

UNIVERSIDAD AUTÓNOMA DE BAJA CALIFORNIA
FACULTAD DE CIENCIAS MARINAS



“Efectos de la activación de *Maspin* mediante factores de transcripción
artificiales en modelos de cáncer de mama y de ovario”

T E S I S

**QUE PARA CUBRIR PARCIALMENTE LOS REQUISITOS NECESARIOS PARA
OBTENER EL GRADO DE**

**DOCTOR EN CIENCIAS EN ECOLOGÍA MOLECULAR Y
BIOTECNOLOGÍA**

PRESENTA

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Efectos de la activación de *Maspin* mediante factores de transcripción artificiales en modelos de cáncer de mama y de ovario.

RESUMEN.

El cáncer de mama y de ovario son las complicaciones ginecológicas mas mortales en las mujeres a nivel mundial. Ambos tipos de cáncer son frecuentemente descubiertos en etapas tardías cuando se han diseminado provocando metástasis. Hasta el momento no existe una terapia efectiva para tratar esta complicación y por lo tanto existe la urgencia de nuevas terapias, así como de nuevos blancos terapéuticos.

Maspin es un gen supresor de tumores que se expresa normalmente en células epiteliales y generalmente su expresión disminuye durante la progresión del cáncer. El gen *Maspin* generalmente no se encuentra mutado y la disminución en su expresión se debe a mecanismos epigenéticos, lo cual lo hace un excelente candidato para la reactivación mediante factores de transcripción artificiales.

En el presente trabajo se desarrollaron factores de transcripción artificiales para reactivar a *Maspin*. Estos factores de transcripción artificiales se unen al promotor de dicho gen y reclutan elementos de la maquinaria transcripcional. En este estudio, los factores ATF-126 y mATF fueron clonados en vectores retrovirales para su expresión estable y transitoria en células humanas y murinas. ATF-126 y mATF permitieron la reactivación de *Maspin* y provocaron un fenotipo menos cancerígeno y metastático en las líneas celulares de mama y de ovario en que fueron transfectados. Adicionalmente, ensayos con microarreglos de DNA de las células transfectadas con estos factores, permitieron la identificación de genes que pudieran estar implicados en el proceso de carcinogénesis, tal como *Tspan12*, el cual parece estar involucrado particularmente con metástasis.

Estos estudios demuestran que los factores de transcripción artificiales son una herramienta útil en la formulación de nuevas terapias para el tratamiento del cáncer y en la identificación de blancos potenciales para la formulación de nuevas terapias.

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Effect of *Maspin* activation by means of artificial transcription factors in breast and ovarian cancer models.

SUMMARY.

Breast and ovarian cancer are the deadliest gynecological complications in women worldwide. Both cancer types are frequently detected in late stages when they have metastasized. Currently there is no effective therapy for the treatment of this complication and therefore there is urgency for new therapies and targets.

Maspin is a tumor suppressor gene normally expressed in epithelial cells and its expression usually decreases during cancer progression. The *Maspin* gene is generally not mutated and its diminished expression is mainly due to epigenetic mechanisms. This characteristic makes Maspin an excellent candidate for reactivation by means of artificial transcription factors.

In these studies, artificial transcription factors for Maspin reactivation were developed. These artificial transcription factors bind to the *Maspin* promoter and recruit elements of the transcriptional machinery. The factors ATF-126 and mATF were cloned in retroviral vectors for stable and transient expression in human and murine cell lines. These factors caused Maspin reactivation and led to a less carcinogenic and metastatic phenotype in the breast and ovarian cell lines in which they were transduced. Moreover, DNA microarray assays on the transfected cells allowed the identification of genes that could be related with the carcinogenesis process, such as *Tspan12*, which might be involved particularly in metastasis.

Our studies demonstrate that artificial transcription factors are a useful tool in the formulation of new therapies and also in the identification of potential targets for cancer treatment.

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TESIS

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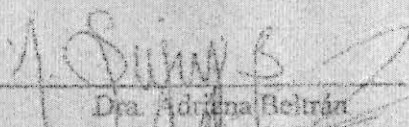
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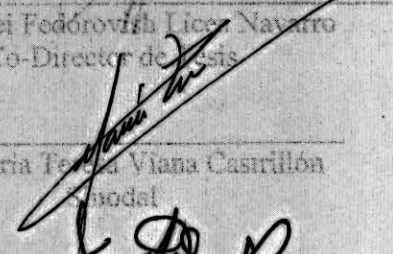
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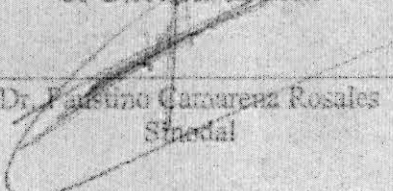
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INTRODUCCIÓN.

Los cánceres de mama y ovario suman en conjunto la cuarta parte del total de muertes por cáncer en mujeres (Jemal *et al.*, 2011). La detección temprana juega un papel esencial en ambos padecimientos. El cáncer de mama puede ser detectado a tiempo, pero una vez que su estadio es avanzado, la supervivencia es alrededor de 40%. En contraste, el cáncer de ovario generalmente es detectado en fases tardías, puesto que no existe un método de detección oportuna. El porcentaje de supervivencia para el cáncer de ovario en etapa tardía es menor al 30%, debido a que en estadios avanzados, produce metástasis hacia otros órganos dificultando de esta manera el tratamiento de la enfermedad (American Cancer Society, 2011). Lo anterior indica que es imprescindible implementar nuevas terapias para tratar la metástasis y enfatiza la necesidad de desarrollar métodos de detección oportuna, principalmente para el cáncer de ovario.

En el presente estudio se utilizaron factores de transcripción artificiales (ATF por sus siglas en inglés, *Artificial Transcription Factor*) para reactivar al gen *Maspin*. *Maspin* (sobrenombre derivado del inglés *mammary serin protease inhibitor*, inhibidor de la serín proteasa mamaria) es un gen supresor de tumores cuya expresión disminuye durante la progresión del cáncer de mama (Zou *et al.*, 1994), ovario (Sopel *et al.*, 2010; Sood *et al.*, 2002), próstata (Zhang *et al.*, 1997 b), pulmón (Beltran y Blancafort, 2011) entre otros (Yasumatsu *et al.*, 2001; Denk *et al.*, 2007). En células normales, *Maspin* es un supresor tumoral encargado de mantener la homeostasis celular. La expresión de *Maspin* en células cancerígenas promueve la apoptosis, la adhesión celular, inhibe la migración de las células tumorales y disminuye el crecimiento de tumores en modelos

animales (Bailey *et al.*, 2006). Una característica importante del gen *Maspin* es que no se encuentra mutado (Zou *et al.*, 1994) y la regulación de su expresión se lleva a cabo mediante mecanismos epigenéticos principalmente la metilación del DNA y la desacetilación de las histonas (Futscher *et al.*, 2002). Existen varias estrategias para revertir dicho silenciamiento epigenético, como el uso de fármacos (Futscher *et al.*, 2002, Beltran *et al.*, 2008) y los ATF (Beltran *et al.*, 2008). La inconveniencia de los fármacos epigenéticos es que actúan en todo el genoma y no específicamente en un gen, reactivando tanto genes supresores de tumores como oncogenes (Kwon y Shin, 2011). Por otro lado, los ATF son diseñados para activar específicamente a un gen (Beerli y Barbas, 2002). Los ATF diseñados en el presente estudio contienen un dominio de unión al DNA y una proteína activadora de la transcripción. El dominio de unión al DNA está compuesto por una proteína dedos de zinc y está ligada al dominio activador VP64, (Beltran *et al.*, 2007).

En el presente trabajo se utilizaron el ATF-126 y el mATF, los cuales fueron diseñados para reconocer 18 pb en el promotor del gen *Maspin* y reactivar su expresión. El ATF-126 es capaz de reactivar la expresión de *Maspin* en líneas celulares de mama y de pulmón (Beltran *et al.*, 2007; Beltran y Blancafort, 2011). Posteriormente, el ATF-126 se subclonó en un sistema retroviral inducible y fue capaz de reactivar a *Maspin* e inhibir el crecimiento de tumores y metástasis en modelos murinos de cáncer de mama (Beltran *et al.*, 2011).

El mATF fue diseñado para reconocer 18 pb en el promotor murino de *Maspin*. El mATF reactivó a *Maspin* en un panel de líneas celulares derivadas de modelos murinos. Esta activación fue excepcionalmente alta en las líneas celulares MOVCAR

derivadas de un modelo murino de cáncer de ovario. Particularmente, cuando mATF es transfectado en la línea celular MOVCAR 5009, disminuye la proliferación, la invasión y la formación de esferas y colonias tumorales. En este mismo modelo, el mATF también propició la desorganización del citoesqueleto y la formación de adhesiones focales en las células, lo cual interfiere con los procesos de migración y adhesión celular. El mATF también se empacó en nanopartículas para probar su eficacia en modelos murinos, inhibiendo el crecimiento de tumores originados con las células MOVCAR 5009 (Lara *et al.*, 2012). Los ensayos con microarreglos de DNA de las células transfectadas con ambos ATF permitieron la identificación de genes que pudieran estar implicados en el proceso de carcinogénesis y metástasis, y por lo tanto, serían blancos potenciales para nuevas terapias. En experimentos posteriores, se encontró que uno de estos genes, *TSPAN12*, está regulado negativamente en los ascites de pacientes con cáncer de ovario (Lara *et al.*, 2012).

Estas investigaciones en modelos de cáncer de mama y ovario, demuestran que los ATF son una herramienta útil en la reactivación genética. También nos permiten perfilar el uso de los ATF como un instrumento en el descubrimiento de nuevos blancos terapéuticos contra el cáncer. En estos modelos en particular, los ATF permitieron la reactivación de *Maspin* e indujeron un fenotipo menos agresivo y metastático en las líneas celulares de mama y de ovario.

ANTECEDENTES.

Estadísticas del cáncer de mama y de ovario. El cáncer de mama y de ovario como problemas de salud.

El cáncer de mama es el tipo de cáncer más frecuente en las mujeres a nivel mundial (Jemal *et al.*, 2011; Ferlay *et al.*, 2008). La esperanza de vida es alta si se detecta de manera temprana, sin embargo, disminuye conforme la enfermedad avanza, descendiendo a entre 15-40% en las etapas más avanzadas (*Tabla I*).

Tabla I. Porcentajes de supervivencia a 5 años para cáncer de mama y ovario. Los datos fueron tomados de la Sociedad Americana de Cáncer (*American Cancer Society*).

Cáncer de mama ¹		Cáncer de ovario ²	
Estadio	Supervivencia a 5 años (%)	Estadio	Supervivencia a 5 años (%)
0	93	I	89
I	88	IA	94
IIA	81	IB	91
IIB	74	IC	80
IIIA	67	II	66
IIIB	41	IIA	76
IIIC	49	IIB	67
IV	15	IIC	57
		III	34
		IIIA	45
		IIIB	39
		IIIC	35
		IV	18

¹ La supervivencia fue estimada de los casos entre 2001 y 2002.

² La supervivencia fue estimada para los casos de cáncer de ovario epitelial de pacientes diagnosticadas entre 1988 a 2001. Los datos fueron extraídos del Instituto Nacional de Cáncer (*National Cancer Institute*) en EE.UU.

En México, el cáncer de seno es el más prevalente y mortal en las mujeres. Según datos del proyecto GLOBOCAN 2008 de la Organización Mundial de la Salud (WHO por sus siglas en inglés, *World Health Organization*), nuestro país presentó en ese año 13,939 casos de cáncer de mama y 5,217 muertes, indicando esto una mortalidad mayor al 30%, mientras que la mortalidad debida al cáncer de ovario es cercana al 70%, siendo mortales 1,891 del total de 2,910 casos estimados, (Ferlay *et al.*, 2008).

El cáncer de ovario es el cáncer de origen ginecológico más mortal a nivel mundial. La mayoría de los casos se detecta en estadios avanzados, cuando la supervivencia es menor al 30% (*Tabla I*). La mortalidad se debe a que es detectado cuando ha invadido las paredes abdominales dando origen a los ascites y la metástasis. A diferencia del cáncer de seno, para el cual si existen métodos de detección temprana como la autoexploración y la mamografía, actualmente no se cuenta con un método de detección temprana de cáncer de ovario. El procedimiento estándar es la vigilancia de los niveles del antígeno CA-125 en sangre y el ultrasonido transvaginal, sin embargo estos métodos no disminuyen significativamente la mortalidad (Slomski, 2012). La dificultad en la detección del cáncer de ovario radica en la falta de conocimiento, la complejidad de su biología y los síntomas confusos. Generalmente es diagnosticado de manera tardía cuando los ascites y la obstrucción intestinal causan dolor agudo (Partridge y Barnes 1999).

Regulación de la expresión de *Maspin*.

El gen *Maspin* es un gen supresor de tumores descubierto en células epiteliales normales de mama en humanos (Zou *et al.*, 1994). Pertenece a la superfamilia de inhibidores de serin proteasas y está localizado en el cromosoma 18q21.3 junto con otros genes de la misma familia como *PAI2* (*Plasminogen Activator Inhibitor type 2*) *SCCA1* y *SCCA2* (*Squamous Cell Carcinoma Antigen* tipo 1 y tipo 2, respectivamente) (Schneider *et al.*, 1995).

La proteína Maspin está compuesta por 376 aminoácidos y tiene un peso de 42 kDa (Zou *et al.*, 1994). Se expresa normalmente en células epiteliales, como mama, próstata y pulmón en humanos. Su expresión generalmente está limitada al componente epitelial y no se expresa en fibroblastos, leucocitos o neuronas (Zhang *et al.*, 1997 a; Futscher *et al.*, 2002). Maspin favorece la adhesión celular y la apoptosis, y disminuye la movilidad celular y la angiogénesis (Khalkhali-Ellis, 2006). Además, cuando *Maspin* se reactiva en células tumorales, inhibe la proliferación y la migración celular, así como el crecimiento de tumores en ratones (Beltran *et al.*, 2007). En general, la disminución en su expresión se asocia con la progresión del cáncer lo cual lo hace un blanco excepcional para terapias antitumorales.

La expresión de Maspin está regulada a nivel transcripcional y epigenético. En el promotor de *Maspin* se encuentran varios elementos *cis* reguladores de la transcripción: ETS, AP-2, AP-1, HRE y p53 (*Figura 1*) (Zhang *et al.*, 1997 a; Zou *et al.*, 2000). Las proteínas de la familia ETS, AP-1 y AP-2 son factores de transcripción que regulan positivamente la expresión de Maspin y se encuentran bajo investigación por sus

probables implicaciones en el desarrollo de cáncer (Seth y Watson, 2005; Leaner *et al.*, 2007; Eckert *et al.*, 2005). El elemento de respuesta a hormonas HRE (por sus siglas en inglés *Hormone Response Element*) es un factor de transcripción dependiente de ligando. HRE regula negativamente la expresión de Maspin y actúa a través del receptor de andrógeno en próstata (Zhang *et al.*, 1997 b) y mediante el receptor de estrógeno en mama (Khalkhali-Ellis *et al.*, 2004).

El factor de transcripción p53, al igual que *Maspin*, es un gen supresor de tumores. La proteína p53 se une al promotor de *Maspin* para activar al gen (Zou *et al.*, 2000). p53 es un supresor de tumores ampliamente estudiado en cáncer puesto que se ha encontrado que en esta patología su expresión siempre está alterada. p53 puede activar o reprimir la expresión de cientos de genes en respuesta a señales de estrés celular. Los genes que p53 regula participan en rutas de señalización celular implicadas en los procesos de apoptosis, senescencia, reparación del DNA y diferenciación celular, entre otros, y es de esta manera que previene el desarrollo del cáncer (Menendez *et al.*, 2009; Riley *et al.*, 2008).

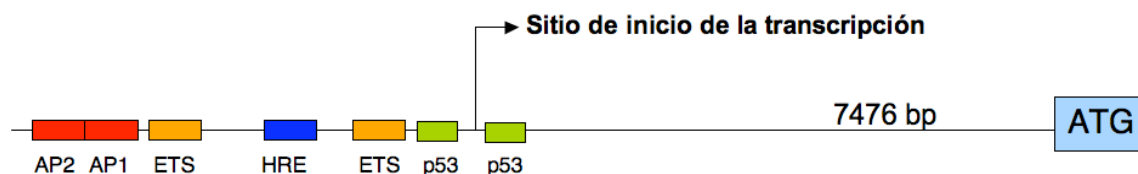


Figura 1. Esquema de la región de 1 kb del promotor de *Maspin*. Se señalan los sitios *cis* AP-2, AP-1, ETS, HRE y p53.

La regulación mediante estos elementos en el promotor de *Maspin*, se pierde en las células tumorales por los mecanismos epigenéticos que silencian al promotor (Zhang

et al., 1997a; Zhang *et al.*, 1997b; Zou *et al.*, 2000). Generalmente, *Maspin* no presenta alteraciones genéticas y los mecanismos epigenéticos involucrados en el silenciamiento son la metilación del DNA y la desacetilación de las histonas (Domann *et al.*, 2000; Maas *et al.*, 2002; Futscher *et al.*, 2004). La metilación del DNA y desacetilación de las histonas son mecanismos epigenéticos íntimamente ligados que participan en la regulación de la expresión génica y ambos provocan un estado de compactación de la cromatina que inhibe la transcripción (*Figura 2*) (Vaissière *et al.*, 2008).

