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RESEARCH ARTICLE

The 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. The aim of this study was 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 attenuates cardiac aging in the myocardium by targeting Par4 (also known as PAWR). This upregulates the downstream proteins CEBPB, FAK, N-cadherin, vimentin, Oct4 and Sca-1 (also known as stem cell antigen-1), and downregulates E-cadherin. Par4 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 enhances the transcription of CEBPB and FAK, which promotes mouse cardiac fibroblast (MCF) epithelial-to-mesenchymal transition (EMT) and self-renewal, resulting in cellular senescence and apoptosis resistance. We conclude that Par4 can bind to the CEBPB promoter and inhibit its transcription. Decreased Par4 expression increases the amount of CEBPB, which binds to the FAK promoter 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 cardiac fibroblast cellular senescence.

KEY WORDS: microRNA, miR-17, Senescence

INTRODUCTION

The aging heart undergoes 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 crucial 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 reduces 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 β-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 that involving p53 and Rb, which leads 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 ~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 those involved in 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-19b1 and miR-92a-1) has been reported to play a fundamental role in heart and lung development and remodeling (Bonauer and Dimmeler, 2009; Dakhlallah et al., 2013; Shan et al., 2009). Furthermore, members of the *miR-17-92* cluster are found to be commonly downregulated 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 (MCF) cell model and in transgenic mice.

We propose that miR-17-3p attenuates cardiac aging in the myocardium by targeting Par4 (also known as 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 upregulated specifically during prostate cancer cell apoptosis (Chaudhry et al., 2014). We found that Par4 is expressed in the mature myocardium and interacts directly with the promoter region of the 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 upregulated by Par4. FAK signaling activates the epithelial-to-mesenchymal transition (EMT) and self-renewal programs that repress cellular senescence in cardiac fibroblasts. We further identified Par4 as a bona fide target of miR-17-3p, which attenuated the senescent phenotype in transfected primary cardiac fibroblasts as well as in a transgenic mouse line overexpressing miR-17-3p. Through this novel Par4–CEBPB–FAK signaling axis that was mediated by miR-17-3p, we have elucidated a novel

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pathway in cellular senescence, with therapeutic implications of senescence-related miRNAs in the aging heart.

RESULTS

Expression of miR-17 decreases MCF senescence and apoptosis

We have reported previously that transgenic mice expressing miR-17 grow more slowly in the early stages of development (Shan et al., 2009), and develop liver tumors as they age (Shan et al., 2013). To investigate the roles of miR-17 in cardiac development, we performed β-galactosidase (β-gal) staining on heart tissues, because β-gal is an enzyme that is highly expressed and accumulates 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 wild-type 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 MCF cell line that was 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 by using real-time PCR (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

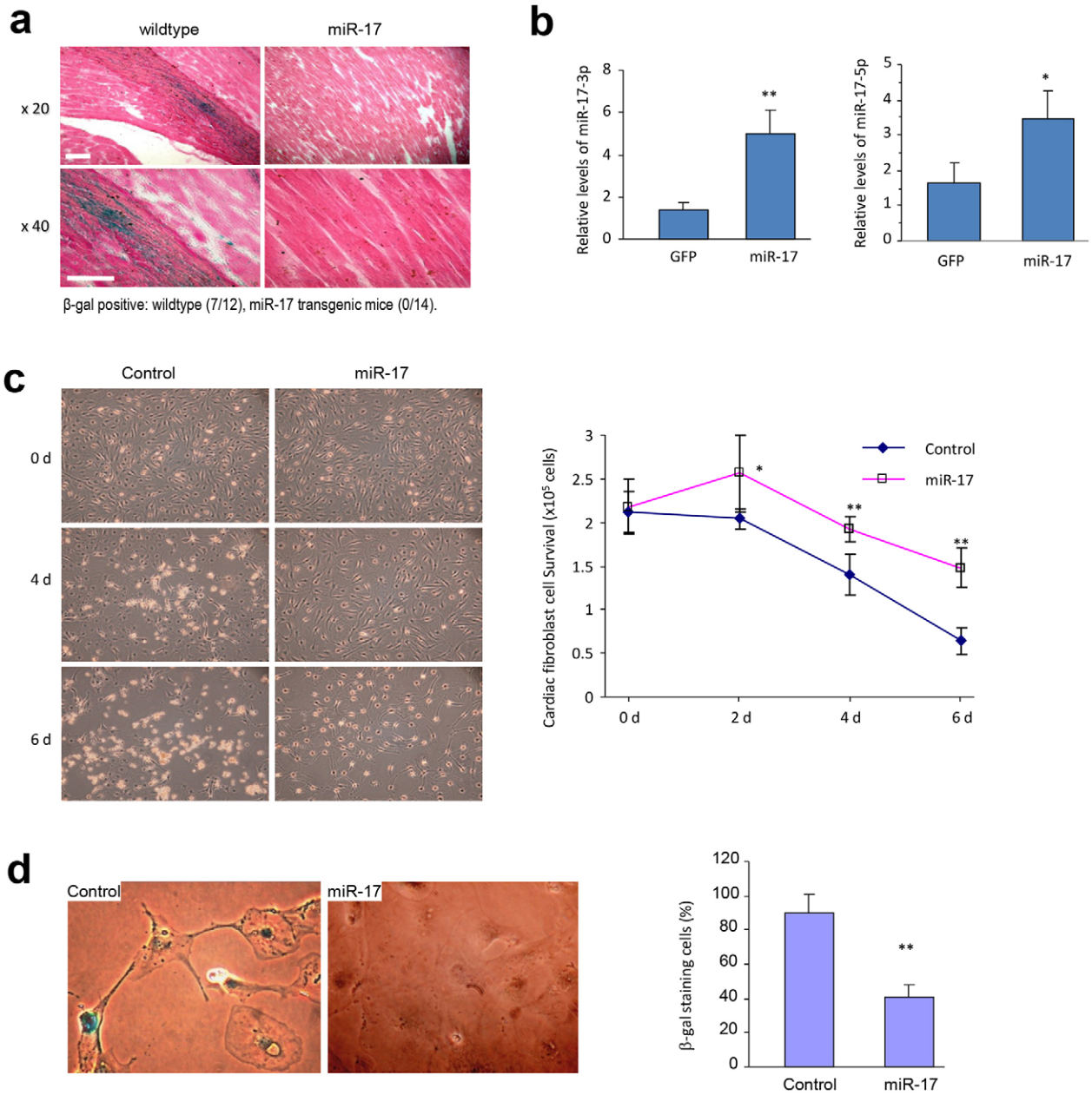

Fig. 1. Expression of miR-17 represses heart senescence. (A) Sections of heart from miR-17 transgenic (14 samples) and wild-type (12 samples) mice were subjected to β-gal staining. Transgenic mice had less β-gal staining in the heart. (B) RT-PCR showed that miR-17-transfected MCFs expressed high levels of miR-17-3p and -5p compared with mock control cells. $n=4$. (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 after 2 days of cell culture (right). $n=6$. (D) Staining for β-gal in the surviving cells from C showed that miR-17 transfection inhibited cell staining (left). $n=6$. All quantitative data show the mean±s.d.; *$P<0.05$; **$P<0.01$. Scale bars: 50 μm.

