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RESEARCH ARTICLE

Bone marrow-fibroblast progenitor cell-derived small extracellular vesicles promote cardiac fibrosis via miR-21-5p and integrin subunit αV signalling

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Abstract

Cardiac fibrosis is the hallmark of cardiovascular disease (CVD), which is leading cause of death worldwide. Previously, we have shown that interleukin-10 (IL10) reduces pressure overload (PO)-induced cardiac fibrosis by inhibiting the recruitment of bone marrow fibroblast progenitor cells (FPCs) to the heart. However, the precise mechanism of FPC involvement in cardiac fibrosis remains unclear. Recently, exosomes and small extracellular vesicles (sEVs) have been linked to CVD progression. Thus, we hypothesized that pro-fibrotic miRNAs enriched in sEV-derived from IL10 KO FPCs promote cardiac fibrosis in pressure-overloaded myocardium. Small EVs were isolated from FPCs cultured media and characterized as per MISEV-2018 guidelines. Small EV's miRNA profiling was performed using Qiagen fibrosis-associated miRNA profiler kit. For functional analysis, sEVs were injected in the heart following TAC surgery. Interestingly, TGFβ-treated IL10-KO-FPCs sEV increased profibrotic genes expression in cardiac fibroblasts. The exosomal miRNA profiling identified miR-21a-5p as the key player, and its inhibition with antagomir prevented profibrotic signalling and fibrosis. At mechanistic level, miR-21a-5p binds and stabilizes *ITGAV* (integrin av) mRNA. Finally, miR-21a-5p-silenced in sEV reduced PO-induced cardiac fibrosis and improved cardiac function. Our study elucidates the mechanism by which inflammatory FPC-derived sEV exacerbate cardiac fibrosis through the miR-21a-5p/ITGAV/Col1α signalling pathway, suggesting miR-21a-5p as a potential therapeutic target for treating hypertrophic cardiac remodelling and heart failure.

KEYWORDS
bone marrow, cardiac fibrosis, fibroblast progenitor cells, miRNA, small extracellular vesicles

1 | INTRODUCTION

Cardiovascular diseases (CVD) are major disease burden worldwide. One of the key features in CVD is excessive deposition of extracellular matrix p, leading to cardiac fibrosis and pathological cardiac remodelling, which ultimately leads to heart failure. In the heart, cardiac fibrosis is mainly mediated by myofibroblasts (activated fibroblasts) (Krieg et al., 2007; Piera-Velazquez et al., 2011). In general, myoFBs have been shown to originate from several sources, including from expanding resident fibroblasts, hematopoietic cells mobilized from bone marrow (BM), and from endothelial cells by the process of endothelial-mesenchymal

transition (EndoMT) (Diez et al., 2010). Recent reports show that a subpopulation of heart-infiltrating BM-derived $CD45^+$ cells (expressing prominin 1, collagen 1a, and other stem cell markers) contribute to myoFB differentiation under the influence of TGFβ (Kania et al., 2008, 2009b; Verma et al., 2017). We have previously shown that TAC induced FPC mobilization and recruitment into the heart and it is further augmented in IL10-KO mice (Verma et al., 2017); however, the precise mechanisms through which these cells contribute to the cardiac fibrosis remain inadequately defined.

Recent reports, including ours, have shown that sEVs secreted by different types of cells enhance post-injury cardiovascular repair (Agarwal et al., 2017; Davis, 2016; Dougherty et al., 2017; Ranjan et al., 2019, 2021; Sahoo & Losordo, 2014; Vandergriff et al., 2018). Exosomes are nanosized vesicles released from the cells, including stem cells, and act as functional paracrine units. It has been reported that the regenerative potential of stem/progenitor cells isolated from diseased animals and patients has been compromised (Caiado et al., 2021; Ho & Takizawa, 2022; Kizil et al., 2015; Sahoo et al., 2021). Importantly, the cardio-protective effect of $CD34^+$ cells is mediated by Shh packaging in CD34+ cell's sEVs, which enhances angiogenic response of the heart (Mackie et al., 2012). Furthermore, Lyu et al., have shown that activated cardiac fibroblast-derived sEVs facilitate Ang II production and its receptor expression in cardiomyocytes and cause pathological cardiac hypertrophy (Lyu et al., 2015). The mechanism of action of sEV-mediated remodelling/repair processes largely includes the transfer of exosomal cargo, including parent cell-specific microRNAs (miRNAs), mRNAs and proteins, to the target cell/tissue resulting in several protective/degenerative processes (Bang et al., 2014; Ibrahim et al., 2014; Ranjan et al., 2019, 2022). Emerging evidence indicates that miRNAs promote fibrosis via altering the expression of genes responsible for proliferation, angiogenesis, and ECM formation (Cardozo et al., 2018; Yang et al., 2016). MiRNAs are small non-coding RNAs that affect gene expression at the posttranscriptional level by altering mRNA stability and inhibiting translation (Bartel, 2004; O'Brien et al., 2018; Ratti et al., 2020). Studies have also demonstrated that miRs may activate gene targets by directly binding to their promoter regions and upregulating target genes (Place et al., 2008; Vasudevan et al., 2007). Previous studies have identified miR-21 as an important contributor to the pathogenesis of cardiovascular diseases, including vascular smooth muscle cell proliferation and apoptosis, cardiac cell growth and death, and cardiac fibroblast functions (Cheng & Zhang, 2010; Nonaka et al., 2021; Surina et al., 2021). Wang et al. showed that miR-21 promotes fibrosis in spinal fibroblasts following trauma (Wang et al., 2018). Furthermore, several studies have shown altered miR-21 expression levels in cardiac hypertrophy and heart failure, revealing its involvement in the cardiac remodelling process (Duygu & Da Costa Martins, 2015; Krzywinska et al., 2020; Watanabe et al., 2020). In contrast, the cardioprotective role of miR-21 has also been shown in acute heart injury model (such as myocardial infarction) to initiate appropriate wound healing process (Luther et al., 2018). Although miR-21 has been studied in the context of fibrotic diseases, its specific mechanism of action in worsening cardiac fibrosis has not been explored. In this study, we investigated the aberrant expression of miR-21a-5p in BM-derived FPC-sEVs under pressure overload and its functional significance in exacerbating cardiac fibrosis in immunocompromised IL-10 KO mice.

2 | MATERIALS AND METHODS

Detailed descriptions of the methods and supporting data are available within the article and in the Data Supplement. All animal experiments conform to the protocols approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Alabama at Birmingham (Birmingham, AL). The data that support the findings of this study are available from the authors, Dr. Suresh K. Verma (sverma@uabmc.edu) upon reasonable request.

2.1 | Bone-marrow fibroblast progenitor cell (FPC) isolation and culture

Both fore and hind limbs were excised from 6- to 8-week-old C57BL/6J and IL10KO adult mice. Bone marrow cells were isolated according to a standard protocol as previously described (Verma et al., 2017; Yamaguchi et al., 2003). Further, FPCs were isolated from bone marrow mononuclear cells with density-gradient centrifugation (Krishnamurthy et al., 2011). For positive cell separation, the prominin-1 magnetic bead selection method was used to collect fibroblast progenitor cells (FPCs) from monocyte/macrophage–depleted BM cells. After serial washing steps, the cells were cultured onto 1% gelatin–coated plates in expansion medium (Iscove modified Dulbecco medium with 20% FBS, 100 μmol/L β-mercaptoethanol, 1% nonessential amino acids, and 1% antibiotic/antimycotic; Mediatech). Ten to 12 days after culture, cells were detached with trypsin (0.05% with EDTA) and seeded for next passage.

2.2 | FPC treatment, sEV isolation and characterization

FPCs were starved in a serum-free medium for 12 h. At about 70% confluent cells were treated with 10 ng/mL TGFβ or BSA for 48 h. The primary challenges encountered in sEVs preparation are associated with issues of reproducibility and variability. In addressing these challenges, our study employs the widely accepted ultracentrifugation method for sEVs preparation (Cheng

et al., 2019). The small EVs were isolated from FPC-conditioned media obtained from multiple biological replicates (as described in respective figure legends), mixed in 50 μL PBS, and stored at −80°C. Briefly, conditioned media collected from FPC culture was centrifuged at 10,000 g for 20 min to remove cells and cellular debris. Supernatant was then transferred into a fresh tube and passed through a 0.22 μm membrane filter. Filtrate was subjected to ultracentrifugation at 100,000 g for 1.5 h at 4°C. This step was repeated by suspending the EV pellet in filtered sterilized PBS. The sEV's quality were validated based on their shape with the transmission electron microscope (TEM; Tecnai Spirit T12, Thermo Fisher) using a single-droplet method-negative stain technique and images were recorded with an AMT CCD camera (Woburn, MA) at UAB core facility (Genschmer et al., 2019). We adjusted the sample concentration until a clear image of a population of approximately 10−100 particles in the scattering volume was obtained. Further, NanoSight analysis (NanoSight NS300, ATA Scientific, Australia) was performed in above dilution range to determine the size and quantity of the sEVs. Briefly, filtered sEVs were adequately vortexed and passed through the syringe pump attached in the NS300 NanoSight (at High Resolution Imaging Core Facility at The University of Alabama at Birmingham, Alabama) and all particles were tracked in solution.

