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Deregulated NFATc1 Activity Transforms Murine Fibroblasts Via an Autocrine Growth Factor-Mediated Stat3-Dependent Pathway

Lucio Lagunas and Neil A. Clipstone*

Department of Pharmacology, Stritch School of Medicine, Loyola University Chicago, Maywood, Illinois 60153

ABSTRACT

The nuclear factor of activated T cells (NFAT) family of transcription factors has recently been implicated with a role in tumorigenesis. Forced expression of a constitutively active NFATc1 mutant (caNFATc1) has been shown to transform immortalized murine fibroblasts in vitro, while constitutive activation of the NFAT-signaling pathway has been found in a number of human cancers, where it has been shown to contribute towards various aspects of the tumor phenotype. Here we have investigated the molecular mechanisms underlying the oncogenic potential of deregulated NFAT activity. We now show that ectopic expression of caNFATc1 in murine 3T3-L1 fibroblasts induces the secretion of an autocrine factor(s) that is sufficient to promote the transformed phenotype. We further demonstrate that this NFATc1-induced autocrine factor(s) specifically induces the tyrosine phosphorylation of the Stat3 transcription factor via a JAK kinase-dependent pathway. Interestingly, this effect of sustained NFAT signaling on the autocrine growth factor-mediated activation of Stat3 is not restricted to murine fibroblasts, but is also observed in the PANC-1 and MCF10A human cell lines. Most importantly, we find that the shRNA-mediated depletion of endogenous Stat3 significantly attenuates the ability of caNFATc1 to transform 3T3-L1 fibroblasts. Taken together, our results afford significant new insights into the molecular mechanisms underlying the oncogenic potential of deregulated NFATc1 activity by demonstrating that constitutive NFATc1 activity transforms cells via an autocrine factor-mediated pathway that is critically dependent upon the activity of the Stat3 transcription factor. J. Cell. Biochem. 108: 237–248, 2009.

KEY WORDS: NFAT; STAT3; ONCOGENE; AUTOCRINE GROWTH FACTORS; CELL TRANSFORMATION; 3T3-L1 FIBROBLASTS

The nuclear factor of activated T cells (NFAT) family of transcription factors encodes four exquisitely calcium-sensitive latent transcription factors whose activity is primarily regulated at the level of their subcellular localization via the actions of the calcium/calmodulin-regulated serine/threonine phosphatase calcineurin [Rao et al., 1997; Hogan et al., 2003]. While NFAT proteins were initially recognized for their central role in the regulation of immune cell function [Macian, 2005], more recent findings have established their importance in regulating a wide variety of other biological processes including the growth and cell type specification of a number of different tissues and cell types [Ranger et al., 2000; Graef et al., 2001; Crabtree and Olson, 2002; Takayanagi et al., 2002; Graef et al., 2003; Hirotani et al., 2004; Schulz and Yutzey, 2004; Koga et al., 2005; Heit et al., 2006].

Given the broad biological role of NFAT proteins in the control of cell growth and differentiation, it is perhaps not surprising that their functional dysregulation has been implicated in disease etiology, including their potential involvement in the development of human cancers. In this regard, constitutively active nuclear forms of NFAT proteins have been found in human breast, colon and pancreatic tumors, as well as in lymphomas and animal models of human T cell leukemia [Jauliac et al., 2002; Marafioti et al., 2005; Pham et al., 2005; Buchholz et al., 2006; Medyouf et al., 2007]. The constitutively active NFAT proteins in these tumors appear to result, not from direct mutations or alterations in the coding regions of the genes encoding NFAT family members, but rather from the constitutive activation of upstream signaling pathways that directly impinge on the regulation of NFAT activity. Importantly, the

Abbreviations used: NFAT, nuclear factor of activated T cells; caNFATc1, constitutively active nuclear factor of activated T cells-c1; Stat, signal transducer and activator of transcription; MSCV, murine stem cell virus; FBS, fetal bovine serum; DMEM, Dulbecco's modified Eagle's medium; CM, conditioned media; SFCM, serum-free conditioned media; MWCO, molecular weight cut off; JAK, Janus kinase; KD, knockdown; CsA, cyclosporin A.

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*Correspondence to: Dr. Neil A. Clipstone, Department of Pharmacology, Loyola University Medical School, 2160 South First Avenue, Maywood, IL 60153. E-mail: nclipstone@lumc.edu

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increased NFAT activity in these tumors has been shown to contribute towards various aspects of the transformed cell phenotype, including the regulation of tumor cell growth and survival [Buchholz et al., 2006; Medyouf et al., 2007], as well as tumor cell migration and invasion [Jauliac et al., 2002]. The notion that deregulated NFAT activity can directly contribute towards tumorigenesis is strongly supported by previous work from our own laboratory directly demonstrating the oncogenic potential of a constitutively active NFATc1 mutant (caNFATc1) [Neal and Clipstone, 2003]. Significantly, we have found that introduction of this caNFATc1 mutant into immortalized murine 3T3-L1 fibroblasts is sufficient to induce these cells to adopt the well-established hallmarks of cell transformation, including loss of contact-mediated growth inhibition, acquisition of anchorage-independent cell growth, reduced serum growth requirements, and the ability to form tumors in nude mice. Other in vitro studies have revealed that NFAT proteins can also contribute towards the TNF-α-induced transformation of murine epidermal cells [Yan et al., 2006] and the induction of cell transformation in response to environmental carcinogens [Ouyang et al., 2007]. Combined, these results have pointed to a new and emerging role for the NFAT family of proteins in the initiation of tumorigenesis and the regulation of the cancer phenotype.

The signal transducer and activator of transcription-3 (Stat3) protein is a latent signal-induced transcription factor that has been implicated with a role in a number of biological processes, including cell transformation and tumorigenesis [Darnell, 1997; Bromberg, 2001; Yu and Jove, 2004]. Like NFAT proteins, Stat3 proteins are also regulated primarily at the level of their subcellular localization [Darnell, 1997; Bromberg, 2001; Yu and Jove, 2004]. In normal resting cells, Stat3 proteins are non-phosphorylated and reside in the cytoplasm. However, in response to the activation of various signaling pathways such as certain growth factors and cytokines, Stat3 proteins are inducibly phosphorylated on a critical regulatory tyrosine residue that promotes their homodimerization and subsequent entry into the nucleus where they can activate transcription. The first hints that Stat3 proteins might play a role in tumorigenesis came from observations that Stat3 proteins are constitutively activated in both v-src oncogene-transformed cells and a number of human cancer cell types [Yu et al., 1995; Bromberg, 2001; Calo et al., 2003; Yu and Jove, 2004]. This correlation between Stat3 activation and cancer was considerably strengthened by the seminal observation that a constitutively active mutant form of Stat3 is sufficient to transform immortalized murine fibroblasts in vitro, thereby providing direct evidence that activation of Stat3 per se is sufficient to directly contribute towards cell transformation [Bromberg et al., 1999]. Furthermore, additional studies have demonstrated that Stat3-deficient cells are less susceptible to the transforming effects of a number of known oncogenes and are more resistant to tumorigenesis in vivo [Chiarle et al., 2005; Ling and Arlinghaus, 2005; Schlessinger and Levy, 2005; Vultur et al., 2005]. Collectively, these results have served to firmly establish Stat3 as a critical nodal point in a number of oncogenic signaling pathways leading to the establishment of the malignant phenotype.

