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Original article

cRel expression regulates distinct transcriptional and functional profiles driving fibroblast matrix production in systemic sclerosis

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Abstract

Objectives. NF-κB regulates genes that control inflammation, cell proliferation, differentiation and survival. Dysregulated NF-κB signalling alters normal skin physiology and deletion of cRel limits bleomycin-induced skin fibrosis. This study investigates the role of cRel in modulating fibroblast phenotype in the context of SSc.

Methods. Fibrosis was assessed histologically in mice challenged with bleomycin to induce lung or skin fibrosis. RNA sequencing and pathway analysis was performed on wild type and *Rel*$^{-/-}$ murine lung and dermal fibroblasts. Functional assays examined fibroblast proliferation, migration and matrix production. cRel overexpression was investigated in human dermal fibroblasts. cRel immunostaining was performed on lung and skin tissue sections from SSc patients and non-fibrotic controls.

Results. cRel expression was elevated in murine lung and skin fibrosis models. *Rel*$^{-/-}$ mice were protected from developing pulmonary fibrosis. Soluble collagen production was significantly decreased in fibroblasts lacking cRel while proliferation and migration of these cells was significantly increased. cRel regulates genes involved in extracellular structure and matrix organization. Positive cRel staining was observed in fibroblasts in human SSc skin and lung tissue. Overexpression of constitutively active cRel in human dermal fibroblasts increased expression of matrix genes. An NF-κB gene signature was identified in diffuse SSc skin and nuclear cRel expression was elevated in SSc skin fibroblasts.

Conclusion. cRel regulates a pro-fibrogenic transcriptional programme in fibroblasts that may contribute to disease pathology. Targeting cRel signalling in fibroblasts of SSc patients could provide a novel therapeutic avenue to limit scar formation in this disease.

Key words: fibroblasts, systemic sclerosis, cRel, extracellular-matrix, skin, lung

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Introduction

SSc is characterized by activation and accumulation of fibroblasts in multiple organs. SSc fibroblasts secrete elevated levels of extracellular matrix (ECM) components: collagens, glycosaminoglycans and fibronectin, which promote scar formation and dermal thickening [1–3]. Fibroblast activation and differentiation into an alpha-smooth muscle actin (αSMA)-positive myofibroblast is associated with increased proliferation, aberrant secretion of ECM proteins and inflammatory chemokines [4, 5]. Phenotypic activation of fibroblasts is mediated by multiple stimuli, including TGF-β, ECM-degrading proteases and inflammation [6, 7]. Canonical NF-κB signalling promotes fibroblast survival in the liver [8, 9], heart [10] and kidney via cell cycle regulation,

BASIC SCIENCE

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Rheumatology key messages

- cRel drives production of extracellular matrix in dermal fibroblasts and is constitutively active in SSc.
- cRel represents a novel therapeutic target in SSc, with potential to address unmet clinical need.

anti-apoptotic pathway activation and modulating matrix turnover [11, 12].

The NF-κB transcription factor family is composed of five members: p50 (NF-κB1), p52 (NF-κB2), RelA (p65), RelB and cRel. Subunits exist as either homodimers or heterodimers in various permutations and are sequestered in an inactive form within the cytoplasm by IκBα. Classically, pro-inflammatory insult or damage stimuli activate the IκB kinase (IKK) complex resulting in IκBα phosphorylation, targeting it for proteasomal degradation thus allowing NF-κB dimers to enter the nucleus and drive target gene transcription [13]. NF-κB signalling plays an essential role in maintaining normal skin physiology, IκBα null mice die of reduced skin barrier function, whilst IKK1 knockout mice are viable but have skin defects [14]. Aberrant NF-κB activation has been implicated in the development of chronic skin diseases; transgenic mice overexpressing active p50 or RelA in skin cells develop epidermal hypoplasia and die prematurely [15]. In SSc patients, a genome-wide association study identified an intronic polymorphism in the *NFKB1* gene encoding p105/p50 as a risk factor for SSc [16]. The link between NF-κB signalling and SSc was further consolidated in separate studies that reporting chemical inhibition of NF-κB or small interfering RNA-targeting of RelA in normal and SSc fibroblasts determined the extent of collagen synthesis [17, 18]. Bioinformatic analysis of tissue-specific cellular modulators of fibrosis has also implicated NF-κB signalling and functionality in both skin and lung fibrosis in SSc patients [19]. Furthermore, risk loci in the *REL* gene have been identified in psoriasis and PsA [20, 21].

A role for cRel-dependent signalling in epidermal disorders has been described in animal models [22–24]. Epidermal thickness is reduced in cRel global knockout mice, and these mice develop less skin fibrosis in response to chronic bleomycin injury [23, 25]. Additionally, a pro-fibrogenic role for cRel has been identified in murine models of cardiac and liver fibrosis, suggesting that cRel regulates fibrogenic processes in multiple organs [26, 27].

This study employs both non-biased transcriptomic and targeted functional approaches to discern the role of cRel in modulating the phenotypic traits of lung and skin fibroblasts. We report that cRel controls the expression of a distinct subset of matrix genes that are elevated in SSc and propose that targeting cRel in SSc patients may lead to novel anti-fibrotic therapies.

