BBA - Molecular Cell Research 1871 (2024) 119640

Contents lists available at ScienceDirect

BBA - Molecular Cell Research

journal homepage: www.elsevier.com/locate/bbamcr

Research paper

Extracellular matrix stiffness controls cardiac fibroblast proliferation via the nuclear factor-Y (NF-Y) transcription factor

Reza Ebrahimighaei, BSc, PhD [a], Nathalie Tarassova [a], Samuel C. Bond [b], Madeleine C. McNeill, BSc, PhD [a], Tom Hathway, BSc [a], Hunaid Vohra, MB, BS, MRCS, MD, FRCS [a], Andrew C. Newby, BSc, PhD [a], Mark Bond, BSc, PhD [a,*]

[a] Department of Translational Health Sciences, Bristol Medical School, Bristol, BS2 8HW, United Kingdom
[b] Clifton High School, Clifton, Bristol, BS8 3JD, United Kingdom

ARTICLE INFO

Keywords:
Cardiac fibroblast
Extracellular matrix
Stiffness
Proliferation
Nuclear factor-Y subunit alpha
Fibrosis

ABSTRACT

The proliferative expansion of cardiac fibroblasts (CF) contributes towards cardiac fibrosis, which results in myocardial stiffening, cardiac dysfunction, and heart failure. CF sense and respond to increased stiffness of their local extracellular matrix, modulating their phenotype towards increased collagen synthesis and higher proliferation, leading potentially to a vicious circle of positive feedback.

Here we describe a novel mechanism that mediates increased CF proliferation in response to a pathologically stiff Exteracellular matrix (ECM). The mechanism we describe is independent of the well-characterised mechanosensitive transcript factors, YAP-TEAD and MKL1-SRF, which our data indicate are only responsible for part of the genes induced by stiffened ECM. Instead, our data identify Nuclear Factor-Y (NF-Y) as a novel mechanosensitive transcription factor, which mediates enhanced CF proliferation in response to a stiff ECM. We show that levels of NF-YA protein, the major regulatory subunit of NF-Y, and NF-Y transcriptional activity, are increased by a stiff ECM. Indeed, NF-Y activity drives the expression of multiple cell-cycle genes. Furthermore, NF-YA protein levels are dependent on FAK signalling suggesting a mechanistic link to ECM composition. Consistent with its role as a mechano-sensor, inhibition of NF-Y using siRNA or dominant negative mutant blocks CF proliferation on plastic in vitro, which models a stiff ECM, whereas ectopic expression of NF-YA increases the proliferation of cells interacting under conditions that model a physiologically soft ECM.

In summary, our data demonstrate that NF-Y is a biomechanically sensitive transcription factor that promotes CF proliferation in a model of pathologically stiffened ECM.

1. Introduction

Cardiac fibrosis, the pathophysiological remodelling of the myocardial extracellular matrix, contributes towards the development of heart failure, which affects 26 million people globally [1]. Some treatments targeting risk factors are available for some conditions that lead to fibrosis, such as volume or pressure overload [2]. These may help limit fibrotic progression. However, new therapeutic strategies are needed that directly target the mechanisms leading to fibrosis, particularly in patients where risk factor management is ineffective.

CF represent a major non-myocyte cell type resident in the myocardium [4] that perform several important functions. In addition to functioning as mechano-electric transducers [5], CF also play an important role in the deposition and maintenance of the myocardial (ECM) and myocardial homeostasis [6]. In a healthy myocardium, CF typically display low rates of proliferation and maintain a slow basal turnover of ECM proteins. However, in response to cardiac injury or overload, CF can trans-differentiate into a myofibroblast phenotype. This is characterised by an increase in their proliferation rate, elevated collagen synthesis and upregulation of the cytoskeletal protein smooth muscle alpha-actin (SMA) expression [7].

The adoption of a myofibroblast phenotype by CF plays an important role in myocardial healing after cardiac injury [8]. Myofibroblasts synthesise increased amount of collagen following myocardial infarction, which is essential for scar formation and structural stabilisation of the infarcted area. However, excessive production of collagen, ECM

* Corresponding author.
E-mail addresses: nathalie.tarassova@bristol.ac.uk (N. Tarassova), scbond2006@gmail.com (S.C. Bond), madeleine.mcneill@bristol.ac.uk (M.C. McNeill), thomas.hathway@bristol.ac.uk (T. Hathway), hv15982@bristol.ac.uk (H. Vohra), a.newby@bris.ac.uk (A.C. Newby), Mark.bond@bris.ac.uk (M. Bond).

https://doi.org/10.1016/j.bbamcr.2023.119640
Received 31 August 2023; Received in revised form 10 November 2023; Accepted 10 November 2023
Available online 22 November 2023

components and increased myofibroblast proliferation have all been implicated in the development of maladaptive fibrosis. This alters the mechanical properties of the myocardium, increasing its stiffness, which impairs cardiac function and accelerates the development of heart failure [9]. Increased CF proliferation expands the population of collagen-secreting cells, thus creating a feed-forward cycle of fibrosis [10]. Therefore, a better understanding of the mechanisms that control CF behaviour is essential for the future development of therapies to limit maladaptive fibrosis and prevent heart failure.

An important consequence of myocardial fibrosis is increased tissue stiffness [11]. Normal heart tissue has a diastolic Young's Modulus of approximately 2 kPa [12], whereas the stiffness of fibrotic myocardium increases tenfold to between 20 and 100 kPa [13,14]. In addition to disrupting normal systolic and diastolic cardiac contraction/relaxation, this increase in stiffness activates intracellular signalling mechanisms that promote further myofibroblast differentiation [15], proliferation, migration and increased ECM production, thus creating a vicious cycle of fibrosis that is both a cause and a consequence of CF activation. Considerable effort has been made to characterise the biomechanical signalling mechanisms that control cell proliferation in response to changes in local tissue stiffness [16]. This research has highlighted important roles for some key transcription factors and their associated co-factors. For example, the transcription factors SRF and TEAD and their respective co-factors MKL1/MKL2 and YAP/TAZ, respectively, are the best-characterised mechanosensitive factors that control gene expression and cell replication in response to local mechanical signals [17,18]. Consistent with this, genetic knockout of MKL1 reduces fibrosis and scar formation after MI [19]. Recent studies have also implicated YAP/TAZ in fibrotic remodelling post cardiac injury [20–23]. However, it is likely that other mechanosensitive factors are also involved. Here we analysed the mechanosensitive transcriptome in CF using transcription factor binding site enrichment analysis to identify novel transcriptional regulators that control cardiac fibroblast proliferation in response to changes in ECM stiffness. We identified the Nuclear Factor-Y (NF-Y) transcription factor as a novel mechanosensitive factor that promotes CF proliferation in response to pathologically stiffened ECM.

2. Methods

2.1. Reagents

All chemicals were obtained from Sigma unless otherwise stated. Antibodies against NF-YA and NF-YB were from Santa Cruz. Antibody to GAPDH (MAB374) was from Merck Millipore. Antibody against Histone-H3 was from Cell Signalling Technologies.

2.2. Cardiac fibroblast culture

Sprague Dawley rats were killed by inhalation of CO_2 in accordance with schedule 1 of the U.K. Animals (Scientific Procedures) Act 1986 and Directive 2010/63/EU of the European Parliament and with the approval of the University of Bristol. Hearts were removed and flushed with PBS before chopping into 2mm^2 pieces and digestion with 2 mg/ml collagenase (Worthington Biochemical Corporation) overnight. The cell suspension was pelleted and resuspended in Advanced DMEM/F12 supplemented with 10 % foetal bovine serum, 100 U/ml penicillin/streptomycin and 2.5 mM L-glutamine. Cells were allowed to adhere to tissue culture plastic for 72 h and non-adherent myocytes washed away. Cultures were expanded by serial passage and used in experiments between passage 4 to 10. Where indicated, cells were cultured on Softwell® collagen coated polyacrylamide hydrogels, purchased from Cell Guidance Systems. Unless otherwise stated, 1 kPa hydrogels were used for soft ECM and 50 kPa gels were used for stiff ECM. Reported substrate stiffness is as described by the manufacturer.

2.3. RNA extraction, quantitative real time PCR and RNAseq

Total RNA was prepared using Qiagen RNeasy mini columns according to the manufacturer's instructions. For RNAseq, RNA was purified with the inclusion of an on column DNAse treatment to ensure removal of all traces of genomic DNA contamination. RNA was quantified by reading the OD_{260} using a nanodrop spectrophotometer and RNAseq samples were assess for integrity using an Agilent Bioanalyzer. RIN scores were all >9. For qPCR, equal amounts of RNA were converted to cDNA using a Qiagen QuantiTect first strand cDNA synthesis kit with random hexamer priming. Quantitative Real Time PCR was performed using KappaFAST SYBR Green using a Qiagen Roto-Gene Q PCR machine (15′@95 °C;15′@62 °C;5′@72 °C). Primers sequences are described in Supplement Fig. 1. Data were normalised to total amount of RNA. RNAseq was performed at Source Biosciences (Cambridge, U.K.). Briefly, libraries were prepared using an Illumina Stranded mRNA Prep kit and 150 bp-paired-end read sequenced using a NovaSeq 6000.

2.4. Western blotting

Total cell lysates were prepared in 1× reducing Laemmli sample buffer (2 % SDS, 10 glycerol, 50 mM Tris pH 6.8, 2.5 % β-mercaptoethanol, 0.002 % bromophenol blue). Proteins were denatured by heating to 95 °C for 5 min before electrophoresis using Bio-Rad 4–15 % polyacrylamide mini-TGX gels in a Mini-Protean II electrophoresis apparatus. Proteins were transferred to TransBlot PVDF membrane (BioRad) using a semi-dry Turbo blotter system (Bio-Rad). Membranes were blocked for 1 h at room temperature in 5 % low-fat milk powder in Tris buffered saline (50 mM Tris-HCl, pH 7.6, 150 mM NaCl, 2 mM KCl) containing 0.2 % Tween20 (1xTBS.T) before incubation with primary antibody overnight at 4 °C. Blots were extensively washed in 1xTBS.T before incubation with HRP-conjugated secondary antibodies (Sigma) for 1 h at room temperature. Specific proteins were detected using Immobilon ECL reagent and a ChemiDoc-MP digital imaging system (Bio-Rad).

2.5. Plasmids, siRNA and adenoviral vectors

Replication deficient adenoviral vector expressing a previously characterised dominant negative mutant of NF-YA [24] was generated by RT-PCR. The resulting NF-YA cDNA was cloned into the multiple cloning sites of the pDC515 adenovirus shuttle vector (Microbix) as previously described [25–27]. Recombinant adenoviruses were generated by co-transfection of HEK293 cells with the pDC515 expression vector and the adenoviral genome vector pBGHfrt, as previously described [28]. Clones of replication-deficient adenoviruses were amplified by infection of four 150 cm^2 flasks of HEK293 cells and the resulting adenovirus was purified using AdEasy adenovirus purification kit (Agilent). Purified adenoviral stocks were titrated by end-point dilution plaque assay in HEK293 cells.

Plasmid NF-Y-sNLUC expressing a secreted version of nanoluciferase was generated by cloning five copies of a consensus NF-Y binding element (CCAAT) upstream of a minimal promoter in reporter plasmid pNL3.3[secNluc/minP] (Promega). Plasmid minP-LUC, containing the Troponin-T minimal promoter was generated by digesting 8xGTIIC-luciferase plasmid (Addgene #34615) with *Kpn1* and *BglII* to remove the TEAD elements, followed by blunt end re-ligation. Reporter plasmid driven by a 400 bp fragment of the wild-type or NF-Y element mutated proximal human CCNA2 promoter was generated by PCR from genomic DNA and cloned into reporter plasmid pNL2.3 [secNLUC]. A wild type or mutant (with two NF-Y elements mutated) 800 bp fragment of the human CCNB1 proximal promoter was synthesized by Invitrogen Life Technologies and cloned into reporter plasmid pNL2.3 [secNLUC]. Synthetic Silencer Select siRNAs targeting YAP, TAZ, RUNX2 and NF-YA were purchased from Invitrogen Life Technologies. Wild-type-NF-YA expression vector was constructed by amplifying the NF-YA cDNA by

RT-PCR and cloning with an N-terminal Myc tag into plasmid pRK-Myc.

2.6. Transient transfection and reporter gene assays

NF-Y reporter gene activity was determined by quantifying secreted nanoluciferase from cells transiently transfected with NF-Y-sNLUC reporter plasmids. Plasmid transfection was performed by electroporation of 1×10^6 cells with a total of 5 μg of plasmid DNA using a Nucleofector-1.5 electroporation device (Lonza) set to program A-024. For gene silencing, cells were transfected with 100 pmoles of siRNA (Silencer Select siRNA; Life Technologies) targeting NF-YA, NF-YB and NF-YC, as indicated. Twenty-four hours post transfection, media were removed from cells, cells washed in PBS and new media conditioned for 4 h. Secreted nanoluciferase activity was assayed in cell conditioned media using the NanoGlo activity assay (Promega) and a Glomax Discover luminometer (Promega) according to the manufacturer's instructions.

