Therapeutic effects of thalidomide in myeloma are associated with the expression of fibroblast growth factor receptor 3

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Abstract: Thalidomide (Thal), a novel agent in the treatment of multiple myeloma, is presumed to act through a variety of mechanisms. In the present study, we examined the relationship between fibroblast growth factor receptor 3 (FGFR3) expression and the therapeutic effect of Thal. The DNA synthesis of KMS-11 clone, which overexpresses FGFR3, was inhibited by Thal in a dose-dependent manner; whereas U266 cells, which lack FGFR3 expression, failed to respond to Thal inhibition. To further examine the intertwining of Thal's therapeutic effects, wild-type human full-length FGFR3 cDNA was transfected into U266 cells. FGFR3 transfected U266 clones revealed increased FGFR3 expression, but resulted in decreased DNA synthesis and increased apoptosis under Thal treatment. Under Thal treatment, the myeloma proliferation-related protein, vascular endothelial growth factor (VEGF), and interleukin-6 (IL-6) were decreased in U266 FGFR3 transfectant as well. These results suggest that Thal inhibits myeloma cell proliferation and may depend on FGFR3 expression status. To further confirm this observation, we transfected a plasmid constructed anti-FGFR3 ribozyme (Rz52) into KMS-11 cells. In the ribozyme transfectant KMS-11 clone, FGFR3 expression was decreased; whereas the effects of Thal in cell growth inhibition were abrogated in KMS-11 Rz52 clone. Further results suggested that Thal inhibition of DNA synthesis, induction of apoptosis, and down-regulation of VEGF and IL-6 might be dependent on FGFR3-associated signal transduction of the ERK and STAT3 phosphorylation pathway. Thus, FGFR3 may be a predictive/surrogate marker for selection of Thal treatment in myeloma.

Keywords: fibroblast growth factor receptor 3, multiple myeloma, thalidomide, vascular endothelial growth factor

Introduction

Multiple myeloma (MM) is characterized by the clonal proliferation of malignant plasma cells in the bone marrow (Vacca et al 1994; Hallek et al 1998). Only 5% of patients achieve a complete response with conventional therapy (Zaidi et al 2001). The relapse is largely due to development of MM resistance to chemotherapeutic agents (Attal et al 1992). Remission following autologous bone marrow transplant (ABMT) can be accomplished in 26%–45% of patients (Bjorkstrand et al 1995; Tricot et al 1995; Attal et al 1996; Barlogie et al 1997, 1999; Tosi et al 2002). However, the majority of MM patients will eventually relapse (Anderson et al 1999; Singhal et al 1999; Stevenson and Anderson 1999).

Vascular endothelial growth factor (VEGF) is a key mediator of tumor angiogenesis and plays a role in the pathogenesis of hematological malignancies, including MM (Folkman 1995; Bellany et al 1999; Dankbar et al 2000; Podar et al 2001; Tartaglia et al 2001). VEGF and its receptors Flt-1 (VEGFR-1) and KDR (VEGFR-2) can be detected in the bone marrow microenvironment (DeVries et al 1992;

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Podar et al 2001). VEGF directly induces proliferation and triggers trans-filter migration of human MM cells expressing Flt-1, suggesting an autocrine VEGF loop in MM (Bellamy et al 1999). VEGF can increase expression of several hematopoietic growth factors in endothelial cells, including interleukin-6 (IL-6) (Terman et al 1992), which plays a critical role in the onset of plasma cell tumors in vivo and in vitro and has an antiapoptotic effect on myeloma cells (Kawano et al 1988; Suematsu et al 1992; Hilbert et al 1995).

The fibroblast growth factor receptor 3 (FGFR3), the high affinity receptor for bFGF, was recognized on the surface of myeloma cells with t(4; 14) translocation (Chesi et al 1998, 2001). The t(4; 14) (p16.3; q32) chromosomal translocation occurs in approximately 20% of MM and leads to the apparent upregulation of FGFR3 (Bergsagel and Kuehl 2001; De Bont et al 2001). The FGFR3 gene is a member of the family of the tyrosine kinase receptor, which binds to a number of related mitogenic fibroblast growth factors resulting in the activation of complex signaling pathways and activation of cell proliferation regulation and cell transformation (Kanai et al 1997). FGFR3 signaling can substitute for IL-6 in directing growth and survival in a myeloma cell line, and inhibition of these signals results in apoptosis (Plowright et al 2000). In our previous report, we demonstrated that ribozyme-induced FGFR3 down-regulation inhibited DNA synthesis in addition to VEGF down-regulation in KMS-11 cells (Qian et al 2005). This finding suggests that FGFR3 might play a role in the pathogenesis of MM associated with the t(4; 14) trans-location, and is a potential therapeutic target for the treatment of MM.

Thal is a glutamic acid derivative that exerts potent antiangiogenic activity in experimental systems and in advanced MM (Singhal et al 1999; Barlogie et al 2001). Although Thal was initially used to treat MM because of its known antiangiogenic effects (D'Amato et al 1994), the mechanism of its activity appears to be more complex and remains unclear. Recent studies suggested that Thal could inhibit MM cell growth in vitro directly (Hideshima et al 2000). Furthermore, Thal has shown a broad spectrum of different effects including inhibiting the production of cytokines, such as IL-6, VEGF, and bFGF in the bone marrow (Gupta et al 2001), and studies propose it may also increase apoptosis (Davies et al 2001). Recent reports of increased angiogenesis in the marrow of multiple myeloma patients (Ribatti et al 1999; Vacca et al 1999) further provided the rationale for its use in treating MM (Singhal et al 1999; D'Amato et al 2000). The plasma levels of bFGF

in patients with progressive MM were high and decreased with subsequent response to Thal therapy but remained controversial in many ways (Kenyon et al 1997; Hideshima et al 2000; Neben et al 2001). The overall mechanism of the Thal effect is not completely understood. In this study, we have begun to characterize the mechanisms of Thal against human MM cells. We demonstrated that Thal-induced cytotoxicity in MM cells is associated with FGFR3 status.

Materials and methods
Cell culture
KMS-11, the human myeloma cell line containing the t(4; 14) translocation and overexpression of FGFR3, was kindly provided by Dr Bergsagel. U266, the human myeloma cell line that lacks FGFR3, was obtained from the American Tissue Culture Collection (ATCC). Cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) and 1% (v/v) penicillin and streptomycin (100 mg/mL), and were maintained at 37 °C in 5% CO_2 atmosphere.

