



LINC01013 Is a Determinant of Fibroblast Activation and Encodes a Novel Fibroblast-Activating Micropeptide

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Abstract

Myocardial fibrosis confers an almost threefold mortality risk in heart disease. There are no prognostic therapies and novel therapeutic targets are needed. Many thousands of unannotated small open reading frames (smORFs) have been identified across the genome with potential to produce micropeptides (< 100 amino acids). We sought to investigate the role of smORFs in myocardial fibroblast activation.

Analysis of human cardiac atrial fibroblasts (HCFs) stimulated with profibrotic TGFβ1 using RNA sequencing (RNA-Seq) and ribosome profiling (Ribo-Seq) identified long intergenic non-coding RNA LINC01013 as TGFβ1 responsive and containing an actively translated smORF. Knockdown of LINC01013 using siRNA reduced expression of profibrotic markers at baseline and blunted their response to TGFβ1. In contrast, overexpression of a codon-optimised smORF invoked a profibrotic response comparable to that seen with TGFβ1 treatment, whilst FLAG-tagged peptide associated with the mitochondria. Together, these data support a novel LINC01013 smORF micropeptide-mediated mechanism of fibroblast activation.

Keywords Myocardial fibrosis · lncRNA · Micropeptide · Small open reading frame · Translation

Abbreviations

CMV	Cytomegalovirus
DMEM	Dulbecco's modified eagle medium
ECM	Extracellular matrix
HCF	Human cardiac fibroblast
HEK	Human embryonic kidney
HUVEC	Human umbilical cord vascular endothelial cell

lincRNA	Long intergenic non-coding ribonucleic acid
lncRNA	Long non-coding ribonucleic acid
Nuc-Seq	Nuclear RNA sequencing
ORF	Open reading frame
RNA	Ribonucleic acid
RNA-Seq	Ribonucleic acid sequencing
RPF	Ribosome-protected fragment
siRNA	Small-interfering ribonucleic acid
smORF	Small open reading frame

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STAR	Spliced transcript alignment to a reference
TGFβ1	Transforming growth factor beta 1
UTR	Untranslated region

Introduction

Myocardial fibrosis is a common final pathology of almost all forms of heart disease. It leads to arrhythmia and haemodynamic failure, and is an independent predictor of mortality [1–3]. No current treatment for myocardial fibrosis has been shown to offer prognostic benefit [4]. Although some major determinants of myocardial fibrosis are recognised, inhibition of upstream molecules, such as transforming growth factor beta-1 (TGFβ1) has detrimental pleiotropic effects, limiting their viability as therapeutic targets [5, 6]. Therefore, identification of novel therapeutic targets, downstream of TGFβ1, is urgently needed.

Cardiac fibroblasts comprise a heterogeneous mesenchymal cell population that produce and remodel extracellular matrix, and whose activation is a precursor to the fibrotic response [7]. The final consequence of fibroblast activation is their transdifferentiation into extracellular matrix (ECM) producing and contractile myofibroblasts, the persistence of which turns the fibrotic response from physiological to maladaptive, with the production of pathological quantities of extracellular matrix. Markers of fibroblast activation include interleukin-11 (*IL11*), as a key determinant of fibroblast activation [8], snail (*SNAIL*), which is necessary for myofibroblast transdifferentiation and fibrosis [9–13], and periostin (*POSTN*) and alpha-smooth muscle actin (α -SMA, *ACTA2*) which are both established markers of myofibroblast transdifferentiation [7, 14].

Small open reading frames (smORFs) are increasingly recognised to play a significant role in regulating cellular processes [15, 16]. These short regions with coding potential can be identified both within the upstream and downstream regions of messenger RNAs traditionally labelled as untranslated (UTRs), and within long non-coding (LNC) RNAs—an abundant class of RNAs with largely undescribed functional roles [17]. In order to visualise smORFs which are actively translated, ribosome profiling (Ribo-Seq) leverages the property of ribosomes to protect RNA from endonuclease digestion. Sequencing and mapping of ribosome-protected fragments (RPFs) of RNAs generate a snapshot of regions of active translation [18–20]. From these studies, it is now clear that many regions, previously annotated as non-coding, in fact contain smORFs which are not only actively translated but can be demonstrated to produce biologically active micropeptides with a diverse range of actions, including in the cardiovascular system [15, 16, 21–25].

LINC01013 is a largely uncharacterised intergenic lncRNA, previously considered to be non-coding, which

has been seen to have a role in chondrogenesis [26], cancer progression [27, 28] and regulation of DNA repair in endothelial cells [29]. A recent epigenomics study found it to be co-ordinately expressed with a key fibrotic gene *CCN2/CTGF* [30] in the context of human umbilical cord vascular endothelial (HUVEC) cells, suggesting a potential role in fibrosis. To date, no study has addressed the possibility of LINC01013 encoding a bioactive peptide.

