Article:
Burke, MJ, Walmsley, R, Munsey, TS et al. (1 more author) (2019) Receptor tyrosine kinase inhibitors cause dysfunction in adult rat cardiac fibroblasts in vitro. Toxicology in Vitro, 58. pp. 178-186. ISSN 0887-2333

https://doi.org/10.1016/j.tiv.2019.03.026

Receptor tyrosine kinase inhibitors cause dysfunction in adult rat cardiac fibroblasts *in vitro*.

Matthew J. Burke[1], Robert Walmsley[1], Tim S. Munsey[1], Andrew J. Smith[1,2*]

[1] School of Biomedical Sciences, Faculty of Biological Sciences, University of Leeds, Woodhouse Lane, Leeds LS2 9JT, United Kingdom

[2] Centre for Human and Applied Physiological Sciences and Centre for Stem Cell and Regenerative Medicine, Guy's Campus, King's College London, London SE1 1UL, United Kingdom

*** = Corresponding author**

A.J.Smith1@leeds.ac.uk

7.52b Garstang Building,

School of Biomedical Sciences,

Faculty of Biological Sciences,

University of Leeds,

Woodhouse Lane,

Leeds, LS2 9JT

United Kingdom

Abstract

The anti-cancer receptor tyrosine kinase inhibitors include known cardiotoxins: a component of this toxicity may be mediated by effects on cardiac fibroblasts (CFs). We hypothesised that imatinib mesylate (imatinib) and sunitinib malate (sunitinib) cause significant dysfunction in adult CFs. Following *in vitro* treatments with imatinib or sunitinib, adult rat CF viability was assessed by fluorescein diacetate assay, proliferation measured by bromodeoxyuridine nuclear incorporation and changes to the expression of CF secretome components determined by real time quantitative RT-PCR. Imatinib and sunitinib significantly reduced cell viability over 48 hours, with EC_{50} values of 11.0 μM (imatinib) and 4.5 μM (sunitinib) respectively. Imatinib reduced CF proliferation from 35.5±3.2% in control to 23.0±5.5% (3 μM; $p<0.001$) and to 9.4±2.5% (10 μM; $p<0.001$), whereas sunitinib reduced proliferation to 22.9±3.1% (1 μM; $p<0.001$) and to 15±1.0% (3 μM; $p<0.001$). Further, 10 μM imatinib increased mRNA expression of TGFB1 7-fold, ($p<0.01$), IL6 6-fold ($p<0.01$), and IL1B 7-fold ($p<0.05$) and reduced PDGFD 15-fold ($p<0.01$); whereas sunitinib specifically reduced IL1B mRNA expression 17-fold ($p<0.01$). Overall, these findings show tyrosine kinase inhibitors cause significant dysfunction in CFs. These data point to an important role for the PDGF pathway in governing CF functions, including survival and proliferation.

Keywords

Fibroblasts, protein kinase inhibitors, cardiotoxins, platelet-derived growth factor receptors (PDGFRs), interleukin (IL)-1β, transforming growth factor (TGF)-β1, cytokines

Highlights

- The receptor tyrosine kinase inhibitors (RTKIs) sunitinib and imatinib cause significant dysfunction in cardiac fibroblasts.
- Sunitinib and imatinib reduce cardiac fibroblast viability.
- Both RTKIs inhibit cardiac fibroblast proliferation.
- Sunitinib markedly reduces IL1B expression in cardiac fibroblasts.
- Imatinib alters the expression of key growth factors and cytokines in cardiac fibroblasts, including TGF-β1, PDGFD, IL6 and IL1β

Introduction

Receptor tyrosine kinase inhibitors (RTKIs) are relatively recently-developed anti-oncogenic drugs that have revolutionized the treatment of several advanced cancers with previously poor prognoses (Gross *et al.*, 2015). Physiologically, receptor tyrosine kinases (RTK) are implicated in cell survival, growth, proliferation, and differentiation (Lemmon and Schlessinger, 2010), with over 60% of identified RTKs shown to be constitutively active in major sarcomas and carcinomas (Sangwan and Park, 2006).

Imatinib mesylate (imatinib) is a highly efficacious RTKI used to treat chronic myeloid leukemia (CML) and gastrointestinal stromal tumors (GIST) (Deininger *et al.*, 2005; Hehlmann *et al.*, 2011). Imatinib's targets are tyrosine kinases with major roles in normal and aberrant cell growth, including the RTK platelet-derived growth factor receptors (PDGFRs) and the cytosolic Abelson protein (c-Abl) (Paul and Mukhopadhyay, 2004; Hayashi *et al.*, 2015). Imatinib inhibition of c-Abl and its mutated isoform (Bcr-Abl) was shown to deplete cancerous cell numbers to undetectable levels in 80% of newly-diagnosed CML cases (Deininger *et al.*, 2005). Sunitinib malate (sunitinib) is an RTKI that inhibits PDGFRs with greater potency than imatinib (Karaman *et al.*, 2008), demonstrating efficacy in treating imatinib-resistant GIST (Telli *et al.*, 2008) and metastatic renal cell carcinoma (RCC) (Motzer *et al.*, 2007; Lankheet *et al.*, 2014). Further, sunitinib potently inhibits angiogenesis via blockade of vascular endothelial growth factor (VEGF) signaling (Roskoski, 2007).

In the clinic, RTKIs are associated with varying levels of cardiotoxicity. Sunitinib treatment leads to significant increases in cardiac events; including hypertension, LV dilation and congestive heart failure (Chu *et al.*, 2007). Recently, a multi-centre trial of sunitinib safety in RCC treatment identified left ventricular dysfunction in 10% of patients (Narayan *et al.*, 2017). Sunitinib causes significant toxicity to cardiomyocytes *in vitro* (Kerkelä *et al.*, 2009) and its use consistently leads to compromised cardiac function *in vivo* (Greineder *et al.*, 2011). Although imatinib-induced declines in cardiac function were reported during the treatment of ten individuals (Kerkelä *et al.*, 2006), a subsequent study of over 1200 patients suggested cardiomyopathy with imatinib treatment may be rare (Atallah *et al.*, 2007). However, imatinib has been shown to have direct cytotoxic effects on cardiomyocytes (Kerkelä *et al.*, 2006) and to exacerbate pathological cardiomyocyte hypertrophy (Barr *et al.*, 2014). Further characterisation of mechanisms of RTKI-induced cardiotoxicity would be of clinical value: considering the key roles of tyrosine kinases in the heart, RTKIs may affect several cardiac cell types, including cardiac fibroblasts (CFs).

Cardiac fibroblasts are mesenchymal cells that are one of the major non-myocyte cellular components of the heart (Pinto *et al.*, 2016) and have extensive interactions with other

cardiac cell populations (Porter and Turner, 2009). These interactions occur largely through extracellular matrix (ECM) regulation or paracrine signaling by growth factors and cytokines (Souders *et al.,* 2009). Myocardial ECM is a connective tissue framework comprised mainly of fibrillar collagens: as the primary regulators of the ECM (Corda *et al.,* 2000), CFs are the major source of collagen types I and III, contributing around 90% of myocardial collagen. A key means to intrinsically police this connective tissue network is control of CF proliferation, collagen synthesis and cell differentiation (Perona, 2006). The CFs secrete and are influenced by a range of growth factors and cytokines, ensuring co-ordinated release of fibrillary collagens and ECM homeostasis.

The platelet-derived growth factor (PDGF) family of ligands induce dimerization of the tyrosine kinase PDGFRs and stimulate extensive downstream signaling, including the activation of phosphatidylinositol-3 kinase (PI3K) and protein kinase B (AKT) (Demoulin and Essaghir, 2014). These pathways are critical to CF survival, proliferation and ECM synthesis (Gao *et al.,* 2005; Donovan *et al.,* 2013). However, increased levels of PDGF ligand in the human myocardium are associated with declines in cardiac function (Zhao *et al.,* 2011; Gallini *et al.,* 2016). In fact, significant increases in the levels of PDGF-D ligand have been shown to potentiate ECM dysfunction (Zhao *et al.,* 2013).

