Deletion of transcription factor AP-2α gene attenuates fibroblast differentiation into myofibroblast

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1 | INTRODUCTION
Excessive fibrosis underlies many critical organ dysfunctions.1,2 Fibrosis emanates from fibroblast trans-differentiation into myofibroblasts,3 marked by increased α-smooth muscle actin (α-SMA) expression and excessive collagen secretion, initiated as a reparative process of normal wound healing and tissue repair in response to injury.4 However, activated myofibroblasts accumulate within pathological lesions of various fibrotic disorders,5 including patchy and interstitial fibrosis in progressive heart failure and cardiac hypertrophy.5 Therefore, attenuation of differentiation to myofibroblasts is expected to mitigate fibrosis. We attempted to find a potential target to extenuate the fibroblast differentiation by analysing the transcription factors in human fibroblasts/myofibroblasts, as transcriptome changes occur in fibroblasts during differentiation.7 Here, we report a novel molecular target, transcription factor AP-2α (TFAP2A), to reduce fibroblasts trans-differentiation.

2 | MATERIALS AND METHODS
Informed consents were obtained from all participants, and the study was carried out according to the World Medical Association Declaration of Helsinki.

Human ventricular fibroblasts (hVFs)-Control hVFs from disease-free trauma victims (Lonza Inc, Allendale, NJ; ScienCell, Carlsbad, CA) were purchased; hVFs were isolated from Heart Patients (Aurora Health Care, Milwaukee, WI), (HF) as reported earlier.8 NIH/3T3 fibroblasts (ATCC, Manassas, VA), Transforming growth factor (TGF)-β1 (Peprotech, Rocky Hill, NJ), angiotensin II (Abcam, Cambridge, MA), miRNeasy Mini Kit, RT2 Profiler PCR Array, RT2 First Strand Kit, RT2 SYBR Green PCR master mix, miScript II RT kit (QIAGEN, Venlo, the Netherlands); Power SYBR Green PCR Master Mix (Thermo Fisher Scientific, Waltham, MA), Antibodies: Anti-α-SMA, Anti-TFAP2A (Abcam, Cambridge, MA), Anti-α/β-tubulin and Anti-GAPDH (Cell Signaling, Danvers, MA) were purchased.

2.1 | Polymerase chain reaction array
The isolated hVFs were grouped into fibroblasts-less differentiated (HF-LD) and fibroblasts-highly differentiated (HF-HD) based on their α-SMA expression (immunoblot), compared to the control hVFs (Figure 1A,B). Polymerase chain reaction (PCR) array was performed with RT2 Profiler™ PCR Array-Human Transcription Factors and compared between HF-LD (n = 3) and HF-HD (n = 3). Mature RNA (miRNeasy Mini kit) was reverse transcribed using RT2 First strand cDNA synthesis kit. The cDNA was used on the real-time RT2 Profiler PCR Array (QIAGEN, Cat# PAHS-075Z) in combination with RT2 SYBR® Green qPCR Mastermix (Roche LightCycler® 480 Instrument). Threshold cycle (Ct) values (excel file) were uploaded onto the data analysis centre web portal (http://www.qiagen.com/geneglobe). Ct values were normalized
based on a Manual Selection of reference genes. The fold change/regulation \(2^{\Delta \Delta CT}\) was calculated using \(\Delta \Delta CT\) method [\(\Delta CT\) was calculated between target gene and an average of reference genes (HKG), followed by \(\Delta \Delta CT\) calculations (\(\Delta CT\) (Test Group) - \(\Delta CT\) (Control Group))].