Compound
Thal was purchased from Sigma and dissolved in DMSO and stored at –20 °C as stock. Drugs were diluted in cultured medium (10^{-2}–10^4 μmol/L) with <0.1% DMSO immediately before use.

Plasmid construction
To construct the FGFR3 expression vector, a HindIII/Kpn1 fragment was released from the vector containing full-length human FGFR3 cDNA (a gift from Dr Podolsky's lab) and subsequently ligated into HindIII/Kpn1 digested pcDNA3.1 plasmid (Invitrogen, San Diego, CA, USA). Anti-FGFR3 ribozyme expression vector, PTV5Rz52, was designed as previously reported (Qian et al 2005).

Transfection
Prior to electroporation, cells were washed twice with serum free RPMI 1640 and resuspended into electroporation buffer (Eppendorf, Germany) at a concentration of 1.4×10^6 viable cells/mL. The cell suspension was mixed with 20 mg/mL plasmid in a 2-mm gap electroporation cuvette (Eppendorf, Germany). The cells were electroporated at a fixed 300 V and the time constant was 50 ms using a multiporator (Eppendorf, Germany). The electroporated cells were left in the cuvette for 10 min at room temperature and transferred

to 60-mm petri dishes containing warm (37 °C), complete medium and incubated at 37 °C, 5% CO_2. The growth medium was replaced 24 hours after electroporation.

[^{3}H]-thymidine incorporation assay

DNA synthesis was determined by measuring the incorporation of radiolabeled thymidine into the nucleic acid of cells. 2×10^4 cells were incubated with $0.5 \mu Ci/mL$ [^{3}H] thymidine for 8 hours at 37 °C and then washed with ice-cold phosphate-buffered saline (PBS). The sample was counted by using the Beckman LS 5000CE Scintillation Counter. All experiments were performed in triplicate.

Apoptosis and flow cytometry analysis

1×10^6 MM cells, cultured 48 hours after treatment with Thal were harvested, washed with PBS, fixed with 70% ethanol, and pretreated with 10 mg/mL of RNase (Sigma). Cells were stained with propidium iodide (PI; 10 mg/mL, Sigma), and analyzed by flow cytometry. The cell cycle profile was determined by using the program M software on an Epics flow cytometer (City of Hope, Core Facility, CA, USA). Apoptosis was assessed by determining the percent of cells staining with hypodiploid DNA (sub-G1) contents. The FTIC labeled Annexin V was stained on the cells treated or without treatment with Thal for apoptosis evaluation.

RNA purification and reverse transcriptase polymerase chain reaction assay

Total RNA was prepared with RNeasy Mini kits (Qiagen, CA, USA) according to the manufacturer's instructions. Complementary DNA (cDNA) was synthesized by using SuperScript™ One-Step Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) system with platinum Taq (Invitrogen, Life Technologies). For amplification of FGFR3 cDNA, the sequences of the primers are 5′-TCC CAT GGG GCC CAC TGC CTG-3′ (FGFR35 sense) and 5′-GCT CGG GCC GTG TCC AGT AAG-3 (FGFR33C antisense). FGFR3 cDNA was amplified for 35 cycles consisting of denaturing at 94 °C for 30 s, annealing at 53 °C for 45 s and extending at 72 °C for 1 min. VEGF primers corresponding to the 3′ and 5′ untranslated regions, 5′-TCG GGC CTC CGA AAC CAT GA-3′ (sense) and 5′-CCT GGT GAG AGA TCT GGT TC-3′ (antisense), were used to amplify the known splice variants, producing products of 516 base pairs (bp) of

VEGF121; 588 bp of VEGF145; 648 bp of VEGF165; 720 bp of VEGF189; and 771 bp of VEGF206. Samples were amplified for 35 cycles at 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 60 s. VEGF receptor primers were used to amplify Fms-like tyrosine kinase-1 (Flt-1) 5′-CAA GTG GCC AGA GGC ATG GAG TT-3′ (sense), 5′-GAT GTA GTC TTT ACC ATCCTG TTG-3′ (antisense), and the kinase insert domain-containing receptor (KDR) 5′-CAA CAA AGT CGG GAG AGG AG-3′ (sense) and 5′-ATG ACG ATG GAC AAG TAG CC-3′ (antisense). Samples were amplified for 35 cycles at 94 °C for 1 min, 60 °C for 2 min, and 72 °C for 3 min. IL-6 was amplified using the primers 5′-GCG CCT TCG GTC CAG TTG-3′ (sense) and 5′-CTC CTT TCT CAG GGC TGA G-3′ (antisense). Samples were amplified for 38 cycles consisting of 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 3 min. For all PCR reactions we used 25 picomoles of each primer. The integrity of mRNA in each sample was confirmed by amplification of β-actin. Polymerase chain reaction (PCR) products were separated on a 1.2% agarose gel, stained with ethidium bromide, and photographed. The intensity of each band was quantified using ImageQuant software (Molecular Dynamics, Sunnyvale, CA, USA), based on the area under each peak. Signal intensities of the VEGF, VEGFR, and IL-6 PCR products were measured and normalized by the signal intensity of the β-actin PCR product.

Antibodies and Western blot analysis

Cells were lysed using lysis buffer: 0.65 mL of ice-cold RIPA buffer ($1 \times$ PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulfate [SDS]) with freshly added inhibitors: 100 μg/mL phenylmethyl-sulfonyl fluoride (PMSF), 1 mmol/L sodium orthovanadate, and 30 μL/mL aprotinin. For detection of VEGF and FGFR3 protein, cell lysates were subjected to 7.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to polyvinylidene difluoride (PVDF) membrane. All antibodies used in this study were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and immuno-blotted with anti-VEGF antibody. The membrane was striped and reprobed with anti-alpha tubulin Ab to ensure equivalent protein loading.