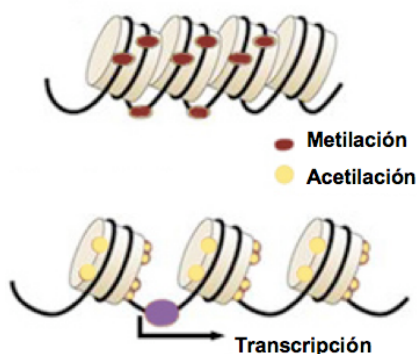


Figura 2. Representación del estado de compactación de la cromatina debido a los mecanismos de metilación y acetilación. La metilación de los dinucleótidos CpG mantiene el estado de compactación de la cromatina (arriba), mientras que la acetilación de la cromatina la mantiene en un estado abierto a la transcripción (abajo) (Modificada de Robertson y Wolffe, 2000).

La metilación del DNA es un evento común en el genoma de los eucariotas y está relacionado con la inactivación genética. La metilación del DNA sucede en los dinucleótidos CpG (C-fosfato-G) donde las DNA metiltransferasas (DNAMT) actúan para convertir la citosina a 5-metilcitosina. De manera global, la frecuencia de los dinucleótidos CpG en el DNA es baja, sin embargo, en los promotores de los genes pueden encontrarse regiones de dinucleótidos CpG concentrados, y se les conoce como

“islas CpG”. Generalmente, las islas CpG no están metiladas y su metilación está asociada con la represión de la transcripción. La represión mediada por la metilación de las islas CpG es importante en los procesos como la inactivación del cromosoma X, la impronta genómica y la expresión específica de tipo celular. No obstante, también se presenta la metilación aberrante de las islas CpG y este fenómeno está relacionado con la inactivación de los genes supresores de tumores en la progresión del cáncer (Strathdee *et al.*, 2004). En el caso de *Maspin* la metilación de las regiones CpG silencia al gen y se ha observado que en células normales el promotor no está metilado y la estructura de la cromatina está abierta a la transcripción, mientras que en células tumorales el promotor está metilado (Domann *et al.*, 2000).

Además de la metilación del DNA, la cromatina sufre otras modificaciones que alteran su estructura, como la modificación postraducciona de las histonas por acetilación, metilación, fosforilación y ubiquitinación. La acetilación de las histonas es el fenómeno más estudiado, puesto que está relacionada con la metilación del DNA (Vaissière *et al.*, 2008). De manera general, los genes activos están asociados con una cromatina hiperacetilada y laxa. El relajamiento de la cromatina es ocasionado por la acetilación del residuo de lisina en las histonas, que le proporciona una carga negativa y produce una disminución en la interacción entre la histona y el DNA (Cloos *et al.*, 2008). La hipoacetilación por otro lado, es un fenómeno recurrente en el silenciamiento de los genes supresores de tumores como *Maspin* ya que crea el efecto opuesto (Dalvai *et al.*, 2010; Cloos *et al.*, 2008; Maas *et al.*, 2002).

Recientemente se ha incluido a los microRNA como elementos de la regulación epigenética, puesto que estos no modifican el DNA *per se*, pero si modifican la

expresión de los genes. Los microRNA son moléculas pequeñas de RNA, que constan de ≈ 22 nucleótidos y regulan la expresión genética de manera negativa a nivel post-traducciona. Algunos de ellos ya se han clasificado como oncogenes o genes supresores de tumores por el impacto que tienen en cáncer y metástasis, sin embargo, aún se desconoce el papel que muchas de estas moléculas desempeñan en otros procesos biológicos (Chuang y Jones, 2007).

Reactivación de genes supresores de tumores y de *Maspin*.

Los genes supresores de tumores están silenciados por mecanismos epigenéticos en cáncer, los cuales pueden reactivarse mediante fármacos epigenéticos. En este rubro existen fármacos que interfieren con el proceso de metilación del DNA y fármacos que interfieren con el proceso de desacetilación de las histonas. Hasta el momento hay cientos de estos fármacos en pruebas clínicas, pero sólo cuatro han sido aprobados por la Administración de Alimentos y Fármacos en Estados Unidos (FDA por sus siglas en inglés, *Food and Drug Administration*), dos son inhibidores de la metilación del DNA: 5-azacitidina (Vidaza de la compañía farmacéutica Celgene) y 5-aza-2'-deoxicitidina (Dacogen de la farmacéutica Eisai); los otros dos son inhibidores de las enzimas histona-desacetilasas: SAHA (Zolinza por Merck) y romidepsin (Istodax por Celgene) (Mack, 2010). Hasta el momento se discute el hecho de que los fármacos no son específicos sino que actúan de manera global, reactivando oncogenes y provocando inestabilidad genómica (Rius y Lyko, 2012).

Como ya se ha venido mencionando, *Maspin* es un gen supresor de tumores y su re-expresión en las células cancerígenas es un aspecto deseable puesto que disminuye las habilidades metastásicas y tumorales de las células cancerígenas. La re-expresión de *Maspin* en las células se ha basado en dos estrategias: la introducción de *Maspin* de manera exógena y la reactivación endógena del gen. De manera exógena, la re-expresión de *Maspin* se ha logrado mediante la transfección con cDNA en células tumorales de mama (Zou *et al.*, 1994;), ovario (Sood *et al.*, 2002) y próstata (Biliran y Sheng, 2001). Una vez que se expresa *Maspin*, las células son menos propensas a la invasión, a la movilidad y a la formación de tumores en ratones.

Puesto que el silenciamiento de *Maspin* se debe a la metilación del promotor, se experimentó con una línea celular de cáncer mamario la cual no expresa esta proteína, para reactivar endógenamente su expresión mediante un fármaco epigenético. El tratamiento con el fármaco 5-aza-2'-deoxicitidina reactiva la expresión de *Maspin* en las células MCF-7 que normalmente no expresan esta proteína y presentan el promotor metilado (Domann *et al.*, 2000). En 2007, Beltran y colaboradores reportaron la reactivación endógena de *Maspin* mediante tres Factores de Transcripción Artificiales (ATF por sus siglas en inglés *Artificial Transcription Factors*): ATF-97, ATF-126 y ATF-452. Estos tres ATF reactivan a *Maspin* cuando son transfectados en líneas celulares de cáncer de mama (MCF-7, MDA-MB-468 y MDA-MB-231). El grado de reactivación de *Maspin* varía entre los ATF y las líneas celulares, y depende de la accesibilidad al promotor y por lo tanto del nivel de metilación en este sitio. La combinación de fármacos epigenéticos que relajan el promotor y ATF-126 conduce a una activación de *Maspin* mayor que la observada al usar estos agentes de manera

separada (Beltran *et al.*, 2008). También se ha demostrado que el ATF-126 reactiva Maspin e induce la desmetilación en el promotor de este gen en la línea celular de cáncer de pulmón NCI-H157 (Beltran y Blancafort, 2011).

Los factores de transcripción artificiales como elementos de regulación genética.

La regulación de los genes puede alterarse en el proceso de transcripción. Esto sucede cuando se modifica la estructura de los promotores o los factores de transcripción por mecanismos genéticos, o cuando los promotores se vuelven inaccesibles a la transcripción por mecanismos epigenéticos. Ya hemos hablado de los mecanismos epigenéticos que silencian *Maspin* y ahora hablaremos de otros factores de transcripción que operan sobre el promotor de este gen: los ATF.

Los ATF son análogos de los factores de transcripción naturales y al igual que estos, se unen a los elementos *cis* en los promotores de los genes y reclutan otras proteínas implicadas en la transcripción. En la última década se han sintetizado ATF con el objetivo de regular la expresión génica en ciertas enfermedades, principalmente el cáncer. Los ATF constan de un dominio de unión al DNA, un dominio efector (que puede ser activador o represor) y una señal de localización nuclear (*Figura 3*) (Sera, 2009). Entre los dominios de unión al DNA, las proteínas dedos de zinc tipo C_2H_2 son el grupo más importante ya que son las más predominantes en humanos y se unen a secuencias específicas de DNA con alta afinidad (Beerli y Barbas, 2002; Blancafort *et al.*, 2003; Segal *et al.*, 1999; Beerli *et al.*, 1998; Liu *et al.*, 1997). Además, los dedos de zinc C_2H_2 son multimodulares, cada “módulo” o dedo de zinc consta de 30 aminoácidos

formando una estructura compuesta de una hélice- α y dos hebras β estabilizadas por un ion de zinc (*Figura 4*). Cada dedo de zinc contiene 4 aminoácidos conservados: dos cisteínas en las hebras β y dos histidinas en el α -hélice, por lo cual se les conocen como dedos de zinc tipo C_2H_2 . Cada módulo reconoce tres nucleótidos y los factores de transcripción naturales contienen tres o más dedos de zinc para unirse específicamente a una región del promotor. Esta misma estrategia de multimodularidad es la que se utiliza en el diseño de los ATF. Mientras más larga o más módulos contiene una proteína dedos de zinc, es más específica. Por lo tanto, una proteína dedo de zinc compuesta por tres módulos se une a nueve nucleótidos y se espera que en el genoma humano encuentre más de 10^4 sitios de unión, mientras que una proteína de seis dedos de zinc se une a una secuencia de 18 nucleótidos teóricamente única en el genoma humano. Debido a la especificidad de las proteínas dedos de zinc, éstas son el dominio de unión al DNA más usado en el diseño de los ATF (Beerli y Barbas, 2002).

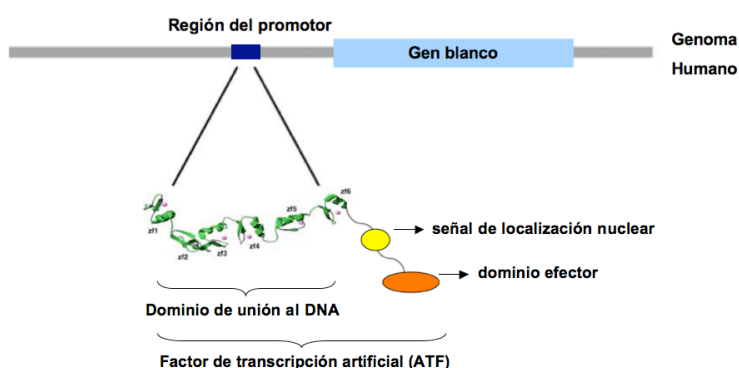


Figura 3. Esquema de los componentes de un factor de transcripción artificial (ATF): señal de localización nuclear, dominio de unión al DNA y dominio efector. El dominio de unión al DNA se une en el promotor y dirige el efecto del ATF a un gen en particular (gen blanco). La señal de localización nuclear es una secuencia que dirige el ATF al núcleo de la célula. El dominio efector puede ser represor, si lo que se desea es suprimir la expresión del gen blanco, o activador, si se desea incrementarla (Modificada de Sera, 2009).

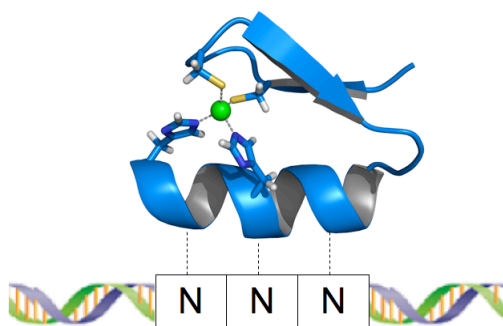


Figura 4. Estructura de un dedo de zinc tipo C_2H_2 . Estas proteínas pueden formar proteínas multimodulares de hasta 9 dedos de zinc, cada módulo es una proteína de 30 aminoácidos formando una estructura de un α -hélice y dos hebras β estabilizadas por un ion zinc. Cada módulo o “dedo” se une a 3 nucleótidos (N) en la secuencia de DNA mediante aminoácidos en el α -hélice (Figura modificada de Wikipedia, autor: Thomas Splettstoesser).

Los ATF además de ser específicos, son altamente afines a sus blancos. Poseen afinidades de hasta $K_d \approx 70$ pM, lo cual se acerca mucho a los factores de transcripción naturales (Beerli *et al.*, 2000; Jamieson *et al.*, 2003; Tan *et al.*, 2003). La *Tabla II* muestra diversos ejemplos de ATF compuestos de proteínas dedos de zinc diseñados para regular la transcripción de los genes de manera endógena, entre ellos *Maspin*.

Tabla II. Genes regulados mediante factores de transcripción artificiales (ATF) compuestos por dedos de zinc. VP16, VP64 y p65 son dominios activadores, mientras que KRAB, RAB y v-ErbA son dominios represores (Bartsevitch y Juliano, 2000; Ren *et al.*, 2002; Jouvenot *et al.*, 2003).

# módulos	Dominio efector	Función del gen que regula	Gen blanco	Referencia
6	VP16, VP64	Blanco terapéutico en cáncer de mama	<i>ErbB-2</i>	Beerli <i>et al.</i> , 2000
5	KRAB	Control de resistencia múltiple a fármacos	<i>MDR1</i>	Bartsevitch y Juliano, 2000
3	VP16	Eritropoyesis	<i>Epo</i>	Zhang <i>et al.</i> , 2000
3, 6	VP16	Angiogénesis	<i>VEGF-A</i>	Rebar <i>et al.</i> , 2002
6	RAB	Adipogénesis	<i>PPARγ</i>	Ren <i>et al.</i> , 2002

3	v-ErbA, VP16	Neoplasias	<i>IGF2 y H19</i>	Jouvenot <i>et al.</i> , 2003
5	VP16	Apoptosis	<i>Bax</i>	Falke <i>et al.</i> , 2003
6	VP16, KRAB	Mantenimiento de la pluripotencialidad	<i>Oct-4</i>	Bartsevich <i>et al.</i> , 2003
6	KRAB	Regula ciclo celular	<i>CHK2</i>	Tan <i>et al.</i> , 2003
3	VP16	Metabolismo óseo	<i>PTHr1</i>	Liu <i>et al.</i> , 2005
6	VP64	Terapia en enfermedades asociadas con β -globina	<i>γ-globina</i>	Gräslund <i>et al.</i> , 2005
6	VP64	Supresor de tumores	<i>maspin</i>	Beltran <i>et al.</i> , 2007
3	VP16	Distrofia muscular de Duchenne	<i>utrofina</i>	Mattei <i>et al.</i> , 2007
6	p65	Produce proteína antiangiogénica en tejido ocular	<i>PEDF</i>	Yokoi <i>et al.</i> , 2007

El ATF-126 fue diseñado para reactivar *Maspin* y es una proteína multimodular de seis dedos de zinc ligada al dominio activador VP64. La proteína de dedos de zinc está diseñada para reconocer una región única de 18 pb en el promotor de *Maspin*, mientras que el dominio VP64, es un tetrámero del dominio activador VP16, un potente activador de la transcripción del virus del herpes simple (Beltran *et al.*, 2007; Sadowski *et al.*, 1988). Además, el ATF-126, contiene la etiqueta de hemaglutinina (HA) para hacer posible su reconocimiento en ensayos inmunológicos como ELISA, Western blot y ChIP (*Figura 5*).

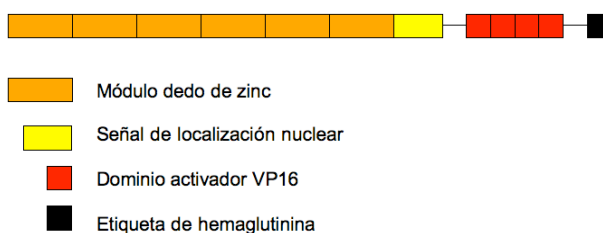


Figura 5. Representación esquemática de la construcción del ATF-126, el cual contiene seis dedos de zinc que constituyen el dominio de unión al DNA. Enseguida, se encuentra la señal de localización nuclear y posteriormente las cuatro repeticiones del dominio activador VP16 y la etiqueta de hemaglutinina (HA).

El dominio activador VP16 potencia la transcripción ya que provoca principalmente dos efectos en su entorno: por un lado, recluta elementos del complejo de iniciación y por el otro, recluta elementos relacionados con la modificación de la cromatina. El VP16 recluta a casi todos los elementos del complejo de iniciación, complejos HAT (Histon-AcetilTransferasas) y complejos Swi/Snf (Hall y Struhl, 2002; Memedula y Belmont, 2003; Herrera y Triezenberg, 2004; Uhlmann *et al.*, 2007). El dominio activador VP16 ha sido usado extensivamente como modelo de estudio de la activación de la transcripción en eucariotas y recientemente se usa como activador de la expresión génica dirigida mediante ATF (Sera, 2009). Para esta última aplicación, el dominio activador VP16 o su tetrámero VP64, se fusionan de manera artificial a dominios de unión al DNA como los dedos de zinc, para reactivar genes de varios organismos, incluyendo genes humanos (v. *Tabla II*).

JUSTIFICACIÓN.

