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Research article

FGF signaling inhibits the proliferation of human myeloma cells and reduces c-*myc* expression

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Abstract

Background: Multiple myeloma is a cancer of antibody producing plasma cells whose etiology is unknown. FGF signaling has been implicated in myeloma pathogenesis but its precise role remains unclear.

Results: Here, we investigate the biochemical and phenotypic consequences of FGF stimulation in several different human myeloma cell lines. We find that FGF signaling inhibits cell cycle progression in two lines and surprisingly, reduces the expression of c-*myc* while turning on c-*fos*. In several other lines, FGF signaling does not affect proliferation rate, including cells harboring translocated FGF Receptor 3. When cells are presented with a growth arrest signal, FGF addition induces cell death.

Conclusions: By showing that FGF signaling inhibits mitogenesis and induces apoptosis, we demonstrate novel effects of activating this ubiquitous signaling pathway in multiple myeloma.

Background

Fibroblast growth factor (FGF) signaling regulates the activity of virtually all higher vertebrate cell types. In addition to controlling normal development and physiology, numerous observations support the concept that FGF signaling is abnormal in most forms of human cancer. A review of this voluminous literature is not possible here, but it should be noted that in many cases FGF signaling inhibits rather than promotes growth of the transformed clone. For example, FGF2 (basic FGF) slows the proliferation of breast cancer cells [1,2], and low levels of FGF2 are associated with accelerated breast cancer progression and worse prognosis [3]. Similarly, mutations in FGF Receptor 3 (FGFR3) are strongly associated with less aggressive forms of bladder cancer, suggesting that FGF signaling may inhibit proliferation in this malignancy [4,5]. Finally, recent studies show that FGF2 induces apoptosis in Ewing's sarcoma cells [6].

Alternatively, in some human cancer models, FGF signaling has no effect on tumor progression. In the SV40 T antigen transgenic model of pancreatic cancer, rigorous genetic analysis using both gain and loss of function alleles of FGF Receptor 4 has demonstrated that FGFR4 has no effect on the growth of transformed islet cells, even though it is invariably expressed in this setting [7]. This finding implies that the defect in cell fate determination seen in cancer is accompanied by the misexpression of genes whose action is neutral with respect to oncogenesis.

Multiple Myeloma (MM) is a neoplasia of transformed plasma cells [8]. Genetic abnormalities, including chromosomal translocations, inversions, and deletions are a common feature of MM [9]. Some of the translocations have been characterized and the genes within the rearranged loci pinpointed. One such translocation is t (4;14) which harbors at least two genes in the displaced interval: Fibroblast Growth Factor Receptor 3 (FGFR3) and Multiple Myeloma Set Protein (MMSET) [10,11]. The presence of FGFR3 in the translocated region has led to the proposal that FGF signaling promotes oncogenesis in myeloma [12].

The effect of activating FGF signaling on MM cell proliferation is unclear. The translocation involving FGFR3 is observed infrequently, being detectable in approximately fifteen percent of patients [11,13-16]. In addition, activating mutations in regulatory domains of FGFR3 have been observed in multiple myeloma cell lines and rarely in patient samples [14,17]. These mutations generally have been shown to augment the signaling potential of the receptor. Interestingly, recent microarray analysis reveals that FGFR3 mRNA is associated with gene expression patterns seen in early, less aggressive sub-groups of multiple myeloma [18]. Indeed, FGFR3 mRNA expression was never observed in more advanced stages of the disease despite translocation of the FGFR3 genomic locus to the IgH gene. This observation indicates that expression of FGFR3 mRNA is disfavored during disease progression. Outcome analysis shows no substantial difference in the progression of patients with translocated FGFR3 [15].

Notably, multiple FGF receptors are expressed in both myeloma patient samples and cell lines [19,20], suggesting that genes in this family other than FGFR3 are important. Although multiple myeloma likely originates from an activated, post-switch B lymphocyte, little data exists on the expression of FGF receptors in mature B cells. One exception is the demonstration that FGF receptor stimulation controls the proliferation of activated normal human peripheral blood B cells [21]. Consistent with a role for FGF signaling in mature B cells, we have found that FGF deficient mice produce more antibody to a Type 1 independent antigen and conversely, antibody production is suppressed in FGF over-expressing mice (manuscript in preparation). Thus, it is likely that FGF receptors play a role in mature B cell function. Finally, in addition to multiple receptors, several different FGF ligands are expressed in patient samples and cell lines. For example, FGF2 expression is higher in the serum of patients with multiple myeloma as compared to controls [22] and this difference is even larger when bone marrow levels are compared [23]. Recent experiments suggest that one source of the FGF2 may be the myeloma cells themselves [20].

Here we examine the role of FGF signaling in human multiple myeloma cell lines. We find that stimulation of the FGF pathway either inhibits growth or has no effect despite the presence of translocated FGFR3. Growth inhibition is accompanied by reduced expression of c-*myc*. Surprisingly, FGF treatment of cells in low serum medium stimulates cell death.