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lines, including NIH3T3 (supplementary material Fig. S1A,B) and BEAS2B cells (supplementary material Fig. S1C). When the cells were treated with H_2O_2 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, fewer miR-17-transfected cells were stained by β-gal (Fig. 2B; supplementary material Fig. S2A), suggesting decreased levels of senescence. The miR-17-transfected cells showed a lower rate of apoptosis (Fig. 2C), when cultured in serum-free medium or treated with H_2O_2, but displayed enhanced proliferation (Fig. 2D; supplementary material Fig. S2B) and a larger side population in flow cytometric analyses (an indication of self-renewal) (Fig. 2E) when cultured in 10% basal medium. Cells with a greater capacity for self-renewal are less likely to undergo senescence.

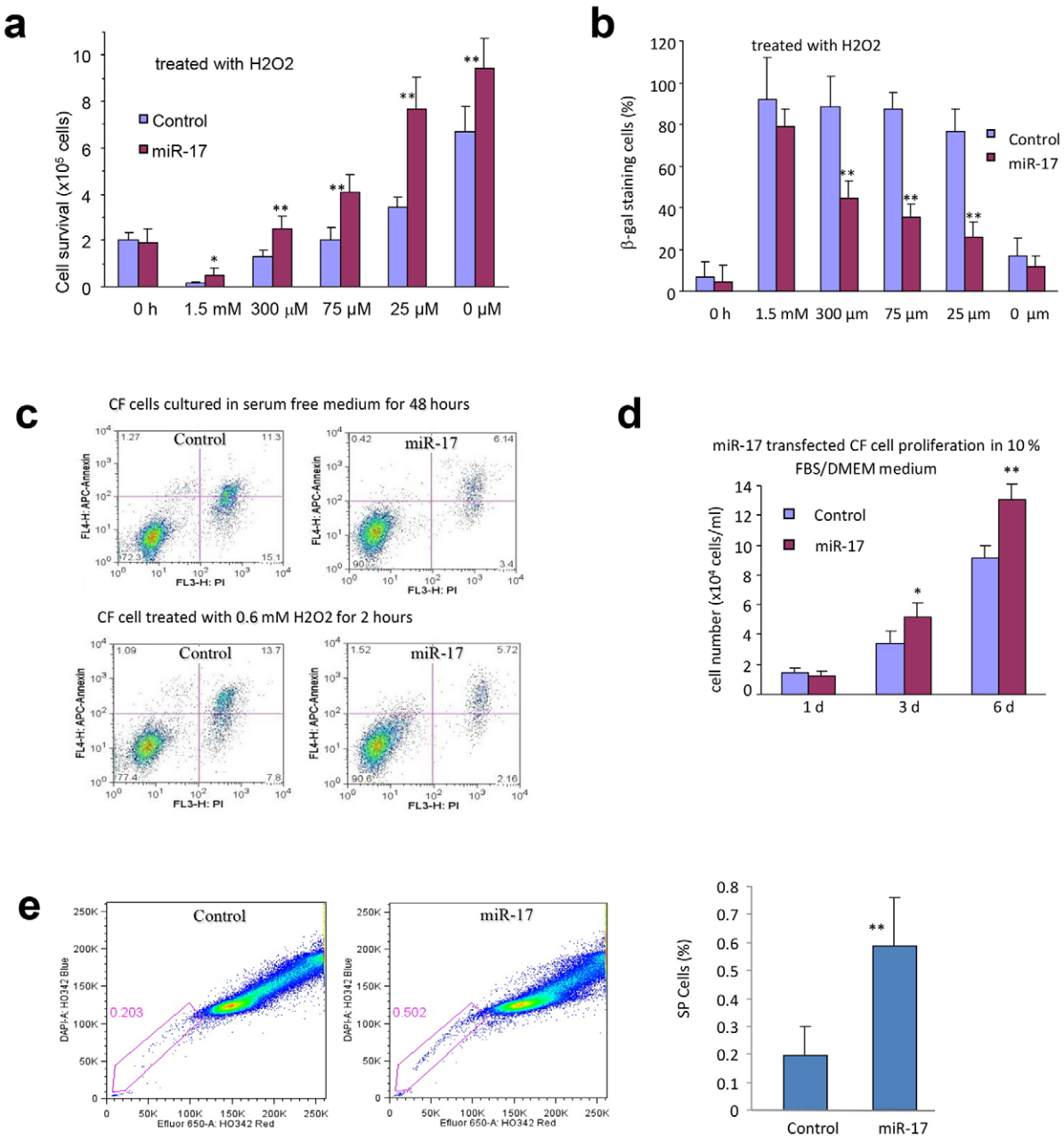

Fig. 2. miR-17 represses cellular senescence and apoptosis in MCFs. (A) Mock- or miR-17-transfected MCFs were treated with the indicated concentrations of H_2O_2 for 2 hours and cultured in basal medium for 48 hours. Enhanced cell survival was detected in the miR-17-transfected MCFs. $n=6$. (B) The number of β-gal-stained MCFs was determined. miR-17 transfection resulted in less β-gal staining when transfected cells were treated with H_2O_2. $n=6$. (C) Mock- or miR-17-transfected MCFs were cultured in serum-free medium for 48 hours or treated with 0.6 mM H_2O_2 for 2 hours, and subjected to annexin V staining, followed by flow cytometric analysis. miR-17 transfection decreased cell apoptosis. Shown is the representative result of three independent experiments. CF, cardiac fibroblast. (D) Mock- or miR-17-transfected MCFs were cultured in basal medium for 6 days. Cell number was counted every day. miR-17 transfection increased cell viability. $n=6$. (E) Mock- or miR-17-transfected MCFs were subjected to Hoechst 33342 staining, followed by flow cytometry for side population (SP) cells (left). There were higher proportions of side population cells in the miR-17-transfected MCF populations compared with the control samples (right). $n=6$. All quantitative data show the mean±s.d.; $^*P<0.05$; $^{**}P<0.01$.

