermore, the protective activity of ASC-CM was also blocked by the furin inhibitor (Fig. 2C). The ASC-CM showed potent furin/PCSK-like peptidase activity, which was inhibited

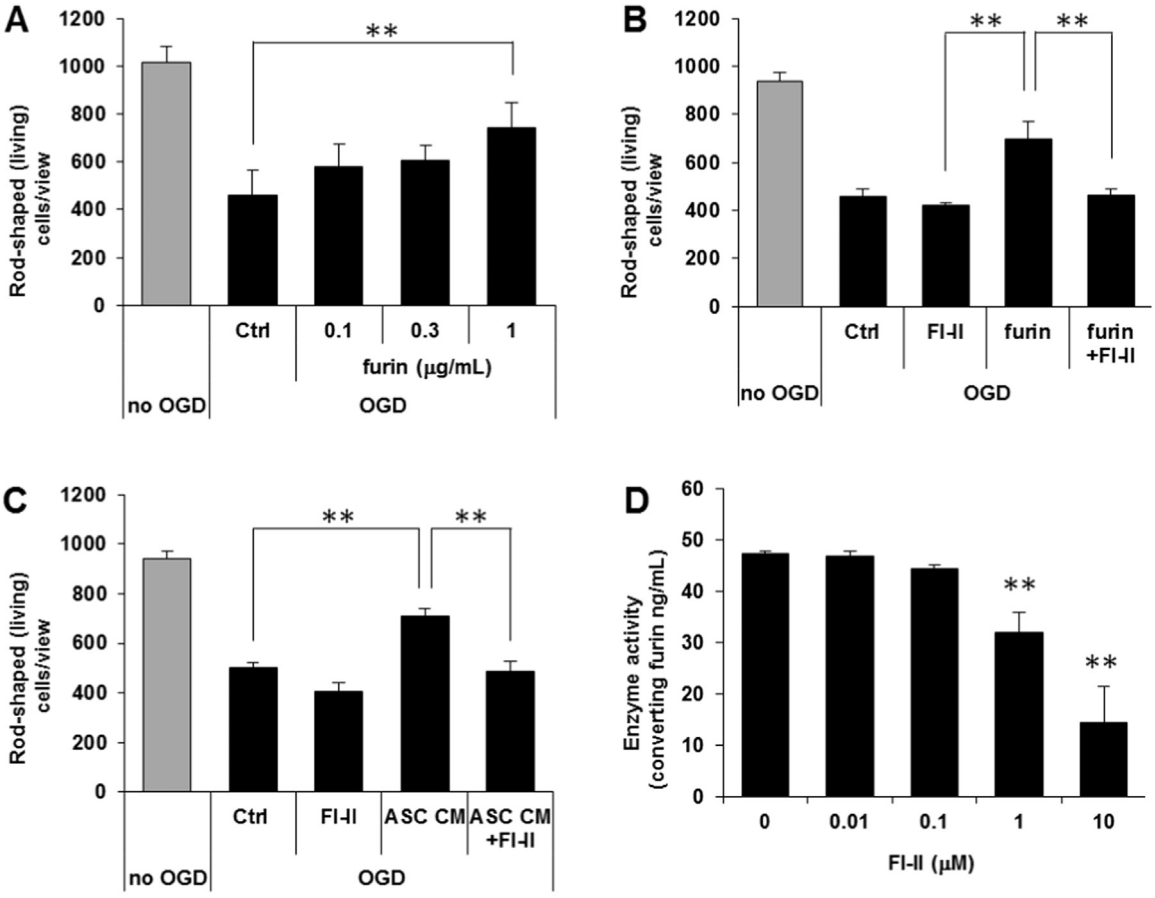

Fig. 2. Furin/PCSK-like enzyme activity provided the protective effects to cardiomyocytes. Live rat cardiomyocytes were counted by an IN Cell analyzer as in Fig. 1. Treatment with recombinant furin increased cell viability (A), which was canceled by Furin inhibitor II (FI-II) (B). FI-II also canceled the protective effect of ASC-CM (C). Furin/PCSK-like enzyme activity was measured as the amount of released AMC from the synthetic substrates Boc-Arg-Val-Arg-Arg-MCA. The activity was expressed as degraded furin (ng/mL) using the standard curve generated by recombinant furin. Furin/PCSK-like enzyme activity of ASC-CM was inhibited by FI- II dose dependently. Data are shown as the means ± SD (N=4); Student's *t*-test for (B)(D), Dunnett's test for (A)(D), $^{**}P < 0.01$.