2.7. Cell proliferation assays

Cell proliferation was quantified using EdU incorporation and counting total cell number/mm^2. Unless otherwise stated, all experiments were conducted using asynchronously proliferating cells cultured in Advanced DMEM/F12 supplemented with 5 % foetal bovine serum, 100 U/ml penicillin/streptomycin and 2.5 mM L-glutamine. For EdU labelling assays, cells were treated as indicated and incubated with 10 μM EdU for 4 h. Cells were fixed in 70 % ethanol and EdU incorporation detected using the EdU-CLICK-488 assay kit (SIGMA). Nuclei were counter stained with DAPI and EdU positive and total nuclei numbers manually counted using ImageJ software. For total cell numbers, cells were fixed in 70 % ethanol, −24, −48- and − 72-h post treatment and nuclei stained with DAPI. Nuclei were counted using Cell Profiler software and expressed as cells/mm^2.

2.8. Data and statistical analysis

Raw experimental data were collated and graphed using Microsoft Excel, with final figures constructed using Microsoft Powerpoint. Statistical analysis was performed using Graphpad Instat software (https://www.graphpad.com/scientific-software/instat/). Reported n numbers indicate the number of independent experiments performed using different preparations of CF. Each cell preparation was derived from pooled myocardial tissue derived from two adult rats. Gene Ontology (GO) analysis was performed using the GO tool at the Gene Ontology Consortium website (https://geneontology.org/) [29]. Transcription factor binding site enrichment analysis was performed using the oPOSSUM3 application ([29]https://github.com/wassermanlab/oPOSSUM3). Data are presented as means ± standard error of the mean. After testing for normal distribution, data were analysed either using one-way ANOVA with Student Newman Keuls post-test, or where appropriate student's t-test, as indicated.

3. Results

3.1. CF proliferation is dependent on ECM-stiffness

We initially confirmed that the proliferation rate of CF is dependent on the stiffness of the ECM. Asynchronously proliferating CF were seeded onto collagen functionalised polyacrylamide hydrogels of increasing stiffness, within the physiological to pathological range. 24 h post cell seeding, cell proliferation was determined by incubation for 4 h with EdU. Proliferation of cells on a soft ECM was low, with only 7.02 ± 0.77 % of cells positively labelled with EdU on the softest ECM, despite the presence of abundant serum mitogens (Fig. 1). However, the percentage of cells incorporating EdU increased as ECM stiffness increased. This data confirms that CF proliferation is dependent on ECM stiffness.

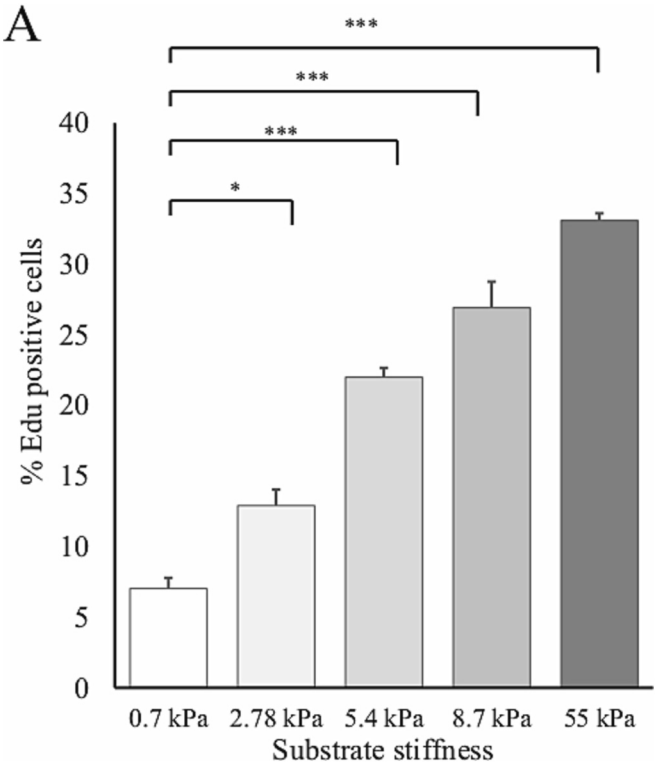
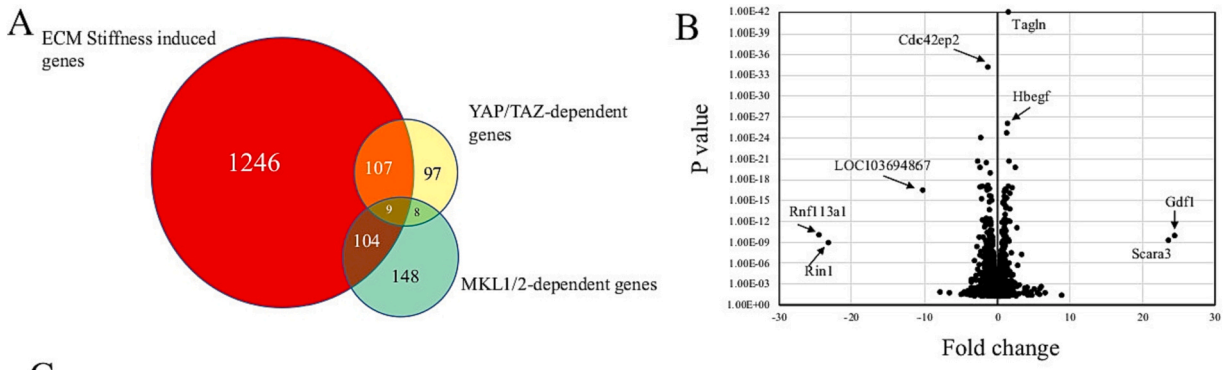


Fig. 1. ECM-stiffness dependent proliferation of CF
CF were seeded onto collagen coated polyacrylamide hydrogels of the indicated stiffness. After 24 h, cells were labelled with 10 μM Edu for 4 h and Edu incorporation quantified. Data is mean ± SEM. $n = 3$; * indicates $p < 0.05$; *** indicates $p < 0.001$. One-way ANOVA with student Newman Keuls post-hoc test.

3.2. Characterisation of the ECM-stiffness dependent transcriptome in CF

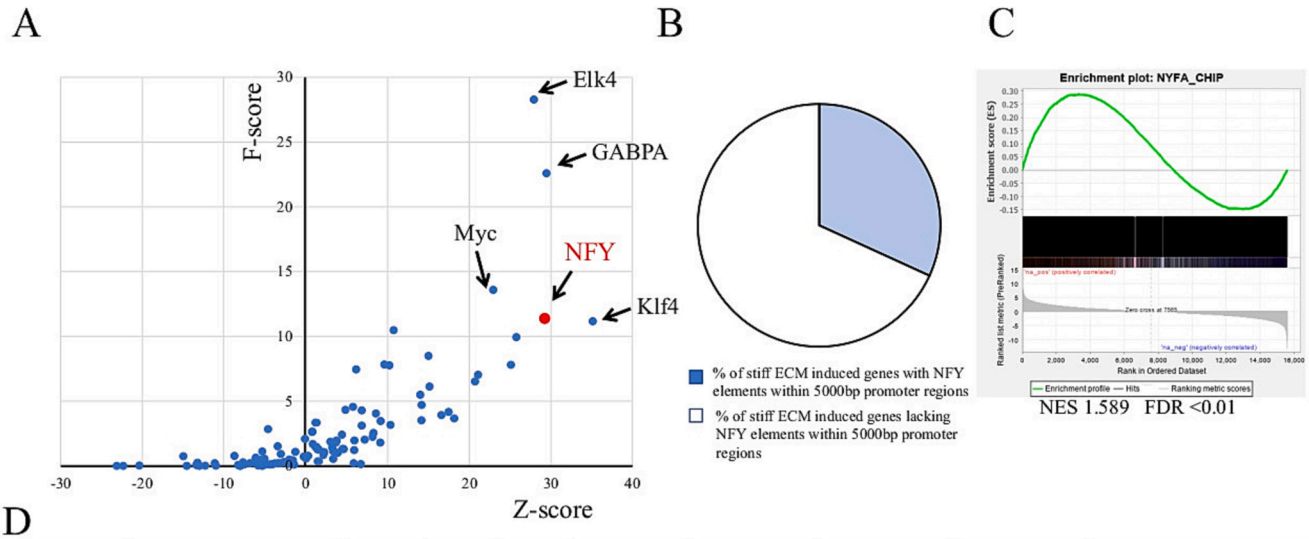
To characterise the ECM-stiffness-dependent transcriptome in CF and to identify novel underlying mechano-sensitive mechanisms, we cultured CF on soft (1 kPa) or stiff (50 kPa) collagen functionalised acrylamide hydrogels and extracted total RNA 24 h post cell seeding. RNA-seq analysis identified 1446 genes significantly upregulated (>1.1 fold increase) and 2568 genes significantly down regulated (>1.1 fold decrease) in cells cultured on stiff ECM compared to cells on a soft ECM (Fig. 2A). The transcriptional co-factors YAP/TAZ [17] and MKL1/2 [31] have previously been implicated in mediating biomechanical regulation of gene expression. We therefore analysed RNA extracted from cells where either YAP/TAZ or MKL1/2 had been silenced by transient transfection with siRNA and cultured on soft or stiff ECM. Efficient silencing of YAP, TAZ, MKL1 and MKL2 protein was confirmed by western blotting (Supplement Fig. 2). Silencing of YAP/TAZ in cells on a stiff ECM significantly repressed expression of 221 genes, including the well characterised YAP/TAZ target gene CCN1 [32,33]. Silencing of MKL1/2 resulted in repression of 269 genes, including the previously characterised MKL1/2 target genes, CCN1, ZYX, MYL9 and ACTA2 [34]. We then compared these transcriptomic changes and found that only 52.5 % and 42 % of the YAP/TAZ and MKL1/2 dependent genes, respectively, were induced by a stiff ECM (Fig. 2A). Moreover, the YAP/TAZ and MKL1/2 dependent genes, together, only represented 15 % of the stiff ECM-induced genes (Fig. 2A). Gene ontology analysis of the stiff-ECM induced genes that were YAP/TAZ and MKL1/2 independent identified significant enrichment of numerous GO-terms associated with cell cell-cycle progression (Fig. 2C). Taken together, these data implicate the involvement of transcriptional regulators, other than YAP/TAZ or MKL1/2, in the expression of genes associated with cell-cycle progression in response to increased ECM-stiffness.



C

GO biological process complete	GO_TERM	Number of 'hit' genes	Expected number of 'hit' genes	Fold Enrichment	P-value	FDR
mitotic cell cycle phase	GO:0098763	65	16.77	3.88	7.23E-18	3.10E-15
cell cycle phase	GO:0022403	65	16.77	3.88	7.23E-18	3.02E-15
cell cycle phase transition	GO:0044770	64	18.44	3.47	1.31E-15	4.50E-13
mitotic M phase	GO:0000087	41	10.62	3.86	1.01E-11	1.75E-09
regulation of G2/M transition of mitotic cell cycle	GO:0010389	43	12.3	3.5	5.02E-11	7.59E-09
G2/M transition of mitotic cell cycle	GO:0000086	33	8.76	3.77	1.76E-09	2.25E-07
G1/S transition of mitotic cell cycle	GO:0000082	29	8.01	3.62	3.58E-08	3.30E-06
cell cycle G1/S phase transition	GO:0044843	29	8.14	3.56	4.78E-08	4.26E-06
mitotic interphase	GO:0051329	19	4.91	3.87	3.40E-06	2.06E-04
S phase	GO:0051320	17	4.04	4.21	4.26E-06	2.51E-04
DNA strand elongation involved in DNA replication	GO:0006271	8	1.18	6.78	1.09E-04	4.47E-03

Fig. 2. CF ECM-stiffness dependent transcriptome
CF were transfected with either siNEG (non-targeting siRNA) or siRNA targeting YAP and TAZ or MKL1 and MKL2, as indicated ($n = 4$). Cells were seeded onto stiff (50 kPa) or soft (1 kPa) ECM and total RNA analysed by RNAseq 24 h later. Venn diagram of overlap between stiff ECM induced genes and genes repressed by siYAP/TAZ or siMKL1/2 (A). Volcano plot of ECM stiffness regulated genes (B). Gene ontology analysis of genes induced by a stiff ECM but YAP/TAZ and MKL1/2 independent (C). FDR indicates false discover rate.