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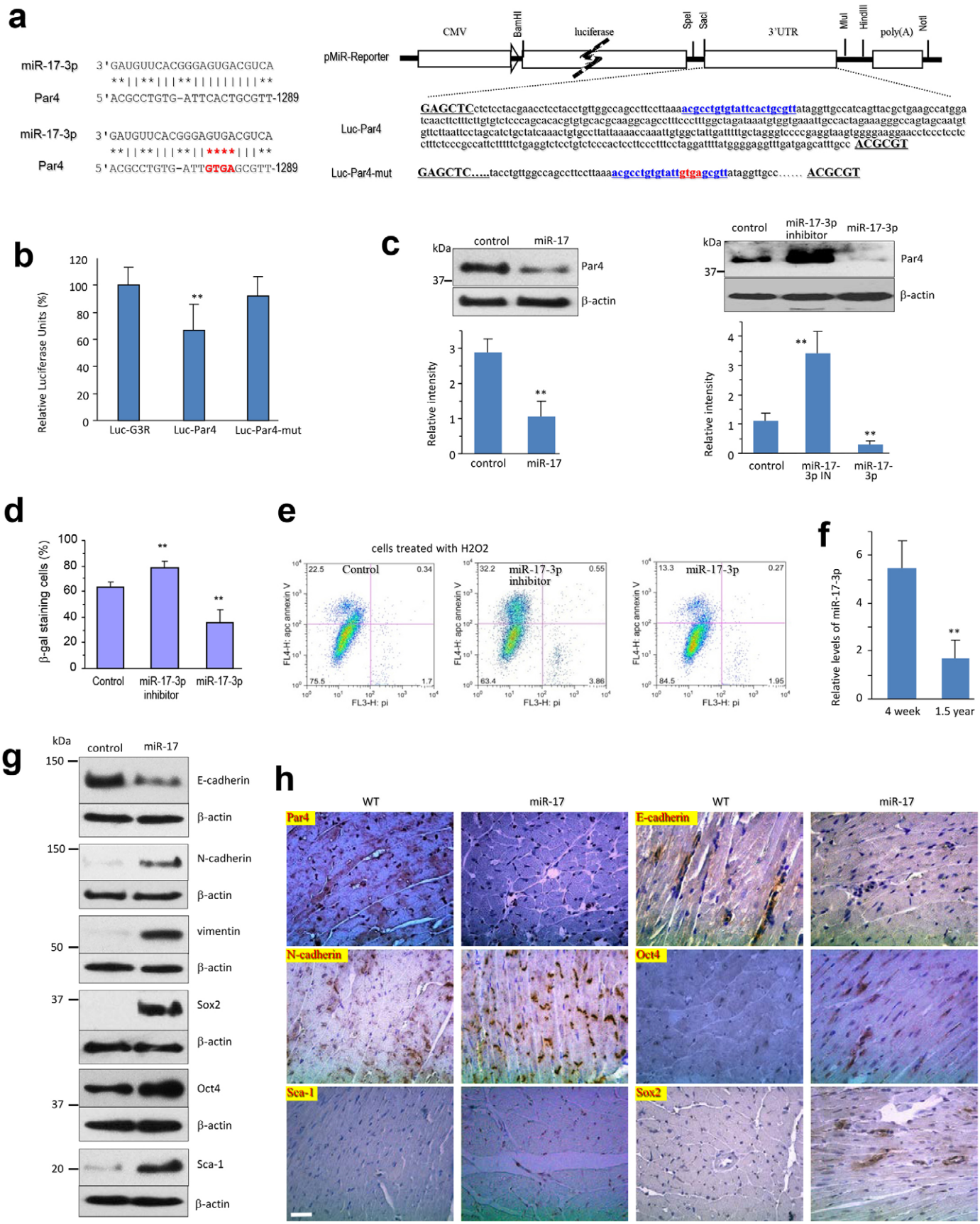

Fig. 3. See next page for legend.

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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 the 3′UTR of mouse Par4, and the mutant construct Luc-Par4-mut, containing a mutation of the miR-17-3p binding site, are shown. (B) 293T cells were co-transfected with miR-17-3p and either the wild-type luciferase reporter construct (Luc-Par4) or the mutant version (Luc-Par4-mut). Luciferase activities of Luc-Par4 were inhibited when it was co-transfected with the miR-17-3p mimic, which was reversed when the miR-17-3p binding site was mutated. $n=4$. (C) Left, protein lysates prepared from miR-17- and vector-transfected MCFs were subjected to immunoblotting with antibodies against Par4 and β-actin. miR-17 expression decreased the amount of Par4. Right, protein lysates of MCFs transfected with control oligos, miR-17-3p inhibitor or miR-17-3p mimic were subject to immunoblotting with antibodies against Par4 and β-actin. The expression of Par4 was repressed by miR-17-3p but enhanced by the inhibitor. Both experiments were repeated three times, followed by densitometric analysis. $n=3$. (D) The cells were treated with 150 μM H_2O_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. $n=6$. (E) The transfected MCFs treated with 0.6 mM H_2O_2 for 12 hours were subjected to an apoptosis assay. Treatment with the miR-17-3p inhibitor increased apoptosis, but treatment with miR-17-3p decreased apoptosis. (F) RNAs were isolated from aging and young hearts and subjected to real-time PCR. The levels of miR-17-3p were significantly higher in the young hearts than in the old hearts. All quantitative data show the mean±s.d.; $^{**}P<0.01$. (G) Protein lysates were subjected to a western blotting assay. miR-17 expression in MCFs resulted in 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 (WT) hearts were subjected to an 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. Scale bars: 50 μm.

miR-17-3p represses senescence and apoptosis by targeting Par4

Computational analysis showed that the miR-17 precursor could produce mature miR-17-5p and miR-17-3p (Lagos-Quintana et al., 2001). Both mature miRNAs potentially target a great number of mRNAs. We looked for genes that play roles in mouse senescence, given that we have previously 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 3′ untranslated regions (3′UTRs) of these genes for potential miR-17-5p and miR-17-3p binding sites using an online target prediction resource (http://bio.sz.tsinghua.edu.cn/) as described previously (Ye et al., 2008), and we 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 same fragment containing a mutation in the binding site (Fig. 3A, right). Luciferase activity was 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, thus confirming direct targeting of Par4 by miR-17-3p (Fig. 3B). Protein lysates prepared from miR-17- and vector-transfected cells were subjected to western blotting, which showed that ectopic transfection of miR-17 repressed Par4 expression significantly (Fig. 3C, left). The target repression appeared to occur post-transcriptionally, because the levels of Par4 mRNAs were not affected by miR-17 transfection (supplementary material Fig. S2C). To confirm targeting by miR-17-3p, we transfected MCFs with miR-17-3p mimic, miR-17-3p anti-sense (a miR-17-3p inhibitor) or a control oligo, and confirmed that Par4 expression was repressed by miR-17-3p, but was promoted by miR-17-3p inhibitor (Fig. 3C, right). Transfection of the miR-17-3p inhibitor enhanced MCF senescence and apoptosis in cells treated with H_2O_2, whereas expression of miR-17-3p repressed 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 levels in hearts from young animals (Fig. 3F).

