Expression of microRNA miR-17-3p inhibits mouse cardiac fibroblast senescence by targeting Par4

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Abstract

The microRNA miR-17-92 cluster plays a fundamental role in heart development. This study aimed to investigate the effect of a member of this cluster, miR-17, on cardiac senescence. We examined the roles of miR-17 in senescence and demonstrated that miR-17-3p attenuated cardiac aging in myocardium by targeting Par4. This up-regulates the down-stream signals CEBPB, FAK, N-cadherin, vimentin, Oct4 and Sca-1, and down-regulates E-cadherin. Par-4 has been reported as a tumor-suppressor gene that induces apoptosis in cancer cells, but not in normal cells. Repression of Par4 by miR-17-3p enhanced transcriptional activity of CEBPB and FAK, which promoted mouse cardiac fibroblast (MCF) epithelial-mesenchymal transition (EMT) and self-renewal, resulting in cellular senescence and apoptosis-resistance. We conclude that Par4 can bind to CEBPB promoter and inhibit its transcription. Decreased Par-4 expression increases CEBPB which binds FAK, and enhances FAK transcription. Par4, CEBPB and FAK form a senescence signaling pathway, playing roles in modulating cell survival, growth, apoptosis, EMT and self-renewal. Through this novel senescence signaling axis, miR-17-3p represses Par4 expression, acting pleiotropically as a negative modulator of cardiac aging and CF cellular senescence.
Introduction

The aging heart is associated with morphological and structural remodeling that lead to functional decline. In particular, cardiovascular aging is understood to be an important risk factor in heart disease. Evidence from clinical and experimental observations has suggested that the aging heart undergoes fibrotic remodeling through an age-dependent accumulation of collagen which increases ventricular stiffness. Understanding the mechanisms of age-associated defects in cardiac function will be critical in designing strategies to prevent adverse remodeling in elderly patients.

As the most populous cell type in the mammalian heart, cardiac fibroblasts are required for extracellular matrix synthesis and deposition. Cellular senescence, concomitant with aging, is a process of growth-arrest that inhibits the proliferative capacity of mammalian cells. Senescent cells are characterized by a large flattened morphology, and several molecular and cytological markers including lysosomal senescence associated beta-galactosidase (SA-β-gal) activity. Several pathways have been shown to induce senescence in cardiac fibroblasts. Replicative senescence is induced by telomeric shortening as a result of different stress signaling, including oncogenesis. Oncogene-induced senescence is caused by the activation of tumor suppressor networks including p53 and Rb, which lead to cell cycle arrest. Loss of mitochondrial function and impaired autophagy are also known to induce cellular senescence.

MicroRNAs (miRs) are short endogenous strands of RNAs of about 18-24 nucleotides in length that are post-transcriptional regulators of gene expression. The discovery of miRs has improved our understanding of post-transcriptional control processes such as development and aging (Deng et al., 2013; Liang et al., 2009; Noren Hooten et al., 2013). A polycistronic miRNA cluster, miR-17~92, consisting of six mature miRNAs (miR-17, miR-18a, miR-19a, miR-20a, miR-19-b1, and miR-92a-1) has been reported to play fundamental role in heart and lung development and remodeling (Bonauer and Dimmeler, 2009; Dakhllallah et al., 2013; Shan et al., 2009). Furthermore, members of miR-17-92 cluster are found to be commonly down-regulated in aging human cells ((Du et al., 2014; Hackl et al., 2010). In this study, we found that transgenic expression of miR-17 could inhibit cardiac senescence and apoptosis in a mouse cardiac fibroblast (CF) cell model and transgenic mouse.
We propose that miR-17-3p attenuates cardiac aging in the myocardium by targeting Par4/PAWR, a Wilm’s tumor 1 (WT-1) interacting protein that acts as a transcriptional repressor. Par4 has been characterized as a tumor suppressor protein that is up-regulated specifically during prostate cancer cell apoptosis (Chaudhry et al.). We found that Par4 is expressed in the mature myocardium and interacted directly with the promoter region of transcription factor CCAAT enhancer-binding protein β (CEBPB), a negative regulator of focal adhesion kinase (FAK) signaling. Thus, FAK is an integrin-mediated regulator of cytoskeletal remodeling whose expression is up-regulated by Par4. FAK signaling activates the epithelial-mesenchymal transition (EMT) and self-renewal programs which repress cellular senescence in cardiac fibroblasts. We further identified Par-4 as a bona-fide target of miR-17-3p, which attenuated the senescent phenotype in transfected primary cardiac fibroblasts as well as a transgenic mouse line over-expressing miR-17-3p. Through this novel Par4/CEBPB/FAK signaling axis that was mediated by miR-17-3p, we elucidated a novel pathway in cellular senescence, with therapeutic implication of senescence-related miRNAs in the aging heart.

