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MicroRNAs targeting the SARS-CoV-2 entry receptor ACE2 in cardiomyocytes

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ABSTRACT

The World Health Organization (WHO) declared coronavirus disease 2019 (COVID-19) as a public health emergency of international concern as more than 15 million cases were reported by 24th July 2020. Angiotensin-converting enzyme 2 (ACE2) is a COVID-19 entry receptor regulating host cell infection. A recent study reported that ACE2 is expressed in cardiomyocytes. In this study, we aimed to explore if there are microRNA (miRNA) molecules which target ACE2 and which may be exploited to regulate the SARS-CoV-2 receptor. Our data reveal that both Ace2 mRNA and Ace2 protein levels are inhibited by miR-200c in rat primary cardiomyocytes and importantly, in human iPSC-derived cardiomyocytes. We report the first miRNA candidate that can target ACE2 in cardiomyocytes and thus may be exploited as a preventive strategy to treat cardiovascular complications of COVID-19.

1. Introduction

The novel coronavirus disease 2019 (COVID-19) outbreak occurred in December 2019 and in the following months this epidemic was declared as a global health emergency by the World Health Organization (WHO) with more than 600 thousand deaths reported till date [1]. Similar to SARS-CoV and the Middle East respiratory syndrome (MERS), severe symptoms such as acute respiratory distress syndrome (ARDS) and multiple organ failure were observed in nearly 20% patients suffering from COVID-19 infection [2].

Angiotensin-converting enzyme 2 (ACE2) helps to maintain the balance of blood pressure and electrolyte in the human body. It also reduces the Angiotensin II levels in the circulation by suppressing the renin-angiotensin-aldosterone system conferring anti-hypertensive effects [3]. Recently, it has also been reported as a receptor for the spike protein of SARS-CoV-2 and plays pivotal roles during the COVID-19 infection [4]. Notably, COVID-19 patients with severe symptoms also seem to suffer from other health conditions including cardiovascular disease (CVD), hypertension and diabetes [5]. Importantly, ACE2 is expressed in cardiomyocytes and elevated in patients with heart diseases [6]. Thus, it is likely that the extent of COVID-19 infection gets more pronounced by ACE2 in patients with comorbidities, which in turn may cause additional myocardial damage. MiRNAs are highly conserved small non-coding RNAs which are ~20–22 nucleotides in length and can negatively regulate gene expression. Several studies have reported that miRNAs could modulate ACE2 expression in a variety of cell types and diseases [7], but whether miRNA could be potential preventive target for SARS-CoV-2 is still unknown.

In our previous review, we summarized the state of art of investigation and potential therapeutic strategies for COVID-19 patients with cardiac disease [8]. In this study, we investigate several in-silico identified miRNAs which could regulate ACE2 expression in vitro and therefore provide a potential strategy to develop novel therapeutic candidates for COVID-19.

2. Results

As one of the main receptors of SARS-CoV-2 [4], ACE2 is considered as a potential target for COVID-19 prevention. We first examined the expression of Ace2 in different mouse organs. Ace2 showed higher expression in kidney, lung and heart when compared to liver or spleen (Fig. 1A). To further investigate ACE2 expression pattern in different
cell types (cardiomyocyte, fibroblast and endothelial cell) from human and rat were tested. Interestingly, Ace2 was highly expressed in neonatal rat cardiomyocytes (NRCMs) compared to neonatal rat cardiac fibroblasts (NRCFs) (Fig. 1B). Similarly, ACE2 demonstrated higher expression in human induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CMs) compared to human cardiac fibroblasts and endothelial cells (Fig. 1C). Our next aim was to identify miRNAs which could potentially regulate ACE2 expression. A bioinformatics search using TargetScan was applied to predict miRNAs binding to the three prime untranslated region (3'UTR) of Ace2. Amongst the in silico candidates, miR-429, −200b, −200c were selected for further validation based on the highest conservation for the 3'UTR binding site (Fig. 1D). Collectively, these data suggest that cardiomyocytes are probably the major cell type which expresses ACE2 in the heart and miR-429, −200b and -200c may present as promising targets to regulate ACE2 expression.