Table 1
The mRNA expression of PCSK family genes in ASCs. Two biological replicates were analyzed by microarray. The scaling signal was compensated by global scaling that set average signal intensity of the each array to a target signal of 500. Detection calls (P: presence or A: absence) and detection p-value were determined by microarray suite software (Affymetrix).

Probe set	Gene Title	Gene Symbol	ASC #1			ASC #2		
			Scale Signal	Detection calls	Detection p-value	Scale Signal	Detection calls	Detection p-value
1387247_at	proprotein convertase subtilisin/kexin type 1	Pcsk1	2.0	A	0.458	13.1	A	0.171
1387155_at	proprotein convertase subtilisin/kexin type 2	Pcsk2	5.4	A	0.970	0.7	A	0.905
1367778_at	furin (paired basic amino acid cleaving enzyme)	Furin	343.2	P	0.001	364.9	P	0.000
1387213_at	proprotein convertase subtilisin/kexin type 4	Pcsk4	90.9	P	0.014	99.4	P	0.019
1392773_at	proprotein convertase subtilisin/kexin type 5	Pcsk5	1924.4	P	0.006	1742.9	P	0.003
1387812_at	proprotein convertase subtilisin/kexin type 6	Pcsk6	715.8	P	0.000	747.4	P	0.000
1367886_at	proprotein convertase subtilisin/kexin type 7	Pcsk7	230.7	P	0.030	206.3	P	0.030

by the furin inhibitor dose dependently (Fig. 2D). These results indicated that ACSs secreted active PCSKs in culture.

Taken together, these results strongly suggest that the secreted furin/PCSK-like enzyme activity plays a critical role in the cardioprotective activity of ASC-CM in the OGD condition.

3.3. *PCSK5A is the main component of cardioprotective activity of ASC-CM*

In order to identify the main source of secreted furin/PCSK-like enzyme activity in the ASC-CM, we analyzed expression profiles of