To characterize growth signaling, immunoblotting was also done with antiphospho-specific STAT3 (Tyr705)-R, anti-STAT3 (F-2), antiphospho-specific ERK (E-4), and anti-ERK1 (C-16) (Santa Cruz, CA, USA). The membrane was stripped and reprobed with anti-alpha tubulin Ab to ensure
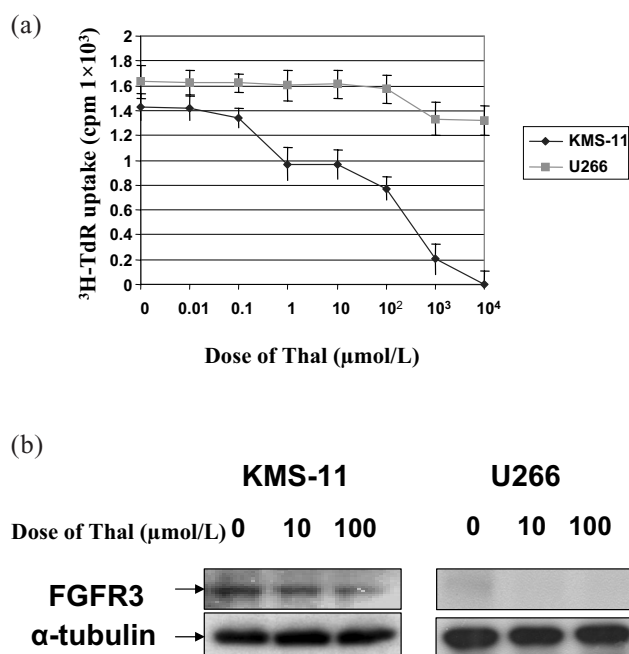
(a)

(b)

Figure 1 Effect of Thal on DNA synthesis of MM cell lines. 2×10^4 cells of KMS-11 or U266 clone were cultured with concentrations ranging from 10^{-2} to $10^4 \mu mol/L$ of Thal for 48 hours. 3H-TdR (cpm) uptake were measured as described in the *Materials and Methods* section. Values represent the mean ($\pm$ SD) of triplicate. The experiments were repeated at least three times. The FGFR3 protein expression was examined under Thal treatment and demonstrated on the lower panel (Figure 1b).

equivalent protein loading. Immunoblotting was performed by incubation with the corresponding antibody (1:200 dilution, 1 hour at room temperature) followed by incubation with alkaline phosphatase conjugated goat anti-mouse (1:5000 dilution, 1 hour at room temperature), or bovine anti-goat (1:2000 dilution, 1 hour at room temperature) secondary antibodies. Immunoreactive bands were visualized by enhanced chemiluminescence.

Statistical analysis

All experiments were repeated at least three times and each sample in triplicate in each experiment. The values reported the mean of triplicate ($\pm$ SD).

Results

FGFR3 deficient U266 cells resistant to Thal in contrast to FGFR3 overexpression KMS-11 cells, which are sensitive to Thal treatment

The effect of Thal on DNA synthesis in MM cells KMS-11 and U266 was determined by measuring ^{3}H-TdR uptake treated with or without various concentrations of Thal from 10^{-2} to $10^4 \mu mol/L$. DNA synthesis was inhibited in a dose-

dependent manner by Thal in FGFR3 overexpressing KMS-11 cells and shown in Figure 1a. The DNA synthesis was inhibited starting from $1 \mu mol/L$ of Thal. Fifty-percent inhibition of DNA synthesis of KMS-11 cells was noted at $100 \mu mol/L$ Thal. Under $10^3 \mu mol/L$ of Thal treatment, DNA synthesis of MS-11 was nearly completely blocked. However, U266 cells, which lack FGFR3, only a slight inhibition of DNA synthesis was observed even in a high concentration of $10^3 \mu mol/L$ Thal and continued to have resistance to $10^4 \mu mol/L$. The FGFR3 expression was decreased on the KMS-11 clone in a dose-dependent manner. Whereas, U266 had no change in its low expression level (Figure 1b).
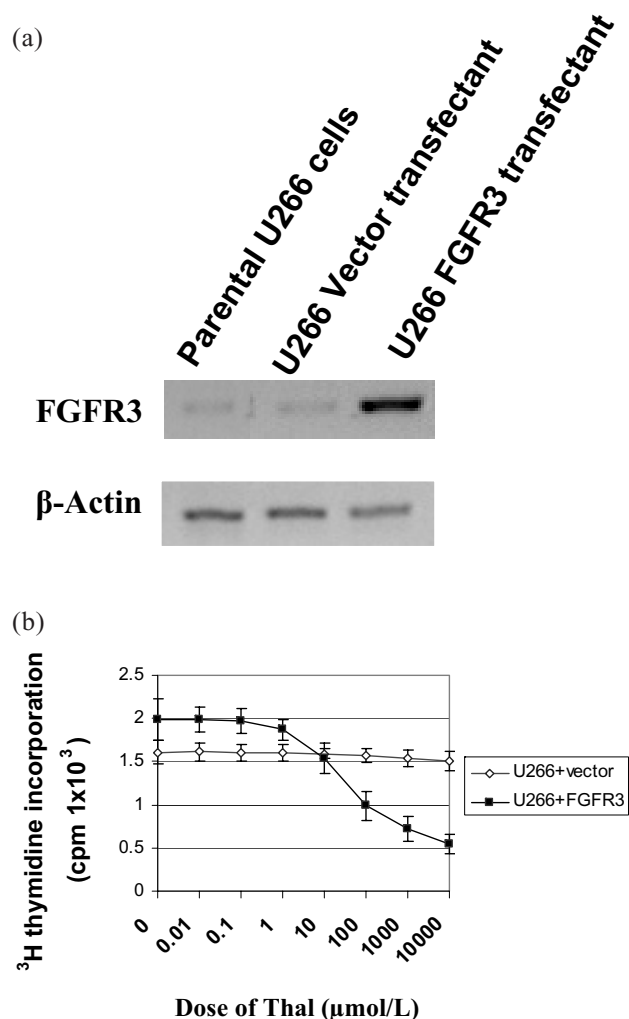
(a)

(b)