2.2 | Quantitative real-time PCR

Total RNA was isolated from hVFs (miRNeasy Mini kit) and reverse transcribed (miScript RT II kit) with the supplied HiFlex buffer. qPCR was performed on the LightCycler 480 Instrument II, using the Power SYBR Green

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**FIGURE 1** Expression of TFAP2A gene in human ventricular (myo)fibroblasts (hVFs) is significantly decreased with decrease in trans-differentiation. Differentiation magnitude was assessed by the expression of \(\alpha\)-SMA, a marker of differentiated myofibroblasts. (A) Immunoblot displaying high and low expressions of \(\alpha\)-SMA in hVF lysates from each heart failure patients (HTV) [*red* label is patients with low differentiation], and control hVFs obtained from disease-free trauma victims. Scattered plot displays the individual expression of \(\alpha\)-SMA normalized to GAPDH. (B) Bar graph displays analysis of grouped samples of high-differentiation (HF-HD, n = 4) and low-differentiation (HF-HD, n = 4), based on the \(\alpha\)-SMA/GAPDH ratio compared to the control (n = 3) group. (C) The Heat Map visualizing the fold changes in expression of genes in the Transcription factor qPCR Array between HF-LD and HF-HD group hVFs. Note that TFAP2A gene (well: G09) expression is significantly different between the two groups (n = 3). (D) Table provides the fold regulation (vs HF-LD) data used for the map associated with each gene. (E) Validation of TFAP2A expression in hVFs from HF-HD and HF-LD patients by qRT-PCR \(2^{\Delta \Delta CT}\). *P = 0.014 vs Control; **P = 0.01 vs HF-HD; one-way-ANOVA followed by Tukey’s multiple comparisons test. *P < 0.05, n = 5; unpaired t test.

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**FIGURE 2** Deletion of TFAP2A gene significantly reduces TGF-\(\beta\)-induced fibroblast differentiation. (A) CRISPR/Cas9-based gene editing in NIH/3T3 fibroblasts deleted the TFAP2A expression, as validated by pooled Real-time PCR data \(2^{\Delta \Delta CT}\) of TFAP2A gene (normalized to B2M gene) and immunoblotting of NIH-3T3 (wild-type) and TFAP2A-knocked out (TFAP2A-KO) fibroblasts. Gene expression of \(\alpha\)-smooth muscle actin (\(\alpha\)-SMA), collagen (COL) 1A1 (COL1A1), COL2A1, and COL3A1 were quantitatively analysed by real-time PCR in wild-type and TFAP2A-KO fibroblasts. Incubation in TGF-\(\beta\)-1(5 ng/ml for 48-72h) significantly increased the expression of \(\alpha\)-SMA (B), COL1A1 (C), COL2A1 (D) in the wild-type with muted effect in the TFAP2A-KO fibroblasts. While TGF-\(\beta\)-1 did not have any significant effect on the COL3A1 expression in the wild-type, COL3A1 expression was significantly down-regulated both at basal level and following TGF-\(\beta\) treatment in the TFAP2A-KO fibroblasts. Immunoblot (F) and the bar graph (G) show that TGF-\(\beta\) significantly increased the \(\alpha\)-SMA protein expression in the wild-type with muted effect in the TFAP2A-KO fibroblasts. TGF-\(\beta\)-1 receptor type1 (TGFBR1) mRNA levels were increased in TFAP2A-KO (H) with no significant difference in type 2 (TGFBR2) (I) compared to wild-type fibroblasts. (J) Proliferation rate (doubling time) was not significantly different between the groups. *P < 0.05 vs wild-type, **P < 0.05 vs wild-type + TGF, *P < 0.05 vs TFAP2A-KO groups; n = 3, One-way ANOVA followed by Tukey’s multiple comparisons test. *P < 0.05, unpaired t test.
PCRMastermixand10ngdilutedcDNAperwell.Followingthehuman
primerswereused:TFA2PA-F:5′-GACCTCTGATCCACTCTTAC-3′
R:5′-GAGACGGCATTTGCTTGAC-3′;β-2-microglobulin(B2M)-
F:5′-CACCCTGAAAAAGATGATGGCCT-3′andR:5′-CCAATCCAAA
ATGCCGACATCT-3′.ThefollowingPrimeTimeqPCRmouse
primerassayswereused:α-SMA(Mm.PT.58.16320644);COL1A1
(Mm.PT.58.7562513);COL2A1(Mm.PT.58.5206680);COL3A1(Mm.
PT.58.13848686),TGFBR1(Mm.PT.58.28402453),TGFBR2(Mm.
PT.58.6358355)andB2M(Mm.PT.39a.22214835).Thecyclingcondi-
tionswere95°Cfor10minutes,followedby40cyclesat95°Cfor15s,
1minuteat60°C,ands72°Cfor40s.Meltcurvemethodwasperformed
byanadditionalsuffixationstepof1cycleat95°Cfor5sfollowedby
65°Cfor1minandrampingdatcollectionto97°C.Relativeexpression
values(ΔCt)wereobtainedbynormalizingCtvalues(RocheLightcycler
480Softwarev1.5.1.62)ofthetestedgenesthatwithofB2M.

