Published in final edited form as:

Ann Thorac Surg. 2015 November ; 100(5): 1817–1825. doi:10.1016/j.athoracsur.2015.05.074.

Triptolide Inhibits Lung Cancer Cell Migration, Invasion, and Metastasis

Theresa A. Reno, PhD, Jae Kim, MD, and Dan Raz, MD

Division of Thoracic Surgery, City of Hope Medical Center, Duarte, California

Abstract

Background—Triptolide is an extract from *Tripterygium wilfordii* used in traditional Chinese medicine to treat autoimmune disorders. Triptolide has anti-cancer effects *in vitro* and is reported to impair cancer cell migration. We studied whether triptolide inhibits lung cancer cell migration and metastasis.

Methods—We determined the microRNA expression profile of triptolide treated cells. We tested the effects of triptolide treatment on migration and invasion of lung cancer cells using Transwell filters coated with fibronectin and Matrigel, respectively. Western blots were used to compare expression of proteins involved in cell migration before and after 10nM triptolide treatment. Tail vein injections using H358 cells were performed. The mice were treated with 1mg/kg triptolide or vehicle by intraperitoneal injection three times per week. Lung and liver metastases were compared at 9 weeks. Means of groups were compared using a t-test.

Results—Triptolide altered the expression of microRNAs involved in cellular movement and significantly decreased migration and invasion of lung cancer cells from approximately 18 to 3 cells per field (p-value <.001). Triptolide decreases Focal Adhesion Kinase (FAK) expression, which leads to impairment of downstream signaling. Finally, triptolide treated mice injected with lung cancer cells significantly decreased metastatic colony formation in the lungs (p-value <.01).

Conclusions—Triptolide decreases lung cancer cell migration and invasion *in vitro* and inhibits metastatic tumor formation in mice. Triptolide suppresses FAK, which causes deregulation of the migration machinery. These results suggest that triptolide inhibits lung cancer metastasis and should be investigated as a new lung cancer therapy.

Keywords

Lung Cancer; Animal Model; Cell Signaling; Cell Biology

Address correspondence to Dr. Dan Raz, Division of Thoracic Surgery, City of Hope Medical Center, 1500 E. Duarte Rd., Duarte, CA 91010. Phone: 626-256-4673 Ext. 67100; Fax: 626-471-7215; draz@coh.org.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Introduction

Cell migration is a fundamental multi-step process that involves the movement of a cell from one location to another [1]. The process of cell migration begins with the response of the cell to a stimulus that leads to the polarization of the cell. The polarized cell then extends actin containing protrusions at the leading edge, which are stabilized by adhering to the extracellular matrix (ECM) through the formation of focal adhesion (FA) complexes comprised mainly of integrin receptors and FA proteins, such as Focal Adhesion Kinase (FAK) [2]. As the cell body moves, the adhesions are disassembled allowing for detachment and repeating of the entire process.

The highly complex and organized events of migration are crucial for normal physiological and morphogenetic processes, including wound healing and embryogenesis [3]. Although normal cells use migration to facilitate their program, as genetic mutations accumulate within the DNA, there is aberrant activation of migration signaling pathways [4]. This abnormal activation is key to multiple pathophysiological events, including cancer cell metastasis. The vast majority of lung cancer deaths are related to metastatic disease. Identifying new therapies that target lung cancer metastasis can potentially reduce mortality and improve quality of life for patients with lung cancer.

Triptolide is an extract from the Thunder God Vine, *Tripterygium wilfordii*, which has been used in traditional Chinese medicine to treat autoimmune disorders, such as rheumatoid arthritis and systemic lupus erythematosus [5]. Recently, triptolide has been shown to have antitumor effects in human cancer cells, including lung cancer, through various mechanisms, including caspase activation and enhancing p53 activity [6]. Though studies show that triptolide can impair colon cancer cell migration through the downregulation of cytokine receptors, the effect of triptolide on lung cancer migration is unknown [7].

The primary aim of this study was to investigate the ability of triptolide to inhibit lung cancer cell migration, invasion, and metastasis. We also explored the effects of triptolide on the expression and activation of specific proteins regulating migration, including FAK.

Material and Methods

Reagents and Antibodies

Dulbecco's Modified Eagle Medium (DMEM) was purchased from Life Technologies (Carlsbad, CA). 8.0 micron Transwell dishes with and without Matrigel coating were purchased from BD Biosciences (San Jose, CA).