Results

To determine whether FGF could influence myeloma cell proliferation, we incubated myeloma lines with FGF1 under various conditions. Although similar results were obtained with FGF2, we performed most experiments with FGF1 since it stimulates the widest number of receptors [24]. We assayed growth rate in lines containing translocated FGFR3, such as OPM1, OPM2, JIM3, KMS11, H929, UTMC2, and KMS18 and also in lines lacking this abnormality. In both the OPM2 and OPM1 cell lines, FGF signaling reduced cell number during log phase growth, whereas in the other cell lines it had no effect (Fig. 1a). Since OPM1 and OPM2 cells are derived from the same patient [25], FGF signaling may slow cell proliferation *in situ*, since a reversal of the signaling pathway would be unlikely to take place upon adaptation to culture in two different cases. These cells express an FGFR3 mutant, K650E, which has significantly enhanced tyrosine kinase activity [26]. In an Interleukin-6 dependent mouse plasmacytoma line constitutively expressing elevated levels of FGFR3 (B9 cells), FGF1 had no effect on cell number (fig. 1a). In addition, no effect was observed in a plasmacytoma variant expressing the hyperactive mutant K650E. Previously, it had been reported that polyclonal populations expressing high levels of the K650E allele proliferate more rapidly compared to polyclonal populations lacking this receptor [27]. While we do not know the precise reason for the discrepancy, this study examined the effects of FGF treatment during apoptosis as a consequence of Il-6 withdrawal.

To investigate how FGF signaling influences cell cycle progression, we examined the cell cycle profile by FACS analysis of DNA content. We observed that progression through all phases of the cell cycle is retarded by treatment with FGF1, as reflected by a shift to the left in the number of cells with a given fluorescence intensity (Fig. 1b). We found three-fold fewer cells in the G2-M phase of the cell cycle after three days in the presence of FGF1 (region A, fig. 1b). These data demonstrate that FGF signaling slows cell cycle progression in human myeloma cells.

The c-MYC oncoprotein is frequently dysregulated in human cancer and c-*myc* plays a central role in the growth and proliferation of lymphocytes, including mature B cells [28]. Moreover, irregularities at the c-*myc* locus have been documented in virtually all human myeloma cell