Las muertes debido al cáncer de mama y de ovario comprenden aproximadamente la cuarta parte de las muertes por cáncer en mujeres. La mortalidad es debida principalmente al proceso de metástasis, que ocurre en las etapas tardías de la enfermedad. Hasta el momento no existe un tratamiento efectivo para la metástasis, por lo que es imprescindible el desarrollo de nuevas estrategias terapéuticas.

En nuestros estudios, buscamos disminuir el potencial metastático de las células cancerígenas mediante la reactivación de Maspin. Se sabe que la expresión de esta proteína disminuye en varios tipos de cáncer incluidos los de mama y ovario. Además, se ha observado que la reactivación de Maspin en células tumorales disminuye las propiedades metastáticas de estas células. Los ATF ofrecen una excelente alternativa para la reactivación de este gen, puesto que este no se encuentra mutado y su silenciamiento se debe a factores epigenéticos. Además, la activación mediante ATF tiene la ventaja de activar de manera específica a un solo gen, a diferencia de otros métodos que actúan de manera global.

HIPÓTESIS.

Los factores de transcripción artificiales diseñados para reconocer 18 pb en el promotor de Maspin, son capaces de unirse a su sitio de reconocimiento y reactivar la expresión del gen en modelos de cáncer de mama y ovario. Dicha unión provoca cambios en las células disminuyendo sus propiedades tumorales y metastáticas.

OBJETIVOS.**General:**

Desarrollar una estrategia para el tratamiento de tumores y metástasis en cáncer de ovario y de mama y demostrar su eficacia en modelos murinos.

Particulares:

1. Utilizar factores de transcripción artificiales como estrategia para reactivar al gen supresor de tumores *Maspin* en modelos murinos de cáncer de mama y de ovario
2. Estudiar los cambios epigenéticos, genéticos y fenotípicos que producen los factores de transcripción artificiales diseñados para Maspin en las células cancerígenas.

CAPÍTULO I: Supresión del crecimiento de tumores y metástasis en mama mediante un factor de transcripción artificial (Ver el artículo original anexo: *Suppression of Breast Tumor Growth and Metastasis by an Engineered Transcription Factor*).

En este trabajo se subclonó el ATF-126 en el sistema Tet-On Advanced. Esta estrategia permite expresar el ATF bajo el control de doxiciiclina y crear líneas celulares estables. En este sistema, el ATF-126 activó a Maspin en la línea celular de cáncer de mama MDA-MB-231 *in vitro*. Una vez que se activó Maspin, disminuyó la viabilidad celular y se incrementó la apoptosis en estas células. Además, las células MDA-MB-231 que contienen el ATF-126 se usaron para establecer tumores en ratones y se demostró que tras el tratamiento con doxiciiclina se activó la producción del ATF-126 y de Maspin en los tumores, al mismo tiempo que su tamaño disminuyó. Más aún, estas células perdieron su habilidad de colonizar los pulmones en un modelo murino de metástasis.

En este mismo trabajo, también se encontró que el ATF-126 confiere a las células una “firma genética” que está relacionada con un mejor pronóstico de cáncer de mama y se identificaron algunos genes y microRNA que podrían estar relacionados con la habilidad de las células para formar metástasis.

CAPÍTULO II: Tratamiento de cáncer ovárico epitelial de tipo seroso mediante factores de transcripción artificiales tipo dedos de zinc (Ver el artículo original anexo: *Targeting Serous Epithelial Ovarian Cancer with Designer Zinc Finger Transcription Factors*).

Este artículo presenta los estudios realizados en un modelo murino de cáncer de ovario. El mATF se unió al promotor de *Maspin* y lo activó en la línea celular MOVCAR 5009, una línea celular altamente metastática de cáncer de ovario. La activación de Maspin en este modelo condujo a la disminución en la habilidad de las células para proliferar, migrar, invadir y formar colonias y esferas tumorales.

Lo más relevante es el empaquetamiento del mATF en nanopartículas para tratar tumores establecidos con las células MOVCAR 5009 en ratones. El tratamiento con las nanopartículas y mATF permitió la expresión de Maspin e inhibió el crecimiento de los tumores en dicho modelo.

En este mismo trabajo, también se encontró que hay un gen que se activa tras el tratamiento con mATF y la activación de Maspin, el gen *Tspan12*. Además, este gen se encontró regulado negativamente en muestras de ascites de pacientes con cáncer de ovario y podría estar relacionado con el proceso de metástasis.

CAPÍTULO III: La técnica de inmunoprecipitación de la cromatina como herramienta para demostrar la unión del ATF y la modificación de las marcas epigenéticas en el promotor de *Maspin* (trabajo sin publicar).

En 2007 Beltran y colaboradores demostraron que era posible la reactivación endógena de *Maspin* mediante ATF. El ATF-126 fue clonado en un vector de expresión transitoria, el plásmido pMX-IRES-GFP. Los vectores de expresión transitoria permiten la expresión de genes foráneos en las células, sin embargo este sistema presenta varias desventajas. Primero, el DNA introducido se va perdiendo en el proceso de mitosis o por desintegración. Además, la expresión del ATF-126 mediante este sistema no puede ser controlada y una vez que el ATF se produce, empieza a activar *Maspin* provocando apoptosis en las células cancerígenas. Existen otros sistemas de expresión que permiten la expresión estable y controlada del gen de interés, uno de ellos es el sistema de expresión Tet-On Advanced (Clontech). El sistema Tet-On Advanced consta de dos plásmidos, pTet-On and pTight. El gen de interés es clonado en pTight, mientras que pTet-On produce una proteína que sólo se une al promotor de pTight en presencia de doxíciclina (Dox) y activa la producción del gen de interés (*Figura 6*).

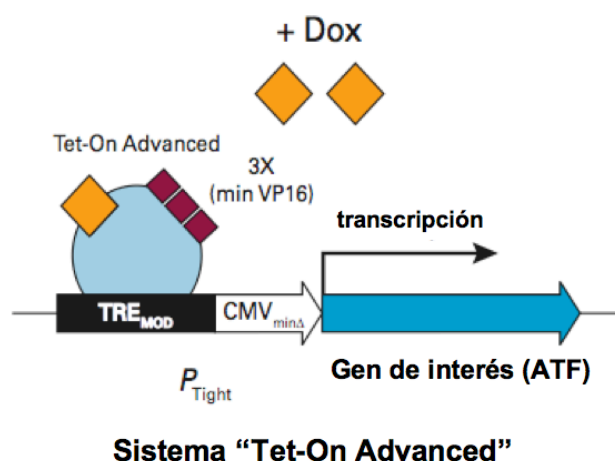


Figura 6. El sistema de inducción "Tet-On Advanced" es dependiente de doxiciclina. Este sistema contiene dos vectores, pTet-On Advanced y pTight. El primero produce una proteína de fusión que contiene tres dominios de activación VP16 y solamente se une al promotor de pTight en presencia de doxiciclina (Dox) para activar la expresión del gen de interés (en nuestro caso ATF) (Figura modificada del Manual de expresión de Clontech: *Tet-On® Advanced Inducible Gene Expression Systems User Manual*).

Mediante este sistema se crearon líneas celulares estables que contienen el plásmido para expresar el ATF-126 bajo el control de doxiciclina. Este sistema presenta dos ventajas: por un lado permite la propagación de las células hasta alcanzar el número masivo que se requiere para los estudios de inmunoprecipitación de la cromatina (ChIP por sus siglas en inglés, *chromatin immunoprecipitation*) ($\approx 1 \times 10^7$) y por otro lado, permite generar tumores en ratones para estudiar, *in vivo*, la activación de Maspín mediante el ATF. Con este sistema, se transfectaron líneas celulares con el promotor de Maspín expuesto como HMEC, SUM149 y SUM159, las cuales expresan Maspín; además de MDA-MB-231 en la que el promotor está metilado, cerrado y no expresa la proteína. De acuerdo a la estructura de los promotores, se consideró que el ATF se uniría y activaría a Maspín en las líneas celulares que presentan el promotor abierto. La Figura 7 muestra los resultados de la inducción del ATF-126 y la reactivación de Maspín en

estas líneas celulares. El ATF-126 se expresa en todas ellas, mientras que la reactivación de Maspin solamente se observa en MDA-MB-231. Estos resultados son opuestos a lo esperado y sería necesario determinar el estado de metilación en el sitio de unión del ATF-126 para determinar la razón por la que el ATF no activa a Maspin en estas líneas celulares.

Puesto que la inducción del ATF y la reactivación de Maspin fue positiva en MDA-MB-231, se procedió a inmunoprecipitar el ATF en este modelo. Las pruebas de PCR con primers para amplificar Maspin revelaron que no era posible inmunoprecipitar el ATF. La causa probable es que la producción del ATF es baja y no es suficiente para que logremos capturar a esta proteína mediante el anticuerpo anti-HA (*Figura 7*), aunque esta producción sea suficiente para reactivar Maspin y producir los efectos deseados en las células. Estos resultados requirieron la búsqueda de un modelo que expresara el ATF y activara a Maspin en niveles más altos.

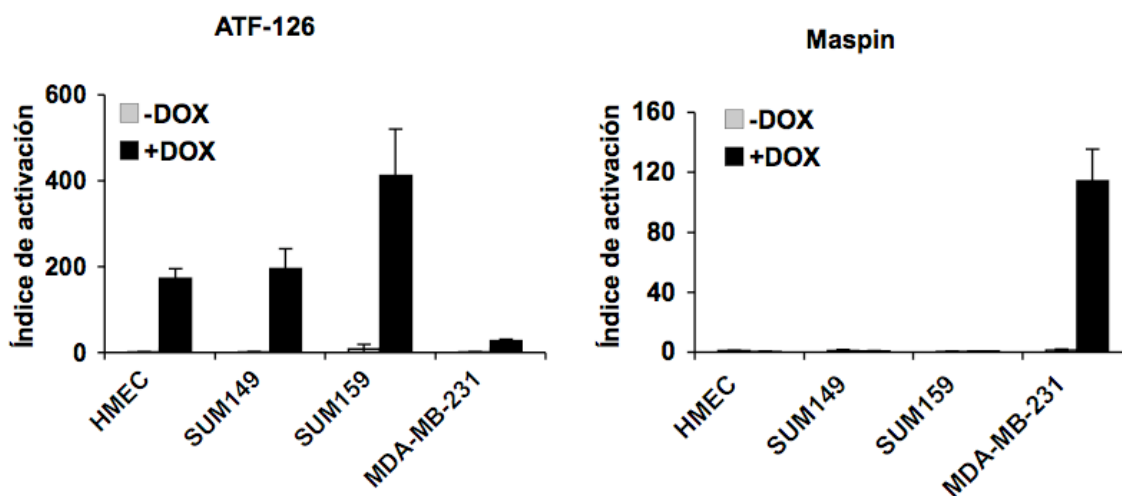


Figura 7. Índice de activación de ATF-126 y Maspin determinado mediante qRT-PCR en las líneas celulares mamarias HMEC, SUM149, SUM159 y MDA-MB-231. El índice es relativo a la muestra control –DOX. Las líneas celulares fueron transducidas con el sistema de expresión Tet-On Advanced para crear líneas celulares estables que contienen el plásmido pTight-ATF-126 y lo expresan bajo la inducción con doxiciplina (+DOX).

Puesto que se contaba con un panel de líneas celulares derivadas de tumores murinos de origen mamario y de ovario, se consideró el diseño y transfección de un ATF que reactivara a Maspin en el promotor murino, este se denominó mATF. El mATF fue capaz de reactivar a Maspin en todas las líneas celulares probadas (Lara *et al.*, 2012). La reactivación de Maspin fue particularmente muy alta en las líneas MOVCAR y además, este modelo produce cantidades elevadas de mATF. La producción alta de estos dos elementos, sugerían que habría una mayor oportunidad para inmunoprecipitar complejos del ATF unido al promotor de Maspin. Para este último propósito se usó la línea celular MOVCAR 5009 como modelo de estudio y fue posible detectar, mediante la técnica de ChIP, que físicamente el mATF se une al promotor de *Maspin* (Lara *et al.*, 2012). El siguiente paso fue demostrar que después de esta unión y antes de la activación de Maspin suceden cambios epigenéticos en el promotor de *Maspin* que permiten esta activación. Con este objetivo, se utilizó la técnica de ChIP para inmunoprecipitar los complejos que se forman por la unión de anticuerpos en las marcas epigenéticas. Los anticuerpos usados están detallados en la *Tabla III*.

Tabla III. Anticuerpos usados en ChIP para detectar marcas de metilación y acetilación de las histonas.

Anticuerpo	Cantidad (μL/IP)	Fabricante	Transcripción
H3K9me1	18	Abcam9045	Activación
H3K9me2	18	Abcam1220	Supresión
H3K9me3	18	Abcam8898	Supresión
H4Ac	5	Active Motif 39179	Activación
H3K27Ac	5	Active Motif 39135	Activación
H3K4me3	5	Active Motif 39159	Activación
H2A-Ac	4	Cell Signaling 2575	Activación
H2A total	4	Cell Signaling 2578	N/A
H2B-Ac	4	Cell Signaling 2574	Activación

H2B total	4	Cell Signaling 2722	N/A
H3Ac	4	Cell Signaling 9649	Activación
H3 total	4	Cell Signaling 4499	N/A
H4Ac	4	Cell Signaling 2594	Activación
H4 total	4	Cell Signaling 2592	N/A

Como se mencionó anteriormente, el ATF-126 remueve la metilación del promotor de *Maspin* en la línea celular de cáncer de pulmón NCI H157 (Beltran y Blancafort, 2011). La metilación es uno de los mecanismos epigenéticos que modifican los promotores de los genes y usualmente precede a otros cambios como la acetilación. Basados en esto se propuso demostrar que el ATF no sólo cambia la metilación del DNA en el promotor de *Maspin*, sino que también suceden cambios en la metilación y la acetilación de las histonas. La estrategia era inmunoprecipitar las marcas epigenéticas (con los anticuerpos que se detallan en la *Tabla III*) en las muestras transfectadas con el control (el vector vacío) y con el mATF. Las muestras obtenidas mediante ChIP después se analizaron mediante PCR convencional. Se esperaba encontrar un incremento en las marcas correspondientes a la activación de la transcripción y una disminución de las marcas de represión (ver *Tabla III*). Respecto a las marcas de acetilación, los PCR resultaron negativos, indicando que la técnica no había sido adecuada para inmunoprecipitar el DNA al que estas marcas se unen. Existen reportes que proponen el uso de cromatina nativa (no fijada con formaldehído) para inmunoprecipitar marcas epigenéticas (Wagschal *et al.*, 2007), sin embargo las muestras usadas para las pruebas de ChIP habían sido fijadas con formaldehído y el protocolo había resultado exitoso para inmunoprecipitar el ATF. Otra posibilidad es que los anticuerpos o las diluciones a las que son empleados no hayan sido los adecuados para lograr nuestro objetivo. Serán necesarias más pruebas para estandarizar la aplicación de esta técnica en este modelo de

estudio. Algunos de los factores que podrían modificarse son el tiempo (o la anulación) en el proceso de fijación, la dilución a la que se usan los anticuerpos y la cromatina (un exceso de cromatina puede resultar inhibitoria) (Carey *et al.*, 2009).

En lo que respecta a las marcas de metilación de las histonas, la PCR resultó positiva para la marca H3K9 (*Figura 8*). Las imágenes se procesaron mediante el software ImageJ (NCBI) para cuantificar el cambio en la amplificación del promotor de *Maspin* entre la muestra control y mATF. El análisis densitométrico mediante ImageJ no mostró diferencias significativas entre control y mATF. Para cuantificar este cambio, se requirió de un método más sensible como PCR en tiempo real. Se probaron varios juegos de oligonucleótidos para esta técnica. El par de oligonucleótidos para amplificar el promotor de *Maspin* con mejores resultados fue F(F5)5'-CGTGCTTGTCGCAGGCCATCAGA-3' y R(BH)'5'-GCTCACCTGTGACCACACCCAG-3' que produce un amplicón de 180 pb. Los datos obtenidos del PCR en tiempo real fueron normalizados y analizados como se describe en el Manual de SABiosciences: Análisis de Inmunoprecipitación de la Cromatina mediante PCR cuantitativo en Tiempo Real (*Quantitative Real-Time PCR Analysis of Chromatin Immunoprecipitation*) Versión 1.2 y en el trabajo de Haring *et al.*, 2007. Las pruebas de ChIP se realizaron tres veces, sin embargo los resultados no fueron reproducibles (*Figura 9*). El anticuerpo H3 es un control interno para detectar la cantidad total de esta histona, la cual no debe variar entre el control y mATF. Contrario a lo esperado, observamos variación en H3 total y más aún el resultado no fue consistente en los tres experimentos. La monometilación de H3K9 (H3K9me1) es una marca que denota activación de los promotores (ver *Tabla III*) y se esperaba ver un incremento en las

muestras transfectadas con mATF, sin embargo se observó el efecto opuesto en dos de los tres experimentos. La dimetilación de H3K9 (H3K9me2) es una marca represiva y efectivamente se observó su disminución en los tres ensayos realizados. Sin embargo, los índices del primer experimento ($\approx 40-70$) fueron mayores a los encontrados en los experimentos posteriores (≈ 1.5). Los resultados de la trimetilación de H3K9 (H3K9me3) también mostraron discrepancias. Se considera que los obstáculos a superar en estos análisis son principalmente dos: la estandarización del protocolo de ChIP para cada una de las marcas epigenéticas que se desea analizar y la introducción de otro método más sensible para la cuantificación de los productos del ChIP. Hasta el momento el método más poderoso y preciso para el análisis de las muestras de ChIP es la espectrometría de masas (Qiu y Wang, 2009). Para determinar los cambios epigenéticos que suceden a la unión del ATF al promotor, tal vez podría hacerse uso de la técnica de PICh (Proteómica de segmentos de cromatina aislados, por sus siglas en inglés *Proteomics of Isolated Chromatin segments*). La técnica de PICh consiste en aislar un segmento de cromatina mediante una sonda de DNA. Las proteínas asociadas al DNA después se analizan por espectrometría de masas (Rusk, 2009; Déjardin y Kingston, 2009).