D

MsigDB	GENE SET	SIZE	ES	NES	NOM P value	FDR q value	FWER p value	RANK at MAX	LEADING EDGE
c3.tft.v7.4	E2F1_Q6	212	0.47	2.12	0	0.001	0.004	4169	tags=55%, list=27%, signal=74%
	E2F_Q4	214	0.46	2.07	0	0.001	0.006	3511	tags-45%, list-23%, signal=38%
	SRF_01	44	0.55	1.92	0	0.001	0.032	1477	tags=34%, list=9%, signal=38%
	NFY_Q6_01	218	0.37	1.68	0	0.012	0.399	2511	tags=30%, list=16%, signal=35%
	TEF1_Q6 (TEAD)	194	0.28	1.22	0.068	0.193	1	1783	tags=19%, list=11%, signal =21%

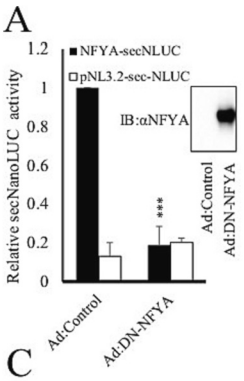
Fig. 3. Transcription factor binding site Enrichment of NF-Y-dependent genes in stiff-ECM DEGs
Transcription factor binding site enrichment analysis of promoter regions of stiff (50 kPa vs 1 kPa) ECM induced by YAP/TAZ and MKL1/2 independent genes (A). Pie chart of proportion of stiff ECM by YAP/TAZ and MKL1/2 independent genes containing a consensus NF-Y binding element within -5kbp of the TSS (B). Gene set enrichment analysis of ENCODE NF-YA-ChiP-seq gene set within the stiff ECM regulated genes (C). Gene set enrichment analysis of molecular signatures gene sets within the stiff ECM regulated genes (D).

3.3. NF-Y-binding elements are enriched in the promoter regions of stiffness-induced genes

We performed transcription factor binding site enrichment analysis of the promoter regions of YAP/TAZ and MKL1/2 independent genes, that were upregulated in cells cultured on a stiff ECM, using oPOSSUM3 [30], to identify novel bio-mechanically sensitive transcription factors (Fig. 3A). This analysis identified significant enrichment of binding elements for multiple transcription factors. Many of the high scoring factors, including Elk4, Myc and Klf4 are downstream targets of pathways e.g., MAPK pathway, which have already been demonstrated to be biomechanically sensitive. There is currently no literature on the biomechanical regulation of GABPA or NF-Y. To investigate this, we constructed GABPA and NF-Y reporter gene plasmids and quantified their activity in CF interacting with 1 kPa or 50 kPa substrates. Both reporters demonstrated stiffness dependent activity, with NF-Y displaying the greater increase in activity on stiff compared to soft substrates (Supplement Fig. 3). We therefore focussed on characterising the regulation and function of NF-Y in CF. We were able to identify at least one consensus NF-Y binding sequence (CCAAT) within the 5000 bp upstream of the transcriptional start site in 31.8 % of the stiff-ECM induced genes (Fig. 3B), suggesting that NF-Y may be functionally important in a large proportion of the genes induced in cells on a stiff substrate. Using gene set enrichment analysis, we detected a significant enrichment of an ENCODE NF-YA-ChIP gene set (Fig. 3C) in the stiff substrate induced genes, demonstrating an association between NF-Y binding and induction by a stiff substrate. Analysis of the Broad Institute Molecular signatures gene sets of regulatory transcription factor targets (C3 transcription factor targets; Molecular Signatures Database) by GSEA, identified enrichment of E2F, SRF, NF-Y and TEAD target genes in the stiff-ECM induced genes (Fig. 3D). This is consistent with the increased proliferation of cells on stiff substrates and involvement of these factors in mechano-sensing. Together, these data suggest that the NF-Y transcription factor is enriched in the promoter regions of stiff-ECM induced genes and hence may play a functional role in regulating their expression in response to changes in ECM compliance.

3.4. Identification of substrate-stiffness sensitive NF-Y target genes

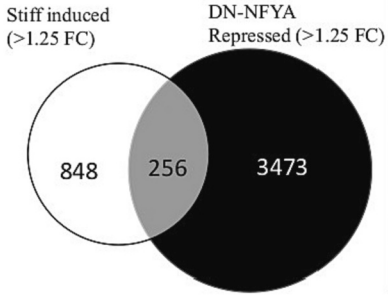
We constructed a recombinant adenoviral vector expressing a previously characterised (mutant YA29) dominant-negative mutant of NF-YA [24], which disrupts the DNA binding ability of the NF-Y complex (referred to as DN-NF-Y hereafter). Infection of CF with adenoviral vector expressing dominant-negative NF-Y (Ad:DN-NF-Y) resulted in overexpression of the mutant NF-YA protein, detected by western blotting (Fig. 4A; inset). Expression of DN-NF-Y also completely suppressed activity of an NF-Y-sNLUC reporter gene, without affecting the activity of a control promoter reporter lacking NF-Y binding elements (Fig. 4A). To characterise the NF-Y-dependent transcriptome in CF, cells were infected with either a control adenovirus (Ad:Control) or dominant-negative NF-Y (Ad:DN-NF-Y) adenovirus and total RNA extracted 24 h later and analysed by RNA-sequencing. We identified 8504 unique genes as significantly upregulated by >1.25-fold and 3725 unique genes significantly down regulated by >1.25-fold in cells expressing DN-NF-Y compared to controls (Supplement Fig. 4). We focussed on the repressed genes as these are likely to be dependent on endogenous NF-Y activity for their expression. Gene Ontology analysis of these repressed genes identified significant enrichment of GO-terms associated with cell-cycle progression (Fig. 4B). Of the 3725 DN-NF-Y repressed genes, 256 were also found to be induced in cells cultured on a stiff substrate in our earlier RNA-seq dataset (Fig. 4C). Gene Ontology analysis of these 256 genes also identified significant enrichment of GO-terms associated with cell-cycle progression (Fig. 4D). We focussed on the 37 genes contributing towards enrichment of the GO-term GO:0051726 (Regulation of



B

Biological Process	GO Term	Genes Submitted	Expected Genes	Fold Enrichment	P value
Positive regulation of mitotic cytokinesis	GO:1903490	7	1.2	5.84	1.88E-03
Mitotic spindle elongation	GO:0000022	9	1.88	4.78	1.09E-03
Mitotic spindle assembly	GO:0090307	31	8.56	3.62	1.41E-07
spindle checkpoint signaling	GO:0031577	16	5.31	3.02	6.86E-04
DNA-templated DNA replication maintenance of fidelity	GO:0045005	25	9.07	2.76	8.07E-05
Regulation of cell cycle checkpoint	GO:1901976	20	8.22	2.43	1.57E-03
Mitotic cell cycle	GO:0000278	243	104.58	2.32	2.54E-26
Mitotic cell cycle process	GO:1903047	206	88.83	2.32	2.15E-22
Positive regulation of cell cycle process	GO:0090068	92	42.79	2.15	2.73E-09
DNA replication	GO:0006260	79	37.48	2.11	8.31E-08
Cell cycle	GO:0007049	417	209.5	1.99	1.03E-32

C



D

Biological Process	GO-TERM	Number of Genes	Expected Genes	Fold Enrichement	P value
mitotic spindle checkpoint signaling	GO:0071174	5	0.38	13.25	6.81E-05
regulation of DNA-templated DNA replication	GO:0090329	6	0.73	8.22	1.42E-04
regulation of mitotic nuclear division	GO:0007088	11	1.48	7.41	6.43E-07
regulation of metaphase/anaphase transition of cell cycle	GO:1902099	7	1.18	5.92	2.68E-04
spindle assembly	GO:0051225	7	1.2	5.86	2.85E-04
mitotic cell cycle checkpoint signaling	GO:0007093	8	1.6	5.01	2.90E-04
cellular response to transforming growth factor beta stimulus	GO:0071560	9	1.81	4.97	1.29E-04
cell cycle checkpoint signaling	GO:0000075	9	1.97	4.56	2.39E-04
Regulation of cell cycle	GO:0051726	37	14.11	2.62	1.29E-07
cell division	GO:0051301	27	6.39	4.23	7.57E-10
mitotic cell cycle	GO:0000278	32	7.69	4.16	2.56E-11

Fig. 4. Analysis of the NF-Y-dependent transcriptome in CF
CF were seeded onto tissue culture plastic and infected with either a control adenoviral vector or an adenovirus expressing dominant-negative NF-YA. NF-Y reporter gene activity ($n = 3$; A) and protein levels (A;inset) were quantified. Total RNA was extracted 24 h later and analysed by RNA-seq. Gene ontology analysis of DN-NF-YA repressed genes (B). Overlap of stiff ECM induced genes and DN-NF-YA repressed genes (C). Gene ontology analysis of the 256 stiff ECM induced NF-Y-dependent genes (D).

cell-cycle). To refine this set of genes further and identify those genes most likely to represent direct NF-Y-target genes, we searched the 5000 bp upstream of the transcription start sites for consensus NF-Y binding elements (CCAAT). This identified 14 genes containing one or more NF-Y binding elements within their promoter regions (Fig. 5A). Interestingly, those genes associated with a promoter NF-Y-binding element displayed a greater fold inhibition by DN-NF-Y than those lacking NF-Y elements within their promoters (Fig. 5A), indicating that these genes may represent direct NF-Y-targets. We next sought to validate the NF-Y-dependence of a subset of these 14 genes using RT-qPCR. We focussed on CCNB, ECT2, SPDL1, DDX11 and CDC7. We also included the previously characterised NF-Y-target CCNA2 [35]. CCNA2 was significantly repressed by DN-NF-Y in our RNA-seq dataset but did not quite reach significance when comparing stiff vs soft substrates. Quantitative PCR analysis detected a strong downregulation of CCNA2, CCNB, ECT2, SPDL1, DDX11 and CDC7 mRNAs by DN-NF-Y, with only minimal changes in the housekeeping genes 36B4 and UBC (Fig. 5B), thus confirming our RNA-seq data. We also tested if the mRNA levels of these genes were elevated in cells interacting with stiff compared to soft substrates. Consistent with our earlier data, we detected significantly higher levels of all putative NF-Y-target gene mRNA in cells interacting with stiff compared to soft substates (Fig. 5C). To gain an insight into whether any of these genes are direct targets for NF-Y, we constructed nano-luciferases reporter plasmids under then control of either wild type CCNA2 or CCNB1 promoter regions, or promoters containing mutated NF-Y binding elements. In cells cultured on tissue culture plastic, mutation of the NF-Y binding element(s) in either promoter significantly reduced promoter activity (Fig. 5D and E).

3.5. ECM-stiffness modulates NF-YA protein levels, activity and target gene expression

The NF-Y transcription factor comprises three subunits, NF-YA, NF-YB and NF-YC [36]. We focussed on NF-YA since this is the major regulatory subunit of the NF-Y transcription factor complex [37]. Levels of NF-YA proteins were significantly higher in cells interacting with a stiff compared to soft substrate (Fig. 6A). This was associated with a concomitant increase in the levels of the myofibroblast differentiation marker alpha-SMA (Fig. 6A). Dual immunofluorescent staining indicated that essentially 100 % of cells interacting with a stiff substrate co-expressed NF-YA and αSMA protein (Supplement Fig. 5). Protein levels gradually increased with ECM-stiffness between 0.1, 1, 4 and 25 kPa (Fig. 6B). To quantify changes in NF-Y transcriptional activity, we constructed a secreted nanoluciferase reporter vector under the control of a synthetic promoter containing five copies of a consensus NF-Y binding element (5'-CCAAT-3′). Cells transfected with NF-Y-sNLUC but not a control reporter lacking NF-Y elements, displayed significantly higher NF-Y activity when interacting with a stiff ECM, compared to a soft ECM (Fig. 6C), with activity gradually increasing with ECM-stiffness between 0.1 kPa and 12 kPa (Fig. 6E). Activity of the control reporter vector, under a minimal promoter lacking any NF-Y elements, was not affected by changes in ECM stiffness (Fig. 6E). Consistent with these changes in NF-YA protein levels and NF-Y activity, we detected a significant increase in the mRNA levels of previously characterised NF-Y-target gene mRNAs CCNA2 (Fig. 6F) and CCNB (Fig. 6G) in cells interacting with stiff compared to soft substrates. As a positive control, we also analysed expression of the classical YAP/TAZ-TEAD target genes, CTGF and CCN1, which displayed a similar ECM stiffness dependent