FAK, pFAK (Y397), Src, pSrc (Y416), Paxillin, pPaxillin (Y118), p130Cas, p-p130Cas (Y249), Erk1/2, pErk1/2 (T202, Y204), and GAPDH antibodies were purchased from Cell Signaling (Danvers, MA). MMP14, pPaxillin (Y31), PYK2, and pPYK2 (Y402) antibodies are from abcam (Cambridge, MA). HRP-conjugated goat anti-rabbit secondary antibody was purchased from Genetex (Irvine, CA). Triptolide is from Sigma (St. Louis, MO).

Cell Culture and Drug Treatment Methods

H460, A549, and H358 cells were acquired from ATCC and cultured in 5% CO₂ at 37°C in DMEM containing 10% FBS, 1% sodium pyruvate, 1% L-glutamine/gentamycin and 1% penicillin/streptomycin.

Triptolide was diluted with DMSO and a 10nM concentration was used for *in vitro* assays.

Small RNA deep sequencing and Ingenuity Analysis

Total RNA was extracted from control and triptolide treated (10nM for 48 hours) H460 cells using the RNeasy Mini Kit from Qiagen (Valencia, CA). Small RNA sequencing was performed using the Illumina HiSeq2500 and the manufacturer's sample preparation protocol (TruSeq Small RNA Sample Prep kit, Illumina, Inc., San Diego, CA), with some modifications. Briefly, 500ng of total RNA was used for smRNA sequencing library construction. The constructed smRNA library was reverse transcribed and then subjected to PCR amplification followed by 6% TBE PAGE gel purification with size selection. The denatured sequencing library was loaded in hybridization buffer to a final DNA concentration of 10 pM and used for single read flow cell cluster generation and 40 cycles of read1. 7 cycles of index read sequencing was performed. The data was normalized using trimmed means of m values.

Ingenuity Pathway Analysis (Qiagen, Valencia, CA) was used to analyze the miRNA sequencing data. Briefly, the top 5% of altered miRNAs were run through the TargetScan database and the results were uploaded into Ingenuity for pathway analysis. The molecular and cellular function option was used with a score cutoff of $-\log(\text{p-value}) = 1.3$ and threshold of .05 with the Fisher's Exact test scoring method.

Western Blotting

Immunoblotting was performed using PVDF membranes and 4–12% Bis-Tris Nupage gels from Life Technologies (Carlsbad, CA). 5% non-fat milk was used for blocking.

Migration and Invasion Assays

Migration was analyzed using Transwell filters coated with 5µg/ml fibronectin. Control or triptolide treated cells (1×10^5) were added to the top chamber and allowed to migrate through the filter for 6 hours. Cells on top of the filter were removed and the cells on the bottom of the filter were then fixed in 4% paraformaldehyde. The filter was mounted on a microscope slide in DAPI containing media and analyzed using fluorescent microscopy. The assay was completed three times in triplicate with nine random images per filter. Invasion assays were performed as the described migration assay, but Matrigel was used to coat the filter and the cells were allowed to invade for 24 hours.

Microscopy

Fluorescence and brightfield imaging were performed using a Zeiss Axio Observer Z1 inverted microscope equipped with Axiocam MRc5 (brightfield) and Hamamatsu Orca CCD (fluorescence) cameras.

Tail Vein Injection Mouse Model

H358 cells (1×10^6) were injected into the tail vein of 6–8 week old NSG mice (9 per group). One day after injection of the cells, one group was given a 1.5mg/kg dose of triptolide. Treatment continued a week after the initial dose with the mice receiving control (PBS) or triptolide (1mg/kg) intraparetoneal injections three times a week for 9 weeks. Mice were then euthanized and the lungs and liver were harvested, fixed in 10% formalin, and paraffin embedded for pathological examination. The mouse tail vein injection experiment was done in accordance with a protocol approved by the Institutional Animal Care and Use Committee at City of Hope and all procedures complied with the Guide for the Care and Use of Laboratory Animals.

Statistical Analysis

All quantified data were plotted and analyzed in GraphPad Prism 6.0 using a Student t-test. Data are representative of at least 3 independent experiments as replicate means $\pm$ SEM. ** or *** are *p* values < 0.01 , or 0.001 , respectively.