Figure 2 Effect of Thal on DNA synthesis in U266 FGFR3 transfectant clone. (a) Expression of FGFR3 in transient transfection U266 clone. U266 cells were transfected with the indicated plasmids as described in the *Materials and Methods* section. RT-PCR assay shows the expression of FGFR3 in FGFR3 transfected U266 clones. (b) The transfectants were cultured with concentrations ranging from 10^{-2} to $10^4 \mu mol/L$ of Thal. U266 parental cells demonstrated similar results as the U266 vector transfectant. The results demonstrated here are only from U266 vector transfectant clone. 3H-TdR (cpm) uptake was measured as described in the *Materials and Methods* section. Values represent the mean ($\pm$ SD) of triplicate cultures. The experiments were performed in triplicate.
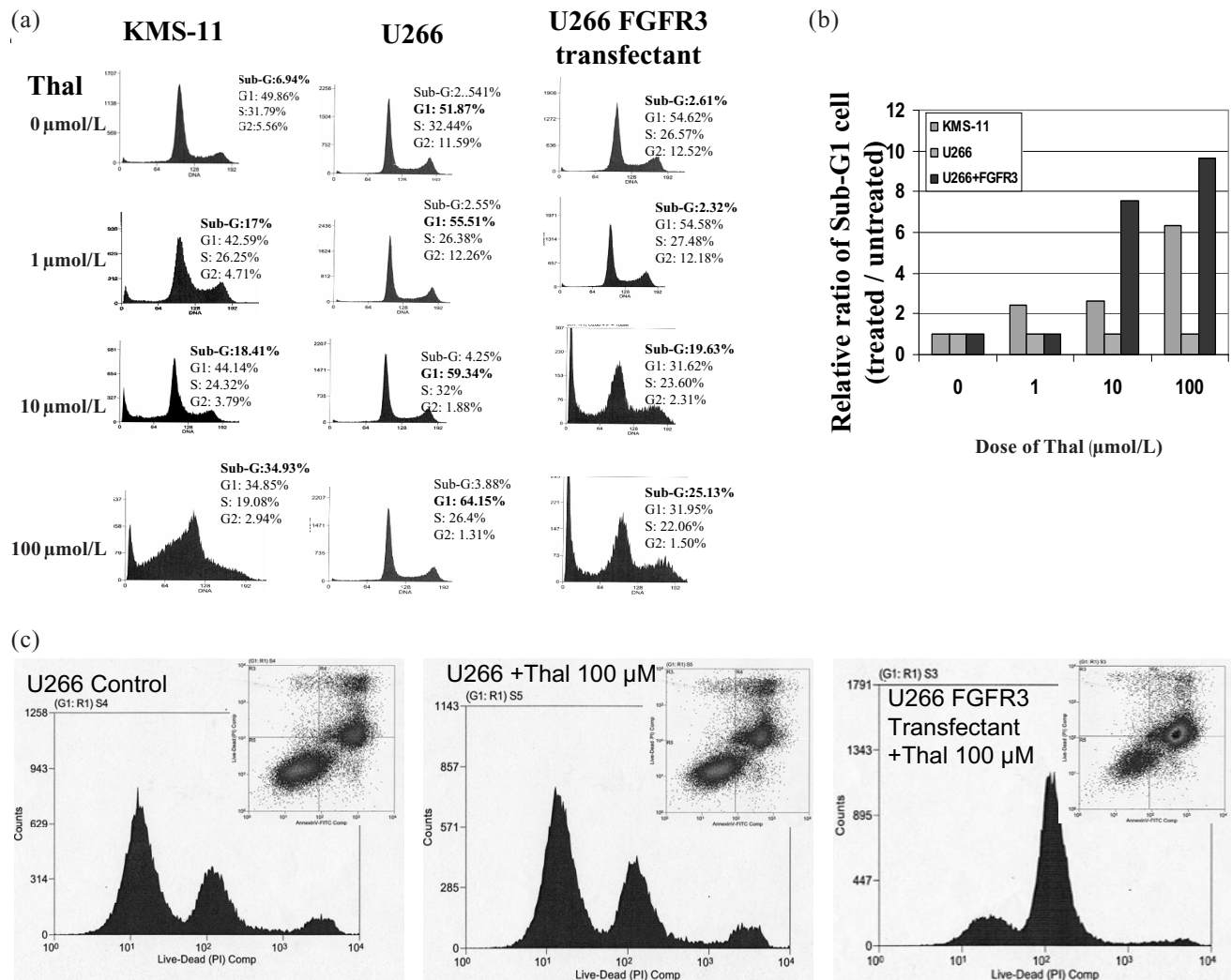
Figure 3 Effect of Thal on the apoptosis and cell cycle profile of flow cytometry analysis in MM cells. KMS-11, U266 parental cells, and U266 FGFR3 transfectant clones were cultured with 1 μmol/L, 10 μmol/L, and 100 μmol/L Thal for 72 hours. Cultures in media control alone served as a negative control. (a) Cells were then stained with PI, and cell cycle profile was determined by flow cytometric analysis. (b) The histogram showing the relative ratio of Thal of Sub-G1 cells treated to untreated of each clone. (c) Annexin staining confirmed the apoptosis related with Thal treatment.

The Thal resistant phenotype of U266 can be circumvented by transfecting FGFR3 into U266 cells

To further confirm the effect of Thal inhibition of MM cell growth as associated with FGFR3, the full-length human FGFR3 cDNA was transfected into U266 cells. The expression of FGFR3 was examined by RT-PCR to confirm the successful transfection (Figure 2a). DNA synthesis was then determined by measuring ^{3}H-TdR uptake for FGFR3 transfected U266 clone cultured from 10^{-2} to 10^4 μmol/L Thal for 48 hours. The FGFR3 transfected U266 clone revealed increased thymidine incorporation compared with U266 and vector transfectant in low dose Thal (<10 μmol/L). However, DNA synthesis was inhibited in a dose-dependent manner from 10 to 10^4 μmol/L concentration by Thal

(Figure 2b). Fifty-percent inhibition of DNA synthesis was noted at 100 μmol/L Thal. In contrast, there is no significant change in ^{3}H-TdR incorporation that could be observed at 100 μmol/L Thal in U266 and vector transfectant. These results further confirm that inducing FGFR3 expression can circumvent U266 resistance to Thal.

Thal-induced apoptosis can be enhanced in FGFR3 transfected U266 clone

To further analyze the mechanism of Thal-induced inhibition of DNA synthesis, flow cytometry was employed to examine KMS-11, parental U266 cells, U266 vector transfectant clones, and U266 FGFR3 transfectant clones. These cells were treated with and without 1 μmol/L, 10 μmol/L, or

100 μmol/L Thal for 48 hours. As shown in Figure 3a, Thal increased the sub-G1 population of KMS-11 cells; in addition there was a dose-dependent increase in apoptosis (from 10 to 100 μmol/L). In contrast, in parental U266 cells as well as U266 vector transfectant (data only show U266 parental clone), Thal only induced G1 growth arrest but did not increase sub-G1 population. This finding confirms that parental U266 cells are resistant to Thal-induced apoptosis. Of interest, U266 FGFR3 transfectant clone also demonstrated increased sub-G1 population in a dose-dependent manner by Thal. In comparing sub-G1 population from KMS-11 to U266 FGFR3 transfectants there was a similar level of apoptosis after Thal treatment. The relative ratio of sub-G1 population after Thal treated to untreated were further summarized in Figure 3b, which suggested FGFR3 could circumvent the Thal resistant phenotype of U266 cells at higher doses of Thal. The FTIC labeled Annexin V stained the cell and confirmed the enhanced apoptosis associated with Thal-induced cell growth inhibition in U266 transfected FGFR3 clones (Figure 3c).