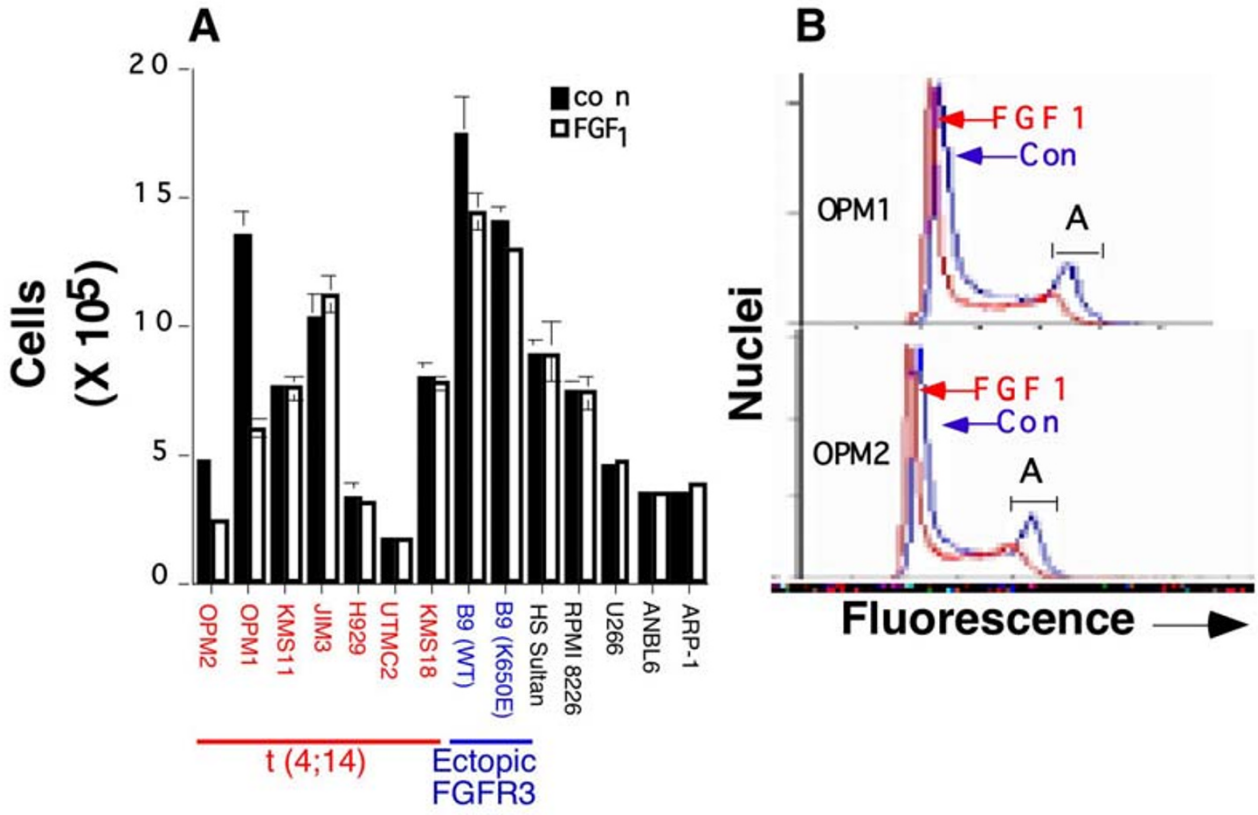

Figure 1

FGF Signaling Either Inhibits Myeloma Cell Proliferation or Has No Effect. A) Myeloma cell lines were treated with FGF1 (100 ng/ml) and heparin (10 ug/ml) or heparin alone (control) for three days and cell number was determined. B9 cells are a mouse plasmacytoma line stably expressing FGF Receptor 3 wild type (wt) or hyperactive mutant K650E [27]. Error bars represent standard deviation of assays performed in triplicate. Results shown are representative of more than four independent determinations for OPM2 cells and three independent determinations for OPM1 cells; $p < .01$, Student's paired t test. B) OPM1 and OPM2 cells were treated with FGF1 (10 ng/ml) and heparin (10 ug/ml) or heparin only for three days and DNA content was measured using FACS analysis of ethidium bromide stained nuclei.

lines [29]. In OPM2 cells, c-*myc* is translocated to the IgH locus [30], suggesting that its expression may be deregulated. To determine whether c-*myc* expression is affected by FGF stimulation in myeloma cells, we examined the levels of c-*myc* mRNA and protein following addition of FGF1. While in some cell types, growth factor addition enhances c-MYC expression, FGF1 stimulation down-regulated c-MYC in OPM2 cells (fig. 2a). To our knowledge, this represents the first example of a decrease in c-MYC expression in response to a classical tyrosine kinase growth factor. The decrease in c-MYC protein levels may be a result of decreased levels of the mRNA which was also lower in the presence of FGF 1 (fig. 2b). We note that decreased *c-myc* mRNA expression is only observed at concentrations of FGF1 greater than 1 ng/ml.

Why is there no effect on proliferation rate in cell lines harboring translocated FGFR3? The FGF signal transduction pathway could be stimulated in these lines but the stimulation could fail to reduce cell number due to inefficient or defective inhibition of the DNA synthesis machinery. To determine whether FGF signaling affects DNA synthesis, we incubated cells with FGF1 and assayed H^3 thymidine incorporation into DNA. We found that FGF1 treatment either modestly inhibited DNA synthesis or had no effect in cell lines containing the 4;14 translocation (Fig. 3). Despite the relatively slow growth rate of myeloma cell lines, the decrease in DNA synthesis in OPM2 cells was observable twenty four hours after growth factor addition.