Results

Triptolide alters the microRNA expression profile of human lung cancer cells

miRNAs are important regulators of gene expression that are deregulated during cancer progression [8]. We assessed triptolide-induced alteration of miRNA expression in non-small cell lung cancer (NSCLC) cells. After triptolide treatment, 126 and 101 miRNAs were significantly upregulated and downregulated, respectively (Figure 1A and Table 1). The differential expression analysis gene selection criteria was an absolute fold change of greater than or equal to 1.5 and a false discovery rate of less than or equal to .01. Using Ingenuity Pathway Analysis of the genes controlled by the top upregulated and downregulated miRNAs, we determined the top molecular and cellular functions regulated by these genes (Figure 1B). “Gene expression” was the top molecular function regulated by the differentially expressed miRNAs. It is well known that triptolide can inhibit gene expression by binding to XPB, a subunit of the transcription factor TFIIH, and by inducing proteasome degradation of RNA polymerase II [20, 21]. “Cellular movement” was the second most highly regulated process. This result led us to evaluate that role of triptolide in regulating cell migration.

Triptolide decreases lung cancer cell migration and invasion

To investigate if triptolide has an effect on lung cancer cell migration, we plated triptolide treated NSCLC cells on Transwell filters coated with the solid substrate chemoattractant fibronectin. Triptolide treatment significantly decreases the migration of all three lung cancer cell lines compared to the DMSO control cells (Figure 2). Cellular adhesion to fibronectin was similar between the control and treatment groups in all three cell lines indicating that triptolide does not affect cell attachment to the extracellular matrix (data not shown).

Cancer cell metastasis requires invasion and degradation of the surrounding extracellular matrix of the basement membrane and surrounding tissues [22]. To study the role of

triptolide in lung cancer cell invasion, we used Transwell filters coated with Matrigel, which is used to mimic the basement membrane. Figure 3 shows that triptolide treatment almost completely inhibits lung cancer cell invasion.

Triptolide decreases lung cancer cell metastasis

Due to the significant decrease in both cancer cell migration and invasion after triptolide treatment, we next wanted to investigate the effect of triptolide in lung cancer cell metastasis *in vivo*. We performed tail vein injections of H358 cells in NOD SCID gamma (NSG) mice as an experimental model to assess metastatic colonization. Mice treated with triptolide had significantly less metastatic colonization of the lungs compared to the vehicle control mice (Figure 4). The control and treatment groups did not have metastatic lesions in the liver after gross and microscopic examination (data not shown). This data suggests that triptolide could be a potential therapeutic for targeting lung cancer progression.

Triptolide decreases FAK protein expression and impairs downstream signaling

Migration and invasion are controlled by the spatiotemporal activation and regulation of a network of proteins [23]. We observed decreased expression of total FAK, a key regulator of integrin signaling in migrating cells, in the triptolide treated cells when compared to the control cells (Figure 5A). Auto-phosphorylation of FAK at tyrosine 397 is decreased after treatment. This is indicative of decreased activation, which could be due, in part, to the decrease in total FAK expression and/or a possible disruption of integrin binding. Due to the downregulation of FAK expression and activation, we next examined if downstream signaling pathways were disrupted. Major downstream signaling components known to interact with FAK and become activated after phosphorylation by FAK are Src, p130Cas, paxillin [24]. Triptolide treatment leads to a decrease in the activation of Src (Y416) and p130Cas (Y247), but, surprisingly, triptolide did not affect the activation state of paxillin (Y31 and Y118) (Figure 5B). The total expression of these proteins was not altered by triptolide treatment. We also observed that triptolide treatment greatly increases Erk1/2 activation.

Due to the inhibition of lung cancer cell invasion and the decrease in FAK expression and activation after triptolide treatment, we looked at the expression of matrix metalloproteinase 14 (MMP14), which participates in the degradation of the ECM and has been shown to regulate the FAK-p130Cas complex [25, 26]. Triptolide treatment decreases MMP14 expression, which may help explain the lack of invasion by lung cancer cells after treatment (Figure 5C). Taken together, these data indicate that triptolide may work to decrease cancer cell migration, invasion, and metastasis through downregulation of FAK and inactivation of its downstream signaling pathways.