In the current study, we have sought to elucidate the pathway by which deregulated NFATc1 activity contributes towards cell transformation. Building upon our previous demonstration that introduction of a caNFATc1 mutant into 3T3-L1 fibroblasts leads to the production of an autocrine growth factor(s) capable of promoting the growth and survival of these cells under serum-free conditions [Neal and Clipstone, 2003], we now provide evidence that chronic exposure of wild-type 3T3-L1 cells to conditioned media (CM) from caNFATc1-expressing cells is in fact sufficient to promote their transformation in vitro. We further demonstrate that the caNFATc1-induced autocrine factor(s) present in the CM specifically activates the Stat3 transcription factor and that this activation of Stat3 is essential for cell transformation induced by caNFATc1. Thus, our results afford significant new insights into the molecular mechanisms underlying the oncogenic potential of constitutive NFATc1 activity.

MATERIALS AND METHODS

MATERIALS

PP1 and AG490 were purchased from Biomol International, while ionomycin, cyclosporin A (CsA), and Jak inhibitor I (P6) were all purchased from Calbiochem, and FK506 was a gift from the Fujisawa Pharmaceutical Co. Pronase E was purchased from Sigma-Aldrich and NanoSpin columns (10,000 molecular weight cut off—MWCO) were from Gelman Sciences. The following antibodies were purchased from Cell Signaling Technology: anti-Stat3 (#9132), anti-phospho-Stat3-Tyr705 (#9131), anti-phospho-Stat3-Ser727 (#9134), anti-phospho-Stat5A/B-Tyr694 (#9351), anti-Jak2 (24B11), and anti-phospho-Jak2-Tyr1007/1008 (#3771). Antibodies directed against Stat5A/B (C-17) and AKT1/2 (H136) were purchased from Santa Cruz, while anti-phospho-AKT-Ser473 (#44-621G) was purchased from Biosource. Horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin and goat anti-mouse immunoglobulin secondary antibodies were both purchased from Zymed Laboratories.

PLASMID CONSTRUCTS

The MSCV-caNFATc1 and MSCV-GFP control retroviral expression vectors have been described previously [Neal and Clipstone, 2003]. The retroviral construct expressing an shRNA specific for murine Stat3 was generated as follows. First, plasmids encoding shRNAs specific to murine Stat3 in the Lentiviral vector pLKO.1 were purchased from Open Biosystems and used as templates in a PCR reaction using the forward primer 5′-CTCTCAATTGTATCGATCAC-GAGACTAGCCTC-3′ and the reverse primer 5′-CCTCAAGCTTG-GATGAATACTGCCATTTGTCTCG-3′. The resulting PCR fragments were digested with *Mfe* I and *Hin*d III and cloned into pSR-GFP/Neo (OligoEngine) previously digested with *Eco* RI and *Hin*d III. After testing for knockdown efficiency, a single highly efficient Stat3-specific shRNA vector (pSR-ST3-456) was chosen for subsequent studies. The pSR-Luc2 shRNA retroviral vector encoding an shRNA specific for firefly luciferase was created by introducing oligonucleotides corresponding to a previously validated luciferase-specific shRNA [Miyagishi et al., 2004] into the Bgl II and *Hin*d III sites of pSR-GFP/Neo. The integrity of all constructs was confirmed by DNA sequencing.

CELL CULTURE AND RETROVIRAL INFECTIONS

3T3-L1, GP2-293 (Clontech) and PANC-1 cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) containing 4.5 g/L glucose and L-glutamine (Cellgro) supplemented with 100 U/ml penicillin (Cellgro), 100 μg/ml streptomycin (Cellgro), and 7.5% (v/v) fetal bovine serum (FBS; Hyclone). MCF10A cells were maintained in F12:DMEM containing 4.5 g/L glucose and L-glutamine (Invitrogen) supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 5% (v/v) heat-inactivated equine serum (Invitrogen), 10 μg/ml insulin (Sigma), 100 ng/ml cholera toxin (Sigma), 500 ng/ml hydrocortisone (Sigma), and 20 ng/ml epidermal growth factor (Sigma). All cells were maintained in a humidified incubator at 37°C with 7.5% CO_2. VSV-G pseudotyped recombinant retroviruses were generated and used to spin-infect target cells exactly as previously described [Neal and Clipstone, 2003].

COLLECTION OF CONDITIONED MEDIA (CM)

CM was collected from cells grown to approximately 80% confluence in DMEM supplemented with 7.5% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin. The collected media was centrifuged at 13,000*g* for 5 min and then either used immediately or stored at −80 °C until further use. To collect serum-free conditioned media (SFCM), cells were seeded onto 90 mm culture plates and allowed to reach 80% confluence. The cells were then washed and maintained in DMEM supplemented with only 100 U/ml penicillin, and 100 μg/ml streptomycin for a further 16 h, at which point the SFCM was collected, centrifuged and stored at −80°C.

TREATMENT OF CELLS WITH CONDITIONED MEDIA

To assess the effects of CM on the transformation of 3T3-L1 cells, the growth media on wild-type 3T3-L1 cells was replaced twice daily for a total of 7 days with undiluted CM from either GFP- or caNFATc1-expressing cells, at which point the cells were briefly expanded in normal growth media and analyzed for their transformed properties. For experiments analyzing the biochemical consequences of treating wild-type 3T3-L1 cells with SFCM, cells were serum-starved overnight then exposed to SFCM for 40 min. For the heat treatment studies, SFCM was heated to the desired temperature for 60 min, then cooled to 37°C before addition to serum-starved cells. For the protease-treatment studies NFATc1-SFCM was incubated with pronase E (0, 1, or 5 μg/ml) at 37°C for 17 h then added directly to serum-starved 3T3-L1 cells. As a control to rule out a non-specific effect of protease treatment on 3T3-L1 cells, a parallel sample of pronase was incubated with media alone then added to serum-starved cells together with NFATc1-SFCM. Crude fractionation of NFATc1-SFCM was performed by centrifugation in a NanoSpin ultracentrifugation spin column. The material retained by the membrane and the flow through were then independently tested for their ability to stimulate the phosphorylation of Stat3 when added to serum-starved 3T3-L1 cells.

FOCUS FORMING ASSAY

The relevant cell populations were diluted 1–20 with wild-type 3T3-L1 cells and then 1×10^5 cells of the resulting cell mixtures were seeded per well of a 6-well plate and maintained in complete growth media with media changes every 2 days. After 10 days, cells were stained with methylene blue, photographed and the visible colonies from triplicate wells were enumerated.

DETERMINATION OF ANCHORAGE-INDEPENDENT GROWTH

Cells (5×10^5) were seeded in 60 mm agarose-coated bacterial petri dishes in complete growth media containing 1.8% methylcellulose (Sigma). Cells were re-fed weekly by the addition of 1 ml of the 1.8% methylcellulose containing growth media and colonies were counted after 4 weeks.

TUMOR FORMATION IN NUDE MICE

Cells (5×10^5) of the relevant cell population was injected s.c. into the right flanks of female NCr nude mice (Taconic). For each cell population five mice were injected per experiment. After approximately 30 days post-injection, animals were sacrificed, the tumors excised and their dimensions determined using digital calipers. Total tumor volume was determined using the equation $0.52 \times l \times w \times h$.

DETERMINATION OF CELL GROWTH

Cell growth was measured by MTS assay (Promega). Briefly, 2×10^3 cells of each cell population ($n = 7$) were seeded per well of a 96-well culture plate. At the indicated times the MTS reagent was added and the plate was returned to the incubator for 2 h, at which point the absorbance at 490 nm was determined.

