c-Jun N-terminal kinase/c-Jun inhibits fibroblast proliferation by negatively regulating the levels of stathmin/oncoprotein 18


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INTRODUCTION

The JNKs (c-Jun N-terminal kinases) are stress-activated serine/threonine kinases that can regulate both cell death and cell proliferation. We have developed a cell system to control JNK re-expression at physiological levels in JNK1/2-null MEFs (murine embryonic fibroblasts). JNK re-expression restored basal and stress-activated phosphorylation of the c-Jun transcription factor and attenuated cellular proliferation with increased cells in G1/S-phase of the cell cycle. To explore JNK actions to regulate cell proliferation, we evaluated a role for the cytosolic protein, STMN (stathmin)/Op18 (oncoprotein 18). STMN, up-regulated in a range of cancer types, plays a crucial role in the control of cell division through its regulation of microtubule dynamics of the mitotic spindle. In JNK1/2-null or c-Jun-null MEFs or cells treated with c-Jun siRNA (small interfering RNA), STMN levels were significantly increased. Furthermore, a requirement for JNK/c-Jun signalling was demonstrated by expression of wild-type c-Jun, but not a phosphorylation-defective c-Jun mutant, being sufficient to down-regulate STMN. Critically, shRNA (small hairpin RNA)-directed STMN down-regulation in JNK1/2-null MEFs attenuated proliferation. Thus JNK/c-Jun regulation of STMN levels provides a novel pathway in regulation of cell proliferation with important implications for understanding the actions of JNK as a physiological regulator of the cell cycle and tumour suppressor protein.

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In addition to its growth-promoting effects, some evidence also suggests that cellular Jun, like the related JunB and JunD proteins, can negatively regulate proliferation. Specifically, JNK/c-Jun signalling can induce apoptosis and so limit cell numbers and tumour development [13]. However, comparatively little is known regarding the ability of JNK/c-Jun signalling to slow cell-cycle progression directly.

The proliferation of cells involves the progression from G1/S through to the G2/M mitotic phase with each checkpoint exquisitely regulated by cell-cycle-regulatory proteins such as p53 and the cyclin–cycillin-dependent kinase complexes [20]. In addition, cytoplasmic microtubule-regulatory proteins, such as the STMN (stathmin) family, are important in cell-cycle regulation [21,22]. STMN is ubiquitously expressed and plays a key role in maintaining dynamics of the MT (microtubule) network through its ability to bind α/β-tubulin heterodimers to control the turnover of microtubules (reviewed in [21]). In this way, STMN is also critically important for mitotic spindle formation, with dysregulated STMN activity or expression resulting in G2/M arrest due to activation of the mitotic spindle checkpoint [23,24]. Indeed, STMN levels are closely correlated with cell proliferation, with enhanced STMN expression observed in highly proliferating cells or during re-initiation of proliferation in quiescent cells [25–27]. Furthermore, elevated STMN levels have been widely reported in a vast range of different tumour types [25,28]. Importantly, targeted down-regulation of STMN levels inhibits the growth of cancer cells [29,30] highlighting a key function for STMN in the regulation of cell proliferation and tumour malignancy.

STMN activity is regulated by multi-site phosphorylation of four highly conserved residues (Ser16, Ser24, Ser37 and Ser63) that negatively impacts on STMN–tubulin association and therefore promotes MT stabilization [21]. During cell-cycle progression, STMN phosphorylation (Ser24 and Ser37) by cyclin-dependent kinases during G2/M transition is critical for proper mitotic progression [31]. In addition to post-translational modification, the overexpression of STMN frequently reported in cancer is regulated by transcription factors such as Egr1, p53 and members of the E2F family [32–34]. These studies highlight the importance of transcriptional events that regulate STMN expression levels in the control of cancer cell proliferation. Interestingly, ectopically expressed c-Jun has been previously shown to phosphorylate STMN-like proteins [36], the contributions of JNK/c-Jun in the regulation of STMN levels have not been investigated in detail.

In the present study, we have characterized JNK-dependent signalling and downstream events with greater control of the timing and levels of JNK protein expression. Our development of a novel lentiviral expression system has facilitated generation of a gain-of-function model to study JNK-dependent events. Specifically, we have established stable cell lines with tamoxifen (4-hydroxytamoxifen)-inducible expression of JNK1 and JNK2 in a JNK-null (JNK1/2−/−) MEF (murine embryonic fibroblast) background. Critically, we showed reconstitution of functional JNK1 and JNK2 isoforms to physiological levels. Using this cell system, we have demonstrated, for the first time, a link between the JNK–c-Jun signalling axis and decreased STMN levels. Furthermore, the shRNA (short hairpin)-directed STMN down-regulation in JNK1/2−/− null MEFs attenuated proliferation. JNK/c-Jun regulation of STMN levels thus provides a novel pathway in the regulation of cell proliferation with important implications for understanding the actions of JNK as a physiological regulator of the cell cycle and tumour suppressor protein.