Comment

Metastasis is a complex series of events whose foundation relies on the aberrant activity of the cellular migration machinery [27]. Metastases form when cancer cells leave the site of the primary tumor and colonize a secondary site in the body. Cancer cells that disperse from the primary tumor undergo a cascade of events, including localized invasion, intravasation

into the blood or lymphatic system, and extravasation from the blood or lymphatic vessel where they colonize and form new tumors [28]. Cancer progression may be inhibited by developing therapies that prevent metastasis. Aberrant microRNA expression has been implicated in metastasis of human cancers. miRNAs are a class of small non-coding regulatory RNAs that play an important role in gene regulation. Using small RNA deep sequencing, we have shown that triptolide significantly alters the miRNA expression profile of lung cancer cells, and, in particular, miRNAs that are associated with cell motility. We have also demonstrated that triptolide strongly inhibits lung cancer cell migration, invasion, and metastasis using *in vitro* cell migration and invasion assays and the *in vivo* tail vein injection model.

Tyrosine kinases are key mediators in signal transduction pathways that catalyze the phosphorylation of target proteins [29]. FAK is a non-receptor tyrosine kinase and a protein scaffold that links integrin and growth factor receptor signaling to cancer cell migration, proliferation, and survival [30]. FAK is activated through binding to integrins after the coupling of integrin receptors with the ECM. Following activation, FAK serves as a kinase and scaffolding protein for several signaling molecules, including p130Cas, Src, paxillin, Grb2, and cortactin. The role of FAK in cancer cell migration and metastasis has been well studied and has shown that FAK expression positively regulates cancer progression [30]. Based on our observations that triptolide decreased cancer cell migration, invasion, and metastasis, we studied FAK phosphorylation and total protein levels and found that triptolide treatment decreased both the expression and activation of FAK. PYK2 is a FAK orthologue that has been proposed to be a prognostic marker for NSCLC progression and has been shown to compensate for the loss of FAK [31]. Additionally, increased PYK2 activity has been seen after treatment with FAK inhibitors [32]. Protein expression of PYK2 was not altered by treatment, which demonstrates that PYK2 is not activated to compensate for decreased FAK activity after triptolide-mediated FAK downregulation. Triptolide treatment also led to the decreased activation of p130Cas and Src, but not paxillin. Although FAK is a major regulator of paxillin activation, other proteins, such as JNK and Brk, have been shown to phosphorylate paxillin and help in maintaining cellular adhesion [33, 34]. Triptolide treated cells are able to adhere to ECM proteins to the same degree as control cells despite the lack of activated FAK, which indicates that other proteins, such as paxillin, are functioning appropriately to regulate this process. Interestingly, after treatment with triptolide, there is considerable activation of Erk1/2. Erk1/2 is a well-studied member of the MAPK family and is a positive regulator of cell migration as a mediator of focal adhesion dynamics [35]. Activated Erk1/2 intricately regulates the FAK-paxillin complex to promote focal adhesion turnover [36]. It is possible that Erk1/2 is being activated through a separate pathway and though Erk1/2 activation is increased, the decreased expression of FAK by triptolide hinders the ability of Erk1/2 to facilitate focal adhesion assembly and disassembly. The activation of Erk1/2 may have more of an effect on regulating cell death and senescence through the p53 tumor suppressor pathway [37]. The subcellular localization of Erk1/2 may also be important since it has been shown that the localization of Erk1/2 in the cell as well as its activity are significant in determining cellular functions, including senescence [37].

The invasion and subsequent metastasis of motile cells is often facilitated by the spatiotemporal expression and activation of MMPs that regulate the association between the stroma and cancer cells. MMPs are often associated with the cellular migration machinery where they localize to the membrane surface and facilitate degradation of the tumor stroma [38]. Molecules involved in cell migration signaling pathways have been shown to regulate the expression and activity of MMPs [39]. The FAK-p130Cas complex has also been shown to regulate the localization of MMP14 [40]. Our studies demonstrate that triptolide treatment leads to the near complete inhibition of lung cancer cell invasion *in vitro* and to the decreased expression of MMP14. The decreased expression of MMP14 may explain the lack of invasion by triptolide treated cells, but there is a possibility that inhibition of other MMPs and other invasion-associated molecules play a role in this downregulation.