Methods

An extended Methods section is available in the supplementary material, section Methods, available at *Rheumatology* online.

Animal experiments were approved following local ethical review and performed under a UK Home Office license. Human foreskin and SSc forearm skin samples were taken under full ethical approval and with patient consent (Dermatology biobank ethics Ref: 08/H0906/95) sponsored by the Newcastle upon Tyne Hospitals Foundation Trust (R&D biobank reference: 4775) and the University of Manchester (Scleroderma and Raynaud's Research Bank REC14/NW/0132) and Sunderland Research Ethics Service (REC approval reference 13/NE/0089). Human lung samples were taken under full ethical approval and patient consent (REC approval reference 11/NE/0291). All SSc patients fulfilled the new ACR/EULAR criteria for SSc [28]. Patient characteristics of the skin biopsy cohort are included in supplementary Table S1, available at *Rheumatology* online.

Animal studies

Wild-type (WT) and $Rel^{-/-}$ mice on a pure C57BL/6 background were from Jorge Caamano (Birmingham University, Birmingham, UK). Adult male mice aged 8–10 weeks old, were used for experimental models. Samples from TSK1 (The Jackson Laboratory: B6.Cg-$Fbn1^{Tsk}$ +/+ $Bloc1s6^{pa}$/J Stock No.: 000305) mice and pa/pa mice were from Oliver Distler (University Hospital Zurich, Zurich, Switzerland). Bleomycin-induced dermal fibrosis by s.c. injection for 4 weeks and lung fibrosis by single intra-tracheal instillation was carried out as previously described [23, 29]. S.c. injections or intra-tracheal instillations of 0.9% saline served as a vehicle control. All studies were performed with full ethical approval and under a UK home office licence.

Cell culture

Dermal fibroblasts from healthy control samples were cultured as previously described [30]. Fibroblasts were isolated from skin biopsies of clinically affected forearm of patients with SSc [31]. Fibroblasts were transfected using Effectene (Qiagen, Manchester, UK) according to standard protocols.

RNA sequencing

RNA was extracted from mouse skin and lung fibroblasts using RNeasy Plus Micro Kit (Qiagen, Manchester, UK).

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After checking RNA integrity, mRNA was purified and libraries were prepared using NEB Next Ultra Directional RNA Library Prep Kit for Illumina (NEB, Hitchin, UK). The libraries were sequenced on an Illumina HiSeq 2000 and quality control was carried out with FASTQC (www.bioinformatics.babraham.ac.uk/projects/fastqc). All of our original sequence data have been deposited in (the GEO data repository; GSE151469). Reads were aligned to the mouse genome and quantified with STAR (doi: 10.1093/bioinformatics/bts635) in two-pass mode, and further sample quality control and differential expression analysis were performed in R with DESeq2 (doi: 10.1186/s13059-014-0550-8).

Plasmids transient transfection

For transient transfection, human dermal fibroblasts cells were seeded in six-well plates at 2×10^5 cells per well. The cells were then transfected for 48 h with 1.0 μg human FLAG-tagged Δ*RID*cRel (Addgene #27265, Teddington, UK) and human Hemagglutinin (HA)-tagged RelA (gift from Neil Perkins). The empty pcDNA3 vector was used as a mock transfection control. Transfection was performed using Effectene reagent according to the instructions specified by the manufacturer (Qiagen, Manchester, UK).

Immunofluorescence

Fibroblasts were seeded at 12.5×10^4 cells per well in an eight-well chamber slide. Cells were fixed in 3.7% formaldehyde (Sigma), permeabilized in 0.1% Triton-X100 and blocked by incubation in 1% BSA (Sigma, Dorest, UK). They were then incubated overnight at 4°C, with an anti-cRel (Santa Cruz sc-70, Heidelberg, Germany) followed by incubation with goat-anti rabbit secondary antibody (Invitrogen A-11035). Nuclei were counterstained with DAPI (Invitrogen, Paisley, UK) and images obtained using a Nikon Inverted Confocal Microscope.

Histology and immunohistochemistry

Deparaffinized and rehydrated sections were incubated in 2% hydrogen peroxide/methanol for 15 min. αSMA staining was performed as described previously [32]. Tris-EDTA antigen retrieval (pH 9) was performed at 95°C for 20 min. Sections were blocked with an avidin/biotin kit (SP-2001; Vector Laboratories, Burlingame, CA, USA) for 20 min, then with 20% pig serum in PBS for 20 min. Slides were incubated overnight at 4°C with rabbit primary antibodies directed against cRel diluted 1:150 (Abcam ab108299, Cambridge, UK) in PBS. Slides were washed with PBS and then incubated with biotinylated swine anti-rabbit (E0353; Dako, Glostrup, Denmark) for 1.5 h. Amplification of antigen was achieved using an R.T.U. Vectastain kit (Vector Laboratories), and positive cells were visualized by 3,3-diaminobenzidine tetrahydrochloride. Masson's Trichrome staining was performed as previously described [33]. All tissue sections were analysed at 200× magnification using a Nikon Eclipse Upright microscope and percentage area of positive stained tissue was quantified using NIS-Elements BR analysis software.