Material and Methods

Materials

The monoclonal antibodies against Par4, Sox2, Oct4, vimentin and senescence β-galactosidase staining kit were obtained from Cell Signaling. The monoclonal antibodies against N-cadherin and E-cadherin were obtained from BD Biosciences. The monoclonal antibodies against FAK and CEBPB were obtained from Santa Cruz Biotechnology. The monoclonal antibody against Sca-1 was obtained from R&D. Horseradish peroxidase-conjugated goat anti-mouse IgG and horseradish peroxidase-conjugated goat anti-rabbit IgG were obtained from Bio-Rad. Immunoblotting was performed using the ECL Western blot detection kit. Real-time PCR kits were obtained from Qiagen. Anti-mouse Par4 siRNAs were designed and synthesized by GenePharma Co. Ltd (Shanghai) as follows: siRNA1 (Par4-mus-682, 5’gugcuuagagauaggaat and 5’uucguacuacuacuagcaatt), siRNA2 (Par4-mus-943, 5’cggcuauucuccuaaccaatt and 5’uugaggacagacagccggtt), siRNA3 (Par4-mus-1038, 5’gcgguagcaagaaactt and 5’uucguacuacuacuacgcttt). The miR-17 transgenic mice were generated previously (Shan et al., 2009). All of the methods were performed following a protocol approved by the Animal Care
Committee of Sunnybrook Research Institute. The animal procedures were performed conform the NIH guidelines.

**Constructs**

The precursor miR-17 sequence was ligated into a mammalian expression vector, BluGFP, which contains a Bluescript backbone, a CMV promoter driving expression of green fluorescent protein (GFP), and a H1 promoter driving miR-17 precursor as described previously (Shan et al., 2009). The pre-miR-17 sequence in this construct is the same as the endogenous sequence, which produces both miR-17-5p and miR-17-3p. The control plasmid was the same as the miR-17 construct except that the pre-miR-17 sequence was replaced with a non-related sequence (5'atcacagtactgtgataactgaagtttttggaaaagctttagttattaa), serving as a mock control. The mouse cardiac fibroblasts (MCFs, generated by ScienCell, Cat #M6300) were transfected with miR-17 and control vector. After cell sorting, the transfected stable cells were obtained and used in this study.

A luciferase reporter vector (pMir-Report; Ambion) was used to generate the luciferase constructs (Rutnam et al., 2014). A fragment of the 3'-untranslated region (3'UTR) of mouse Par4 (Pawr) was cloned by RT-PCR, using two primers, musPar4-SacI (5'cccgagctctagaagctgcacgggcggcttc) and musPar4-MluI (5'ccacgcgttaatatatacagtcctctcaacaa). The PCR products were digested with SacI and MluI and inserted into a SacI- and MluI-opened pMir-Report vector to obtain the Luc-Par4 luciferase construct. Mutation of the miR-17-3p binding site was created by PCR, using two primers musPar4-SacI and musPar4-MluI-mut (5'ccacgcgttaatatatacagtcctctcaacaaacgtgatga). To serve as a negative control, a non-related sequence was amplified from the coding sequence of the chicken versican G3 domain using two primers, chver10051SpeI and chver10350SacI (Du et al., 2013a). We do not expect any endogenous miRNA to bind to this fragment as it is in a coding region.

The plasmid PCB6 of Par4 was kindly provided by Dr. Vivek M. Rangnekar (Chakraborty et al., 2001), while the plasmid pcDNA3.1 (-)-mCEBPB of CEBPB was kindly provided by Dr. Robert C. Smart (Zhu et al., 1999).

**Cell proliferation assay**

Cells (4 x 10^4) were seeded onto 6-well dishes in 10% FBS/DMEM medium and maintained at 37°C overnight. Cells were harvested daily and cell number was analyzed by coulter counter.

**Cell survival assay**
Cells (2 x 10^5) were cultured in 10% FBS/DMEM medium in culture dishes and maintained at 37°C. After 12 hours of culture, culture medium was removed, and the cultures were washed with PBS, followed by addition of serum-free DMEM. Cells were harvested daily and cell number was counted by coulter counter.

**Hydrogen peroxide senescence assay**

Cells in 10% FBS/DMEM medium were treated with indicated concentrations of hydrogen peroxide for 2 hrs. After washing with PBS, the treated cells were incubated in fresh growth medium without H_2O_2 for 48 hours, and processed to β-gal staining.

**Side Population (SP) cell analysis**

Cells (2x10^6) were harvested from tissue culture dishes, washed, suspended at 10^6 cells per ml in DMEM containing 2% FCS, and pre-incubated at 37°C for 10 minutes. Samples were then cultured with 2.5 μg/ml Hoechst 33342 dye for 90 min, either alone or in combination with 50 μM Verapamil. The stained cells were analyzed by flow cytometry using a dual-wavelength analysis (blue, 424–444 nm; red, 675 nm) after excitation with 350-nm UV light. SP cell analysis was processed as previously described (Du et al., 2013b; Fang et al., 2013).

**Annexin V assay**

An Annexin V-FITC apoptosis detection kit (Biovision Inc) was used to detect apoptotic activity. Cells (1×10^6) were collected and resuspended in binding buffer, and incubated with Annexin V-FITC and propidium iodide in the dark for 15 min. Annexin V-FITC binding was determined by flow cytometry (Ex=488 nm; Em=530 nm) using FITC signal detector (FL1) and propidium staining by the phycoerythrin emission signal detector (FL2).