A

GENE	FOLD CHANGE	NFYA promoter element (-5000bp)
Ccnb1	0.387	YES
Iho1	0.400	NO
Ect2	0.422	YES
Tom1l1	0.434	NO
Spdl1	0.459	YES
Sik1	0.460	YES
Ddx11	0.479	YES
Mad2l1	0.508	NO
Cdc7	0.546	YES
Src	0.556	YES
Cit	0.558	YES
Clspn	0.561	YES
Mybbp1a	0.571	YES
Cited2	0.584	YES
Cks1b	0.592	YES
Zwint	0.597	YES
Mdm1	0.597	YES
Nup62	0.607	NO
Trip13	0.613	NO
Aurka	0.616	NO
Rad1	0.625	NO
Cdca8	0.628	NO
Chmp4c	0.633	NO
Numa1	0.642	NO
Spc24	0.666	NO
Chmp6	0.672	NO
Prc1	0.678	NO
Rad51	0.678	NO
E2f7	0.687	NO
Cdca5	0.689	NO
Dapk3	0.690	NO
Tipin	0.699	NO
Ppp2r3b	0.701	NO
Fgfr2	0.702	NO
Ruvbl1	0.716	NO
Tgfb2	0.746	NO
Ets1	0.749	NO

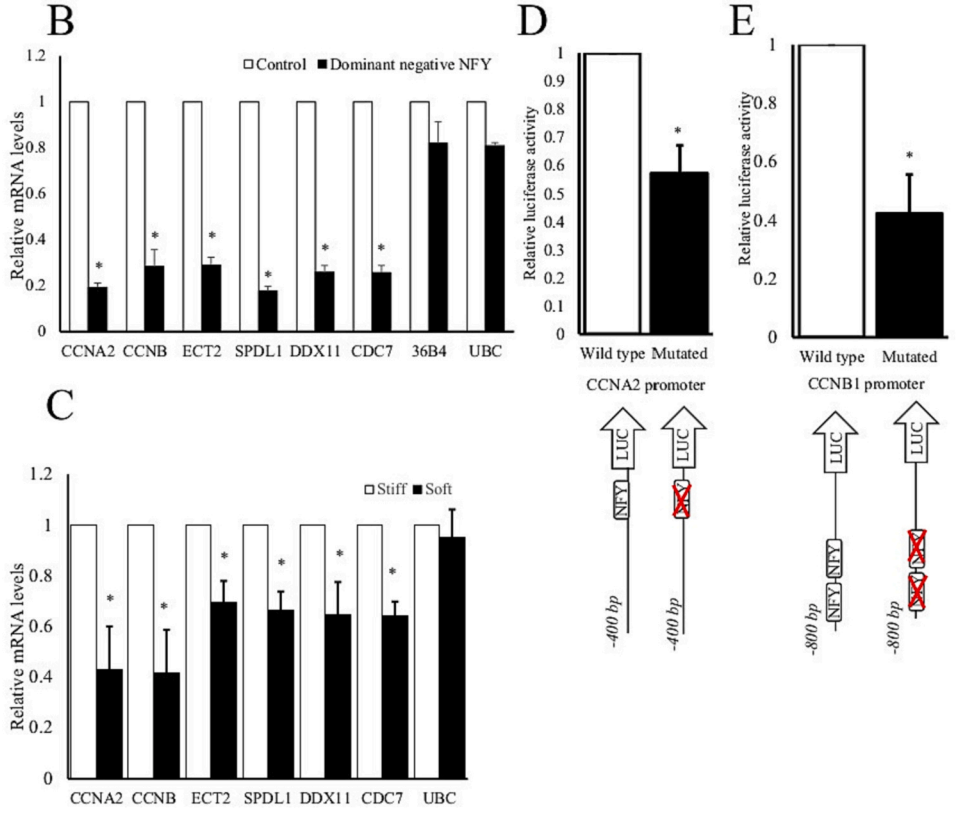


Fig. 5. NF-Y-dependent regulation of proliferation related genes
Table of DN-NF-Y repressed genes tagged with the GO-term "regulation of cell cycle" (A). Levels of mRNAs of indicated genes was quantified by qRT-PCR ($n = 4$; B). CF were seeded onto soft (1 kPa) or stiff ECM (50 kPa) as indicated and mRNA levels of indicated genes was quantified by qRT-PCR ($n = 5$; C). CF were transfected with a reporter gene plasmid under the control of wild-type CCNA2 (n = 4; D) or CCNB1 (n = 3; E) promoter regions or promoter regions containing mutated NF-Y elements. Data is mean ± SEM. One-way ANOVA with student Newman Keuls post-hoc test (B and C) or paired student's *t*-test (D and E). * Indicates $p < 0.05$.

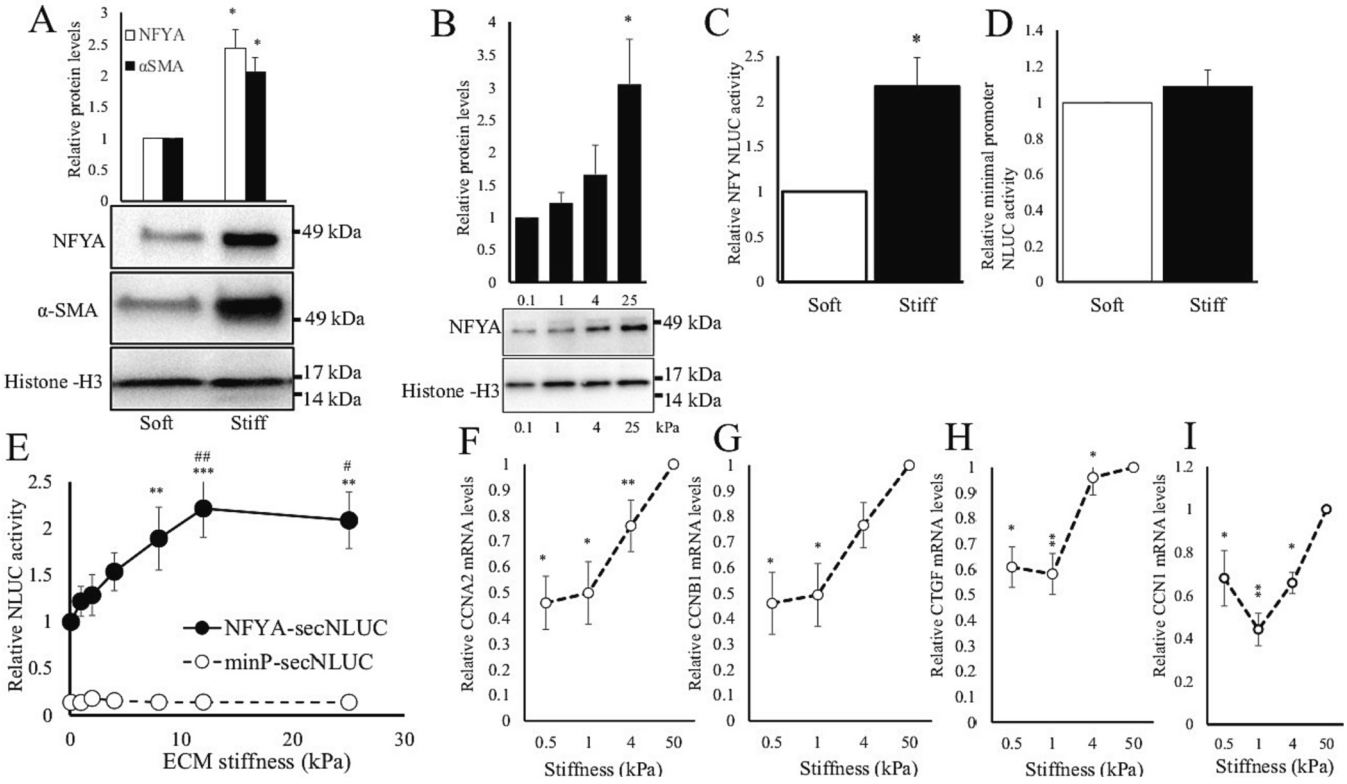


Fig. 6. Pathologically stiff ECM induces NF-YA protein, activity and target gene expression. CF were seeded onto stiff (50 kPa) or soft (1 kPa) substrates and total cell lysate analysed for NF-YA, αSMA and Histone H3 protein levels by western blotting (n = 3; A). Cells were seeded onto hydrogels of indicated stiffness and NF-YA and Histone-H3 protein levels determined by western blotting (n = 3; B). Cells were transfected with a NF-Y-reporter plasmid (C) or a reporter regulated by a minimal promoter (lacking NF-Y elements; D). After transfection, cells were seeded onto stiff or soft substrates (n = 3; C and D) or substrates of indicated stiffness ($n = 7$; E) and secreted reporter gene activity quantified. Cells were seeded onto substrates of indicated stiffness and total RNA analysed for mRNA levels of the NF-Y-target genes, CCNA2 ($n = 6$; F), CCNB1 (n = 6; G) or the classical YAP/TAZ and MKL1/2 target gene mRNAs CTGF (n = 6; H) or CCN1 (n = 6; I). Data is mean ± SEM. Student t-test (A, C, D). One-way ANOVA with Student Newman Keuls post-hoc test (B, *E*-I). * Indicates $p < 0.05$, ** indicates $p < 0.01$, *** indicates $p < 0.001$ vs control value.

regulation, in terms of magnitude and stiffness sensitivity (Fig. 6H and I). Together, these data demonstrate that ECM-stiffness enhanced NF-YA protein levels, NF-Y transcriptional activity and NF-Y-dependent expression of cell-cycle regulatory genes.

3.6. NF-YA protein levels are dependent on actin cytoskeleton integrity and focal adhesion kinase activity

Bio-mechanical signals are typically transmitted into the cell via focal adhesion signalling proteins to actin stress fibres [38,39]. Focal adhesion maturation is dependent on tensional forces generated in the actin stress fibres, indicating a complex interplay between actin and focal adhesion signalling proteins. We therefore asked whether NF-YA protein levels were dependent on actin polymerisation and activity of Focal Adhesion Kinase (FAK). To test the functional importance of FAK, cells were treated with the pharmacological FAK inhibitors Defactinib [40] or PF573228 [41]. Efficient inhibition of FAK activity was assessed by monitoring levels of FAK phosphorylation at the Y397 autophosphorylation site (Supplement Fig. 6). Treatment with either of these inhibitors significantly reduced NF-YA protein levels (Fig. 7A and B) without affecting levels of the housekeeping protein histone-H3. To further confirm the importance of FAK signalling in NF-YA regulation, we over expressed the endogenous FAK inhibitor, FRNK [18]. Over-expression of FRNK also significantly reduced NF-YA protein levels (Fig. 7C), supporting the conclusion that NF-YA protein levels in CF are dependent on FAK activity. Moreover, FAK inhibition, using Defactinib, significantly reduced NF-Y-reporter gene activity (Fig. 7D) consistent with our observed reductions in NF-YA protein in response to FAK inhibition. Furthermore, the mRNA levels of the NF-Y-target genes, CCNA2 and CCNB1, were also strongly down regulated in response to FAK inhibition by Defactinib treatment for 8 or 18 h (Fig. 7E). We next tested if NF-YA levels are dependent on actin cytoskeleton integrity and actin polymerisation. We treated cells with low doses of the actin binding drug latrunculin-B [42] and quantified NF-YA levels. Latrunculin-B treatment dose dependently (from 0.05 to 0.25 μg/ml) inhibited NF-YA protein levels (Fig. 7F and G) without affecting histone-H3 levels. Consistent with this reduction in NF-YA protein levels, latrunculin-B also significantly reduced NF-Y reporter gene activity (Fig. 7H) and reduced the mRNA levels of CCNA2 and CCNB1 (Fig. 7 I). Taken together, these data demonstrate that the levels of NF-YA protein and NF-Y activity are dependent on actin-cytoskeleton integrity and signalling via FAK.