Here, we demonstrate a direct role of LINC01013 in the response of cardiac fibroblasts to TGFβ1. Moreover, we use ribosome profiling to identify a small open reading frame (LINC01013ORF) with strong evidence of translation. LINC01013ORF is independently able to activate cardiac fibroblasts and encodes a small peptide that localises to the mitochondria. Taken together, these data show that LINC01013 and its encoded micropeptide are likely to play a key role in fibroblast activation and represent novel targets for antifibrotic therapy.

Methods

Cell Culture of HCF Cells

Human atrial cardiac fibroblasts (passage 1) for in vitro analyses were obtained commercially from iBiologics (lines 1125, 1153, 1156, 1162, 1164, 1166). Culture medium for all lines was Dulbecco's modified eagle medium (DMEM, Gibco), with 10% foetal bovine serum (Gibco) and 1% penicillin/streptomycin (Gibco). Cells were maintained at 37° with 95% air and 5% CO₂. Tissue culture plates were pre-treated with poly-D-lysine (2 μg/cm², Sigma). Cells were passaged by trypsinisation with 0.05% trypsin (ThermoFisher) and centrifuged at 1000 rpm for 5', before resuspension, counting and re-plating at appropriate concentration.

Cell Culture of HeLa Cells

Human HeLa cells (ATCC) were cultivated in a humidified incubator at 37 °C with 5% CO₂ using Dulbecco's modified eagle medium (DMEM) with high glucose (4.5 g/l), 10% foetal bovine serum (FBS), 2 mM L-glutamine and 1 mM sodium pyruvate. Medium was changed after 48 h and cells were split at confluency rates of 80–90% using standard trypsinization techniques.

RNA-Seq and Ribo-Seq

Ribo-Seq and RNA-Seq datasets were obtained using human cardiac fibroblasts treated with TGFβ1 as previously described in [31]. The adapters were trimmed using Trimmomatic V0.36 [32] and reads that aligned to

mitochondrial RNA, ribosomal RNA and transfer RNA using Bowtie [33] were discarded as previously described in [31]. The remaining reads were aligned to the human genome (hg38) using STAR [34] and read coverage on known coding genes was calculated using featureCounts from the Subread package [35]. Differential expression analysis was carried out using DESeq2. Ribotaper [36] was used to detect actively translated small open reading frames (smORFs) based on Ribo-Seq and RNA-Seq reads.

siRNA Knockdown

HCFs (iBiologics) were seeded at 2.4×10^5 per well of 6 W plates. After 24 h, cells were washed twice with OptiMEM (ThermoFisher) and transfected with pooled anti-LINC01013 or nontargeting control siRNAs (Dharmacon) using Lipofectamine RNAiMax (Invitrogen) following manufacturer's protocols and to a final siRNA concentration of 100 nM for 24 h. Transfection media was then changed to serum-free DMEM +/- 5 ng/ml TGF β 1 (Bio-Rad).

qPCR

Cells were lysed with Trizol (Invitrogen) and phase-separated with chloroform by centrifuging at 12,000 g for 15' at 4 °C. RNA was isolated using the RNeasy kit (Qiagen), following manufacturer's guidelines. cDNA generation was with Superscript VILO (Invitrogen), following manufacturer's guidelines. LINC01013ORF was amplified by primer pair: 5' GCTGCCCTAAACGCATTCT 3' (forward) and 5' GGGCTTTTGGTGGGGATTCTT 3' (reverse) in SYBR green real-time PCR master mix. Markers of fibroblast activation were measured by Taqman probes: ACTA2: Hs00909449_m1; POSTN: Hs01566734_m1; COL1A1: Hs00164004_m1; IL11: Hs01055413_g1; MMP2: Hs01548727_m1, FN1: Hs01549976_m1 and GUSB endogenous control: Hs99999908_m1, analysed with TaqMan Fast Advanced Master Mix (ThermoFisher). Quantitative PCR was performed on a QuantStudio 7 Flex Real-Time PCR System. Fold changes in gene expression were calculated using the $2^{-(\Delta\Delta C(T))}$ method [37].

LINC01013ORF3 Overexpression

A codon optimised sequence with identical coding potential but altered RNA sequence to LINC01013ORF was cloned into expression vector pcDNA3.1 (Addgene) and expressed under control of a CMV promoter/enhancer. Control vector contained eGFP also expressed from CMV promoter/enhancer in the pMaxGFP vector (Lonza).