**Western blot**

Previous methods were used in this study (Fang et al., 2012; Siragam et al., 2012).

**Real-time PCR**

Total RNAs were extracted from 1x10^6 cells with mirVana miRNA Isolation Kit (Ambion) according to the manufacturer’s instructions. Real-time PCR was performed as described (Liu et al., 2013; Yang et al., 2013).

**Senescence β-Galactosidase staining**

Senescence β-gal staining was performed using senescence-galactosidase staining kit (Cell Signaling) according to manufacturer’s instructions. Briefly, cells were fixed for 5 min in 3.7 % formaldehyde, washed with PBS, and stained in β-galactosidase solution at 37°C until the
intensity of staining was visible in either experimental or control plates. The number of positive cells was counted under light microscope.

In tissue staining, mice were euthanized using carbon dioxide followed by isolation of the hearts. The heart tissues of wide type and transgenic mice were freshly excised, rapidly frozen in liquid nitrogen, and mounted in OCT. Sections (4 um) were cut, mounted onto glass slides, fixed in 2% formalin in PBS for 1 min at room temperature, and immersed in β-gal staining solution at 37°C for 24 hours. The samples were counterstained with eosin, and viewed under bright field at 40 x magnification.

**Immunohistochemistry (IHC)**

Heart tissues were freshly excised and fixed in 10% formalin overnight, immersed in 70% ethanol, embedded in paraffin, and sectioned. The sections were de-paraffinized with xylene and ethanol and then boiled in a pressure cooker. After washing with Tris-buffered-saline (TBS), the sections were blocked with 10% goat serum and incubated with primary antibody at 4°C overnight. The sections were incubated with biotinylated secondary antibody at 25°C for 2 hours, followed by avidin conjugated horseradish peroxidase provided by the Vectastain ABC kit (Vector, PK-4000). The slides were then stained with DAB, and Mayer’s Hematoxylin for counter staining.

**Immunoprecipitation assay**

Cells were washed in ice-cold phosphate-buffered saline, and lysed in 1 ml lysis buffer. Equal amounts of protein were incubated with 5 μg primary antibody and 40 μl of 50% slurry of protein A-Sepharose at 4°C for 4 h. The pellet was washed 3 times with PBS and were resuspended in 2× Laemmli buffer (0.125 M Tris HCl, 4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.004% bromphenol blue, pH6.8), followed by Western blot analysis.

**Chromotin immunoprecipitation (ChIP) assay**

ChIP was performed using SimpleChIP chromatin IP kit (Cell Signaling) according to the manufacturer’s instructions. Briefly, cells were treated with formaldehyde solution, and the chromatin was isolated, digested, and immunoprecipitated with antibody against Par4, CEBPB or FAK. 10% of inputs were applied to immunoblotting. The captured chromatin was eluted, uncross-linked, and the DNA was recovered. ChIP DNA was subject to PCR using specific primers flanking a piece of DNA sequence in the promoter region of CEBPB (using primers Cebpb-promo-F, 5’gttcaggagccaaagtcagagga, and mus.Cebpb-promo-R,
5’cttgccagcaagctacaccccag or FAK (using primers mus.Ptk2.promo-F, 5’agggcaagacggaggacaggtg, and mus.Ptk2.promo-R, 5’ttggtgcggccggactagaag)

**Statistical analysis**

All experiments were performed in triplicate and numerical data were subject to independent sample t-test. The levels of significance were set at *p<0.05 and **p<0.01.

**Results**

**Expression of miR-17 decreases MCF senescence and apoptosis**

We have previously reported that transgenic mice expressing miR-17 grew slower in the early stages of development (Shan et al., 2009), and developed liver tumors as being aged (Shan et al., 2013). To investigate the roles of miR-17 in cardiac development, we performed β-galactosidase (β-gal) staining on the heart tissues, which is an enzyme highly expressed and accumulated in the lysosomes in senescent cells. We found that the miR-17 transgenic mice displayed lower intensities of β-gal staining in the heart than the wildtype mice (Fig 1a), suggesting an inhibitory effect of miR-17 on cardiac senescence. To discover the role of miR-17 in senescence, we developed a mouse cardiac fibroblast (MCF) cell line stably transfected with either a miR-17 expression construct or a control vector. Expression of mature miR-17-5p and miR-17-3p was confirmed in Fig 1b. MCFs transfected with miR-17 showed extended survival (Fig 1c) and lower intensities of β-gal staining, when the cells were cultured in serum-free medium (Fig 1d). Expression of miR-17 also enhanced cell survival in other cell lines, including NIH3T3 (Supplementary Fig S1a, S1b) and BEAS2B (Fig S1c). When the cells were treated with H2O2 for 2 hours and cultured in basal medium for 48 hours, significantly more miR-17-transfected cells survived as compared with the vector-transfected cells (Fig 2a). However, less miR-17-transfected cells were stained by β-gal (Fig 2b, Fig S2a), suggesting decreased capacity in senescence. The miR-17-transfected cells showed a lower rate of apoptosis (Fig 2c), when cultured in serum-free medium or treated with H2O2, but enhanced proliferation (Fig 2d, Fig S2b) and increased population of side population (SP, an indication of cell self-renewal) cells (Fig 2e) when culture in 10% basal medium. Cells with greater capacity of self-renewal are less likely to undergo senescence.