3.7. NF-Y activity is required for CF proliferation

Since our RNAseq data demonstrated that NF-Y-dependent genes display an enrichment of GO-terms associated with cell-cycle progression, we asked if NF-Y activity is required for CF proliferation. We initially used DN-NF-Y to block NF-Y function and quantify effects on cell replication and cell cycle progression. Cells infected with a control adenovirus lacking a transgene (Ad:Control)) increased in cell number over the 72 h post infection (Fig. 8A). However, cells expressing DN-NF-Y did not increase in cell number and total cell number 48 and 72 h post infection were significantly lower than Ad:Control infected cells (Fig. 8A). To gain an insight into how NF-Y activity is involved in cell cycle progression, we analysed propidium iodide-stained cells by flow

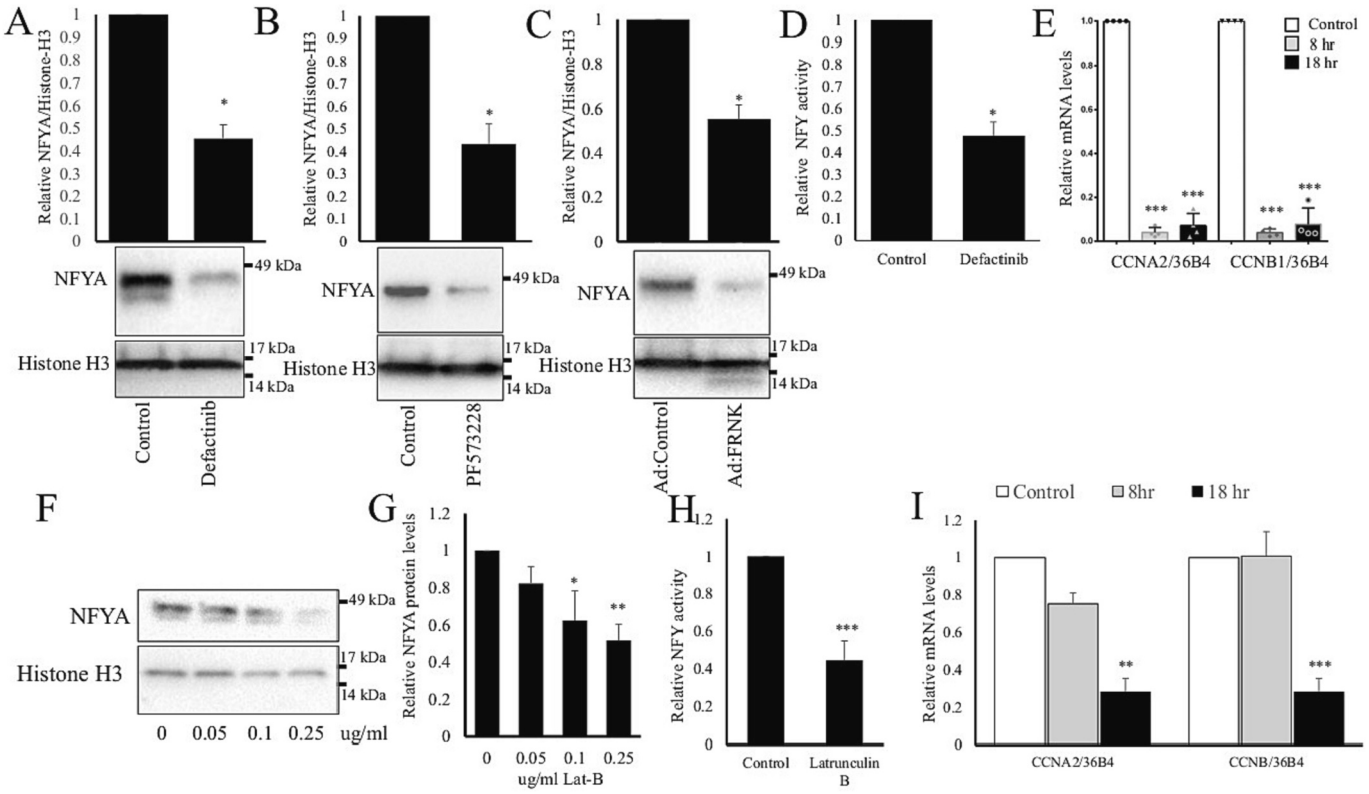


Fig. 7. FAK and actin cytoskeleton dependent regulation of NF-YA protein levels and activity
CF were seeded onto tissue culture plastic and treated with 10 μM Defactinib (n = 3; A), 10 μM PF573228 (n = 3; B) or infected with either Ad:Control or Ad:FRNK (n = 4; C). Total cell lysates were analysed for NF-YA and Histone-H3 protein levels by western blotting (A-C). Cells were transfected with NF-Y reporter plasmid and treated with 10 μM Defactinib for 8 or 18 h as indicated, and NF-Y reporter activity was quantified after 18 h (D) and CCNA2 and CCNB1 mRNA analysed after 8 and 18 h (n = 4; E). CF seeded on plastic were treated with the indicated concentrations of latrunculin-B (LatB) for 18 h (F and G). NF-YA and Histone H-3 protein levels were quantified by western blotting (n = 7; F and G). NF-Y reporter gene activity was quantified after 18 h (n = 3; H). Cells were treated with 0.25 μg/ml latrunculin-B (n = 4; I) for 8 or 18 h. Total RNA was analysed for CCNA2 and CCNB1 mRNA by qRT-PCR. Data is mean ± SEM. Students paired t-test (A-D, H). One-way ANOVA with Student Newman Keuls post-hoc test (E,G,I).

cytometry. We found that expression of DN-NF-Y increased the percentage of cells in S-phase of the cell cycle (Fig. 8 B and C), even though total cell numbers were reduced after 48 h. This may indicate that cells are arresting in mid to late S-phase. To test this, we labelled cells with EdU, to quantify the percentage of cells actively progressing through S-phase and synthesising new DNA. This demonstrated that cells infected with DN-NF-Y adenovirus displayed significantly reduced EdU incorporation (Fig. 8D). To specifically test if DN-NF-Y was causing cells to arrest within S-phase, and hence reduce the numbers of cells reaching late-S phase, we quantified only those cells staining brightly with EdU, after a 4 h labelling period (half the typical time to complete S phase [43]). These EdU bright cells will have progressed through early and mid S-phase and hence have incorporated high levels of EdU. This analysis demonstrated a significant reduction in EdU bright cells (i.e., less cells reaching late S-phase cells) in response to DN-NF-Y adenovirus. This reduction in the percentage of cells reaching late-S phase was more robust than the reduction in the total percentage of EdU positive cells. Taken together, these data indicate the NF-Y activity is required for cardiac fibroblast proliferation and specifically required for efficient progression through S-phase.

We next sought to further confirm the importance of NF-Y for CF proliferation, this time employing siRNA-mediated silencing of the three NF-Y subunits, NF-YA, NF-YB and NF-YC (referred to as siNF-Y). Transfection of cells with siNF-Y resulted in a significant partial reduction in the protein levels of NF-YA and NF-YB ($n = 3$; Fig. 7F and G). We were unable to validate the efficacy of NF-YC silencing due to the lack of a good quality commercial antibody. However, siNF-Y transfection partially but significantly reduced the activity of an NF-Y-dependent reporter gene compared to siNEG transfected cells (Fig. 8H), confirming a functional reduction of NF-Y activity. Cells transfected with a non-targeting control siRNA (siNEG) increased in cell number between 24 and 48 h and 48 to 72 h post transfection (Fig. 8I). Cells transfected with siNF-Y increased in cell numbers between 24 and 48 h but not between 48 and 72 h post transfection. This resulted in the total cell number of siNF-Y transfected cells being significantly lower than siNEG cell at the 72-h timepoint (Fig. 8I). Analysis of cell cycle phase by flow cytometry revealed that NF-Y silencing significantly increased the percentage of cells in G1 phase and reduced the percentage in S-phase (Fig. 8J and K). We next tested if NF-Y silencing reduced the percentage of cells actively progressing through S-phase by quantifying EdU incorporation. NF-Y silencing significantly reduced the percentage of cells positively labelled for EdU incorporation (Fig. 8L). Quantification of cells progressing into late S-phase during the labelled period (i.e., EdU-bright) demonstrated a more pronounced reduction in siNF-Y cells compared to siNEG transfected cells (Fig. 8M).

3.8. NF-Y mediates ECM stiffness-dependent proliferation of CF

To test if NF-Y is responsible for mechano-sensitive proliferation of CF, we attempted to rescue the proliferation of cells interacting with a soft ECM by ectopic expression of NF-YA. In cells transiently transfected with a control plasmid, endogenous NF-YA levels were significantly elevated by interaction with a stiff ECM (Fig. 9A). Ectopic expression of wild-type NF-YA in cells grown on a soft ECM increased total NF-YA protein to levels equal to that present in control cells cultured on a stiff ECM (Fig. 9A). Culture of NF-YA plasmid transfected cells on a stiff

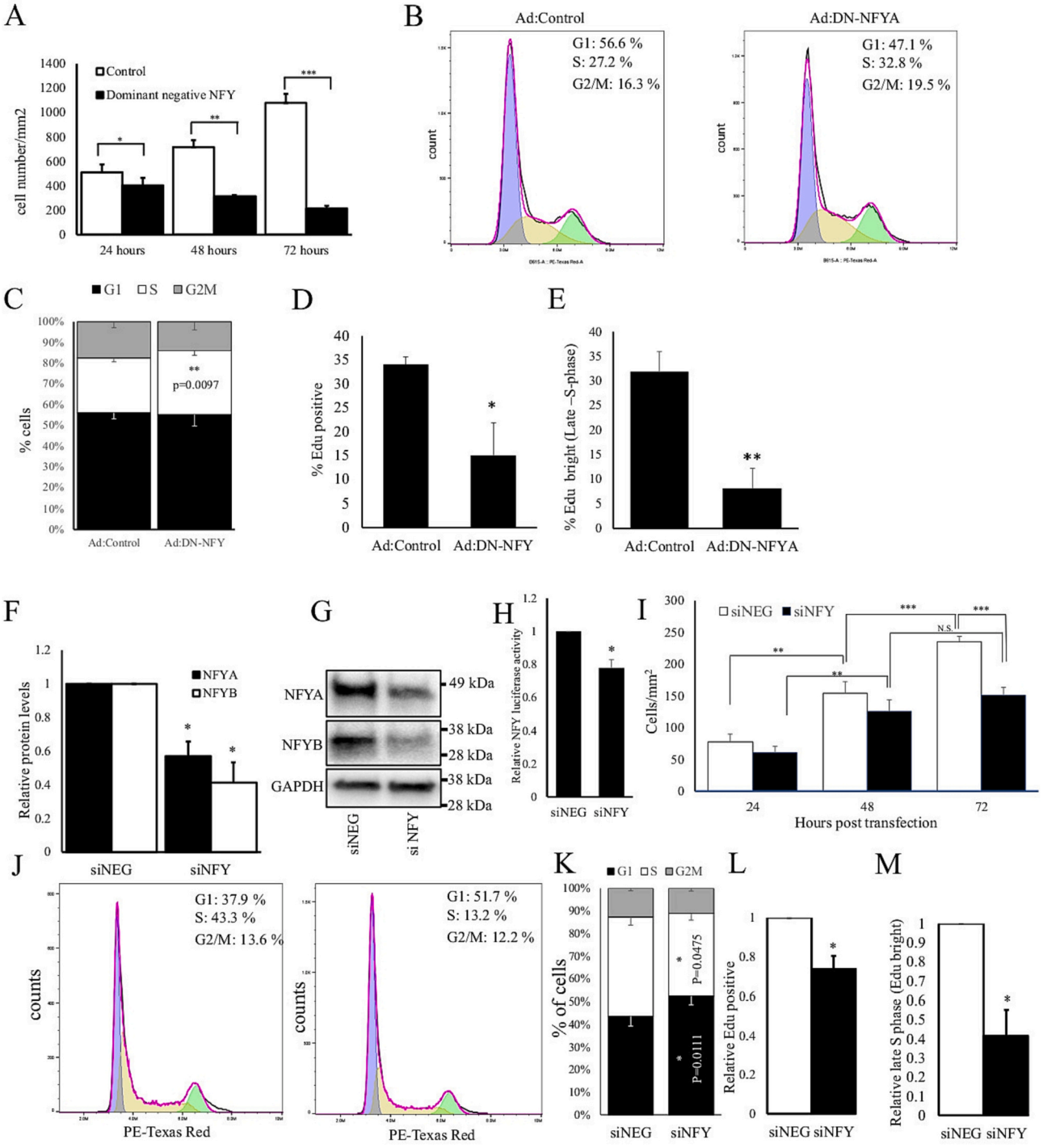


Fig. 8. CF proliferation is NF-Y-dependent.

CF cultured on plastic were infected with either Ad:Control or Ad:DN-NF-Y. Total cell number was determined by manual cell counting after 24, 28 or 72 h ($n = 9$; A). CF were infected with either Ad:Control or Ad:DN-NF-Y. Cells were PI labelled and analysed for cell cycle phase by flow cytometry (n = 4; B and C). Cells were Edu labelled for 4 h, 20 h post-infection. Percentage Edu positive (n = 5; D) and percentage of Edu bright (late S-phase) cells (n = 5; E) was quantified. CF were transfected with siNEG or siNF-Y. Protein levels of NF-YA, NF-YB and GAPDH were quantified by western blotting (n = 3; F and G). Cells were transfected with either siNEG or siNF-Y together with and NF-Y-reporter gene plasmid (H). NF-Y reporter activity was quantified (H) and total cell number counted after 24, 48 and 72 h (n = 6; I). Cells were transfected with either siNEG or siNF-Y and analysed for cell cycle phase by PI staining and flow cytometry (n = 3; J and K). Cells were Edu labelled for 4 h. Percentage Edu positive (n = 6; L) and percentage Edu bright (n = 4; M) was quantified. One-way ANOVA with student Newman Keuls post-test (A, I) or Student's t-test (C-F, H, K-M). * Indicates $p < 0.05$; ** indicates $p < 0.01$; *** indicates $p < 0.001$.