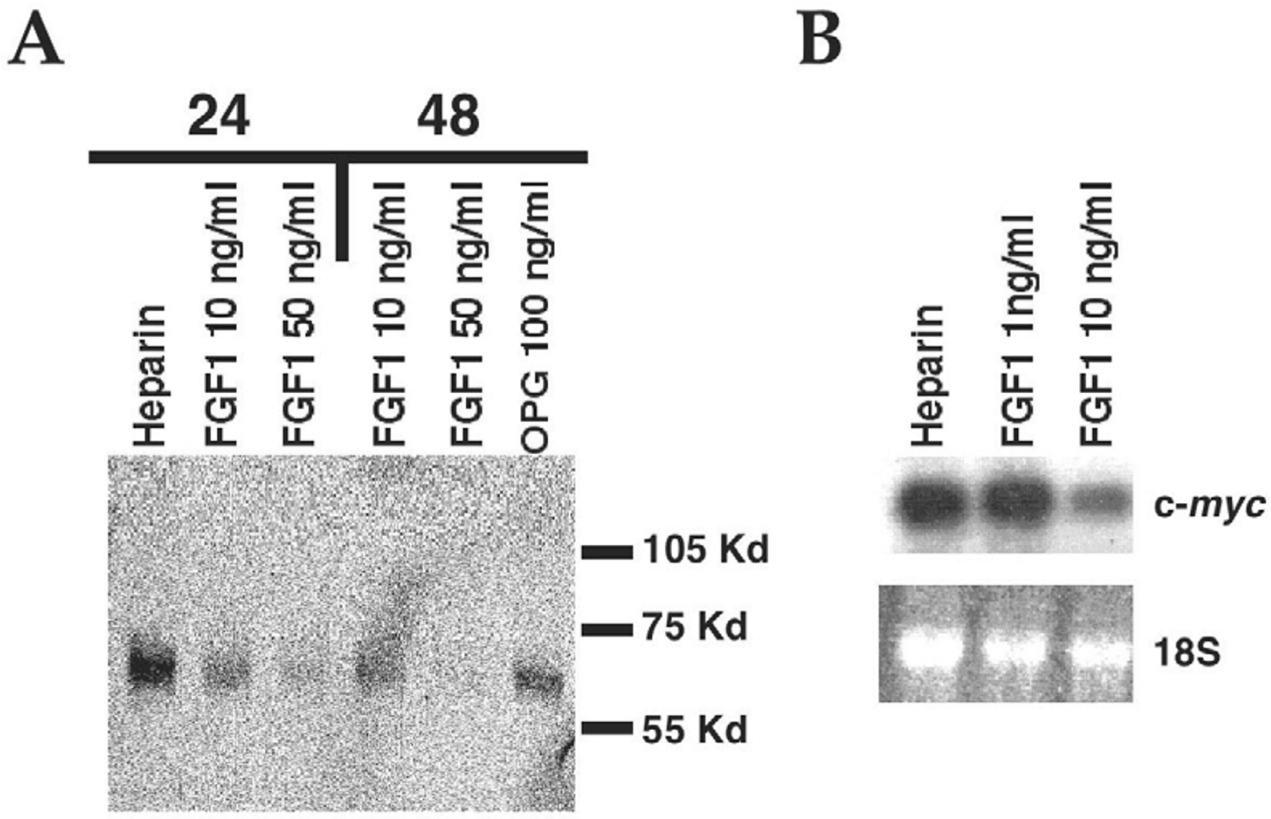

Figure 2
FGF Signaling Inhibits c-*myc* expression. A) Immunoblot of RIPA lysates from OPM2 cells using monoclonal anti-c-MYC antibody. Cells were treated with FGF1 (or Osteoprotegerin as negative control) for 24 or 48 hours at the indicated concentrations. Cells were harvested in RIPA lysis buffer containing protease inhibitors. B) Expression of c-*myc* mRNA levels. Cells were treated with FGF1 at the indicated concentrations for 3 days and c-*myc* mRNA levels were measured by Northern blot. Bottom panel shows ethidium bromide stained 18S ribosomal RNA.

One of the earliest transcriptional events in response to FGF signaling is the induction of c-*fos*. To determine the effect of FGF signaling on c-*fos* expression, we examined the levels of c-*fos* mRNA after FGF stimulation of serum starved cells. The cell lines OPM2, KMS11, and UTMC2 all strongly activated c-*fos* transcription in response to FGF2 (Fig. 4). These lines carry the 4;14 translocation. We conclude that early nuclear events in response to FGF stimulation are properly activated in myeloma cell lines whose proliferation rate is unchanged by FGF stimulation (Fig. 1). In addition, these results have significant implications for models of FGFR3 function. Hyperactive mutant forms of FGFR3 are thought to be constitutively active and ligand independent. Experiments with KMS11 and OPM2 cells have shown that basal auto-phosphorylation of FGFR3 and MAP kinase activity are higher under serum starved conditions, suggesting that FGF signaling is persistently active in these cells [16]. We find that FGFR3 mutants respond strongly to ligand and with respect to nuclear signaling events, are not constitutively activated. Therefore, activating mutations in a cell surface receptor in the absence of ligand do not necessarily lead to the expected changes in gene expression from stimulation of the receptor's signal transduction pathway.

Since it is possible that FGF signaling could influence survival without affecting proliferation rate, we explored whether it could modulate either serum starvation or dexamethasone-induced cell death. FGF stimulation did not protect myeloma cell lines from either death inducing stimulus whereas IL-6 did inhibit cell death under the same conditions (Fig. 5A,5B). While we did not directly