2.3CRISPR/CAS9-baseddeletionofTFAP2Agene

ThETFAP2AknockoutcelllinewithNIH/3T3fibroblasts(TFA2PA-
KO)wasestablishedusingCRISPR/CAS9technologythrough
CreativeBiogene,Shirley,NY.

2.4Invitrotrans-differentiationprotocol

Fibroblastsfromwild-typeorTFAP2AKOgroupswereplatedat
4000cells/cm²withDMEMmedia(10%BCS)andincubatedat
37°Cunder5%CO₂.Following24hours,hVFsweiteitherreated
withTGF-β1(5ng/mL),angiotensininII(100nM)orkeptascontrolinn
DMEMmedia(2.5%BCS).After48-72hours,thefibroblasts/myofi-
broblastswerrinsedwithDulbecco’sPBSandassayed.

2.5Immunoblotting

Standardwesternprotocolswerefollowed⁹withrespectiveprimary
(dilutions:α-SMA,1:500,TFA2PA,1:100)andsecondaryantibod-
ies(1:2000).Allsampleswereimmunoblottedsimultaneouslyand
repeatedatleasttwice.

2.6Proliferation

BothWTandKOfibroblastswereslatedasstatedbeforeintripli-
cate(perstime-point)in6-wellplatesandcountedbyCellometerAuto
2000(NexcelomBioscience,Lawrence,MA)at24,48,and72hours
post-plating.Doublingtimecalculatedby[t−t₀]/[[log(N₀)−log(N₀)]/
log(2)],where_t₀refers timen(initialcount),_trepresents time(sect-
count),N₀referscountattime_t₀,andN₀representscountattime_t.