ECM resulted in a further increase in NF-YA protein levels. This shows that exogenously expressed NF-YA protein is also subject to ECM-stiffness dependent regulation and that exogenous expression of NF-YA can reverse the reduction in endogenous NF-YA levels in response to a soft ECM. A similar pattern was observed when quantifying NF-Y reporter gene activity (Fig. 9B). Activity was significantly elevated in control plasmid transfected cells on a stiff compared to a soft ECM. Overexpression of NF-YA increased reporter activity in cells on a soft

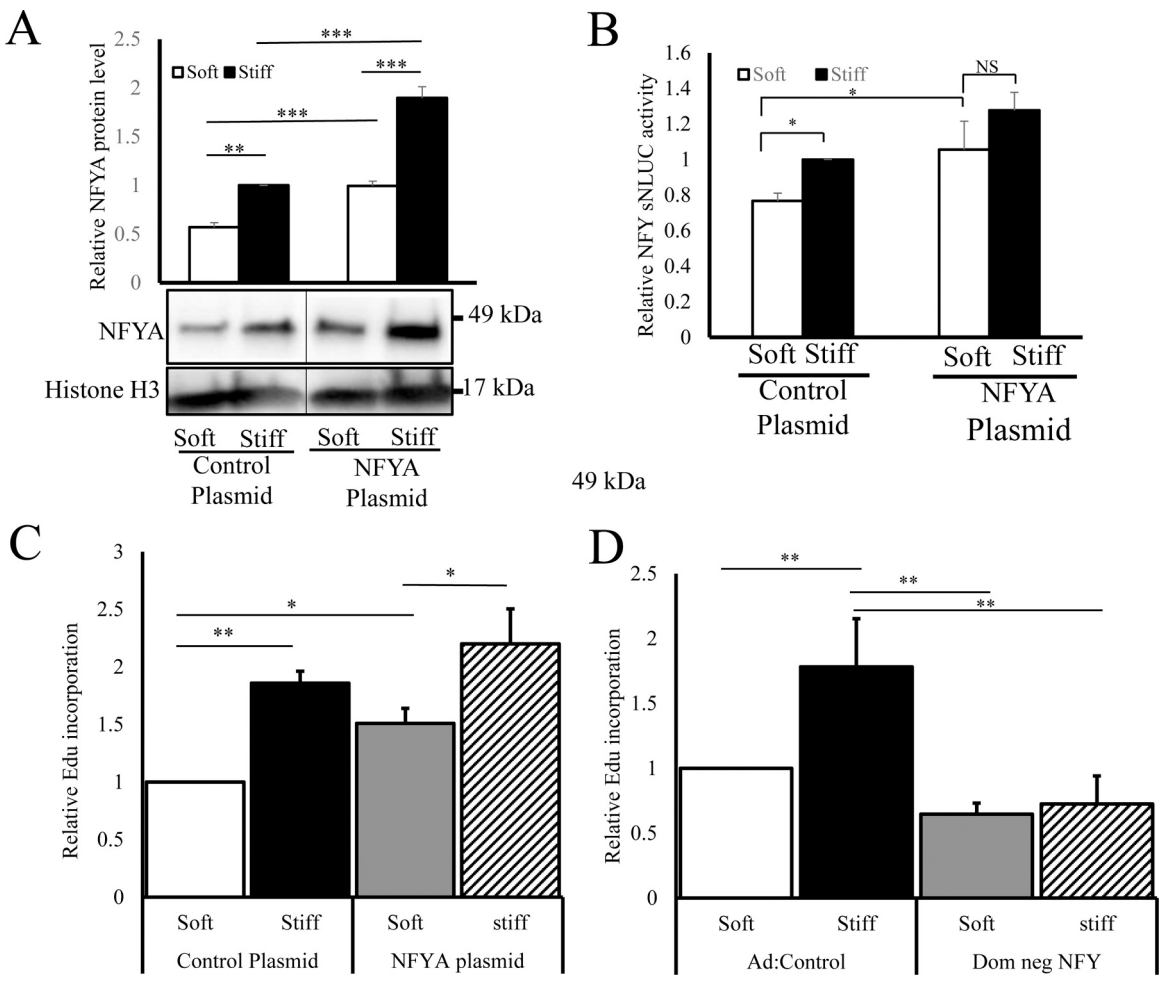


Fig. 9. ECM-stiffness mediated proliferation of CF is NF-Y-dependent.
CF were transfected with either control plasmid or plasmid expressing wild-type NF-YA and seeded onto soft or stiff ECM. Total cell lysates were analysed by western blotting for NF-YA and Histone-H3 protein levels (n = 3; A). Cells were co-transfected with NF-Y-reporter plasmid together with either control plasmid or plasmid expressing wild-type NF-YA and seeded onto soft or stiff ECM. NF-Y reporter activity was quantified 24 h later (n = 6; B). Cells were transfected with either control plasmid or wild-type NF-YA plasmid (n = 4; C) or infected with either control or dominant-negative-NF-Y adenovirus (n = 4;D) and Edu labelled for 4 h. * Indicates $p < 0.05$; ** indicates $p < 0.01$; One-way ANOVA with student Newman Keuls post-test.

ECM to levels that were not statistically different from control plasmid cells on a stiff ECM, demonstrating that ectopic expression of NF-YA is able to fully rescue NF-Y activity (Fig. 9B). Analysis of EdU incorporation demonstrated that proliferation of control plasmid cells was significantly greater in cells interacting with a stiff, compared to a soft ECM (Fig. 9C), consistent with our earlier data (Fig. 1A). Importantly, overexpression of NF-YA significantly increased proliferation of cells on a soft ECM to levels not statistically different from that observed in control cells cultured on stiff ECM (Fig. 9C). Proliferation of NF-YA plasmid transfected cells on a stiff ECM was further increased and significantly higher than NF-YA plasmid transfected cells on a soft ECM. Finally, we tested in expression of DN-NF-Y can prevent the induction of proliferation in response to an increase in ECM stiffness. Proliferation of Ad:Control virus infected cells was significantly increased by a stiff ECM, but proliferation of Ad:DN-NF-Y infected cells on a soft ECM was significantly lower than Ad:Control cells (Fig. 9D). Furthermore, an increase in ECM stiffness had no effect on the proliferation on Ad:DN-NF-Y infected cells in contrast to control infected cells.

4. Discussion

Here we demonstrate for the first time that the level of NF-YA, the regulatory component of the NF-Y transcription factor, is increased in response to greater stiffness of the extracellular matrix surrounding CFs. The resulting increased NF-Y activity is responsible for 23 % the ECM-stiffness induced transcriptome in CF, independently of known mechano-sensitive factors YAP/TAZ and SRF/MKL1/2. NF-Y stimulates expression of several cell cycle genes and promotes CF proliferation in response to a stiffened ECM.

The stiffness of the myocardium is increased in patients with cardiac fibrosis [79]. This results from greater deposition of a collagen-rich ECM, enhanced collagen crosslinking via the activity of enzymes such as LOX [44] and PLOD [45], and a proliferative expansion of the CF population. CF proliferation is important as this increases the number of collagen producing cells. In humans, healthy diastolic human myocardium has been reported to have a stiffness of 1.7 kPa [46]. This increases in fibrotic myocardium to stiffnesses measurements in the range of 20–100 kPa. CF resident within the myocardium sense and respond to this increased stiffness of their local ECM by differentiating into activated myofibroblasts [47], characterised by elevated expression of α-SMA and increased proliferation. Here we show that levels of NF-YA are associated with expression of αSMA, a marker of a myofibroblast phenotype, implying that NF-YA levels increase during myofibroblast differentiation and contribute towards their increased proliferation.

Many adhesion dependent cell types display increased proliferation rates in response to stiffening of the ECM [48–51], although the effects

on CF have not been extensively characterised. For example, Herum et al. reported that static mechanical stretching, but not increased ECM stiffness, promoted CF proliferation [15]. This contrasts with the data that we present here showing an increase in proliferation when CF interact with a pathologically stiff, compared to a physiologically soft, ECM. This may simply reflect the different cell type studied (embryonic heart compared to adult heart used here) or differences in the compliance of the tissue studied. Importantly, increased proliferation of CFs occurs in vivo in regions of fibrosis [52,53], consistent with the paradigm of enhanced proliferation in regions of increased stiffness. As CF are the primary source of interstitial matrix in the heart, it follows that proliferative expansion of CF population will further enhance ECM synthesis, thus contributing towards the creation of a positive feedback loop, driving progression of fibrosis [54,55]. However, the mechanisms involved in transducing tissue stiffness into changes in CF proliferation are poorly understood.

Several mechanisms have been demonstrated to play a role in transducing extracellular bio-mechanical signals into the nucleus and hence gene transcription. One of the best characterised is the Hippo pathway, centred around the core Hippo kinases, LATS and MST, which when activated, phosphorylate and inactivate the transcriptional co-factors YAP and TAZ [17]. In their hypo-phosphorylated state YAP and TAZ translocate into the nucleus where they interact with TEAD-family transcription factors to mediate a transcriptional response. Other transcriptional co-factors, including MKL1 and MKL2 have also been implicated in mechano-transduction [56]. Biomechanical signals that promote actin polymerisation induce nuclear translocation of MKL1 and MKL2, where they activate SRF-dependent gene expression [57]. Here we show that although these pathways do mediate changes in gene expression in response to an increase in ECM stiffness in CF, together they are only responsible for a relatively small part (8 % each) of the ECM-stiffness induced transcriptome. For example, we identified 1466 genes as being induced by culture on a stiff compared to a soft ECM. However, only 116 and 113 were dependent on YAP/TAZ or MKL1/2, respectively (9 genes were dependent on both YAP/TAZ and MKL1/2). The implication is that remaining 1246 genes in CF are regulated by at least one additional mechano-sensitive transcription factor/co-factor. Our data presented here identified NF-Y as another important mechanosensitive factor. We found that NF-Y binding elements are significantly enriched in the promoters of stiffness induced genes. Furthermore, NF-YA concentration and NF-Y transcriptional activity is enhanced in CF interacting with a pathologically stiff ECM. To our knowledge, this is the first demonstration that the NF-Y transcription factor participates in ECM-stiffness-dependent gene expression. Consistently, however, a previous study demonstrated that NF-Y elements in the promoter of the pro-collagen-α1 and pro-collagen-α2 genes mediate high levels of expression and are responsible for the induction of collagen expression in response to cyclic strain, supporting a role for NF-Y as a regulator of mechanically sensitive transcription [58]. Furthermore, published data on NF-Y-dependent regulation of collagen expression taken together with our data on NF-Y-dependent CF proliferation support a role for NF-Y in fibrotic disease [59,60]. Also consistent with this, reduction in the protein levels of NF-YA has been shown to mediate the anti-fibrotic effects of branched chain amino acids in hepatic fibrosis [61].

Very little is currently understood regarding the mechanisms regulating NF-YA protein levels. Lindahl demonstrated that NF-Y DNA binding is increased by TGFβ stimulation [60]. Another study demonstrated that levels of NF-YA are regulated post translationally by ubiquitination and acetylation, which control the stability of the NF-YA protein [37]. Here we demonstrate a role for focal adhesion kinase (FAK) signalling. FAK inhibition using two different pharmacological inhibitors, or overexpression of the endogenous FAK inhibitor, FRNK, reduced NF-YA protein levels and activity in CF. We also show that NF-YA protein levels are dependent on the integrity of the actin cytoskeleton. Treatment of cells with the actin binding drug, Latrunculin-B significantly reduced NF-YA protein levels, activity and target gene expression. Previous studies have demonstrated that FAK is recruited to and activated at focal adhesions in cells interacting with stiff substrates [62]. These develop and mature in a stiffness dependent manner, in part via the tensional forces generated in actin stress fibres, which connect with these focal adhesions. In cells cultured on soft substrates, stress fibre tensional forces are diminished, due to the compliance of the substrate, and hence FAK activity is also lower in these cells. Future studies should investigate further how FAK, actin cytoskeleton remodelling and bio-mechanical signals control NF-YA protein levels.