IMMUNOBLOT ANALYSIS

Whole cell lysates were prepared by rinsing cells with ice-cold phosphate-buffered saline, then scraping into sample buffer (62.5 mM Tris–HCl pH 6.8, 2% sodium dodecyl sulfate, 10% glycerol, 50 mM dithiothreitol). Lysates were then sonicated for 10 s using a Digital Sonifier (Branson) at an amplitude of 11%, heated to 95°C for 10 min and stored at −80°C. Samples were resolved by sodium dodecyl–polyacrylamide gel electrophoresis, transferred to nitrocellulose (BioRad), and subjected to immunoblot analysis with the relevant primary antibodies followed by the appropriate horseradish peroxidase-coupled secondary antibodies. All antibody incubations were per the manufacturers instructions. Immunoblots were visualized by chemiluminescence using Super Signal West Pico Chemiluminescent Substrate (Pierce).

RESULTS

EXPOSURE OF 3T3-L1 FIBROBLASTS TO CM FROM caNFATc1-EXPRESSING CELLS IS SUFFICIENT TO CAUSE THESE CELLS TO ADOPT A TRANSFORMED CELL PHENOTYPE

In previous studies we have demonstrated that ectopic expression of caNFATc1 in murine 3T3-L1 fibroblasts leads to the secretion of a factor(s) that is able to promote the survival and proliferation of these cells under serum-free conditions [Neal and Clipstone, 2003]. Given that production of autocrine growth factors is a well-established mechanism of cell transformation [Sporn and Roberts, 1985], we initially decided to investigate whether the activity of the caNFATc1-induced secreted factor(s) was sufficient to induce normal immortalized murine 3T3-L1 fibroblasts to undergo cell transformation. Towards this end, CM was first collected from 3T3-L1 cells stably expressing either caNFATc1 (NFATc1-CM) or

GFP (GFP-CM). Wild-type 3T3-L1 cells were then treated twice daily for a 7-day period with either NFATc1-CM or GFP-CM, at which point the cells were re-plated under normal growth conditions and their cellular phenotype was assessed. As expected, when GFP-CM-treated 3T3-L1 cells were allowed to reach confluence they stopped dividing and formed a single cell monolayer as a consequence of contact-mediated growth inhibition (Fig. 1A). Conversely, we observed that NFATc1-CM-treated 3T3-L1 cells consistently overgrew the monolayer and formed foci with a transformed cell appearance (Fig. 1A,B). Such foci were never detected in GFP-CM-treated cells. Furthermore, we found that these later NFATc1-CM-treated cells readily formed colonies when grown in methylcellulose culture, indicating that they are capable of anchorage-independent cell growth (Fig. 1C). In contrast, no such colonies were observed in cultures from control GFP-CM-treated cells. Taken together, these results demonstrate that the continuous long-term exposure of normal wild-type 3T3-L1 cells to CM from caNFATc1-expressing cells is sufficient to cause these cells to lose contact-mediated growth inhibition and acquire anchorage-independence, both hallmarks of transformed cells [Hanahan and Weinberg, 2000]. Thus, it appears that caNFATc1 induces the production of a secreted factor(s) that is sufficient to promote the phenotypic conversion of 3T3-L1 murine fibroblasts to the transformed cell phenotype.

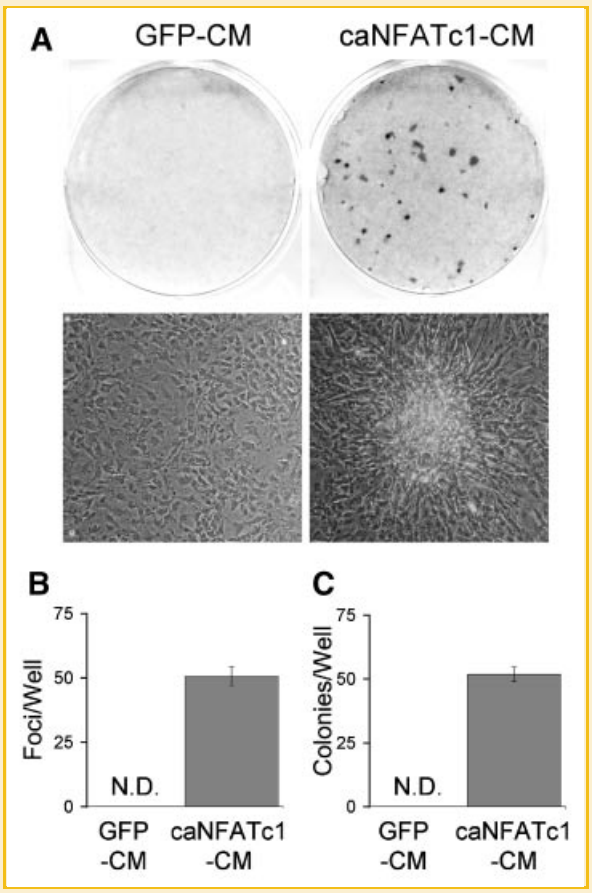

Fig. 1. 3T3-L1 cells acquire a transformed phenotype when constantly exposed to CM from caNFATc1-expressing cells. A: Sustained exposure of wild-type 3T3-L1 to caNFATc1-CM promotes formation of transformed cell foci. Wild-type-3T3-L1 cells were treated twice daily over a 7-day period with CM from either GFP- or caNFATc1-expressing cells, at which point cells were diluted 1–20 with wild-type 3T3-L1 cells and a total of 1×10^5 cells from each population were seeded in triplicate in wells of a 6-well plate in normal growth media. After 10 days, cells were then either stained with methylene blue to document the formation of transformed foci (upper panel), or were photographed at 10× magnification to reveal changes in cell morphology (lower panel). B: Quantification of the number of transformed foci formed per well from the experiment described in (A). Plates were counted in triplicate and the standard deviation is indicated by the error bars. ND, none detected. C: The effects of caNFATc1-CM on the promotion of anchorage-independent cell growth. Cells treated with either GFP-CM or caNFATc1-CM as in (A) were plated in methylcellulose-containing media and the mean number of colonies formed from five independent plates were quantified after 4 weeks. The standard deviation is indicated. ND, none detected. Results are representative of at least three independent experiments with similar outcomes.

TREATMENT OF 3T3-L1 FIBROBLASTS WITH CM FROM caNFATc1-EXPRESSING CELLS SPECIFICALLY STIMULATES THE PHOSPHORYLATION OF THE STAT3 TRANSCRIPTION FACTOR

In order to further investigate the underlying molecular mechanisms by which sustained NFATc1 activity acts to promote cell transformation, we first examined both caNFATc1-expressing cells and control GFP-transduced cells for the activation status of a number of downstream signaling pathways previously implicated in promoting the cancer phenotype. Strikingly, we found that the Stat3 transcription factor was significantly activated in caNFATc1-expressing cells, but not in control cells (see Fig. 2A), as determined by immunoblotting with an antibody specific for pStat3-Y705, a post-translational modification known to be responsible for triggering the nuclear translocation of latent Stat3 proteins [Zhong et al., 1994]. In order to determine whether this activation of Stat3 was stimulated by a caNFATc1-induced secreted factor(s) we analyzed the effects on Stat3 phosphorylation following treatment of serum-starved wild-type 3T3-L1 cells with SFCM isolated from either caNFATc1-expressing cells (NFATc1-SFCM) or control GFP cells (GFP-SFCM). As shown in Figure 2B, we found that treatment with NFATc1-SFCM, but not GFP-SFCM, was able to induce the marked phosphorylation of Stat3-Y705, demonstrating that the activation of Stat3 is indeed caused via the action of an NFATc1-induced secreted factor(s). In fact, not only do we observe an effect of NFATc1-SFCM on the phosphorylation of Stat3-Y705, we also observe an increase in the phosphorylation of Stat3-S727, a post-translational modification that is believed to be required for the maximal activation of Stat3-dependent transcriptional activity [David et al., 1995; Wen et al., 1995]. Importantly, the effect of NFATc1-SFCM on the activation of Stat3 appears to be specific, as no effect on the phosphorylation of the related Stat protein, Stat5, was observed (Fig. 2B). Taken together, these results demonstrate that sustained NFATc1 activity results in the secretion of factor(s) capable of acting in an autocrine fashion to induce the activation of Stat3, a transcription factor that is known to play a pivotal role in the transformation of a variety of different cell types [Bromberg, 2001; Calo et al., 2003; Yu and Jove, 2004].