**EXPERIMENTAL**

**Antibodies and reagents**

Anti-phospho-JNK1/2 and -JNK1/2 antibodies were from BD Biosciences. Anti-STMN, -c-Jun and -phospho(Ser38)-c-Jun antibodies were from Cell Signaling Technology. Anti-phospho(Ser54)-STMN and phospho(Ser58)-STMN antibodies were from Abcam. Anti-GAPDH (glyceraldehyde-3-phosphate dehydrogenase) and -PARP-1 [poly-(ADP-ribose) polymerase 1] antibodies were from Santa Cruz Biotechnology. The JNK inhibitor VIII [N-(4-amino-5-cyano-6-ethoxypyridin-2-yl)-2-(2,5-dimethoxyphenyl)acetamide] and the MEK [MAPK (mitogen-activated protein kinase)/ERK (extracellular-signal-regulated kinase) kinase] inhibitor U0126 were from Calbiochem. All other reagents, including 4HT, were obtained from Sigma-Aldrich.

**Plasmid expression and lentiviral constructs**

pF-5x-UAS-MCS-SV40-puroGEV16-W (Figure 1A) was created by generating a fusion product containing the SV40 (simian virus 40) early promoter, puromycin resistance and GAL4 ERT2 (third generation oestrogen receptor mutant) and VP16 transcription factor (GEV16) by overlap PCR and cloning into pF-5x-UAS-MCS-W [37]. Puromycin resistance and the inducibility of the GEV16 were confirmed using GFP (green fluorescent protein) expression from this new single-vector system. This system simplifies the previously described double-vector system [37] and provides a single infection procedure to rapidly generate selectable 4HT-inducible cell lines with high efficiency.

Myc-tagged JNK1 (JNK1α1), JNK2 (JNK2α2) or a GFP control insert were cloned into pF-5x-UAS-MCS-SV40-puro-GEV16-W, using Agel/Nhel restriction sites. Lentiviral particles were then generated by transfection (LipofectamineTM 2000; Invitrogen) of JNK expression plasmids [along with pCMV ΔR8.2 and pCMV-BSV encoding lentiviral structural components] into HEK (human embryonic kidney)-293T cells. Cell transfections were performed in serum-replacement medium without antibiotics (OptiMEM; Invitrogen) for 5–6 h routinely before returning to growth medium. Virus was then collected 48 h post-transfection and used for subsequent transduction into JNK1/2−/− MEFs [100 MOI (multiplicity of infection), 24 h] in the presence of Polybrene (1 μg/ml). Lentiviral-infected cells were then maintained in normal growth medium for a further 24 h before selection with puromycin (8 μg/ml) for 3 days. Polyclonal populations of puromycin-resistant MEFs expressing GFP, JNK1 or JNK2 alone, or combined JNK1 and JNK2, were then expanded and used for subsequent analyses.

**Cell culture**

**JNK1/2−/−, JNK1/2−/− or JNK1/2−/− lines with iJNK (inducible JNK; iJNK1/2; iJNK1 and iJNK2) or iGFP (inducible GFP) expression, BE colon cancer cells, c-Jun−/− and stably reconstituted [c-Jun wt (wt is wild-type), c-Jun-AA] MEFs and STMN shRNA stable-knockdown MEFs were maintained in a humidified 5% CO2 environment in DMEM (Dulbecco’s modified Eagles medium) supplemented with 10% fetal calf serum and penicillin/streptomycin (100 units/ml). Inducible MEF cells were induced with 4HT (10 nM) for 24 or 48 h to reconstitute JNK or GFP expression.**

**Cell lysis and immunoblotting**

Total cell lysates were prepared in RIPA buffer [50 mM Tris/HCl (pH 7.3), 150 mM NaCl, 0.1 mM EDTA, 1% (w/v) sodium
JNK/c-Jun downregulates stathmin and cell proliferation

**Figure 1** Induction of JNK expression in JNK-null fibroblasts recapitulates signalling to c-Jun

(A) Schematic diagram of the novel 4HT-inducible JNK expressing lentiviral vector, shown here specifically for JNK1 (JNK1α1). JNK expression, driven by a GAL4-5 UAS promoter, is repressed by Hsp90 (heat-shock protein 90) binding of the SV40 promoter-driven GAL4-ERt2-VP16 transactivating protein. Induction, as driven by the exposure to 4HT, releases GAL4-ERt2-VP16 from Hsp90 and so promotes JNK expression. The vector also contains a puromycin-resistance gene (PuroR). hCMV, human cytomegalovirus promoter sequence; HIV-1 flap, HIV DNA flap; U3, promoter region; U5, recognition site for viral integrase; WRE, woodchuck hepatitis B virus RNA regulatory element.

(B) The expression of Myc-tagged JNK1 and JNK2 (iJNK1/2) was induced with 4HT (10 nM for 24 h) and levels of JNK compared with wild-type (JNK1/2+) and JNK-null (JNK1/2−) MEFs by immunoblotting with an anti-pan-JNK (JNK1/2) antibody. Immunoblotting with the anti-Myc antibody confirmed lentiviral-driven Myc–JNK expression. c-Jun phosphorylation at the activating Ser73 (pS73) and expression (c-Jun) were also determined by immunoblotting. GAPDH levels were used as a loading control.