Immunofluorescence

Immunofluorescence experiments were performed as described previously [25] with slight adjustments. HeLa cells were grown on 15 mm glass slides for 24 h and transfected with 3xFLAG tagged LINC01013ORF (LINC01013ORF-3xFLAG) in a pcDNA3.1 vector, using Lipofectamine 2000 reagent. Twenty-four-hour post transfection, cells were fixed with 4% paraformaldehyde for 10 min at room temperature and washed three times with ice-cold phosphate-buffered saline (PBS). Cells were permeabilized and blocked for 1 h at room temperature using 2.5% bovine albumin serum, 10% anti-goat serum and 0.1% Triton X or 30 mg/mL digitonin followed by 3 washing steps. Whilst Triton X permeabilizes the outer and inner mitochondrial membrane and therefore allows the staining of mitochondrial matrix proteins, digitonin leaves the inner mitochondrial membrane intact and prevents staining of mitochondrial matrix proteins. Overexpressed LINC01013ORF-3xFLAG was stained for 1 h at room temperature using an anti-FLAG mouse monoclonal antibody (1:500, F1804, Sigma Aldrich) and co-stained with the mitochondrial matrix protein ATP1F (1:1000, rabbit ATP1F #13,268, Cell Signaling Technology; Danvers, MA, USA) or the mitochondrial outer membrane protein TOM20 (1:200, rabbit TOM20 #42,406, Cell Signaling Technology; Danvers, MA, USA). Slides were then washed and incubated with fluorescently-labelled secondary antibodies (1:500, Alexa Fluor 488 anti-rabbit & Alexa Fluor 594 anti-mouse; Invitrogen, Carlsbad, CA, USA) for 30 min at room temperature. Cells were washed again, stained with 4–6-diamidino-2-phenylindole (NucBlue Fixed Cell ReadyProbes Reagent, R37606, Thermo Fisher) for 5 min at room temperature and mounted onto glass slides using ProLongTM Gold antifade reagent (Molecular Probes; InvitrogenTM). Images were visualised using a LEICA SP8 confocal microscope using a $\times 63$ objective. Image analysis was performed using the Leica confocal software Las X (v3.5.2) and ImageJ (v1.52a).

In Vitro Translation of LINC01013 RNA

The full LINC01013 RNA sequence (including 5'UTR, ORF region and 3'UTR) was synthesized and inserted into an expression plasmid by Genewiz Europe (Leipzig, Germany; construct available upon request). Linearized plasmid DNA (0.5 μ g) was transcribed and translated in vitro as described previously [25] using the TnT Coupled Wheat Germ Extract system (Promega, Mannheim, Germany) in the presence of 10 mCi/mL [³⁵S]-methionine (Hartmann Analytic, Braunschweig, Germany) according to manufacturer's instructions. To visualise the translation products, 5 μ L lysate was denatured for 2 min at 85 °C in 9.6 μ L Novex Tricine SDS Sample Buffer (2X) (Thermo Fisher Scientific)

and 1.4 μ L DTT (500 mM). Proteins were separated on 16% Tricine gels (Invitrogen) for 1 h at 50 V followed by 3.5 h at 100 V and blotted on PVDF-membranes (Immobilon-PSQ Membrane, Merck Millipore).

Statistics

Continuous data were expressed as mean \pm SEM. For two groups with normal distribution, data were compared using Student's *t*-test with multiple test correction if required. For normally distributed data with more than two groups and one dependent variable, one-way ANOVA was used, with Tukey's multiple test correction. For normally distributed data with more than two groups and two dependent variables, two-way ANOVA was used with Tukey's multiple test correction. Nonparametric data of more than two groups was analysed using Friedmann's test. Statistical analysis was performed with Prism 8.4.3.

Results

LINC01013 Is Expressed in Activated Human Cardiac Fibroblasts and Contains an Actively Translated smORF

In order to establish the profile of LINC01013 RNA expression in cardiac fibroblasts, we first evaluated transcripts using data previously derived from primary human cardiac fibroblasts (HCFs) activated with TGF β 1 and analysed by RNA-Seq [8]. This identified two alternate transcripts: a major form corresponding to NR_038981.1 and a shorter, minor form corresponding to NR_146223.1 (Fig. 1a). We interrogated transcripts for potentially translated regions using ribosome profiling (Ribo-Seq) data likewise obtained from TGF β 1-stimulated HCFs [31]. This identified four smORFs: three of these had only limited evidence of translation but one, located in exon 4 of and within the major NR_038981.1 transcript, had a notably high density of ribosome footprints with consistent trinucleotide periodicity, highly indicative of active translation (Fig. 1b-c). This smORF spans 168 bp, which has an ATG in a moderate Kozak context (AAGATGA), with coding potential for a 56AA micropeptide of a predicted mass of 6.3KDa. The NR_038981.1 transcript (hereon termed LINC01013) was therefore prioritised for further study together with the smORF contained therein (hereon named LINC01013ORF).