Scoring of clinical samples

Semi-quantitative analysis (by blinded observer) of cRel staining was performed using a scoring system based on the number of fibroblasts that were positively stained in both the papillary and reticular dermis (0–3) in both anatomical layers. No staining scored 0, 0–40% scored 1, >40–60% scored 2 and >60% scored 3.

Statistical analysis

Data were analysed using GraphPad Prism version 5.01 for Windows (GraphPad, San Diego, CA, USA). For two-group comparisons, Student's *t*-test was applied if the pre-test for normality (D'Agostino-Pearson normality-test) was not rejected at 0.05 significance, in which case a Mann–Whitney *U* test for non-parametric data was used. *P*-values <0.05 were considered significant.

Results

cRel is a pro-fibrogenic driver of matrix production in chronic fibrosis models

Immunohistochemistry confirmed cRel expression was elevated in the skin and lung after bleomycin challenge. Nuclear localization of cRel was observed in the epidermis and dermal fibroblasts of fibrotic skin, whereas cRel was visible in fibroblasts, epithelial cells and alveolar macrophages of the lung (Fig. 1A and B). We previously reported that cRel knockout ($Rel^{-/-}$) mice are protected from developing dermal fibrosis [23]; however, the role of cRel in lung fibrosis had not previously been explored. Intra-tracheal bleomycin challenge is associated with severe acute lung injury followed by inflammation and fibrosis [34]. Bleomycin elicited the expected structural changes, accumulation of collagen and activation of αSMA+ myofibroblasts in the lungs of WT mice (Fig. 1C and D). Conversely, fibrosis and myofibroblast activation were significantly attenuated in $Rel^{-/-}$ mice after bleomycin challenge (Fig. 1C and D). Histological improvement in fibrosis was accompanied by a significant reduction in fibrogenic gene expression and hydroxyproline levels (supplementary Fig. S1A and B, available at *Rheumatology* online). These data highlight a pro-fibrogenic role of cRel in the pathogenesis of chronic lung fibrosis.

cRel regulates a distinct transcriptional profile in lung and skin fibroblasts

To test if cRel signalling modulates fibroblast phenotype we performed RNA-sequencing on un-passaged WT and $Rel^{-/-}$ lung and skin fibroblasts. cRel deletion in fibroblasts was confirmed by western blot (supplementary Fig. S2A, available at *Rheumatology* online). A total of 19 genes were differentially expressed (DE) in $Rel^{-/-}$

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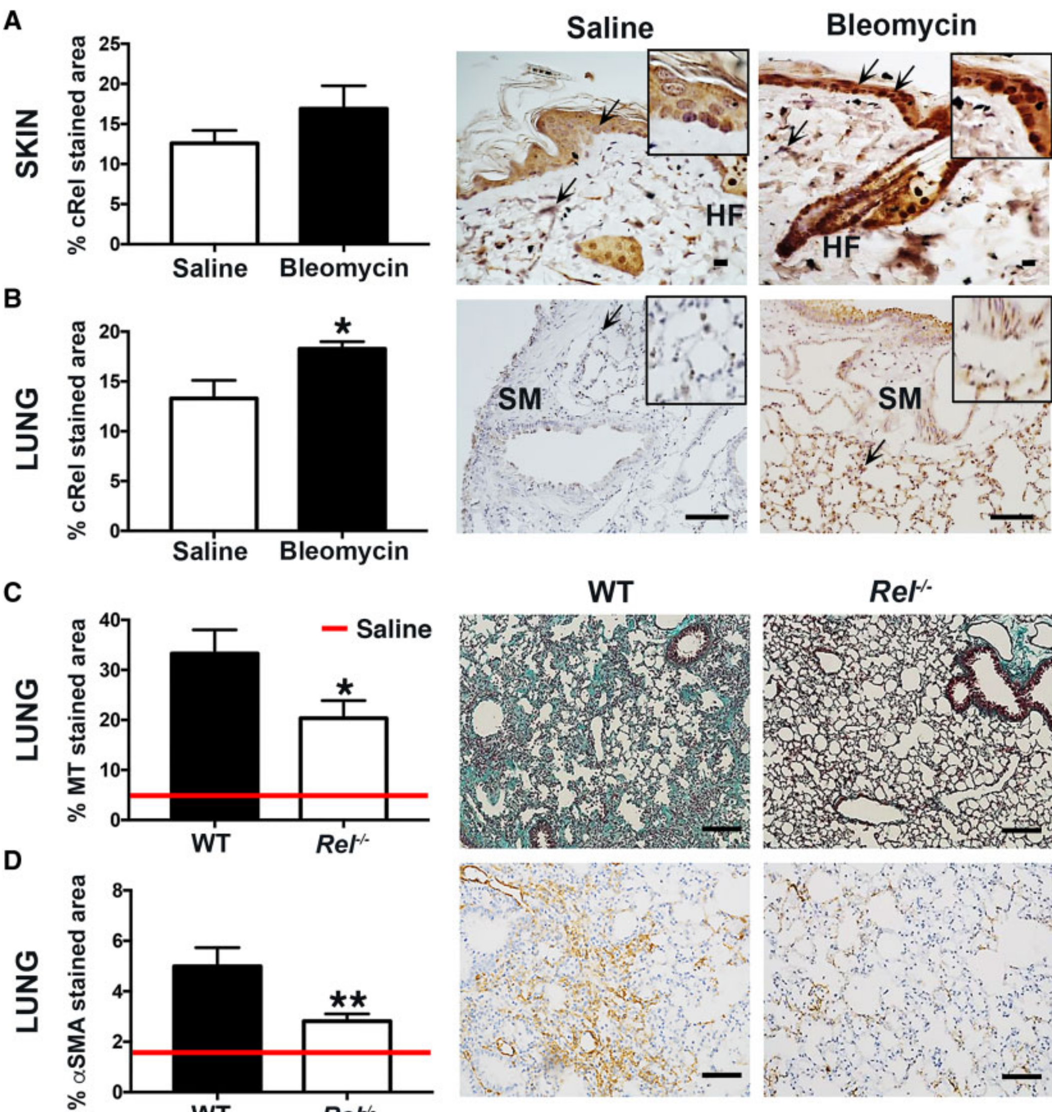
FIG. 1 cRel in chronic models of skin and lung fibrosis