(C) Immunoblot analysis as in (B), but with JNK1 or JNK2 isoform-specific inducing MEFs (iJNK1 and iJNK2). JNK1/2−/−, JNK1/2+/+ and 4HT induced (10 nM for 24 h) iJNK1 and iJNK2 MEFs were treated with sorbitol (0.5 M for 30 min). An anti-pan-JNK (JNK1/2) antibody showed JNK expression levels and active JNK was detected through immunoblotting with a phospho-specific anti-JNK antibody (p-JNK1/2). Immunoblotting with the anti-Myc antibody confirmed lentiviral-driven JNK expression. c-Jun phosphorylation (pS73) and expression (c-Jun) were also determined by immunoblotting. GAPDH levels were used as a loading control.

**siRNA (small interfering RNA)**

c-Jun ON-TARGETplus™ Smart Pool siRNA and non-targeting control siRNA (Dharmacon) were used according to the manufacturer’s instructions. Briefly, siRNAs (20 nM) were transiently transfected with Lipofectamine™ 2000, washed and cultured for a further 48 h before preparing protein lysates and immunoblotting.

Expression vectors encoding STMN shRNA were constructed by ligating annealed oligonucleotides into pSuper.neo (Oligoengine). The double-stranded oligonucleotide sequences targeting STMN were: 5′-GATCCCCAATTGAGGCTACTACGTTCAAGAGACTCTTTTGTAGCCTCAATTTTTT-3′ and 5′-TGGAGAAAATATGGAGGCTACTACTTTTGTAGCCTCAATTTTTT-3′ and 5′-TGGAGAAAATATGGAGGCTACTACTTTTGTAGCCTCAATTTTTT-3′ and 5′-TCGAGAAAAATATGGAGGCTACTACTTTTGTAGCCTCAATTTTTT-3′. A non-targeting sequence (5′-GATCCCCAATTGAGGCTACTACGTTCAAGAGACTCTTTTGTAGCCTCAATTTTTT-3′ and 5′-TCGAGAAAATATGGAGGCTACTACTTTTGTAGCCTCAATTTTTT-3′) was used as a control.

**Real-time PCR**
iJNK1/2 MEFs were induced with 4HT or an ethanol vehicle for 24 h before total RNA was isolated using the PureLink RNA mini kit (Invitrogen). In total, 1 µg of RNA was used to synthesize cDNA using the Omniscript RT kit (Qiagen). cDNA was synthesized at 37 °C for 1 h. Real-time PCR was then performed using the mouse STMN Taqman assay (Applied Biosystems) and the Taqman Gene Expression Master Mix on the ABI 7300 Analyser using a two-step PCR protocol of 95 °C for 15 s and 60 °C for 1 min. PCR data was analysed using the ABI 7300 software and further analysis was performed in Microsoft Excel. STMN mRNA expression is calculated as the reverse log of C and normalized against the standard 18S rRNA.

**Cell proliferation**

Cell proliferation was determined by labelling metabolically active cells with the yellow tetrazolium salt, XTT (sodium 3-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis-(4-methoxy-6-nitro) benzene sulfonic acid hydrate), using the XTT Cell Proliferation Kit (Roche) according to the manufacturer’s
protocol. Briefly, 2 × 10^3 MEFs were seeded per well in a 96-well plate, treated with 4HT and cell numbers assayed after 24 and 48 h by the addition of tetrazolium dye. STMN-shRNA-expressing MEFs were assayed after 24 and 48 h without 4HT. The XTT labelling reagent was added during the final 4 h then the absorbance at 492 nm and 690 nm was measured to indicate cell density \((A_{492} − A_{690})\).

**Cell-cycle analysis**

iJNK MEFs, treated with 4HT (10 nM, 24 h) or a vehicle control, were trypsinized, washed with PBS, collected and fixed in 80% ice-cold ethanol for 1 h on ice. Cells were then pelleted, washed in PBS and then stained with propidium iodide (50 μg/ml) in PBS supplemented with RNase A (25 μg/ml) for 30 min at room temperature (24°C). Stained cells were then analysed on a FACS Calibur flow cytometer (BD Biosciences).

**RESULTS**

A novel JNK expression system reveals that c-Jun serine phosphorylation can be equivalently regulated by JNK1 or JNK2

In addition to its well-characterized role as a stress-induced regulator of apoptotic cell death, JNK is recognized as a physiologically important activator of the c-Jun transcription factor, with the JNK–c-Jun signalling axis characterized as a critical regulator of cell proliferation [12,13]. However, studies evaluating whether the individual JNK isoforms, JNK1 and JNK2, contribute differently to the regulation of cell proliferation have yielded different conclusions that may reflect additional specific details of the system investigated, such as the level of JNK isoform expression, the levels of suppression of JNK isoform expression achieved by siRNA approaches, or the broader context of altered signalling and other cellular processes in normal or transformed cells [15,16].

Thus, to address the contribution of JNKs to proliferation in a defined cell system and to study JNK-dependent signalling and downstream events with greater control on the timing and levels of JNK protein expression, we developed a novel gain-of-function cell system. Specifically, we developed a lentiviral system by cloning JNK1α or JNK2α sequences into the pF5x-UAS-MCS-SV40-puroGEV16-W vector (Figure 1A). Throughout the present study, these JNK isoforms are simply referred to as JNK1 and JNK2 respectively. This system simplified the previously described double-vector system [37] because it provided a single infection procedure to rapidly generate selectable 4HT-inducible stable cell lines with high efficiency.