A diverse array of factors, including cytokines, polypeptide growth factors, peptide hormones, and lipid molecules, have been previously shown to be capable of activating the Stat3 pathway

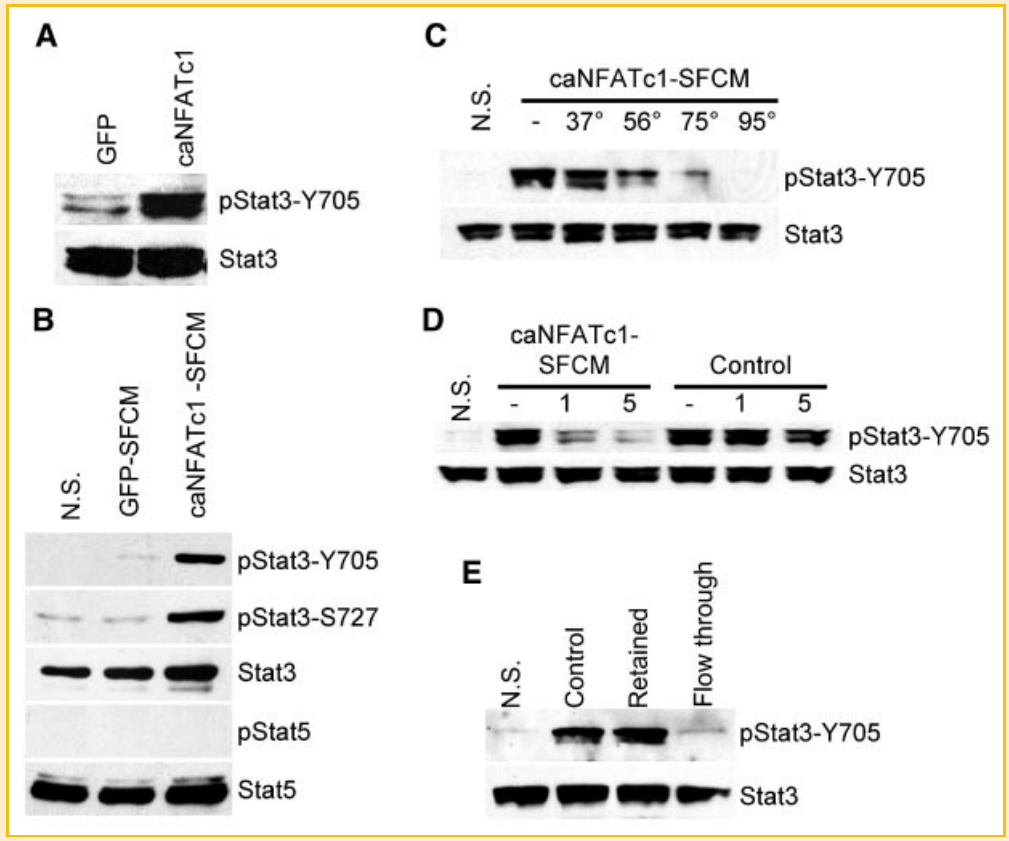

Fig. 2. CM from caNFATc1-expressing cells promotes the phosphorylation of Stat3. A: Stat3 is selectively phosphorylated on tyrosine 705 in caNFATc1-expressing cells. Whole cell lysates from equivalent numbers of either control GFP- or caNFATc1-expressing cells were analyzed by immunoblot analysis with antibodies specific for pStat3-Y705. As a loading control, lysates were also immunoblotted with a pan anti-Stat3 antibody. B: Treatment of wild-type 3T3-L1 cells with SFCM from caNFATc1-expressing cells induces the phosphorylation of Stat3. Wild-type 3T3-L1 cells were serum starved for 16 h, then treated with SFCM from either control GFP- or caNFATc1-expressing cells. After 40 min, cell lysates were prepared and analyzed by immunoblotting with an anti-pStat3-Y705, anti-pStat3-S727 and a pan anti-Stat3 antibody. Lysates were also immunoblotted with an anti-pStat5 and a pan anti-Stat5 antibody to illustrate specificity. NS, non-stimulated; representing cells that were cultured under similar conditions, but were not treated with SFCM. C: The factor present in NFATc1-SFCM that is responsible for inducing the activation of Stat3 is heat-sensitive. NFATc1-SFCM was heated at the indicated temperature for 60 min, then cooled and used to treat serum-starved 3T3-L1 cells. After 40 min, cell lysates were prepared and analyzed by immunoblotting with the indicated antibodies. D: The factor present in caNFATc1-SFCM that is responsible for inducing the activation of Stat3 is sensitive to protease-treatment. NFATc1-SFCM was incubated overnight at 37°C with either vehicle, 1 or 5 μg/ml pronase E and then used to treat serum-starved 3T3-L1 cells. As a control to rule out a non-specific effect of pronase treatment, pronase E was incubated with media alone, then added to serum-starved 3T3-L1 cells simultaneously with NFATc1-SFCM. In both cases, protein lysates were prepared 40 min post-stimulation and analyzed by immunoblotting with the indicated antibodies. E: NFATc1-SFCM was fractionated using a NanoSpin ultracentrifuge spin column (10,000 MWCO) and the flow through and retained fractions were individually tested for their ability to induce the activation of Stat3 in serum-starved 3T3-L1 cells. Results are representative of more than three independent experiments.

[Zhong et al., 1994; Darnell, 1997; Liang et al., 1999; Lo and Wong, 2004; Yu and Jove, 2004; Han et al., 2006; Frias et al., 2007; Cheranov et al., 2008]. In order, therefore, to gain some initial insights into the nature of the NFATc1-induced factor(s) responsible for inducing the phosphorylation of Stat3, we next investigated the physiochemical properties of the activity present in NFATc1-SFCM. First, we examined the effects of heat-treatment on the ability of the NFATc1-SFCM to promote the phosphorylation of Stat3. For this experiment NFATc1-SFCM was incubated at various temperatures for 60 min then used to stimulate serum-starved 3T3-L1 cells. As shown in Figure 2C, the activity present in NFATc1-SFCM was progressively lost with increasing heat treatment. Second, we examined the sensitivity of the NFATc1-SFCM to exogenous protease digestion. For this experiment the NFATc1-SFCM was first incubated with the broad specificity protease, pronase, prior to its addition to serum-starved 3T3-L1 fibroblasts. As a control to rule out a non-specific effect of the protease on the cells, pronase was incubated in parallel with media alone and then added to serum-starved cells concomitantly with NFATc1-SFCM. As shown in Figure 2D, we found that incubation of NFATc1-SFCM with pronase resulted in a dose-dependent decrease in its ability to activate Stat3, whereas conversely, in control samples, the addition of pronase to cells at the same time as NFATc1-SFCM was without effect. Essentially similar results were also obtained with an independent protease, proteinase K (data not shown). These results therefore indicate that the Stat3 inducing activity present in NFATc1-SFCM is specifically sensitive to the actions of a protease and is therefore likely to be a polypeptide. Finally, we performed a crude fractionation of the NFATc1-SFCM using an ultrafiltration centrifugation device with a MWCO of >10,000 Da.