To evaluate LINC01013 expression in vivo, we interrogated single cell nucleus RNA sequencing (Nuc-Seq) data previously compiled from 14 healthy human hearts [38]. LINC01013 was detected almost exclusively in fibroblasts, myeloid cells and neuronal cells (Fig. 1e). Within the fibroblast population, LINC01013 was associated with

an extracellular matrix-producing cluster of fibroblasts (described as 'FB4' in Litviňuková et al. [38]), which express a range of markers of TGF β 1 stimulation and fibroblast activation (Fig. 1f). Conversely, LINC01013 was not associated with the quiescent 'FB1' fibroblast subcluster, which has the lowest expression of TGF β 1- and fibroblast activation-associated genes. LINC01013 was expressed in fibroblasts across all regions of the healthy human heart (Supplementary Fig. 1; information on fibroblast subtypes can be found in Supplementary Table 1).

Upregulation of the LINC01013 transcript under the experimental conditions used here was confirmed using cultured primary HCFs analysed by qPCR. LINC01013 expression was readily detectable and consistently and robustly upregulated after treatment with 5 ng/ml TGF β 1 for 24 h (Fig. 1d).

siRNA-Mediated Knockdown of LINC01013 Reduces the Fibrotic Phenotype at Baseline and After TGF β 1 Stimulation

To evaluate the effect of knocking down LINC01013 RNA on the fibrotic phenotype, we used a model of fibroblast activation: HCFs were serum-starved for 16 h, then treated with 5 ng/ml of TGF β 1 for 24 h, after which RNA markers of fibroblast activation were quantified: α -smooth muscle actin (*ACTA2*), interleukin 11 (*IL11*), periostin (*POSTN*), fibronectin (*FNI*) and collagen 1 α 1 (*COL1A1*) and were robustly increased as determined by qPCR, whilst matrix metalloproteinase (*MMP2*) remained unchanged (Supplementary Fig. 2).

We first assessed the effect of siRNA-mediated LINC01013 knockdown at baseline: HCFs were serum-starved for 16 h, before treatment with either anti-LINC01013 siRNA or nontargeting control (NC) siRNA. qPCR demonstrated effective knockdown of the endogenous LINC01013 RNA (Fig. 2a) and reduced fibroblast activation markers *ACTA2*, *IL11* (Fig. 2b, c) and *MMP2* (Supplementary Fig. 3) but not *POSTN* (Fig. 2d) at 24 h.

To evaluate the effect of LINC01013 knockdown on TGF β 1-mediated fibroblast activation, HCFs were serum starved for 16 h before treatment with either anti-LINC01013 siRNA or nontargeting control siRNA for 24 h and further cultured in either standard media or standard media + 5 ng/ml TGF β 1. LINC01013 knockdown blunted the response to TGF β 1, reducing *ACTA2* and *IL11* (Fig. 2b, c), but not *POSTN* (Fig. 2d) expression.

Overexpression of the LINC01013ORF Leads to Fibroblast Activation

We first used in vitro translation to validate production of the LINC01013ORF protein product (Supplementary