(**A** and **B**) Quantification and representative images of cRel staining in the skin (A) and lung (B) of saline control and bleomycin-injured mice ($n = 6$ mice/group). Arrows indicative of cRel nuclear positivity, SM denotes smooth muscle and HF highlights hair follicle. (**C** and **D**) Histological quantification and representative images of (C) Masson's Trichrome and (D) α-smooth muscle actin (αSMA) staining in 21-day bleomycin-injured lungs from wild type (WT) and *Rel*$^{-/-}$ mice. Red line on graph denotes levels in control saline treated animals. (C) Images were taken at 200× magnification, scale bars equal 100 μm. Data are presented as percentage stained area of tissue sections ($n = 12$) for WT and ($n = 9$) for *Rel*$^{-/-}$ mice. Data are presented as mean (S.E.M.). *$P < 0.05$, **$P < 0.01$; statistical analysis performed using Mann–Whitney *U* test.

compared with WT lung fibroblasts (Fig. 2A), while 181 genes were DE in *Rel*$^{-/-}$ compared with WT skin fibroblasts (Fig. 2B). Significantly down-regulated genes in *Rel*$^{-/-}$ lung fibroblasts included key regulators of ECM: collagens *Col4a1*, *Col5a2*, *Col11a1*, *Col18a1*, MMP 3 (*Mmp3*) and Tenascin C (*Tnc*) (Fig. 2A). Similarly, down-regulated genes in *Rel*$^{-/-}$ skin fibroblasts included ECM components/regulators: *Col4a1*, *Col5a2*, fibronectin (*Fn1*), plexin D1 (*Plxd1*) and fibrillin-1 (*Fbn1*) (Fig. 2B). Validation of RNA-sequencing data was performed using qPCR (Fig. 2C and supplementary Fig. S2B and C, available at *Rheumatology* online). *Col4a1* and *Col5a2* were the only DE genes conserved between lung and skin fibroblasts. Expression of known drivers of fibrosis *Fn1* and *Tnc* were significantly down-regulated in *Rel*$^{-/-}$ skin and lung fibroblasts respectively (Fig. 2C).

cRel chromatin immunoprecipitation assays were performed on WT lung and skin fibroblasts. Binding was

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Fig. 2 cRel deletion in lung and skin fibroblasts alters matrix gene expression

(**A** and **B**) Heat map of differentially expressed (DE) genes in un-passaged wild type (WT) versus $Rel^{-/-}$ (A) lung and (B) skin fibroblasts ($P < 0.05$). Expression across each gene (or row) scaled so that mean expression is zero and S.D. is one. Samples with relatively high expression of a given gene are marked in red and samples with relatively low expression are marked in blue. Hierarchical clustering has reordered samples and genes. Dendrogram shows sample clustering. (**C**) qPCR validation of matrix gene expression in lung and skin fibroblasts *Col4a1*, *Col5a2*, *Fn1* and *Tnc*. Data are presented as mean (S.E.M.). $*P < 0.05$, $**P < 0.01$ by Mann–Whitney U test ($n = 6$ per group). (**D**) Conserved gene ontology (GO) classifications that are significantly enriched in both lung (black bars) and skin fibroblasts (clear bars) ($P < 0.05$). (D) Ingenuity Pathways Analysis (IPA) Diseases and Functions Annotation for skin fibroblasts (functionally different clusters with lowest *P*-values and highest z-scores).

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significantly enriched at *Col1a1*, *Col4a1* and *Fn1* promoters in lung fibroblasts (supplementary Fig. S2D, available at *Rheumatology* online). Consistent with the cultured fibroblast RNA sequencing data, a significant decrease in expression of matrix genes *Col4a1*, *Fn1*, *Tnc* was observed in the skin of bleomycin injured $Rel^{-/-}$ mice (supplementary Fig. S2E, available at *Rheumatology* online).DE genes identified in murine skin fibroblasts were compared with four publicly available SSc patient transcriptomic microarray datasets. Overlap of DE genes with three disease subsets: diffuse, limited and morphea (supplementary Fig. S2F, available at *Rheumatology* online), *FN1* was elevated in all disease subsets, *COL5A2* (limited and morphea), while *COL4A1* was elevated in diffuse disease. The *IKBKB* gene, which encodes IKKβ, an NF-κB activating kinase, was significantly elevated in diffuse disease and identified as an upstream regulator of cRel-dependent genes in skin fibroblasts (supplementary Table S2, available at *Rheumatology* online).