As shown in Figure 2E, we found that the activity present in NFATc1-SFCM responsible for inducing the activation of Stat3 was fully retained by the membrane, and was not present in the flow through. Taken as a whole, therefore, these results are consistent with the notion that the activity present in NFATc1-SFCM that is responsible for inducing the activation of Stat3 is most likely a protein and not a low molecular weight peptide or lipid molecule.

SIGNALING REQUIREMENTS FOR THE ACTIVATION OF STAT3 INDUCED BY NFATC1-SFCM

To further explore the mechanism by which NFATc1-induced autocrine factors induce the activation of Stat3, we next examined the signaling pathways involved. The activation of the Stat3 transcription factor by growth factors and cytokines is believed to proceed via one of two principal signaling pathways: either via the activation of Src family kinases or via the activation of the JAK family of kinases [Yu et al., 1995; Darnell, 1997; Bromberg, 2001; Yu and Jove, 2004]. We therefore sought to determine the potential role of these pathways in the activation of Stat3 induced by NFATc1-SFCM. Towards this end, serum-starved wild-type 3T3-L1 cells were first treated with NFATc1-SFCM, in the presence or absence of the specific src kinase inhibitor, PP1, and the phosphorylation status of Stat3 was assessed. As shown in Figure 3A, we found that PP1 did not inhibit the ability of NFATc1-SFCM to induce Stat3 phosphorylation, although it did effectively inhibit the ability of NFATc1-SFCM to induce the specific activation of the Akt protein kinase. Hence, these results indicate that the activation of Stat3 by NFATc1-SFCM is not dependent upon activity of the Src tyrosine kinase. Similar negative results were obtained using specific inhibitors of the platelet-derived growth factor receptor tyrosine kinase, the insulin-like growth factor receptor 2 tyrosine kinase, fibroblast growth factor receptors and the tumor growth factor β-signaling pathway (data not shown). Conversely, we found that treatment of serum-starved wild-type 3T3-L1 cells with two independent JAK kinase inhibitors, either the pan-JAK kinase inhibitor, JAK inhibitor I (P6), or the JAK2-specific inhibitor, AG490, both acted in a dose-dependent fashion to inhibit the ability of NFATc1-SFCM to induce the phosphorylation of Stat3 (see Fig. 3B,C). Concomitant with this effect we found that treatment of serum-starved wild-type 3T3-L1 cells with NFATc1-SFCM resulted in the increased phosphorylation of JAK2 on amino acids tyrosine 1007 and 1008 (Fig. 3D), residues previously shown to play a role in the activation of JAK2 kinase activity [Feng et al., 1997]. Hence, these results indicate that the autocrine factor(s) present in NFATc1-SFCM most likely induce the activation

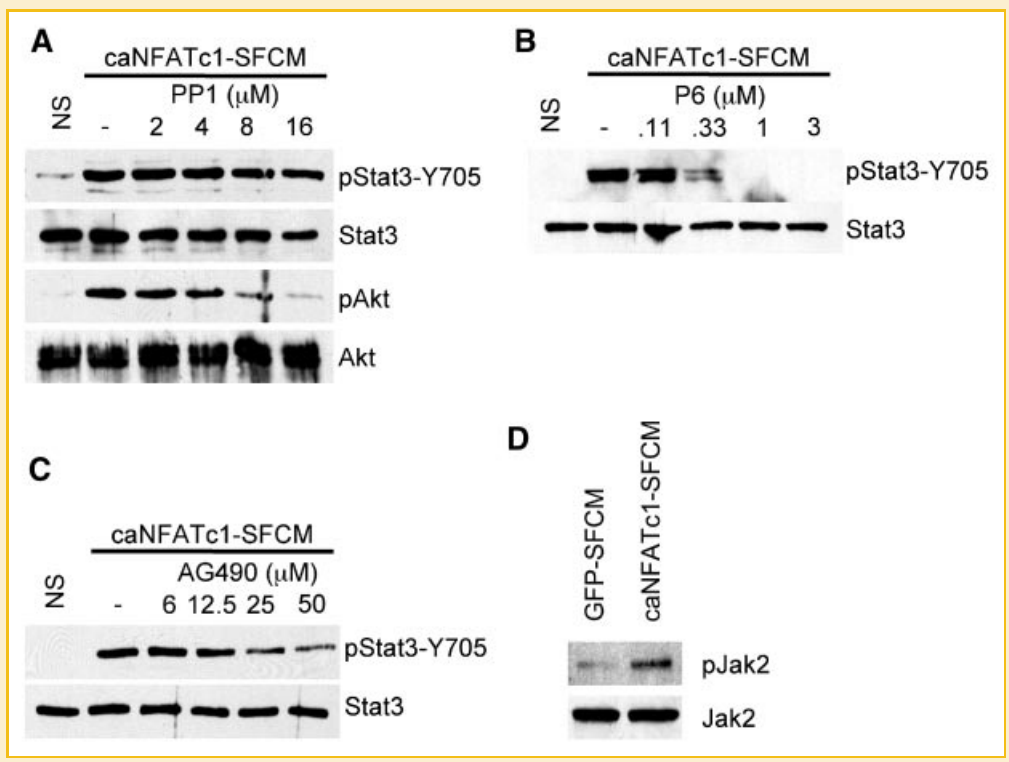

Fig. 3. Analysis of the signaling pathways involved in mediating the effects of caNFATc1-induced autocrine factors on the phosphorylation of Stat3. A–C: Wild-type 3T3-L1 cells were serum-starved for 16 h then treated with caNFATc1-SFCM in the presence or absence of various concentrations of either the c-src kinase inhibitor PP1 (A), the pan-JAK Inhibitor I (B), or the JAK2 kinase inhibitor AG490 (C). After 40 min, cell lysates were prepared and analyzed by immunoblotting with anti-pStat3-Y705, pan anti-Stat3, anti-pAkt, and pan anti-Akt antibodies. D: SFCM from caNFATc1-expressing cells selectively induces the phosphorylation of Jak2. Serum-starved 3T3-L1 cells were treated with SFCM from either control GFP cells (GFP-SFCM) or caNFATc1-expressing cells (caNFATc1-SFCM) and cell lysates were analyzed by immunoblotting with anti-pJAK2 and pan anti-JAK2 antibodies. Results are representative of more than three independent experiments with similar outcomes.

of Stat3 via a pathway involving the activation of JAK family kinases.