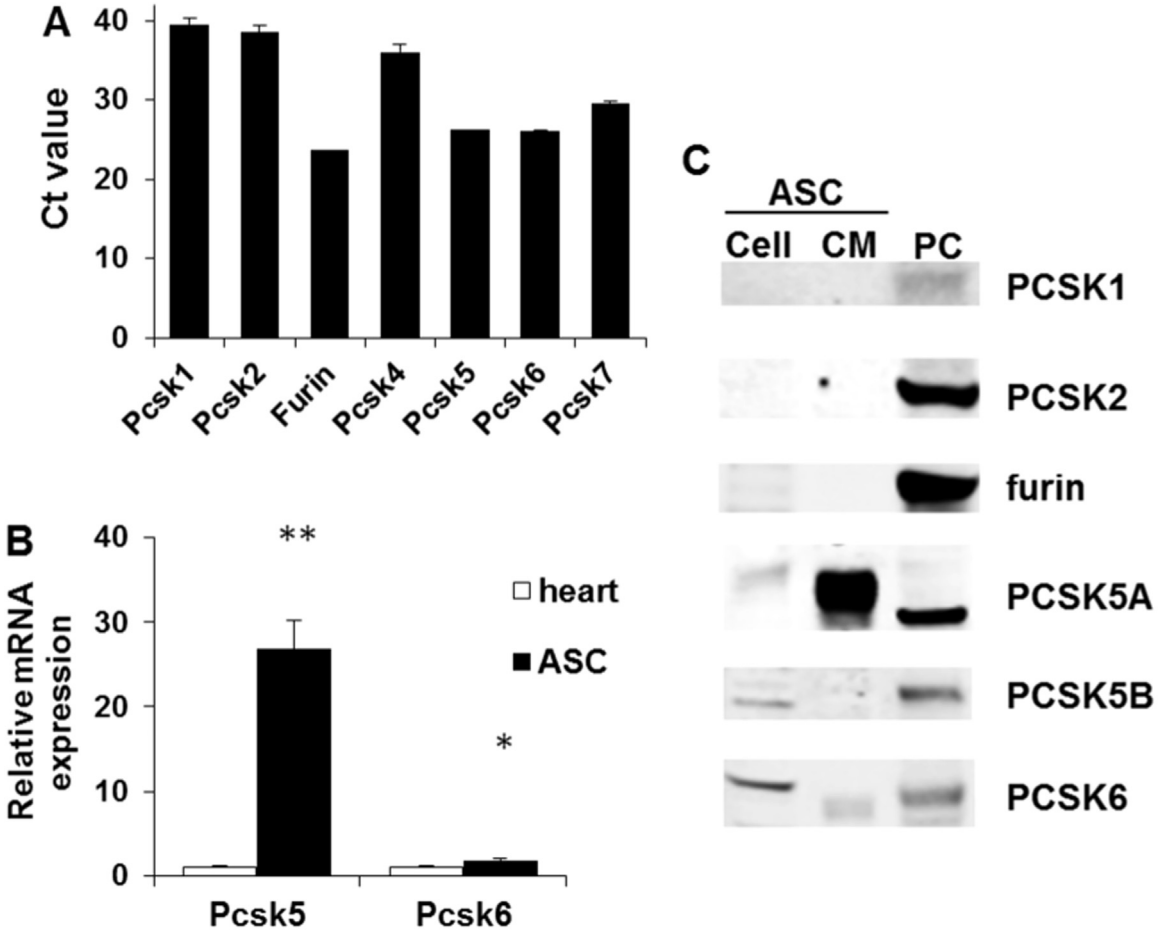

Fig. 3. The expression of PCSKs in ASCs. The expressions of PCSKs at the mRNA level were examined by qRT-PCR. Ct values of the genes of the PCSK family (A) and relative gene expression in ASCs and rat hearts' left ventricles (control) were normalized to GAPDH, and fold changes relative to control are shown as mean ± SD (N=3, Student's *t*-test, $^{*}P < 0.05$, $^{**}P < 0.01$) (B). Total protein from cell lysates and conditioned media of ASCs were loaded on each lane and subjected to Western blot analysis (C). Rat kidney tissue was used as a positive control for PCSK1, PCSK2, furin and PCSK6, and rat brain tissue lysate was used as a positive control for PCSK5A and PCSK5B (PC lane). Note that PCSK5A and PCSK6 in the conditioned media were slightly smaller in size, possibly because of the processing before secretion.

PCSK family members in ASCs. The PCSK family is comprised of at least nine endoprotease enzymes [21]. Among them, seven PCSKs cleave the same site downstream of unique paired basic amino acids within the motif (R/K Xn R/K↓ downward arrow indicates the cleavage site) and it has been reported that PCSK1, PCSK2, PCSK5 and PCSK6 are secretory types [22]. First, we evaluated the mRNA expression of the seven PCSKs. Microarray analyses of ASCs revealed that among the four secretory PCSKs, PCSK5 and PCSK6 showed high signal intensity (Table 1). Quantitative RT-PCR analysis also revealed that, in accordance with the microarray results, PCSK5 and PCSK6 were highly expressed (Fig. 3A) The PCSK5 expression level of the ASCs was over 25-fold higher than that of in rat hearts (Fig. 3B). We then conducted protein expression analysis in the CM. In our previous comprehensive proteomic analysis by LC-MS we conducted previously [17], both PCSK5 and PCSK6 were identified. There are two forms of PCSK5, PCSK5A and PCSK5B which are a secretory form and cytosolic form respectively [19]. Western blot analysis revealed that only PCSK5A existed in the CM, and PCSK5B existed in the cell lysate (Fig. 3C). On the other hand, the expression of PCSK6 in the CM was lower than that in the cell lysate (Fig. 3C). PCSK1 and PCSK2, the other two secreted type of PCSKs, and furin in either the cell lysate or the CM were not detected. These results indicate that PCSK5 would be the main source of the furin/PCSK-like activity in the ASC-CM. To confirm the importance of PCSK5 in the cardioprotective activity of the ASC-CM, CM were prepared from PCSK5A knockdown ASCs and tested in the OGD-assay. The siRNA against PCSK5A dramatically decreased the PCSK5A protein (Fig. 4A) and the Furin/PCSK-like enzyme activity (Fig. 4B), which were consistent with the decrease of mRNA expression (data not shown). The PCSK5A KD-CM significantly decreased the protective effect against OGD–induced cardiomyocyte cell death (Fig. 4C). Collectively, our results indicated that PCSK5A is one of the critical components of ASC-CM that mediates anti-OGD activity for adult cardiomyocytes.