**miR-17-3p represses senescence and apoptosis via targeting Par4**
Computational analysis showed that miR-17 precursor could produce mature miR-17-5p and miR-17-3p (Lagos-Quintana et al., 2001). Both mature miRNAs target a great number of mRNAs potentially. We looked for genes that play roles in mouse senescence, since we have found that ectopic expression of miR-17 decreases mouse aging (Du et al., 2014), by focusing on genes involved in mouse aging (http://genomics.senescence.info GENES/search.php?organism=Mus+musculus&show=4). We searched the potential binding sites of these genes for miR-17-5p and miR-17-3p using their 3’UTRs as described (http://bio.sz.tsinghua.edu.cn/) (Ye et al., 2008) and found that Par4 was a candidate targeted by miR-17-3p (Fig 3a, left). To confirm targeting of Par4 by miR-17-3p, we generated luciferase constructs harboring either a fragment of Par4 or the fragment containing mutation of the binding site (Fig 3a, right). Luciferase activities were repressed when the construct (Luc-Par4) was co-transfected with miR-17-3p mimic, and the repression was abolished when the target site was mutated confirming direct targeting of Par4 by miR-17-3p (Fig 3b). Protein lysates prepared from miR-17- and vector-transfected cells were subject to Western blot, which showed that ectopic transfection of miR-17 repressed Par4 expression significantly (Fig 3c, left). The target repression appeared to occur post-transcriptionally, since the levels of Par4 mRNAs were not affected by miR-17 transfection (Fig S2c). To confirm targeting by miR-17-3p, we transfected MCFs with miR-17-3p mimic, miR-17-3p anti-sense (miR-17-3p inhibitor), or a control oligo, and confirmed that Par4 expression was repressed by miR-17-3p, but promoted by miR-17-3p inhibitor (Fig 3c, right). Transfection of miR-17-3p inhibitor repressed MCF senescence and apoptosis when treated with H2O2, while expression of miR-17-3p enhanced senescence (Fig 3d) and apoptosis (Fig 3e). Consistent with these results, we found that the aging hearts expressed significantly lower levels of miR-17-3p compared with the young hearts (Fig 3f).

Expression of proteins in the related signaling pathway was examined to uncover the functions of miR-17-3p in cellular senescence. We found that miR-17-transfected MCFs expressed low levels of Par4 and E-cadherin, but high levels of N-cadherin, vimentin, Oct4, and Sca-1 (Fig 3g), suggesting that miR-17-3p might enhance MCF epithelial-mesenchymal transition (EMT) and self-renewal by targeting Par4. We then examined the relevance of these proteins in the heart tissues of the miR-17 transgenic mice and found that their levels were consistent with those detected in the miR-17-transfected cells (Fig 3h).
To corroborate the roles of Par4 in the system tested, we silenced endogenous Par4 with siRNAs targeting Par4. Par4 silencing produced low levels of Par4 and E-cadherin, and increased expression of N-cadherin, vimentin, Sox2, Oct4, and Sca-1 (Fig 4a). It also enhanced cell survival (Fig 4b, Fig S3a) and repressed senescence when cultured in serum-free medium or treated with H2O2 (Fig 4c, Fig S3b). It was noted that although three siRNAs targeting Par4 could silence Par4 expression in the protein level, only siRNA-1 and siRNA-2 displayed strong functions in cell survival and senescence, reaching the levels of significance. Silencing Par4 also alleviated serum-free medium or H2O2-induced apoptosis (Fig 4d). Consistent with these results, silencing Par4 promoted MCF proliferation in basal medium (Fig S3c). Par4 siRNA expressing MCFs showed high proportion of SP cells, indicating silencing Par4 enhanced self-renewal (Fig S4a).

To validate the roles of Par4, overexpression of Par4 in the miR-17-transfected MCFs repressed cell survival when the cells were cultured in serum-free medium and treated with H2O2 (Fig 5a) or cultured in serum-containing medium (Fig S4b). Ectopic expression of Par4 also promoted senescence when the cells were cultured in serum-free medium or treated with H2O2 (Fig 5b, Fig S4c). Expression of Par4 enhanced cell apoptosis induced by serum-free medium or H2O2 induced (Fig 5c). The cells were also analyzed for the population of SP cells, which showed that Par4 transfection decreased SP cell population significantly (Fig 5d). We then analyzed proteins involved in cell self-renewal and found that ectopic transfection of Par4 increased E-cadherin level but decreased expression of N-cadherin, vimentin, Sox2, Oct4 and Sca-1 (Fig 5e).