To test this hypothesis, NRCMs were first transfected with miRNA mimics pre-miR-429, pre-miR-200b and pre-miR-200c. High efficiency of miR-429, −200b and −200c overexpression was observed by miRNA specific TaqMan qPCR (Supplementary Fig. 1A). We then evaluated the Ace2 mRNA and Ace2 protein level in transfected NRCMs. Compared to the negative control, pre-miR-200c strongly diminished the expression of Ace2 in human and heart tissue biopsy from patients infected with SARS-CoV-2 [10]. Another multi-organ autopsy study of COVID-19 patients revealed the presence of SARS-CoV-2 viral RNA in the heart tissue [11]. Surprisingly, the SARS-Cov-2 mRNA was found in the heart tissue biopsy of patients who had already recovered from SARS-CoV-2 infection [12]. Moreover, several recent studies described that hiPSC-CMs can be efficiently infected with SARS-CoV-2 particles and such a platform can be further utilized for screening of potential anti-viral medicines [9,13]. To fight the COVID-19 pandemic in the absence of vaccines, it is necessary to identify promising drugs which act against SARS-CoV-2 infection in cardiomyocytes and such in vitro translational platforms can immensely aid in this process.

3. Discussion

In this study, high expression of Ace2 was observed in mouse kidney, lung and heart. A recent study employed single cell sequencing analysis of heart tissue showing that ACE2 expression is enriched in the cardiomyocyte fraction and is further elevated specifically in cardiomyocytes of patients with heart disease [6]. Our findings reaffirm these observations that ACE2 is abundant in cardiomyocytes compared to other cardiac cell types. Strikingly, viral particles were observed in the endo-myocardial biopsy from patients infected with SARS-CoV-2 [10]. Another multi-organ autopsy study of COVID-19 patients revealed the presence of SARS-CoV-2 viral RNA in the heart tissue [11]. Surprisingly, the SARS-Cov-2 mRNA was found in the heart tissue biopsy of patients who had already recovered from SARS-CoV-2 infection [12]. Moreover, several recent studies described that hiPSC-CMs can be efficiently infected with SARS-CoV-2 particles and such a platform can be further utilized for screening of potential anti-viral medicines [9,13]. To fight the COVID-19 pandemic in the absence of vaccines, it is necessary to identify promising drugs which act against SARS-CoV-2 infection in cardiomyocytes and such in vitro translational platforms can immensely aid in this process.

Vaccine is one of the most efficient tools to prevent COVID-19. By April 2020, 115 vaccine candidates for COVID-19 were reported, out of which 73 targets were selected for pre-clinical investigation. In addition to protein targeting vaccines, the list of most promising vaccine also includes an mRNA based vaccine from Moderna (mRNA-1273) which is
currently in Phase I clinical trial [14]. However there have been no efforts, so far, to investigate the role of non-coding genome in the progression of SARS-CoV-2. It is well known that the human genome mostly comprises of non-coding transcripts which are master regulators of several physiological processes. In our study, we identified that indeed miRNAs can modulate ACE2, the key molecule for cellular SARS-CoV-2 infection. Thus, the miRNA mediated inhibition of the coronavirus cell-entry receptor ACE2 could be an alternative treatment for a potential future coronavirus pandemic.

The miR-200 family, comprising of miR-200b, miR-200c and miR-429, is a well-known miRNA cluster which is highly investigated in anti-cancer studies [15]. Also, it has been previously shown that miR-200c is upregulated in CVDs [16]. Here, we elucidated the role of miR-200c in SARS-CoV-2 infection where the overexpression of miR-200c represses ACE2 expression in both rat and human cardiomyocytes. Further experiments to investigate the potential of miR-200c to lower the ACE2-mediated infection of hiPSC-CMs with SARS-CoV-2 or SARS-CoV-2 spike pseudotype viruses are warranted. However, considering the correlation of miR-200c and CVDs, the activation of miR-200c should be carefully monitored.

Of note, patients with pre-existing CVDs are more likely to suffer from severe symptoms of COVID-19 [5]. Our study highlights that miR-200c can regulate the expression of ACE2 in cardiomyocytes. However, the contradicting effects of ACE2 in CVDs and COVID-19 infection should be considered carefully. On one hand, angiotensin-converting enzyme inhibitors and angiotensin receptor blockers are widely administered to patients with heart failure, which reduces stress on the heart and leads to enhanced ACE2 expression and activity. On the other hand, elevated levels of ACE2 could also render the cells more susceptible to get infected by SARS-CoV-2 [17]. Thus, it is a big challenge to ascertain the therapeutic time window for targeting ACE2 in COVID-19 patients with CVDs. Nevertheless, a miR-200c based therapy may help to bridge the time till an effective COVID-19 vaccine is available to the world. Additionally, considering that SARS-CoV-2 employs ACE2, which is also the receptor for HCoV-NL63, MERS-CoV and SARS-CoV, it does not seem too far-fetched to speculate that a novel coronavirus outbreak in future will again utilize ACE2, thus making further investigations worthwhile.