Although quiescent in the healthy heart, CFs can be activated by injury and differentiate into myofibroblasts (myoFbs), cells with accelerated secretions of growth factors, inflammatory cytokines and ECM components (Turner and Porter, 2013). The key stimulus behind this phenotypic switch is transforming growth factor (TGF)-ß1, a multifunctional cytokine shown to potentiate cardiac fibrosis, a co-morbidity of virtually all cardiac pathologies. *In vitro*, TGF-ß1 ligand application results in a marked increase in CF collagen expression, while transfection of the TGFB1 gene is sufficient to induce rapid myocardial fibrosis *in vivo* (Eghbali *et al.,* 1991; Roberts, 1999, Verrecchia and Mauviel, 2002). The binding of TGF-ß1 ligand to TGFß cell surface receptors promotes SMAD 2/3 association with SMAD 4 and its translocation to the nucleus (Massague *et al.,* 2005; Saito *et al.,* 2013). Several SMAD independent pathways are also stimulated downstream of TGFßR, with the upstream catalytic activity of PI3K common to each (Zhang, 2009). In CFs, two of these distinct pathways are mediated by AKT and the non-receptor tyrosine kinase c-Abl, with evidence suggesting these modulate separate facets of the TGFß phenotype (Wilkes and Leof, 2006; Hong *et al.,* 2011). The downstream transcription factors involved in transducing these signals into cellular effects include nuclear factor kappa B (NF-κB) (Hogan *et al.,* 2013). Overall, TGFß signaling drives maladaptive cardiac remodeling: promoting CF proliferation, whilst upregulating numerous genes associated with the myoFb phenotype.

This fibrogenic process is also intrinsically linked to the expression of pro-inflammatory mediators (Kania *et al.*, 2009), including interleukin (IL)-6 and IL-1β (Jonsson *et al.*, 2016). The highly pleiotropic IL-1β plays an important role in myocardial repair, directly enhancing ECM turnover through increased degradation (Turner, 2014). Further, the absence of IL-1β following infarction attenuates active fibrotic remodeling, largely due to a reduction in the inflammatory response (Bujak and Frangogianis, 2009). However, in a non-reperfusion model of infarction, in which inflammation is suppressed, the absence of IL-1β is detrimental to myocardial repair and compromises cardiac function (Hwang *et al.*, 2001). Moreover, its blockade was not associated with improved functional recovery in congestive heart failure patients or following acute myocardial infarction (Mann, 2005; Abbate *et al.*, 2013). In fact, a recent study identified distinct protective effects of IL-1β in late stage atherosclerosis; its blockade reduced the smooth muscle cell-mediated deposition of collagen and resulted in increased plaque rupture (Gomez *et al.*, 2018). Paracrine signaling mediated by inflammatory cytokines also mediates extensive cross-talk between CFs and cardiomyocytes, particularly in the disease setting; typically promoting CF activation whilst also directly inducing cardiomyocyte hypertrophy (Fujiu and Nagai, 2014). Consequently, changes to the CF phenotype has widespread, and often context dependent, impacts on the dynamic environment of the myocardium in health and disease.

Tyrosine kinases are critical to myocardial function, constituting a significant proportion of the receptors and intracellular signaling proteins that mediate basal and pathological CF activity. This study investigated whether imatinib and sunitinib cause dysfunction in adult CFs, examining impacts on CF viability, proliferation and changes to CF secretome components. Characterising the effects of these RTKIs on CFs identifies the impacts of known cardiotoxins on a principal cardiac cell population and further improves our understanding of the roles of tyrosine kinases in governing CF functions.

Methods

Cell culture

Adult rat CFs were donated from Professor Derek Steele's group by Dr. Hannah Kirton (University of Leeds). Briefly, hearts were dissected from adult male Wistar rats and subjected to collagenase digestion on a Langendorff apparatus under constant perfusion and collected using filter-sterilised solution (Duncan *et al.*, 2010). The collected sample was centrifuged (20 *g* for 3 minutes) to separate small cells from cardiomyocytes and larger debris. The small cell fraction was isolated and re-suspended in 10 ml of medium, then applied to uncoated 10 cm Petri dishes (Corning) and incubated at 37°C for 45 mins to allow CF adherence. Residual cell suspension was then aspirated and unattached cells removed by

washes with PBS with antibiotics (1% penicillin-steptomycin; 0.1% gentamicin; 0.1% Fungizone). Fresh growth medium was then added (Dulbecco's Modified Eagles Medium (DMEM, cat.no: 21969) supplemented by: foetal bovine serum (10%), glutamax (1%) penicillin/streptomycin (1%) and Fungizone (0.1%); all ThermoFisher Scientific). Cells were cultured in a humidified incubator (Binder) at 37°C, 5% CO_2 and passaged at confluency, up to a maximum of 5 passages. All solutions were sterilised through 0.45 µm pore filters (VWR) and all work carried out in a sterile Class II flow cabinet.

Cell characterisation

$5x10^3$ CFs/well in growth medium were cultured on chamber slides. After 5 days the medium was removed and CFs were fixed in 4% paraformaldehyde (PFA) at room temperature for 10 minutes. Following PBS washes, the chamber apparatus was detached from the slide. The fixed cells were blocked with 10% donkey serum in PBS with 0.1% tween (PBS-T) at room temperature for 30 minutes. The primary antibody (vimentin, Santa Cruz Biotechnology) was applied at a dilution of 1:50 in PBS-T and incubated at 4°C overnight. Then cells were incubated with the secondary antibody (donkey anti-goat IgG Alexa 488, ThermoFisher Scientific) at 1:100 in PBS-T, 37°C for 1 hour before counter-staining with a DAPI nuclear stain. For dual-marker staining, an additional primary antibody (alpha smooth muscle actin, Sigma-Aldrich) was applied at a dilution of 1:50 in PBS-T and incubated at 37°C for 1 hour, then cells were incubated with the secondary antibody (donkey anti-mouse IgG Alexa 548, ThermoFisher Scientific) at 1:100 in PBS-T, 37°C for 1 hour (these steps were added between the first round of secondary antibody staining and DAPI staining). Immunofluorescent images were obtained using the EVOS FL Auto 2 imaging system (ThermoFisher Scientific). Cultured cell morphological images were captured with a light microscope (Nikon Eclipse TS-100).

Cell viability

$1x10^3$ CFs/well were seeded into 96-well plates and treated with imatinib mesylate (Synkinase), sunitinib malate (Cayman Chemicals) in growth medium (controls were 'treated' by replacement of fresh growth medium) for 24 or 48 hours. At treatment end-points, medium was aspirated and replaced with fluorescein diacetate solution (FDA, Sigma Aldrich) at a final concentration of 5µg/ml (in 1% ethanol, 9% PBS, 90% DMEM) and the plate incubated at 37°C for 10 minutes. Fluorescence was analysed using a VarioskanFlash plate-reader (v.4.00.53) at wavelengths of 485 nm (excitation) and 520 nm (emission). A background fluorescence value was obtained using FDA solution alone and cell viability for treatment groups calculated as a percentage of the no-treatment group.

Cell proliferation

$1x10^5$ CFs/well in 6-well plates were treated with imatinib, sunitinib or growth medium only for 48 hours, with BrdU (final concentration of 1µg/ml) added at 8 hour intervals. Cell proliferation was assessed using the BrdU labelling and detection kit (Roche Applied Sciences). After 48 hours, medium was removed, cells were washed in PBS and harvested using trypsin. Cells were fixed onto microscope slides using double cell spot funnels (300 rpm for 6 minutes) then fixed and wax-coated using Cell Fixx (ThermoFisher) pending immunostaining. Immediately prior to immunostaining, wax was removed with 95% ethanol at room temperature for 15 minutes, cells permeabilised with Triton-X100 at room temperature for 10 minutes and blocked with 10% donkey serum PBS-T at room temperature for 30 minutes. Cells were immunolabelled with an anti-BrdU antibody (diluted 1:50 in manufacturer's buffer) at 37°C for 45 minutes, then with donkey anti-mouse IgG Alexa 488 secondary antibody (1:100 in PBS-T) at 37°C for 1 hour, (ThermoFisher). Cells were counter-stained with DAPI and stored at 4°C pending fluorescent microscopy. Images were obtained using the EVOS FL Auto 2 Imaging system (ThermoFisher). Five random fields were examined per cell preparation, then images analysed using ImageJ (NIH) and the percentage of $BrdU^{+ve}$ nuclei calculated.

RNA isolation

10 cm dishes were seeded with $4x10^5$ CFs in growth medium and treated with growth medium only (control), imatinib or sunitinib for 72 hours. Post-treatment, cells were harvested, centrifuged (300 *g* for 5 minutes) and re-suspended in ice-cold PBS. This suspension was pelleted at 14000 *g*, 4°C for 5 minutes, then PBS aspirated and the pellet stored at -80°C pending RNA isolation. Cells were lysed and homogenised in lysis buffer containing 1% β-mercaptoethanol (Qiagen), RNA isolated using the RNEasy mini-kit (Qiagen), with RNA concentration and purity assessed by a Nanodrop spectrophotometer (ThermoFisher), measuring absorbance ratios 260:280 nm and 260:230 nm. Isolated RNA was stored at -80°C.

cDNA synthesis

cDNA was synthesised from isolated total RNA using the iScript™ cDNA synthesis kit (Bio-Rad), with 1µg of RNA per 50 µl of reaction mix. The following protocol was used: 25°C (5 mins), 46°C (20 mins), 95°C (1 min) on a thermal cycler (Bio-Rad CFX96 Touch™). The generated cDNA was used immediately for real time quantitative PCR (qPCR).