2.3 | Adult mice cardiac fibroblasts isolation and treatment with sEVs

Cardiac fibroblasts (CFs) were isolated from the heart of 6- to 8-week-old C57BL/6J mice and cultured in DMEM 4.5 g/L glucose media (Corning, Catalogue #10-017-CV) containing 10% foetal bovine serum (Thermofisher Scientific, Catalogue #11-965-118). At 60%–70% confluence, CFs were treated with TGFβ-FPC sEVs (30,000 particles/mL) or PBS in DMEM containing 2.5% sEV-depleted FBS media (Gibco#A2720801) for 48 h before using them in the outlined assays.

2.4 | Fibrotic genes expression analysis and exosomal miRNA profiling

Total RNA was isolated from cells (CFs) using RNeasy Mini Kit (Qiagen). To measure the gene expressions, first cDNA was synthesized using high-capacity cDNA reverse transcription kit (Applied Biosystems) and then quantitative reverse transcription polymerase chain reaction was performed on QuantStudio3. The 18S small nucleolar RNA or GAPDH was used to normalize the threshold delta-delta CT method. Further, for sEV's miRNA measurement, total RNA was isolated from FPCs using miRNeasy Mini Kit, as per the manufacturer's instructions. The RNA was reverse transcribed using miScript II RT kit and PCR array was performed using the miScript SYBR Green PCR Kit on custom printed 96-well miScript miRNA PCR fibrosis array (Qiagen) as per the manufacturer's instructions. TaqMan miRNA assay kit (Applied Biosystems, Waltham, MA) was used to validate the target miRNAs. The expression of each miRNA was normalized to the expression of U6 snRNA (Cat# 4427975, ThermoFisher), as per the manufacturer's protocol.

2.5 | Cell migration assay

A monolayer of cardiac fibroblast was grown in six well plates in complete medium. At full confluence, the cell layer was scratched using 200 μL micro-pipette tip. After scratching under different treatment conditions, the cells were allowed to grow/migrate for 48 h at 37°C in the incubator. To evaluate the migration potential, the picture of scratched area was taken using light microscope at 0, 24, and 48 h. and healed area was calculated using NIH ImageJ software.

2.6 | Immunostaining assay

Cells (FPCs or CFs) were seeded in chambered slides for treatment and immunostaining. FPCs were treated with BSA and TGFβ; however, CFs were treated with sEVs (obtained form FPC-conditioned media following different treatments). After 48 h of treatment, cells were washed with PBS and fixed with 4% paraformaldehyde (PFA) and permeabilized with 0.2% Triton-X 100 in 1X PBS for 20 min. Further, cells were stained with fibrosis associated markers (αSMA/Col1α/DAPI). Finally, 5–6 sets of data were collected from different biological replicates.

2.7 | Protein isolation and western blot analysis

For protein isolation, cells were lysed in cell lysis buffer (Cat# 9803, Cell Signalling Technology, MA, USA), centrifuged at 14,000 rpm for 15 min at 4°C, and the supernatant containing the proteins was stored at −80°C. Further, protein concentration was estimated by using BCA Protein Assay Kit (Thermo Fisher Scientific, USA). Proteins were separated using SDS-PAGE

and transferred to polyvinylidene difluoride membranes (BioRad). Membranes were blocked for 1 h at room temperature with Intercept (TBS) blocking buffer (Cat# 927–60001, LI-COR Biosciences) as per manufacturer instruction and then incubated with the antibodies overnight at 4°C. Subsequently, the membranes were incubated with IRDye secondary antibodies [LI-COR Biosciences] at 37°C for 1 h. Signals were detected and quantified using Odyssey® Fc Imaging System (LI-COR Biosciences, model number 2800). Antibodies used to validate the sEVs and signal pathways were recombinant anti-Flotillin 1 (Abcam#ab133497), anti-CD81 (System Biosciences#EXOAB-CD81A-1), TSG101 (Proteintech#67381-1-Ig), Col1α (Santa Cruz Biotechnology#sc-293182), recombinant anti-TGFβ (Abcam#ab215715), Integrin αv (Novus Biologicals# NBP2-67557), GAPDH (Cell Signalling Technology#5174), and β-actin (Cell Signalling Technology#3700). In addition, Calnexin (Proteintech#81938-1-RR), and cytochrome c (Santa Cruz# sc-13156) antibodies were used as negative controls for endoplasmic reticulum and mitochondria, respectively.

2.8 | miR-modulation in small EV

The 70% confluent FPCs were transfected with miR-21a-5p inhibitor (or control scramble miRNA) (Thermo Fisher Scientific, Waltham, MA) using Lipofectamine-2000 Transfection Reagent (Thermo Fisher #11668030) in Opti-MEM I Reduced Serum Medium (Thermo Fisher# 31985070). After 8 h, the culture medium was changed to incomplete FPC medium without antibiotic/antimycotic. Cells were treated with TGFβ or BSA for 48 h, followed by collecting the culture media for isolating sEVs and cell lysate for biochemical analyses.

2.9 | Heart failure induction, small EV treatment, cardiac function and histology

Six- to 8-week-old WT (C57BL/6J) mice were procured from Jackson Laboratories (Bar Harbor, Maine). Cardiac hypertrophy and subsequent heart failure were induced by constricting the transverse aortic arch using 27G needle as described previously (Verma et al., 2012). TAC-induced mobilization of FPCs was determined by fluorescence-activated cell sorter (FACS) analysis of peripheral blood mononuclear cells, as described previously (Krishnamurthy et al., 2011). To study miRNA-mediated fibrogenesis, FPC-sEVs were injected into the mouse's jugular vein during TAC surgery. Transthoracic 2-dimensional M-mode echocardiography was performed before (baseline), at 14 and 28 days after Sham/TAC surgery using Vevo 3100 VisualSonics, Toronto, ON, Canada. Percent fractional shortening and percent ejection fraction were calculated as described previously (Krishnamurthy et al., 2009). Finally, mice were euthanized, and their hearts were harvested for biochemical analyses. For histology, whole hearts were perfused with sterile, cold PBS followed by 10% formalin for 5–10 min. After removing the atria, the ventricles were fixed in 10% phosphate-buffered formalin for 4 h at room temperature. Tissue sections (4 μm each) made from each heart were subjected to Masson's trichrome staining and quantitated using Nikon NIS software.

2.10 | Statistical analyses

GraphPad Prism software (version 9.5.1) was used to perform statistical analyses and experimental data are expressed as mean ± S.E.M. of five to seven independent experiments. A 2-sided p value of <0.05 was considered statistically significant. Data distribution were assessed by the Shapiro–Wilk test for normality. If values were normally distributed, unpaired, two-tailed Student's t-test was used to determine significant difference between two groups, while ANOVA followed by Turkey post hoc test was applied to calculate significance if there were more than two groups. If values were not normally distributed, nonparametric Mann–Whitney test was used for two groups or Kruskal–Wallis test for >2 groups.

3 | RESULTS

3.1 | TGFβ activates adult mice FPC cells

We isolated bone marrow FPC cells from C57BL/6j and IL10-KO mice as a subpopulation of lineage-negative hematopoietic cells ($CD45^+$) expressing prominin-1 (Verma et al., 2017). To test the hypothesis that TGFβ treatment augments pro-fibrotic signalling in IL10-KO FPCs, FPCs isolated from WT and IL10-KO mice were treated with TGFβ and the expression of fibrotic markers were measured. Real-time qPCR (RT-qPCR) analysis showed an increased expression of profibrotic genes Col1a, fibronectin, periostin, and αSMA in IL-10KO-FPC following TGFβ treatment compared to WT-FPC (Figure S1A–D). These results indicate that TGFβ activates FPCs to profibrotic (myofibroblast) phenotype and this process is further exacerbated in the absence of IL-10.