Using JNK1/2−/− MEFs, we generated 4HT-inducible lines to reconstitute JNK1 and JNK2 together (iJNK1/2, Figure 1B) or JNK1 or JNK2 alone (iJNK1 or iJNK2 respectively, Figure 1C).

In all cases, an N-terminal Myc-epitope tag was additionally included to allow specific detection of exogenous protein expression. Thus we confirmed JNK expression following 4HT induction (10 nM, 24 h) by blotting for the Myc-tag of the expressed JNK construct (Figures 1B and 1C, middle panels). Furthermore, immunoblotting with the anti-pan-JNK antibody (Figures 1B and 1C, upper panels) revealed a key feature of our system: the re-expression of the JNK1 and JNK2 proteins to levels comparable with those observed for the endogenous JNK isoforms in the wild-type cells (JNK1/2−/+). Reprobing for GAPDH was performed as a control and confirmed equal protein loading (Figures 1B–1D).

With the success of the JNK isoform expression in the iJNK1 or iJNK2 MEFs to levels comparable with those observed physiologically in the JNK1/2−/+ cells, we next examined phospho-c-Jun levels as an indicator of reconstituted signalling events downstream of JNK. The re-expression of either JNK1 or JNK2 in these cells was sufficient to restore basal c-Jun phosphorylation (Figure 1C). Similarly, for iJNK1 or iJNK2 cells under conditions of hyperosmotic stress, the phosphorylation of c-Jun, JNK1 and JNK2 proteins was comparable with those levels observed in the JNK1/2−/+ MEFs following hyperosmotic stress (Figure 1D). Thus our results highlight the overlapping function of JNK isoforms JNK1 and JNK2 in the regulation of cell proliferation under both basal and stress-stimulated conditions.

**JNK1 or JNK2 expression is sufficient to negatively regulate cell proliferation**

To evaluate the contribution of JNK more broadly in the regulation of cell proliferation, we next compared the proliferation of the immortalized JNK1/2−/+ and JNK1/2−/− MEFs. Using the XTT assay as an estimate of viable cell numbers after 48 h in culture, we noted that JNK1/2−/− MEF numbers increased at a significantly greater rate when compared with wild-type cells (Figure 2A). Importantly, the proliferation rates following the re-expression of either JNK1 and/or JNK2 in the iJNK MEFs also decreased to levels comparable with those observed in the wild-type (JNK1/2−/+) MEFs (Figure 2B). This effect was not due to exposure to 4HT alone as control experiments showed no significant change in cell proliferation of the JNK1/2−/+ MEFs that lacked iJNK expression constructs, when exposed to equivalent 4HT treatment (10 nM for 48 h; Figure 2B). Furthermore, proliferation of an inducible JNK1/2−/− MEF line expressing GFP as a control did not change significantly following 4HT treatment (Figure 2B), indicating that the decreased proliferation in JNK1/2−/− MEFs was the result of JNK re-expression. Immunoblot analysis confirmed JNK expression in iJNK MEFs after 48 h of 4HT treatment (Figure 2C).

We next examined whether the lower cell numbers upon JNK re-expression may result from JNK1/2-dependent triggering of cell death. We examined cleavage of PARP-1, an early marker of apoptotic caspase activation and cell death (Figure 3A). Although hyperosmotic stress treatment of JNK1/2−/+ cells decreased the levels of full-length PARP-1 (113 kDa) with the concomitant increased formation of a cleavage product (89 kDa), there were no observed changes in full-length or cleaved PARP-1 in iJNK1/2−/− MEFs following 4HT exposure (Figure 3A). These results show that JNK expression did not contribute significantly to cell death under these basal conditions.

To evaluate the influence of JNK expression on cell-cycle distribution, we used propidium iodide staining followed by flow cytometry. The induction of JNK1 and JNK2 expression in the iJNK1/2−/− MEFs significantly decreased the population of cells in G1/M (28.6 ± 0.9% to 16.6 ± 0.6%, P < 0.05, n = 3) and increased the cells in G2/S (71.4 ± 0.9% to 83.4 ± 0.6%) (Figure 3B). Therefore JNK re-expression was sufficient to trigger the slowing in cell proliferation with a reduction in cells actively undergoing mitosis. When we repeated these cell-cycle distribution experiments with the iJNK1 or iJNK2 cells, expression of either JNK1 or JNK2 isoforms was sufficient to decrease the population of cells in G1/M and increase in the cells in G2/S (Figure 3B). In Figure 3(C), representative histograms show the effect of 4HT induction on cell-cycle distribution in iJNK1/2 MEFs. Taken together, these results indicate that the expression of JNK1 or JNK2 at physiological levels is sufficient to negatively regulate fibroblast proliferation under normal conditions.
JNK negatively regulates STMN levels