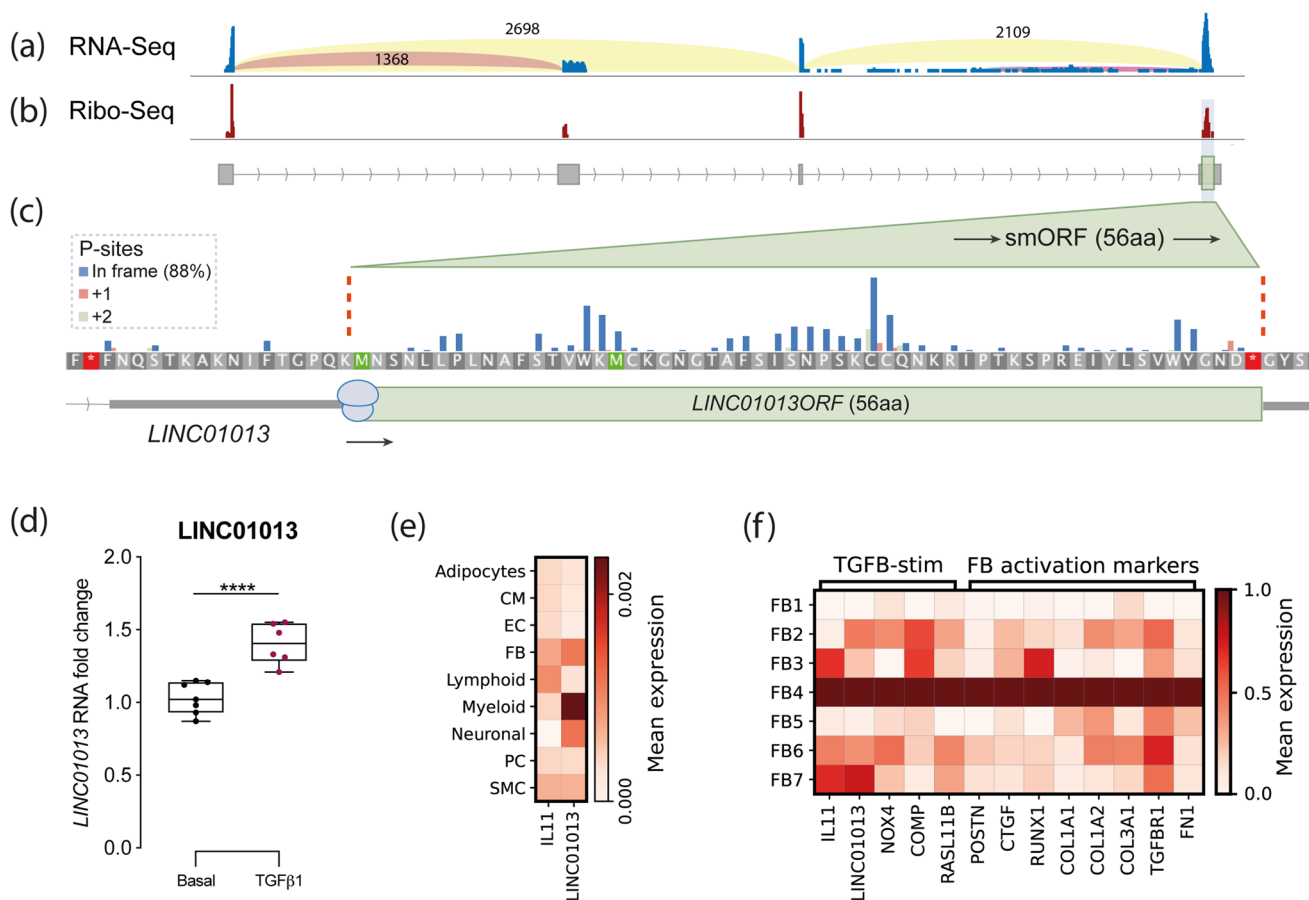


Fig. 1 LINC01013 is expressed in cardiac fibroblasts and encodes a novel micropeptide: LINC01013ORF. **a** RNA-Seq sashimi plot of RNA transcripts derived from the *LINC01013* gene identified in human cardiac atrial fibroblasts. Light-blue vertical bars indicate RNA read depth within exons, and arced lines quantify reads across high-confidence exon-exon junctions. Two transcripts were identified corresponding to NR_038981.1 (e1+e3+e4; herein named LINC01013) and NR_146223.1 (e1+e2). **b**, **c** Ribo-Seq analysis of identifies a high-confidence translated smORF within exon 4 contained in the LINC01013 transcript. Red bars in **b** show high densities of Ribo-Seq reads, of which are 88% in frame (blue bars, **c**) of

a translated smORF: LINC01013ORF (green box, **c**). High ribosome drop-off at the LINC01013ORF stop codon further supports active translation of this ORF (data not shown). Arrowed lines: intron; grey boxes: exon, green: translated region. **d** LINC01013 expression in HCFs is increased by TGFβ1 stimulation in vitro, using primers amplifying LINC01013ORF. $N=6$. ****= $p<0.0001$. **e** Nuc-Seq data showing that LINC01013 is expressed in fibroblasts, myeloid and neuronal cells. CM: cardiomyocyte, EC: endothelial cell, FB: fibroblast, PC: pericyte, SMC: smooth muscle cell. **f** LINC01013 expression is associated with markers of TGFβ1 stimulation and fibroblast activation, and ECM-producing fibroblast subtype 'FB4'

Fig. 4). To evaluate its functional role and to distinguish peptide driven effects, we used a codon-optimised form of LINC01013ORF, cloned into a pcDNA3.1 vector (pcDNA3.1-LINC01013ORF), driven by a constitutive CMV promoter. Control HCFs were transfected with an eGFP expression construct and transfection validated by visualisation of eGFP (Supplementary Fig. 5). In control cells, TGFβ1 treatment increased myofibroblast transdifferentiation markers: *ACTA2* and *POSTN*, profibrotic transcription factor *SNAIL* and its target ECM protein, *FNI*. Overexpression of LINC01013ORF increased these same markers (Fig. 3a-d) to the same level as seen with TGFβ1.