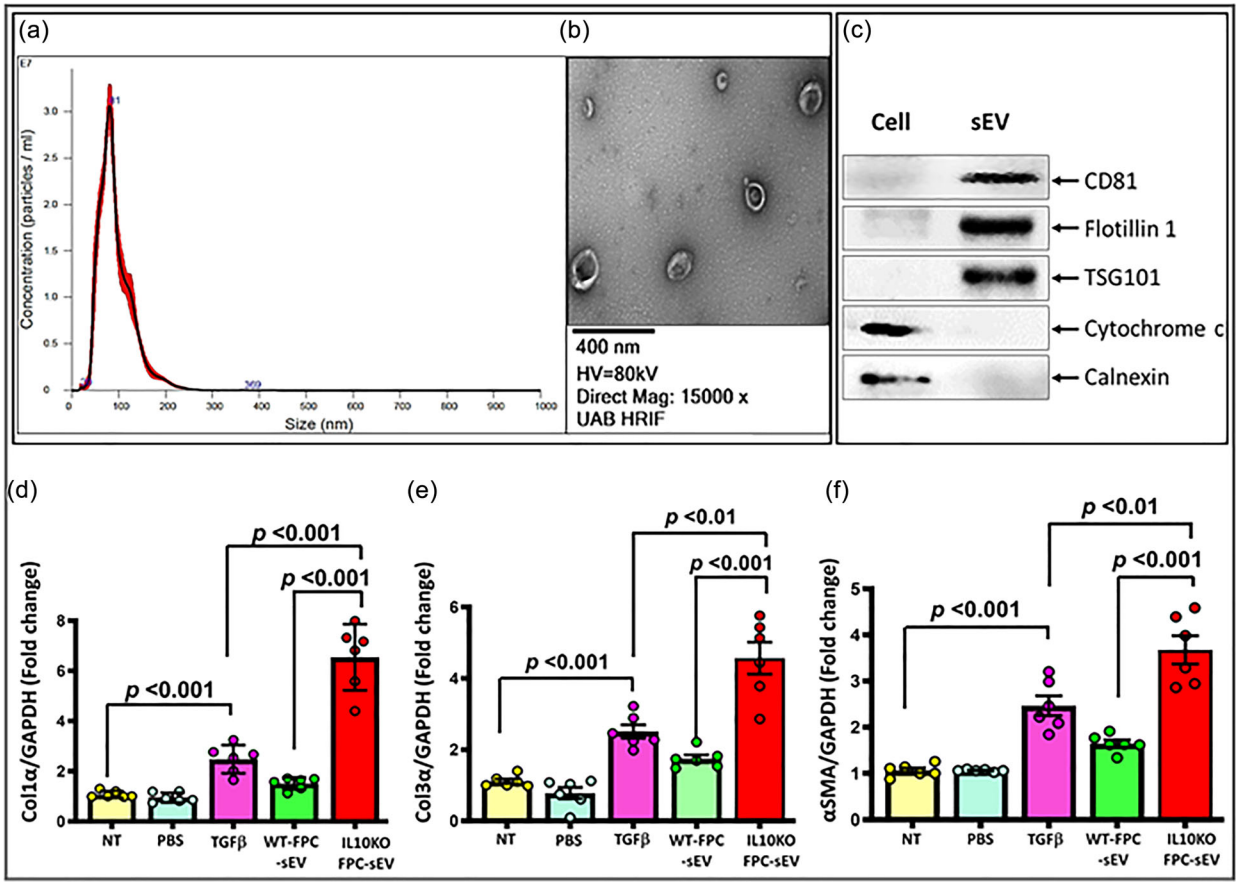

FIGURE 1 (a–c) Small EVs isolation and characterization. sEVs were isolated from the WT and IL10 KO FPC-conditioned media following PBS or TGFβ treatment using ultracentrifugation method. (a) Size of sEV was determined by NanoSight and showed that the average EV size in our preparation is 90 nm. (b) Representative images captured by transmission electron microscopy (magnification 30,000X) revealed the presence of double-membrane cup-shaped morphology of small EV. (c) FPC-derived sEVs express surface protein markers Flotilin-1, CD81, TSG101 and negative markers calnexin, and cytochrome c. (d–f) sEVs-derived from TGFβ-treated FPC activated fibrotic genes expression in naïve cardiac fibroblasts. Wild-type adult mice heart cardiac fibroblasts were treated with the respective sEVs for 48 h. At the end of treatments, fibrotic gene expression was measured in the RNA using qPCR technique. IL10KO-FPC-Exo significantly enhanced Collagen 1α (d), Collagen 3α (e), and α-smooth muscle actin (f) genes expression as compared to WT-FPC sEV. Relative mRNA expression of target genes was normalized with the 18S. $p < 0.05$ was considered as statistical significance, $N = 5–7$.

3.2 | FPCs show paracrine effects on cardiac fibroblasts via their sEVs

To investigate the potential paracrine effects mediated by small extracellular vesicles (sEVs), we initially extracted sEVs from the conditioned media of FPCs through ultracentrifugation. Validation of the sEVs' quality was performed using NanoSight and electron microscopy, revealing a consistent double-membrane cup-shaped structure with an average size of <90 nm (Figure 1a, b). For a comprehensive characterization of the sEVs, we examined the expression of common transmembrane protein markers. Our findings indicate exclusive expression of CD81 and flotillin-1 in the sEVs, as illustrated in Figure 1 (c). Notably, our sEV preparation showed no expression of mitochondrial (cytochrome C) or endoplasmic reticulum (calnexin) marker proteins. This comprehensive analysis underlines the purity of our sEVs.

To determine the role of activated FPC-sEVs on CFs activation, WT (C57BL/6j) mice heart CFs (cardiac fibroblasts) were isolated and treated with TGFβ (as a positive control) or sEVs obtained from TGFβ treated FPCs of WT (control for IL10-KO FPC sEV group) or IL10-KO mice and analysed the fibrosis-associated genes expression. RT-qPCR data revealed that TGFβ treatment alone (with no sEV exposure) significantly enhanced the expression of fibrosis-associated genes compared to respective control. Interestingly, when the CFs were treated with IL10-KO-FPC-TGFβ-sEVs, it substantially increased the expression of profibrotic genes, that is, Col1α, Col3α, and αSMA, compared to CFs exposed to WT-FPC-TGFβ-sEVs (Figure 1d–f). These data indicate that TGFβ-treated IL10-KO-FPC-sEVs contain ample profibrotic factors that induce resident fibroblast activation.

3.3 | FPC-sEVs contain fibrosis-associated miRNAs upon TGFb treatment

To investigate how sEVs trigger fibroblast activation and profibrotic signalling, it is important to determine the contents of the sEVs. MicroRNAs (miRNAs) are small, noncoding RNAs that are evolutionarily conserved in most plants and animals. Intriguingly, prior research has strongly linked miRNAs to profibrotic signalling; thus, we hypothesized that profibrotic miRNAs packaged in FPC-sEVs facilitate heart fibroblast activation and fibrosis in pressure-overloaded myocardium. We measured the fibrosis-associated miRNAs in the sEVs-derived from the conditioned media from the TGFβ treated WT-FPCs and IL10-KO-FPCs using pathway-based miRNA array kit. We isolated exosomal miRNAs and ran miScript miRNA PCR Array, a mouse Fibrosis Pathway-focused array, using a 96-well format (Qiagen#MIMM-117Z). Subsequent data analysis with "GeneGlobe Data Analysis Center (Qiagen)" revealed that several fibrosis-related miRNAs were upregulated in TGFβ-treated WT-FPC- and IL10-KO-FPC-derived sEVs compared to control sEVs (Figure 2a). Interestingly, data revealed that miR-21a-5p, miR-338-5p, and 34a-5p were highly upregulated in IL-10KO FPC's sEVs as compared to WT FPC's sEVs following TGFβ treatment. We further validated these miRs expression via RT-qPCR using selective miR's primers. While TGFβ-treated WT-FPC-sEVs showed a modest increase, the TGFβ-treated IL10-KO-FPC-sEVs had a remarkably high level of miR-21a-5p compared to untreated FPC sEVs (Figure 2b). Other miRs (MiR-338-5p and 34a-5p) did not show significant differences in this validation experiment (Figure 2c, d). These findings strongly suggest that an altered packaging of miRNAs occurs within TGFβ-activated IL-10 KO FPC-derived sEVs, potentially triggering resident fibroblast activation and exacerbating fibrosis in IL-10KO mice hearts. Based on these findings, we decided to investigate the impact of miR-21a-5p-enriched FPC-derived sEVs on cardiac fibroblast activation and cardiac fibrosis.

3.4 | MiR-21a-5p packaged in TGFβ-activated FPC-sEVs is responsible for fibrosis

To evaluate whether miR-21a-5p present in the FPC-sEVs facilitates cardiac fibroblast to myofibroblast transition, we inhibited miR-21a-5p in FPC using antagomir (anti-miR) and then collected sEVs (Figure S2A, B). Our results showed that sEVs derived from anti-miR-21a-5p-transfected-FPCs significantly reduced fibrosis-associated genes, that is, αSMA and Col1α expression in CFs compared to the cells treated with control sEVs (Figure 3a, b). We also observed a corresponding trend in the protein levels of Col1α and TGFβ in the CFs following respective sEVs treatments (Figure 3c–e). Furthermore, we performed immunostaining with Col1α antibody to verify the above results after treating CFs with FPC-sEVs isolated from above-described treatment groups. In consistent with the qPCR and western blot data, the Col1α immunostaining (green fluorescence) was dramatically reduced when the CFs were treated with miR-21a-5p-inhibited FPC-sEVs (Figure 3f–g). In addition to molecular changes, CFs also displayed structural changes during myofibroblast transition. These changes include the larger, flattened cell shape due to the accumulation of more stressed fibers in TGFβ treatment. Taken together, these data confirmed that TGFβ enhanced miR-21a-5p packaging into the sEVs, which is responsible for activation of CFs and cardiac fibrosis. In addition, we also found that IL-10 reduced miR21a-5p packaging in sEV.