In summary, we found that triptolide changes the expression profile of miRNAs in lung cancer cells and these modifications are predicted to alter cell motility pathways. In addition, after treating cells with triptolide, we demonstrated an inhibition of cancer cell migration, invasion, and metastasis. The inhibition of these processes correlated with a decrease in FAK expression and activation of downstream pathways. The extent of the decrease in migration, invasion, and cell motility machinery expression does appear to partly correlate with the metastatic potential of the lung cancer cell lines. H460 cells tend to be more invasive and form tumors more readily in mice than A549 and H358 cells. Based on our data, H460 is more sensitive to triptolide treatment than the other two NSCLC lines. Though further work needs to be completed to more fully understand the mechanism of the inhibitory effect of triptolide on FAK expression and lung cancer cell progression, these studies establish that triptolide is a potential novel lung cancer therapy.

References

1. Ridley AJ, Schwartz MA, Burridge K, et al. Cell migration: integrating signals from front to back. *Science*. 2003; 302:1704–1709. [PubMed: 14657486]
2. Wozniak MA, Modzelewska K, Kwong L, Keely PJ. Focal adhesion regulation of cell behavior. *Biochimica et biophysica acta*. 2004; 1692:103–119. [PubMed: 15246682]
3. Friedl P, Gilmour D. Collective cell migration in morphogenesis, regeneration and cancer. *Nat Rev Mol Cell Biol*. 2009; 10:445–457. [PubMed: 19546857]
4. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. *Cell*. 2011; 144:646–674. [PubMed: 21376230]
5. Liu Q. Triptolide and its expanding multiple pharmacological functions. *International immunopharmacology*. 2011; 11:377–383. [PubMed: 21255694]
6. Jiang XH, Wong BC, Lin MC, et al. Functional p53 is required for triptolide-induced apoptosis and AP-1 and nuclear factor-kappaB activation in gastric cancer cells. *Oncogene*. 2001; 20:8009–8018. [PubMed: 11753684]
7. Johnson SM, Wang X, Evers BM. Triptolide inhibits proliferation and migration of colon cancer cells by inhibition of cell cycle regulators and cytokine receptors. *The Journal of surgical research*. 2011; 168:197–205. [PubMed: 19922946]
8. Palmero EI, de Campos SG, Campos M, et al. Mechanisms and role of microRNA deregulation in cancer onset and progression. *Genet Mol Biol*. 2011; 34:363–370. [PubMed: 21931505]
9. Pichiorri F, Suh SS, Rocci A, et al. Downregulation of p53-inducible microRNAs 192, 194, and 215 impairs the p53/MDM2 autoregulatory loop in multiple myeloma development. *Cancer Cell*. 2010; 18:367–381. [PubMed: 20951946]