The 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 (also known as stem cell antigen-1) (Fig. 3G), suggesting that miR-17-3p might enhance EMT and self-renewal of MCFs by targeting Par4. We then examined the relevance of these proteins in the heart tissues of the miR-17 transgenic mice, and we 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 small interfering (si)RNAs. Par4 silencing resulted in reduced 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; supplementary material Fig. S3A) and repressed senescence when cells were cultured in serum-free medium or treated with H_2O_2 (Fig. 4C; supplementary material Fig. S3B). It was noted that although three siRNAs targeting Par4 could silence Par4 expression at the protein level, only siRNA-1 and siRNA-2 displayed strong and statistically significant effects on cell survival and senescence. Silencing Par4 also reduced the amount of apoptosis induced by culturing cells in serum-free medium or in the presence of H_2O_2 (Fig. 4D). Consistent with these results, silencing Par4 promoted MCF proliferation in basal medium (supplementary material Fig. S3C). MCF cultures that were transfected with Par4 siRNA showed a high proportion of side population cells, indicating that silencing Par4 enhanced self-renewal (supplementary material Fig. S4A).

To validate the roles of Par4, we overexpressed Par4 in miR-17-transfected MCFs. We found that this repressed cell survival when the cells were cultured in serum-free medium or treated with H_2O_2 (Fig. 5A) or cultured in serum-containing medium (supplementary material Fig. S4B). Ectopic expression of Par4 promoted senescence when the cells were cultured in serum-free medium or treated with H_2O_2 (Fig. 5B; supplementary material Fig. S4C). Expression of Par4 enhanced cell apoptosis induced by serum-free medium or H_2O_2 (Fig. 5C). The number of side population cells was analyzed by flow cytometry, which showed that Par4 transfection decreased the size of this population significantly (Fig. 5D). We then analyzed proteins involved in cell self-renewal and found that transfection of Par4 increased the level of E-cadherin but decreased the expression of N-cadherin, vimentin, Sox2, Oct4 and Sca-1 (Fig. 5E).

Expression of Par4 negatively regulates 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 to be highly expressed in the miR-17- and Par4-siRNA-transfected MCFs (Fig. 6A), and the effect appeared to occur at the transcriptional level (Fig. 6B). To examine how CEBPB and FAK expression was upregulated, we isolated chromatin from the miR-17- and vector-transfected MCFs, and performed a chromatin immunoprecipitation (ChIP) assay. The precipitated DNA was subjected to PCR with specific primers flanking a DNA sequence

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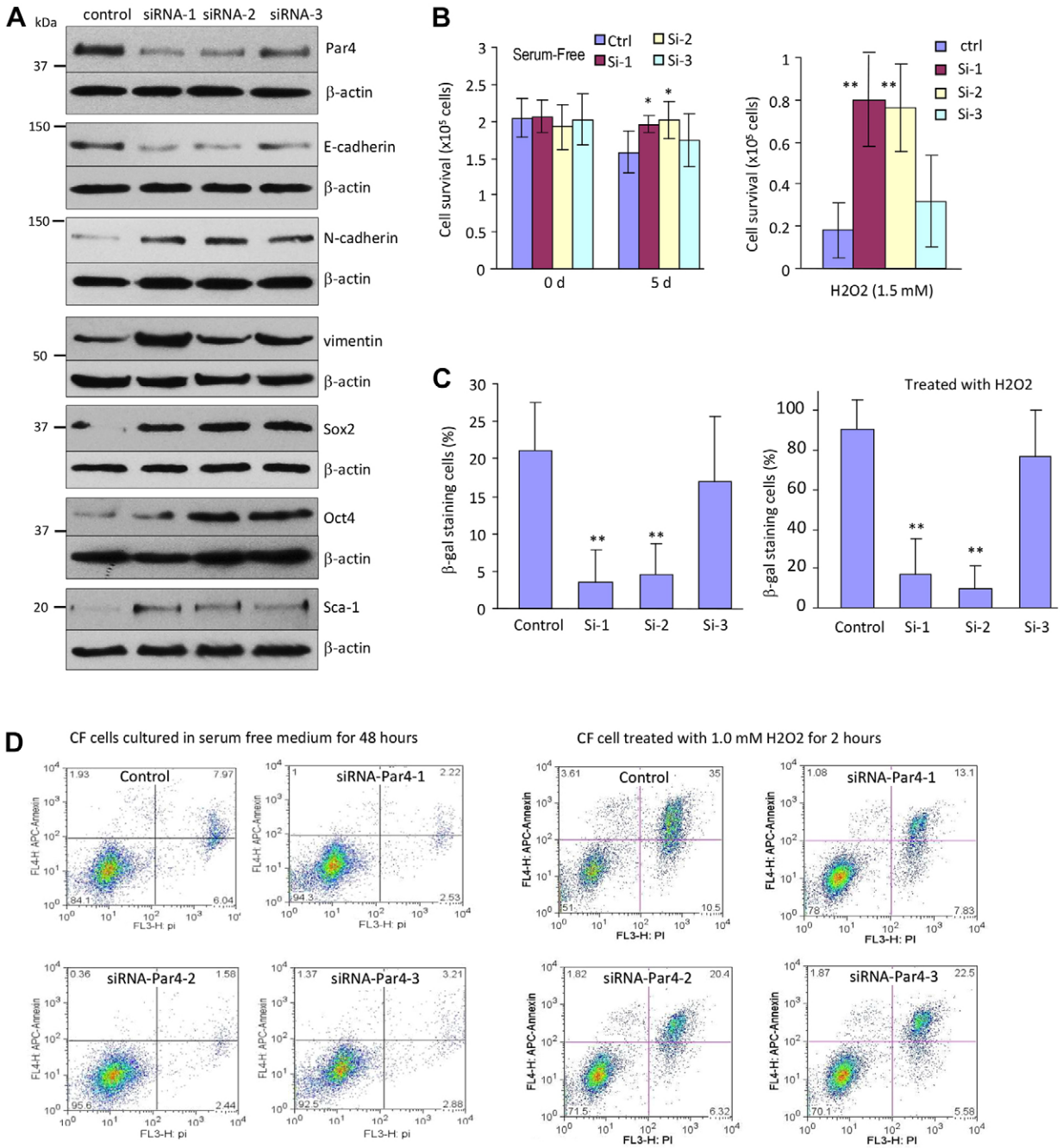

Fig. 4. Silencing Par4 enhances MCF senescence and apoptosis. (A) Protein lysates prepared from MCFs transfected with siRNAs targeting Par4 (sequences as described in Materials and Methods) and control oligo were subjected to immunoblotting. Transfection of MCFs with Par4 siRNAs decreased the levels of Par4 and E-cadherin, but increased the levels of N-cadherin, vimentin, Sox2, Oct4 and Sca-1. (B) The Par4-siRNA-transfected MCFs (Si-1–3) were cultured in basal medium for 6 days or treated with H_2O_2. Transfection with two of the three Par4 siRNAs resulted in increased viability. Ctrl, control. $n=6$. (C) The number of β-gal-stained MCFs was quantified. Par4-siRNA-transfected MCFs showed less β-gal staining. $n=6$. All quantitative data show the mean±s.d.; $^*P<0.05$; $^{**}P<0.01$. (D) The Par4-siRNA-transfected MCFs were cultured in serum-free medium for 48 hours (left) or in 1.0 mM H_2O_2 for 2 hours (right) followed by annexin V staining. Par4 siRNA transfection resulted in decreased levels of annexin V staining. CF, cardiac fibroblast.