LINC01013ORF Encodes a Micropeptide that Localises to the Mitochondrial Matrix

To investigate the subcellular localisation of LINC01013ORF peptide, we overexpressed a LINC01013ORF-3×FLAG fusion peptide in HeLa cells, and co-stained with DAPI (nuclei), anti-FLAG (LINC01013ORF-FLAG) and anti-ATPIF (mitochondrial matrix). When cells were permeabilised with triton-X, the peptide encoded by LINC01013ORF (LINC01013ORF-pep) clearly co-localised with ATPIF (Fig. 4) indicating its mitochondrial localisation. Additionally, we permeabilised cells with digitonin which does not penetrate the

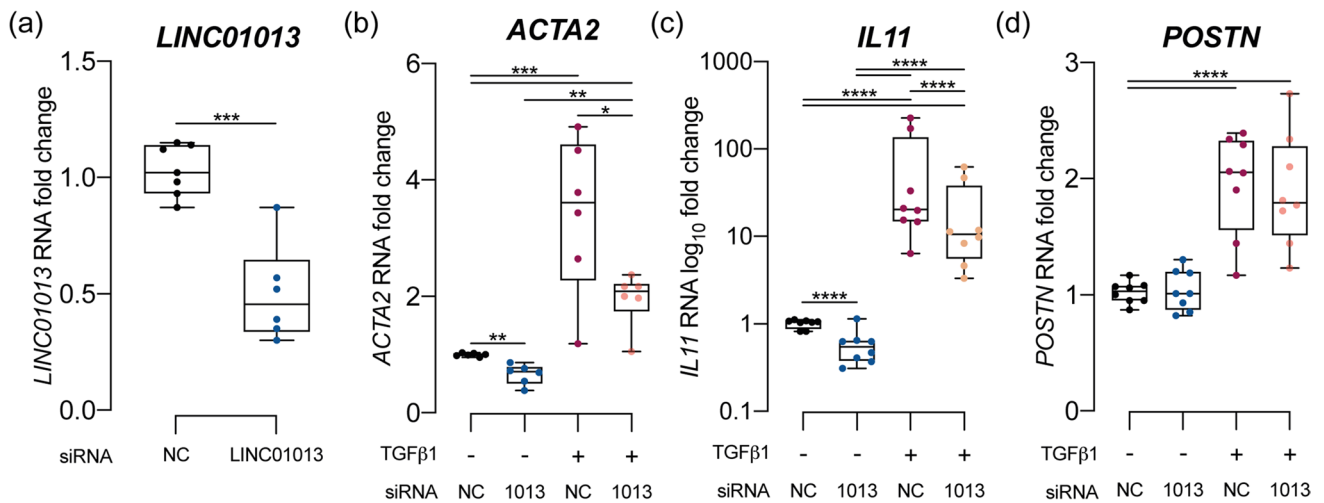


Fig. 2 LINC01013 knockdown using siRNA reduces fibroblast activation and blunts the response to TGFβ1. **a** LINC01013 RNA was knocked down effectively by anti-LINC01013 siRNA. **b–d** siRNA-mediated LINC01013 knockdown reduces markers of fibro-

blast activation *ACTA2* and *IL11* at baseline, and blunts their response to TGFβ1 stimulation. *POSTN* expression was not affected by LINC01013 knockdown. $N=6$. $*=p<0.05$, $**=p<0.01$, $***=p<0.001$, $****=p<0.0001$. NC: nontargeting control siRNA