Effect of Thal dependence on VEGF, VEGF receptor, and IL-6 expression in U266 FGFR3 transfectant clone

Thal dependent on FGFR3 status may regulate downstream expression of VEGF and corresponding receptors associated with MM cells. U266 parental and FGFR3 transfectant clones were cultured with 1 μmol/L, 10 μmol/L, or 100 μmol/L Thal for 48 hours to determine the effect of Thal dependency. VEGF expression was determined by RT-PCR, as shown in Figure 4a. VEGF mRNA expression was similar in the treated and untreated U266 cells (Figure 4a, left panel). The U266 FGFR3 transfectant demonstrated higher VEGF mRNA expression than U266 parental cell in the non-treated control group (Figure 4a, right panel). However, VEGF mRNA was inhibited in a dose-dependent manner by Thal in the U266 FGFR3 transfectant clone. The VEGF mRNA decreased to 50% of the control in 10 μmol/L Thal and further decreased in 100 μmol/L Thal treated (Figure 4a, right panel). VEGF protein levels were detected by Western bolt, as shown in Figure 4b. In the left panel, parental U266 cells revealed no changes in VEGF protein expression under Thal treatment. VEGF protein expression was higher in the U266 FGFR3 transfectant clone than in the parental cell, but inhibited in a dose-dependent manner by Thal. In the U266 FGFR3 transfectant clone, decreased VEGF parental expression started from 10 μmol/L then further decreased to undetectable levels at 100 μmol/L of Thal (Figure 4b,

right panel). The VEGF receptor Flt-1mRNA was further examined by RT-PCR and is shown in Figure 4c. Flt-1 mRNA was equally expressed, but was not inhibited by Thal in both FGFR3 transfected U266 clone and parental U266 cells. IL-6 mRNA expression, which is known to be associated with FGFR3 expression, was also determined by RT-PCR and is shown in Figure 4c. Interestingly, the expression of IL-6 mRNA was slightly increased by high-dose treatment of Thal in the parental U266 cells, but was significantly inhibited in a dose-dependent manner by Thal in FGFR3 transfected U266 clone. The IL-6 mRNA expression decreased to 30% of the control with 10 μmol/L Thal and approximately 10% of the control with 100 μmol/L of Thal in the U266 FGFR3 transfectants. These findings confirmed our previous observation that FGFR3 expression is associated with VEGF expression. Furthermore, the FGFR3 overexpression in U266 clone enhanced Thal inhibition of VEGF and IL-6 expression.

Ribozyme cleaved overexpressing FGFR3 mRNA confers KMS-11 cells resistance to Thal treatment

We previously reported that Rz52 ribozyme could effectively cleave overexpressing FGFR3 mRNA of KMS-11 cells (Qian et al 2005). To further examine whether eliminating FGFR3 expression may desensitize KMS-11 to Thal, the
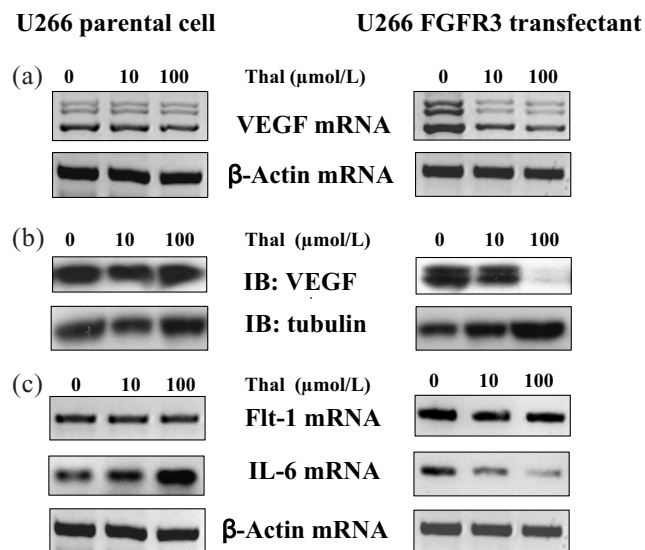

U266 parental cell   **U266 FGFR3 transfectant**

Figure 4 Effect of Thal on VEGF, VEGF receptor-1 (Flt-1), and IL-6 expression in FGFR3 transfected U266 clone. The U266 cells and FGFR3 transfected U266 clone were cultured with 10 μmol/L and 100 μmol/L Thal for 48 hours. Cultures in media control alone served as a negative control. (a) RT-PCP assay of VEGF mRNA expression was performed as described in the *Materials and Methods* section. (b) Western blot analysis, probed with anti-VEGF, shows the expression of VEGF in MM cell lines. (c) Expression of IL-6 and Flt-1 mRNA was examined by RT-PCT as described in the *Materials and Methods* section. β-actin was probed as a control for RT-PCR assay and α-tubulin was the control for Western blot.
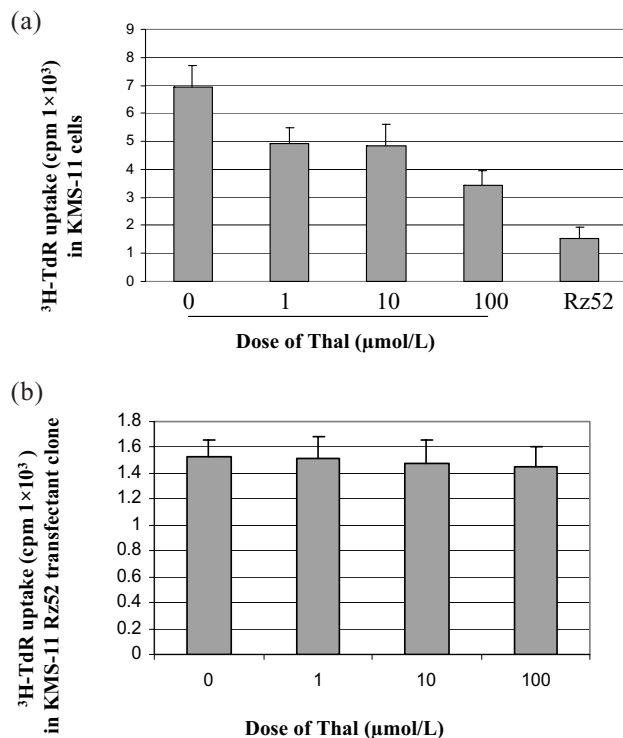
(a)