10. Chen G, Umelo IA, Lv S, et al. miR-146a inhibits cell growth, cell migration and induces apoptosis in non-small cell lung cancer cells. *PLoS ONE*. 2013; 8:e60317. [PubMed: 23555954]
11. Ohyagi-Hara C, Sawada K, Kamiura S, et al. miR-92a inhibits peritoneal dissemination of ovarian cancer cells by inhibiting integrin alpha5 expression. *The American journal of pathology*. 2013; 182:1876–1889. [PubMed: 23499550]
12. Lee C, He H, Jiang Y, et al. Elevated expression of tumor miR-222 in pancreatic cancer is associated with Ki67 and poor prognosis. *Med Oncol*. 2013; 30:700. [PubMed: 24026657]
13. Ell B, Qiu Q, Wei Y, et al. The microRNA-23b/27b/24 cluster promotes breast cancer lung metastasis by targeting metastasis-suppressive gene prosaposin. *The Journal of Biological Chemistry*. 2014; 289:21888–21895. [PubMed: 24966325]
14. Fang C, Zhao Y, Guo B. MiR-199b-5p targets HER2 in breast cancer cells. *Journal of cellular biochemistry*. 2013; 114:1457–1463. [PubMed: 23296799]
15. Zhang Z, Liu S, Shi R, Zhao G. miR-27 promotes human gastric cancer cell metastasis by inducing epithelial-to-mesenchymal transition. *Cancer Genet*. 2011; 204:486–491. [PubMed: 22018270]
16. Yang T, Chen T, Li Y, et al. Downregulation of miR-25 modulates non-small cell lung cancer cells by targeting CDC42. *Tumour biology : the journal of the International Society for Oncodevelopmental Biology and Medicine*. 2014
17. Luo W, Huang B, Li Z, et al. MicroRNA-449a is downregulated in non-small cell lung cancer and inhibits migration and invasion by targeting c-Met. *PLoS ONE*. 2013; 8:e64759. [PubMed: 23734217]
18. Hung TM, Ho CM, Liu YC, et al. Up-regulation of microRNA-190b plays a role for decreased IGF-1 that induces insulin resistance in human hepatocellular carcinoma. *PLoS ONE*. 2014; 9:e89446. [PubMed: 24586785]
19. Liu X, Chen Q, Yan J, et al. MiRNA-296-3p-ICAM-1 axis promotes metastasis of prostate cancer by possible enhancing survival of natural killer cell-resistant circulating tumour cells. *Cell death & disease*. 2013; 4:e928. [PubMed: 24263102]
20. Titov DV, Gilman B, He QL, et al. XPB, a subunit of TFIIH, is a target of the natural product triptolide. *Nature chemical biology*. 2011; 7:182–188. [PubMed: 21278739]
21. Manzo SG, Zhou ZL, Wang YQ, et al. Natural product triptolide mediates cancer cell death by triggering CDK7-dependent degradation of RNA polymerase II. *Cancer Research*. 2012; 72:5363–5373. [PubMed: 22926559]
22. Joyce JA, Pollard JW. Microenvironmental regulation of metastasis. *Nature reviews Cancer*. 2009; 9:239–252. [PubMed: 19279573]
23. Tsai JH, Donaher JL, Murphy DA, Chau S, Yang J. Spatiotemporal regulation of epithelial-mesenchymal transition is essential for squamous cell carcinoma metastasis. *Cancer Cell*. 2012; 22:725–736. [PubMed: 23201165]
24. Schlaepfer DD, Hauck CR, Sieg DJ. Signaling through focal adhesion kinase. *Prog Biophys Mol Biol*. 1999; 71:435–478. [PubMed: 10354709]
25. Wang YZ, Wu KP, Wu AB, et al. MMP-14 overexpression correlates with poor prognosis in non-small cell lung cancer. *Tumour biology : the journal of the International Society for Oncodevelopmental Biology and Medicine*. 2014; 35:9815–9821. [PubMed: 24986569]
26. Wang Y, McNiven MA. Invasive matrix degradation at focal adhesions occurs via protease recruitment by a FAK-p130Cas complex. *The Journal of cell biology*. 2012; 196:375–385. [PubMed: 22291036]
27. Fidler IJ. The pathogenesis of cancer metastasis: the 'seed and soil' hypothesis revisited. *Nature reviews Cancer*. 2003; 3:453–458.
28. Pantel K, Brakenhoff RH. Dissecting the metastatic cascade. *Nature reviews Cancer*. 2004; 4:448–456. [PubMed: 15170447]
29. Schlessinger J. Cell signaling by receptor tyrosine kinases. *Cell*. 2000; 103:211–225. [PubMed: 11057895]
30. Sulzmaier FJ, Jean C, Schlaepfer DD. FAK in cancer: mechanistic findings and clinical applications. *Nature reviews Cancer*. 2014; 14:598–610. [PubMed: 25098269]