Activated fibroblast (myoFBs) shows immense potential to migrate, a basic feature needed for efficient wound healing. Therefore, we tested whether inhibiting miR-21a-5p in FPC sEVs has any effect on CF's migration. We performed cell migration (scratch) assay in the culture plate following 100% CFs confluency. Before respective exosomal treatments, we captured CF's pictures (0 h). We then treated CFs with sEVs obtained from different treatments and captured pictures again at 24 and 48 h post treatment. Our results showed that the TGFβ-FPC-sEV treatment group has tremendous migration property, especially in the IL10-KO group. However, CFs migration was substantially reduced when treated with sEVs isolated from miR-21a-5p depleted IL-10KO FPCs (Figure S3A, B).

3.5 | In silico analysis reveals integrin subunit alpha V (ITGAV) as a potential downstream target of miR-21a-5p

Micro RNAs usually bind with a critical target molecule to exert their effect. To identify the miR-21a-5p potential targets, we utilized miRDB, an online database for miRNA targets binding prediction (https://mirdb.org/). We screened the genes with >70 target score. Among the potential candidates, we selected *Integrin Subunit Alpha V (ITGAV)* for further investigation due to its role in fibrosis in other organs (Bouvet et al., 2020; Chen et al., 2016; Henderson et al., 2013). Predicted pairing with the target region in *ITGAV* and conserved binding sites for miR-21a-5p as identified by TargetScanMouse and protein-protein interactions by STRING (Search Tool for the Retrieval of Interacting Genes/Proteins) are shown in Figure 4 (a, b). Our data analysis has confirmed that *ITGAV* has multiple miR-21a-5p binding sites (Figure 4b). To further confirm the clinical significance of selected target, we assessed the expression of *ITGAV* using the available GSE182985 data set (Li et al., 2022) in sham and TAC groups and generated a heatmap by given Fragments Per Kilobase of transcript per Million mapped reads (FPKM) values above 10 and

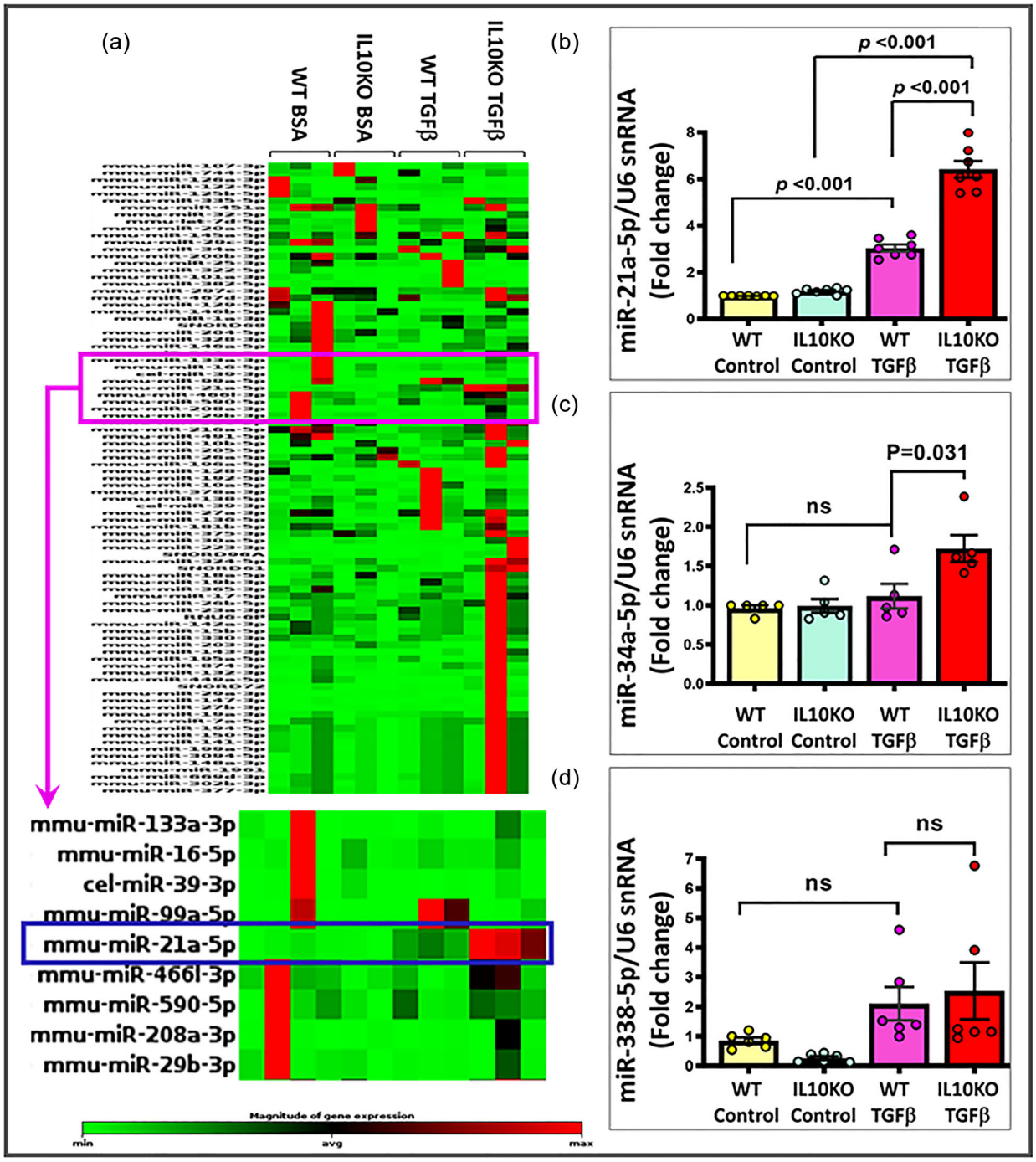

FIGURE 2 TGFβ activated IL-10 KO FPC's small EV are enriched with miR-21a-5p. To determine the sEVs miRNA contents, a biased approach was adopted where miRNAs were isolated from the sEV to perform fibrosis-associated miRNAs array from Qiagen. (a) The heat map of the differentially expressed miRNAs between the groups showed enhanced expression of fibrosis-associated miRNAs in the TGFβ-activated sEVs as compared to the control. miRNA array data suggest that miR-21a-5p is highly expressed in the sEVs derived from IL-10KO FPC sEVs following TGFβ treatment. (b–d) The miR-21a-5p expression was further validated by qPCR in the sEV's miRNAs from all groups that showed significantly upregulated expression in TGFβ-activated IL-10KO FPC derived sEVs. In contrast, the qPCR expression results of miR-34a-5p and miR-338-5p did not show significant difference ($N = 3–5$).

<50 (Figure S4A). Interestingly, *ITGAV* expression was significantly upregulated post-TAC (Figure S4B). Furthermore, we used miRNet (miRNA-centric network visual analytics platform) and confirmed the miR-21a-5p-ITGAV interaction (Figure S4C). The gene enrichment analysis using PANTHER showed biological adhesion group in the pie diagram, which confirmed *ITGAV* expression after TAC (Figure S4D). Our Insilco data analysis suggests that miR-21a-5p has strong binding potential with *ITGAV* and IL-10KO FPC-sEVs mediated increased fibroblast activation could be due to interaction of miR-21a-5p with *ITGAV* and its downstream signalling.