Real time quantitative PCR

Real-time quantitative PCR (qPCR) was performed on a thermal cycler (Bio-Rad CFX96 Touch™) using SYBR green (Bio-Rad). The PCR reaction mix contained 300 nM of forward and reverse primers and 2 µl synthesised cDNA template per well. Data were extracted

using CFX manager™ (Bio-Rad, V3.1). For analysis, the deltaCT method was used and values were normalized to the reference gene Actb. Products from PCR reactions were resolved on a 2% agarose gel infused with SYBR™ Gold nucleic acid gel stain (1:10000; ThermoFisher). All primers were designed using primer BLAST and synthesised by Sigma Aldrich (**Table 1**).

Gene targets	Forward sequence (5'- 3')	Reverse sequence (5' - 3')	Predicted product size (bp)	mRNA accession number
Actb	TGGAGCAAACATCCCCCAAA	TGCCGTGGATACTTGGAGTG	104	NM_031144.3
COL3A1	TGGAGGTGAAAAGTCTGGCG	AGCATCCATCTTGCAGCCTT	244	NM_032085.1
FGF2	GCCTGGACAGAAGGCCATAC	CGGACACAGGGAAGGGTTTG	173	NM_019305.2
IGF1	CAGTTCGTGTGTGGACCAAG	TCAGCGGAGCACAGTACATC	151	NM_001082477.2
IL1B	GGGCGGTTCAAGGCATAACA	GTCGAGATGCTGCTGTGAGA	247	NM_031512.2
IL6	CCCACCAGGAACGAAAGTCA	ACTGGCTGGAAGTCTCTTGC	81	NM_012589.2
PDGFD	CAGAGCGCATCCATCAAAGC	TCTTCCTCTGACAACGGTGC	312	NM_023962.2
TGFB1	CACTCCCGTGGCTTCTAGTG	GGACTGGCGAGCCTTAGTTT	145	NM_021578.2
VIM	GATGTGGACGTTTCCAAGCC	CTGTCTCCGGTATTCGTTGAC	196	NM_031140.1

Table 1. Primer sequences. Forward and reverse primer sequences used for real time qPCR.

Statistical analysis

One-way ANOVA was used for comparison of mean data between groups for analyses of cell viability and proliferation, whereas two-tailed, unpaired Student's *t*-test on deltaCT values was employed to compare target gene expression between each treatment group and their control. Images were processed using ImageJ (V1.47t) and graphs were made using OriginPro (9.1). EC_{50} values were calculated using non-linear regression. All statistical testing was carried out using the IBM® SPSS® Statistics software (V21.0).

Results

Isolation and identification of CFs

Isolated cells exhibited a clearly spindle-shaped morphology, characteristic of CFs (Souders *et al.*, 2009) **(Fig. 1A).** To confirm cell identity, the presence of the CF marker vimentin was confirmed by real-time qRT-PCR, showing robust expression across multiple passages, expressed relative to beta-actin mRNA levels **(Fig. 1B)**. In addition, extensive vimentin protein expression and absence of alpha smooth muscle actin protein expression were identified by immunocytochemistry staining of both freshly-isolated CFs and from CFs

following maintenance over 7 days and passaging **(Fig. 1C)**. This identified characteristic fibroblast phenotypic features and their maintenance in culture.

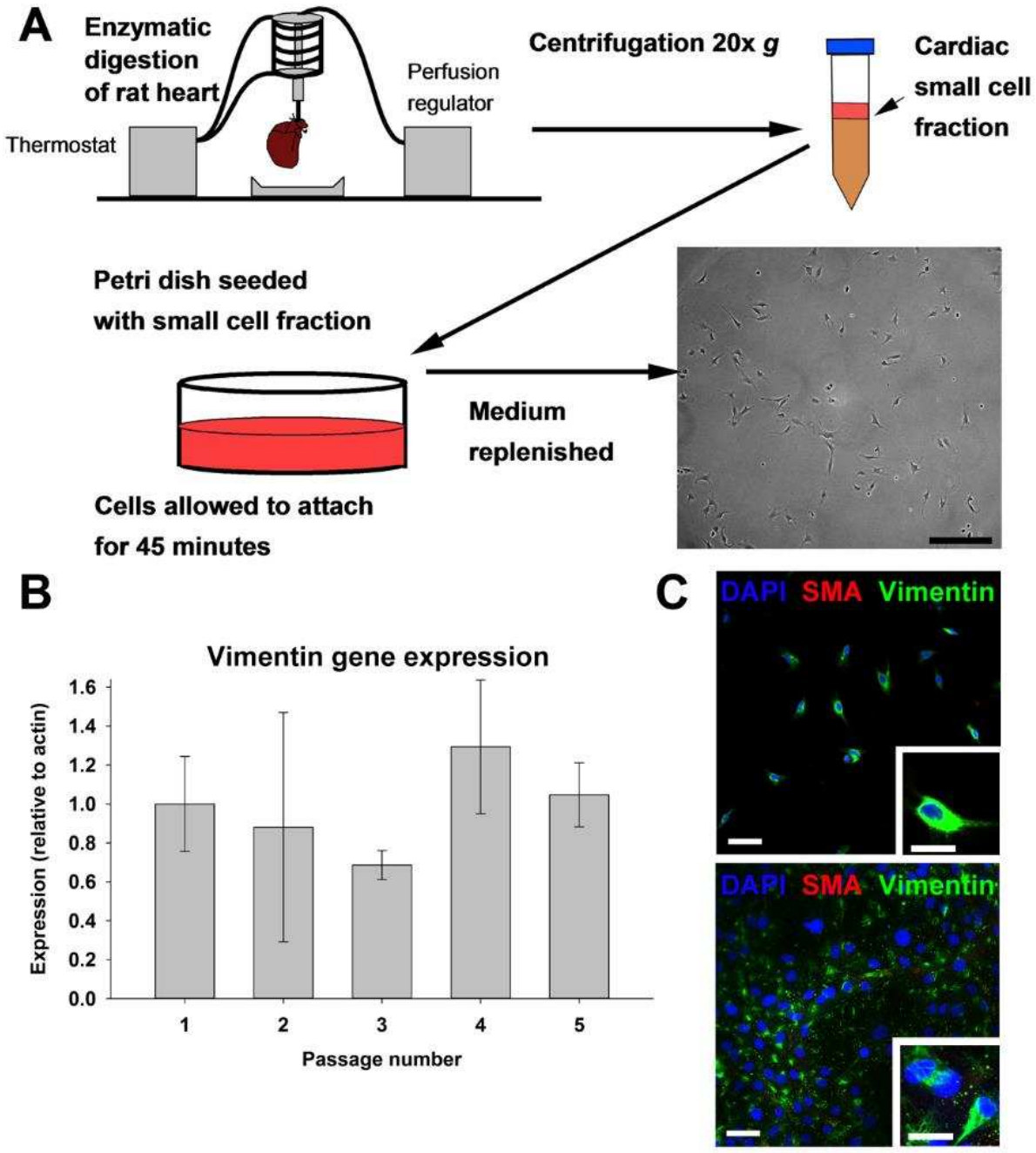

Figure 1. Isolation and characterisation of adult rat cardiac fibroblasts. (A) Schematic detailing the isolation of CFs from adult male Wistar rat hearts (bar=200 μm). **(B)** Confirmation of active vimentin mRNA expression in CFs by real-time qPCR relative to β-actin, consistently expressed over multiple passages (P1 to P5); data are mean±SEM (n=3). **(C)** Immunofluorescent staining confirms vimentin protein expression (green) and absence of alpha smooth muscle actin protein expression (red) in CFs, with these patterns of expression seen consistently in CFs: both freshly-isolated and maintained in culture for several days with passaging (main image bars=50 μm; inset bars=20 μm).