at the CEBPB or FAK promoter. Transfection with miR-17 decreased Par4 binding to the CEBPB promoter (Fig. 6C). In cells transfected with Par4 siRNA, antibody against CEBPB pulled down higher levels of FAK promoter DNA compared with that pulled down from the control MCFs (Fig. 6D). However, Par4-siRNA-transfected MCFs did not show any FAK binding to the CEBPB promoter, as compared with the control oligo-transfected cells (data not shown). The ChIP assay indicated that Par4 might bind to the CEBPB promoter directly and repress CEBPB transcription, whereas CEBPB could bind to the FAK promoter and enhance FAK transcription. By using western blotting, we confirmed that silencing Par4 promoted the expression of CEBPB and FAK (Fig. 6E).

To validate the interaction of Par4, CEBPB and FAK, a Par4 plasmid was transfected into miR-17-expressing MCFs. The Par4-expressing cells showed high levels of Par4 and low levels

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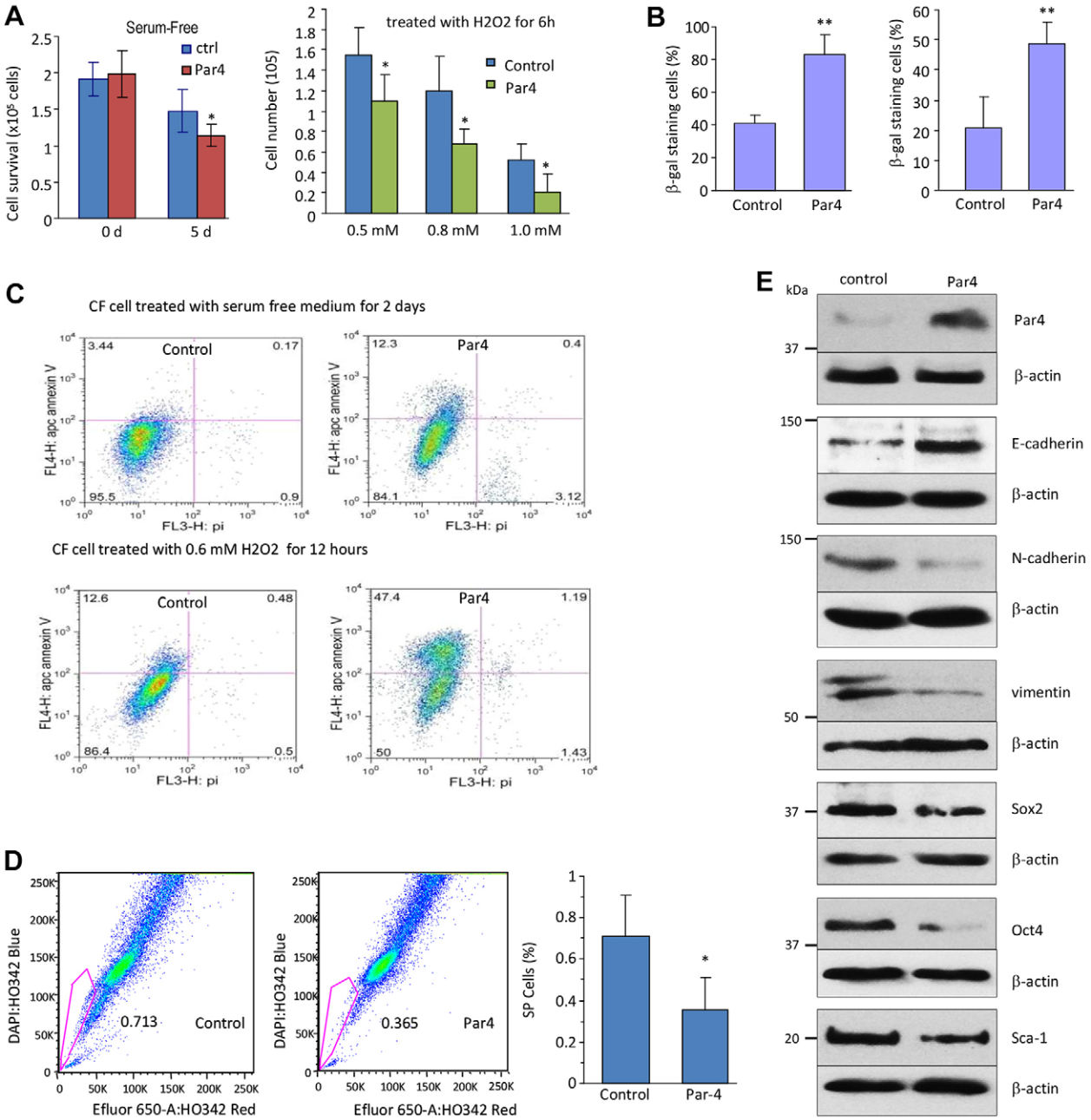

Fig. 5. Expression of Par4 promotes senescence and apoptosis. (A) The miR-17-transfected MCFs were transfected with Par4 or a control (ctrl) vector. The cells were cultured in serum-free medium for 2 days (left) or treated with H_2O_2 for 6 hours (right). Exogenous expression of Par4 decreased cell viability. $n=6$. (B) The cells were subjected 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 H_2O_2 for 2 hours and then cultured in basal medium for 48 hours (right). $n=6$. (C) The cells were also subject to annexin V staining. Exogenous expression of Par4 increased cell apoptosis when the cells were cultured in serum-free medium for 48 hours (upper panel) or treated with 0.6 mM H_2O_2 for 12 hours (lower panel). CF, cardiac fibroblast. (D) The cells were subjected to Hoechst 33342 staining, followed by flow cytometry for side population (SP) cells. Par4-transfected MCFs showed a lower proportion of side population cells than control samples (left), and this difference reached significant levels (right). $n=6$. All quantitative data show the mean±s.d.; $^*P<0.05$; $^{**}P<0.01$. (E) Protein lysates were subjected to immunoblotting. Transfection of Par4 increased E-cadherin level but decreased the expression of N-cadherin, vimentin, Sox2, Oct4 and Sca-1.

of CEBPB and FAK, and the regulation appeared to occur at the transcriptional level (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). By ChIP, immunoprecipitated exogenous Par4 was shown to pull down more CEBPB promoter DNA (Fig. 7C), whereas immunoprecipitated exogenous CEBPB could pull down more FAK promoter DNA (Fig. 7D). This confirmed that Par4 can bind to the CEBPB promoter, repressing CEBPB transcription, and that CEBPB can bind to the FAK promoter, enhancing FAK transcription.