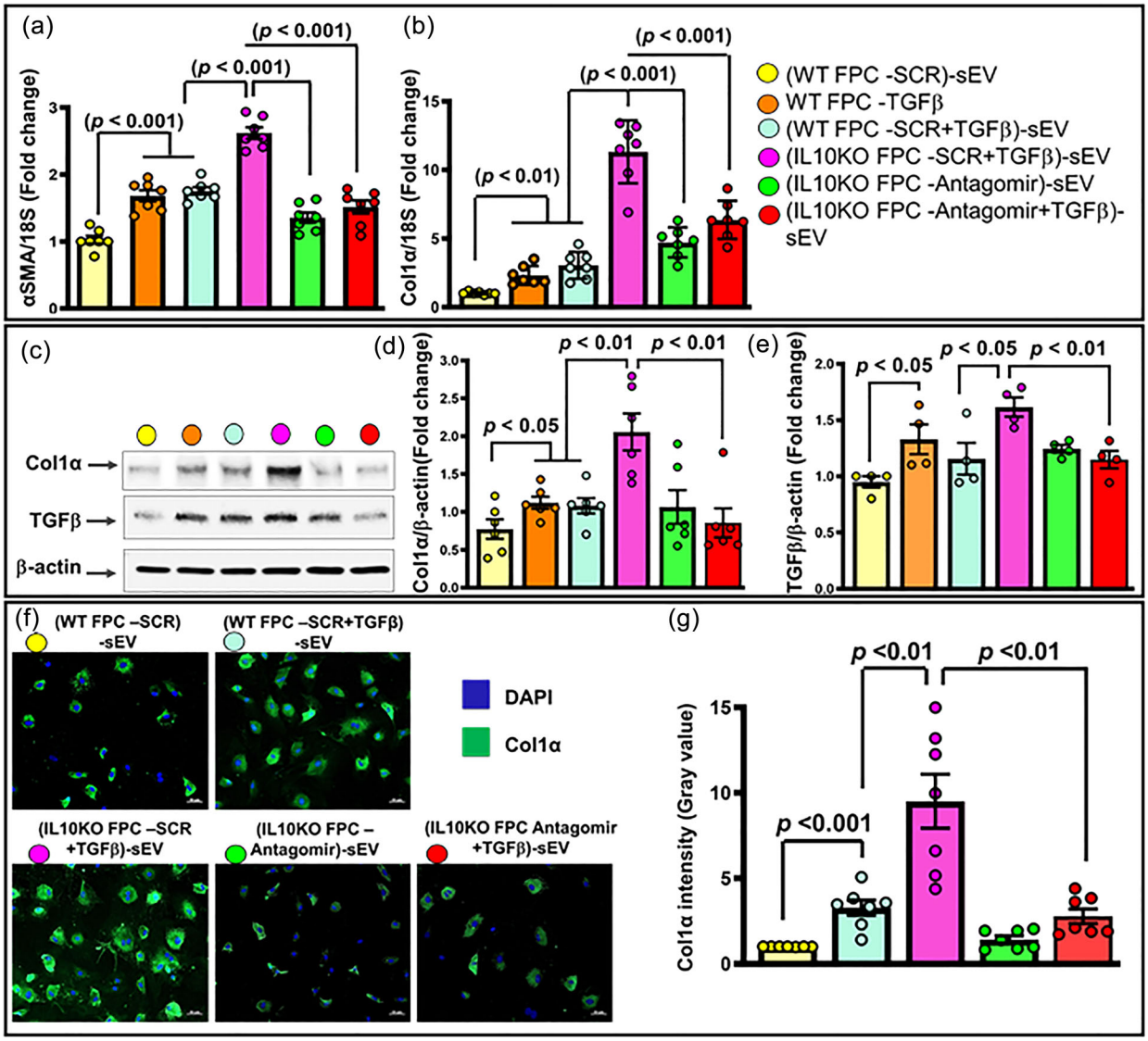

FIGURE 3 IL-10 KO FPCs exosomal miR-21a-5p exacerbates fibroblast activation. Further, to determine the contribution of sEV's miR-21a-5p on fibrotic signalling, both WT and IL-10 KO FPCs were transfected with scrambled or anti-miR-21a-5p microRNA using lipofectamine 2000 followed by TGFβ treatment for 48 h. The sEVs were collected from the respective conditioned media. WT adult cardiac fibroblasts were stimulated with respective modified sEVs (miR-21a-5p modified) for 48 h. (a–b) Interestingly, miR-21a-5p inhibition in IL-10 KO FPC significantly reduced the IL-10 KO FPC sEVs induced αSMA and Collα gene expression in cardiac fibroblasts. (c–e) In corroboration to RNA data, miR-21-5p inhibition significantly reduced collα and TGFβ protein levels as well. (f–g) Furthermore, immunostaining was performed in cardiac fibroblasts treated with different FPC-sEVs. As observed Collα expression was upregulated in the CFs after TGFβ-FPC-sEV treatment. However, miR-21a-5p inhibition in FPC- sEV rescued Collα expression significantly. Images were taken at 100X and scale bar is 100 μm. $p < 0.05$ was considered as statistical significance, $N = 4–6$.

3.6 | miR-21a-5p stabilizes integrin subunit alpha V (ITGAV) mRNA and aggravates col1α expression and fibrotic signalling following TGFβ-treatment

Previous studies have suggested that miR binding with its target transcripts can regulate their expression and downstream signalling via regulating its stability (Coenen-Stass et al., 2019; Shukla et al., 2011; Zhuang et al., 2013). To determine whether exosomal miR-21a-5p regulates *ITGAV* mRNA stability and expression in cardiac fibroblast, we first treated mouse CFs with sEVs derived from miR-21a-5p-antimiR-treated FPCs and untreated FPCs. After 48 h, we performed an *ITGAV* mRNA stability assay by blocking its *de nova* transcription using actinomycin-D chase experiment. We found that *ITGAV* mRNA half-life was significantly reduced in CFs treated with FPC-miR-21a-5p-antagomir-sEVs compared to control and FPC-miR-21a-5p-mimic-sEVs groups. These results confirmed that miR-21a-5p has potential to stabilize *ITGAV* mRNA (Figure 4c) in cardiac fibroblasts, which may lead to fibroblast activation and cardiac fibrosis. To further confirm whether increased *ITGAV* mRNA stability facilitated integrin av expression and its interaction with Col 1a, ITGAV/Collα immunostaining was performed in CFs following

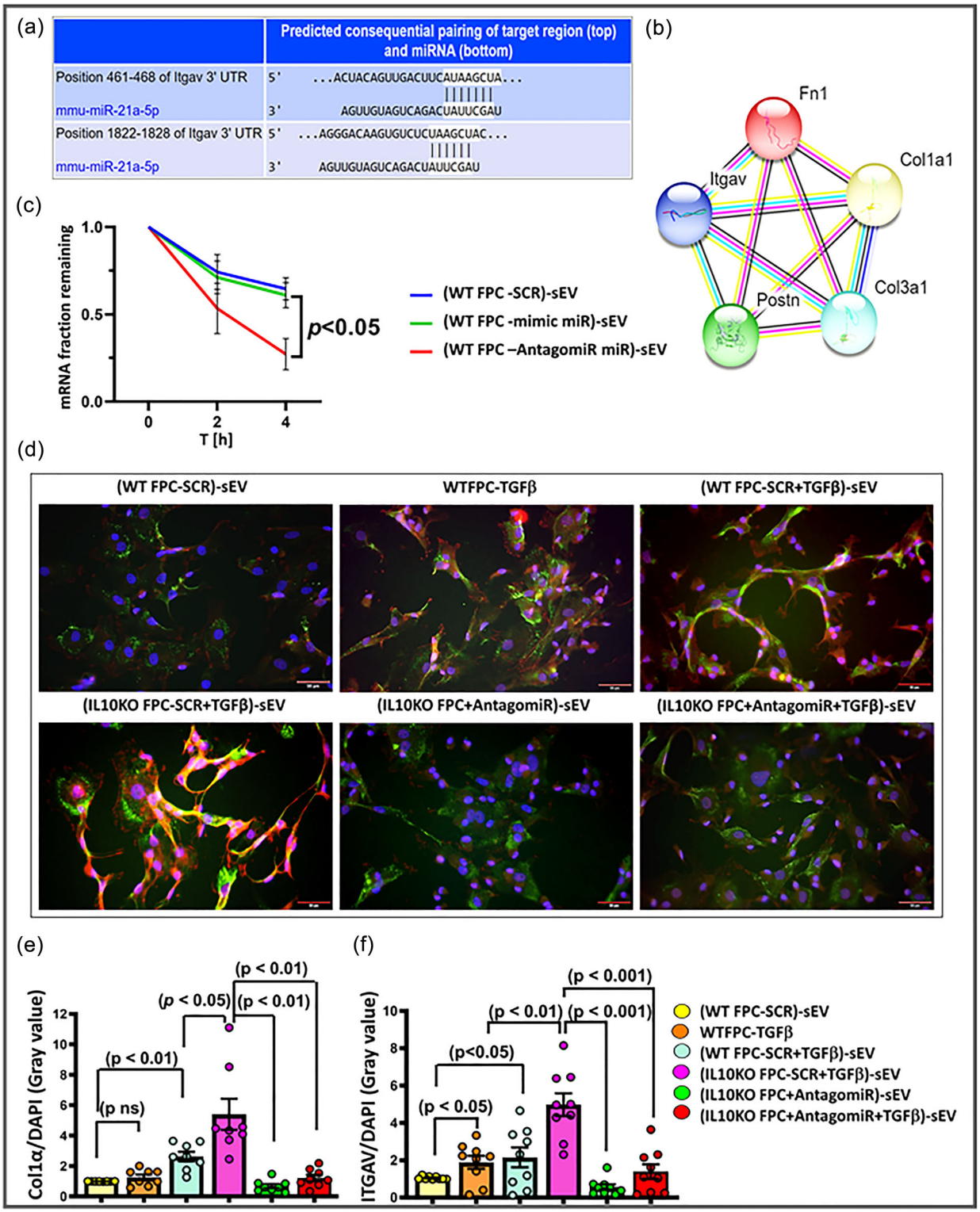

FIGURE 4 miRNA target prediction and mRNA stability assay. miRNA 21a-5p target prediction analysis using computational approach suggests Integrin Subunit Alpha V (ITGAV), a profibrotic integrin receptor, contains multiple miR-21a-5p binding sites. (a) The image depicts the predicted consequential pairing of the target region (ITGAV) and conserved sites for miR-21a-5p using TargetScanMouse tool. (b) Further functional association of fibrosis-associated proteins with ITGAV by STRING analysis suggests that it interacts with multiple profibrotic proteins, namely, periostin, Collagen 1a, fibronectin etc. (c) Actinomycin-D chase assay showed that ITGAV stability was significantly decreased in CFs following miR-21a-5p inhibition as compared to miR-21a-5p overexpression. (d–f) To determine whether TGFβ induced miR-21a-5p mediated ITGAV expression enhances its interaction with Col1a, cardiac fibroblasts were treated with FPC (treated with scrambled, TGFβ, and miR-21 antagomir) derived sEVs. Immunostaining of Integrin Subunit Alpha V (ITGAV) (Red), Col1α (Green), and DAPI (Blue) were performed in these cells. The data clearly suggests that reduced miR-21a-5p packaging in sEVs significantly reduced both ITGAV and Col1α expression and their interaction compared to respective controls. The scale bar is 100 μm. $p < 0.05$ was considered as statistical significance, $N = 4–8$.