4. Methods

4.1. Cell culture

Neonatal rat cardiomyocytes (NRCMs) and Neonatal rat cardiac fibroblasts (NRCFs) were isolated from one to three-day old rat pups by using the Neonatal Heart Dissociation Kit (Miltenyi). NRCMs were cultured in MEM (BioConcept) medium with 5% fetal bovine serum (FBS) (Gibco), 100 μM BrdU (Sigma) and 1% penicillin/streptomycin
(Gibco). NRCFs were cultured in DMEM (Gibco) medium with 10% FBS (Gibco). Human primary cardiac fibroblasts (HCFs, Promocell) were cultivated in Fibroblast Basal medium (Promocell) supplemented with 5% FBS (Gibco). Human Umbilical Vein Endothelial Cells (HUVECs) (ATCC, US) were cultured in EBM-2 (Lonza supplemented with hEGF, hydrocortisone, VEGF, hFGF-B, R-3-IGF-1, ascorbic acid, gentamicin/amphotericin-B and 10% FBS (Gibco). The cord blood derived human induced pluripotent stem cell line (hiPSCs) [18] was maintained on feeder-free culture conditions using Geltrex (Thermo Scientific) coated polystyrene plates (Greiner CELLSTAR) and StemMACS full medium with supplements (Miltenyi). HiPSCs were differentiated into mero-dermal lineage by modulation of Wnt pathway, followed by a metabolic selection process to obtain purified population of hiPSC-CMs. Pre-miR-negative control#2 (Life Technologies), −200c (Life Technologies, PM11714) and -200b (Life Technologies, PM10492) were transfected for 48 h with Lipofectamine 2000 (Life Technologies) in OptiMEM medium.

4.2. RNA isolation and qRT-PCR

RNA isolation from in vitro and mouse organ tissue was performed by using Trifast (Peqlab) as described in manufacturer’s instructions. Isolated RNA (500 ng) was reversed transcribed with random primer using iScript Select cDNA synthesis kit (Bio-Rad). Real-Time quantitative PCR was done with iQ SYBR Green mix (Bio-Rad) on C1000 Touch Thermocycler (Bio-Rad) using specific primer pairs.

The specific primers for target genes are as follows (5’–3’): rno_Ace2 forward: (ACCGGAAATGTTGCAGCAC) and reverse: (AGTGACATGA TTTCTCCACCGGC); hsa_ACE2 forward: (GGGAGAATTAAAACGAGA AGC) and reverse: (TGAGGACCTGAAGACCCATT); mm_Hprt forward: (CAGTCACGGGGCAGCATTA) and reverse: (GCTGATGCCTGACAGGA GG); hsa_HPRF2 forward: (AGGAGTACGCTTGTGCTCG) and reverse: (GTCCCCTGTGGACTGGTCA).

Taq-man assay was applied to measure miR-429 (Assay ID: 001077), −200b (Assay ID: 002251) and -200c (Assay ID: 002300) expression in NRCM and hiPSC-CM. SnRNA (Assay ID: 001718) and RNU48 (Assay ID: 001006) were used as house keepers, respectively in rat and human.

4.3. Western blotting

Cell pellets were lysed in 1X Cell lysis buffer (Cell Signaling) and isolated protein was measured by Bradford (Bio-Rad) method. 30 μg of protein was loaded for each sample on SDS-polyacrylamide gel to resolve the proteins. Proteins were transferred to polyvinylidene fluoride membrane in Mini PROTEAN Tetra cell (Bio-Rad). Specific proteins were identified by following antibodies: ACE2 (Proteintech), and Actb (Cell Signaling). HRP conjugated secondary antibody (Cell Signaling) was used for detection of bands. Band intensity was calculated by Image J software.

4.4. Luciferase reporter assay

Luciferase reporter vector was constructed using pMIR-Report plasmid (Ambion# Am5795) containing the binding site (or mutated) with miR-200c in 3’UTR of ACE2 (179–185). HEK 293 T cells were transfected with wild type (WT) or mutated (MUT) luciferase reporter plasmid with pre-miR-negative control#2 (Life Technologies), pre-miR-200c (Life Technologies, PM11714) and beta-Gal control plasmid (Promega). Luciferase activity was performed according to manufacturer’s instructions to study the direct binding of miR-200c in 3’UTR of ACE2 normalizing with beta-Gal values via utilizing beta-Gal kit (Promega) and Luciferase Assay System (Promega).

4.5. Statistics

All data were analyzed using GraphPad Prism software. Data are presented as mean ± SEM, and an unpaired 2-tailed t-test was performed to calculate the significance between groups.

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