4. Discussion

Previous studies have shown that the application of MSCs could improve heart function after MI by promoting angiogenesis and/or suppressing cardiomyocyte cell death [10,23,24]. The therapeutic effect is more likely to be based on an indirect mechanism provided by MSCs, such as paracrine factors and/or cell–cell interactions, rather than MSCs being a part of regenerated tissues after differentiation into cardiomyocytes or endothelial cells by themselves. Two lines of evidence support this notion. First, almost all engrafted MSCs rapidly disappear from the sites, and there is no solid evidence that those MSCs reside long enough to contribute to the improvement of heart functions [16,25]. Second, MSCs can secrete a wide range of bioactive molecules and trophic factors that stimulate myocyte differentiation and angiogenesis, and MSC-CM actually demonstrated a similar efficacy to MSCs themselves [14]. Among these secretions are VEGF, IFG-1, and TSG-6 [14,16]. Although the importance of MSC trophic factors is gaining more attention, not many factors have been clearly defined to be able to substitute for MSCs' functions. In this study, we have demonstrated that furin/PCSK-like enzymes are important MSC trophic factors aiding in adult cardiomyocytes' protection.

Furin/PCSK-like activity played a critical role in the adult cardiomyocyte protective activity of ASC-CM (Fig. 2C). A diversity of biological functions is known as being attributable to furin/PCSK family members. Furin, one of the most studied family members, has been known to cleave various secreted proteins, including IGF-1, EGF, TGF-β, Lefty, BMP10, MMPs, and ADAMs, and convert them