A CRITICAL ROLE FOR STAT3 IN CANFATC1-INDUCED CELL TRANSFORMATION

Our above data demonstrate that the production of an NFATc1-induced autocrine factor(s) is sufficient to both promote 3T3-L1 cell transformation and lead to the activation of the Stat3 transcription factor. Since Stat3 is known to play a key oncogenic role in many cell types [Bromberg, 2001; Calo et al., 2003; Yu and Jove, 2004], we next sought to determine what role this transcription factor might play in mediating the oncogenic effects of caNFATc1. For these experiments we adopted an shRNA-mediated knockdown strategy. Wild-type 3T3-L1 cells were retrovirally transduced with either a Stat3-specific shRNA (Stat3-KD cells), or a control firefly luciferase-specific shRNA (Luc-KD cells). As shown in Figure 4A, expression of the murine Stat3-specific shRNA was able to effectively knockdown the expression of the endogenous Stat3 protein in Stat3-KD cells, as determined by immunoblotting. This knockdown of Stat3 was specific, as the Stat3-specific shRNA did not affect the expression

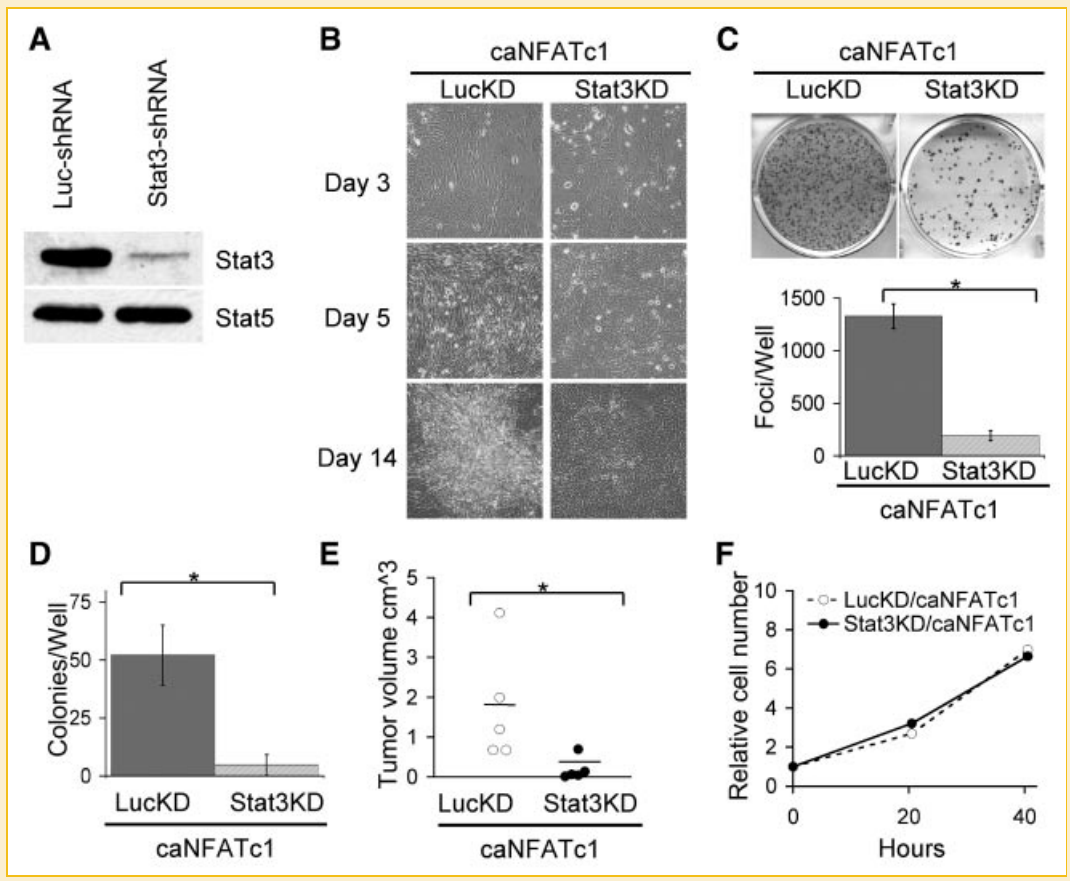

Fig. 4. Depletion of endogenous Stat3 attenuates caNFATc1-induced cell transformation. A: Ectopic expression of a Stat3-specific shRNA significantly reduces expression of the endogenous Stat3 protein in 3T3-L1 cells. Wild-type 3T3-L1 cells transduced with retroviruses expressing either the Stat3-specific shRNA or a control firefly luciferase shRNA were analyzed by immunoblotting with an antibody specific for Stat3 and a pan anti-Stat5 antibody as a loading control. B: Depletion of Stat3 prevents ectopic expression of caNFATc1 from inducing the acquisition of a transformed cell phenotype. 3T3-L1 cells expressing either control firefly luciferase shRNA (Luc-KD) or the Stat3-specific shRNA (Stat3-KD) were transduced with the caNFATc1-encoding retrovirus, briefly expanded and then plated into 6-well plates. Bright field view photographs were then taken at the indicated times. caNFATc1-expressing Stat3-KD cells undergo contact-mediated growth inhibition at confluence, whereas caNFATc1-expressing Luc-KD cells overgrow the monolayer and form clearly definable transformed foci. C: Depletion of Stat3 attenuates the ability of caNFATc1 to induce the formation of transformed cell foci. Stat3-KD and Luc-KD cells each transduced with the caNFATc1 retrovirus were diluted 1–20 with wild-type 3T3-L1 cells and a total of 1×10^5 cells from each population was plated in wells of a 6-well plate. After 10 days cells were stained with methylene blue, photographed (upper panel) and the mean number of transformed cell foci per plate from triplicate determinations were determined (lower panel). The standard deviations are indicated by the error bars. * Denotes $P = 0.0001$. D: Depletion of Stat3 attenuates the ability of caNFATc1 to promote anchorage-independent cell growth. Stat3-KD and Luc-KD cells transduced with the caNFATc1 retrovirus were each plated into methylcellulose-containing media (5×10^5 cells/well) and the mean number of colonies formed from six independent plates after 4 weeks was determined. The standard deviations are indicated by the error bars. * Denotes $P < 0.0001$. E: Depletion of Stat3 attenuates the ability of caNFATc1-expressing cells to form tumors in nude mice. Aliquots (5×10^5 cells) of Stat3-KD and Luc-KD cells each transduced with the caNFATc1 retrovirus were injected s.c. into female nude mice ($n = 5$). After 30 days the mean tumor volume was calculated using the equation $0.52 \times l \times w \times h$. * Denotes $P < 0.05$. Results are representative of at least three independent experiments with similar outcomes. F: Depletion of Stat3 does not affect the intrinsic proliferation of 3T3-L1 cells. Aliquots (2×10^3) of Stat3-KD and Luc-KD cells each transduced with the caNFATc1 retrovirus ($n = 7$) were plated per well of a 96-well plate and their proliferation was monitored daily by MTS assay.

levels of Stat5, nor was Stat3 expression levels affected by expression of the control luciferase-specific shRNA in Luc-KD cells (Fig. 4A).