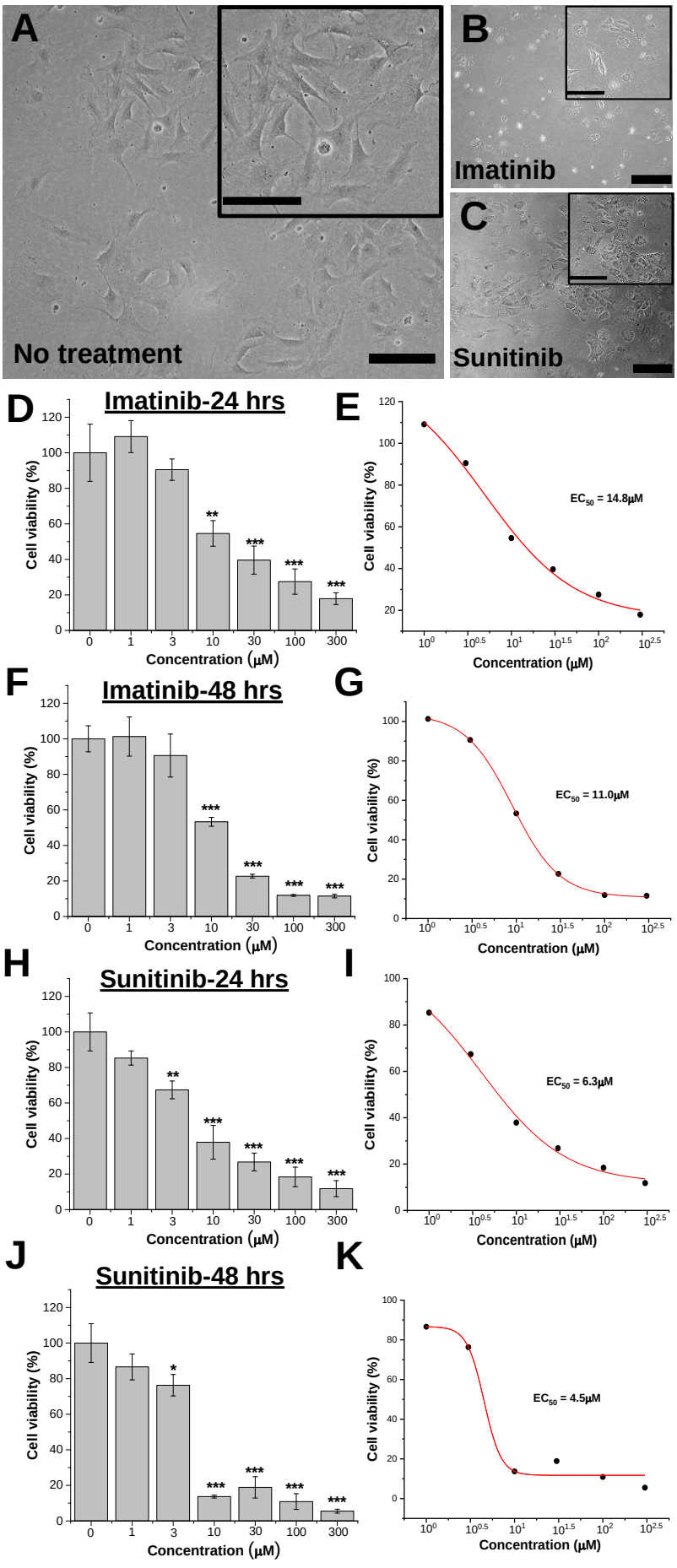
A
No treatment
B
Imatinib
C
Sunitinib
D
Imatinib-24 hrs
Cell viability (%)
Concentration (μM)
E
EC50 = 14.8μM
Cell viability (%)
Concentration (μM)
F
Imatinib-48 hrs
Cell viability (%)
Concentration (μM)
G
EC50 = 11.0μM
Cell viability (%)
Concentration (μM)
H
Sunitinib-24 hrs
Cell viability (%)
Concentration (μM)
I
EC50 = 6.3μM
Cell viability (%)
Concentration (μM)
J
Sunitinib-48 hrs
Cell viability (%)
Concentration (μM)
K
EC50 = 4.5μM
Cell viability (%)
Concentration (μM)

Figure 2. Imatinib and sunitinib significantly impact on CF viability. Representative images of CFs after 48 hour treatments with growth medium only **(A),** 10 μM imatinib **(B)** or 3 μM sunitinib **(C)** (full image bar=200 μm; inset bar=100 μm). Cell viability was assessed by FDA assay **(D-K)**. Cells were treated with imatinib for 24 **(D)** or 48 hours **(E)**, or with sunitinib for 24 **(H)** or 48 hours **(J)**. Treatment group values were calculated as a percentage of control (0 μM; growth medium only). Data presented are mean±SEM (n=5-6). Significant differences tested by one-way ANOVA and post-hoc Tukey's test (*p<0.05, ** p<0.01, ***p<0.001 *vs.* control). Mean cell viabilities for each treatment were plotted *vs.* log(concentration) and EC_{50} values were calculated for imatinib 24 **(E)** and 48 hours **(G),** as well as sunitinib 24 **(I)** and 48 hours **(K)**.

RTKIs significantly impact on CF viability

We first determined the effects of imatinib and sunitinib on CF viability over a range of concentrations. Morphological examination of control CFs **(Fig. 2A)** and CFs following treatment for 48 hours with 10 μM imatinib **(Fig. 2B)** or 3 μM sunitinib **(Fig. 2C)**, showed both drugs reduced cell numbers and affected CF morphology. Over 48 hours, cultured CFs formed dense clusters and an extensive number of membrane protrusions, whereas imatinib-treated CFs retained a smaller spindle-like morphology with fewer membrane protrusions, while sunitinib-treated CFs had fewer membrane protrusions and did not preserve a spindle shape.

At 1μM, neither drug had a significant impact on viability at any time point compared to control **(Fig. 2D-K)**. Imatinib and sunitinib reduced CF viability in a concentration-dependent manner, over 24 (imatinib EC_{50}=14.8 μM **Fig.2D:E**, sunitinib EC_{50}=6.3 μM **Fig.2H:I**) and 48 hours (imatinib EC_{50}=11.0 μM **Fig.2F:G**, sunitinib EC_{50} = 4.5 μM **Fig.2I:K).** These findings are consistent with apparent cell density during morphological analyses. Across the examined concentration ranges, reductions in cell viability were linear and concentration-dependent over 24 and 48 hours, with the greatest drop in viability between 3 μM and 10 μM for both drugs, at both time points.

Imatinib and sunitinib concentration-dependently reduce CF proliferation

Increased CF proliferation is implicated in pathological myocardial remodeling and is typically associated with cardiac functional decline; therefore RTKI effects on CF proliferation were examined using nuclear BrdU incorporation assays. Based on the cell viability results, two different concentrations were used for each drug, one representing a lower concentration with which effects on viability were not observed and a second higher concentration immediately below the estimated EC_{50}. After 48 hours, 35.5±3.2% of control cells were $BrdU^{+ve}$ **(Fig. 3A, 3E)**, identifying them as newly-formed. Imatinib treatments significantly reduced CF proliferation compared to control, with $BrdU^{+ve}$ nuclei accounting

for 23±5.5% of total nuclei at 3 μM (p<0.001, **Fig. 3B**) and 9.4±2.5% at 10 μM **(Fig. 3C)** (p<0.001). Sunitinib treatments also significantly reduced CF proliferation, reducing $BrdU^{+ve}$ nuclei to 22.9±3.1% at 1 μM **(Fig. 3F)** (p<0.001) and to 15±1.0% at 3 μM **(Fig. 3G)** (p<0.001).

Sunitinib and imatinib impact on CF growth factor and cytokine expression

A major CF function is to synthesise and release a broad range of growth factors and cytokines into the myocardium. We therefore investigated the effects of sunitinib (3 μM) and imatinib (10 μM) on CF secretome components, using qRT-PCR to examine expression of growth factor and cytokine genes after 72 hour RTKI treatments. Both compounds caused consistent but non-significant reductions in COL3A1 mRNA expression compared to control **(Fig. 4B)**. Interestingly, imatinib treatment resulted in the significant upregulation of TGFB1 (7.1±1.6-fold, p<0.01), IL6 (5.6±0.9-fold, p<0.01), VIM (5.4±1-fold, p<0.001) and IL1B (7.1±2.3-fold, p<0.05, **Fig. 4A**) mRNA, while significantly reducing PDGF-D mRNA expression (15.3±0.2-fold, p<0.01, **Fig. 4A**). Notably, sunitinib had an opposing effect on IL1B mRNA expression, decreasing expression 17.4±1.6-fold (p<0.01, **Fig. 4A**), but did not significantly impact the other genes examined. When resolved on an agarose gel (2%), PCR products exhibited single bands that corresponded to predicted PCR product size, (excepting FGF2: this target was therefore omitted from PCR analysis).