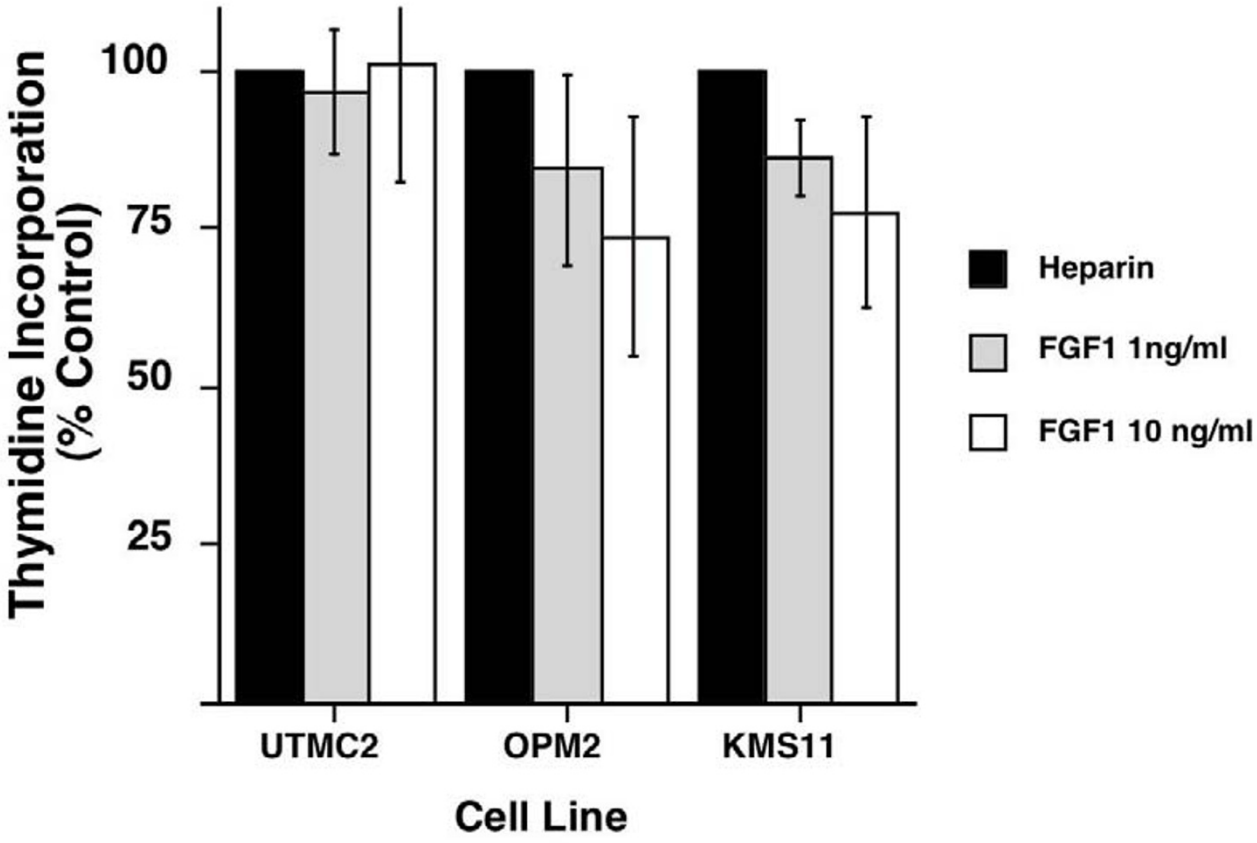

Figure 3
FGF Signaling Reduces DNA Synthesis in Myeloma Cells. Myeloma cell lines containing t(4;14) were incubated in serum free medium with FGF1 (10 or 100 ng/ml) plus heparin (10 ug/ml) and incorporation of H^3 thymidine into DNA was measured one day later. Data are expressed as percent of control (heparin only) and represent the mean of five independent experiments, measured in triplicate; $p < .01$ Student's paired t test.

measure apoptosis in these lines, others have shown that dexamethasone and serum starvation activate an apoptotic program in these cells [31,32]. Unexpectedly, we observed that FGF1 induces apoptosis in OPM2 cells. When these cells are cultured in reduced serum, FGF1 produced a dose-dependent acceleration of apoptosis, leading to a three-fold increase in sub G_0 DNA content 24 hours after transfer to serum free conditions (Fig. 5C). This effect is accompanied by reduced DNA synthesis, as reflected by a shift to the left in the number of cells with a given DNA content. Consequently, FGF signaling slows cell cycle progression during serum starvation, demonstrating that cell death is not a result of a conflict between the growth arrest signal from serum starvation and a putative growth stimulatory signal from FGF1.

Discussion

We have investigated the role of FGF signaling in human multiple myeloma cell lines. Examination of numerous lines reveals that FGF treatment either reduces cell number or has no effect. Moreover, when OPM2 cells are presented with a strong growth arrest signal by culturing in low serum medium, FGF addition stimulates apoptosis. These studies demonstrate that the function of FGF signaling in multiple myeloma is more complex than previously appreciated. Since reduced proliferation is observed in two different cell lines derived from the same patient, FGF signaling may inhibit mitogenesis *in vivo* and thus retard disease progression. This hypothesis is consistent with the silencing of FGFR3 mRNA expression in advanced myeloma, even though the FGFR3 locus is translocated to the actively expressed IgH region [18]. In