To investigate the functional relevance of cRel signalling in fibroblasts, we performed Gene Ontology (GO) analysis of DE genes identified in both skin and lung data sets. GO analysis revealed significant enrichment for terms such as supramolecular fibre organization, ECM structure and organization, all of which belong to the cellular component ontology (Fig. 2D). Further interrogation of shared cellular component GO terms identified enrichment in ECM components, collagen trimers, fibrils and basement membrane (supplementary Fig. S3A, available at *Rheumatology* online). Ingenuity Pathway Analysis was used to identify predicted diseases and functions associated with DE genes in $Rel^{-/-}$ skin fibroblasts. Up-regulated functions included cellular growth, movement and proliferation, whereas dermatological diseases and conditions are reduced in $Rel^{-/-}$ fibroblasts (Fig. 2E). Upstream canonical pathways associated with fibrosis were also activated (supplementary Fig. S3B, available at *Rheumatology* online).

In a non-inflammatory fibrosis model; the tight skin mouse (TSK1), cRel expression was elevated and localized to regions of dense fibril formation (supplementary Fig. S4A, available at *Rheumatology* online), accompanied by significantly increased cRel and matrix gene expression (supplementary Fig. S4B, available at *Rheumatology* online).

cRel null fibroblasts have distinct functional capabilities

Matrix production and secretion, assessed by the Sircol assay, was significantly lower in the cellular supernatants of both $Rel^{-/-}$ lung and skin fibroblasts (Fig. 3A and B). Whilst 5′-bromo-2′-deoxyuridine (BrdU) assay and scratch assays confirmed $Rel^{-/-}$ lung and skin fibroblasts display increased proliferation and migration rates compared with their WT counterparts (Fig. 3C–F). Significant increases in wound closure were observed as early as 6 h in $Rel^{-/-}$ lung fibroblasts, whereas in $Rel^{-/-}$ skin fibroblasts accelerated migration was evident from 24 h (Fig. 3E and F). One hundred percent wound closure was observed at 48 h in $Rel^{-/-}$ fibroblasts, compared with WT fibroblasts with only 50% wound closure at the same timepoint. These functional observations were also validated in 3D spheroids (supplementary Fig. S5A–E, available at *Rheumatology* online). These findings illustrate the biological effects of cRel signalling in fibroblasts are to increase the fibrogenic potential and suppress the migratory and proliferative phenotype.

Constitutively active cRel drives expression of matrix genes in human dermal fibroblasts and cRel activity is increased in SSc dermal fibroblasts

We next asked if cRel regulates matrix production in human dermal fibroblasts. Immunofluorescence staining revealed cRel (red) expression is relatively low in normal human dermal fibroblasts (NHDF) but markedly elevated in SSc fibroblasts. Localization of cRel in SSc fibroblasts was both nuclear (arrows) and cytoplasmic, suggesting that under disease conditions, cRel is active, whereas cRel in NHDF is predominantly inactive and confined to the cytoplasm (Fig. 4A). These data indicate that during disease, cRel signalling becomes persistently activated.

To model this *in vitro*, plasmid constructs containing a constitutively active cRel (cRelΔRID) were transiently transfected into NHDF and matrix gene expression was assessed. cRel overexpression was confirmed by qPCR (supplementary Fig. S6A, available at *Rheumatology* online) and western blot (FLAG-tag) (supplementary Fig. S6B, available at *Rheumatology* online). cRelΔRID significantly increased *COL4A1*, *COL5A2*, *TNC* and *FN1* gene expression (Fig. 4B) and soluble collagen production (Fig. 4C) compared with empty vector controls. There was no difference in expression of these genes or in soluble collagen production when the RelA was overexpressed. These data confirm that in human dermal fibroblasts cRel regulates a subset of matrix genes and collagen production.

Normal and SSc fibroblast gene expression was compared and consistent with a highly activated phenotype (supplementary Fig. S6C, available at *Rheumatology* online). Genes identified to be regulated by cRel in this study including *TNC* and *FN1* were also significantly increased in SSc fibroblasts, although *COL4A1* and *COL5A2* gene expression were elevated but did not reach significance (Fig. 4D).

cRel expression is elevated in fibroblasts of SSc skin and lung

We examined cRel expression and cellular localization in the skin of 8 non-fibrotic controls and 20 SSc patients, with either dcSSc or lcSSc. cRel expression was markedly increased in SSc skin compared with control, non-fibrotic skin. Consistent with our previous study [23], the epidermis of non-fibrotic skin showed infrequent nuclear-positive cRel staining in keratinocytes of the basal layer, while there was an increase in nuclear positivity of keratinocytes in the basal layer and stratum