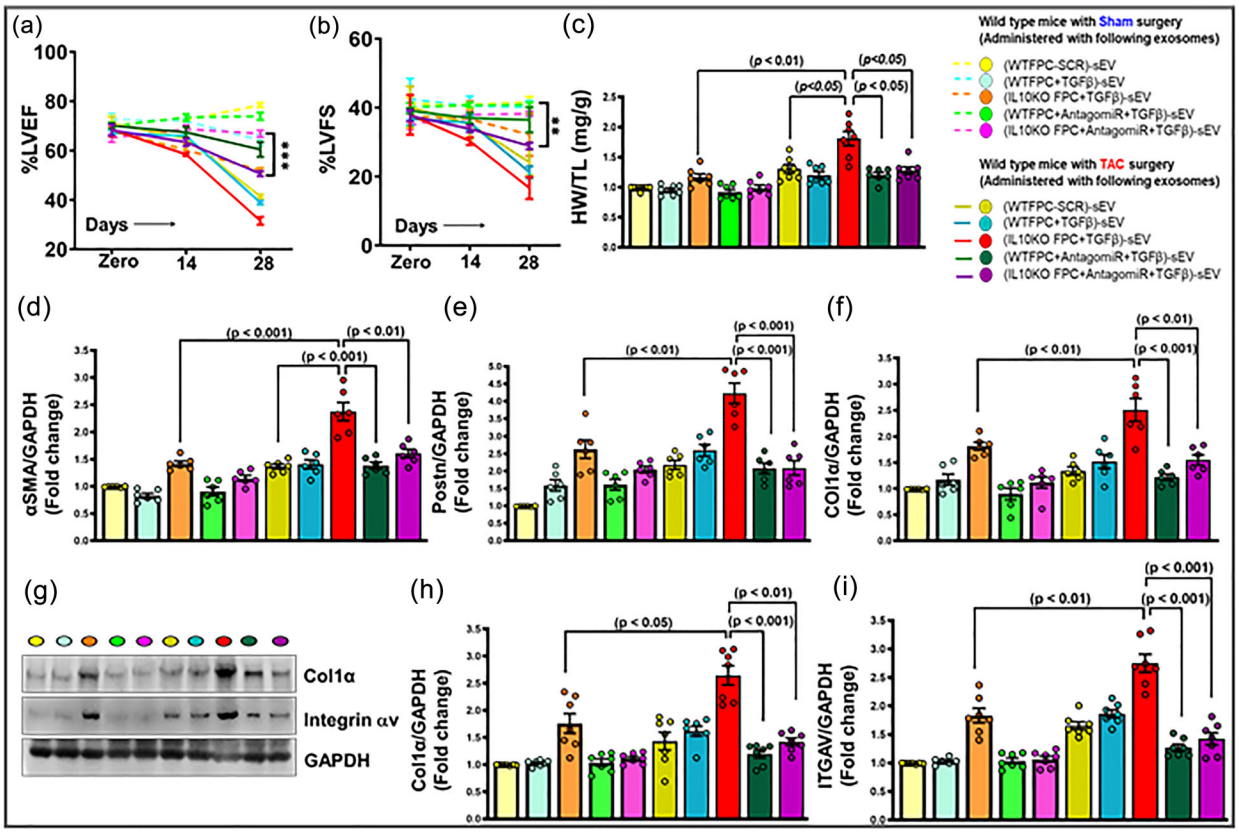

FIGURE 5 Reduced miRNA-21a-5p packaging in BMFPC-small EVs improves cardiac function and attenuates TAC-induced fibrotic genes expression. WT mice were treated with WT or IL10 KO BMFPC-sEVs (miR modified sEVs) following sham/TAC surgery. After serial echocardiographic analysis, mice were euthanized, and heart tissue was extracted for biochemical analysis. (a–b) TAC-induced exacerbated cardiac dysfunction in the mice treated with IL-10 KO BMFPC's sEVs as compared to WT BMFPC sEVs. Interestingly, TAC-induced decrease in ejection fraction (%LVEF) and fractional shortening (%LVFS) were significantly restored following miR-21a-5p inhibited sEVs (from both WT and IL-10KO FPC) treatment. (c) Furthermore, the TAC-induced heart weight to tibia length ratio was significantly reduced following mice treated with miR-21 inhibited sEVs. (d–f) To determine the effect of WT and IL-10 FPC sEVs (with or without inhibitor) on cardiac fibrosis, we performed fibrotic genes expression in the heart tissues 28 days following TAC and sEVs treatment. TAC-induced exacerbated fibrosis associated genes expression was significantly reduced in mice that received miR-21a-5p inhibited sEVs. (g–i) Further, we performed western analysis for ITGAV and Col1α protein levels following sEV treatment. miR-21a-5p inhibition in sEV markedly abolished TAC-induced ITGAV and Col1α protein levels. $p < 0.05$ was considered as statistical significance, $N = 6–8$.

sEVs treatment. Noticeably, CFs that were exposed to TGFβ-treated FPC-derived sEVs showed increased ITGAV (Red) and Col1α (Green) expression, which suggests that miR-21a-5p involved in the profibrotic signalling (Figure 4d–f). At the same time, inhibiting miR-21a-5p with antagomir abrogated the aberrant overexpression of ITGAV and Col1α in CFs. Our data confirm that IL-10-KO FPC-derived sEVs facilitate ITGAV stability and expression.

3.7 | C57BL/6J mice under pressure-overload regain normal cardiac function when treated with FPC-(anti-miR-21a-5p)-small EVs

To determine whether antagomir-mediated reduction in exosomal miR-21a-5p packaging reduced ITGAV-mediated fibroblast activation and cardiac fibrosis in vivo, we performed TAC surgery in WT mice and injected WT and IL-10 KO FPC-sEVs (1×10^9 sEVs/mice) via jugular vein injection (Pang et al., 2021). As our previous experiences, a single exosomal injection is enough to facilitate signalling in heart. Cardiac function was assessed by echocardiography at baseline (before surgery and treatment), days 14 and 28 post-TAC (Figure S5A). The heart function was significantly reduced in the mice which received sEVs from IL-10 KO FPC sEVs as compared to WT-FPC sEVs (Figures 5a, b **and** S5B). Excitingly, the WT TAC mice injected with IL10-KO-FPC-antagomir-sEVs showed a significant improvement in their cardiac functions compared to respective control mice. The heart weight to tibia length ratio was significantly increased (Figure 5c) in TAC mice that received IL-10KO FPC sEVs. Interestingly, the ratio was reduced in the mice that received miR-21a-5p inhibited sEVs. The ratio of heart weight to body weight also followed a similar trend (Figure S5C).

Considering the profound effect of miR-21a-5p on cardiac function in vivo, next, we assessed whether inhibition of miR-21a-5p leads to reduced cardiac fibrosis and better cardiac function. To do so, first, we evaluated the expression of fibrosis-associated genes (αSMA, periostin, and Col1α) in mice heart tissues. All three markers were considerably overexpressed in IL-10 KO FPC-TGFβ-sEV-treated TAC-mice. Importantly, this effect was significantly rescued in the mice injected with miR-21a-5p-inhibited sEVs, even if it is derived from TGFβ-treated IL10-KO-FPC (Figure 5d–f). Next, we measured ITGAV and Col1α protein levels. Our results showed that administration of TGFβ-treated FPC-sEV overexpressed Col1α and integrin av expression by approximately 2- to 3-fold, whereas miR-21a-5p-inhibited sEVs significantly suppressed TAC-induced ITGAV and Col1α expression compared to control-sEVs groups (Figures 5g–i **and** S6). Further, we evaluated extracellular matrix protein level in mouse hearts sections using Masson's trichrome staining (blue). We found that the percentage of fibrosis was significantly increased in the TGFβ-treated FPC-sEVs (*i.e.*, miR-21a-5p-enriched sEVs) (Figure 6a, b). Strikingly, administration of sEVs with inhibited miR-21a-5p significantly reduced the left ventricular fibrosis suggesting improved left ventricular cardiac function. The WT mice that underwent TAC surgery but received IL10-KO-FPC-(Antagomir+TGFβ)-sEV also showed significant improvement from pressure overload and cardiac fibrosis, suggesting that miR-21a-5p-inhibition is an effective therapeutic strategy to reverse cardiac fibrosis in general.