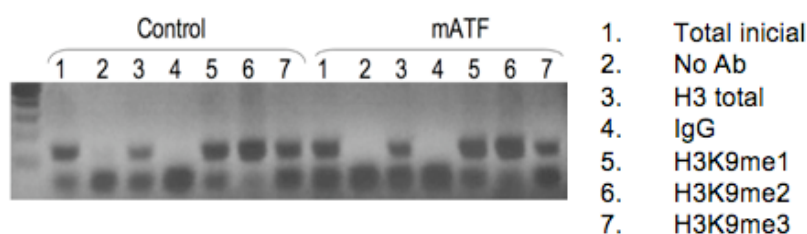


Figura 8. Gel de agarosa mostrando los productos de la PCR de las muestras de ChIP. Las células MOVCAR 5009 fueron transfectadas con el control y el mATF y procesadas mediante ChIP con los anticuerpos que se enlistan. Los productos obtenidos de ChIP se emplearon para la PCR con un oligonucleótido que amplifica una región en el promotor de *Maspin*. H3K9me1: monometilación de H3K9; H3K9me2: dimetilación de H3K9; H3K9me3: trimetilación de H3K9.

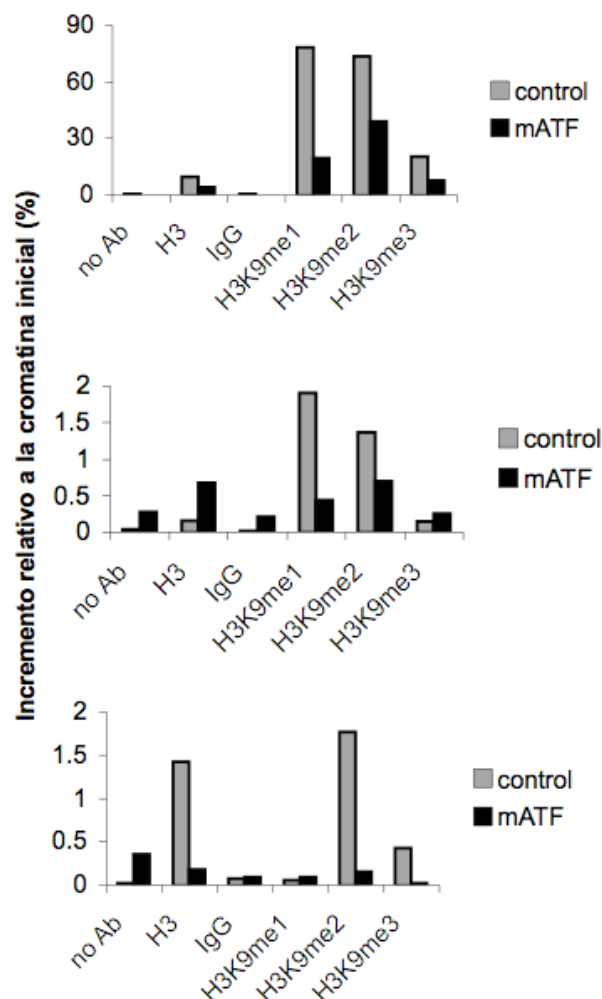


Figura 9. Análisis de las muestras de ChIP mediante PCR en tiempo real de las células MOVCAR 5009 transfectadas con el control (vector vacío) y mATF. Los datos obtenidos del PCR en tiempo real fueron normalizados y analizados como se describe en el Manual de SABiosciences: Análisis de Inmunoprecipitación de la Cromatina mediante PCR cuantitativo en Tiempo Real (*Quantitative Real-Time PCR Analysis of Chromatin Immunoprecipitation*) Versión 1.2 y en el trabajo de Haring *et al.*, 2007. H3K9me1: monometilación de H3K9; H3K9me2: dimetilación de H3K9; H3K9me3: trimetilación de H3K9.

DISCUSIÓN GENERAL.

Los ATF son diseñados para unirse a una secuencia específica de DNA (Beerli y Barbas, 2002). En el presente estudio, el ATF-126 y el mATF se diseñaron para unirse específicamente al promotor de *Maspin* e hipotéticamente sólo deben unirse y activar a dicho gen. Mediante la técnica de ChIP se demostró que el mATF se une físicamente en el promotor de este gen y los microarreglos de DNA demostraron que *Maspin* es el gen que se regula principalmente. Aún se debate el hecho de la especificidad de la unión, puesto que existen otros genes que se regulan tras la transfección con los ATF mencionados. Para demostrar la especificidad de los ATF, será necesario analizar sus sitios de unión a escala genómica mediante la técnica de ChIP-seq (Mardis, 2007).

Aunque el análisis de los microarreglos de DNA de las células transfectadas con el ATF-126 demuestran que *Maspin* es el blanco principal, el mATF muestra una regulación alta de otros genes además de *Maspin* (Lara *et al.*, 2012). Si bien cada módulo de la proteína dedos de zinc se une con alta afinidad a tres nucleótidos, aún existe la posibilidad de su unión a otras secuencias (Mandell y Barbas, 2003). Barbas y colaboradores desarrollaron la biblioteca de dedos de zinc a partir de la cual se diseñaron los ATF empleados en el presente estudio y demostraron que mientras un triplete de DNA es reconocido con gran afinidad por un dedo de zinc, existen otros tripletes a los que el dedo de zinc se puede unir con menor afinidad. El software “Zinc Finger Tools” (disponible en línea <http://www.scripps.edu/mb/barbas/zfdesign/zfdesignhome.php>) fue usado para determinar las secuencias en el promotor de *Maspin* a las que se une cada

dedo de zinc del mATF con menor probabilidad, las secuencias se muestran en la *Tabla IV*.

Tabla IV. Secuencias de DNA a las que el mATF se une hipotéticamente, determinadas mediante el software en línea “Zinc Finger Tools”.

Principal secuencia blanco del mATF: (en negritas, los posibles cambios en especificidad para cada triplete)
5'- GAA/T GAC CTG GGT/C GT/AG GTC -3'
Otras secuencias blanco menos probables:
5'- GAT GAC CTG GGT GTG GTC -3'
5'- GAT GAC CTG GGC GTG GTC -3'
5'- GAT GAC CTG GGT GAG GTC -3'
5'- GAT GAC CTG GGC GAG GTC -3'
5'- GAA GAC CTG GGC GTG GTC -3'
5'- GAA GAC CTG GGT GAG GTC -3'
5'- GAA GAC CTG GGC GAG GTC -3'

Estas secuencias se usaron para realizar alineamientos con el genoma murino (usando la herramienta BLAST de NCBI). El alineamiento de cada una de estas secuencias nos proveyó con una lista de genes en los que esta secuencia pudiera encontrarse. Siendo la secuencia de 18 pb, se buscaron alineamientos con un mínimo de hasta 13 nucleótidos y se determinó manualmente que ninguna de dichas secuencias corresponde a los genes principalmente regulados tras la transducción con el mATF. Ninguno de los genes validados mediante PCR en tiempo real, *Tspan12*, *Cib2*, *Kcnmb4*, *Kctd12*, *Nid1*, *Wnt10a* o *Cebpd* se encontró en estas listas. Debido a lo anterior, se sugiere que la regulación de estos genes se debe a la reactivación de Maspin y no al hecho de que el mATF se esté uniendo a secuencias para las que no fue diseñado (sitios inespecíficos). Un estudio más contundente para demostrar que esta unión es específica sería el ChIP-seq. La prueba de ChIP-seq acopla el método de ChIP a la secuenciación

de los fragmentos obtenidos mediante esta técnica y su alineación con genomas completos para determinar los sitios de unión. Esta técnica nos permitiría dilucidar todos los sitios del genoma a los que el ATF se está uniendo.

Por otro lado, el empleo de los ATF acoplado con los microarreglos de DNA también ha resultado una excelente herramienta en el descubrimiento de funciones de los genes. Los estudios de Beltran y colaboradores (2011) demuestran que tras la reactivación de Maspin mediante cDNA y ATF-126 se reactiva un grupo de 19 genes. Estos genes pudieran estar implicados en el proceso de metástasis y potencialmente son regulados por una cascada de señales que procede a la reactivación de Maspin. Los análisis en las células transfectadas con mATF también evidencian varios genes que se activan tras la transducción con mATF y la reactivación de Maspin. La reactivación excepcionalmente alta de uno de estos genes *Tspan12*, sugería que este gen guardaba una relación estrecha con Maspin o que podría haberse activado de manera inespecífica mediante el mATF. Como mencionamos anteriormente, se verificó mediante el software Zing Finger Tools que el mATF no se une al promotor de *Tspan12*. Esto también pudiera determinarse analizando las muestras de ChIP que se obtuvieron para determinar la unión del mATF en el promotor de *Maspin*. Estas mismas muestras pudieran analizarse mediante PCR con primers específicos para el promotor de *Tspan12*.

Por otro lado, para verificar que la producción de Tspan12 se activa debido a Maspin, se realizaron experimentos para inhibir Maspin en las células transfectadas con el mATF. Se encontró que en estas células una vez inhibido Maspin, Tspan12 se inhibe también. La reactivación de Tspan12 en nuestro modelo de estudio y el hecho de que otras proteínas de la familia de las tetraspaninas están involucradas en el proceso de

metástasis, también plantea la posibilidad de que este gen esté implicado en este proceso, tal como Maspin. Para confirmarlo, se analizaron los ascites de pacientes de cáncer de ovario y encontramos que la expresión de *Tspan12* y Maspin es deficiente en estas muestras (Lara *et al.*, 2012). Más estudios en muestras derivadas de pacientes nos permitirían confirmar esta hipótesis. Es posible que *Tspan12* esté regulado de manera negativa en la progresión del cáncer y particularmente en metástasis. Sería necesario analizar la expresión de este gen en muestras derivadas de otras clases de cáncer, particularmente muestras de tumores secundarios. Los estudios que se realizaron en el presente trabajo sugieren que *Tspan12* es un gen supresor de tumores. Para determinar su función, sería indispensable realizar estudios de inhibición y sobre-expresión genética y definir el fenotipo que se produce en las células.

Además de los cambios genéticos derivados de la transfección con los ATF y la activación de Maspin, se deduce que hay cambios epigenéticos en el promotor, puesto que el activador VP16 recluta elementos modificadores de la cromatina. Sin embargo, el protocolo de ChIP aplicado en los presentes estudios no nos permitió demostrar cambios en las marcas de acetilación y metilación de las histonas en el promotor de Maspin, sin embargo si fue posible detectar cambios en la expresión de los microRNA (Beltran *et al.*, 2011). La reactivación de Maspin mediante cDNA y ATF-126 en la línea celular de cáncer mamario MDA-MB-231 modificó significativamente la expresión de miR-1, miR-34a, miR-10b, miR-363 y miR-124. Actualmente no se sabe la relación exacta que guardan estos microRNA con Maspin. Es sabido que miR-10b es un agente mediador de la metástasis (Ma *et al.*, 2007) y por lo tanto su disminución tras la activación de Maspin concuerda con la función anti-metastásica reportada para esta proteína. Por otro lado, se

ha reportado el incremento de la expresión de miR-363 en líneas celulares de cáncer de cabeza y cuello (Wald *et al.*, 2011) y muestras de pacientes de cáncer faríngeo (Lajer *et al.*, 2011), mientras que en las células MDA-MB-231, este microRNA se reduce tras la reactivación de Maspin. Estos resultados y los estudios previos sugieren que miR10b y miR-363 funcionan como oncogenes.

Así mismo, el estudio de la activación de Maspin y la concomitante activación de miR-34a en este modelo, soportan la función de este microRNA como supresor de tumores. miR-34a está usualmente regulado negativamente en cáncer y se relaciona con un mejor pronóstico cuando se encuentra expresado en los tumores pancreáticos de pacientes (Chang *et al.*, 2007). miR-34a es directamente transactivado por el supresor de tumores p53 y su expresión promueve la apoptosis (Yamakuchi y Lowenstein, 2009).

No existen reportes de la función de miR-1 en cáncer, sin embargo, se conoce que en células musculares, miR-1 provoca la inhibición de KLF-4 (Xie *et al.*, 2011). KLF-4 por su parte, es un factor de transcripción que coopera en la des-diferenciación celular (Takahashi y Yamanaka, 2006). Existen teorías que relacionan a la des-diferenciación celular con el origen de las células cancerosas y bajo esta premisa pudiera sugerirse que miR-1 funciona como un supresor de tumores. En nuestro sistema de estudio, la expresión de este microRNA se incrementa posteriormente a la reactivación de Maspin.

Los resultados de la regulación negativa de miR-124 mediante la activación de Maspin, son contradictorios a los estudios existentes respecto a este microRNA. Básicamente los estudios previos lo califican como un gen supresor de tumores. miR-124 se encuentra regulado de manera negativa en algunos tipos de cáncer, como hígado y piel (Furuta *et al.*, 2010; Yamane *et al.*, 2012), mientras que su expresión inhibe la

proliferación en células de glioblastoma multiforme y cáncer gástrico (Silber *et al.*, 2008; Xia *et al.*, 2012).

Previo a este estudio, no existía ningún reporte que relacionara la expresión de estos microRNA con Maspin. Serán necesario más estudios para determinar cuáles son los genes que estos microRNA están silenciando y cuáles son las cascadas de señalización en que participan, así como la relación directa que estos eventos guardan con el gen supresor de tumores *Maspin*.

Por último, es importante destacar que los cambios genéticos y epigenéticos que los ATF y la activación de Maspin producen en las células conducen a un fenotipo menos agresivo tanto *in vitro* como *in vivo*. También es de particular importancia el hecho de que los ATF estén diseñados para reactivar o suprimir genes de manera específica contrastando con el efecto global de las terapias actuales para el tratamiento del cáncer. Además, los ATF empacados en nanopartículas son aún más específicos, puesto que es sabido que estas se dirigen particularmente a los tejidos tumorales debido al efecto de incremento de la permeabilidad y retención (*EPR effect*, por sus siglas en inglés *Enhanced Permeability and Retention*) (Fang *et al.*, 2011).

CONCLUSIONES.

En el presente trabajo se hizo uso de las proteínas dedos de zinc para crear ATF capaces de unirse al promotor y reactivar a Maspin con selectividad. Las pruebas de ChIP demuestran que el mATF se une al promotor de *Maspin* en las células MOVCAR 5009. El ATF-126 y el mATF activan a Maspin en todos los modelos de cáncer de mama y ovario empleados en estos estudios. Los resultados de los microarreglos de DNA y PCR en tiempo real demostraron que el gen *Maspin* se regula a un mayor nivel en comparación con otros genes. Es posible que la regulación de los otros genes no se derive propiamente de los ATF, sino de cascadas de señalización originadas por la activación de Maspin. Además, la activación de Maspin no solo provoca cambios genéticos en las células, sino también epigenéticos, particularmente cambios en la expresión de los microRNA.

Independientemente del método de transfección, sea esta mediante retrovirus o nanopartículas, los ATF y la activación de Maspin provocan un fenotipo menos tumoral y metastásico en los modelos empleados en este estudio. Este estudio demuestra que los ATF son una herramienta útil en la formulación de nuevas terapias para el tratamiento del cáncer y su empaquetamiento en nanopartículas sienta las bases para su uso en futuras aplicaciones clínicas.

RECOMENDACIONES GENERALES.

1. Continuar los estudios de los cambios epigenéticos que los ATF inducen en los promotores de los genes.
2. Estudiar la función de Tspan12 y su relación con los procesos de carcinogénesis y metástasis.
3. Demostrar que los ATF se unen únicamente al sitio del genoma para el que fueron diseñados.

Para continuar los estudios de los cambios epigenéticos, es indispensable estandarizar la técnica de ChIP para cada una de las marcas que desea estudiarse. También es necesario encontrar una técnica más precisa para analizar las muestras que se obtengan, como la espectrometría de masas. También mencionamos que es posible aplicar otra técnica para este estudio como el PICH, que permitiría analizar todas las marcas a partir de una sola muestra obtenida con una sonda de DNA.