**Expression of Par4 negatively regulated CEBPB and FAK transcription.**

Proteins in the signaling pathway mediating Par4 repression of EMT and self-renewal were examined. Both CEBPB and FAK were found highly expressed in the miR-17- and Par4 siRNA-transfected MCFs (Fig 6a), and the effect appeared to occur at transcriptional level (Fig 6b). To examine how CEBPB and FAK expression was up-regulated, we isolated chromatin from the miR-17- and vector-transfected MCFs, followed by protein-DNA binding assay. The precipitated DNA was subject to PCR with specific primers flanking a piece of DNA sequence at the CEBPB or FAK promoter. Transfection with miR-17 decreased Par4 binding to CEBPB promoter (Fig 6c). In cells transfected with Par4 siRNA, antibody against CEBPB pulled-down higher levels of FAK promoter compared with the control (Fig 6d). However, with more FAK
pulled-down, Par4 siRNA-transfected MCFs did not show any FAK binding to CEBPB promoter, as compared with the control oligo-transfected cells (data not shown). The above ChIP assay indicated that Par4 might directly combine with CEBPB promoter, and repress CEBPB transcription, while CEBPB could bind FAK promoter, and enhance FAK transcription. On Western blotting, we confirmed that silencing Par4 promoted expression of CEBPB and FAK (Fig 6e).

To validate the interaction of Par4, CEBPB and FAK, a Par4 plasmid was transfected to miR-17 expressing MCFs. The Par4 expressing cells showed high levels of Par4 and low levels of CEBPB and FAK, and the modification appeared to occur at transcriptional levels (Fig 7a). Nevertheless, there could be translational and/or post translational modification, as CEBPB protein was hardly detected. In addition, ectopic transfection of CEBPB enhanced FAK expression at the mRNA and protein levels (Fig 7b). In protein-DNA binding assay, ectopic transfection of Par4 was shown to pull-down more CEBPB promoter DNA (Fig 7c), while ectopic transfection of CEBPB could pull-down more FAK promoter DNA (Fig 7d). This confirmed that Par4 could bind to CEBPB promoter, repressing CEBPB transcription, while CEBPB could bind to FAK promoter, enhancing FAK transcription.

In summary, we show that miR-17-3p targets Par4, repressing Par4 expression in MCFs. Down-regulation of Par4 promotes CEBPB transcription, which further enhances FAK transcription. Expression of CEBPB and FAK promote EMT and self-renewal. Pheno-typically, the miR-17 expressing MCFs show repressed senescence and apoptosis, and enhanced cell growth and survival (Fig 7e).

Discussion

In this study, we found that transgenic expression of miR-17 suppressed mouse cardiac senescence (organismal senescence) and MCF senescence (cellular senescence). Although multiple miRNAs have been reported to regulate senescence positively or negatively (Boehm and Slack, 2005; Boehm and Slack, 2006; Ibanez-Ventoso and Driscoll, 2009; Liu et al., 2012; Yu et al., 2013), most studies focused on the effect of miRNAs on senescence of tumor cells at the cellular level, or investigated the longevity role of miRNAs in lower organisms (Bilsland et al., 2013; Jung and Suh, 2013; Smith-Vikos and Slack, 2012). Uniquely, our study systematically describes the functions of one specific miRNA, mir-17, in controlling senescence and apoptosis.
of mouse heart and cardiac fibroblasts. We found that miR-17 played these roles by targeting Par4, and promoting CEBPB/FAK senescence related signaling pathway in the transgenic mice and transfected cells.

Par-4, encoded by the Pawr gene, is a pro-apoptotic protein that is up-regulated in response to apoptotic stimuli and is required for cell apoptosis (Hebbar et al., 2012; Zhao and Rangnekar, 2008). Par-4 expresses in low levels in a variety of human cancers, and increased expression of Par4 may induce cell death in some cancer cell lines (Cook et al., 1999; Garcia-Cao et al., 2005; Ranganathan and Rangnekar, 2005). Thus, Par-4 is believed to be a tumor suppressor and a critical regulator of tumor cell survival. Emerging evidence has implicated Par-4 down-regulation as a prognostic factor in cancer (Alvarez et al., 2013). Further study indicated that the SAC domain of Par-4 conferred cancer resistance in transgenic mice without compromising normal viability or aging, and may have therapeutic significance (El-Guendy et al., 2003).

Our study demonstrated that Par4 played important roles in cardiac senescence, which is related to its negative regulation of transcript factors, CEBPB and FAK. Silencing Par4 markedly promoted CEBPB transcription, while expression of CEBPB could bind to FAK promoter and enhanced its transcription. CEBPB is essential for mammary gland growth and development and has been associated with poor prognosis in breast cancer (Russell et al., 2010; Zahnow, 2009). Overexpression of CEBPB in MCF10A cells was reported to result in EMT and ErbB independence (Russell et al., 2010). Focal Adhesion Kinase (FAK) has kinase-dependent and kinase independent scaffolding, cytoplasmic and nuclear functions (Golubovskaya, 2014). It was found to be over-expressed in many types of human cancer (Ben Mahdi et al., 2000; Golubovskaya, 2014; Golubovskaya et al., 2008; Sood et al., 2004). FAK plays an important role in cell adhesion, spreading, motility, invasion, and angiogenesis (Schlaepfer et al., 2004).