31. Kuang BH, Zhang MQ, Xu LH, et al. Proline-rich tyrosine kinase 2 and its phosphorylated form pY881 are novel prognostic markers for non-small-cell lung cancer progression and patients' overall survival. *British Journal of Cancer*. 2013; 109:1252–1263. [PubMed: 23922106]
32. Cabrita MA, Jones LM, Quizi JL, Sabourin LA, McKay BC, Addison CL. Focal adhesion kinase inhibitors are potent anti-angiogenic agents. *Molecular oncology*. 2011; 5:517–526. [PubMed: 22075057]
33. Huang C, Rajfur Z, Borchers C, Schaller MD, Jacobson K. JNK phosphorylates paxillin and regulates cell migration. *Nature*. 2003; 424:219–223. [PubMed: 12853963]
34. Chen HY, Shen CH, Tsai YT, Lin FC, Huang YP, Chen RH. Brk activates rac1 and promotes cell migration and invasion by phosphorylating paxillin. *Molecular and Cellular Biology*. 2004; 24:10558–10572. [PubMed: 15572663]
35. Webb DJ, Donais K, Whitmore LA, et al. FAK-Src signalling through paxillin, ERK and MLCK regulates adhesion disassembly. *Nature cell biology*. 2004; 6:154–161. [PubMed: 14743221]
36. Kolch W. Coordinating ERK/MAPK signalling through scaffolds and inhibitors. *Nat Rev Mol Cell Biol*. 2005; 6:827–837. [PubMed: 16227978]
37. Cagnol S, Chambard JC. ERK and cell death: mechanisms of ERK-induced cell death--apoptosis, autophagy and senescence. *The FEBS journal*. 2010; 277:2–21. [PubMed: 19843174]
38. Kessenbrock K, Plaks V, Werb Z. Matrix metalloproteinases: regulators of the tumor microenvironment. *Cell*. 2010; 141:52–67. [PubMed: 20371345]
39. Kuo L, Chang HC, Leu TH, Maa MC, Hung WC. Src oncogene activates MMP-2 expression via the ERK/Sp1 pathway. *Journal of Cellular Physiology*. 2006; 207:729–734. [PubMed: 16453304]
40. Lu H, Hu L, Yu L, et al. KLF8 and FAK cooperatively enrich the active MMP14 on the cell surface required for the metastatic progression of breast cancer. *Oncogene*. 2014; 33:2909–2917. [PubMed: 23812425]

Figure 1. Triptolide alters the microRNA expression profile of human lung cancer cells
 (A) heatmap representing the significantly upregulated and downregulated miRNAs after 48 hours of treatment with 10nM triptolide in H460 human NSCLC cells. (B) Ingenuity Pathway Analysis of the top altered cellular and molecular functions of the genes that are regulated by the top 5% differentially regulated miRNAs after triptolide treatment.

Figure 2. Triptolide decreases NSCLC migration

Triptolide treatment decreases migration in H460, A549, and H358 cells using Transwell filters mounted in DAPI containing Prolong Gold $n = 27$ fields per group. *** represents a p value of <0.001 , as determined by a Student T-test.

Figure 3. Triptolide inhibits NSCLC invasion

Triptolide treatment inhibits invasion of H460, A549, and H358 cells through Matrigel. $n = 27$ fields per group. *** represents a p value of <0.001 as determined by a Student T-test.

Figure 4. NSCLC cell metastasis is decreased by triptolide treatment

Triptolide treatment (1 mg/kg) three times a week for nine weeks of NSG mice injected with H358 cells decreases metastatic lesions compared to control mice (right). H&E staining of lung tissue in control and triptolide treated mice at 5× (top) and 20× (bottom) magnifications. $n = 9$ mice per group. ** represents a p value of <0.01 as determined by a Student T-test.

Figure 5. Triptolide treatment decreases FAK expression and activity and alters signaling downstream of FAK

H460, A549, and H358 cells treated with 10nM triptolide on DMSO control for 48 hours were harvested for western blot analysis of FAK and phosphorylated FAK (Y397) (A). (B) Tyrosine phosphorylation of Src and p130CAS is decreased, the activity of Erk1/2 is increased, and there is no change paxillin activation after triptolide treatment. (C) MMP14 total protein expression decreased by triptolide treatment. Triptolide slightly alters PYK2 activation. Con- Control; Trp- Triptolide.

Table 1

Differentially Expressed miRNAs After Triptolide Treatment

miRNAs	Log Fold Change	Biological Effect	References
mir-215	6.31	Positive regulator of the human tumor suppressor p53	[9]
mir-146a	6.01	Inhibits NSCLC cell growth and migration	[10]
mir-92a	-5.24	Inhibits the dissemination of ovarian cancer cells	[11]
mir-222	-4.70	Elevated expression in pancreatic cancer	[12]
mir-23b	-6.56	Enhances breast cancer metastasis to the lung	[13]
mir-199b	6.06	Targets HER2 and decreases migration of breast cancer cell	[14]
mir-27a	-4.58	Promotes human gastric cancer cell metastasis	[15]
mir-25	-3.85	Downregulation inhibits NSCLC cell proliferation	[16]
mir-449a	4.96	Inhibits NSCLC cell migration and invasion	[17]
mir-190b	4.64	Up-regulated in hepatocellular carcinoma	[18]
mir-296	-4.26	Promotes metastasis of prostate cancer cells	[19]