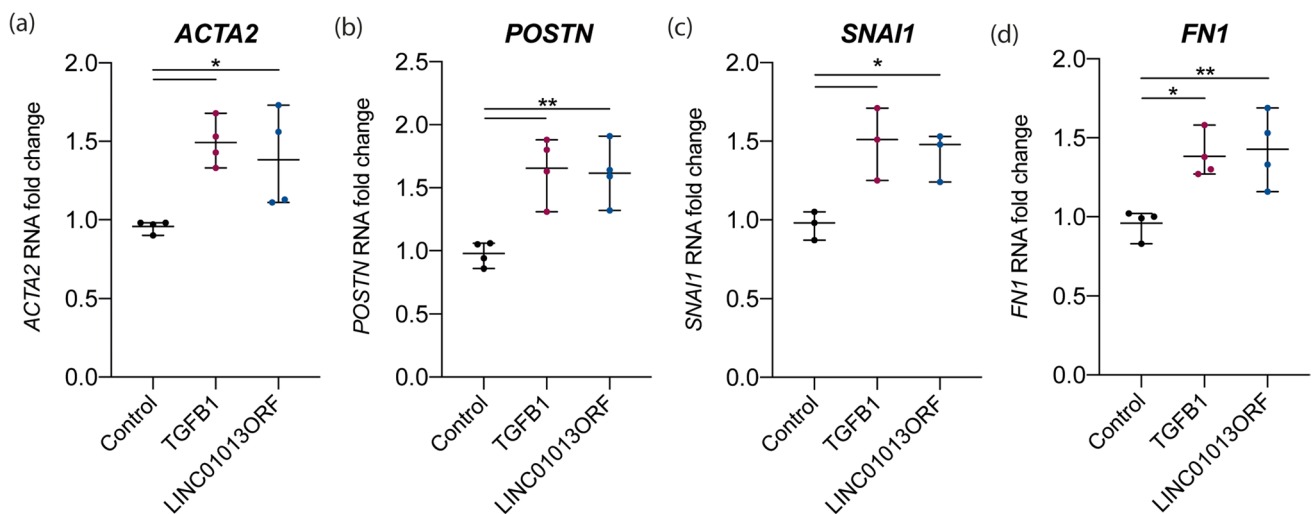


Fig. 3 Overexpression of LINC01013ORF activates fibroblasts. **a–d** HCFs were transfected with either eGFP (control) or codon-optimised LINC01013ORF expression constructs. Cells transfected with control constructs were further treated with 5 ng/ml TGFβ1 for 24 h, whilst LINC01013ORF-transfected cells were cultured in media alone.

TGFβ1 increased expression of *ACTA2*, *POSTN*, *SNAI1* and *FN1* in control cells. In the absence of TGFβ1, LINC01013ORF expression increased expression of *ACTA2*, *POSTN*, *SNAI1* and *FN1* to the same level as TGFβ1 stimulation. $N=4$, $*=p<0.05$, $**=p<0.01$, $***=p<0.001$

mitochondrial inner membrane and thus prevents antibody staining of mitochondrial matrix proteins, but permits that of those in the intermembrane space or outer mitochondrial membrane (OMM). Dropout of both ATP1F and FLAG signals, but not TOMM20 (OMM protein) with digitonin permeabilisation (Supplementary Fig. 6), indicates that LINC01013ORF micropeptide, like ATP1F, localises to the mitochondrial matrix.

Discussion

We have demonstrated that LINC01013 is upregulated in the response of cardiac fibroblasts to TGFβ1, and that it harbours a previously undocumented smORF that is translated and encodes a biologically active peptide. Knockdown of LINC01013 reduces markers of fibroblast activation at baseline and blunts the response to TGFβ1, whilst

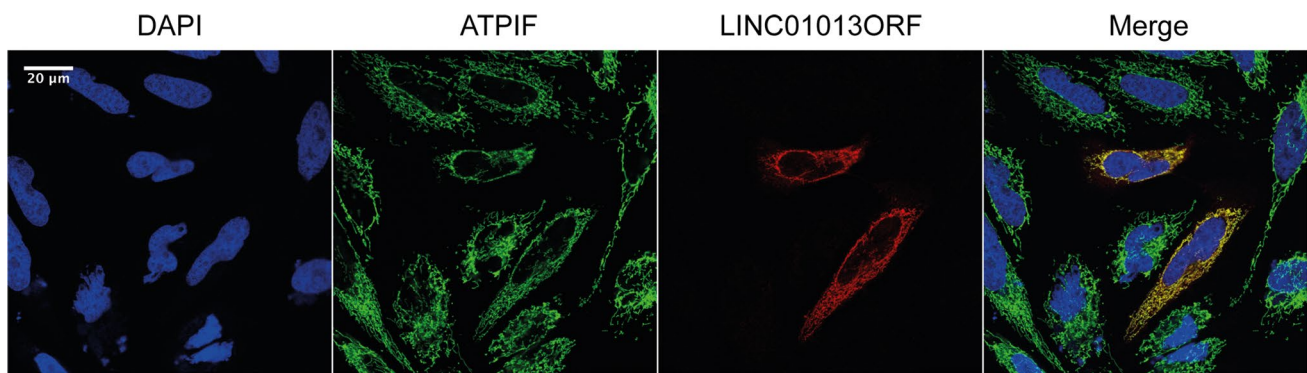


Fig. 4 LINC01013ORF localises to the mitochondrial matrix. LINC01013-ORF-3×FLAG was expressed in HeLa cells. Permeabilisation with triton-X and co-staining the mitochondrial matrix

protein ATPIF (GFP) and LINC010FLAG (mCherry) demonstrated co-localisation of LINC01013ORFpep and mitochondria. $N=4$, representative images are shown

overexpression of LINC01013ORF induces markers of activation to the same level as seen with TGF β 1 stimulation. To our knowledge, LINC01013ORF is the only known translated smORF described to date to be associated with TGF β 1 signalling.