Para estudiar Tspan12, se requiere de la inhibición y la sobre-expresión de este gen. Los estudios de inhibición pueden llevarse a cabo mediante el silenciamiento de Tspan12 con siRNA o shRNA *in vitro*, y también con knock-down en modelos murinos. Los estudios de sobre-expresión generalmente se hacen introduciendo el cDNA que codifica para la proteína, sin embargo, si resultara que Tspan12 está silenciado por mecanismos epigenéticos, sería posible reactivarlo con fármacos o con un ATF.

Por último, los experimentos de ChIP-seq demostrarán la unión exclusiva de los ATF a sus respectivos promotores.

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Suppression of Breast Tumor Growth and Metastasis by an Engineered Transcription Factor

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Abstract

Maspin is a tumor and metastasis suppressor playing an essential role as gatekeeper of tumor progression. It is highly expressed in epithelial cells but is silenced in the onset of metastatic disease by epigenetic mechanisms. Reprogramming of *Maspin* epigenetic silencing offers a therapeutic potential to lock metastatic progression. Herein we have investigated the ability of the Artificial Transcription Factor 126 (ATF-126) designed to upregulate the *Maspin* promoter to inhibit tumor progression in pre-established breast tumors in immunodeficient mice. ATF-126 was transduced in the aggressive, mesenchymal-like and triple negative breast cancer line, MDA-MB-231. Induction of ATF expression *in vivo* by Doxycycline resulted in 50% reduction in tumor growth and totally abolished tumor cell colonization. Genome-wide transcriptional profiles of ATF-induced cells revealed a gene signature that was found over-represented in estrogen receptor positive (ER+) "Normal-like" intrinsic subtype of breast cancer and in poorly aggressive, ER+ luminal A breast cancer cell lines. The comparison transcriptional profiles of ATF-126 and *Maspin* cDNA defined an overlapping 19-gene signature, comprising novel targets downstream the *Maspin* signaling cascade. Our data suggest that *Maspin* up-regulates downstream tumor and metastasis suppressor genes that are silenced in breast cancers, and are normally expressed in the neural system, including *CARNS1*, *SLC8A2* and *DACT3*. In addition, ATF-126 and *Maspin* cDNA induction led to the re-activation of tumor suppressive miRNAs also expressed in neural cells, such as miR-1 and miR-34, and to the down-regulation of potential oncogenic miRNAs, such as miR-10b, miR-124, and miR-363. As expected from its over-representation in ER+ tumors, the ATF-126-gene signature predicted favorable prognosis for breast cancer patients. Our results describe for the first time an ATF able to reduce tumor growth and metastatic colonization by epigenetic reactivation of a dormant, normal-like, and more differentiated gene program.

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Introduction

Mammary Serine Protease Inhibitor (Maspin, SERPINB5) is a multifunctional protein possessing tumor and metastasis suppressive functions [1,2]. Additionally, *Maspin* over-expression inhibits *in vivo* angiogenesis [3]. The multifaceted nature of *Maspin* affecting many molecular mechanisms during neoplastic disease progression makes it a very attractive target in cancer biology. Importantly, clinical data shows that high *Maspin* levels are associated with better prognosis in breast, lung and prostate carcinomas [4,5,6].

As a class II tumor suppressor gene, *Maspin* is not mutated, rearranged or deleted in tumor cells. Instead, its expression is regulated by means of transcription factors [7] and epigenetic modifiers [8,9]. While *Maspin* is expressed at high levels by epithelial cells, it is down-regulated in mesenchymal cells, such as stromal fibroblasts. In breast cancer cell lines and cancer specimens, silencing of *Maspin* correlates with acquisition of invasive and metastatic behavior. Epigenetic mechanisms controlling *Maspin* silencing include both, DNA [9] and H3K9 histone

methylation [10]. Hence epigenetic mechanisms are reversible yet inherited during cell division, blockade of *Maspin* promoter silencing offers a potent strategy to reactivate tumor suppressor function. To this end, we have previously described the construction of Artificial Transcription Factors (ATFs) made of sequence-specific six Zinc Finger (ZF) domains [11] designed to bind unique 18-base pair recognition sites in the *Maspin* proximal promoter [12]. The ZFs were linked to a VP64 transactivator domain, which mediates a strong promoter up-regulation by recruitment of the *polIII* transcriptional complex. In cell systems, both in lung and breast cancer cell lines, retroviral transduction of one of the ATFs, ATF-126, led to a potent induction of apoptosis and inhibition of cell invasion [12,13]. Furthermore, these ATFs were able to directionally demethylate the *Maspin* promoter and this effect depended on upon the orientation of the ATF along the DNA [13]. Consistently, we found that ATFs synergized with both methyltransferase and histone deacetylase inhibitors to reactivate silenced *Maspin* [13,14,15].

These previous observations suggested that ATF-126 was able to partially reprogram or revert the epigenetic state of the *Maspin* promoter, resulting in a re-activation of the endogenous gene. However, the impact of ATF-126 in inhibiting tumor progression in preexisting tumors and/or metastases *in vivo* has never been addressed. Herein, we have taken advantage of an inducible viral vector system to control the expression of ATF-126 in pre-existing breast tumor growths and experimental metastases in immunodeficient mice. Chemical induction of ATF-126 *in vivo* resulted in tumor suppression as well as in inhibition of breast tumor cell colonization. Furthermore, genome-wide DNA microarrays of MDA-MB-231 cells induced with ATF-126 revealed that breast tumor cells acquired a 550-gene signature that was found over-represented in estrogen receptor positive (ER+) breast cancer cell lines and in the normal-like intrinsic subtype of breast cancer. Our data indicates that ATF-126 up-regulates novel *Maspin*-dependent targets possessing tumor and metastasis suppressive functions, which are found epigenetically silenced in aggressive tumors. Our results outline a possible mechanism by which ATF-126 reprograms aggressive tumor cells towards a more “normal-like”, more benign, and more differentiated “epithelial-like” phenotype.

Results

Induction of ATF-126 by DOX results in endogenous reactivation of *Maspin* in MDA-MB-231 breast cancer cells

In order to monitor the effect of ATF-126 in inhibiting tumor progression in pre-existing tumors, we cloned the ATF-126 gene into an inducible TetOn retroviral vector (**Fig. S1A**). In this expression system the ATF expression was activated only in presence of the chemical inducer, Doxycycline (DOX). The MDA-MB-231-LUC cell line stably engineered with a luciferase (LUC) gene was transduced with either a control (empty retroviral vector) or the same vector expressing ATF-126. The LUC gene allowed the non-invasive monitoring of tumor growth and dissemination in a mouse model, using bioluminescence imaging, BLI (**Fig. S1B**).

The effects of inducing ATF-126 with DOX were first monitored in cell culture assays. As shown in **Fig. 1A**, induction of the full length ATF-126 (comprising the specific 6ZF DNA-binding domains and the VP64 transactivator domain) resulted in a dose-dependent ATF-126 expression, as assessed by qRT-PCR. The induction of the ATF was accompanied by a concomitant up-regulation of the *Maspin* target (**Fig. 1B**). For subsequent studies we used a concentration of DOX of 100 ng/ml at which the expression of both, ATF-126 and *Maspin*, reached saturation.

Efficient ATF-126 induction was also verified by immunoprecipitation (using an anti-HA antibody recognizing the C-terminal HA epitope of the ATF; **Fig. 1C**, left panel). *Maspin* up-regulation in +DOX cells was also verified by immunoprecipitation (**Fig. 1C**, right panel). As shown in **Fig. 1D**, ATF-126 +DOX cells up-regulated *Maspin* quickly after ATF-126 expression (6–12 hours after addition of DOX), as expected from direct transcriptional regulation. A saturation of ATF expression was reached 48 hours after the addition of the drug, whereas that *Maspin* up-regulation achieved a maximum at 72 hours post-induction (**Fig. 1D**). Removal of DOX from the cell culture of ATF-126 cells also resulted in a decay of ATF expression that was evident 24 hours upon the retrieval of the drug. However, 72 hours after DOX removal, substantial *Maspin* expression was still detected in the cells even though ATF-126 mRNA expression was not. These results suggested that ATF-126 was able to transiently impact the epigenetic and transcriptional status of the *Maspin* promoter. Previously we have reported that ATF-126 was able to demethylate the *Maspin* proximal promoter [13]. Moreover, It is

possible that the endogenous mechanisms responsible for *Maspin* silencing (including endogenous DNA-methylation processes) would be restored 96 hours after removal of DOX in ATF-126 transduced cells.

ATF up-regulation by DOX results in *Maspin*-dependent induction of apoptosis

Endogenous expression of tumor suppressors, including *Maspin*, has been associated with reduction of tumor cell viability by induction of apoptosis [12,16]. Next, we studied if ATF-126 induction resulted in a reduction of tumor cell growth. As shown in **Fig. 2A**, the reactivation of ATF-126 (ATF-126 +DOX) led to 70% reduction in tumor cell viability relative to the same cells in absence of DOX (ATF-126 –DOX). As expected, no difference in cell viability was observed between CONTROL and ZF-126 (inactive ATF-126 lacking the activator domain) cells upon DOX induction. Interestingly, removal of DOX in ATF-126 cells led to a maintenance of the cell proliferation defect for 96 hrs after DOX removal (**Fig. 2B**), and approximately over four cell generations. This maintenance of tumor suppression correlates with the time window of *Maspin* transcriptional activation of **Fig. 1E**. In addition to cell proliferation defects, ATF-126 +DOX cells exhibited a 40% induction of apoptosis, as assessed by Annexin-V staining, which monitors early apoptosis (**Fig. 2C**). Similarly, 30–40% of ATF-126 +DOX cells were positive for the Hoechst staining, which labels apoptotic nuclei (**Fig. 2D**).

To verify that the apoptotic response triggered by ATF-126 was due to *Maspin* reactivation, we challenged ATF-126 cells with either a *Maspin*-specific or scramble shRNA constructs. The *Maspin*-shRNA in ATF-126 +DOX cells led to a 60% reduction of *Maspin* mRNA expression (**Fig. 2E**). As expected, a scramble shRNA construct did not significantly impact *Maspin* expression. Consistently, the scramble-shRNA in ATF-126 +DOX cells induced similar levels of cell death relative to the original ATF-126 +DOX cell line (**Fig. 2F**). However, the *Maspin*-shRNA in ATF-126 +DOX cells was able to rescue the phenotype, and a significant reduction of cell death (55% of reduction) was observed in the *Maspin* knock-down relative to the scramble and the ATF-126 parental line +DOX (**Fig. 2F**). Although the rescue of the cell death phenotype was not 100%, our results suggest that ATF-126 mediate reduction in tumor cell growth by primarily activation of its designed target *Maspin*. The incomplete rescue of the phenotype could be due the fact that the *Maspin* shRNA did not completely knocked down the mRNA transcript levels of *Maspin*, as shown in **Fig. 2E**. A siRNA approach further confirmed that the tumor suppressive phenotype observed in ATF-126 +DOX cells was due to *Maspin* re-activation. As shown in **Figs. 2G–H**, a *Maspin*-specific siRNA but not a non-specific mismatch (scramble) siRNA rescued the proliferation defect of ATF-126 +DOX cells. Overall these results support the conclusion that the cell death phenotype induced by ATF-126 was dependent on the *Maspin* target.

ATF-126 reduces xenograft tumor growth and suppresses metastatic colonization of MDA-MB-231 cells upon DOX induction in immunodeficient mice

To investigate the ability of ATF-126 to reduce tumor cell growth in pre-existing breast tumor xenografts, we implanted either control 1×10^6 MDA-MB-231-LUC cells or ATF-126 cells in SCID mice (N=8 animals per group). Animals were kept in DOX-free conditions until tumors reached 100–200 mm³ (**Fig. 3A**). Eighteen days post-induction, half of the animals for control and ATF-126 groups were maintained in DOX-free diet, whereas the other half was switched to a +DOX diet. Tumor

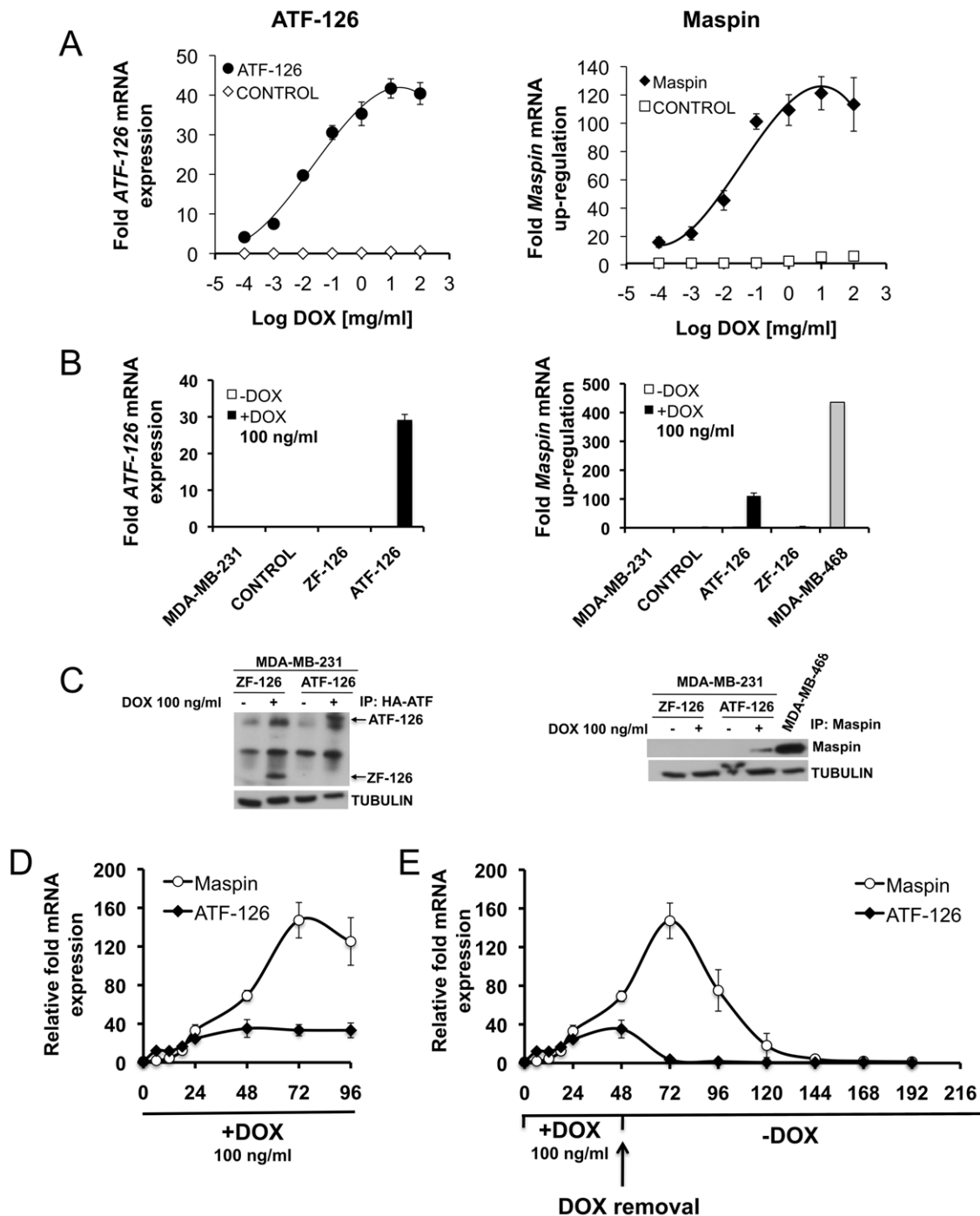


Figure 1. Induction of ATF-126 by DOX results in reactivation of the target gene *Maspin*. **A.** Dose-response plot monitoring *ATF-126* (left panel) and *Maspin* (right panel) mRNA levels upon treatment with increasing concentrations of DOX. CONTROL and ATF-126 cells were treated for 72 hours and mRNA was measured by quantitative real-time PCR (qRT-PCR). **B–C.** *ATF-126* and *Maspin* mRNA expression levels by qRT-PCR (**B**) and western blot (**C**) induced with 100 ng/ml of DOX. MDA-MB-468 is a poorly aggressive ER- breast cancer cell line expressing endogenous *Maspin* as a reference control [12]. **D.** Time course kinetics of *ATF-126* and *Maspin* mRNA levels by qRT-PCR upon DOX treatment. ATF-126 cells were induced with DOX and collected at 0, 6, 12, 18, 24, 48, 72 and 96 hours. **E.** Time course kinetics of *ATF-126* and *Maspin* expression levels by qRT-PCR upon DOX treatment and removal. ATF-126 cells were induced with DOX for 48 hours, then DOX was removed from the media and cells were maintained in DOX-free media for an additional 168 hours. Gene expression levels were normalized to the –DOX cells. Data represents the mean $\pm$ SD of three independent biological replicates. MDA-MB-231-LUC are un-transduced cells; CONTROL, cells transduced with an empty vector; ATF-126, a full length ATF containing the 6 ZF DNA-binding domains and VP64 activator domain; ZF-126, a truncated or inactive ATF lacking the VP64 activator domain.