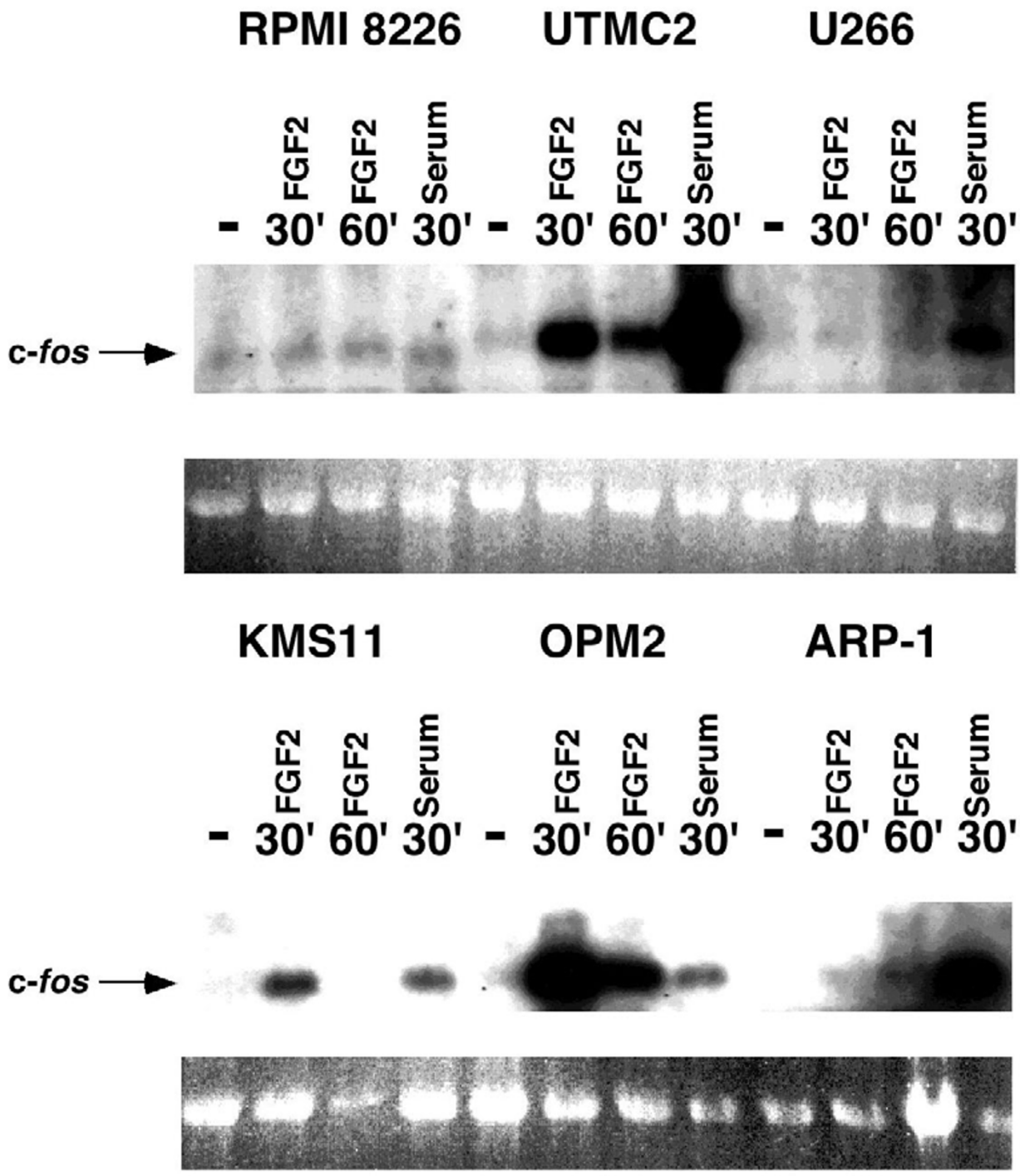

Figure 4
FGF Signaling Induces *fos* in Myeloma Cell Lines containing t(4;14). Cell lines were cultured in serum free medium for twenty four hours and FGF2 (50 ng/ml) or 10% serum was added. Total RNA was isolated and the expression of c-*fos* was measured at the indicated times. Ethidium bromide stain of 28 S RNA is shown.