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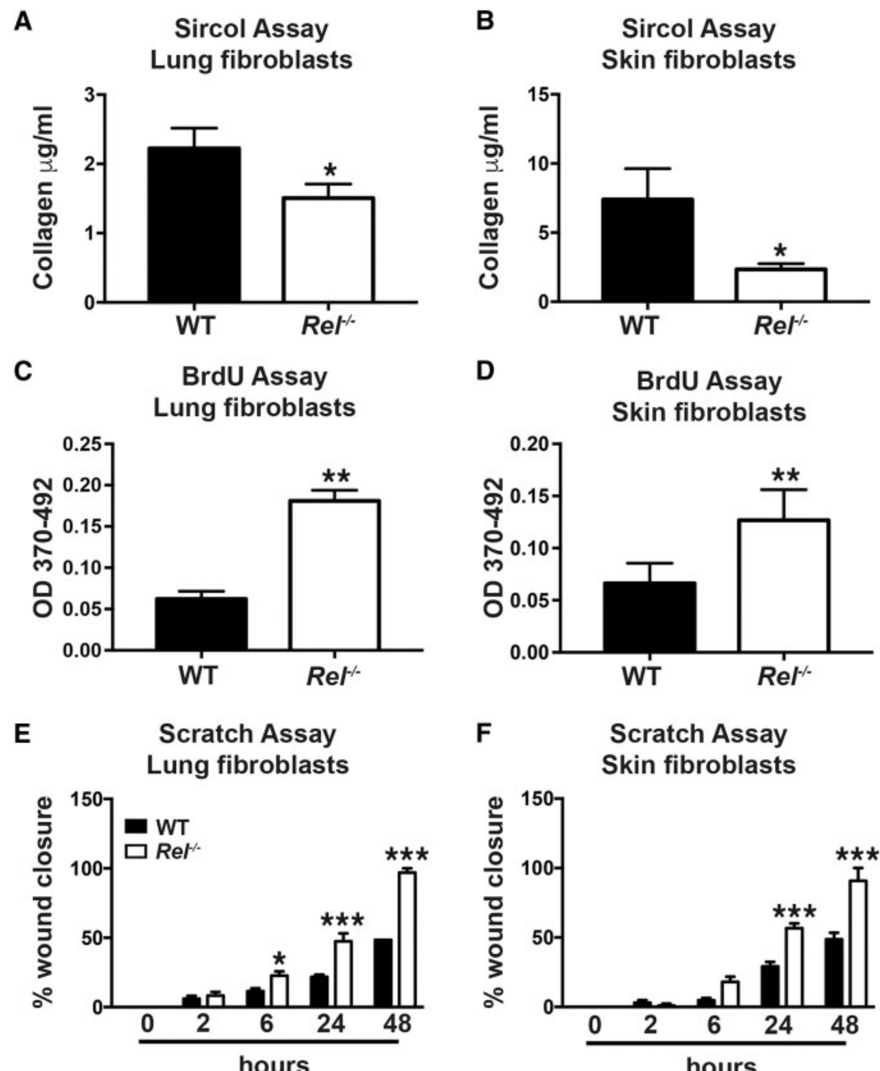
FIG. 3 cRel knockout fibroblasts have distinct functional capabilities



Fibroblasts isolated from WT and *Rel*$^{-/-}$ lung and skin. (**A** and **B**) Sircol Assay measuring soluble collagen production (μg/ml); (**C** and **D**) 5′-bromo-2′-deoxyuridine (BrdU) assay measuring cellular proliferation, (**E** and **F**) scratch assay measuring cellular migration over time. Assays were performed at 24 h post-plating unless otherwise stated. Experiments performed on fibroblasts at passage 1–4. Data are presented as mean (S.E.M.) and are representative of at least three independent experiments. **P* < 0.05, ***P* < 0.01, ****P* < 0.001 by Mann–Whitney *U* test.

spinosum in SSc skin (Fig. 5A). Semi-quantitative analysis of the cRel IHC confirmed a statistically significant upregulation of cRel in fibroblasts of SSc skin compared with control skin (Fig. 5B). High cRel expression was more pronounced in fibrotic SSc patient skin, based on histological skin score, compared with control skin. There was a trend towards higher cRel levels in the dermis of dcSSc compared with lcSSc (data not shown) but no correlation with disease duration (supplementary Table S1, available at *Rheumatology* online). Expression of cRel-regulated matrix and fibrogenic genes was evaluated in a small cohort of non-fibrotic and SSc skin. REL, *αSMA*, *COL5A2*, *FN1* and *TNC* gene expression was increased in all three SSc donors compared with control skin (Fig. 5C). Interestingly, plasma levels of FN1 were significantly higher in SSc patients compared with healthy controls (supplementary Fig. S6D, available at *Rheumatology* online).