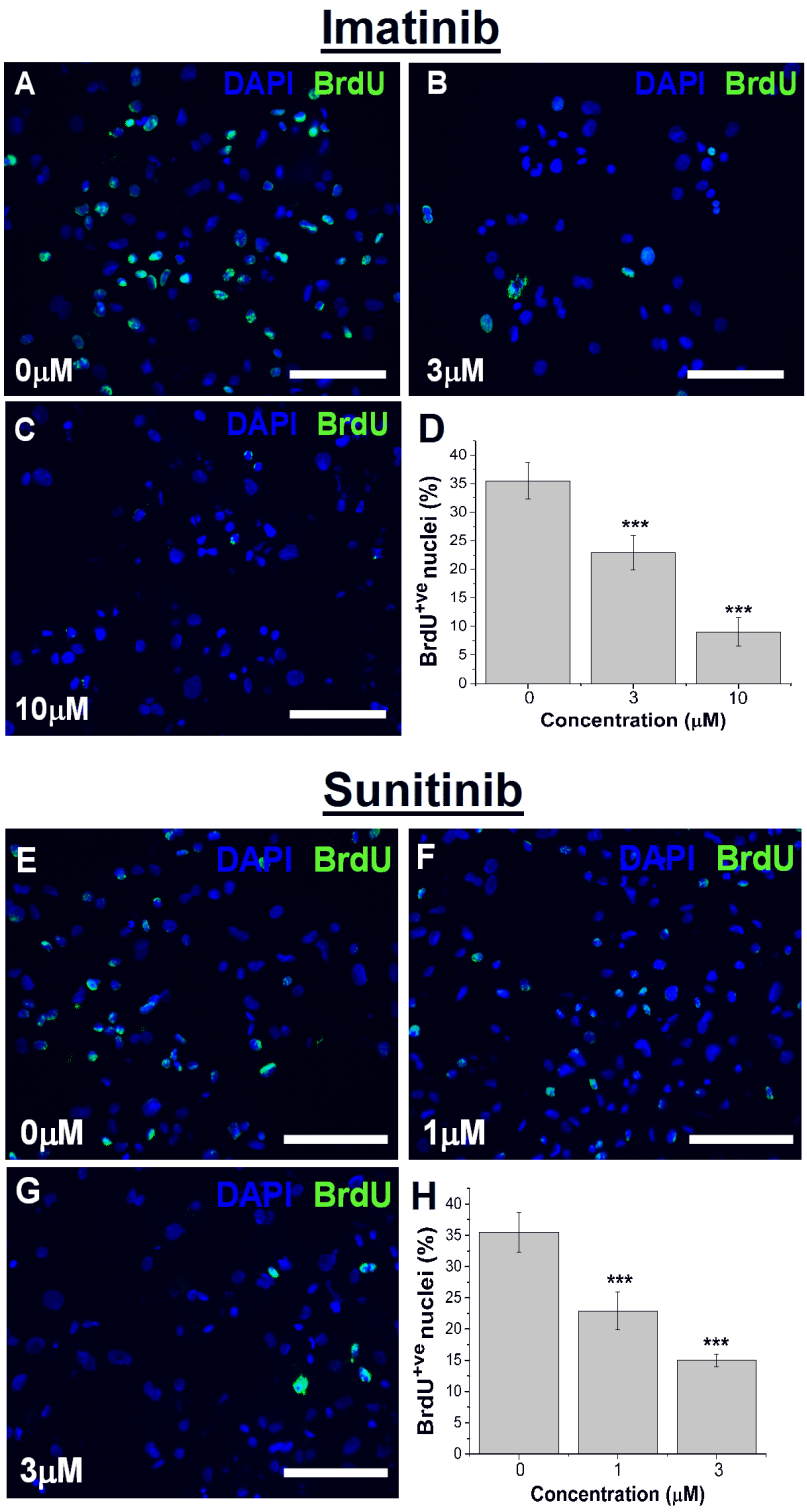

Figure 3. Imatinib and sunitinib concentration-dependently reduce CF proliferation. Representative images of cells treated with imatinib **(A, B, C)** or sunitinib **(E, F, G)**. Data presented for imatinib **(D)** and sunitinib **(H)** are mean $BrdU^{+ve}$ cells as a percentage of total cells±SEM (n=3). Significance of difference between groups tested by one-way ANOVA and post-hoc Tukey's test (***p<0.001 *vs.* control). Scale bar=100 μm.

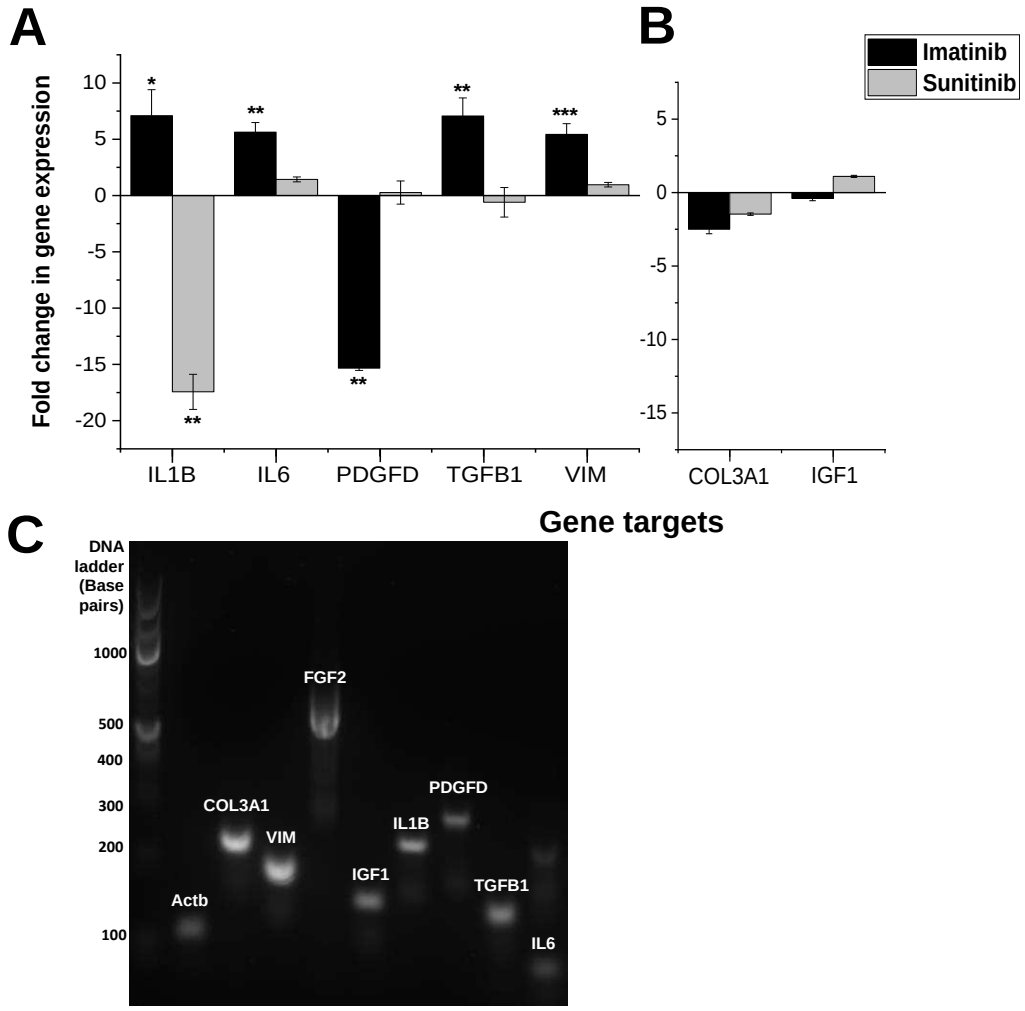

Figure 4. Imatinib and sunitinib showed distinct impacts impact on CF growth factor and cytokine expression secretome. CFs were treated with imatinib (10 μM, black bars) or sunitinib (3 μM, grey bars) for 72 hours, prior to qRT-PCR analysis of mRNA transcript levels for genes relevant to CF paracrine and autocrine signaling. Data presented are mean fold changes in gene expression±SEM relative to control (growth medium only) (n=3). Gene targets are separated and presented as >5 fold change **(A)** and <5 fold change **(B)**. Significant difference from control assessed by a two-tailed Student's *t*-test on deltaCT values (**p*<0.05, ***p*<0.01, ****p*<0.001 *vs*. control). **(C)** Single PCR products were identified by agarose gel electrophoresis.

Discussion

Cardiac fibroblasts regulate myocardial structure and allow it to respond to changing environmental and physiological demands. Furthermore, CFs act as sentinel cells, contributing to a synergistic whole-heart response to stimuli through autocrine/paracrine growth factor and cytokine signaling (Souders *et al.*, 2009). The RTKIs include known cardiotoxins and inhibit a broad range of targets, including those tyrosine kinases with

critical roles in major CF functions. However, the effects of these two prominent RTKIs on healthy CFs have not been comprehensively investigated previously. This study has demonstrated that imatinib and particularly sunitinib cause significant dysfunction to adult CFs *in vitro*; reducing cell viability, proliferation and altering expression of genes associated with the physiological role of the CF.