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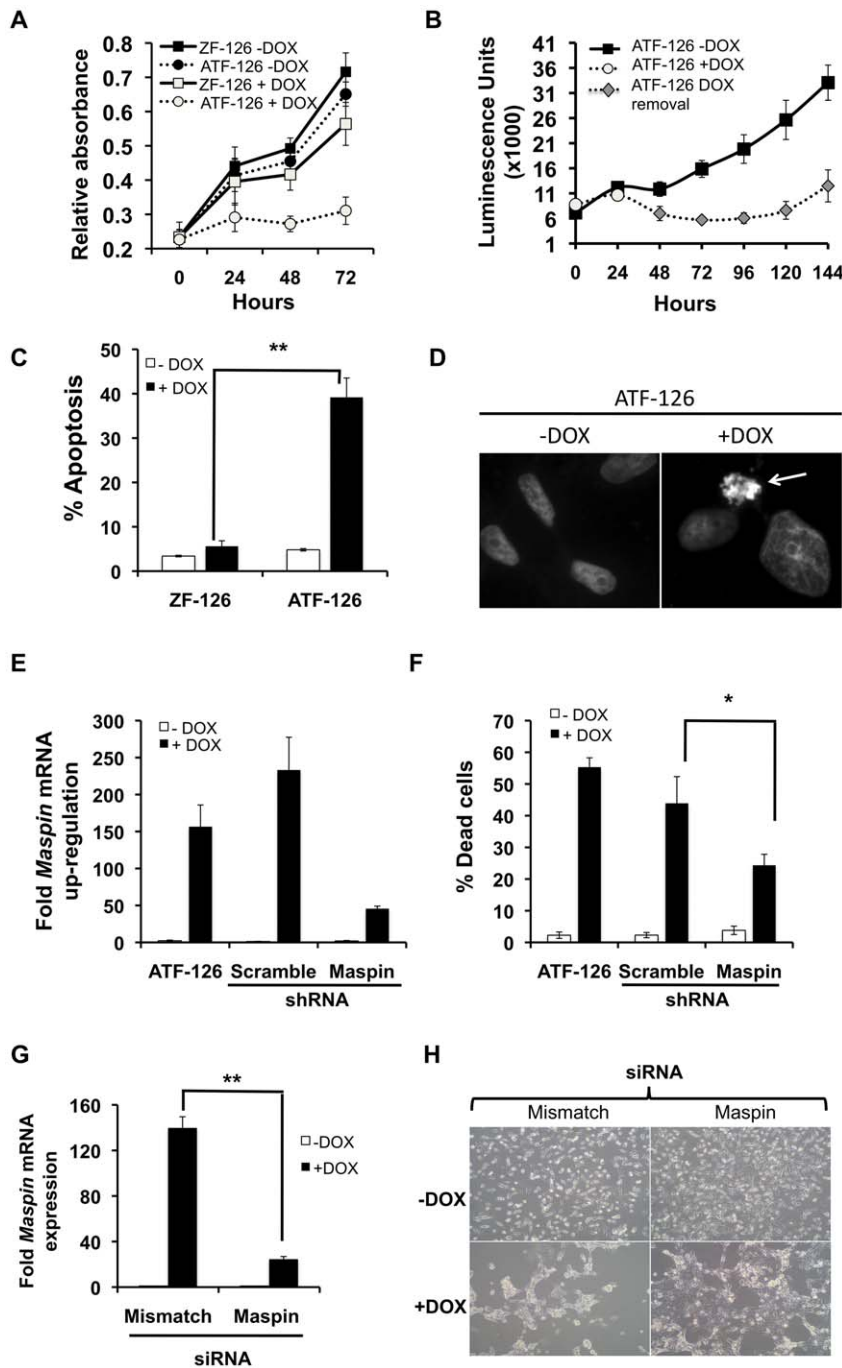


Figure 2. ATF-126 induced apoptosis upon DOX treatment. **A.** Cell viability plot of ATF-126 and ZF-126 cells treated with vehicle (–DOX) or DOX (+DOX) for 72 hours. ATF-126 refers to a full length, active ATF and ZF-126 is a truncated, transcriptionally inactive construct lacking the VP64 activator domain. **B.** Cell viability plot of ATF-126 transduced cells after DOX removal. Cells were induced with DOX for 48 hours (open circles). DOX was removed from the media and cells were kept in DOX-free media for an additional period of 96 hours. Viability of ATF-126 cells growing in absence of DOX (–DOX, filled squares) was plotted as reference control. Cell viability was measured with an XTT assay [12]. **C.** ATF-126 induced apoptosis upon DOX treatment. Either ATF-126 or ZF-126 cells were kept in vehicle-treated media (–DOX) or DOX (+DOX) and collected at 72 hours after induction. The percentage of apoptosis was quantitatively analyzed using an Annexin-V staining [12]. **D.** Hoechst staining of ATF-126 –DOX and +DOX cells at 72 hours post-induction. Arrow points to a positive apoptotic cell, showing nuclear condensation. **E.** Expression of *Maspin* by qRT-PCR in ATF-126 cells retrovirally transduced with either a scramble or with a *Maspin*-specific shRNA construct. **F.** Percentage of cell death by trypan-blue exclusion assay in ATF-126 cells transduced with either a scramble or a *Maspin*-specific shRNA. **G.** *Maspin* expression as assessed by qRT-PCR in ATF-126 cells transfected with either a mismatch or a *Maspin*-specific siRNA, and treated with vehicle (–DOX) or DOX (+DOX). **H.** Representative pictures of the *Maspin* siRNA knock-down experiments of cells treated with vehicle (–DOX) or DOX (+DOX). Bar graphs in C, E, F and G represent the average of 3 independent experiments. Differences between samples were calculated with a student t test with level of significance * $p \leq 0.05$ and ** $p \leq 0.01$. doi:10.1371/journal.pone.0024595.g002

volume was assessed from the day before induction until tumor collection (day 41 post-injection), and BLI imaging was performed once a week. One of the animals belonging to the ATF-126 group previously induced with DOX was switched back to a DOX-free diet and sacrificed at day 53 post-injection (**Fig. 3A**). As shown in **Fig. 3B–C** (left panels), no statistical differences in tumor growth were observed between CONTROL +DOX and -DOX animals. In contrast, ATF-126 animals induced with DOX exhibited a significant reduction of tumor volume (approximately 50% reduction) that was stably maintained until the mice were sacrificed at day 41 (**Fig. 3B–C**, right panels).

Interestingly, induced ATF-126 animals that were removed from DOX at day 41 experienced a tumor relapse (**Fig. 3D**). This tumor recovery suggests that, like we have observed *in vitro*, long-term absence of ATF expression results in a re-establishment of *Maspin* silencing. Analysis of tumors recovered from the animals at day 41 demonstrated that the ATF-126 +DOX animals retained efficient ATF mRNA up-regulation (500-fold relative to un-induced animals). This induction of ATF mRNA was accompanied by a 20-fold *Maspin* up-regulation relative to -DOX animals (**Fig. 3E**). Overall these results demonstrate that ATF-126 was properly up-regulated in the tumor xenograft experiment and that this induction correlated with *Maspin* reactivation and with the maintenance of tumor suppressive functions.

It is well documented for breast, prostate and lung carcinomas that high *Maspin* expression correlates with a less aggressive or metastatic behavior [17,18,19]. We next investigated the ability of ATF-126 to inhibit breast tumor colonization or experimental metastasis formation in immunodeficient mice. To address this, either 1×10^5 MDA-MB-231-LUC CONTROL or ATF-126 cells were injected tail vein in SCID mice (N = 24). Twelve mice per group were maintained in DOX-free diet and 12 in +DOX diet. Because the need of the animals to adapt to a DOX diet, mice were feed three days prior to the injection (**Fig. 4A**). **Fig. 4B** (top panel) shows that CONTROL +DOX and -DOX cells effectively colonized the lungs and metastases were evident at day 14 post-injection. Similarly, the majority of the animals injected with ATF-126 maintained in DOX-free diet effectively colonized the lungs with similar signal intensities than CONTROLS. In contrast, ATF-126 animals in +DOX diet completely suppressed experimental metastatic colonization at day 21 (**Fig. 4B**, bottom panel). Moreover, removal of DOX from the diet of ATF-126 animals maintained these mice free of any detectable lung colonization even 54 days post-injection (**Fig. 4B**, bottom right panel). Overall these results indicated that ATF-126 effectively suppressed metastatic colonization. Whether the ATF-126 is equally effective in suppressing or reducing secondary tumor growth in well-established macrometastases will require further investigation.

ATF-126 up-regulate a gene signature over-represented in the normal-like intrinsic subtype of breast cancer and ER+ cancer cell lines

The above results demonstrated that ATF-126 reduced tumor growth and suppressed metastatic colonization of the MDA-MB-231-LUC line. We next began the investigation of potential mechanisms by which ATF-126 could mediate its suppressive functions by performing genome-wide microarray analyses. CONTROL and ATF-126 cells were induced with DOX for 72 hours. Genes differentially regulated between -DOX and +DOX groups were determined by SAM analyses, with three independent arrays performed for each group. These analyses generated a robust 550-gene signature, defined as group of genes differentially up-regulated by ATF-126 (see **Table S1**). A Gene

Ontology (GO) analysis revealed that multiple *Maspin* downstream pathways were reactivated by ATF-126, including tight junction and cell invasion, TGF-beta, and p53 signaling (**Table S2**).

We next examined this signature across intrinsic breast cancer subtypes, using the UNC337 tumor database comprising 337 breast tumor cases [20]. Our analysis shows that ATF-126 up-regulated targets that are found over-represented in the “Normal-like” intrinsic subtype of breast cancer (**Fig. 5A**). Both Normal-like and Luminal A are ER+ tumors associated with the best prognosis of all breast tumor subtypes [21]. In contrast, the Basal-like and Claudin-low carcinomas are mostly triple negative breast cancers (ER-PR-Her2-) associated with high resistance to chemotherapy and poor prognosis [20]. Claudin-low tumors have been recently discovered through large-scale microarray analysis of breast cancer specimens [20,22]. The MDA-MB-231 cell line used in this study was originally described as Basal B and has been recently characterized as Claudin-low [20,21]. Our finding that ATF-126-responsive genes are enriched in ER+ “Normal-like”, poorly aggressive tumors, correlates with the fact that ATF-126 induction in MDA-MB-231 confers a more benign, less tumorigenic phenotype. Consistent with this, we found that the ATF-126-up-regulated gene signature was indicative or a predictor of a better prognosis in breast cancer patients (**Fig. 5B**).

The relationship between the ATF-126-gene signature and ER status was further observed in available DNA-microarrays data of breast cancer cell lines [21]. **Fig. 5C** shows that the ATF-126-gene signature was found under-represented in Claudin-low and Basal-like ER- lines with the lowest enrichment found in the original MDA-MB-231 cell line. The same signature was over-represented in luminal ER+ cell lines, including the poorly aggressive lines MCF-7 and ZR75. Overall our microarray analysis provides support that ATF-126 initiates a transcriptional gene program resulting in a reprogramming of the original ER-aggressive Claudin-low MDA-MB-231 towards a less aggressive, and more “normal-like” breast cancer cell line. This is also consistent with the fact that high expression of *Maspin*, the primary target gene of ATF-126, is also associated with epithelial-like features [18].

To dissect “bona-fide” downstream targets of *Maspin* in the 550-gene signature we cloned the *Maspin* cDNA into the same DOX-inducible retroviral vector, and gene expression microarrays were performed in the *Maspin* cDNA -DOX and +DOX transduced cell populations. 123 genes (**Table S3**) were found differentially up-regulated upon *Maspin* cDNA induction. Among these, 19 targets were shared with ATF-126 +DOX (**Fig. 6A**, **Table 1**). Nine of the most up-regulated *Maspin*-dependent candidate targets were further validated by qRT-PCR (**Fig. 6B**). These genes included potential therapeutic targets normally expressed in the neural system, such as negative regulators of oncogenic signaling (the epigenetic regulator of Wnt/ β -catenin signaling (*DACT3*) and the SRC kinase signaling inhibitor 1 (*SRCIN1*)), putative tumor suppressors in cancer (*DACT3*, *SLC8A2*, *CARNS1*, *GNG4*, *END2*), and the apoptosis-associated tyrosine kinase *ATTK*. *Solute carrier family 8 (sodium/calcium exchanger), member 2 (SLC8A2)*, *Carnosine synthase 1 (CARNS1)*, and *Dapper, antagonist of beta-catenin, homolog 3 (DACT3)* were up-regulated by more than 10-fold in both, ATF-126 and *Maspin* cDNA cells, and thus, could represent novel “bona-fide” *Maspin*-dependent targets.

We next took advantage of ATF-126 and *Maspin* cDNA inducible cell lines to examine whether oncogenic and tumor suppressive microRNAs were differentially regulated upon DOX induction. We analyzed the expression of 90 miRNAs often dys-regulated in cancers, using a miRNA array platform by qRT-PCR. The expression of each miRNA was normalized to the

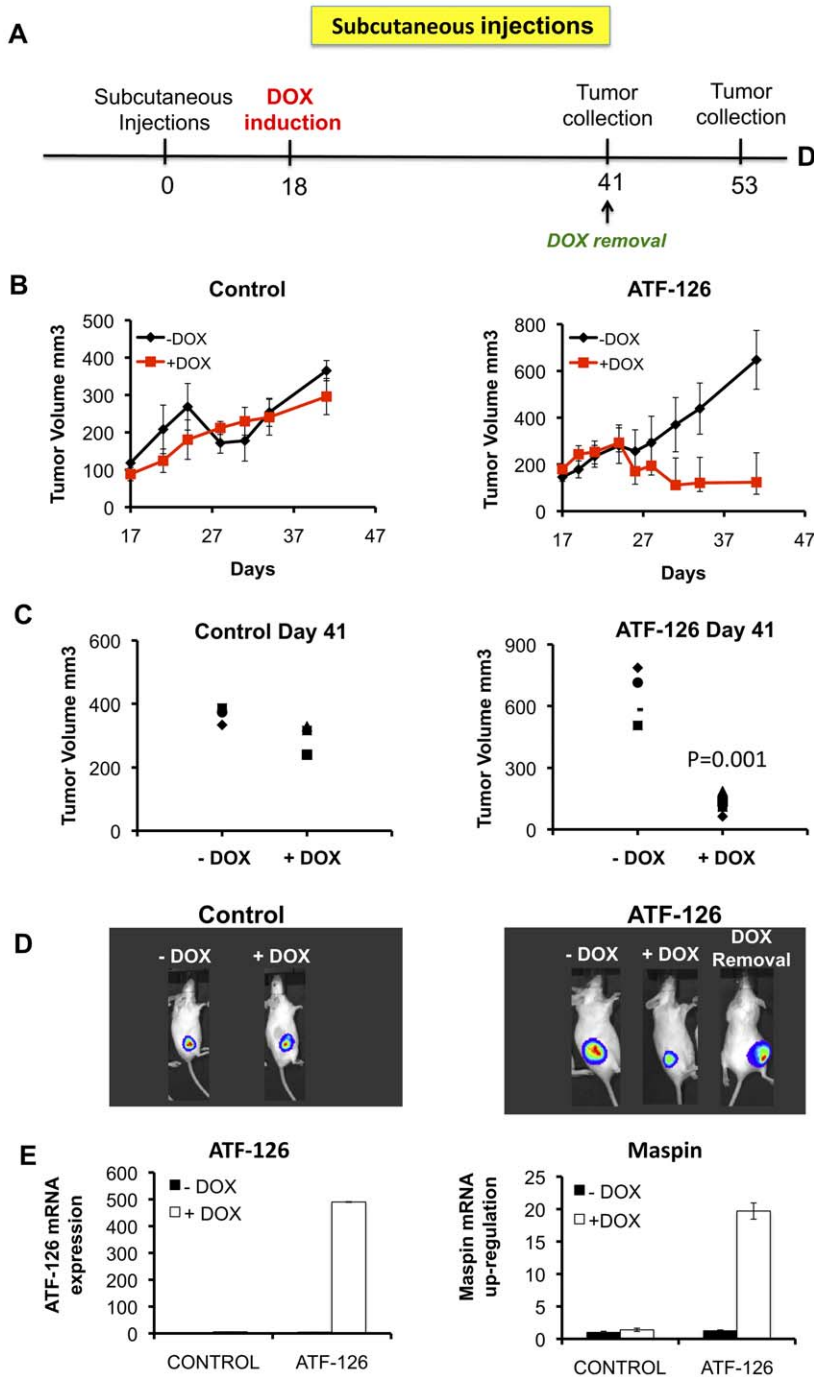


Figure 3. ATF-126 induced tumor suppression in SCID mice. **A.** Time-line of the experiments involving subcutaneous tumor injections illustrating the time of injection of tumor cells, induction and removal of DOX, and tumor collection. **B.** Time course plots monitoring tumor volumes of CONTROL and ATF-126 groups, N = 8 animals per group. Four animals per group were maintained in DOX-free diet (–DOX), whereas the other half was treated with DOX (+DOX). The tumor growth was measured by caliper the day before induction (day 17) until tumor collection (day 41). **C.** Tumor volume measurements at the day of tumor collection for both CONTROL (left panel) and ATF-126 animals (right panel). Differences in tumor growth were calculated with a student t test (**p = 0.001). **D.** Representative bioluminescence images comparing signal intensities of luciferase photon counts from the subcutaneous growths at day 41 for CONTROL (left) and ATF-126 animals (right), treated in absence (–DOX) and presence (+DOX) of DOX. “DOX removal” indicates an ATF-126-injected animal previously induced with DOX, subsequently removed from DOX (day 41), and imaged at day 53 (right). **E.** ATF-126 and *Maspin* expression levels by qRT-PCR in tumor samples collected at day 41 from both, CONTROL and ATF-126 animals. doi:10.1371/journal.pone.0024595.g003