To examine the potential involvement of Stat3 in mediating the oncogenic effects of caNFATc1, both Stat3-KD cells and control Luc-KD cells, were superinfected with a caNFATc1-encoding retrovirus and a number of cellular parameters associated with cell transformation were analyzed. First, we investigated the effects of Stat3 knockdown on the ability of caNFATc1 to induce cells to lose contact-mediated growth inhibition. As shown in Figure 4B, introduction of caNFATc1 into Luc-KD control cells promoted their growth beyond the point of confluence, causing them to form multiple cell layers and adopt a typical transformed spindle-like morphological appearance. In contrast, when caNFATc1 was introduced into Stat3-KD cells, the cells stopped growing once they reached confluence, where they formed a single cell monolayer and maintained a normal cellular architecture (Fig. 4B). Thus, depletion of endogenous Stat3 attenuates the ability of caNFATc1 to promote loss of contact-mediated growth inhibition in 3T3-L1 fibroblasts. Second, we observed that the knockdown of Stat3 significantly impaired the ability of caNFATc1 to induce Stat3-KD cells to form transformed cell foci (Fig. 4C). Third, we investigated the effects of Stat3 knockdown on the ability of caNFATc1 to promote anchorage-independent cell growth. As shown in Figure 4D, while caNFATc1-expressing Luc-KD cells readily formed colonies in semi-solid methylcellulose-containing media, this effect was significantly impaired in the caNFATc1-expressing Stat3-KD cells. Finally, we examined whether knockdown of Stat3 could affect the ability of caNFATc1-expressing cells to form tumors in vivo. For this experiment both Luc-KD and Stat3-KD cells were transduced with the caNFATc1-expressing retrovirus and aliquots of the resulting cell populations were each subcutaneously injected into nude mice and the ability of these two cell populations to form tumors was assessed. As expected, we found that caNFATc1-expressing Luc-KD cells readily formed large, rapidly growing tumors (Fig. 4E). Although we also observed tumor formation in mice injected with the caNFATc1-expressing Stat3-KD cells, by comparison these tumors were significantly smaller and slower growing than those formed by the caNFATc1-expressing Luc-KD control cells. Similar results were obtained in three independent experiments using three independently derived Stat3 knockdown parent cells. Interestingly, when the small tumors from mice injected with caNFATc1-expressing Stat3-KD cells were further analyzed, we found that in each case, unlike the parental caNFATc1-expressing Stat3-KD cells used for injection, the tumors all expressed significant levels of Stat3 (data not shown), suggesting that there was likely in vivo selection for rare cells in the population in which Stat3 was not effectively knocked down. Thus, our data argue that knockdown of Stat3 appears to significantly attenuate the ability of sustained NFATc1 activity to promote the in vivo growth of tumors in nude mice. Taken together, these combined data demonstrate that knockdown of Stat3 significantly attenuates the ability of caNFATc1 to induce a number of well characterized cellular hallmarks of transformation, including loss of contact-mediated growth inhibition, formation of transformed cell foci, anchorage-independent cell growth and tumor growth in nude mice. Importantly, these effects of Stat3 knockdown on the transforming ability of caNFATc1 are not caused by an indirect inhibitory effect of Stat3 depletion on the ability of Stat3-KD cells to proliferate, as consistent with previous findings [Schlessinger and Levy, 2005], we find that knockdown of Stat3 has no effect on intrinsic cell growth (see Fig. 4F). Consequently, these collective results provide compelling evidence that Stat3 plays a critical role in mediating the oncogenic effects of caNFATc1 in 3T3-L1 murine fibroblasts.

SUSTAINED NFATC1 ACTIVITY PROMOTES STAT3 ACTIVATION IN BOTH HUMAN PANC-1 AND MCF10A CELLS

To determine whether the activation of Stat3 is likely to be a general response to deregulated NFAT activity that might be of relevance to human cancer, we examined the effects of sustained NFATc1 activity on the activation of Stat3 in two physiologically relevant human cell lines. First, we chose the human pancreatic cancer cell line, PANC-1, as recent studies have implicated sustained NFATc1 activity in the etiology of pancreatic cancer [Buchholz et al., 2006]. As shown in Figure 5A, under serum-starved conditions, ectopic expression of caNFATc1 in PANC-1 cells results in a marked increase in the phosphorylation of Stat3. Like in our 3T3-L1 model,

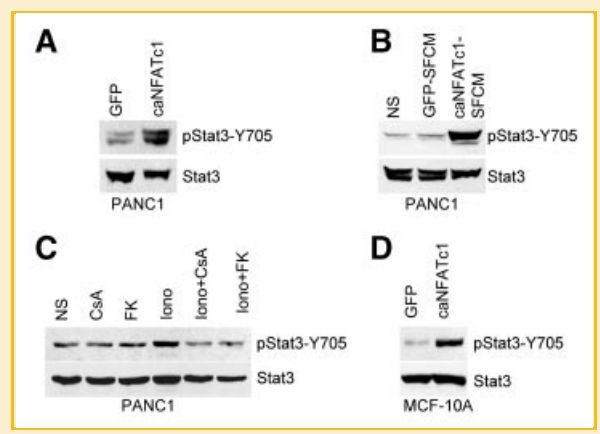

Fig. 5. Sustained NFATc1 activity promotes Stat3 phosphorylation in both human PANC-1 and MCF10A cells. A: PANC-1 cells transduced with either the control GFP or caNFATc1 retrovirus were serum starved for 16 h and whole cell lysates were prepared and analyzed by immunoblotting with anti-pStat3-Y705 and pan anti-Stat3 antibodies. B: Immunoblot analysis of serum-starved PANC-1 cells treated with SFCM from PANC-1 cells transduced with either the control GFP virus (GFP-SFCM) or the caNFATc1 virus (caNFATc1-SFCM). Cells were treated with SFCM for 15 min and whole cell lysates were analyzed by immunoblotting with anti-pStat3-Y705 and the pan anti-Stat3 antibody as a loading control. C: Effects of the activation of the endogenous calcineurin/NFAT-signaling pathway on the phosphorylation of Stat3 in PANC-1 cells. PANC-1 cells were either left non-stimulated (NS) or treated with cyclosporin A (CsA; 2.5 μg/ml), FK506 (FK; 5 ng/ml), ionomycin (Iono; 1 μM), ionomycin + cyclosporin (Iono + CsA) and ionomycin + FK506 (Iono + FK). After 16 h cell lysates were prepared and analyzed by immunoblotting with anti-pStat3-Y705 and the pan anti-Stat3 antibodies. D: Ectopic expression of caNFATc1 induces phosphorylation of Stat3 in MCF10A cells. Cell lysates from MCF10A cells transduced with either the control GFP or caNFATc1 retrovirus were analyzed by immunoblotting with anti-pStat3-Y705 and pan anti-Stat3 antibodies. Results are representative of at least three independent experiments with similar outcomes.

this effect appears to be caused by the secretion of NFATc1-induced autocrine growth factors, as we found that treatment of serum-starved non-transduced PANC-1 cells with SFCM from caNFATc1-expressing PANC-1 cells was able to induce Stat3 phosphorylation (Fig. 5B). Interestingly, activation of the endogenous calcineurin/NFAT-signaling pathway in PANC-1 cells also appears to lead to activation of Stat3. For this experiment PANC-1 cells were treated with the calcium ionophore, ionomycin, a known activator of the calcineurin/NFAT-signaling pathway. As shown in Figure 5C, we found that ionomycin-treatment of PANC-1 cells resulted in a small but reproducible increase in the phosphorylation of Stat3, an effect that was reduced to background levels by the presence of either CsA or FK506, two specific inhibitors of the calcineurin/NFAT-signaling pathway. Second, we analyzed the effects of caNFATc1 in the normal human mammary epithelial cell line MCF10A, as constitutive NFAT signaling has previously been implicated in playing a role in human breast cancer [Jauliac et al., 2002]. As shown in Figure 5D, we find that ectopic expression of caNFATc1 in MCF10A cells also results in the marked phosphorylation of the Stat3 transcription factor. Thus, these data demonstrate that the ability of deregulated NFATc1 activity to activate Stat3 is not simply restricted to murine fibroblasts, but is also conserved in a number of physiologically relevant human cell lines suggesting that this phenomenon is likely to be a general consequence of sustained NFAT signaling in a range of different tissues and cell types.

DISCUSSION

Considerable evidence now points towards an emerging role for the NFAT family of transcription factors in cell transformation and cancer [Jauliac et al., 2002; Marafioti et al., 2005; Pham et al., 2005; Buchholz et al., 2006; Medyouf et al., 2007]. However, the underlying molecular mechanisms by which deregulated NFAT signaling leads to cell transformation have remained poorly understood. Our current work now directly addresses this question and provides evidence that sustained NFATc1 signaling mediates its oncogenic activity in immortalized murine fibroblasts by inducing an autocrine factor-mediated growth loop that acts to transforms cells via the essential actions of the Stat3 transcription factor.