Recently, FAK was reported to play an important role in epithelial to mesenchymal transition (EMT) (Fan et al., 2012; Gjorevski et al., 2011; Golubovskaya, 2014). As a cytoplasmic tyrosine kinase, FAK is identified as a key mediator of intracellular signaling by integrins, a major family of cell surface receptors for extracellular matrix, in the regulation of different cellular functions in a variety of cells (Aplin et al., 1998; Mehta, 2011). Both FAK and integrins were reported to play crucial roles in the maintenance of stemness of mammary stem cells (MaSCs), and serve as a functional marker for MaSCs (Guan, 2010; Luo and Guan, 2009).
Thus we identified miR-17-3p as senescence related miRNA which represses mouse cardiac fibroblast senescence and apoptosis under stresses. In addressing the role of miR-17-3p in cellular senescence, we identified Par4, CEBPB, and FAK as key regulators of senescence and apoptosis. The novel Par4/CEBPB/FAK signaling pathway was uncovered to repress senescence and apoptosis via enhanced EMT and self-renewal phenotype. Identifying and understanding senescence miRNAs and genes will be important in uncover the mechanisms of senescence and application of targeting therapy.

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**Conflict of Interest:** All authors declare no conflict of interest for the study.

**Reference**


Figure Legends

Fig 1. Expression of miR-17 repressed heart senescence.

(a) Sections of heart of miR-17 transgenic (14 samples) and wild type (12 samples) mice were subject to β-gal staining. Transgenic mice had less β-gal staining in the heart.

(b) RT-PCR showed that miR-17 transfected mouse cardiac fibroblasts (MCFs) expressed high levels of miR-17-3p and -5p compared with mock control cells. Asterisks indicate significant differences. *, p<0.05, **, p<0.01. Error bars, SD (n=4).

(c) Mock- and miR-17-transfected MCFs were cultured in serum-free medium for 6 days. Transfection with miR-17 increased cell viability compared with control (left) reaching significant differences two days after cell culture (right). Asterisks indicate significant differences. *, p<0.05, **, p<0.01. Error bars, SD (n=6).

(d) Staining with β-gal of the above survival cells after showed that miR-17 transfection inhibited cell staining (left). Asterisks indicate significant differences (right). **, p<0.01. Error bars, SD (n=6).

Fig 2. miR-17 repressed CFs cellular senescence and apoptosis

(a) Mock- or miR-17-transfected MCFs were treated with indicated concentrations of H₂O₂ for 2 hours, and cultured in basal medium for 48 hours. Enhanced cell survival was detected in the miR-17-transfected MCFs. *, p<0.05, **, p<0.01. Error bars, SD (n=6).

(b) The number of β-gal stained MCFs were quantified. miR-17 transfection showed less β-gal staining when treated with H₂O₂. **, p<0.01. Error bars, SD (n=6).

(c) Mock- or miR-17-transfected MCFs were culture in serum-free medium for 48 hours, or treated with 0.6 mM H₂O₂ for 2 hours, and subject to Annexin V staining, followed by flow cytometry analysis. miR-17 transfection decreased cell apoptosis. Shown is the representative result of three independent experiments.

(d) Mock- or miR-17-transfected MCFs were culture in basal medium for 6 days. Cell number was counted every day. miR-17 transfection increased cell viability. *, p<0.05, **, p<0.01. Error bars, SD (n=6).

(e) Mock- or miR-17-transfected MCFs were subject to Hoechst 33342 staining, followed by flow cytometry for SP cells (left). There were higher proportions of SP cells in the miR-17-transfected MCFs compared with the control (right). **, p<0.01. Error bars, SD (n=6).

Fig 3. miR-17 targets Par4, and promotes EMT and self-renewal.
(a) Left, Computational analysis indicated that miR-17-3p potentially targets Par4. Right, The luciferase construct Luc-Par4, with a fragment of 3'UTR of mouse Par4, and mutant construct Luc-Par4-mut, mutation of the miR-17-3p binding site, were generated.

(b) 293T cells were co-transfected with miR-17-3p and luciferase reporter construct (Luc-Par4) or a mutant (Luc-Par4-mut). Luciferase activities of Luc-Par4 were inhibited when co-transfected with miR-17-3p mimic, which was reversed when the miR-17-3p binding site was mutated. **, $p<0.01$. Error bars, SD ($n=4$).

(c) Left, Protein lysates prepared from miR-17- and vector-transfected MCFs were subject to immunoblotting probed with antibodies against Par4 and β-actin. miR-17 expression decreased Par4 level. Right, Protein lysates of MCFs transfected with control oligos, miR-17-3p inhibitor or miR-17-3p mimic were subject to immunoblotting probed with antibodies against Par4 and β-actin. Par4 level was repressed by miR-17-3p but enhanced by the inhibitor. Both experiments were repeated three times, followed by densitometry analysis. **, $p<0.01$. Error bars, SD ($n=3$).

(d) The cells were treated with 150 µM H$_2$O$_2$ for 2 hours, and then cultured in basal medium for 48 hours followed by β-gal staining. Treatment with miR-17-3p inhibitor increased senescence but treatment with miR-17-3p decreased senescence. **, $p<0.01$. Error bars, SD ($n=6$).