4 | DISCUSSION

Fibrosis is a natural process that is necessary for wound healing. However, deregulated fibrosis can lead to abnormal organ function and eventual loss of the fibrotic organ. Although substantial efforts have been made to elucidate the molecular mechanisms of cardiac fibrosis, much remains to be known. Persistent stress-induced hypertrophy increases the risk of cardiovascular diseases, and inflammation plays an important role in accelerating the pathogenesis (Kania et al., 2009a; Verma et al., 2012). Therefore, it is necessary to know the contribution of profibrotic factors of FPC-derived sEVs homing to the heart. To establish the role of FPC-derived sEVs in cardiac fibrosis in mice, we used an IL10-KO mouse. We have previously shown that IL-10 deletion exacerbates mobilization and homing of FPCs in the heart following pressure overload (Verma et al., 2017). Interestingly, when FPCs isolated from IL10-KO mice were treated with TGFβ, the secreted sEVs were potent in provoking cardiac fibrosis. This drew our attention, and further investigation led us to the contents of the sEVs that induce cardiac fibrosis upon entering the heart. When we treated IL10-KO-FPCs with TGFβ and then used the secreted sEVs to treat wild-type fibroblast cells, the WT cells developed fibrotic phenotype. These key findings motivated the present study to delineate the contribution of FPCs-derived exosomal contents to cardiac fibrosis and cardiac dysfunction.

TGFβ exerts its role through an autocrine or paracrine manner and promotes fibroblast activation and differentiation to myofibroblasts (Frangogiannis, 2020; Kaplan et al., 2007; Shi et al., 2020). Previously, we reported that TGFβ activates FPCs and can enhance the expression of fibrosis-associated miRNAs (Verma et al., 2017). Compared with normal resident fibroblasts, TGFβ-activated myofibroblasts in fibrotic lesions display robust expression of collagen and αSMA (Klingberg et al., 2013; Shinde et al., 2017). Besides stimulating transcription of the collagen 1a, TGFβ possibly stabilizes its mRNA via enhanced cross-linking (McAnulty et al., 1991; Raghow et al., 1987). Our results are consistent with these observations, and TGFβ significantly activates the expression of Col1α, fibronectin, periostin, and αSMA genes, and even higher if IL10 is knocked out (Figure S1). These results indicate that TGFβ treatment significantly activates FPCs that contribute to fibrosis.

SEVs are one of the most important ways for cell to cell communication, and their role in regulating fibroblast activation and tissue fibrosis has been well established (Hohn et al., 2021). Several reports suggest that cancer cell's exosomes or sEVs trigger fibroblasts and mesenchymal stem cells to become myofibroblasts (Cho et al., 2011; Gu et al., 2012; Webber et al., 2010). SEVs stimulate the expression of proteins involved in matrix remodelling and contraction in fibroblasts differentiated towards myofibroblasts (Tutuianu et al., 2021). Therefore, we studied the sEVs-mediated relationship between FPCs and cardiac fibroblasts to see if they were involved in activation of resident fibroblasts. The data presented in this study show that TGFβ- treatment significantly enhanced fibrosis-associated genes expression, which was in line with the aforementioned reports. Interestingly, IL10-KO-FPC-sEVs substantially increased fibrotic gene expression, that is, Col1α, Col3α, and αSMA in CFs as compared to the WT-FPC-sEVs. It is well documented that sEVs carry variety of payloads, especially miRs, which have been shown to activate cardiac fibroblasts and induce fibrosis (Hohn et al., 2021; Ranjan et al., 2019; Wang et al., 2021). Bang et al. (2014) demonstrated that cardiac fibroblasts secrete sEVs that contain fibroblast-derived miR-21, a mediator of cardiomyocyte hypertrophy (Bang et al., 2014). In addition, we reported that myoFB-derived sEVs carrying miRs induced cardiac endothelial cell dysfunction (Ranjan et al., 2021). Interestingly, in this study, the miRs profiling using fibrosis-associated miRNA array kit along with miR-21a-5p qPCR data in the sEVs indicated that miR-21a-5p levels were significantly elevated in TGFβ-treated IL10-KO-FPC-sEVs compared to both control and TGFβ-treated WT-FPC-sEVs. The presence of exogenous TGFβ as a contaminant in the EV preparation might contribute to fibrotic activation. However, considering its shorter half-life, TGFβ is unlikely to be found in sEVs (Wakefield et al., 1990; Hermonat et al., 2007). Therefore, we posit that only TGFβ-induced signalling molecules (miRs in our case), packaged in the sEVs, are implicated in the sEVs-mediated profibrotic signalling observed in this study.

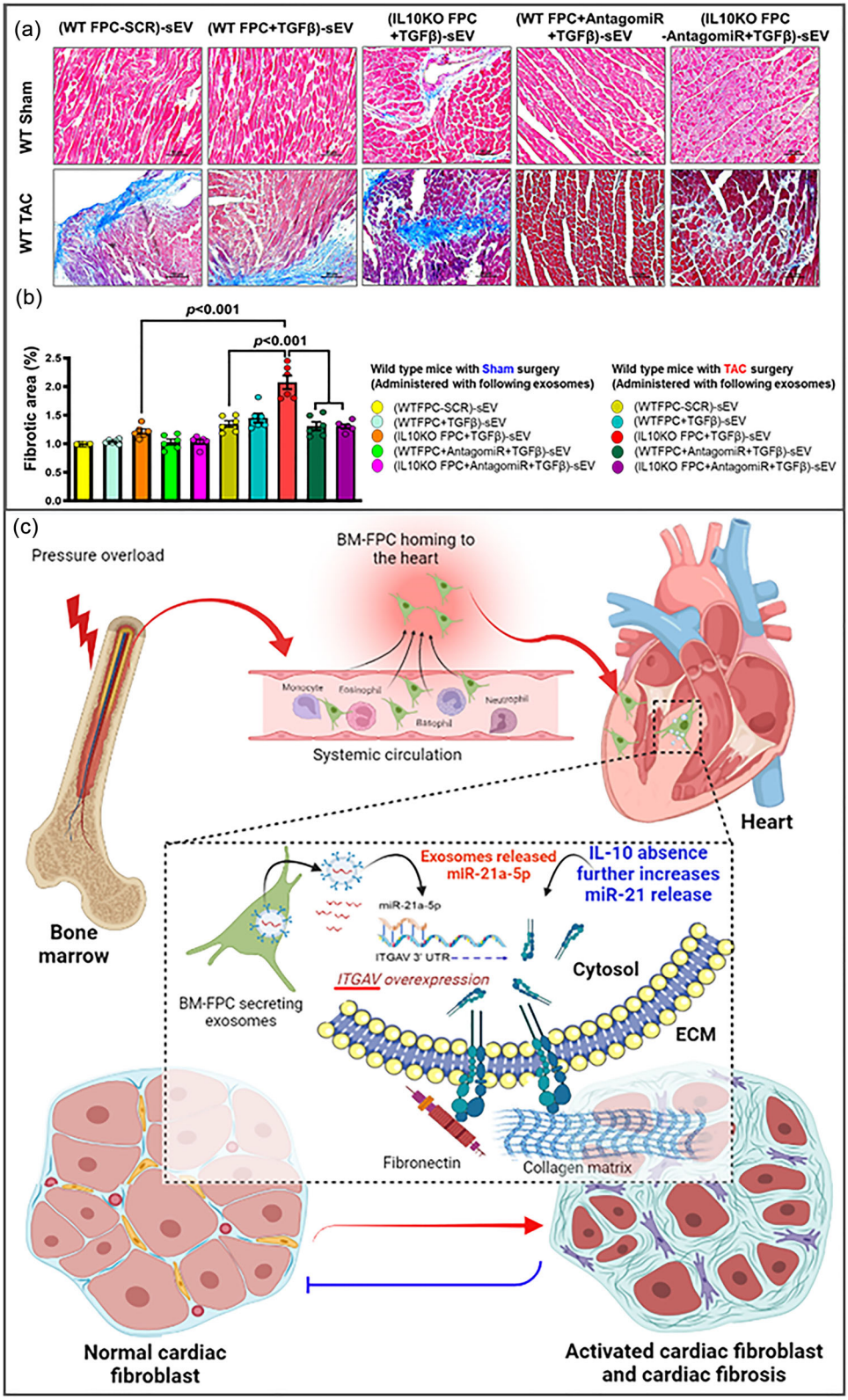

FIGURE 6 miRNA-21a-5p depleted FPC-sEVs treatment attenuates TAC-induced cardiac fibrosis. WT mice were treated with control or IL10 KO FPC-sEVs (miR modified sEVs) following sham/TAC surgery. Massan trichome staining were performed in the heart tissue. (a–b) As we expected, mice which received IL-10KO FPC-sEVs showed tremendous fibrosis following TAC. In contrast, TAC-induced fibrosis was significantly reduced when animal received miR-21 depleted sEVs ($p < 0.05$ was considered as statistical significance, $N = 4–7$). (c) Schematic illustration of the proposed role of bone marrow-derived sEVs miR-21a-5p/ITGAV/Collα signalling cascade in promoting cardiac fibrosis. Activated FPC-derived sEVs enriched with miR-21a-5p following TAC induced cardiac fibrosis by altering the ITGAV/Colα signalling under pressure-overload. Alteration in miR-21a-5p expression using antagomir therapy reduces cardiac fibrosis and IL-10 (anti-inflammatory singling) plays important role in this process.