3RESULTSANDDISCUSSION

Fromleftventriclesofhumanheart,fibroblastswereisolatedand
groupedintodifferentiated(HF-LD)andhighlydifferentiated
(HF-HD)basedontheirα-SMAexpression,comparedcontrol
hVFsastshowninFigure1A,B.PCBarrayofhumantranscrip-
tionfactorsuncoveredthattheTFAP2Aexpression,alongwith
ELK1,wasdecreasedwithdecreaseindifferentiationastheseenin
theheatmap(Figure1C)andfoldregulationdata(Figure1D)
(n=3).ThisdecreasedexpressionofTFAP2AinHF-LDfibro-
blastscomparedtoHF-HDmyofibroblastsnoticedinPCRarray
wasvalidatedbyquantitativereversetranscriptase-PCR(n=5)
(Figure1E).Basedonthesedatas,wehaveduggestedthatTFAP2A
iscrucialforthetrans-differentiationoffibroblastsintomyofib-
broblasts.WeappliedCRISPR/Cas9-basedgeneeditingtoknockout
TFA2AfromNIH/3T3fibroblasts(Figure2A)andanalsedthe
differentiation- and pro-fibrotic parameters at both basal level
andfollowingTGF-β1treatment.TGF-β1significantlyincreased
thearnaexpressionofα-SMA(Figure2B),collagen(COL)1A1
(Figure2C),COL2A1(Figure2D)inthewild-typewhiletheTGF-β1
effectwassignificantlylowintheTFA2A-KOfibroblasts.Even
atbasallevel,thexpressionsofα-SMA(Figure2B)andCOL3A1
(Figure2E)weresignificantlydecreasedintheTFA2A-KOfibro-
blastscomparedtothewild-type.ThissuggeststhatTFAP2Ais
importantforthetrans-differentiationoffibroblaststomyofi-
broblasts.ThisreduceddifferentiationofTFA2A-KOfibroblasts
observedinquPCRwasfurtherconfirmedatproteinlevelby
immunoblottingwheretheα-SMAexpressionwalessignificantboth
atbasallevelandafterTGF-β1administration(Figure2F,G).
ThebluntedeffectofTGF-β1intheTFA2A-KOfibroblastsdose
appear to be due to changes in the upstream TGF-β1 receptor
levels, as the mRNA levels of TGF-β1 receptor type1 (TGFBR1) is
increased in TFA2A-KO fibroblasts (Figure 2H) without any sig-
ifican difference in the type2 receptors (TBFR2) compared to
thewild-type(Figure2I).Interestingly,deletionofTFAP2Agene
attenuatesnotonlyTGF-β1-inducedfibroblastdifferentiation,
butalsoangiotensininII(AngII)-induceddifferentiationaswell,
as
evidentfromlackofincreaseinα-SMAexpressionintheTFA2A-
KO fibroblasts (Supplemental Figure). This suggests that TFAP2A
couldserveasacommondownstreamregulatorofgenesassoci-
ated with fibroblast differentiation. Importantly, the knockdown
ofTFA2A did not adversely affect the basal proliferation capacity
(Figure 2J). The TFA2A-KO fibroblasts proliferated like that of
thewild-typewithadoublingtimeof21±6hr(TFA2A-KO)vs
25±7hr(wild-type)(n=3).TFA2AisanownDNA-bindingtrans-
scriptionfactor to have both repressive and enhancing effects
on various genes and complete knockout of which is embryonically
lethal.¹⁰Theexactmechanismforthereducedtrans-differentia-
tion of TFA2A-KO fibroblasts in response to TGF-β1 is unclear.
Chromatin immunoprecipitation studies of Smad2/3, important
factors in TGF-β1 signalling, revealed abundant TFAP2A binding
elements in Smad2/3 binding sites of the promoter regions of vari-
ous genes in keratinocytes and knockdown of TFA2A changed
the TGF-β1-inducedtranscriptions.¹¹Whether similarmechanisms
underlieinfibroblastsisnotknown.Inhumansertoliclels,Bone
MorphogeneticProtein(BMP)6,amemberofTGF-βsuperfam-
ily,targetsTFA2Atopositivelyregulatetheirgrowth.¹²In
contrast, the basal proliferation of fibroblasts did not reduce following
TFAP2A knockdown in our study. This is in accordance with the observation in another study where TFAP2A can induce cell cycle arrest\textsuperscript{13} while reduced TFAP2A expression was suggested to impair p21cip-mediated growth arrest resulting in increased proliferation.\textsuperscript{14} These properties found in the TFAP2A-KO fibroblasts suggest that TFAP2A could emerge as a useful molecular target to mitigate excessive fibrosis by inhibiting fibroblast differentiation. As evident from the isolated human cardiac fibroblasts from left ventricles, the decrease in TFAP2A expression when cardiac fibroblast differentiation is decreased, suggest that TFAP2A is crucial for the trans-differentiation of cardiac fibroblasts into myofibroblasts which can lead to excessive cardiac fibrosis underlying many cardiac dysfunctions. Therefore, selective inhibition of TFAP2A could develop as a novel therapeutic strategy to reduce cardiac fibroblast differentiation into myofibroblast, mitigate cardiac fibrosis and preserve cardiac function.