Finally, we assessed cRel expression in sections from non-fibrotic and SSc lungs. The expected histological features of SSc and fibrosis were confirmed using haematoxylin and eosin- and Masson's Trichrome-stained lung tissue sections (Fig. 5D). cRel levels were elevated in SSc lung compared with non-fibrotic controls, with positive staining observed in immune cells (alveolar macrophages and inflammatory infiltrate) and surrounding vessels. All SSc patients had numerous fibroblastic foci visible within the lung accompanied by

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FIG. 4 cRel activity is increased in SSc dermal fibroblasts and drives the expression of matrix genes

(**A**) Immunofluorescence staining for cRel in NHDF and SSc fibroblasts, images taken at 200× magnification, scale bars equal 100 μm. Arrows denote nuclear positivity. (**B**) Relative mRNA expression of matrix genes *COL4A1*, *COL5A2*, *FN1* and *TNC* in normal human dermal fibroblasts (NHDF) following transient transfection with cRel ΔRID, RelA or empty plasmid for 48 h ($n = 5$ donors). (**C**) Sircol Assay measuring soluble collagen production (μg/ml) in NHDF following transient transfection with cRelΔRID, RelA or empty plasmid for 48 h ($n = 5$ donors). (**D**) Relative mRNA levels of *COL4A1*, *COL5A2*, *FN1* and *TNC* in NHDF ($n = 3$) and SSc fibroblasts ($n = 4$) per group. Experiments performed on fibroblasts at passage 2–6. Data are presented as mean (S.E.M.). *$P < 0.05$ by Mann–Whitney *U* test.

dense bundles of ECM. Fibroblasts located within the fibroblastic foci stained positively for cRel (Fig. 5D). SSc lung pathology and localization of cRel staining was consistent between the three SSc donors (supplementary Fig. S7, available at *Rheumatology* online). Localization of cRel in fibrotic areas and SSc lung fibroblasts further highlight the relevance of this NF-κB subunit in dcSSc.

Discussion

This study provides multiple lines of evidence that cRel expression is elevated in fibrotic skin and lung, in both murine fibrosis models and human disease. In cultured murine and human fibroblasts, cRel regulates fibroblast phenotype and ECM production. Activation of canonical NF-κB signalling has been linked with ECM production in dermal fibroblasts [18]; however, these studies focussed on signalling via p50 and p65 (RelA) heterodimers. This is the first study to directly assess the role of cRel in fibroblast biology and functionality. Here, we identify a distinct transcriptomic profile of cultured $Rel^{-/-}$ fibroblasts. The fibrogenic phenotype and production of ECM components is suppressed in $Rel^{-/-}$ cells, favouring a shift towards a pro-proliferative and migratory behaviour. Fibroblasts are a heterogeneous population of highly plastic cells, exhibiting a spectrum of phenotypic traits during their transition from a fibroblast to an activated

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Fig. 5 cRel expression is elevated in fibroblasts of SSc skin and lung

(**A**) Representative images of cRel staining in non-fibrotic skin ($n = 8$) and scleroderma (SSc) skin tissue ($n = 20$), showing dermal and epidermal positivity. (**B**) cRel staining scores illustrating the relative staining intensity of fibroblasts in the dermis. (**C**) Relative mRNA levels of *αSMA*, *COL4A1*, *FN1* and *REL* was quantified in normal skin and SSc skin ($n = 3$) per group. (**D**) Representative images of haematoxylin and eosin (H&E), Masson's Trichrome (MT) and cRel staining in non-fibrotic lung ($n = 3$) and SSc lung tissue ($n = 3$). SM denotes smooth muscle and FF represents fibroblastic foci; arrows indicate fibroblasts. All images taken at 200× magnification and scale bars are representative of 100 μm. Data presented as mean (s.e.m.). *$P < 0.05$ by Mann–Whitney *U* test.

myofibroblast during wound healing and fibrosis. Our data implicate cRel as a key modulator of this transition.

We predicted *IKBKB* as an upstream regulator of cRel-dependent gene transcription in skin fibroblasts. Previous studies by Milano *et al.* identified an intrinsic SSc 'gene signature' [35]; we validated *IKBKB* (IκBβ) as a member of the 'diffuse-proliferative' or 'fibro-proliferative' subset using publicly available microarray data. The IKK/IKBKB/NF-κB axis is important in other collagen induced disorders, *Ikkb*$^{-/-}$ mice are protected from developing CIA [36] and IκBβ loss dramatically reduces TNF-α-mediated inflammation despite normal NF-κB activation. In human disease, two novel single nucleotide polymorphisms mapping to the REL locus are associated with RA [37].

Lung involvement in SSc significantly contributes to disease morbidity and mortality [38]. Lung and skin fibroblasts from scleroderma patients exhibit elevated matrix production, increased contractility and increases in 'activation markers', and subtle differences have been observed in migratory capacity on 3D substrates. It is likely that in addition to regulating a core matrix function, cRel also regulates discrete biological functions in lung

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and skin. RNA-sequencing revealed that expression of only two genes (*Col4a1* and *Col5a2*) was conserved between fibroblasts isolated from the two organs. However, a previous transcriptomic study reported that fibroblasts isolated from different tissues display similar morphology but can exhibit different functional properties, a concept termed 'positional memory' [39]. Interestingly, pathways identified in this study included ECM synthesis, cell proliferation and cell migration, functional phenotypes that are modulated by cRel in both skin and lung fibroblasts. Therefore, despite the ability of cRel to regulate discrete genes within anatomically distinct fibroblasts, cRel deletion conveys similar functional outcomes.