Our RNA-seq analysis of NF-Y-dependent genes, highlights a clear association with genes involved in cell proliferation. Out of the 1246 genes induced by a stiff ECM, independently of YAP/TAZ or MKL1/2, 256 were found to be NF-Y. Gene ontology analysis of these 256 genes demonstrated enrichment of multiple gene ontology terms linked to cell proliferation. Interestingly, no enrichment of GO-terms related to ECM deposition or organisation were found, implying that stiff-substrate induced, NF-Y-dependent genes regulate cell proliferation but not ECM remodelling. As our approach to identify NF-Y dependent genes was to express a dominant-negative mutant of NF-YA for 24 h it is possible that some of the genes we identified are not direct NF-Y targets. However, approximately half of the genes with the GO term tag “regulation of cell cycle” had a consensus NF-Y binding element (CCAAT) in the 5000 bp promoter region upstream of the transcriptional start. Furthermore, those genes with a NF-Y promoter element displayed the largest fold inhibition by dominant-negative NF-Y. Furthermore, mutation of the NF-Y binding elements in two examples of these genes (CCNA2 and CCNB1) inhibited promoter-reporter gene activity. Taken together, this implies that at least some of the proliferation related genes are direct targets of NF-Y. Importantly, the mRNA levels of a shortlist of these putative NF-Y-dependent target genes also displayed sensitivity to the stiffness of the ECM. The implication is that ECM-remodelling, which results in an increase in stiffness, activates NF-Y. This, in turn, promotes expression of genes required for cell proliferation. Consistent with this, we found that NF-Y activity to be essential for maximal proliferation of CF. Inhibition of NF-Y using a dominant-negative mutant, which disrupts NF-Y DNA binding, reduced total cell number and reduced DNA synthesis. Our data demonstrates an important role for NF-Y in regulating CF proliferation and adds to growing evidence that supports a role for NF-Y-dependent transcription in the regulation of cell cycle progression and proliferation of other cells. For example, NF-Y has been reported to regulate the expression of multiple cell cycle genes, including E2F1, CCNA, CCNB1, CDK1, CHK2, CDKN1C, and CDC25A [63–65] and to regulate the proliferation of colorectal cancer cells [66], cervical cancer cells [67] and stem cells [68,69]. Our new data, demonstrates for the first time, that NF-Y activation is both necessary and sufficient for the maximal stimulatory effects of a pathologically stiffened ECM on the proliferation of CF. Consistent with this, overexpression of NF-YA alone in CF interacting with a physiologically soft ECM increased NF-Y activity to levels equal to that in cells interacting with a stiff ECM and significantly restores their proliferation. Given that this experiment is conducted with a plasmid transfection efficiency of approximately 50 %, our measure of the rescue of proliferation is likely to be a minimum estimate.

In conclusion, we demonstrate that the NF-Y transcription factor is a novel bio-mechanically sensitive transcription factor, which plays an important role in the regulation of CF proliferation in response to an increase in the stiffness of the local ECM. Targeting the NF-Y complex may represent a viable target for future therapies designed to limit the progression of fibrotic diseases, such as cardiac fibrosis.

Sources of funding

This work was funded by British Heart Foundation research project number PG/19/39/34415.

CRediT authorship contribution statement

Reza Ebrahimighaei: Investigation, Formal analysis, Data curation, Supervision. **Nathalie Tarrasova:** Investigation, Formal analysis. **Samuel C. Bond:** Investigation. **Madeleine C. McNeill:** Investigation, Supervision. **Tom Hathway:** Resources. **Hunaid Vohra:** Resources. **Andrew C. Newby:** Writing – review & editing, Supervision. **Mark Bond:** Conceptualization, Funding acquisition, Project administration, Supervision, Visualization, Writing – review & editing, Resources.

Declaration of competing interest

None of the authors of this manuscript have any conflicts of interest to declare.

Data availability

Data will be made available on request.

Acknowledgments

This work was supported by the British Heart Foundation, grant PG/19/39/34415. We gratefully acknowledge the molecular biology technical support and assistance provided by Dr. Graciela B Sala-Newby.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbamcr.2023.119640.

References

[1] P. Kong, P. Christia, N.G. Frangogiannis, The pathogenesis of cardiac fibrosis, Cell. Mol. Life Sci. 71 (2014) 549–574.
[2] S.A. Mandras, S. Desai, Right Heart Failure, StatPearls, StatPearls Publishing Copyright © 2023, StatPearls Publishing LLC., Place Published, 2023.
[4] W. Chen, N.G. Frangogiannis, Fibroblasts in post-infarction inflammation and cardiac repair, BBA-Mol. Cell. Res. 2013 (1833) 945–953.
[5] C.A. Souders, S.L.K. Bowers, T.A. Baudino, Cardiac fibroblast the renaissance cell, Circ. Res. 105 (2009) 1164–1176.
[6] D. Fan, A. Takawale, J. Lee, Z. Kassiri, Cardiac fibroblasts, fibrosis and extracellular matrix remodeling in heart disease, Fibrogenesis Tissue Repair 5 (2012) 15.
[7] A.A. Gibb, M.P. Lazaropoulos, J.W. Elrod, Myofibroblasts and fibrosis: mitochondrial and metabolic control of cellular differentiation, Circ. Res. 127 (2020) 427–447.
[8] L. Li, Q. Zhao, W. Kong, Extracellular matrix remodeling and cardiac fibrosis, Matrix Biol. 68-69 (2018) 490–506.
[9] P. Kong, P. Christia, N.G. Frangogiannis, The pathogenesis of cardiac fibrosis, Cell. Mol. Life Sci. 71 (2014) 549–574.
[10] J.G. Travers, F.A. Kamal, J. Robbins, K.E. Yutzey, B.C. Blaxall, Cardiac fibrosis: the fibroblast awakens, Circ. Res. 118 (2016) 1021–1040.
[11] B. Lopez, A. Gonzalez, R. Querejeta, M. Larman, J. Diez, Collagen cross-linking but not collagen amount associates with elevated filling pressures in heart failure patients of hypertensive origin. Potential role of lysyl oxidase, Circulation 126 (2012) 1.
[12] M. Couade, M. Pernot, E. Messas, A. Bel, M. Ba, A. Hagege, M. Fink, M. Tanter, In vivo quantitative mapping of myocardial stiffening and transmural anisotropy during the cardiac cycle, IEEE Trans. Med. Imaging 30 (2011) 295–305.
[13] J.G. Jacot, J.C. Martin, D.L. Hunt, Mechanobiology of cardiomyocyte development, J. Biomech. 43 (2010) 93–98.
[14] K.M. Herum, I.G. Lunde, A.D. McCulloch, G. Christensen, The soft- and hard-heartedness of cardiac fibroblasts: mechanotransduction signaling pathways in fibrosis of the heart, J. Clin. Med. 6 (2017).
[15] K.M. Herum, J. Choppe, A. Kumar, A.J. Engler, A.D. McCulloch, Mechanical regulation of cardiac fibroblast profibrotic phenotypes, Mol. Biol. Cell 28 (2017) 1871–1882.
[16] T. Mammoto, D.E. Ingber, Mechanical control of tissue and organ development, Development 137 (2010) 1407–1420.
[17] S. Dupont, L. Morsut, M. Aragona, E. Enzo, S. Giulitti, M. Cordenonsi, F. Zanconato, J. Le Digabel, M. Forcato, S. Bicciato, N. Elvassore, S. Piccolo, Role of YAP/TAZ in mechanotransduction, Nature 474 (2011) 179–U212.
[18] L. Montel, A. Sotiropoulos, S. Hénon, The nature and intensity of mechanical stimulation drive different dynamics of MRTF-A nuclear redistribution after actin remodeling in myoblasts, PloS One 14 (2019), e0214385.
[19] E.M. Small, J.E. Thatcher, L.B. Sutherland, H. Kinoshita, R.D. Gerard, J. A. Richardson, J.M. Dimaio, H. Sadek, K. Kuwahara, E.N. Olson, Myocardin-related transcription factor-a controls myofibroblast activation and fibrosis in response to myocardial infarction, Circ. Res. 107 (2010) 294–304.
[20] D. Bugg, R. Bretherton, P. Kim, E. Olszewski, A. Nagle, A.E. Schumacher, N. Chu, J. Gunaje, C.A. DeForest, K. Stevens, D.H. Kim, J. Davis, Infarct collagen topography regulates fibroblast fate via p38-yes-associated protein transcriptional enhanced associate domain signals, Circ. Res. 127 (2020) 1306–1322.
[21] J. Francisco, Y. Zhang, J.I. Jeong, W. Mizushima, S. Ikeda, A. Ivessa, S. Oka, P. Zhai, M.D. Tallquist, D.P. Del Re, Blockade of fibroblast YAP attenuates cardiac fibrosis and dysfunction through MRTF-A inhibition, JACC Basic Transl Sci 5 (2020) 931–945.
[22] A.R. Perestrelo, A.C. Silva, J. Oliver-De La Cruz, F. Martino, V. Horváth, G. Caluori, O. Polanský, V. Vinarský, G. Azzato, G. de Marco, V. Žampachová, P. Skládal, S. Pagliari, A. Rainer, P. Pinto-do-Ó, A. Caravella, K. Koci, D.S. Nascimento, G. Forte, Multiscale analysis of extracellular matrix remodeling in the failing heart, Circ. Res. 128 (2021) 24–38.
[23] M.M. Mia, D.M. Cibi, S. Ghani, A. Singh, N. Tee, V. Sivakumar, H. Bogireddi, S. A. Cook, J. Mao, M.K. Singh, Loss of Yap/Taz in cardiac fibroblasts attenuates adverse remodelling and improves cardiac function, Cardiovasc. Res. 118 (2022) 1785–1804.
[24] R. Mantovani, X.Y. Li, U. Pessara, R. Hooft van Huisjduijnen, C. Benoist, D. Mathis, Dominant negative analogs of NF-YA, J. Biol. Chem. 269 (1994) 20340–20346.
[25] T.E. Kimura, A. Duggirala, M.C. Smith, S. White, G.B. Sala-Newby, A.C. Newby, M. Bond, The hippo pathway mediates inhibition of vascular smooth muscle cell proliferation by cAMP, J. Mol. Cell. Cardiol. 90 (2016) 1–10.
[26] A. Duggirala, T.E. Kimura, G.B. Sala-Newby, J.L. Johnson, Y.-J. Wu, A.C. Newby, M. Bond, cAMP-induced actin cytoskeleton remodelling inhibits MKL1-dependent expression of the chemotactic and pro-proliferative factor, CCN1, J. Mol. Cell. Cardiol. 79 (2015) 157–168.
[27] M.C. McNeill, J. Wray, G.B. Sala-Newby, C.C.T. Hindmarch, S.A. Smith, R. Ebrahimighaei, A.C. Newby, M. Bond, Nuclear actin regulates cell proliferation and migration via inhibition of SRF and TEAD, Biochim Biophys Acta Mol Cell Res 1867 (2020), 118691.
[28] M. Bond, Y.-J. Wu, G.B. Sala-Newby, A.C. Newby, Rho GTPase, Rac(1), regulates Skp(2) levels, vascular smooth muscle cell proliferation, and intima formation in vitro and in vivo, Cardiovasc. Res. 80 (2008) 290–298.
[29] S.A. Aleksander, J. Balhoff, S. Carbon, J.M. Cherry, H.J. Drabkin, D. Ebert, M. Feuermann, P. Gaudet, N.L. Harris, D.P. Hill, R. Lee, H. Mi, S. Moxon, C. J. Mungall, A. Muruganugan, T. Mushayahama, P.W. Sternberg, P.D. Thomas, K. Van Auken, J. Ramsey, D.A. Siegele, R.L. Chisholm, P. Fey, M.C. Aspromonte, M. V. Nugnes, F. Quaglia, S. Tosatto, M. Giglio, S. Nadendla, G. Antonazzo, H. Attrill, G. Dos Santos, S. Marygold, V. Strelets, C.J. Tabone, J. Thurmond, P. Zhou, S. H. Ahmed, P. Asanitthong, D. Luna Buitrago, M.N. Erdol, M.C. Gage, M. Ali Kadhum, K.Y.C. Li, M. Long, A. Michalak, A. Pesala, A. Pritazahra, S.C. C. Saverimuttu, R. Su, K.E. Thurlow, R.C. Lovering, C. Logie, S. Oliferenko, J. Blake, K. Christie, L. Corbani, M.E. Dolan, H.J. Drabkin, D.P. Hill, L. Ni, D. Sitnikov, C. Smith, A. Cuzick, J. Seager, L. Cooper, J. Elser, P. Jaiswal, P. Gupta, P. Jaiswal, S. Naithani, M. Lera-Ramirez, K. Rutherford, V. Wood, J.L. De Pons, M. R. Dwinell, G.T. Hayman, M.L. Kaldunski, A.E. Kwitek, S.J.F. Laulederkind, M. A. Tutaj, M. Vedi, S.J. Wang, P. D'Eustachio, L. Aimo, K. Axelsen, A. Bridge, N. Hyka-Nouspikel, A. Morgat, S.A. Aleksander, J.M. Cherry, S.R. Engel, K. Karra, S.R. Miyasato, R.S. Nash, M.S. Skrzypek, S. Weng, E.D. Wong, E. Bakker, T. Z. Berardini, L. Reiser, A. Auchincloss, K. Axelsen, G. Argoud-Puy, M.C. Blatter, E. Boutet, L. Breuza, A. Bridge, C. Casals-Casas, E. Coudert, A. Estreicher, M. Livia Famiglietti, M. Feuermann, A. Gos, N. Gruaz-Gumowski, C. Hulo, N. Hyka-Nouspikel, F. Jungo, P. Le Mercier, D. Lieberherr, P. Masson, A. Morgat, I. Pedruzzi, L. Pourcel, S. Poux, C. Rivoire, S. Sundaram, A. Bateman, E. Bowler-Barnett, A.J.H. Bye, P. Denny, A. Ignatchenko, R. Ishtiaq, A. Lock, Y. Lussi, M. Magrane, M.J. Martin, S. Orchard, P. Raposo, E. Speretta, N. Tyagi, K. Warner, R. Zaru, A.D. Diehl, R. Lee, J. Chan, S. Diamantakis, D. Raciti, M. Zarowiecki, M. Fisher, C. James-Zorn, V. Ponferrada, A. Zorn, S. Ramachandran, L. Ruzicka, M. Westerfield, The Gene Ontology knowledgebase in 2023, Genetics 224 (2023).
[30] S.J.H. Sui, J.R. Mortimer, D.J. Arenillas, J. Brumm, C.J. Walsh, B.P. Kennedy, W. W. Wasserman, oPOSSUM: identification of over-represented transcription factor binding sites in co-expressed genes, Nucleic Acids Res. 33 (2005) 3154–3164.
[31] K. Wagh, M. Ishikawa, D.A. Garcia, D.A. Stavreva, A. Upadhyaya, G.L. Hager, Mechanical regulation of transcription: recent advances, Trends Cell Biol. 31 (2021) 457–472.
[32] D. Lai, K.C. Ho, Y. Hao, X. Yang, Taxol resistance in breast cancer cells is mediated by the hippo pathway component TAZ and its downstream transcriptional targets Cyr61 and CTGF, Cancer Res. 71 (2011) 2728–2738.
[33] A. Duggirala, A.C. Newby, M. Bond, cAMP inhibits expression of the proangiogenic factor CCN1/CYR61 in vascular smooth muscle cells by inhibiting RhoA and actin-dependent regulation of MRTF, J. Mol. Cell. Cardiol. 79 (2014) 157–168.
[34] M.C. Smith, C.A. Hudson, T.E. Kimura, S.J. White, G.B. Sala-Newby, A.C. Newby, M. Bond, Divergent regulation of actin dynamics and Megakaryoblastic Leukemia-1 and-2 (Mkl1/2) by cAMP in endothelial and smooth muscle cells, Sci. Rep. 7 (2017) 13.
[35] G. Donati, R. Gatta, D. Dolfini, A. Fossati, M. Ceribelli, R. Mantovani, An NF-Y-dependent switch of positive and negative histone methyl marks on CCAAT promoters, PloS One 3 (2008), e2066.
[36] D. Dolfini, R. Gatta, R. Mantovani, NF-Y and the transcriptional activation of CCAAT promoters, Crit. Rev. Biochem. Mol. Biol. 47 (2012) 29–49.
[37] I. Manni, G. Caretti, S. Artuso, A. Gurtner, V. Emiliozzi, A. Sacchi, R. Mantovani, G. Piaggio, Posttranslational regulation of NF-YA modulates NF-Y transcriptional activity, Mol. Biol. Cell 19 (2008) 5203–5213.