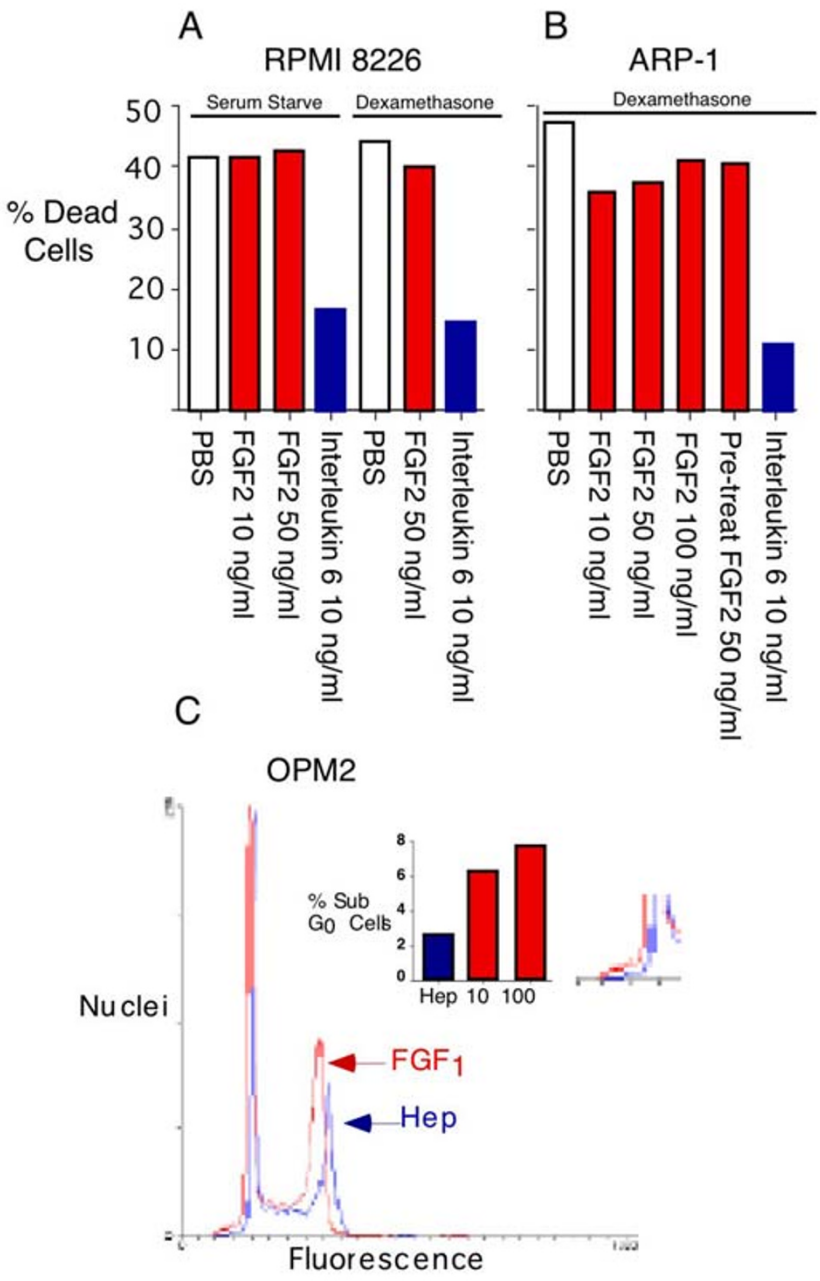

Figure 5

FGF Signaling Induces Cell Death in OPM2 Cells. A) RPMI 8226 myeloma cells were cultured in serum free medium for 24 hours or serum containing medium plus 10 uM dexamethasone (last three columns) in the presence of the indicated factors. The number of dead cells was evaluated using trypan blue exclusion two days after dexamethasone treatment. B) FGF signaling does not protect ARP-1 myeloma cells from dexamethasone-induced apoptosis. Conditions as in panel A. Pre-treatment consisted of pre-incubation of the cells with IL-6 for one hour, followed by death inducer. C) OPM2 cells were cultured in serum free medium for one day in the presence or absence of FGF1 and cell cycle analysis of ethidium bromide stained nuclei was performed. Results are representative of four independent determinations; $p < .01$ Student's paired t test. Red trace shows results with 100 ng/ml FGF1 as compared to heparin only (blue trace). Inset shows quantification of sub G_0 DNA content for 10 and 100 ng/ml FGF1 as compared to heparin alone and a magnified version of the sub G_0 region shown in the FACS.

addition, since FGF fails to inhibit proliferation in most myeloma cell lines despite expression of other FGF receptors [19,20], FGF-induced growth inhibition may need to be silenced for the transformed clone to expand. Since most myeloma cell lines are derived from cells which have escaped the bone marrow, we speculate that FGF signaling may serve to inhibit proliferation in early stages of the disease, when the transformed clone is confined to the bone marrow microenvironment. Although we can not exclude the possibility that FGF signaling may stimulate proliferation in multiple myeloma cells at some stage of their development, our data indicate that no effect or slower proliferation are more likely outcomes. Studies with primary myeloma cells may shed further light on this issue.

We find that FGF-induced growth inhibition is accompanied by reduced c-MYC expression. To our knowledge, this is the first demonstration of a reduction in c-MYC expression by a classical tyrosine kinase growth factor since FGF stimulation has the opposite effect in many other cell types. Aberrations at the c-*myc* locus are almost always observed in multiple myeloma cell lines, implying that cis-deregulation is required for growth. In OPM2 cells, the c-*myc* gene is fused to the IgH locus [30]. Despite this abnormality, it is surprising to observe that FGF stimulation overcomes whatever destabilizing effect it introduces and suppresses c-MYC expression. While activation of the c-*myc* pathway may not be an initiating event in multiple myeloma, numerous studies have revealed that c-*myc* contributes to many different human cancers, including multiple myeloma. Therefore, signals which counteract c-MYC expression are of paramount importance.

Studies of the effect of activating the FGFR3 pathway in both fibroblasts and hematopoietic cells have shown that FGFR3 poorly stimulates proliferation. For example, despite abundant expression, both wild type and hyperactive mutant alleles of FGFR3 were unable to transform NIH 3T3 cells [33]. In another study, only the K650E allele produced tumors in nude mice while the wild-type receptor was inert [17]. Recently, a novel FGFR3 mutation (G380D) was shown to weakly transform NIH 3T3 cells. This mutant displayed a similar potency to the K650E allele and was more than 300 times less active than the standard receptor tyrosine kinase/oncogene *trk* [16]. In BaF3 pro B cells, overexpression of FGFR1 stimulates DNA synthesis while overexpression of FGFR3 does not [24,26].

On the other hand, the K650E allele has been shown to induce pre-B cell tumors when retrovirally expressed in the bone marrow of transgenic mice [34]. While this is the strongest evidence to date that FGFR3 can positively affect proliferation in hematopoietic cells, over-expression experiments using retroviral vectors which are not easily down regulated might best be interpreted with caution. Indeed, the very early pre-B cell phenotype of the tumors in this report raise the possibility that FGF signaling may regulate immature rather than mature B cell proliferation.

Why does FGF have no effect on the rate of cell division of some myeloma cell lines containing the 4;14 translocation, such as KMS11 and UTMC2? One possibility is that early steps in signaling are not activated normally as a result of mutation. For example, previous studies in plasmacytoma cell lines have demonstrated a block in TGF beta signaling due to inefficient receptor binding at the cell surface [35]. Another possibility is that early steps in FGF signaling are activated but later steps are blocked. To assay for defects in the signal transduction pathway, we examined expression of c-*fos* in response to FGF stimulation. Since c-*fos* is normally induced, we conclude that the FGF pathway leading to changes in gene expression remains unperturbed by mutation.