Persistence of fibroblasts creates a pro-fibrotic microenvironment and drives SSc pathogenesis. Work by Xie *et al.* identified the percentage of matrix fibroblast expansion in bleomycin-induced lung fibrosis as greater than the αSMA+ myofibroblast population, implicating matrix-producing fibroblasts as the dominant driver of lung fibrosis [40]. The transcriptomic profile of matrix-producing fibroblasts in this study was consistent with our findings; *Cxcl14* was the most distinct extracellular expressed gene, with enrichment of transcription factor *Aebp* [40]. cRel could be a 'genetic switch' that promotes the ECM-producing fibrotic fibroblast fate; a similar role has recently been identified for transcription factor PU.1 in multiple models of lung and skin fibrosis [41]. Functionally, *Rel*$^{-/-}$ fibroblasts may be more migratory/contractile due to alterations in the matrix they deposit or because the balance between contractile and matrix producing fibroblasts has been altered.

Deposition and accumulation of matrix is regulated by a network of matricellular proteins, including fibronectin and tenascin-C. Many matricellular proteins are directly regulated by TGF-β, altered signalling drives the production/accumulation of matrix [42], in the context of our findings it is plausible that *TGF-β* is a target of cRel. This regulatory role of cRel in fibroblasts could be via modulation of both fibrillogenesis and matrix organization; previous studies in TSK1 models identified *Col5a2* [43] and *Tnc* [44] as regulators of matrix formation and fibril formation. Alteration of Col1/ColV fibres in *Rel*$^{-/-}$ mice may render the fibres more accessible to proteolytic enzymes or ease accessibility to MMP degradation. In human disease, elevated expression of *TNC* [45], *FN1* [46], *COL4A1* [47] and *COL5A2* [48] have all been described in diffuse SSc. *TNC* [49] and *FN1* [50] are known NF-κB target genes, but to our knowledge this is the first study to link these genes to cRel-dependent signalling.

In mice treated with bleomycin, cRel induction is followed by its persistence in lesional tissue, while *Rel*$^{-/-}$ mice show reduced αSMA+ cells and less fibrosis. Elevation and activation of cRel was observed in fibroblasts and skin of SSc patients, although this did not correlate with disease progression. Our SSc patient study cohort contained only three male patients, consistent with an overall female predominance of the disease [51]. Although cRel staining was elevated in the dermis of male ($n = 3$) patients, when compared with female patients ($n = 17$), the sample size was too small to interpret this further. It should also be noted that the median age of male patients was higher than that of female patients (67 *vs* 54 years). A small number of patients received immunosuppressant therapy (AZA, CYC or MMF); however, there was no relationship between immunosuppressant treatment and cRel dermal staining intensity. Two patients who received CS in combination with immunosuppressants had reduced levels of cRel staining in the dermis. Due to the small overall sample size of our patient cohort ($n = 20$), the biological/clinical significance of these findings cannot currently be further interpreted. This is a limitation of this study but represents an interesting avenue to follow in future studies.

cRel activation might represent an earlier step in disease pathogenesis, by eliciting changes in ECM composition and creating a fibrogenic microenvironment. The ECM microenvironment provides cells with physical support for adhesion and can regulate cell position, cell cycle, metabolism and differentiated state [52]. Altered ECM production is not only a consequence but also an active driver of fibrosis. One hypothesis is that progressive fibrosis in the absence of on-going injury occurs in a fibrogenic niche, e.g. fibrogenic progenitors and fibrogenic progeny reside in an ECM that is itself fibrogenic [53]. Elevated cRel in SSc fibroblasts may perpetuate the deposition of ECM, thus initiating a fibrotic cascade within an injured microenvironment. This more broadly supports the concept of 'para-inflammation' where fibroblasts perpetuate a self-sustaining feedback loop of matrix deposition [54].Targeting core fibrogenic signalling pathways, like cRel, that regulate matrix re-modelling and deposition could prove beneficial in multi-organ conditions such as SSc. Previous studies have focussed on the role of canonical (RelA/p50) signalling in tissue fibrosis using murine models. Deletion of the p65/RelA subunit is embryonic lethal [55] and p65/RelA-dependent signalling is critical for cell survival. Meanwhile, *Nfkb1*$^{-/-}$ mice, which lack p50/p105, have an accelerated ageing phenotype, driven by chronic low-level inflammation [56], and p50 signalling is essential to maintain an appropriate anti-inflammatory response [57]. Studies by Karin and colleagues utilizing animal models of disease [58–60] have focussed on the upstream NF-κB kinase IKKβ. Potent IKKβ inhibitors, which blunt canonical NF-κB signalling, resulted in a variety of adverse effects in humans including immunodeficiencies, hepatotoxicity and an increased pre-disposition to liver and skin malignancies [61].

Rel$^{-/-}$ mice develop normally [62] and do not exhibit signs of gross epidermal defects such as those seen in *Relb*$^{-/-}$ mice [63]. Deletion of cRel limits inflammation and fibrosis in murine models of fibrotic disease in multiple organs and this study has revealed that cRel exerts discrete functions on fibroblast phenotype. Strategies to selectively target the cRel subunit of NF-κB have been successful in graft versus host disease

and melanoma [64, 65] and therapeutic targeting of cRel using commercially available small molecule inhibitors; IT-603 or IT-609 have not demonstrated adverse

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effects in pre-clinical models[64, 65]. There are currently no effective anti-fibrotic therapies, and the crucial role of cRel in controlling ECM production and fibrosis in both, mouse and human tissues could potentially address this unmet clinical need.

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Supplementary data

Supplementary data are available at *Rheumatology* online.

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