To date LINC01013 has remained largely uncharacterised and the presence of a smORF encoded peptide has not been previously reported. Our data show that LINC01013 is directly implicated in TGF β 1-mediated fibroblast activation and that this effect is mediated, at least in part, due to the presence of a smORF encoded peptide. Whilst this study was under review, others have reported similar activation of LINC01013 by TGF β 1 in aortic valve fibroblasts [39].

TGF β 1-mediated fibroblast activation can occur both via canonical (SMAD-mediated) and noncanonical (SMAD-independent) pathways [40]. We propose that the effects of LINC01013ORF peptide may act primarily through the canonical pathway: siRNA-LINC01013 mediated knock-down reduced expression of SMAD-dependent genes *ACTA2* and *IL11*, but did not reduce *POSTN* (Fig. 2) the expression of which is upregulated by noncanonical p38 MAPK [41]. LINC01013ORF overexpression activated the canonical target genes *ACTA2* and *SNAI1* (Fig. 3). It also resulted in increased *FN1* and *POSTN* expression which is likely due to direct *SNAI1*-mediated transcription [42], or activation of the Wnt/ β -catenin, pathway which is known to act on a broad range of genes involved in fibroblast activation [43]. Our data therefore align with previous findings of a LINC01013-SNAI1-FN1 pathway [27], and suggest the translated ORF within LINC01013 may be the active player involved.

Further supportive of its role in TGF β 1 signalling, we show that in the human heart, LINC01013 is positively associated with the previously described [38] subpopulation of fibroblasts that express markers of TGF β 1 activation and ECM production, and negatively associated with a subpopulation of quiescent fibroblasts.

The mechanisms by which LINC01013ORF encoded peptide acts upon TGF β 1 signalling remain uncertain. When overexpressed, LINC01013ORF peptide localises to the mitochondria. Mitochondria-mediated metabolic drivers that broadly activate fibroblasts are well described, acting via reactive oxygen species (ROS) activating p38 and ERK1/2 pathways [44]. It is therefore possible that the profibrotic effect of LINC01013ORF peptide may be through overall metabolic stress. Of note, overexpression can influence a protein localisation and future work should resolve the localisation of the endogenous protein to substantiate the connection to mitochondria and fibroblast activation. FLAG-tag fusion can also potentially influence subcellular localisation, though has not typically induced mitochondrial localisation in the study of other smORFs [25].

More broadly, our data demonstrate how Ribo-Seq can be leveraged in the discovery of novel translated targets. Many lncRNAs have been implicated in cardiac disease [45], including myocyte dysfunction [46] and as potential biomarkers post infarct [47], and our work highlights the importance of considering an active role of biologically active translated smORFs within these: there is a growing body of evidence for functional smORF peptides [22, 23, 48]. lncRNAs harbouring smORFs may thus potentially act via coding and non-coding mechanisms, with synergistic or independent functions.

In conclusion, we propose that LINC01013 functions downstream of TGF β 1 and may represent a potential therapeutic target in limiting fibroblast activation. We have demonstrated it to be both necessary and sufficient for fibroblast activation at the RNA level, acting potentially via the canonical TGF β 1 pathway. Fibrosis is a determinant of clinical outcome in the heart, liver, lung and kidneys, and so large cohorts of patients with established fibrosis, or who are at risk of its development, can potentially benefit from antifibrotic therapy. To our knowledge, LINC01013ORF is the first smORF to be directly implicated in fibroblast activation,

and our work highlights the importance to consider biologically active smORFs within putatively non-coding regions. Finally, whilst our observations have been made in cardiac fibroblasts, due to mechanistic homology, they are potentially translatable to fibrosis in other tissues.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s12265-022-10288-z>.

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Declarations

Ethics Approval No human or animal studies were carried out by the authors for this article.

Conflict of Interest S.A.C and S.S are cofounders and shareholders of Enleofen Bio Pte. Ltd. All other authors declare no competing interests.

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