(e) The transfected MCFs treated with 0.6 mM H$_2$O$_2$ for 12 hours were subject to apoptosis assay. Treatment with miR-17-3p inhibitor increased apoptosis but treatment with miR-17-3p decreased apoptosis.

(f) RNAs were isolated from aging and young hearts and subject to real-time PCR. The levels of miR-17-3p were significantly higher in the young hearts than in the old hearts.

(g) Protein lysates were subject to western blot assay. miR-17 expression in MCFs showed decreased expression of E-cadherin, and increased expression of N-cadherin, vimentin, Sox2, Oct4, and Sca-1.

(h) Sections of miR-17 transgenic and wild type hearts were subject to immunohistochemistry assay. miR-17 transgenic mice showed increased expression of N-cadherin, Oct4, Sox2, and Sca-1, but decreased expression of Par4 and E-cadherin.

**Fig 4. Silencing Par4 enhanced CFs senescence and apoptosis.**
(a) Protein lysates prepared from MCFs transfected with siRNAs targeting Par4 (sequences provided in Methods) and control oligo were subject to immunoblotting. Transfection with Par4 siRNAs decreased levels of Par4 and E-cadherin, but increased levels of N-cadherin, vimentin, Sox2, Oct4, and Sca-1.

(b) The Par4 siRNA-transfected MCFs were culture in basal medium for 6 days or treated with H2O2. Transfection with two of the three Par4 siRNAs showed increased viability. *, p<0.05, **, p<0.01. Error bars, SD (n=6).

(c) The number of β-gal stained MCFs were quantified. Par4 siRNA-transfected (Si-1 and Si-2) MCFs showed less β-gal staining. **, p<0.01. Error bars, SD (n=6).

(d) The Par4 siRNA-transfected MCFs were cultured in serum-free medium for 48 hours (left), or cultured in 1.0 mM H2O2 for 2 hours (right) followed by Annexin V staining. Par4 siRNAs (Si-1 and Si-2) transfection showed decreased levels of Annexin V staining.

Fig 5. Expression of Par4 promotes senescence and apoptosis.

(a) The miR-17-transfected MCFs were transfected with Par4 or a control vector. The cells were cultured in serum-free medium for 2 days (left) or treated with H2O2 for 12 hours (right). Ectopic expression of Par4 decreased cell viability. *, p<0.05. Error bars, SD (n=6).

(b) The cells were subject to β-gal staining. Par4 transfection increased β-gal staining when the cells were cultured in serum-free medium (left) or treated with 150 µM H2O2 for 2 hours and then cultured in basal medium for 48 hours (right). **, p<0.01. Error bars, SD (n=6).

(c) The cells were also subject to Annexin V staining. Ectopic expression of Par4 increased cell apoptosis when the cells were cultured in serum-free medium for 48 hours (left) or treated with 0.6 mM H2O2 for 12 hours (right).

(d) The cells were subject to Hoechst 33342 staining, followed by flow cytometry for SP cells. Par4-transfected MCFs showed low proportion of SP cells (left), which reached significant levels (right). *, p<0.05. Error bars, SD (n=6).

(e) Protein lysates were subject to immunoblotting. Ectopic transfection of Par4 increased E-cadherin level but decreased expression of N-cadherin, vimentin, Sox2, Oct4 and Sca-1.

Fig 6. Silencing Par4 enhanced CEBPB and FAK transcription.

(a) Protein lysates from miR-17-transfected (left) or Par4 siRNA (siRNA-1)-transfected (right) MCFs were subject to immunoblotting. Transfection with miR-17 or Par4 siRNA decreased expression of Par4, but increased expression of CEBPB and FAK.
(b) RT-PCR showed that anti-Par4 siRNA (siRNA-1)-transfected MCFs expressed high mRNA levels of CEBPB (upper) and FAK (lower) compared with control cells. *, $p<0.05$, **, $p<0.01$. Error bars, SD ($n=4$).

(c) Upper, Chromatinns from miR-17- or control vector-transfected MCFs were isolated, digested and immuno-precipitated with rabbit IgG (negative control) and antibodies against Histone H3 (positive control) or Par4, followed by PCR with primers flanking a DNA sequence at the CEBPB or FAK promoter. Lower, in miR-17-transfected MCFs, there had less Par4 binding to CEBPB promoter. **, $p<0.01$. Error bars, SD ($n=4$).

(d) Upper, Chromatinns from Par4 siRNA (siRNA-1)- or control oligo-transfected MCFs were processed as above, followed by PCR with primers flanking a DNA sequence at the FAK or CEBPB promoter. Lower, anti-Par4 siRNA-transfected MCFs had more CEBPB binding to FAK promoter. **, $p<0.01$. Error bars, SD ($n=4$).

(e) Protein lysates from Par4 siRNA (siRNA-1)- or control oligo-transfected MCFs were subject to immuno-precipitation as indicated, followed by Western blotting. Less Par4 was pulled-down in the Par4 siRNA-transfected MCF. However, more CEBPB and FAK were pulled-down in the Par4 siRNA-transfected MCF lysates.