In summary, we show that miR-17-3p targets Par4, repressing Par4 expression in MCFs. The downregulation of Par4 promotes CEBPB transcription, which further enhances FAK transcription. Expression of CEBPB and FAK promote EMT and self-renewal.

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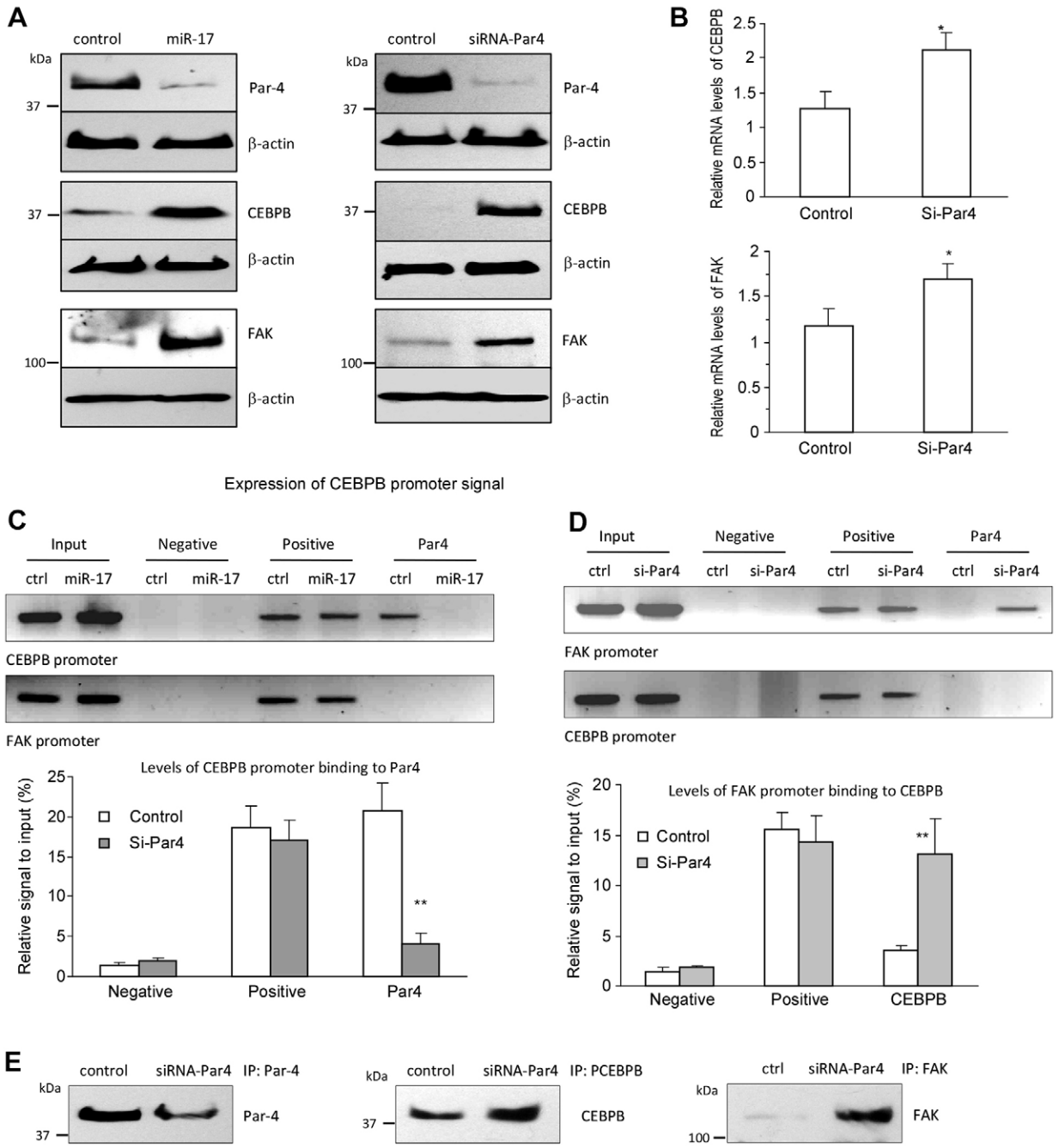

Fig. 6. Silencing Par4 enhances CEBPB and FAK transcription. (A) Protein lysates from miR-17-transfected (left) or Par4 siRNA (siRNA-1)-transfected (right) MCFs were subjected to immunoblotting. Transfection with miR-17 or Par4 siRNA decreased the expression of Par4, but increased the expression of CEBPB and FAK. (B) RT-PCR showed that Par4 siRNA (siRNA-1)-transfected MCFs expressed higher levels of CEBPB mRNA (upper panel) and FAK mRNA (lower panel) compared with that expressed by control cells. $n=4$. (C) Upper panel, chromatin from miR-17- or control-vector-transfected MCFs was isolated, digested and immunoprecipitated 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 panel, in miR-17-transfected MCFs, there was less Par4 binding to the CEBPB promoter. Ctrl, control. $n=4$. (D) Upper panel, chromatin from Par4 siRNA (siRNA-1)- or control-oligo-transfected MCFs was processed as for C, followed by PCR with primers flanking a DNA sequence at the FAK or CEBPB promoter. Lower panel, anti-Par4 siRNA-transfected MCFs had more CEBPB binding to the FAK promoter than did control cells. $n=4$. All quantitative data show the mean±s.d.; $^{*}P<0.05$; $^{**}P<0.01$. (E) Protein lysates from Par4 siRNA (siRNA-1)- or control-oligo-transfected MCFs were subjected to immunoprecipitation (IP) as indicated, followed by western blotting. Less Par4 was pulled down in the Par4-siRNA-transfected MCF sample. However, more CEBPB and FAK were pulled down in the Par4-siRNA-transfected MCF lysates.