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CONFLICT OF INTEREST

There is no conflict of interest.

AUTHORS’ CONTRIBUTIONS

GRR initiated, designed, executed, analysed the study and wrote the manuscript; SE executed the real-time PCR and PCR array; CW and PH implemented the cell culture, immunoblotting and proliferation assays; FXD, LE, FR and AJ interpreted data and proof-read the manuscript.

DATA AVAILABILITY STATEMENT

All data sets are publicly available from the Dryad Digital Repository at https://doi.org/10.6084/m9.figshare.7898168.

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REFERENCES

1. Rockey DC, Bell PD, Hill JA. Fibrosis—a common pathway to organ injury and failure. \textit{N Engl J Med}. 2015;373:96.

2. Leaf IA, Duffield JS. What can target kidney fibrosis? \textit{Nephrol Dial Transplant}. 2017;32:189-197.

3. Rosenbloom J, Mendoza FA, Jimenez SA. Strategies for anti-fibrotic therapies. \textit{Biochim Biophys Acta}. 2013;1832:1088-1103.

4. Ichiki T, Schirger JA, Huntley BK, et al. Cardiac fibrosis in end-stage human heart failure and the cardiac natriuretic peptide guanylyl cyclase system: regulation and therapeutic implications. \textit{J Mol Cell Cardiol}. 2014;75:199-205.

5. Ryu JH, Daniels CE. Advances in the management of idiopathic pulmonary fibrosis. \textit{F1000 Med Rep}. 2010;2:28.

6. Conrad CH, Brooks WW, Hayes JA, Sen S, Robinson KG, Bing OH. Myocardial fibrosis and stiffness with hypertrophy and heart failure in the spontaneously hypertensive rat. \textit{Circulation}. 1995;91:161-170.

7. Wettlaufer SH, Scott JP, McEachin RC, Peters-Golden M, Huang SK. Reversal of the transcriptome by prostaglandin E2 during myofibroblast dedifferentiation. \textit{Am J Respir Cell Mol Biol}. 2016;54:114-127.

8. Ross GR, Bajwa T Jr, Edwards S, et al. Enhanced store-operated Ca\textsuperscript{2+} influx and ORAI1 expression in ventricular fibroblasts from human failing heart. \textit{Biol Open}. 2017;6:326-332.

9. Mitchell PJ, Wang C, Tijan R. Positive and negative regulation of transcription in vitro: enhancer-binding protein AP-2 is inhibited by SV40 T antigen. \textit{Cell}. 1987;50:847-861.

10. Schorle H, Meier P, Buchert M, Jaenisch R, Mitchell PJ. Transcription factor AP-2 essential for cranial closure and craniofacial development. \textit{Nature}. 1996;381:235-238.

11. Koinuma D, Tsutsumi S, Kamimura N, et al. Chromatin immunoprecipitation on microarray analysis of Smad2/3 binding sites reveals roles of ETS1 and TFAP2A in transforming growth factor beta signaling. \textit{Mol Cell Biol}. 2009;29:172-186.

12. Wang H, Yuan Q, Sun M, et al. BMP6 regulates proliferation and apoptosis of human sertoli cells via Smad2/3 and cyclin D1 pathway and DACH1 and TFAP2A activation. \textit{Sci Rep}. 2017;7:45298.

13. Wajapeyee N, Somasundaram K. Cell cycle arrest and apoptosis induction by activator protein 2alpha (AP-2alpha) and the role of p53 and p21WAF1/CIP1 in AP-2alpha-mediated growth inhibition. \textit{J Biol Chem}. 2003;278:52093-52101.

14. Scibetta AG, Wong PP, Chan KV, Canosa M, Hurst HC. Dual association by TFAP2A during activation of the p21cip/CDKN1A promoter. \textit{Cell Cycle}. 2010;9:4525-4532.

SUPPORTING INFORMATION

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

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