(b)

Figure 5 Effects of Thal on inhibiting DNA synthesis were abrogated by anti-FGFR3 ribozyme in KMS-11 clone. (a) ^{3}H-TdR uptake was measured in 2×10^5 KMS-11 cells. The cells were cultured with concentrations ranging from 1 μmol/L to 100 μmol/L of Thal for 48 hours and compared with KMS-11 PTV5Rz52 transfectant (Rz52). (b) 2×10^5 Rz52 transfected KMS-11 clones were cultured with 1 μmol/L, 10 μmol/L, and 100 μmol/L of Thal. Cultures in media alone served as a negative control. 3H-TdR (cpm) uptake was measured 48 hours after treatment with Thal. Values represent the mean (±SD) of triplicate cultures.

Rz52 transfected KMS-11 cell designated KMS-11 Rz52 transfectant clone was examined. After treatment with 100 μmol/L Thal, the parental KMS-11 cells demonstrated a 50% decrease in ^{3}H thymidine incorporation compared with control. The KMS-11 Rz52 transfectant clone demonstrated an 80% decrease in thymidine incorporation as shown in Figure 5a. In Figure 5b, the DNA synthesis of Rz52 transfected KMS-11 clone was determined by measuring 3H-TdR uptake treated with or without 1 μmol/L, 10 μmol/L, or 100 μmol/L Thal for 48 hours and revealed no further inhibition. This result suggested that Thal inhibition of DNA synthesis of KMS-11 cells could be abrogated by anti-FGFR3 ribozyme (Rz52). Thal inhibition of myeloma cell growth associated with FGFR3 status was confirmed.

Ribozyme cleaved FGFR3 could further block Thal induced inhibition of VEGF and IL-6 expression in KMS-11 cells

We previously reported that VEGF and IL-6 expression in Rz52 transfected KMS-11 clone decreased to 50% of the parental KMS-11 cells (Qian et al 2005). To further confirm that Thal inhibition of VEGF and IL-6 expression was associated with FGFR3 status, Rz52 transfected KMS-11 clones were treated with or without 1 μmol/L, 10 μmol/L, or 100 μmol/L Thal for 48 hours before examination. VEGF expression was determined by RT-PCR, as shown in Figure 6a. VEGF mRNA expression was inhibited by Thal and decreased to 50% of the control in KMS-11 parental cells; whereas, Rz52 transfected KMS-11 clone decreased VEGF mRNA expression as compared with KMS-11 parental cell in the control group and it remained unchanged under Thal 100 μmol/L treatment. VEGF protein levels were detected by Western blot and demonstrated consistent findings with RT-PCR as shown in Figure 6b. Flt-1mRNA was detected by RT-PCR as shown in Figure 6c. Flt-1 mRNA was expressed but was not inhibited by Thal in both KMS-11 parental cells and PTV5Rz52 transfected KMS-11 clone. IL-6 mRNA expression was determined by RT-PCR and shown in Figure 6c. Expression of IL-6 mRNA was inhibited in a dose-dependent manner by Thal in KM-11 cells and decreased to 30% of the control in 100 μmol/L Thal treatment. IL-6 expression in Rz52 transfected KMS-11 clones was unchanged even at the higher concentration of 100 μmol/L. These findings suggested that Thal decreased
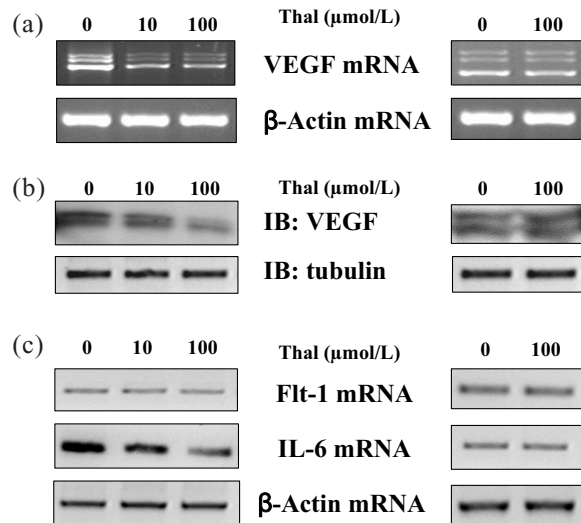