Phenotypically, 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)

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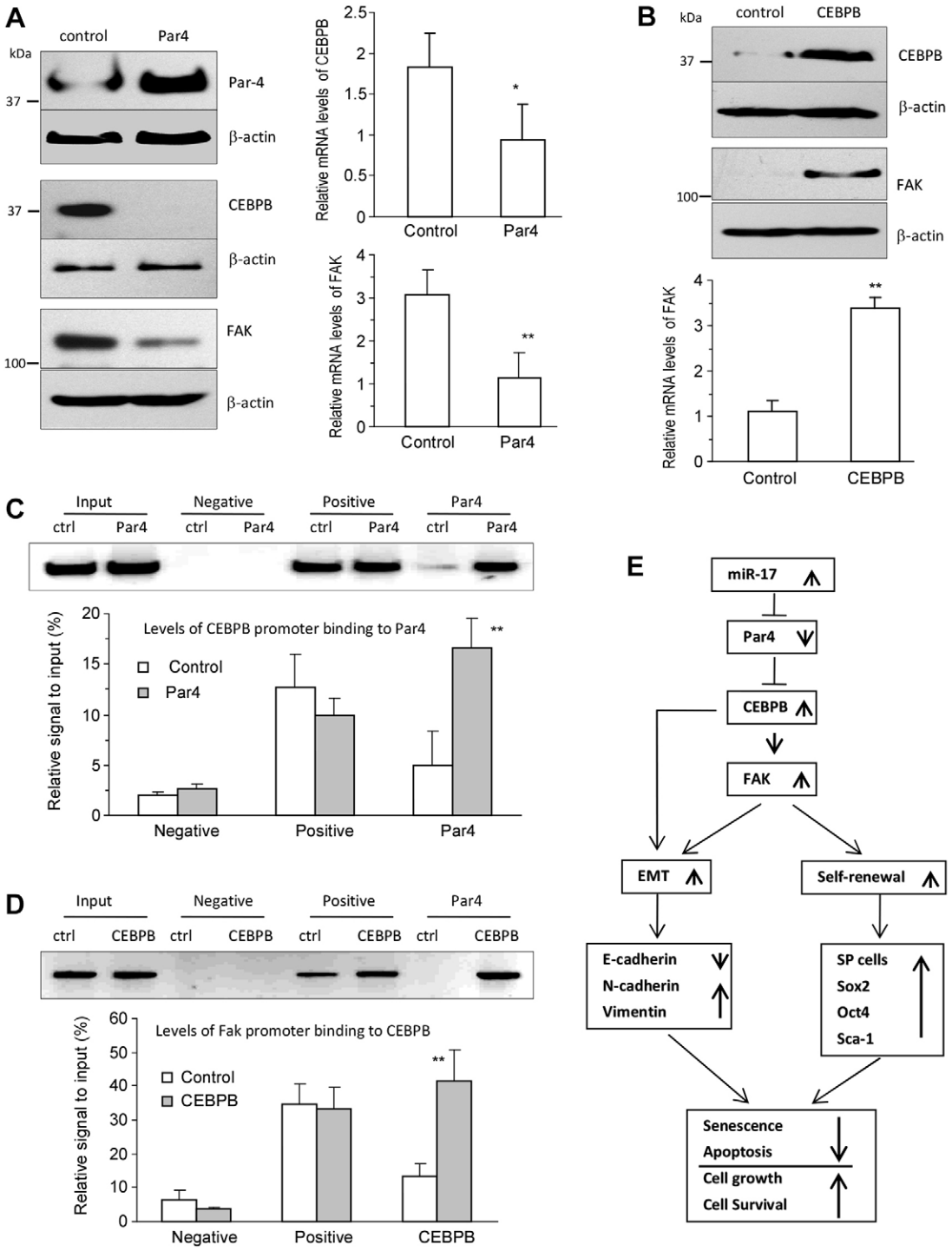

Fig. 7. Expression of Par4 represses CEBPB and FAK transcription. (A) Par4 or vector was transiently transfected into the miR-17-transfected MCFs. Protein lysates were prepared for immunoblotting. Par4 expression decreased CEBPB and FAK expression (left). Real-time PCR analysis showed that Par4 expression decreased the levels of CEBPB and FAK mRNA compared with those of the control (right). $n=4$. (B) Protein lysates prepared from CEBPB- or vector-transfected MCFs were subject to immunoblotting. CEBPB transfection increased CEBPB and FAK expression (upper panel). Real-time PCR analysis showed that CEBPB expression increased the mRNA level of FAK compared with that of control cells. $n=4$. (C) Upper panel, chromatin from vector- and Par4-transfected MCFs was isolated, digested and immunoprecipitated 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 panel, Par4 transfection resulted in higher levels of CEBPB promoter binding to the Par4 protein. $n=4$. (D) Upper panel, chromatin from vector- and CEBPB-transfected MCFs was processed as above, followed by PCR with primers flanking a DNA sequence at the FAK promoter. Lower panel, CEBPB transfection resulted in higher levels of FAK promoter binding to CEBPB protein. $n=4$. All quantitative data show the mean±s.d.; $^{*}P<0.05$; $^{**}P<0.01$. (E) Mechanisms by which miR-17 represses MCF senescence and apoptosis. miR-17 targets Par4, repressing Par4 expression. Downregulation of Par4 promotes EMT and self-renewal, which represses MCF senescence and apoptosis, and enhances cell growth and survival.

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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; Ibáñez-Ventoso and Driscoll, 2009; Liu et al., 2012; Yu et al., 2013), most studies have focused on the effect of miRNAs on the senescence of tumor cells at the cellular level, or have investigated the role of miRNAs in longevity in lower organisms (Bilsland et al., 2013; Jung and Suh, 2012; Smith-Vikos and Slack, 2012). Uniquely, our study systematically describes the functions of one specific miRNA, mir-17, in controlling senescence and apoptosis in mouse heart and cardiac fibroblasts. We found that miR-17 acts by targeting Par4 and promoting CEBPB and FAK senescence-related signaling in the transgenic mice and transfected cells.

Par4, encoded by the *Pawr* gene, is a pro-apoptotic protein that is upregulated in response to apoptotic stimuli and is required for cell apoptosis (Hebbar et al., 2012; Zhao and Rangnekar, 2008). Par4 is expressed at low levels in a variety of human cancers, and increased expression of Par4 might induce cell death in some cancer cell lines (Cook et al., 1999; García-Cao et al., 2005; Ranganathan and Rangnekar, 2005). Thus, Par4 is believed to be a tumor suppressor and a crucial regulator of tumor cell survival. Emerging evidence has suggested that Par4 downregulation could be used as a prognostic factor in cancer (Alvarez et al., 2013). Another study indicated that the SAC domain of Par4 confers cancer resistance in transgenic mice without compromising normal viability or aging, and that it might have therapeutic significance (El-Guendy et al., 2003).

Our study demonstrated that Par4 plays important roles in cardiac senescence, which is related to its negative regulation of the transcription factor CEBPB and of FAK. Silencing of Par4 markedly promoted CEBPB transcription, and CEBPB could bind to the FAK promoter and enhance 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). FAK has kinase-dependent and kinase-independent scaffolding, cytoplasmic and nuclear functions (Golubovskaya, 2014). It has been found to be overexpressed 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., 2013; Gjorevski et al., 2012; Golubovskaya, 2014). As a cytoplasmic tyrosine kinase, FAK has been 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, 2012). Both FAK and integrins have been 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, 2010).

Thus, we have identified miR-17-3p as senescence-related miRNA that represses MCF senescence and apoptosis under stress. 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 found to repress senescence and apoptosis through enhanced EMT and self-renewal. Identifying and understanding senescence-associated miRNAs and genes will be important to allow us to uncover the mechanisms of senescence and to discover targeted therapies.