To examine potential mediators of the JNK-induced repression of cell proliferation, we considered the regulation of STMN, a cell-cycle-regulatory protein highly expressed in many rapidly proliferating cells [22]. Although members of the STMN-like proteins, specifically SCG10, have been shown to be JNK substrates involved in neurogenesis [36], STMN has not previously been shown to be directly targeted by JNK during cell proliferation. Therefore we first tested the possibility of a JNK–STMN interaction. We did not find evidence of JNK binding of STMN using a yeast two-hybrid assay (Supplementary Figure S1 at http://www.BiochemJ.org/bj/430/bj4300345add.htm). Similarly, STMN did not co-immunoprecipitate with anti-JNK antibody incubation and affinity isolation from JNK1/2+/+ MEF lysates (results not shown) which is in agreement with a previous report [36]. Consistent with these findings, the chemical inhibition of JNK did not prevent growth-factor-stimulated STMN phosphorylation which was completely ablated with a MEK/ERK inhibitor (Supplementary Figure S2 at http://www.BiochemJ.org/bj/430/bj4300345add.htm). These results indicated that JNK1/2 did not directly regulate STMN in the context of mitogen-stimulated cellular proliferation.

Instead, we considered the possibility that JNK signalling may contribute to the regulation of STMN levels. Indeed, the levels of STMN protein were strikingly higher in the more rapidly proliferating JNK1/2−/− MEFs with levels barely detectable in the wild-type (JNK1/2+/+) counterparts (Figure 4A). Importantly, we showed that the induction of JNK1 and/or JNK2 expression was sufficient to substantially reduce STMN protein levels, whereas the levels of the control protein, GAPDH, remained constant (Figure 4A). In control iGFP MEF cells, we showed only a
Figure 4  JNK1 or JNK2 negatively regulates STMN levels in JNK-inducible fibroblasts

(A) Stable inducible JNK MEF cells (JNK1/2, JNK1 or JNK2) were treated with 4HT (10 nM for 24 h). Stable inducible GFP MEFs (iGFP), wild-type (JNK1/2+/+) and JNK-null MEFs (JNK1/2−/−) were examined as controls. Protein lysates were blotted for STMN. Lysates from iGFP cells were additionally immunoblotted for GFP expression. GAPDH levels were blotted as a loading control.

(B) JNK1/2 MEFs were treated with 4HT (10 nM) or an ethanol (0.1 %) vehicle control for the times indicated. Protein lysates were then prepared. Following transfer on to PVDF membranes, the upper portion was blotted for JNK and the lower portion blotted for STMN levels as indicated.

(C) JNK1/2 MEFs were induced with 4HT (24 h) or an ethanol (0.1 %) vehicle control before RNA was extracted and STMN mRNA levels determined by quantitative real-time PCR. Values are expressed relative to 18S rRNA and are means ± S.E.M. (n = 5, *P < 0.01).

marginal decrease in STMN protein levels following 4HT-induced GFP expression (Figure 4A). However, this was not to the extent of STMN protein decrease from 4HT induction in JNK MEFs (Figure 4A).

To define more closely the links between JNK expression and loss of STMN protein levels, we took advantage of the inducible expression of JNK1 and JNK2 and examined the time course of changes in JNK and STMN levels in greater detail. First, although we loaded equivalent protein for each sample, STMN levels in uninduced cells was increased at 16 h indicating an increase in the proportion of STMN as a percentage of total protein (Figure 4B). This increase may be linked to the increased cell density during continuous culture. A previous study in C2 myoblasts demonstrated that the cellular content of STMN was increased when cell density exceeded a certain threshold [39]. With detailed evaluation of changes over a 1–16 h period following induction of JNK1/2 with 4HT, we showed detectable JNK1/2 expression within 4 h of 4HT induction, highlighting the rapid responses in expression possible in this system (Figure 4B). A modest decrease in STMN levels observed 8 h post-4HT induction became more evident with 16 h exposure to 4HT (Figure 4B). Thus the reduction in STMN protein levels followed JNK1/2 re-expression in iJNK1/2 MEF cells.

We next investigated whether JNK regulation of STMN was transcriptional by measuring STMN mRNA levels with quantitative real-time PCR. The induction of JNK1/2 expression in iJNK1/2 MEFs with 4HT was sufficient to significantly reduce STMN mRNA levels (Figure 4C). Furthermore, we measured STMN protein turnover in JNK1/2−/− and wild-type MEF counterparts by inhibiting de novo protein synthesis with cycloheximide. We found that the rate of STMN loss following cycloheximide treatment was not substantially different between the two cell types which suggests that JNK expression does not have an impact on STMN protein stability (Supplementary Figure S3 http://www.BiochemJ.org/bj/430/bj4300345add.htm). Taken together, our results indicate that JNK1/2 reduced STMN transcript levels to negatively regulate STMN protein levels.