Figure 6 Effects of Thal on inhibition of VEGF and IL-6 were halted by anti-FGFR3 ribozyme in KMS-11 cells. The KMS-11 cells were cultured with 10 μmol/L and 100 μmol/L Thal, and KMS-11 Rz52 transfectant clone were cultured only with 100 μmol/L Thal for 48 hours. Cultures in media control alone served as a negative control. (a) RT-PCP assay of VEGF mRNA expression was performed as described in the *Materials and Methods* section. (b) Western blot analysis, probed with anti-VEGF, shows the expression of VEGF in MM cell lines. (c) Expression of IL-6 and Flt-1 mRNA was examined by RT-PCT as described in the *Materials and Methods* section in MM cell lines. β-actin mRNA was used as a control RT-PCR. Whereas, α-tubulin was used as a control for the Western blot.
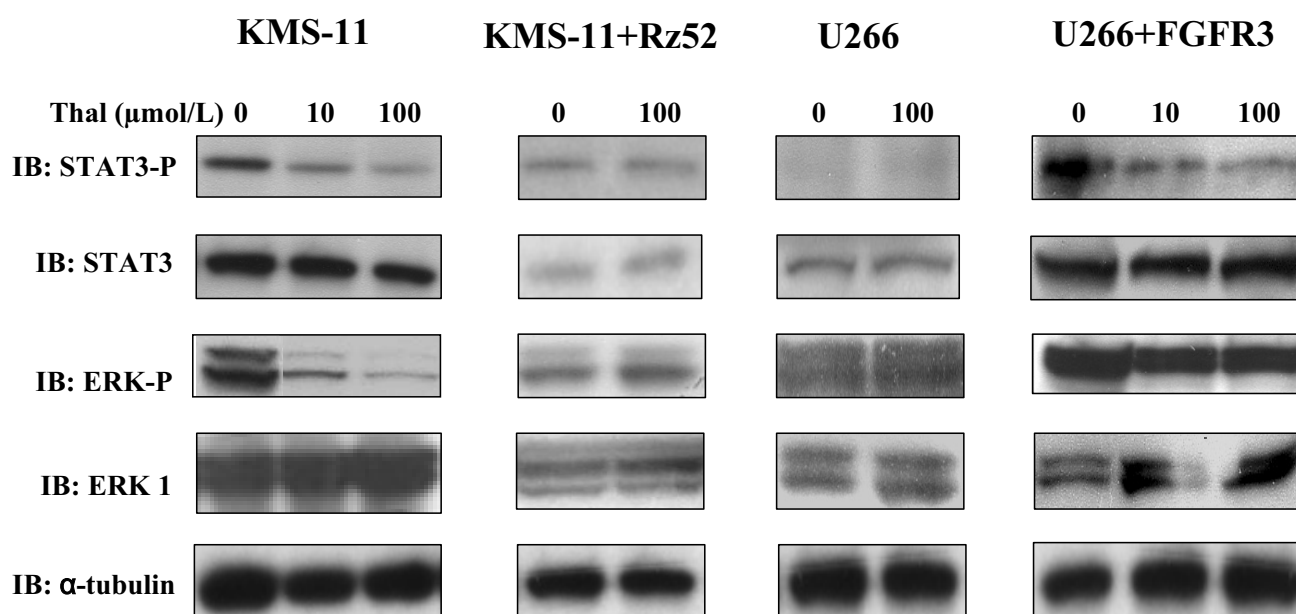
Figure 7 Effect of Thal on phosphorylation of STAT3, ERK, and ERK1 protein. Cells were cultured with 1 μmol/L, 10 μmol/L, and 100 μmol/L Thal for 48 hours and then were harvested. Whole cell lysates (40 ng) were analyzed by Western blot with specific antiserum against (from the top): STAT-phosphorylated (STAT-P), STAT3, ERK-phosphorylated (ERK-P), and ERK1. Western blot analysis for α-tubulin confirmed equal protein loading as a control. Detailed methodology is described in the *Materials and Methods* section.

FGFR3 associated with VEGF and IL-6 inhibition. The ribozyme down-regulation of FGFR3 could block Thal induced down-regulation of VEGF and IL-6 expression in KMS-11 cell. This observation is consistent with our above finding.

Phosphorylation of STAT3 and ERK is responsible for Thal dependent on FGFR3 status

STAT3 is a key mediator involved in cytokine signaling. After Thal treatment, Western blot analysis was performed using a phospho-specific antibody that binds to phosphorylated STAT3 and total STAT3. As shown in Figure 7, phosphorylation of STAT3 was inhibited in a dose-dependent manner by Thal in KMS-11 parental cells. Ribozyme-cleaved FGFR3 expression, led to the decreased expression of phosphorylated STAT3. Phosphorylated STAT3 has lower expression in KMS-11 Rz52 transfectant clone than in parental KMS-11 cells (Figure 7, top panel). The expression of phosphorylated STAT3 in U266 parental cells revealed extremely low levels with no change after Thal treatment. Whereas in U266 FGFR3 transfectant clone, the same expression level of phosphorylated STAT3 was the same in the KMS-11 cells. Thal inhibits phosphorylated STAT3 in U266 FGFR3 transfectant clone in the same manner as KMS-11 cells. Total STAT3 was expressed in all

the above clone and was not inhibited by Thal in any dose range.

Since cytokines, such as VEGF and IL-6, are not only activated by the JAK-STAT signaling pathway, but also regulate members of MAPK family, we then examined phosphorylation of ERK and ERK1 after Thal treatment. Phosphorylated ERK was inhibited by Thal in a dose-dependent manner in both parental KMS-11 cells and U266 FGFR3 transfectant clones, but the KMS-11 Rz52 transfectant clone and parental U266 cells revealed no change in phosphorylated ERK after Thal treatment. ERK1 expression was unchanged in all cell lines after Thal treatment. These results confirmed that the therapeutic effect of Thal dependent on FGFR3 status, may send transduction signals through phosphorylation of STAT3 and ERK. Western blot analysis with anti-tubulin antibody confirmed the equivalent protein loading.

Discussion

Our results demonstrate that the anti-MM effect exerted by Thal is associated with FGFR3 status. The t(4; 14) translocation occurs in 20% of multiple myeloma and results in ectopic expression of FGFR3 (Bergsagel and Kuehl 2001; De Bont et al 2001). Ectopic expression of FGFR3 promotes myeloma cell proliferation and prevents apoptosis (Kanai et al 1997). The effect of Thal in MM has been investigated both in vivo and in vitro settings. At high concentrations

(100 μmol/L), DNA synthesis is inhibited in MM cells in vitro (Hideshima et al 2000). In our study, FGFR3 overexpressing KMS-11 cells were exposed to Thal, with the observation that it inhibited DNA synthesis and induced apoptosis in a dose-dependent fashion. Our findings further suggested that the therapeutic effects of Thal-induced apoptosis might be associated with FGFR3 status resulting in down-regulation of VEGF and IL-6. The lack of FGFR3 expression in U266 cells demonstrated resistance to Thal. This was confirmed in a previous finding (Sampaio et al 1991). To investigate whether the Thal therapeutic effect is associated with FGFR3 status, the wild-type human FGFR3 cDNA was transfected into U266 cells in addition to the ribozyme cleaved FGFR3 in KMS-11 cells. Our results suggested that Thal inhibition of MM cell proliferation may be dependent with FGFR3 expression status in vitro.