[38] T. Shemesh, B. Geiger, A.D. Bershadsky, M.M. Kozlov, Focal adhesions as mechanosensors: a physical mechanism, Proc. Natl. Acad. Sci. U. S. A. 102 (2005) 12383–12388.
[39] J. Colombelli, A. Besser, H. Kress, E.G. Reynaud, P. Girard, E. Caussinus, U. Haselmann, J.V. Small, U.S. Schwarz, E.H. Stelzer, Mechanosensing in actin stress fibers revealed by a close correlation between force and protein localization, J. Cell Sci. 122 (2009) 1665–1679.
[40] S.F. Jones, L.L. Siu, J.C. Bendell, J.M. Cleary, A.R. Razak, J.R. Infante, S.S. Pandya, P.L. Bedard, K.J. Pierce, B. Houk, W.G. Roberts, S.M. Shreeve, G.I. Shapiro, A phase I study of VS-6063, a second-generation focal adhesion kinase inhibitor, in patients with advanced solid tumors, Invest. New Drugs 33 (2015) 1100–1107.
[41] J.K. Slack-Davis, K.H. Martin, R.W. Tilghman, M. Iwanicki, E.J. Ung, C. Autry, M. J. Luzzio, B. Cooper, J.C. Kath, W.G. Roberts, J.T. Parsons, Cellular characterization of a novel focal adhesion kinase inhibitor, J. Biol. Chem. 282 (2007) 14845–14852.
[42] I. Spector, N.R. Shochet, Y. Kashman, A. Groweiss, Latrunculins: novel marine toxins that disrupt microfilament organization in cultured cells, Science 219 (1983) 493–495.
[43] M. Bialic, B.A. Nachar, M. Kozlak, V. Coulon, E. Schwob, Measuring S-phase duration from asynchronous cells using dual EdU-BrdU pulse-chase labeling flow cytometry, Genes 13 (2022).
[44] K. Csiszar, Lysyl oxidases: a novel multifunctional amine oxidase family, Prog. Nucleic Acid Res. Mol. Biol. 70 (2001) 1–32.
[45] T. Hautala, M.G. Byers, R.L. Eddy, T.B. Shows, K.I. Kivirikko, R. Myllylä, Cloning of human lysyl hydroxylase: complete cDNA-derived amino acid sequence and assignment of the gene (PLOD) to chromosome 1p36.3——p36.2, Genomics 13 (1992) 62–69.
[46] M. Pernot, M. Couade, P. Mateo, B. Crozatier, R. Fischmeister, M. Tanter, Real-time assessment of myocardial contractility using shear wave imaging, J. Am. Coll. Cardiol. 58 (2011) 65–72.
[47] X. Huang, N. Yang, V.F. Fiore, T.H. Barker, Y. Sun, S.W. Morris, Q. Ding, V. J. Thannickal, Y. Zhou, Matrix stiffness-induced myofibroblast differentiation is mediated by intrinsic mechanotransduction, Am. J. Respir. Cell Mol. Biol. 47 (2012) 340–348.
[48] J. Schrader, T.T. Gordon-Walker, R.L. Aucott, M. van Deemter, A. Quaas, S. Walsh, D. Benten, S.J. Forbes, R.G. Wells, J.P. Iredale, Matrix stiffness modulates proliferation, chemotherapeutic response, and dormancy in hepatocellular carcinoma cells, Hepatology 53 (2011) 1192–1205.
[49] Y.T. Yeh, S.S. Hur, J. Chang, K.C. Wang, J.J. Chiu, Y.S. Li, S. Chien, Matrix stiffness regulates endothelial cell proliferation through septin 9, PloS One 7 (2012), e46889.
[50] R.W. Tilghman, C.R. Cowan, J.D. Mih, Y. Koryakina, D. Gioeli, J.K. Slack-Davis, B. R. Blackman, D.J. Tschumperlin, J.T. Parsons, Matrix rigidity regulates cancer cell growth and cellular phenotype, PloS One 5 (2010), e12905.
[51] E.A. Klein, L. Yin, D. Kothapalli, P. Castagnino, F.J. Byfield, T. Xu, I. Levental, E. Hawthorne, P.A. Janmey, R.K. Assoian, Cell-cycle control by physiological matrix elasticity and in vivo tissue stiffening, Curr. Biol. 19 (2009) 1511–1518.
[52] J.I. Virag, C.E. Murry, Myofibroblast and endothelial cell proliferation during murine myocardial infarct repair, Am. J. Pathol. 163 (2003) 2433–2440.
[53] E. Lutgens, M.J. Daemen, E.D. de Muinck, J. Debets, P. Leenders, J.F. Smits, Chronic myocardial infarction in the mouse: cardiac structural and functional changes, Cardiovasc. Res. 41 (1999) 586–593.
[54] S.R. Ali, S. Ranjbarvaziri, M. Talkhabi, P. Zhao, A. Subat, A. Hojjat, P. Kamran, A. M. Müller, K.S. Volz, Z. Tang, K. Red-Horse, R. Ardehali, Developmental heterogeneity of cardiac fibroblasts does not predict pathological proliferation and activation, Circ. Res. 115 (2014) 625–635.
[55] T. Moore-Morris, N. Guimarães-Camboa, I. Banerjee, A.C. Zambon, T. Kisseleva, A. Velayoudon, W.B. Stallcup, Y. Gu, N.D. Dalton, M. Cedenilla, R. Gomez-Amaro, B. Zhou, D.A. Brenner, K.L. Peterson, J. Chen, S.M. Evans, Resident fibroblast lineages mediate pressure overload-induced cardiac fibrosis, J. Clin. Invest. 124 (2014) 2921–2934.
[56] A. Angelini, J. Trial, J. Ortiz-Urbina, K.A. Cieslik, Mechanosensing dysregulation in the fibroblast: a hallmark of the aging heart, Ageing Res. Rev. 63 (2020), 101150.
[57] F. Miralles, G. Posern, A.I. Zaromytidou, R. Treisman, Actin dynamics control SRF activity by regulation of its coactivator MAL, Cell 113 (2003) 329–342.
[58] G.E. Lindahl, R.C. Chambers, J. Papakrivopoulou, S.J. Dawson, M.C. Jacobsen, J. E. Bishop, G.J. Laurent, Activation of fibroblast procollagen alpha 1(I) transcription by mechanical strain is transforming growth factor-beta-dependent and involves increased binding of CCAAT-binding factor (CBF/NF-Y) at the proximal promoter, J. Biol. Chem. 277 (2002) 6153–6161.
[59] B. Saitta, S. Gaidarova, S.A. Jimenez, CCAAT binding transcription factor binds and regulates human COL1A1 promoter activity in human dermal fibroblasts: demonstration of increased binding in systemic sclerosis fibroblasts, Arthritis Rheum. 43 (2000) 2219–2229.
[60] G.E. Lindahl, R.C. Chambers, J. Papakrivopoulou, S.J. Dawson, M.C. Jacobsen, J. E. Bishop, G.J. Laurent, Activation of fibroblast procollagen alpha 1(I) transcription by mechanical strain is transforming growth factor-beta-dependent and involves increased binding of CCAAT-binding factor (CBF/NF-Y) at the proximal promoter, J. Biol. Chem. 277 (2002) 6153–6161.
[61] K. Takegoshi, M. Honda, H. Okada, R. Takabatake, N. Matsuzawa-Nagata, J. S. Campbell, M. Nishikawa, T. Shimakami, T. Shirasaki, Y. Sakai, T. Yamashita, T. Takamura, T. Tanaka, S. Kaneko, Branched-chain amino acids prevent hepatic fibrosis and development of hepatocellular carcinoma in a non-alcoholic steatohepatitis mouse model, Oncotarget 8 (2017) 18191–18205.
[62] M. Gałdyszyńska, R. Zwoliński, L. Piera, J. Szymański, R. Jaszewski, J. Drobnik, Stiff substrates inhibit collagen accumulation via integrin α2β1, FAK and Src kinases in human atrial fibroblast and myofibroblast cultures derived from patients with aortal stenosis, Biomed. Pharmacother. 159 (2023), 114289.
[63] J. Zwicker, F.C. Lucibello, L.A. Wolfraim, C. Gross, M. Truss, K. Engeland, R. Muller, Cell-cycle regulation of the cyclin-a, CDC25C and CDC2 genes is based on a common mechanism of transcriptional repression, EMBO J. 14 (1995) 4514–4522.
[64] K.S. Katula, K.L. Wright, H. Paul, D.R. Surman, F.J. Nuckolls, J.W. Smith, J.P. Y. Ting, J. Yates, J.P. Cogswell, Cyclin-dependent kinase activation and S-phase induction of the cyclin B1 gene are linked through the CCAAT elements, Cell Growth Differ. 8 (1997) 811–820.
[65] K. Korner, V. Jerome, T. Schmidt, R. Muller, Cell cycle regulation of the murine cdc25B promoter - essential role for nuclear factor-Y and a proximal repressor element, J. Biol. Chem. 276 (2001) 9662–9669.
[66] P. Benatti, D. Dolfini, A. Vigano, M. Ravo, A. Weisz, C. Imbriano, Specific inhibition of NF-Y subunits triggers different cell proliferation defects, Nucleic Acids Res. 39 (2011) 5356–5368.
[67] I. Androic, A. Krämer, R. Yan, F. Rödel, R. Gätje, M. Kaufmann, K. Strebhardt, J. Yuan, Targeting cyclin B1 inhibits proliferation and sensitizes breast cancer cells to taxol, BMC Cancer 8 (2008) 391.
[68] G. Rigillo, V. Basile, S. Belluti, M. Ronzio, E. Sauta, A. Ciarrocchi, L. Latella, M. Saclier, S. Molinari, A. Vallarola, G. Messina, R. Mantovani, D. Dolfini, C. Imbriano, The transcription factor NF-Y participates to stem cell fate decision and regeneration in adult skeletal muscle, Nat. Commun. 12 (2021).
[69] G. Bungartz, H. Land, D.T. Scadden, S.G. Emerson, NF-Y is necessary for hematopoietic stem cell proliferation and survival, Blood 119 (2012) 1380–1389.