**Fig 7. Expression of Par4 repressed CEBPB and FAK transcription.**

(a) Par4 or vector was transiently transfected into the miR-17-transfected MCFs. Protein lysates were prepared for immunoblotting. Par4 expression decreased CEBPB and FAK expression (left). Real-time PCR analysis showed that Par4 expression decreased levels of CEBPB and FAK compared with the control. *, $p<0.05$, **, $p<0.01$. Error bars, SD ($n=4$).

(b) Protein lysates prepared from CEBPB- or vector-transfected MCFs were subject to immunoblotting. CEBPB transfection increased CEBPB and FAK expression (upper). Real-time PCR analysis showed that CEBPB expression increased mRNA levels of FAK compared with control cells. **, $p<0.01$. Error bars, SD ($n=4$).

(c) Upper, Chromatinns from vector- and Par4 -transfected MCFs were isolated, digested and immuno-precipitated with rabbit IgG (negative control) and antibodies against Histone H3 (positive control) or Par4, followed by PCR with primers flanking a DNA sequence at the CEBPB promoter. Lower, Par4 transfection showed higher levels of CEBPB promoter binding to Par4 protein. **, $p<0.01$. Error bars, SD ($n=4$).
(d) Upper, Chromatins from vector- and CEBPB-transfected MCFs were processed as above, followed by PCR with primers flanking a DNA sequence at the FAK promoter. Lower, CEBPB transfection showed higher levels of FAK promoter binding to CEBPB protein. **, $p<0.01$. Error bars, SD ($n=4$).

(e) Mechanisms of miR-17 repressing MCFs senescence and apoptosis. miR-17 targets Par4, repressing Par4 expression. Down-regulation of Par4 promotes EMT and self-renewal, which represses MCFs senescence and apoptosis, and enhances cell growth and survival.
Fig 1

**a**

Wildtype and miR-17 samples at x20 and x40 magnification. 

β-gal positive: wildtype (7/12), miR-17 transgenic mice (0/14).

**b**

Bar charts showing the relative levels of miR-17-3p and miR-17-5p. 

**c**

Images of cardiac fibroblast cell survival over 0, 4, and 6 days for control and miR-17 groups. 

**d**

Images of β-gal positive staining for control and miR-17 groups. 

β-gal positive: wildtype (7/12), miR-17 transgenic mice (0/14).
Fig 2

(a) miR-17 transfected CF cell proliferation in 10% FBS/DMEM medium. 

(b) CF cell treated with H2O2 for 48 hours. 

(c) CF cells cultured in serum free medium for 48 hours. 

(d) miR-17 transfected CF cell proliferation in 10% FBS/DMEM medium. 

(e) SP Cells (%) treated with 0.6 mM H2O2 for 2 hours.
**miR-17-3p**

*Par4*

GAGCTC

5'ACGCCTGTG-ATTGTAGGCTTT-1289

Luc-Par4

Luc-Par4-mut

**β-actin**

**E-cadherin**

**N-cadherin**

**vimentin**

**Sox2**

**Oct4**

**Sca-1**

**β-actin**

**Control miR-17-3p IN**

**miR-17-3p inhibitor**

**kDa**

37

150

50

37

20

**Relative levels of miR-17-3p**

4 week

1.5 year

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Fig 4

**a**

Par4

β-actin

E-cadherin

β-actin

N-cadherin

β-actin

vimentin

β-actin

Sox2

β-actin

Oct4

β-actin

Sca-1

β-actin

**b**

Serum-Free

Cell survival (x10^5 cells)

Control Si-1 Si-2 Si-3

** **

Cell survival (x10^5 cells)

H2O2 (1.5 mM)

** **

**c**

β-gal staining cells (%)

Control Si-1 Si-2 Si-3

** **

β-gal staining cells (%)

Treated with H2O2

** **

CF cells cultured in serum free medium for 48 hours

CF cell treated with 1.0 mM H2O2 for 2 hours
Fig 5

a) Cell survival (x10^5 cells) and cell number (10^5 cells) for control and Par4-treated cells after 0 and 5 days (Serum-Free) and treated with H2O2 for 6 hours.

b) β-gal staining cells (%) for control and Par4-treated cells with varying H2O2 concentrations.

c) Flow cytometry analysis of CF cells treated with serum-free medium for 2 days and with 0.6 mM H2O2 for 12 hours.

d) Flow cytometry with DAPI and Efluor 650-A:HO342 showing SP cells (%).

e) Western blot analysis of control and Par4-treated cells for various proteins.
Expression of CEBPB promoter signal

Levels of CEBPB promoter binding to Par4

Levels of FAK promoter binding to CEBPB

Fig 6
Fig 7

Levels of Fak promoter binding to CEBPB

Levels of CEBPB promoter binding to Par4

Relative mRNA levels of FAK

Relative mRNA levels of CEBPB

Control Par4

Control CEBPB

Control Par4

Control Par4

E-cadherin  N-cadherin  Vimentin

SP cells  Sox2  Oct4  Sca-1

Senescence

Apoptosis

Cell growth

Cell Survival