Both RTKIs caused dose-dependent reductions in CF viability, an effect that can partially be accounted for by anti-proliferative effects at lower drug concentrations. However, at doses above 10 μM imatinib or 3 μM sunitinib there was a marked reduction in cell viability, clearly demonstrating cytotoxicity. The PDGF pathway is critical to fibroblast proliferation and survival (Lindahl and Betsholtz, 1998), and disruption of PDGF signaling has been shown to increase fibroblast sensitivity to toxic stimuli, significantly reducing cell survival (Gao *et al.*, 2005). Sunitinib has greater potency than imatinib for the inhibition of PDGFRs in activity-based assays (Kitagawa *et al.*, 2013), explaining why reductions in CF viability with lower sunitinib concentrations were apparent here. Previously, inhibition of PDGFR activity with the sunitinib analogue SU9518 significantly impaired human lung fibroblast survival while potently inhibiting proliferation (Li *et al.*, 2006). Considering the vital role of the PDGF pathway in CF survival, it can be postulated that the RTKI-induced viability reductions observed here are linked to the extent of PDGFR inhibition.

There were striking differences seen in the effects of the two drugs on CF growth factor and cytokine expression. Sunitinib, but not imatinib, markedly reduced IL1B expression. Although the broad range of sunitinib targets means there are numerous possible mechanisms underlying this effect, sunitinib has been shown to potently inhibit the activation and subsequent signaling of NF-κB (Miller *et al.*, 2010), a transcription factor pivotal for inducing multiple pro-inflammatory genes in CFs, including IL1B and tumour necrosis factor alpha (TNF-α) (Cogswell *et al.*, 1994; Turner *et al.*, 2007; Lawrence, 2009; Rivera-Serrano and Sherry, 2017). Upregulation of this pro-inflammatory axis is typically associated with maladaptive ventricular remodeling following myocardial injury, but evidence suggests low physiological levels of these mediators play a significant role in beneficial tissue repair (Mann, 2005; Frangogiannis, 2015). A comprehensive blockade of IL1B signaling with a recombinant IL1R antagonist following acute myocardial infarction was not associated with improved recovery of cardiac function (Abbate *et al.*, 2013). Similarly, antibody-mediated inhibition of IL1B signaling was shown to reduce collagen accumulation and compromise the preservation of ventricular architecture following coronary artery occlusion in mice, leading to exacerbated ventricular dilation (Hwang *et al.*, 2001). Indeed, interleukin signaling is highly pleotropic and significantly impacts many

aspects of CF physiology; it stimulates the release of pro-angiogenic factors, including substantial levels of nitrous oxide, as well as promoting ECM turnover and cell migration (Turner, 2014). In the clinic, sunitinib is associated with significant adverse cardiac events; including hypertension, LV dilation and LV dysfunction (Chu *et al.*, 2007). Together with the significant impairment of CF proliferation, the sunitinib-mediated inhibition of IL1B gene expression identified here represents significant perturbation to basal CF physiology. Similar effects *in vivo* may reduce inherent CF ability to maintain ventricular architecture in response to increased biomechanical stresses or direct cardiac insult. Therefore CF dysfunction, along with VEGF receptor inhibition and direct cytotoxic action on cardiomyocytes (Kerkelä *et al.*, 2009), could combine to induce the adverse cardiac events associated with sunitinib treatment.

Growing clinical evidence indicates the relative cardiac safety of imatinib (Atallah *et al.*, 2007), with its direct cytotoxic action on cardiomyocytes occurring at high concentrations (Kerkelä *et al.* 2006). In the current study, this RTKI had a marked effect on the secretome targets examined, including those highly relevant to physiological CF behaviours. Imatinib was associated with a significant increase in TGFB1 expression, suggesting the direct regulation of this cytokine at the transcriptional level by an imatinib-specific target in CFs. Previously, a similar increase in TGFB1 expression was induced in human renal epithelial cells by imatinib treatment (Torsello *et al.*, 2016). In that study, the increase in TGFB1 was recapitulated by Abl2 inhibition, a known imatinib target, and was associated with increased VIM expression; the latter finding consistent with our data in CFs.

Increased TGFB1 expression following imatinib treatment here was not associated with increased proliferation or COL3A1 expression, indicating blockade of this cytokine's fibrogenic effects by imatinib. This supports previous work demonstrating a TGF-ß1-mediated upregulation of collagen III protein requires c-Abl (Wilkes and Leof, 2006). The importance of c-Abl in the development of progressive CF-driven fibrosis has been extensively demonstrated (Wang *et al.*, 2017), with c-Abl also acting as an effector downstream of PDGFR stimulation during CF activation (Jang *et al.*, 2014). Thus, imatinib blockade of PDGFRs and c-Abl was the likely rationale behind exploration of the drug as an anti-fibrotic agent, although trials proved unsuccessful (Daniels *et al.*, 2009). The upregulation of inflammatory cytokines in the presence of imatinib is consistent with previous work demonstrating imatinib failed to reduce their expression in hepatic fibrosis or isolated hepatic stellate cells (Kim *et al.*, 2012; Shiha *et al.*, 2014). Although a primary target of imatinib is c-Abl, this protein is distinct from the SMAD-dependent pathway thought to be the primary driving force behind TGFB-induced inflammatory gene expression in fibroblasts (Leask, 2010). Thus, increases in TGFB1 can theoretically

stimulate inflammatory gene expression in CFs while in the presence of imatinib; a potential compounding factor in its ineffectiveness at attenuating advanced fibrosis. Imatinib treatment of CFs and the associated increase in TGFB1 expression resulted in downregulated expression of PDGFD. A consistent inhibitory link between TGFB1 and PDGFD gene expression has been established in human lung fibroblasts (Chaabane *et al.*, 2014). This TGFB1-dependent downregulation of PDGFD did not require SMAD-independent pathways, as it was attenuated by SMAD4 blockade (Chaabane *et al.*, 2014).

This study demonstrates that tyrosine kinase inhibition significantly perturbs CF functions, reducing both viability and proliferation, while also causing diverse alterations in the expression of CF secretome components. We have identified here novel adverse impacts induced by RTKIs on a major cardiac cell type: although the effects are seen more clearly at higher doses, it would be of interest in future work to identify the cumulative effects of lower doses over extended periods of time, more closely mimicking the clinical exposure pattern. While this study does not imply CF impacts are the primary mediator of RTKI-induced cardiotoxicity, it does identify effects on CF phenotype of potential pathological importance, particularly sunitinib-induced decreases in IL1B expression. The disparate effects of these drugs on a principal cardiac cell type reveal potential new insights into the increased prevalence of cardiotoxicity occurring in the clinical use of sunitinib compared with imatinib.

Conflicts of interest

The authors declare no conflicts of interest.

Funding sources

This study was supported by funding from the University of Leeds.

Acknowledgements

We wish to thank Dr. Neil Turner (University of Leeds) for the provision of antibodies for immunocytochemistry and for his advice on the preparation of this manuscript, and Dr. Brian Jackson of the Protein Production Facility (University of Leeds) for the use of imaging facilities.

References

1) Abbate, A., Van Tassell, B.W., Biondi-Zoccai, G., Kontos, M.C., Grizzard, J.D., Spillman, D.W., Oddi, C., Roberts, C.S., Melchior, R.D., Mueller, G.H. and Abouzaki, N.A., 2013. Effects of interleukin-1 blockade with anakinra on adverse cardiac remodeling and heart failure after acute myocardial infarction [from the Virginia Commonwealth University-

Anakinra Remodeling Trial (2)(VCU-ART2) pilot study]. *The American journal of Cardiology*, *111*(10), pp.1394-1400.

2) Atallah, E., Durand, J.B., Kantarjian, H. and Cortes, J., 2007. Congestive heart failure is a rare event in patients receiving imatinib therapy. *Blood*, *110*(4), pp.1233-1237.

3) Barr, L.A., Makarewich, C.A., Berretta, R.M., Gao, H., Troupes, C.D., Woitek, F., Recchia, F., Kubo, H., Force, T. and Houser, S.R., 2014. Imatinib activates pathological hypertrophy by altering myocyte calcium regulation. *Clinical and Translational Science*, *7*(5), pp.360-367.

4) Bujak, M. and Frangogiannis, N.G., 2009. The role of IL-1 in the pathogenesis of heart disease. *Archivum Immunologiae et Therapiae Experimentalis*, *57*(3), pp.165-176.

5) Chaabane, S.C., de Brachène, A.C., Essaghir, A., Velghe, A., Re, S.L., Stockis, J., Lucas, S., Khachigian, L.M., Huaux, F. and Demoulin, J.B., 2014. PDGF-D expression is down-regulated by TGFß in fibroblasts. *PloS One*, *9*(10), p.e108656.