MATERIALS AND METHODS

Materials

The monoclonal antibodies against Par4, Sox2, Oct4 and vimentin, and the senescence β-galactosidase staining kit were obtained from Cell Signaling Technology. 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′-GUGCUU-AGAUGAGUACGAATT-3′ and 5′-UUCGUACUCAUCUAAGCACTT-3′; siRNA2 (Par4-mus-943), 5′-CCGGCUAGUUUCUCCUCAATT-3′ and 5′-UUGAGGAGAAACUAGCCGGTT-3′; siRNA3 (Par4-mus-1038), 5′-GGCUGAUGCAAGAUAAAGATT-3′ and 5′-UCUUUAU-CUUGCAUCAGCCTT-3′. The miR-17 transgenic mice were generated as described 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 in accordance with the National Institutes of Health 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 GFP and an H1 promoter driving expression of the 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′-ATAC-AGTACTGTGATAACTGAATTTTTGGAAAAGCTTTAGTTATTAA-3′), serving as a mock control. The MCFs (generated by ScienCell, Cat #M6300) were transfected with miR-17 and control vector. After cell sorting, the stably transfected 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′UTR of mouse Par4 was cloned by RT-PCR, using two primers – musPar4-SacI (5′-CCCGAGCTCTAGAAGCTGCACGGGCGGCTTC-3′) and musPar4-MluI (5′-CCACGCGTTAAATATACAGTCCTCTCA-ACAA-3′). The PCR products were digested with *Sac*I and *Mlu*I and inserted into a *Sac*I- and *Mlu*I-digested pMir-Report vector to obtain the Luc-Par4 luciferase construct. Mutation of the miR-17-3p binding site was performed by PCR, using two primers – musPar4-SacI and musPar4-MluI-mut (5′-CCACGCGTTAAATATACAGTCCTCTCAACAAGCGTGTGA-3′). 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 (University of Kentucky Chandler Hospital, Lexington, KY; Chakraborty et al., 2001), and the plasmid pcDNA3.1 (-)-mCEBPB of CEBPB was kindly provided by Dr Robert C. Smart (North Carolina State University, Raleigh, NC; Zhu et al., 1999).

Cell proliferation, cell survival and H_2O_2 senescence assays

To assay cell proliferation, 4×10^4 cells were seeded onto six-well dishes in 10% FBS-DMEM medium and maintained at 37℃ overnight. Cells were harvested daily and cell number was analyzed by using a Coulter counter. To determine cell survival, 2×10^5 cells were cultured in 10% FBS-DMEM medium in culture dishes and maintained at 37℃. After 12 hours of culture, the 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 using a Coulter counter. For the H_2O_2 senescence assays, cells in 10% FBS-DMEM medium were treated with the indicated concentrations of H_2O_2 for 2 hours. After washing with PBS, the treated cells were incubated in fresh growth medium without H_2O_2 for 48 hours, and processed for β-gal staining.

Side population analysis

Cells (2×10^6) were harvested from tissue culture dishes, washed, suspended at 10^6 cells per ml in DMEM containing 2% FBS, and pre-incubated at 37℃ for 10 minutes. Samples were then cultured with 2.5 µg/ml Hoechst 33342 dye for 90 minutes, 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. Side population cell analysis was performed 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 minutes. Annexin-V–FITC binding was determined by flow cytometry (excitation wavelength of 488 nm; emission wavelength of 530 nm) using the FITC signal detector (FL1), and propidium iodide staining was detected by the phycoerythrin emission signal detector (FL2).

Western blotting and real-time PCR

Western blotting was performed as described previously (Fang et al., 2012; Siragam et al., 2012). For real-time PCR, total RNAs were extracted from 1×10^6 cells by using the mirVana miRNA Isolation Kit (Ambion) according to the manufacturer's instructions. Real-time PCR was performed as described previously (Liu et al., 2013; Yang et al., 2013).

Senescence-associated β-galactosidase staining

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

For tissue staining, mice were killed using carbon dioxide followed by isolation of the hearts. The heart tissues of wild-type and transgenic mice were excised, rapidly frozen in liquid nitrogen and mounted in OCT. Sections (4 µm) were cut, mounted onto glass slides, fixed in 2% formalin in PBS for 1 minute at room temperature, and immersed in β-gal staining solution at 37℃ for 24 hours. The samples were counterstained with eosin and viewed under brightfield at 40× magnification.

Immunohistochemistry

Heart tissues were excised and fixed in 10% formalin overnight, immersed in 70% ethanol, embedded in paraffin and sectioned. The sections were deparaffinized 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℃ overnight. The sections were incubated with biotinylated secondary antibody at 25℃ for 2 hours, followed by incubation with avidin-conjugated horseradish peroxidase provided in the Vectastain ABC kit (Vector, PK-4000). The slides were then stained with DAB, and Mayer's Hematoxylin was used for counterstaining.

Immunoprecipitation assay

Cells were washed in ice-cold PBS and lysed in 1 ml of lysis buffer. Equal amounts of protein were incubated with 5 µg of primary antibody and 40 µl of 50% Protein-A–Sepharose slurry at 4℃ for 4 hours. The pellet was washed three times with PBS and was resuspended in 2× Laemmli buffer (0.125 M Tris-HCl, 4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.004% Bromophenol Blue pH 6.8), followed by western blot analysis.

ChIP assay

ChIP was performed using the SimpleChIP chromatin IP kit (Cell Signaling Technology) 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. A total of 10% of the inputs were used for immunoblotting. The captured chromatin was eluted, crosslinking was reversed and the DNA was recovered. ChIP DNA was subjected to PCR using specific primers flanking a DNA sequence in the promoter region of either CEBPB, using the primers Cebpb-promo-F (5′-GTTCAGGG-ACCCAAGTTCAGAGGA-3′) and mus.Cebpb-promo-R (5′-CTCTGCC-AGCAAGCTACACCCCAG-3′), or FAK, using the primers mus. Ptk2.promo-F (5′-AGGGCAAGACCGGGAGGACAGGTG-3′ and mus. Ptk2.promo-R (5′-TGTGTGCGGCCGGGACTAGAAGAG-3′).

Statistical analysis

All experiments were performed in triplicate and numerical data were subjected to independent sample *t*-test. The levels of significance are shown as $*P<0.05$ and $**P<0.01$.

Competing interests

The authors declare no competing or financial interests.

Author contributions

W.W.D. performed and coordinated the project. X.L. and T.L. were involved in immunostaining. H.L. was involved in annexin V assays. A.K. was involved in RNA analysis. F.L. was involved in the generation of luciferase constructs. W.W.D. and B.B.Y. designed the project, analyzed the results and wrote the paper.

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Supplementary material

Supplementary material available online at
http://jcs.biologists.org/lookup/suppl/doi:10.1242/jcs.158360/-/DC1

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