JNK signalling to c-Jun negatively regulates the cell-cycle-regulatory protein STMN

With the well-established actions of JNK in signalling to c-Jun and a previous report indicating that overexpressed c-Jun increased STMN expression [35], we evaluated whether c-Jun may be involved in regulating STMN levels in our studies. Indeed, we confirmed higher levels of STMN in c-Jun−/− MEFs when compared with their wild-type (c-Jun+/+) controls (Figure 5A). Similarly, the down-regulation of c-Jun levels by transient expression of c-Jun siRNA in JNK1/2−/− MEFs (Figure 5B) and colon cancer BE cells (Figure 5C) increased STMN levels, whereas a non-targeting control siRNA sequence was without effect. These results indicate that the levels of c-Jun are critical negative regulators of STMN levels in the cell under normal conditions and thus suggest that the JNK–c-Jun signalling axis may provide a critical point of control under basal conditions.
JNK/c-Jun downregulates stathmin and cell proliferation

Figure 5  JNK regulation of STMN levels is dependent on signalling via c-Jun

(A) Lysates isolated from c-Jun-null (c-Jun<sup>−/−</sup>) and wild-type (c-Jun<sup>+/+</sup>) MEFs were blotted for STMN, c-Jun and GAPDH. (B) JNK1/2<sup>−/−</sup> MEFs were transfected with c-Jun siRNA (10–50 μM), maintained for 48 h before cell lysis and then blotted for STMN, c-Jun and GAPDH. Mock transfection without siRNA or with non-targeting siRNA served as controls. (C) BE colon cancer cells, transfected with c-Jun siRNA or control siRNA, were similarly blotted for STMN, c-Jun and GAPDH. (D) c-Jun<sup>−/−</sup> MEF or c-Jun<sup>−/−</sup> MEFs stably expressing wild-type (c-Jun-wt) or c-Jun S63A/S73A mutant (c-Jun-AA) were analysed for STMN, c-Jun and c-Jun Ser73 phosphorylation levels by immunoblotting. Levels of STMN were quantified and the values indicated below the upper panel.

To evaluate the role of JNK-regulated c-Jun activity directly in STMN regulation, we analysed c-Jun<sup>−/−</sup> MEFs reconstituted to stably express the wild-type c-Jun (c-Jun-wt) protein or a c-Jun mutant with the JNK target sites Ser<sup>63</sup> and Ser<sup>73</sup> mutated to non-phosphorylatable alanine residues (c-Jun-AA). When we restored expression with c-Jun-wt, STMN levels were reduced by 48% (Figure 5D) consistent with the actions of c-Jun to negatively regulate STMN levels. In contrast, STMN levels were increased by 26% following the re-expression of the c-Jun-AA mutant in the c-Jun<sup>−/−</sup> MEFs (Figure 5D). These results indicated that the JNK-mediated phosphorylation of c-Jun (at Ser<sup>63</sup> and Ser<sup>73</sup>) is critical in the negative regulation of STMN levels by c-Jun and supports a model in which the JNK–c-Jun signalling axis is a critical negative regulator of STMN levels under basal conditions.

Finally, to confirm that the elevated STMN levels contributed to proliferation in JNK1/2<sup>−/−</sup> MEFs, we generated JNK1/2<sup>−/−</sup> lines stably expressing either a STMN shRNA or a non-targeting control sequence. We confirmed by immunoblot analysis that STMN expression was down-regulated in STMN shRNA-expressing cells, whereas the non-targeting control shRNA was without effect (Figure 6A). When we measured cell proliferation, we observed that the numbers of STMN shRNA-expressing cells increased at a significantly lower rate when compared with cells expressing control shRNA (Figure 6B). This result confirms that elevated STMN expression is sufficient to increase proliferation in the JNK-null fibroblasts.

DISCUSSION

JNK1 or JNK2 expression reveals common actions for JNK isoforms in c-Jun phosphorylation

Studies utilizing isoform-specific JNK1- or JNK2-knockout MEFs can be subject to concerns that the long-term loss of JNK isoforms may lead to compensatory changes that indirectly have an impact on cellular function. In the present study, we have developed a gain-of-function model to explore JNK-regulated signalling and downstream events. We developed stable fibroblast cell lines for the controlled expression of JNK1 and JNK2 isoforms, alone and in combination, at physiological levels, in a JNK-null background. This has allowed us to study the direct consequences of reconstituting JNK expression in cells. Furthermore, we have confirmed that the re-expressed JNK isoforms were responsive to stress stimulation and were sufficient for c-Jun signalling, indicating redundancy of the JNK1 and JNK2 isoforms in these direct events to phosphorylate c-Jun.

Our results, that JNKs can act to suppress cell proliferation, support conclusions drawn from previous studies using a range of different experimental approaches including a chemical genetics
approach that directly inhibited specific JNK isoforms [15],
transient overexpression of individual JNK isoforms in TNFα
(tumour necrosis factor α)-treated JNK-null MEFS [40], and
siRNA silencing of either JNK1 or JNK2 [41]. The agreement
of these results from diverse studies support common roles
for JNK1 and JNK2 in regulating c-Jun, a conclusion also
supported by biochemical and structural evidence indicating
that JNK1 and JNK2 can bind and efficiently phosphorylate c-Jun in
vitro. As c-Jun plays critical roles in the up-regulation of gene
expression following exposure to growth factors, phorbol esters
and transforming oncogenes [42], it is important to consider how
c-Jun might exert its effects as a transcriptional regulator.