6) Cogswell, J.P., Godlevski, M.M., Wisely, G.B., Clay, W.C., Leesnitzer, L.M., Ways, J.P. and Gray, J.G., 1994. NF-kappa B regulates IL-1 beta transcription through a consensus NF-kappa B binding site and a nonconsensus CRE-like site. *The Journal of Immunology*, *153*(2), pp.712-723.

7) Corda, S., Samuel, J.L. and Rappaport, L., 2000. Extracellular matrix and growth factors during heart growth. *Heart Failure Reviews*, *5*(2), pp.119-130.

8) Daniels, CE., Lasky, JA., Limper, AH., Mieras, K., Gabor, E. and Schroeder, DR., 2009. Imatinib treatment for idiopathic pulmonary fibrosis: randomized placebo-controlled trial results. *American Journal of Respiratory and Critical Care Medicine*. Vol (181)6. pp.604-610

9) Deininger, M., Buchdunger, E. and Druker, B.J., 2005. The development of imatinib as a therapeutic agent for chronic myeloid leukemia. *Blood*, *105*(7), pp.2640-2653.

10) Demoulin, J.B. and Essaghir, A., 2014. PDGF receptor signaling networks in normal and cancer cells. *Cytokine & Growth Factor Reviews*, *25*(3), pp.273-283.

11) Donovan, J., Shiwen, X., Norman, J. and Abraham, D., 2013. Platelet-derived growth factor alpha and beta receptors have overlapping functional activities towards fibroblasts. *Fibrogenesis & Tissue Repair*, *6*(1), p.10.

12) Duncan, D.J., Yang, Z., Hopkins, P.M., Steele, D.S. and Harrison, S.M., 2010. TNF-α and IL-1ß increase Ca2+ leak from the sarcoplasmic reticulum and susceptibility to arrhythmia in rat ventricular myocytes. *Cell Calcium*, *47*(4), pp.378-386.

13) Eghbali, M., Tomek, R., Woods, C. and Bhambi, B., 1991. Cardiac fibroblasts are predisposed to convert into myocyte phenotype: specific effect of transforming growth factor beta. *Proceedings of the National Academy of Sciences*, *88*(3), pp.795-799.

14) Essaghir, A., Dif, N., Marbehant, C.Y., Coffer, P.J. and Demoulin, J.B., 2009. The transcription of FOXO genes is stimulated by FOXO3 and repressed by growth factors. *Journal of Biological Chemistry*, *284*(16), pp.10334-10342.

15) Frangogiannis, N.G., 2015. Interleukin-1 in cardiac injury, repair, and remodeling: pathophysiologic and translational concepts. *Discoveries*, *3*(1).

16) Fujiu, K. and Nagai, R., 2014. Fibroblast-mediated pathways in cardiac hypertrophy. *Journal of Molecular and Cellular Cardiology*, *70*, pp.64-73.

17) Gallini, R., Lindblom, P., Bondjers, C., Betsholtz, C. and Andrae, J., 2016. PDGF-A and PDGF-B induces cardiac fibrosis in transgenic mice. *Experimental Cell Research*, *349*(2), pp.282-290.

18) Gao, Z. Sasaoka, T. Fujimori, T. Oya, T. Ishii, Y. Sabit, H. Kawaguchi, M. Kurotaki, Y. Naito, M. Wada, T. and Ishizawa, S. 2005. Deletion of the PDGFR-B gene affects key fibroblast functions important for wound healing. *Journal of Biological Chemistry,* 280(10), pp.9375-9389.

19) Gomez, D., Baylis, R.A., Durgin, B.G., Newman, A.A., Alencar, G.F., Mahan, S., Hilaire, C.S., Müller, W., Waisman, A., Francis, S.E. and Pinteaux, E., 2018. Interleukin-1ß has atheroprotective effects in advanced atherosclerotic lesions of mice. *Nature Medicine*, *24*(9), p.1418.

20) Greineder, C.F., Kohnstamm, S. and Ky, B., 2011. Heart failure associated with sunitinib: lessons learned from animal models. *Current Hypertension Reports*, *13*(6), pp.436-441.

21) Gross, S. Rahal, R. Stransky, N. Lengauer, C. and Hoeflich, K.P. 2015. Targeting cancer with kinase inhibitors. *The Journal of Clinical Investigation,* 125(5), pp. 1780-1789.

22) Hayashi, Y., Bardsley, M.R., Toyomasu, Y., Milosavljevic, S., Gajdos, G.B., Choi, K.M., Reid-Lombardo, K., Kendrick, M.L., Bingener-Casey, J., Tang, C.M. and Sicklick, J.K., 2015. Platelet-derived growth factor receptor-α regulates proliferation of gastrointestinal stromal tumor cells with mutations in KIT by stabilizing ETV1. *Gastroenterology*, *149*(2), pp.420-432.
23) Hehlmann, R., Lauseker, M., Jung-Munkwitz, S., Leitner, A., Müller, M.C., Pletsch, N., Proetel, U., Haferlach, C., Schlegelberger, B., Balleisen, L. and Hänel, M., 2011. Tolerability-adapted imatinib 800 mg/d versus 400 mg/d versus 400 mg/d plus interferon-α in newly diagnosed chronic myeloid leukemia. *Journal of Clinical Oncology*, *29*(12), pp.1634-1642.
24) Hogan, K. Ravindran, A. Podolsky, M.A. and Glick, A.B. 2013. The TGFß1 pathway is required for NF-kB dependent gene expression in mouse keratinocytes. *Cytokine,* 64(3), pp.652-659.
25) Hong, M., Wilkes, M.C., Penheiter, S.G., Gupta, S.K., Edens, M. and Leof, E.B., 2011. Non-Smad transforming growth factor-ß signaling regulated by focal adhesion kinase binding the p85 subunit of phosphatidylinositol 3-kinase. *Journal of Biological Chemistry*, *286*(20), pp.17841-17850.
26) Hwang, M.W., Matsumori, A., Furukawa, Y., Ono, K., Okada, M., Iwasaki, A., Hara, M., Miyamoto, T., Touma, M. and Sasayama, S., 2001. Neutralization of interleukin-1ß in the acute phase of myocardial infarction promotes the progression of left ventricular remodeling. *Journal of the American College of Cardiology*, *38*(5), pp.1546-1553.
27) Jang, S.W., Ihm, S.H., Choo, E.H., Kim, O.R., Chang, K., Park, C.S., Kim, H.Y. and Seung, K.B., 2014. Imatinib Mesylate Attenuates Myocardial Remodeling Through Inhibition of Platelet-Derived Growth Factor and Transforming Growth Factor Activation in a Rat Model of HypertensionNovelty and Significance. *Hypertension*, *63*(6), pp.1228-1234.
28) Jonsson, M.K., Hartman, R.J., Ackers-Johnson, M., Tan, W.L., Lim, B., van Veen, T.A. and Foo, R.S., 2016. A transcriptomic and epigenomic comparison of fetal and adult human cardiac fibroblasts reveals novel key transcription factors in adult cardiac fibroblasts. *JACC: Basic to Translational Science*, 1(7), pp.590-602
29) Kania, G., Blyszczuk, P. and Eriksson, U., 2009. Mechanisms of cardiac fibrosis in inflammatory heart disease. *Trends in Cardiovascular Medicine*, *19*(8), pp.247-252.
30) Karaman, M.W., Herrgard, S., Treiber, D.K., Gallant, P., Atteridge, C.E., Campbell, B.T., Chan, K.W., Ciceri, P., Davis, M.I., Edeen, P.T. and Faraoni, R., 2008. A quantitative analysis of kinase inhibitor selectivity. *Nature Biotechnology*, *26*(1), p.127.
31) Kerkelä, R., Grazette, L., Yacobi, R., Iliescu, C., Patten, R., Beahm, C., Walters, B., Shevtsov, S., Pesant, S., Clubb, F.J. and Rosenzweig, A., 2006. Cardiotoxicity of the cancer therapeutic agent imatinib mesylate. *Nature Medicine*, *12*(8), p.908.
32) Kerkelä, R., Woulfe, K.C., Durand, J.B., Vagnozzi, R., Kramer, D., Chu, T.F., Beahm, C., Chen, M.H. and Force, T., 2009. Sunitinib-induced cardiotoxicity is mediated by off-target inhibition of AMP-activated protein kinase. *Clinical and Translational Science*, *2*(1), pp.15-25.
33) Kim, Y., Fiel, M.I., Albanis, E., Chou, H.I., Zhang, W., Khitrov, G. and Friedman, S.L., 2012. Anti-fibrotic activity and enhanced interleukin-6 production by hepatic stellate cells in response to imatinib mesylate. *Liver International*, *32*(6), pp.1008-1017.
34) Kitagawa, D. Yokota, K. Gouda, M. Narumi, Y. Ohmoto, H. Nishiwaki, E. Akita, K. and Kirii, Y. 2013. Activity-based profiling of approved tyrosine kinase inhibitors. *Genes to Cells,* 18, pp.110-122.
35) Lankheet, N.A., Kloth, J.S., Gadellaa-van Hooijdonk, C.G., Cirkel, G.A., Mathijssen, R.H., Lolkema, M.P., Schellens, J.H., Voest, E.E., Sleijfer, S., De Jonge, M.J.A. and Haanen, J.B.A.G., 2014. Pharmacokinetically guided sunitinib dosing: a feasibility study in patients with advanced solid tumours. *British Journal of Cancer*, 110(10), p.2441.
36) Lawrence, T., 2009. The nuclear factor NF-κB pathway in inflammation. *Cold Spring Harbor Perspectives in Biology*, *1*(6), p.a001651.
37) Leask, A., 2010. Potential therapeutic targets for cardiac fibrosis: TGFß, angiotensin, endothelin, CCN2, and PDGF, partners in fibroblast activation. *Circulation Research*, *106*(11), pp.1675-1680.
38) Lemmon, M.A. and Schlessinger, J., 2010. Cell signaling by receptor tyrosine kinases. *Cell*, *141*(7), pp.1117-1134.
39) Li, M. Ping, G. Plathow, C. Trinh, T. Lipson, K.E. Hauser, K. Krempien, R. Debus, J. Adbollahi, A. and Huber, P.E., 2006. Small molecular receptor tyrosine kinase inhibitor of