MDA-MB-231 cell line. Interestingly, both, ATF-126 and *Maspin* cDNA, up-regulated miRNAs with potential tumor suppressive functions, such as miR-1 [23,24] and miR-34 [25], while down-regulating oncogenes and metastasis promoters, including miR-

10b [26] (**Fig. 6C**). In addition, we found that miR-124 which is required for somatic cells to reprogram to neural cells [27], and miR-363 (**Fig. 6C**), were down-regulated in both ATF-126 and *Maspin* cDNA. Thus, our target analysis suggests that ATF-126

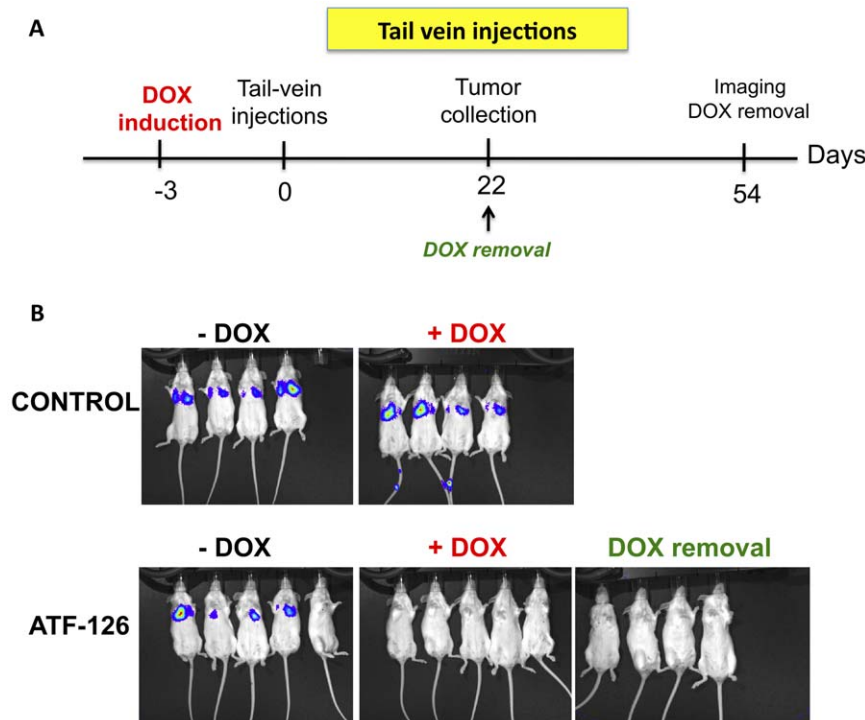


Figure 4. ATF-126 inhibits breast tumor cell colonization in the lungs. A. Time-line of the tail-vein injections experiments, indicating the times of the DOX induction and removal, and tumor collection. **B.** Bioluminescence images of CONTROL and ATF-126 mice groups maintained in DOX-free (–DOX, left panel) and DOX containing diet (+DOX, right panel). Mice were injected via tail-vein with either CONTROL or ATF-126 cells and imaged every week to assess lung colonization. Images shown were taken at day 22 after injection of the tumor cells. DOX removal indicates ATF-126 animals previously induced by DOX and next placed in DOX-free diet. doi:10.1371/journal.pone.0024595.g004

activates downstream targets of *Maspin* resulting in an up-regulation of potential tumor suppressors, and down-regulation of oncogenic and pro-metastatic pathways.

Claudin-low carcinomas and representative cell lines are characterized by a down-regulation of epithelial junction proteins, such as cadherins and claudins [20]. Interestingly, multiple panels of epithelial markers, such as E-Cadherin (*CDH1*), Claudin 3 and 7, Occludins, and keratins, were re-activated upon ATF-126 expression (Fig. 7A). In addition, ATF-126 led to the generation of a CD24 positive population (Fig. 7B). CD24 is expressed in many ER+ tumor cell lines, while its expression is absent in some basal and Claudin-low cell lines (Fig. S2). CD44+/CD24– is considered a cancer stem cell or tumor initiating cell signature, and high CD44/CD24 ratios are characteristic of aggressive Claudin-low tumors and cell lines [20]. The reactivation of CD24 by ATF-126 suggests that ATF-126 could decrease the tumorigenic potential of MDA-MB-231 cells. In summary, the above results suggested that ATF-126 was able to initiate a transcriptional program resulting in a reprogramming of a more mesenchymal, Claudin-low phenotype, towards a more normal-like, epithelial-like, and less aggressive breast tumor line (Fig. 7C).

Discussion

In this paper we have investigated the ability of the ATF-126, designed to up-regulate *Maspin*, to decrease tumor growth and colonization of an aggressive MDA-MB-231 line. We found that induction of ATF expression *in vivo* resulted in 50% reduction in tumor growth. In addition, ATF expression abolished the capability of the breast cancer to colonize the lungs.

The approach used in this work facilitated the tight control of the ATF expression both in cell culture and in a breast cancer xenograft model. As a direct target of the ATF, we found that the *Maspin* transcript was induced with very similar time course kinetics than the ATF. Interestingly, removal of DOX from the cell culture media resulted in a quick decay of ATF-126 expression. However, we found that *Maspin* expression was still retained for at least four cell generations even in complete absence of ATF-126 mRNA expression. In the same time window of *Maspin* transcriptional activation, the ATF-126 +DOX cells maintained their proliferation defects, suggesting that the growth inhibition phenotype could be propagated for several cell generations upon DOX removal. The slow decay of *Maspin* expression and the maintenance of growth inhibition in absence of ATF expression could be the result of the modification of the epigenetic status of the *Maspin* promoter upon binding of the ATF. We have reported that two ATFs, ATF-126 and ATF-97, reduced DNA methylation levels in the *Maspin* promoter [13]. In addition, this demethylation effect was directional and depended upon de orientation of VP64 along the promoter. In this regard, it is possible that the slow *Maspin* decay in absence of ATF expression could reflect a time delay by which the endogenous epigenetic and/or transcriptional mechanisms restore *Maspin* silencing. To address this possibility, we are presently analyzing methylation patterns upon removal of DOX at different time points. Future engineering of ATFs should maximize this demethylation effect, thereby increasing the potency and therapeutic window of ATFs targeting tumor suppressor gene promoters. This unique engineering aspect of ATFs could facilitate the long-term, hereditary, and stable transmission of tumor suppression (“phenotypic

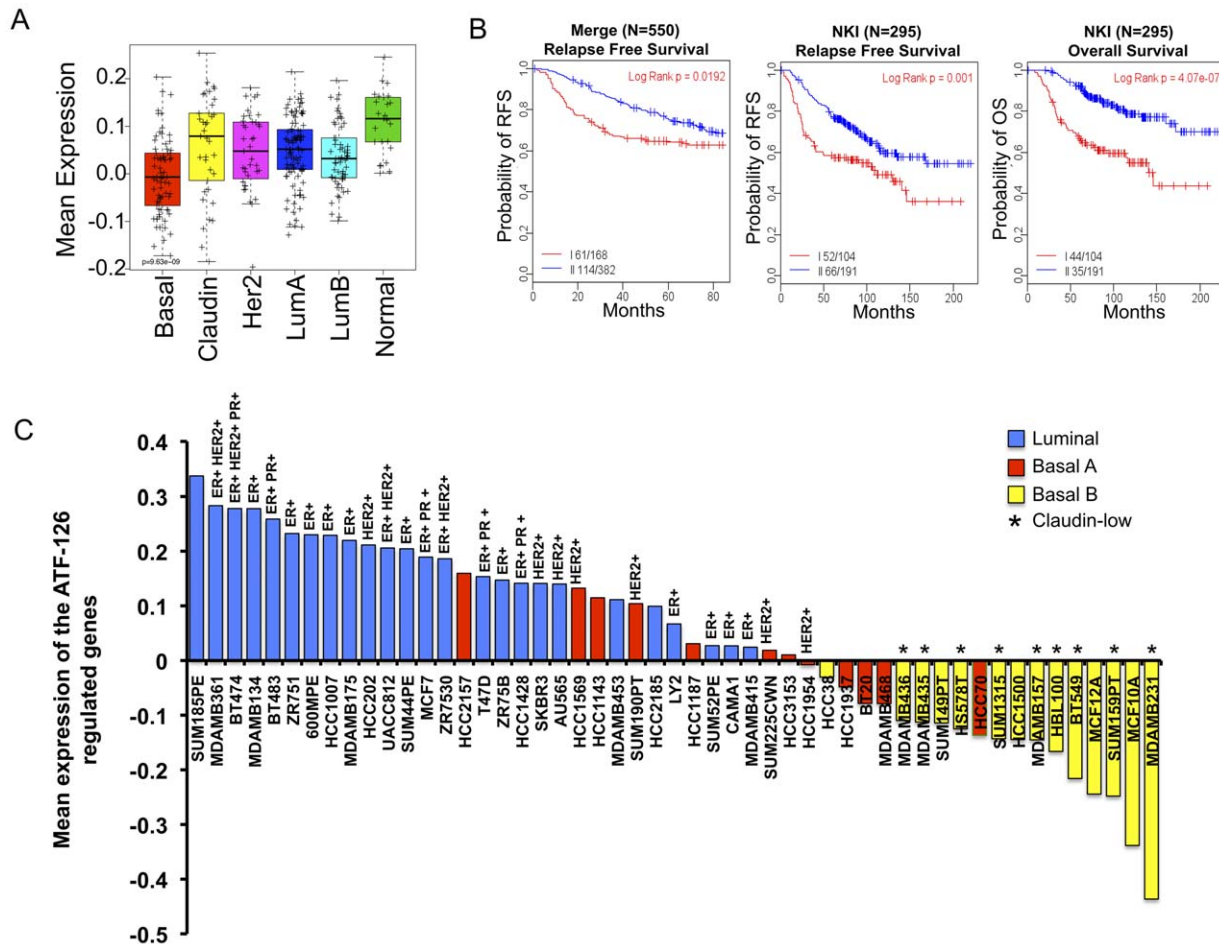


Figure 5. ATF-126 up-regulates a gene signature over-represented in Normal-Like breast cancers. A. Box-and-whisker plot for the mean expression of the 550 up-regulated gene signature (Table S1). This signature represents the number of genes significantly up-regulated in ATF-126 cells exposed to DOX for 72 hours relative to the same cells in absence of DOX. The prevalence of this signature was evaluated across the intrinsic molecular subtypes of breast cancers using the previously published UNC breast cancer patient database (UNC337). P values were calculated by comparing gene expression means across all breast tumor subtypes. **B.** Kaplan–Meier survival estimates of relapse-free survival for the Merge 550 database (left panel), relapse-free survival and overall survival for the NKI 295 database (center and right panel, respectively). Patients were stratified into group I (red curves) and group II (blue curves) based on 2-way hierarchy clusters. P-values were obtained from the log-rank test. **C.** Mean expression analysis of the ATF-126 gene signature across breast cancer cell lines [21], showing the expression status of estrogen receptor (ER), Human Epidermal growth factor Receptor 2 (HER2) and progesterone receptor (PR).
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memory”) over cell generations by targeted remodeling of silenced chromatin.

Our results *in vivo* demonstrate that induction of ATF-126 in animal models led to a 50% reduction in breast cancer cell growth. Long-term removal of DOX from the diet of the animals resulted in a re-establishment of the tumors *in vivo*. As it was observed in our analysis *in vitro*, it is possible that long-term absence of ATF expression results in re-establishment of *Maspin* silencing. This is in contrast with our colonization model of breast cancer, in which removal of DOX from the ATF-126 induced animals did not result in a recurrence of metastasis. These results could be explained because most circulating tumor cells or cells that underwent an early colonization in the lungs were effectively targeted by the ATF. Nevertheless, we do not know at present if the ATF-126 will be also effective in suppressing well-established metastases and this will require further investigation.

In order to investigate potential downstream genetic signatures mediating the inhibition of tumor growth and colonization, we performed DNA arrays. Our genome-wide analysis revealed that

ATF-126 up-regulated a 550-gene signature that was found over-represented in the Normal-like intrinsic subtype of breast cancer, and as well as in luminal A breast cancer cell lines. Together with Luminal A, Normal-like breast cancers are associated with ER expression. These tumors are small, mostly found in post-menopausal women, tend to have normal p53 status, and are known to be genetically more stable than other tumor subtypes [17,18]. Hence, Normal-like and Luminal A tumors both have a significant higher survival upon endocrine adjuvant therapy treatments after surgery as compared to other subtypes of breast cancers. As expected from its over-representation in Normal-like ER+ tumors, we found that the 550-gene signature predicted favorable prognosis for breast cancer patients.

In our cell line analysis, the 550-gene signature was under-represented in the original MDA-MB-231 line as well as in ER-breast cancer lines, while it was highly over-represented in ER+ luminal lines. Although Normal-like cancer cell lines were absent in the cell line database of Neve *et al.* [21] it is highly possible that this signature will also be over-expressed in normal breast and

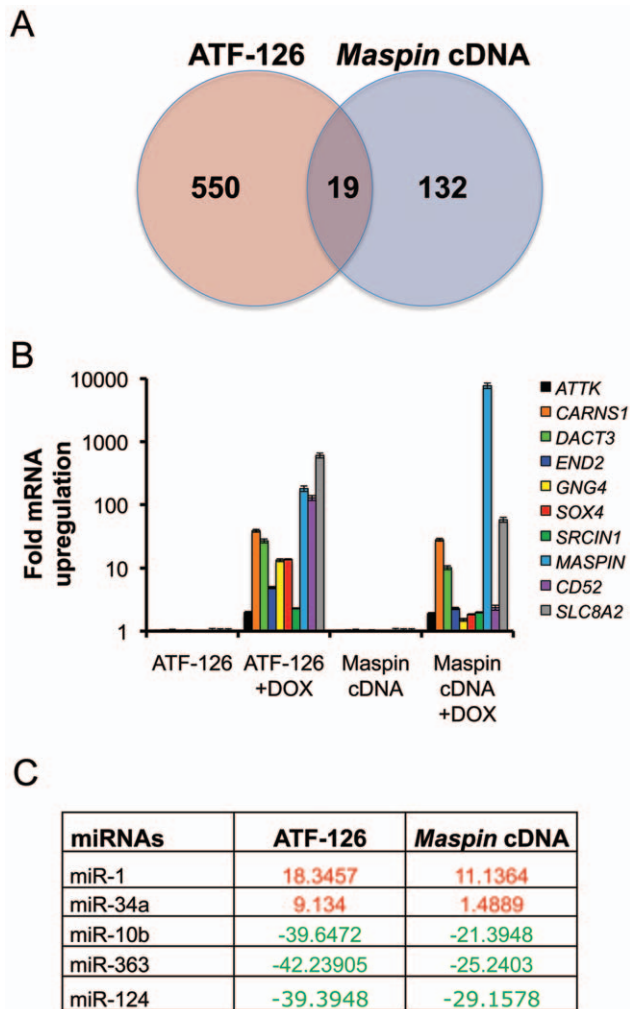


Figure 6. ATF-126 and Maspin cDNA co-regulated targets. **A.** Venn diagram indicating the intersecting up-regulated genes between the ATF-126 and the Maspin cDNA groups in DOX induced cells. **B.** Gene expression analyses of nine differentially up-regulated genes. The ATF-126 and Maspin cDNA stable cell lines were treated with either vehicle (–DOX) or DOX (+DOX) for a period of 72 hrs, and differences in expression quantified by qRT-PCR. Data was normalized to the –DOX cells, and represent an average of three independent experiments. **C.** Common MicroRNAs differentially regulated in the ATF-126 and the Maspin cDNA cell lines, as assessed by qRT-PCR. Data was normalized to the –DOX cells. Up-regulated miRNAs are indicated in red, and down-regulated miRNAs in green.
doi:10.1371/journal.pone.0024595.g006

Normal-like tumor cell lines. Consistent with this idea, we found that many junction proteins highly expressed in normal breast epithelial preparations, such as claudins, cadherins and occludins, were induced upon DOX treatment. This reactivation of junction proteins suggests that ATF-126 was able to reprogram a highly invasive, Claudin-low, and mesenchymal breast cancer line towards a more Normal-like or epithelial-like, and less invasive cancer cell line. Furthermore, flow cytometric analysis of ATF-126 induced cells revealed a new CD24 positive population. Since the CD44⁺/CD24[–] signature has been associated with tumor initiation, the up-regulation of CD24 population suggest that ATF-126 could decrease the tumor initiating ability of the original line MDA-MB-231.