**JNK1 or JNK2 are negative regulators of proliferation**

Our inducible gain-of-function cells provide a new system in
which we can identify and characterize JNK-isoform-specific
processes important in tumour development and progression,
such as cell proliferation, cell death, autophagy and or migration
[5,43,44]. As a first step, we have conducted more detailed
studies on the role of JNK and c-Jun signalling as a cell-cycle
regulator. This is of particular interest as JNK has been previously
considered as either an oncogene or a tumour suppressor.

Previously, JNK-null primary MEFs showed slower pro-
iferation when compared with the proliferation of wild-type
cells [16], but in a subsequent study, the reduced proliferation
in primary JNK-null MEFS was attributed to enhanced cellular
senesence [45]. In contrast, our results, in immortalized MEFS,
are consistent with tumour suppressor functions previously
reported for JNK in transformed cells [7,14]. This emphasizes that
the functions of JNK to regulate the fundamental processes of cell
proliferation may critically depend on cell context, particularly
when considering normal and transformed states. This possibility is
further supported by the results of studies with loss-of-
function mutations in the JNK signalling pathway that correlated
with enhanced tumour formation and metastasis [6], but also
contradicts other studies indicating that JNK activity can enhance
proliferation in cancer [46]. At least part of the resolution of the
roles that JNK can play will depend on more critical evaluation of
the targets immediately downstream of JNK and subsequent
signalling changes that are JNK-dependent. Thus, in the context
of JNK signalling to suppress cell proliferation, a key question
remains on the immediate downstream events that modulate JNK-
dependent cell-cycle progression.

Our findings also contribute to our understanding of the role
of c-Jun in cell proliferation. Previous reports have shown that
c-Jun-null fibroblasts are deficient in proliferation indicating the
requirement of c-Jun expression for normal cell division [19].
However, multiple signalling events regulate c-Jun activity and
the precise gene expression profile regulated by c-Jun depends on
the specific cellular context. In contrast with c-Jun-null cells,
the proliferation defect is more moderate in primary MEFS
expressing c-Jun-AA [13]. Our results point to a novel negative
regulatory role for JNK-mediated c-Jun activity in regulating
cellular proliferation through the control of STMN expression.

**c-Jun can act as a negative regulator of STMN levels**

A striking finding of our study was the very low levels of STMN
observed in the control MEFS, the high levels observed in the
JNK-null MEFS that correlated with their increased proliferation
rates, and the rapid decrease in STMN levels in JNK1, iJNK2 and
iJNK1/2 cells upon JNK re-expression. Furthermore, the decrease
in STMN levels paralleled a decrease in cellular proliferation
upon JNK re-expression. These observations are consistent with
previous reports of enhanced STMN protein levels in rapidly
proliferating cells [22,25,27]. This suggests that high STMN
levels, as observed in the absence of JNK, contribute to the
enhanced proliferation phenotype.

To establish a causal relationship between JNK signalling and
STMN levels required direct manipulation of STMN levels in
JNK-null cells. Previously, siRNA silencing of STMN was shown
to have tumour suppressive effects in breast cancer cells [29].
Similarly, we demonstrated that STMN silencing in JNK-null cells
slowed their proliferation. Therefore changes in STMN levels
play a critical role in determining cell proliferation in JNK1/2−/−
fibroblasts.

The enhanced STMN levels found in fibroblasts with c-Jun
deleted or down-regulated further indicated a negative role for
JNK/c-Jun-mediated signalling in the regulation of STMN levels.
In addition, through our combined use of c-Jun-null cells, c-
Jun siRNA and the re-expression of c-Jun and c-Jun-AA mutant
proteins, we revealed the specific requirement for JNK/c-Jun
signalling in the negative control of STMN levels. Previously,
increased STMN levels have been reported in cells overexpressing
JNK via the transcriptional actions of c-Jun at a characterized
AP-1 site within the STMN promoter sequence [35]. In contrast,
our inducible JNK cell model maintains endogenous c-Jun levels
and is not subject to the overexpression of c-Jun to study its
effects on STMN levels. In addition, c-Jun is targeted by multiple
signalling events distinct from JNK, and the c-Jun contribution to
the regulation of STMN expression may differ in these contexts
[47].

STMN expression is increased in rapidly proliferating and
highly motile cells. Furthermore, enhanced expression of STMN
has been reported in many tumour types highlighting the
importance in understanding the regulatory mechanisms that
control STMN levels. The E2F family of transcription factors has
been shown to promote STMN overexpression observed in
prostate cancer [34]. Negative regulators such as Egr1 and p53
transcription factors can lead to a repression of STMN expression
and cell-cycle arrest [32,33]. We have shown that JNK signalling
to c-Jun can act as an additional negative regulator of cellular
STMN levels to prevent rapid fibroblast proliferation and may
represent a key mechanism contributing to the tumour suppressive
actions of JNK. Importantly, the present study has provided the
first links between JNK signalling and the regulation of STMN
levels in proliferating cells.