In a previous study we had demonstrated that ectopic expression of a constitutively active NFATc1 mutant in murine 3T3-L1 fibroblasts was sufficient to induce growth factor autonomy by inducing the secretion of autocrine growth factors capable of promoting cell growth and survival in the complete absence of exogenously added serum [Neal and Clipstone, 2003]. In the current study we have now extended this observation to show that the continuous long-term exposure of wild-type 3T3-L1 fibroblasts to caNFATc1-induced autocrine factors is sufficient to cause these cells to both lose their contact-mediated growth inhibition and acquire the ability to grow in an anchorage-independent fashion, both critical hallmarks of transformed cells [Hanahan and Weinberg, 2000]. Our results therefore provide evidence that the autocrine action of NFATc1-induced growth factors is sufficient to promote the phenotypic conversion of 3T3-L1 murine fibroblasts to the transformed cell phenotype.

The involvement of autocrine growth factors in promoting cell transformation is a well-established paradigm in cancer biology and is believed to participate in the etiology of a number of human cancers [Sporn and Roberts, 1985; Lang and Burgess, 1990]. Accordingly, we feel that the generation of an NFAT-induced autocrine growth factor signaling pathway is likely to play a key role in mediating the oncogenic potential of deregulated NFAT activity. In this respect, it is worth noting that the regulation of cytokine and growth factor gene expression is the best described function of NFAT proteins in vivo. Indeed, there is a striking parallel between the observed effects of caNFATc1 in promoting the autocrine growth factor-mediated transforming pathway in murine fibroblasts that we describe here, and the well-established functional role of endogenous NFAT proteins in promoting T lymphocyte growth during the immune response via the autocrine production of the primary T lymphocyte growth factor, interleukin-2 [Rao et al., 1997; Macian, 2005]. Moreover, NFAT binding sites are present in the promoter regions of numerous cytokines and growth factor genes and the activation of the NFAT signaling pathway has been implicated in the expression of an array of secreted factors, many of which are known to act in an autocrine fashion [Rao et al., 1997; Boss et al., 1998; Abbott et al., 2000; Hogan et al., 2003; Yang and Chow, 2003; Reinhold et al., 2004; Alfieri et al., 2007]. Hence, it is tempting to speculate that the increased expression and subsequent autocrine action of growth promoting cytokines and growth factors maybe a common mechanism by which deregulated NFAT activity is able to contribute towards initial tumor growth and malignant transformation. Unfortunately, we currently do not know the identity of the critical NFATc1-induced autocrine factor(s) that is responsible for promoting cell transformation. But given the fact that NFAT is known to be involved in promoting the expression of a large array of cytokine and growth factor genes, we anticipate that deregulated NFAT activity will likely induce the expression of a number of independent factors capable of acting in unison to promote tumorigenesis.

Despite not knowing the identity of the critical caNFATc1-induced autocrine growth factor(s) responsible for promoting cell transformation, we were nonetheless able to gain significant insights into the molecular mechanisms underlying the oncogenic activity of sustained NFATc1 signaling through our observation that the autocrine factor(s) induced by caNFATc1 is able to selectively activate the Stat3 transcription factor. This was of prime interest, as Stat3 has previously been strongly implicated with a role in cancer: the activation of Stat3 per se has been shown to be sufficient to promote the transformation of certain cell types [Bromberg et al., 1999] and Stat3 is known to represent a critical molecular conduit for many independent oncogenic signaling pathways [Bromberg, 2001; Calo et al., 2003; Yu and Jove, 2004]. This led us to directly test the possibility that Stat3 might play a role in mediating the transforming effects of sustained NFATc1 signaling. Towards this end, we were able to demonstrate that the shRNA-mediated depletion of endogenous Stat3 protein expression significantly inhibited the ability of ectopic caNFATc1 to induce the phenotypic conversion of immortalized 3T3-L1 cells to the transformed phenotype in vitro. Moreover, we found that depletion of endogenous Stat3 also significantly attenuated the ability of

caNFATc1-expressing 3T3-L1 cells to form tumors in nude mice in vivo. Collectively, therefore, our data provide compelling evidence that Stat3 plays a critical role in mediating the oncogenic effects of sustained NFATc1 activity by acting as an essential downstream effector of NFATc1-induced autocrine growth factors. In fact, the autocrine growth factor-induced activation of Stat3 appears to be a relatively common molecular module in the etiology of cancer, as it is observed in numerous cancers and a number of known oncogenes have been shown to transform cells through a similar mechanism [Schuringa et al., 2000; Faruqi et al., 2001; Li and Shaw, 2002; Park et al., 2003; Sriuranpong et al., 2003; Yeh et al., 2006].

Importantly, our results also demonstrate that the effect of sustained NFAT activity on the activation of Stat3 is not limited simply to murine fibroblasts, but is also observed in a number of human cell types that are relevant to the proposed role of NFAT in human cancer, such as the pancreas and breast. This observation that the activation of Stat3 is likely to be a common biological response to sustained NFAT signaling in a number of distinct cell lineages, makes it tempting to speculate that the deregulated constitutive NFAT signaling that is observed in a number of human cancer cell types, may be able to directly contribute towards tumorigenesis via the induced expression of autocrine growth factors and the subsequent downstream activation of Stat3. In this regard, it is interesting to note that constitutive NFAT activity, production of autocrine growth factors and activation of Stat3 have each been independently implicated in the etiology of both human pancreatic and breast cancer development [Dickson et al., 1992; Rasmussen and Cullen, 1998; Jauliac et al., 2002; Scholz et al., 2003; Ling and Arlinghaus, 2005; Prokopchuk et al., 2005; Buchholz et al., 2006]. However, it remains to be seen to what extent an NFAT-driven, autocrine growth factor-mediated, Stat3-dependent signaling pathway is involved in promoting malignant transformation in vivo.

In summary, this study has afforded significant new insights into the molecular mechanisms underlying the oncogenic effects of sustained NFAT activity. We have demonstrated that forced expression of a constitutively active NFATc1 mutant induces an autocrine growth factor-mediated pathway that acts to transform cells via the essential action of the Stat3 transcription factor. Interestingly, Stat3 has been implicated not only in the initial stages of cell transformation, but also in promoting tumor cell invasion and metastasis [Yu and Jove, 2004; Huang, 2007]. This leads to the intriguing possibility that Stat3 may mediate not only the transforming effects of sustained NFAT signaling that we describe here, but also the effects of constitutive NFAT proteins in promoting tumor cell migration and invasion that have been described in human breast and colon carcinomas [Jauliac et al., 2002]. Finally, while our current study has emphasized the role of autocrine growth factor-mediated Stat3-dependent pathway in cell transformation, we do not rule out the possibility that deregulated NFAT activity may also directly contribute towards promoting tumorigenesis in a cell autonomous fashion via the regulation of other independent genetic pathways. As an example, the constitutive activation of NFATc1 in human pancreatic cancer cells has recently been associated with increased expression of the c-myc proto-oncogene [Buchholz et al., 2006], while the increased expression of other known NFAT target genes such as cycloxygenase-2 and cyclin A [Iniguez et al., 2000; Karpurapu et al., 2008] are also known to contribute towards cell transformation in certain cell types. Indeed, while our current data demonstrate that Stat3 is critically required for caNFATc1-induced transformation, we feel it is likely that this pathway will co-operate with other NFATc1 target genes in promoting the fully transformed cell phenotype. Certainly, therefore, further investigation into the role of sustained NFAT activity in the etiology of tumorigenesis and its crosstalk with the Stat3-signaling pathway is clearly warranted.

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