IMiDs, and to a lesser extent Thal, induced apoptosis of the myeloma MM.IS clone, which is consistent with our current finding (Hideshima et al 2000). Both MM.IS and KMS-11 cells treated with Thal increased sub-G1 cells on flow cytometry in vitro. However, it has been demonstrated that the MM.IS clone lacks FGFR3 expression. Therefore, whether the MM.IS clone response to Thal may be secondary to a non-FGFR3-derived mechanism requires further exploration. Moreover, the dexamethasone resistant clone, myeloma Hs Sultan cells, demonstrated resistance to Thal as well. The Hs Sultan clone is also known to possess low FGFR3 expression that is similar to the U266 clone. Both of these clones are resistant to Thal treatment, suggesting that low FGFR3 might play a role in Thal resistance, at least in part. P53 may also play an important role in Thal sensitivity. The P53 mutant status may also confer resistance to Thal. The cells that possess wild-type P53 may down-regulate p21, thereby facilitating G1 to S transition and enhance susceptibility to apoptosis (Hideshima et al 2000). Whether, FGFR3 expression related Thal efficacy was independent from p53 requires further elucidation.

It has been known that the inhibiting effects of Thal in MM are associated with the down-regulation and decreased secretion of cytokines: tumor necrosis factor (TNF)-α (Sampaio et al 1991), IL-6, and VEGF (D'Amato et al 1994). In MM patients, VEGF is expressed and secreted by tumor cells as well as bone marrow stromal cells (Bellamy et al 1999; Dankbar et al 2000). This may account for, at least in part, the increased micro vessel density observed in the bone marrow of patients with MM and correlates with disease progression and poor prognosis (Vacca et al 1994, 1999). Clinical responses to Thal were observed in MM patients

who were refractory to conventional and high-dose therapy (Ribatti et al 1999). In addition to inhibition of VEGF-induced angiogenesis in bone marrow, Thal may also block VEGF-induced direct effects on cell growth and proliferation of MM (D'Amato et al 1994). Recent clinical studies suggested that there is a correlation with Thal treatment and decreased VEGF levels in clinical responders. Whereas, in nonresponders, the VEGF level in plasma remain steady after Thal treatment (Tosi et al 2002). Our results apparently support this observation that Thal suppresses VEGF expression dependent on FGFR3 status. However, whether FGFR3 expression may play an important role in determining the response of Thal treatment requires further investigation. Of interest, IL-6 is also associated with the growth regulation of human MM (Anderson et al 1989; Hallek et al 1998). It has been shown that FGFR3 overexpressing in the B9 clone exhibits enhanced proliferation in the presence of IL-6. Furthermore, this transfectant exhibits IL-6 independent cell growth (Plowright et al 2000). In some studies, exogenous IL-6 demonstrated resistance to Thal, as well as the IMiDs on some MM clone, suggesting that these novel drugs may, at least in part, function by inhibiting IL-6 production (Hideshima et al 2000). In our studies, expression of IL-6 was inhibited by Thal in KMS-11 cells, coupled with inhibition of cell growth, further suggesting that IL-6 is involved in Thal-induced MM inhibition. In the U266 FGFR3 transfectant clone, Thal inhibited IL-6 expression (see Figure 4). These findings suggested that IL-6 inhibition is also dependent on FGFR3 status. This result may suggest FGFR3 complement IL-6 in certain MM cells. However, the IL-6 expression increased in parental cells under high dose Thal treatment suggested a more complicated regulation involving IL-6. Thus, the different effects seen with Thal treatment in MM patients and the various MM cell lines may be explained by the expression of FGFR3.

Interestingly, bFGF (the ligand of FGFR3) was also noticed to be a prognostic factor in MM patients after Thal treatment (Kenyon et al 1997; Neben et al 2001). It has been reported that bFGF levels in MM patients decreased and is associated with response to Thal treatment (Kenyon et al 1997). In recent studies however, no decline in bFGF levels after a 6-month follow-up was found in Thal treated patients (Neben et al 2001). However, adding bFGF into the medium of MM cells increased MM cell proliferation, suggesting that bFGF might lead to progression of MM cells. Moreover, the effect of bFGF in MM cells is FGFR3 dependent. It has been reported that overexpression of mutant FGFR3 led to

bFGF independent growth in L6 clones, whereas wild-type FGFR3 is bFGF dependent (Plowright et al 2000). Thus, the functional role of bFGF is dependent on FGFR3 status. Most likely, an effect of Thal on cell surface receptors or intracellular signaling events rather than bFGF level could explain its efficacy in patients.

STAT3 has been classified as an oncogene because the constitutively active STAT3 can mediate oncogenic transformation in MM cells and tumor transformation in mice (Bromberg et al 1999; Catlett-Falcone et al 1999; Funamoto et al 2000). Constitutive activation of STAT3 signaling can confer resistance to apoptosis in human myeloma cells 46 and can also up-regulate the expression of VEGF and promote tumor angiogenesis through the up-regulation of VEGF (Funamoto et al 2000). Overexpression of FGFR3 or mutant FGFR3 displayed increased phosphorylation of STAT3. In our study, the Western blot of ERK1, STAT3, and their phosphorylation were examined. The phosphorylation of ERK and STAT3 was decreased in a dose-dependent manner after Thal treatment in parental KMS-11 cells and the FGFR3 transfected U266 clones, but not in parental U266 cells. These findings suggest that the FGFR3 mediated Thal effect occurs via phosphorylation of ERK and STAT3 in MM cells. Other studies have demonstrated that VEGF and IL-6-induced proliferation of MM cells is mediated through the MAPK cascade. Using either MAPK antisense oligonucleotide or the MEK1 inhibitor PD98059 can abrogate MAPK signal transduction, which confirmed this hypothesis (Ogata, Chauhan, Teoh, et al 1997; Ogata, Chauhan, Urashima, et al 1997; Hideshima et al 2000). In our prior experiments, ribozyme-induced down-regulation of FGFR3 inhibited proliferation of KMS-11 cells and inhibited phosphorylation of ERK and STAT3. Ribozyme-induced FGFR3 down-regulation in KMS-11 cells negated the effects of Thal on the phosphorylation status of ERK and STAT3. These results suggest that Thal inhibition of DNA synthesis, induction of apoptosis, and down-regulation of VEGF may be mediated via a FGFR3-associated signal transduction of the ERK and STAT3 phosphorylation pathway. These studies support the hypothesis that Thal inhibition of MM is, at least in part, dependent on FGFR3 status in myeloma cells. The limitation of our observation is that in vitro findings may not directly imply in vivo or in human therapeutic settings. Since Thal requires the liver to activate its metabolites, the dose of Thal in clinical use is different as in vitro investigation. Therefore, the in vitro finding may not directly correspond to clinical scenarios. To further confirm our observations, the Xenograft animal study will be tested next. After confirmation using this model, FGFR3 may become a predictive/surrogate marker for selection of Thal treatment in myeloma.

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