platelet-derived growth factor signalling (SU9518) modifies radiation response in fibroblasts and endothelial cells. *BMC Cancer,* 6(1), p.79.
40) Lindahl, P. and Betsholtz, C. 1998. Not all myofibroblasts are alike: revisiting the role of PDGF-A and PDGF-B using PDGF targeted mice. *Current Opinion in Nephrology and Hypertension,* 7(1), pp.21-26.
41) Massague, J. Seoane, J. and Wotton, D. 2005. Smad transcription factors. *Genes and development,* 19(23), pp.2783-2810.
42) Mann, D.L., 2005. Targeted anticytokine therapy and the failing heart. *The American Journal of Cardiology*, *95*(11), pp.9-16.
43) Miller, S.C., Huang, R., Sakamuru, S., Shukla, S.J., Attene-Ramos, M.S., Shinn, P., Van Leer, D., Leister, W., Austin, C.P. and Xia, M., 2010. Identification of known drugs that act as inhibitors of NF-κB signaling and their mechanism of action. *Biochemical Pharmacology*, *79*(9), pp.1272-1280.
44) Motzer, R.J., Hutson, T.E., Tomczak, P., Michaelson, M.D., Bukowski, R.M., Oudard, S., Negrier, S., Szczylik, C., Pili, R., Bjarnason, G.A. and Garcia-del-Muro, X., 2009. Overall survival and updated results for sunitinib compared with interferon alfa in patients with metastatic renal cell carcinoma. *Journal of Clinical Oncology*, *27*(22), p.3584.
45) Narayan, V., Keefe, S., Haas, N., Wang, L., Puzanov, I., Putt, M., Catino, A., Fang, J., Agarwal, N., Hyman, D. and Smith, A.M., 2017. Prospective evaluation of sunitinib-induced cardiotoxicity in patients with metastatic renal cell carcinoma. *Clinical Cancer Research*.
46) Paul, M.K. and Mukhopadhyay, A.K., 2004. Tyrosine kinase–role and significance in cancer. *International Journal of Medical Sciences*, *1*(2), p.101.
47) Perona, R., 2006. Cell signalling: growth factors and tyrosine kinase receptors. *Clinical and Translational Oncology*, *8*(2), pp.77-82.
48) Pinto, A.R., Ilinykh, A., Ivey, M.J., Kuwabara, J.T., D'Antoni, M., Debuque, R.J., Chandran, A., Wang, L., Arora, K., Rosenthal, N. and Tallquist, M.D., 2015. Revisiting cardiac cellular composition. *Circulation Research*, pp.115
49) Porter KE, Turner NA., 2009. Cardiac fibroblasts - at the heart of myocardial remodeling. *Pharmacological Therapy* 123, pp.255-278.
50) Rivera-Serrano, E.E. and Sherry, B., 2017. NF-κB activation is cell type-specific in the heart. *Virology*, *502*, pp.133-143.
51) Roberts, A.B., 1999. TGF-β signaling from receptors to the nucleus. *Microbes and Infection*, *1*(15), pp.1265-1273.
52) Roskoski Jr, R., 2007. Sunitinib: a VEGF and PDGF receptor protein kinase and angiogenesis inhibitor. *Biochemical and biophysical research communications*, *356*(2), pp.323-328.
53) Saito, A., Suzuki, H.I., Horie, M., Ohshima, M., Morishita, Y., Abiko, Y. and Nagase, T., 2013. An integrated expression profiling reveals target genes of TGF-β and TNF-α possibly mediated by microRNAs in lung cancer cells. *PloS One*, *8*(2), p.e56587.
54) Sangwan, V. and Park, M., 2006. Receptor tyrosine kinases: role in cancer progression. *Current Oncology*, *13*(5), p.191.
55) Shiha, G.E., Abu-Elsaad, N.M., Zalata, K.R. and Ibrahim, T.M., 2014. Tracking anti-fibrotic pathways of nilotinib and imatinib in experimentally induced liver fibrosis: An insight. *Clinical and Experimental Pharmacology and Physiology*, *41*(10), pp.788-797.
56) Souders, C.A., Bowers, S.L. and Baudino, T.A., 2009. Cardiac fibroblast: the renaissance cell. *Circulation Research*, *105*(12), pp.1164-1176
57) Telli, M.L., Witteles, R.M., Fisher, G.A. and Srinivas, S., 2008. Cardiotoxicity associated with the cancer therapeutic agent sunitinib malate. *Annals of Oncology*, *19*(9), pp.1613-1618.
58) Torsello, B., Bianchi, C., Meregalli, C., Di Stefano, V., Invernizzi, L., De Marco, S., Bovo, G., Brivio, R., Strada, G., Bombelli, S. and Perego, R.A., 2016. Arg tyrosine kinase modulates TGF-β1 production in human renal tubular cells under high-glucose conditions. *Journal of Cell Sci*ence, *129*(15), pp.2925-2936.
59) Turner, N.A., 2014. Effects of interleukin-1 on cardiac fibroblast function: relevance to post-myocardial infarction remodelling. *Vascular Pharmacology*, *60*(1), pp.1-7.
60) Turner NA, Mughal RS, Warburton P, O'Regan DJ, Ball SG, Porter KE. 2007. Mechanism of TNFα-induced IL-1α, IL-1ß and IL-6 expression in human cardiac fibroblasts: effects of statins and thiazolidinediones. *Cardiovascular Research*, 76, pp.81-90.
61) Turner NA, Porter KE., 2013. Function and fate of myofibroblasts after myocardial infarction. *Fibrogenesis and Tissue Repair*, 6, pp.5.

62) Verrecchia, F. and Mauviel, A., 2002. Transforming growth factor-ß signaling through the Smad pathway: role in extracellular matrix gene expression and regulation. *Journal of Investigative Dermatology*, 118(2), pp.211-215.
63) Wang, L.X. Yang, X. Yue, Y. Fan, T. Hou, J. Chen, G.X. Liang, M.Y. and Wu, Z.K. 2017. Imatinib attenuates cardiac fibrosis by inhibiting platelet-derived growth factor receptors activation in isoproterenol induced model. *PLoS One,* 12(6).
64) Wilkes, M.C. and Leof, E.B., 2006. Transforming growth factor ß activation of c-Abl is independent of receptor internalization and regulated by phosphatidylinositol 3-kinase and PAK2 in mesenchymal cultures. *Journal of Biological Chemistry*, *281*(38), pp.27846-27854.
65) Zhang, Y.E., 2009. Non-Smad pathways in TGF-ß signaling. *Cell Research*, 19(1), p.128.
66) Zhao, W., Zhao, T., Huang, V., Chen, Y., Ahokas, R.A. and Sun, Y., 2011. Platelet-derived growth factor involvement in myocardial remodeling following infarction. *Journal of Molecular and Cellular Cardiology*, 51(5), pp.830-838.
67) Zhao, X., Lu, Y., Nie, Y. and Fan, D., 2013. MicroRNAs as critical regulators involved in regulating epithelial-mesenchymal transition. *Current Cancer Drug Targets*, 13(9), pp.935-944.