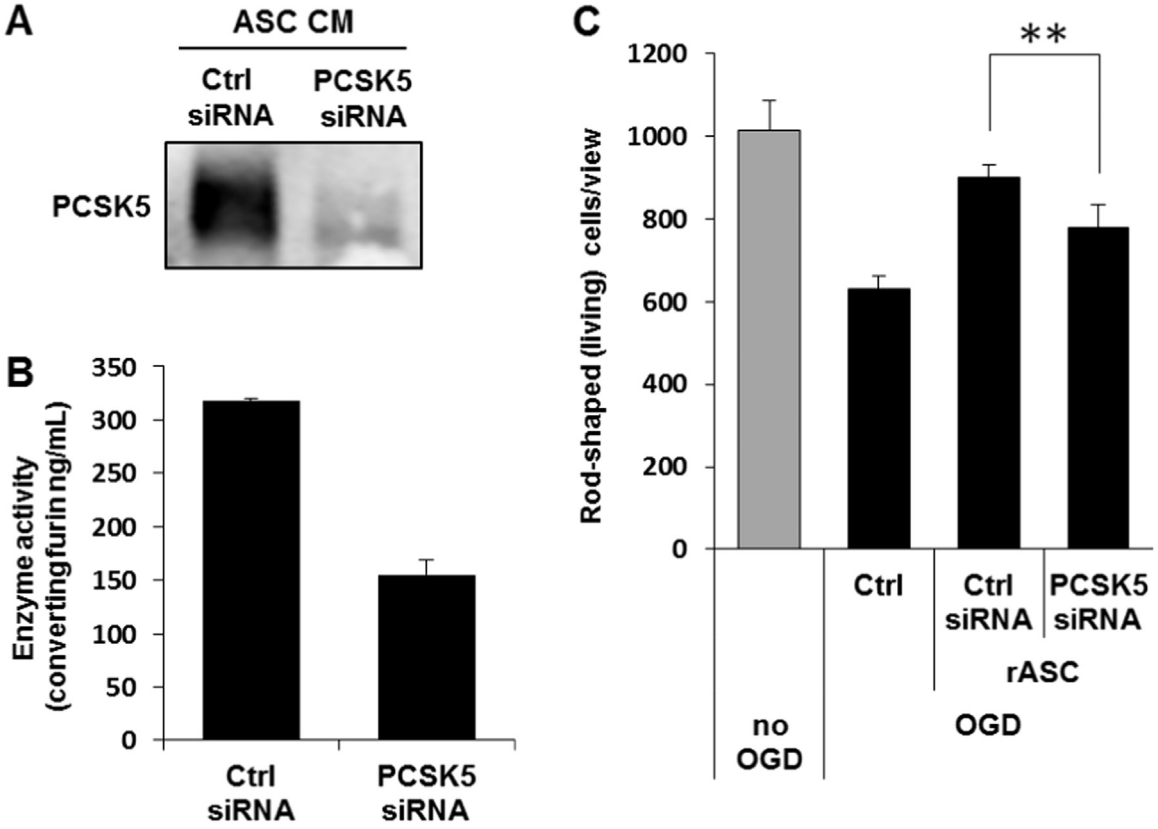

Fig. 4. PCSK5 mediated the effect of ASC-CM on cardiomyocytes. The expressions of PCSK5 and furin/PCSK-like activity were significantly reduced in conditioned medium from ASC treated by PCSK5 targeted siRNA (A, B). In parallel, the cardioprotective effect of ASC-CM was attenuated by PCSK5 knockdown (C). $^{**}P < 0.01$ versus Ctrl siRNA, Student's *t*-test.

into active forms [22]. Furin cleaves proteins at the site after unique paired basic amino acids within the motif (R/K Xn R/K↓, downward arrow indicates the cleavage site), which is also recognized by PCSK1, PCSK2, PCSK4, PCSK5, PACE4 and PCSK7. Therefore, the factors known as furin substrates could also be cleaved by these other PCSKs. Normally, furin cleaves its substitute within cell (Trans-Golgi network, cell surface [21]). We confirmed that furin/PCSK5-like enzyme activity in the ASC-CM showed a cardiomyocyte protective effect and cell-impermeable furin inhibitor blocked the effect (Fig. 2C), suggesting that furin/PCSK5-like enzyme activity exerts its effects outside of cells. It has been estimated that PCSK1, PCSK2, PCSK5, and PCSK6 are secreted types of PCSK, and our results indicated that PCSK5 is the most abundant PCSK (Fig. 3A–C) and would be the main source of PCSK activity in ASC-CM (Fig. 4B). Therefore, among the seven PCSK family members, PCSK5 is the most promising candidate. It is intriguing to speculate that the furin/PCSK5-enzyme activity of PCSK5 also plays a critical role in protecting cardiomyocytes in vivo and in a clinical setting. Further analysis such as identification of PCSK5 substrates and evaluation of PCSK5's function in an in vivo MI model would be required.

Accumulating evidence suggests that MSCs exert therapeutic effects through secreting a variety of bioactive molecules, which prevent organ damage or cell death, and/or stimulate regeneration of injured tissues. Our findings that indicate the importance of secreted furin/PCSK5-like enzyme activity support this notion, and further suggest an intriguing hypothesis that multiple molecules secreted after ischemic injury can be cleaved and activated by furin/PCSK-type protease(s) to give rise to endogenous molecules important for tissue regeneration and recovery. This hypothesis reasonably explains the various biological effects of MSCs that are difficult to accomplish by any one sole factor alone, but could be accomplished by the combination of several factors. Adrenomedullin has been shown to have angiogenic and cardioprotective effects in MI injury model by activating the PI3K and Akt pathways [26]. As pro-adrenomedullin is a HIF1α target gene and induced in hypoxic hearts, it is possible that pro-adrenomedullin is produced by cardiomyocytes in the OGD-condition and is processed to active adrenomedullin by furin or PCSKs in ASC-CM. Key molecules in the anti-OGD activity in our system still remain to be determined, but concerted multiple mediators might be necessary to produce the full activity. Therefore, the therapeutic application of a furin/PCSK-type enzyme, such as PCSK5, may be an alternative approach for treating acute MI or chronic heart failure after MI.

We identified PCSK5A as the important protective factor in the unique OGD-assay by taking advantage of comprehensive proteomic analysis of different types of MSCs. Although further validation is needed to prove PCSK5A as key trophic factor in MSCs' cardiomyocyte protection, focusing on the commonly secreted factors would be a powerful and efficient way to find other promising candidates. Identification of such trophic factors would provide us with deep understanding of the mechanisms of MSC functions and could lead to a complete substitution of the MSC therapy with recombinant protein(s).

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Appendix A. Transparency document

Transparency document associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bbrep.2016.07.001.

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