Supporting our findings, several reports also show that miR-21 is involved in profibrotic signalling in other organs such as kidney, liver, skin, and lung (Hinkel et al., 2020; Liu et al., 2016; Ramanujam et al., 2016; Thum et al., 2008). Ramanujam et al. (2021) demonstrated that macrophage miR-21 promotes myocardial fibrosis through paracrine signalling (Ramanujam et al., 2021). Thum et al. (2008) also showed that miR-21 induces ERK–MAP kinase signalling in cardiac fibroblasts, and the use of antagomir-21 completely reverses it (Thum et al., 2008); however, in their studies, the miR-21 expression was very low in cardiomyocytes and did not increase in the failing hearts. Another study identified highly upregulated miR-21a-5p using miRNAome analysis in murine chronic renal allograft dysfunction and its inhibition had beneficial effects on kidney function (Schauerte et al., 2017). Nonaka et al. (2021) reported that the intensity of fibrosis in Chagas disease cardiomyopathy was caused by miR-21, the only miRNA that showed consistent upregulation across all their GEO datasets (Nonaka et al., 2021). Although a growing number of studies reiterate the miR-21′s role in promoting fibrosis, they are not without controversy. Qiao et al. (2019) showed that miR-21a-5p-containing sEVs-mediated heart repair involved enhancing angiogenesis and cardiomyocyte survival through phosphatase and tensin homolog/Akt pathway; however, it had no effect on fibroblast survival (Qiao et al., 2019). The major difference between our and Qiao's studies in the experimental model used as well as the source of sEVs. They studied the role of miR-21 on cardiomyocytes survival in acute ischemic injury model. In addition, Patrick et al. (2010) found that miR-21-null mice were completely normal, and there was no cardiac remodelling (Patrick et al., 2010). Paradoxically, some miRs have been shown to produce opposing effects under different cellular conditions (Svoronos et al., 2021).

Since fibroblast has immense potential to proliferate, we treated them with different FPC-derived sEVs. Our result showed that miR-21a-5p enriched sEVs derived from IL10-KO FPC greatly increased the proliferative capacity of cardiac fibroblast cells within 48 h, which was effectively reversed in the antagomir-treated group. When we immunostained these cells, we observed increased Col1α expression in the miR-21a-5p-enriched sEV-treated group and the lowest expression of Col1α was seen in the antagomir-treated group. These results are consistent with the study by Cui et al. (2020), in which they showed that BM macrophage-derived sEVs with miR-21a-5p activate fibroblasts and Col1α expression in tendon cells (Yavropoulou et al., 2020). Our *in-silico* analysis identified integrin subunit alpha V (ITGAV) as its likely target. ITGAV has strong interactions with fibrosis-associated proteins, that is, Col1α, fibronectin, and periostin, as evidenced by Protein-Protein Interaction Networks Functional Enrichment Analysis and strongly regulates fibrosis through the TGFβ-signalling pathway (Bouvet et al., 2020; Chen et al., 2016; Henderson et al., 2013). When we investigated how miR-21a-5p binding affects ITGAV expression, our result showed that ITGAV mRNA expression level was significantly higher in those cardiac fibroblasts which were treated with BMFPCs-miR-21a-5p-mimic-sEVs than those treated with BMFPCs-miR-21a-5p-antagomir-sEVs. A volume of literature suggests that miRs induce target gene degradation by interacting with their 3′ untranslated region (3′ UTR) (Bartel, 2009; O'Brien et al., 2018). It has been shown that miR-21 directly binds cytochrome b mRNA and enhances its translation in mitochondria (Li et al., 2016). Another report finds that miR-107 increases CLOCK gene expression by binding to its 3′-UTR (Daimiel-Ruiz et al., 2015). They explained that the miR inhibits protein translation and as compensatory response cells upregulate mRNA transcription. In this study, miR-21′s binding to ITGAV and the expressions of both ITGAV and Col1α are being reflected in the same way. More importantly, when miR-21 was inhibited, it reduced the expression of both ITGAV and Col1a.

To perceive the clinical importance of FPC-derived exosomal miR-21 on stress-induced cardiac fibroblast activation and cardiac fibrosis, we induced hypertrophic heart failure in mice. As mentioned above, FPC mobilization occurs in the peripheral blood during TAC-induced cardiac hypertrophy, and these FPCs are recruited into the mouse heart (Verma et al., 2017). TGFβ-treated FPC-derived sEVs mount an exaggerated response to pressure overload compared to WT mice, and the administration of miR-21-inhibited sEVs significantly alleviates cardiac fibroblast activation and fibrosis in these mice. Increased expression of Col1α in IL10-KO-FPC-TGFβ-sEV-treated mice subjected to TAC surgery suggests significant cardiac fibrosis. Col1α expression was significantly attenuated by miR-21 inhibition. Furthermore, miR-21-mediated Col1α gene expression was dependent on ITGAV. Our in vivo results show that the FPC-TGFβ-sEVs significantly enhance the mRNA expression level of cardiac fibroblast markers, that is, αSMA, periostin, and Col1α, and this enhancement is even higher with IL10-KO-derived FPCs. Furthermore, miR-21-5p preserves the fibrotic memory of mesenchymal cells, as shown in our immunostaining data in vitro and in vivo (Li et al., 2017). The data presented in this study established a direct link between miR-21a-5p-mediated fibroblast activation and cardiac fibrosis. The controlled cardiac fibroblast activation is a fundamental cornerstone of adequate cardiac repair following injury. The current study indicates that the exacerbated miR-21a-5p level from FPC derived sEVs disrupts the tight regulation of fibroblast activation specially during inflammatory condition. Previous studies suggest that ITGAV following binding with Col1α exacerbates fibroblast activation and that the resulting in deregulated collagen deposition and fibrosis. In this study we also found that bone marrow-FPC secreted miR-21a-5p maintains ITGAV-Col1α -mediated collagen deposition in chronic conditions that drive to continued fibrosis. Thus, the present study provides new insights into the mechanistic events of cardiac fibrosis and sheds light on novel therapeutic options focused on targeting miR-21-5p. This study provides valuable insights into the mechanism of cardiac fibrosis. However, it is crucial to acknowledge certain inherent limitations. Beyond microRNAs (miRs), small extracellular vesicles (sEVs) also contain various other biological and signalling molecules, such as protein aggregates, lipids, and nucleic acids. In this investigation, we exclusively focus on elucidating the mechanism by which miR-21-5p, packaged in sEVs, induces cardiac fibrosis. The role of other biological molecules will be explored in future studies. Another limitation pertains

to the downstream targets of miR-21-5p. It is conceivable that miR-21-5p has multiple downstream targets. However, our study concentrates on investigating ITGAV as a primary target in the regulation of cardiac fibrosis.

5 | CONCLUSION

In summary, activated bone-marrow cells homing to the heart secretes small EVs carrying miR-21a-5p during pressure overload. Our result supports the notion that miR-21a-5p acts via Integrin αV and enhances the expression of extracellular matrix proteins, especially Col1α (Figure 6c). Specifically, miR-21a-5p irreversibly transforms fibroblast into contractile myofibroblast by promoting the expression of ITGAV. Collectively, the outcome of the studies highlights a new miR-21a-5p /ITGAV/Col1α axis in cardiac fibroblast activation and cardiac fibrosis, suggesting miR-21a-5p could serve as a potential therapeutic target for treating hypertrophic cardiac remodelling and heart failure using miR modified small EVs.

AUTHOR CONTRIBUTIONS

Prabhat Ranjan: Data curation; formal analysis; methodology; writing—original draft. **Roshan Kumar Dutta**: Data curation; methodology; validation; writing—review and editing. **Karen Colin**: Methodology; validation; writing—original draft. **Jing Li**: Investigation; methodology. **Qinkun Zhang**: Methodology. **Hind Lal**: Methodology; resources. **Gangjian Qin**: Data curation; methodology; resources; visualization. **Suresh Kumar Verma**: Conceptualization; funding acquisition; investigation; project administration; resources; supervision; visualization; writing—review and editing.

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CONFLICT OF INTEREST STATEMENT

The authors declare that the research was conducted in the absence of any commercial or financial relationships without any potential conflict of interest.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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