The induction of apoptosis by FGF stimulation in OPM2 cells reveals that in sharp contrast to acting as a survival factor, FGF signaling transmits a death signal. Death induction by classical growth and survival factors is not without precedent. FGF signaling in human chondrocytes expressing the K650E allele activates cell death without affecting proliferation rate [36]. Moreover, in FGF2 transgenic mice, FGF induced cell death is inhibited by reducing *stat1* gene dosage, suggesting that *stat1* mediated growth inhibition may precede the execution of the apoptotic program [37]. Notably, FGF stimulation of multiple myeloma cell lines harboring t(4;14) does not stimulate stat activity [16]. The accumulating evidence for death induction by receptor tyrosine kinases implies that further investigation of this phenomenon may reveal new pathways of cell suicide. Future research will be directed at understanding in greater detail the molecular mechanism by which FGF signaling controls these processes.

Conclusion

We have demonstrated that FGF signaling inhibits the proliferation of human myeloma cells.

Inhibition is accompanied by reduced DNA synthesis and surprisingly, reduced expression of the proto-oncogene c-*myc*. In many other cell lines FGF signaling did not affect proliferation rate, including cell lines with translocated FGFR3. Under growth suppressed conditions, FGF signaling induced apoptosis. Taken together, our data reveal unanticipated effects of FGF signaling in multiple myeloma and suggest novel conclusions concerning its role in the oncogenic process.

Methods

Cell Culture

OPM1 cells were from Edward Thompson (University of Texas). UTMC2, OPM2, KMS11, and JIM3 cells were the kind gift of P. Leif Bergsagel (Cornell University Medical College). KMS18 cells were from Antonino Neri (University of Milan), ARP-1 cells were from Joshua Epstein (University of Arkansas), and ANBL6 lines were from Diane Jelinek (Mayo Clinic). B9 plasmacytoma cells were the generous gift of A. Keith Stewart (University of Toronto) and were supplemented with 1–10 ng/ml Il-6. All other lines were from ATCC. All cell lines were cultured in RPMI 1640 media with 10% FCS, except UTMC2 cells which were supplemented with 1–10 ng/ml Il-6 (R and D Systems). For serum starvation experiments, cells were replated in media lacking serum but containing insulin, transferrin and selenium (Gibco Serum Supplement) for twenty four hours. Recombinant human FGF1 and 2 were from R and D (Minnesota).

Cell Proliferation Experiments

Cell number following FGF stimulation during log phase growth was determined either by counting the number of trypan blue negative cells or by Coulter Counter with identical results. For B9 plasmacytoma derivatives, proliferation rate was measured in the presence of 1–10 ng/ml Il-6. For thymidine incorporation studies, cells were incubated in serum free medium for 24 hours in the presence or absence of FGF1. Cells were harvested 24 hours later. 4–16 hours before harvest, 0.5 uCi H^3 thymidine per 100 ul was added. Experiments were performed in triplicate and counted on a Beta plate reader (LKB Wallac).

Cell Cycle Experiments

FACS analysis was performed on nuclei isolated by NP-40 lysis [38]. Briefly, cells were centrifuged at 1000 × G and incubated in NP-40 lysis buffer with RNAse (10 ug/ml) at room temperature for 30 minutes followed by citric acid/sucrose buffer containing ethidium bromide. Successful isolation of nuclei was confirmed by microscopic examination. Cell cycle analysis was performed on a Coulter Model XL flow cytometer, gating on signals indicative of intact, non-clumped nuclei.

Immunoblotting and Northern analysis

Cells in log phase growth were harvested by centrifugation and lysed at 4° in RIPA buffer (20 mM Tris pH 8.0, 137 mM NaCl, 10% v/v glycerol, 1% Nonidet P-40, 0.1% Sodium Dodecyl Sulfate, 0.5% Sodium Deoxycholate, 2 mM EDTA) containing protease inhibitors (aprotinin, leupeptin, pepstatin and PMSF). Lysates were clarified by centrifugation at 14, 000 × G. Protein concentration was determined using the BCA method (Pierce). c-MYC was visualized by ECL (Amersham) using the monoclonal C-33 antibody at 1:1000 (Santa Cruz) and HRP coupled Goat anti-Mouse secondary at 1:10,000 (Jackson).

For studies of *fos* and *myc* mRNA expression, total RNA was isolated by guanidium isothiocyanate lysis (Trizol, GIBCO/BRL) or Qiagen mRNA easy (Qiagen) and separated on 1% agarose gels containing formaldehyde. RNA was transferred to nylon membranes (Schleicer and Schuell) and probed with random primer generated P^{32} labeled probes from gel purified fragments of mouse cDNA encoding c-*fos* and c-*myc*. Washing was performed at a minimum stringency of 55°C in 0.1X SSC and 0.1% SDS.

Authors' Contributions

LF performed cell proliferation studies, including thymidine incorporation and cell viability experiments. In addition, LF assisted with Western and Northern blots. AB drafted the manuscript and conducted some of the experiments, including FACS analysis.

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