The c-Jun-dependent down-regulation of STMN is an inter-
esting observation as c-Jun is generally regarded as a positive
regulator of cell cycle [47]. Our findings point to a novel negative
regulation mechanism under the control of the JNK–
c-Jun signalling axis in the regulation of cell proliferation.
Specifically, the level of regulation was found to be transcriptional
as the re-expression of JNK1/2 in the inducible MEFS resulted in
a reduction in STMN mRNA levels. The negative regulation of
STMN mRNA transcript levels specifically by JNK signalling
to c-Jun, compared with JNK-independent c-Jun activation, may
involve c-Jun dimerizing with different AP-1 partners to directly
repress the expression of STMN [47]. In addition, indirect
mechanisms regulated by JNK/c-Jun, such as the regulation of
microRNAs that subsequently have an impact on STMN levels
remain a possibility [48].

In conclusion, a novel gain-of-function cell model system
allowing controlled expression of specific JNK isoforms at
physiological levels has facilitated the study of JNK-specific sig-
alling events, the characterization of JNK-regulated cellular pro-
cesses, and closer evaluation of JNK isoform-specific cellular
events. Using this inducible cell system, we have revealed JNK/c-
Jun signalling to negatively regulate STMN as a novel mechanism
to control the proliferation of fibroblasts. Our findings highlight the actions of c-Jun to down-regulate gene expression under basal conditions and suggest that additional evaluation of JNK/c-Jun-dependent processes in tumour development will be necessary to understand fully the possible scope for targeting JNK actions in the treatment of cancer.

**AUTHOR CONTRIBUTION**

Yvonne Yap performed the majority of the experiments. Bahareh Badrani performed real-time PCR analysis to determine STMN mRNA levels. Ivan Ng generated inducible MEFs. Tuong-Vi Nguyen provided assistance in cell culture and cell proliferation assays and Yan Yip performed c-Jun siRNA studies. John Silke generated the inducible expression vector. Amardeep Dhillon provided critical input into c-Jun regulation of STMN. Steve Mutsaers provided critical analysis of STMN mRNA expression results. Marie Bogoyevitch provided critical input into c-Jun regulation of STMN. Steve Mutsaers

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SUPPLEMENTARY ONLINE DATA

c-Jun N-terminal kinase/c-Jun inhibits fibroblast proliferation by negatively regulating the levels of stathmin/oncoprotein 18

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EXPERIMENTAL

Yeast two-hybrid analysis

STMN, JNK1, c-Jun and JIP-1 (JNK-interacting protein 1; amino acids 127–281) were cloned into pGBK7 (bait) or pGADT7 (prey) vectors to generate in-frame fusion proteins with DNA-binding domains (BD) or activation domains (AD) according to the manufacturer’s protocol (Clontech). All cloned constructs were confirmed by DNA sequencing. Yeast cultures, AH109 (MATa) and Y187 (MATα) (Clontech) were grown and used as hosts in the two-hybrid assay. The mated yeast was grown on synthetically defined yeast medium (SD/-Trp/-Leu) for 4 days at 30°C. Colonies were picked and re-plated several times on low- (SD/-Trp/-Leu/-His) and high-stringency selection medium (SD/-Trp/-Leu/-His/-Ade) with X-α-Gal (5-bromo-4-chloroindol-3-yl-α-D-galactopyranoside) (10 mg/ml) to select for positive induction of reporter genes (His, Ade and α-galactosidase gene MEL1). Positive transformants, indicating the interaction between bait and prey constructs, were visualized after 3–4 days at 30°C. pGADT7 vector alone was used as a negative control.

Figure 1 JNK1 does not bind STMN in a yeast two-hybrid assay

A GAL4-based yeast two-hybrid protein interaction assay was performed by mating full-length JNK1 wild-type or STMN as bait with the indicated prey constructs. Yeast transformants were selected on low- (SD/-Trp/-Leu/-His) and high-stringency (SD/-Trp/-Leu/-His/-Ade) medium with observed growth indicating interaction of the tested bait and prey proteins. Known JNK-binding proteins c-Jun and JIP were used as positive controls. Empty prey vector was used as a negative control.

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Figure S2 MEK/ERK phosphorylates STMN following growth factor stimulation

JNK1/2\(^{+/+}\) MEFs were pre-treated with MEK inhibitor, U0126 (10 \(\mu\)M), or JNK inhibitor (inh.) VIII (50 \(\mu\)M) for 30 min before stimulation with EGF (epidermal growth factor; 10 ng/ml, 30 min) or 0.1 % BSA in PBS as a control. Protein lysates were then prepared and immunoblotted to determine STMN protein levels. Immunoblotting for JNK expression served to confirm MEF genotype.

Blotting with anti-pan-STMN antibody revealed total STMN protein levels.

Figure 3 Turnover of STMN protein in wild-type and JNK1/2\(^{-/-}\) MEFs is comparable

(A) JNK1/2\(^{+/+}\) or JNK1/2\(^{-/-}\) MEFs were treated with cycloheximide (CHX, 100 \(\mu\)M) for the times indicated. Protein lysates were then prepared and immunoblotted to determine STMN protein levels. Immunoblotting for JNK expression served to confirm MEF genotype.

(B) STMN bands were measured by densitometry and expressed as a percentage of untreated controls (i.e. 0 h). Values are means \pm S.E.M. (\(n=3\)). Statistical testing indicated no significant differences in STMN levels between JNK1/2\(^{+/+}\) or JNK1/2\(^{-/-}\) MEFs at any time points of cycloheximide treatment investigated.