The above phenotypic consequences of ATF-126 reactivation, inhibition of tumor growth and metastatic potential as well as gain of epithelial features, are all consistent with the documented functions of the target gene *Maspin*. The fact that both the *Maspin* shRNA and the *Maspin* siRNA were able to rescue the cell growth phenotype in ATF-126 transduced cells demonstrated that the phenotype of ATF-transduced cells was dependent on *Maspin*, and not on potential off target effects.

The molecular targets by which *Maspin* exerts its tumor/metastasis suppressive functions are still under investigation. *Maspin* cDNA over-expression results in re-activation of multiple pathways involved in tumor suppression and apoptosis, motility, and cell adhesion [18],[16]. In agreement with these functions of *Maspin*, we have found that tight junction, cell adhesion, and multiple tumor suppressive pathways were activated by ATF-126.

To dissect the downstream targets of ATF-126 that were “bona fide” downstream targets of *Maspin*, the *Maspin* cDNA was cloned into the same inducible retroviral vector, and genome-wide transcriptional profiles were compared between the un-induced and induced cells. An overlapping 19-target gene signature between the ATF-126 and the *Maspin* cDNA expressing cells was built, which represents “high confidence” hits by which *Maspin* could exert its mechanism of function in Claudin-low breast cancer cells. It is important to note that ATF-126 and *Maspin* cDNA represent two different mechanisms to up-regulate target gene expression; whereas the *Maspin* cDNA over-expresses the exogenous transgene form of *Maspin*, the ATF reactivates the endogenous gene, which results in up-regulation the physiologically relevant isoform, at “normal” cellular levels. In addition, unlike cDNA over-expression, which is predominantly cytoplasmic, the ATF-126 also results in substantial activation of the nuclear form of *Maspin* (*manuscript in preparation*), which mediates tumor and metastasis suppressive functions [28]. Thus, it is not surprising that transcriptional profiles of ATF-126 and *Maspin* cDNA were overlapping, yet not identical. Definitive analysis of genome-wide binding specificity of ATF-126 will require the integration of ChIP-seq data, which is underway. Overall, our results outlined the importance of using several methodologies to up-regulate *Maspin*, which allowed the dissection of potential therapeutic targets downstream the *Maspin* cascade.

Three targets belonging to this overlapping 19-gene signature were up-regulated by 10–100 fold over un-induced cells: *Solute carrier family 8 (sodium/calcium exchanger), member 2 (SLC8A2)*, *Carnosine synthase 1 (CARNS1)*, and *Dapper, antagonist of beta-catenin, homolog 3 (DACT3)*. These genes represent novel *Maspin* targets with potential tumor suppressor activity. *SLC8A2* encodes a Na⁺/Ca²⁺ exchanger, participating in intracellular Ca²⁺ homeostasis, and its expression is restricted to the brain. Interestingly, *SLC8A2* is found silenced by methylation in human gliomas where it has been proposed to act a tumor suppressor gene [29]. *CARNS1* belongs to the ATP-grasp family of ATPases, and catalyzes the formation of carnosine (beta-alanyl-L-histidine) and homocarnosine (gamma-aminobutyryl-L-histidine). Carnosine is a naturally occurring substance discovered more than a hundred years ago, and is present in the mammalian brain and skeletal muscle [30]. Although its function is still under investigation, Carnosine has been proposed to have a protective effect against oxidative stress and represents a potential therapeutic agent for treatment of aging and Alzheimer’s disease [31]. More recently Carnosine has been shown to inhibit proliferation of human brain cancer cells *in vitro* [32]. In 2010 a report showed that Carnosine retarded tumor growth *in vivo* in a NIH3T3-HER2/neu mouse model [33]. Thus, our results suggest that *CARNS1* could potentially represent a *Maspin*-dependent tumor suppressor enzyme in Claudin-low

Table 1. Genes regulated with ATF-126 and Maspin cDNA.

GeneID	Name	Symbol
6543	solute carrier family 8 (sodium/calcium exchanger), member 2	SLC8A2
1043	CD52 molecule	CD52
6659	SRY (sex determining region Y)-box 4	SOX4
147906	dapper, antagonist of beta-catenin, homolog 3 (<i>Xenopus laevis</i>)	DACT3
1013	cadherin 15, type 1, M-cadherin (myotubule)	CDH15
162494	rhomboid, veinlet-like 3 (<i>Drosophila</i>)	RHBDL3
4093	SMAD family member 9	SMAD9
6615	snail homolog 1 (<i>Drosophila</i>)	SNAI1
285489	docking protein 7	DOK7
9148	neuronalized homolog (<i>Drosophila</i>)	NEURL
3726	jun B proto-oncogene	JUNB
51207	dual specificity phosphatase 13	DUSP13
80725	SRC kinase signaling inhibitor 1	SRCIN1
57571	carnosine synthase 1	CARN51
2786	guanine nucleotide binding protein (G protein), gamma 4	GNG4
29993	protein kinase C and casein kinase substrate in neurons 1	PACSIN1
9625	apoptosis-associated tyrosine kinase	AATK
126567	C2 calcium-dependent domain containing 4C	C2CD4C
1907	endothelin 2	EDN2

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carcinomas. *DACT3* is expressed in the embryonic CNS, and is a negative regulator of Wnt/ β -catenin signaling [34]. *DACT3* is down-regulated in colorectal cancers by epigenetic mechanisms including histone methylation and deacetylation [35]. Given the importance of Wnt/ β -catenin in the mammary gland tumorigenesis in triple negative, mesenchymal breast cancers [36], we speculate that *DACT3* represents a potential therapeutic target for these carcinomas.

ATF-126 and in less extent *Maspin* cDNA +DOX cells up-regulated the *protein gamma-4 subunit/guanine nucleotide-binding protein 4* (*GNG4*) gene, a brain-specific protein [37] with potential tumor suppressor activity, which, like *Maspin* [38], is up-regulated by hypoxia factors [39]. Another unexplored potential target of *Maspin* in the 19-gene signature is the *Apoptosis-Associated Tyrosine kinase* (*AATK*), a protein kinase predominantly expressed in the nervous system. *AATK* regulates apoptosis, neurite growth, and differentiation of cerebellar granule cultures [40]. Lastly, ATF-126, and in less extent *Maspin* cDNA, up-regulated the *SRC kinase signaling inhibitor 1* (*SRCIN1*), a gene encoding a novel Src-binding protein that regulates Src activation. Gain and loss of function approaches in breast and colon cancer cells demonstrated that *SRCIN1* inhibits EGFR and Erk1/2 signaling, blocking scatter and proliferation of cancer cells [41]. In neurons, *SRCIN1* is predominantly localized to dendritic spines and enriched in the postsynaptic density, where it modulates spine shape via regulation of the actin cytoskeleton [42].

In addition to potential tumor/metastasis suppressor ORFs naturally expressed in neural cells, both ATF-126 and *Maspin* cDNA up-regulated miRNAs previously associated with tumor suppression in many types of cancers, including miR-1 [23,24] and miR-34 [25]. Intriguingly again, miR-1 is naturally expressed in dorsal root ganglion neurons where it has a role in modulating neurite outgrowth [43]. Similarly, miR-34 has been shown to target Actin in mouse neuronal cells [44]. In addition to activation of potential tumor suppressive miRNAs, both ATF-126 and

Maspin cDNA down-regulated putative oncogenes, including miRNA-10b.

The up-regulation of epigenetically silenced genes and miRNAs normally expressed in the nervous system is intriguing. Once over-expressed in tumors, these targets could be involved in differentiation of tumor cells, and metastasis inhibition. As a member of the *SERPIN* gene superfamily, nuclear *Maspin* is physically associated with Histone Deacetylase 1 (HDAC1) and functions as an HDAC inhibitor, remodeling chromatin and mediating gene reactivation [28,45]. This is in agreement with our data, and could mechanistically explain the epigenetic reactivation of multiple tumor suppressors epigenetically silenced in poorly differentiated tumor cells, and the overall reprogramming of a mesenchymal line towards a more benign, more differentiated, and less aggressive cell line. Subsequent analysis in our identified *Maspin* targets will reveal whether *Maspin* directly binds the promoter of these genes, resulting in tumor and metastasis suppression. We are presently addressing the potential of ATFs to be delivered in breast tumors and metastasis, using nanoparticle-based strategies (*manuscript in preparation*). Another line of investigation will ascertain if ATFs can increase the sensitivity of ER- tumors to anti-cancer drugs used in the clinic, such as anti-estrogens.

Materials and Methods

Cell lines

The human breast cancer cell line MDA-MB-231 (ATCC, Cat. No. HTB-26) and all stable cell lines derived from it were cultured in DMEM medium supplemented with 10% fetal bovine serum and grown at 37°C in a humidified 5% CO₂ incubator.

Development of a double stable Tet-On cell lines

The ZF-126, the ATF-126, and the *Maspin* cDNA were cloned into the p-RetroX-Tight (Cat. Num. 632104, CloneTech, Moun-

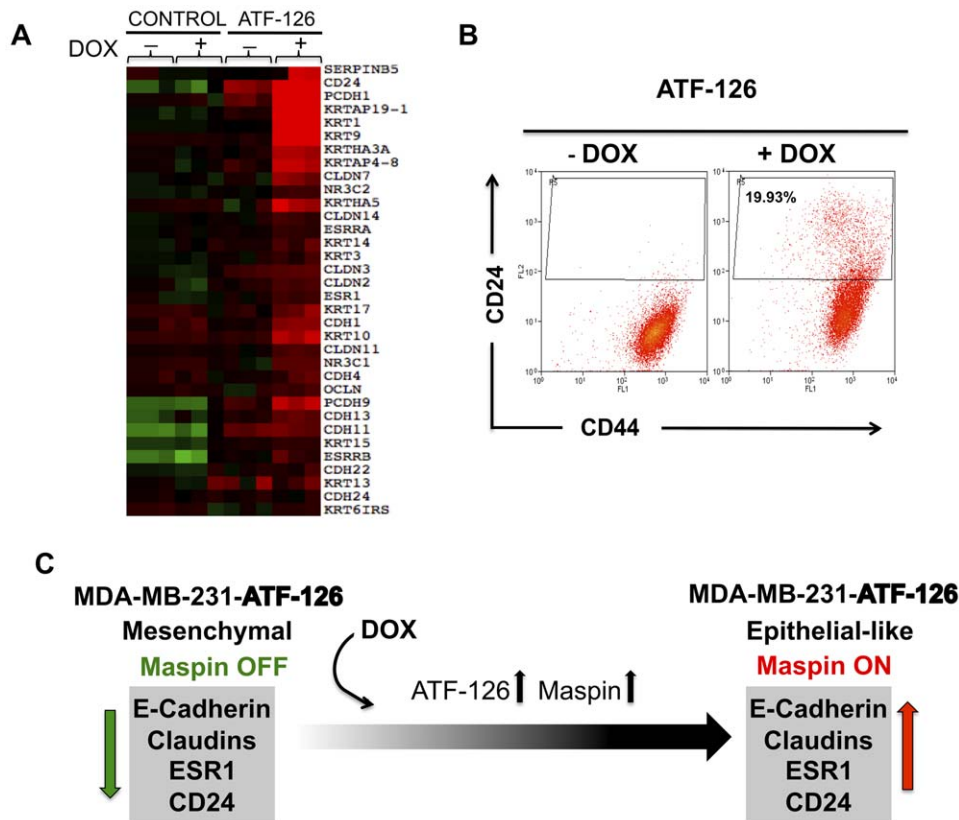


Figure 7. ATF-126 regulates markers associated with decreased tumorigenicity and metastasis. **A.** Microarray expression analysis of selected epithelial markers in CONTROL and ATF-126 cells, in presence or absence of DOX. Cells were collected 72 hours after DOX induction. Arrays were performed in triplicate with three different biological replicates using Agilent 44 k arrays. The Array tree was derived from an unsupervised hierarchical clustering and the gene list is shown in **Table S1**. Each colored square on the upper right represents the relative mean transcript abundance (in log2 space) with highest expression being red, average expression being black, and lowest expression being green. **B.** Induction of ATF-126 by DOX modifies the tumor initiating cell signature CD44⁺CD24⁺. Representative flow cytometric analysis of CD44 and CD24 expression levels in ATF-126 –DOX and +DOX cells collected 72 hours after treatment. The forward scatter (FCS) channel was plotted in y-axis and the fluorescence of the cell surface antigens in the x-axis. The gate in the +DOX panel illustrates the generation of a novel CD24⁺ population upon induction of ATF-126. **C.** A model illustrating a potential mechanism by which ATF-126 could reprogram a mesenchymal, Claudin-low MDA-MB-231 cell line towards a more epithelial-like phenotype.
doi:10.1371/journal.pone.0024595.g007

tain View, CA, US), and delivered into the MDA-MB-231-LUC cells by retroviral transduction. After transduction the cells were placed under selection with puromycin (1 µg/ml) and geneticin (800 µg/ml) for ten days. The CONTROL (p-RetoX-Tight empty vector), ZF-126 (p-RetoX-Tight-ZF-126), the ATF-126 (p-RetoX-Tight-ATF-126), and the *Maspin* cDNA (p-RetoX-Tight-*Maspin*) stable cell lines were expanded and used for *in vitro* and *in vivo* experiments.

ShRNA stable cell lines

Scramble and *Maspin* shRNAs (Open Biosystems) were co-transfected with pMDG.1 into 293TGag-Pol cells to produce retroviral particles. Transfection was done using Lipofectamine system. The viral supernatant was used to infect the MDA-MB-231-ATF-126 stable cell line. Cells were treated with hygromycin B (100 µg/ml) for 10 days.

siRNA *Maspin* gene knock-down

The p-RetoX-Tight-ATF-126 stable cell line was reverse-transfected with either 50 nM of *Maspin* siRNA smart pool (4 siRNAs/pool) or mismatch siRNA, and complexed with dharmafect reagent (all siRNAs and reagents from Dharmacon,

Chicago, IL, USA). Transfection conditions were optimized using a cytotoxic siRNA targeted against human ubiquitin B (Dharmacon). Cells were induced with DOX for 24 hours, then 2.5×10^5 cells/well were transfected in 6-well plates, and DOX was added. Cells were maintained in transfection media for 72 hours, and subjected qRT-PCR analysis.

Gene expression, western blot, proliferation, Immunofluorescence and apoptosis assays were performed as described [13]. Primers and probes for gene expression assays are shown in **Table S4**. Primary and secondary antibodies from the corresponding companies were applied at the concentrations shown in **Table S5**.

Quantification of cell death

Cells were collected and centrifuged 5 min at 100×g, then resuspended in 0.2% trypan blue. Cells were incubated 3 min at room temperature and counted on a hemacytometer. The percentage of cell death was calculated by comparing all samples counts with the ATF-126 –DOX cells.

Subcutaneous Injections

Female SCID mice (age 4 weeks) were purchased from Taconic Farms and housed under pathogen-free conditions. The Institu-

tional Animal Care and Used Committee (IACUC) at the University of North Carolina at Chapel Hill approved all experiments described herein. MDA-MB-231-Control-LUC or MDA-MB-231-ATF126-LUC cells (1×10^6) were collected and re-suspended with matrigel (BD Bioscience, San Diego, CA, US) 1:1 volume ratio in a total volume of 100 μ l. The cell-matrigel mixture was injected into the mouse flank. Tumor growth was monitored by caliper twice a week. When the tumor reached a size of approximately 0.5 cm, doxycycline (+DOX) was administered to mice in form of green food pellet (200 mg/Kg of mice chow) for a period of 25 days. For the DOX removal group, the DOX food was replaced by normal food. During the entire experiment the mice weight was monitored to verify whether toxicity occurred. After DOX treatment, the tumor volume was monitored both by caliper and Bioluminescence imaging (BLI) as described [13]. Assessment of tumor shrinkage was monitored one day previous to DOX induction and the day of tumor collection.

Tail-vein

Three days before the injections mice were kept in normal or DOX+ diet. Animals were injected via tail-vein with either 1×10^5 MDA-MB-231-Control-LUC or MDA-MB-231-ATF126-LUC cells in 100 μ l of PBS. BLI imaging was performed as described [13]. Assessment of tumor growth was monitored *in vivo* once a week for up to 8 weeks.

Flow cytometry

Cells were stained with primary antibodies anti-CD24-conjugated with phycoerythrin (PE) and anti-CD44 conjugated with Fluorescein isothiocyanate (FITC) (BD Bioscience, San Diego, CA, US) following the manufacturer's recommendations. Analysis was performed using a FACScalibur flow cytometer and the CellQuestTM software.

Gene expression microarrays

A total of six cell lines were used for gene expression analyses: CONTROL -DOX, CONTROL +DOX, ATF-126 -DOX, ATF-126 +DOX (all with 3 technical replicates), p-RetoX-Tight-Maspin -DOX, and p-RetoX-Tight-Maspin +DOX (with 2 technical replicates). For each cell line, total RNA was purified, amplified, labeled, and hybridized [46] using Agilent Agilent 4 $\times$ 44 K oligo microarrays (Agilent Technologies, United States). All microarray data is available in the Gene Expression Omnibus (GEO) Database (<http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=nnolvkqsosuwlm&acc=GSE27842>). The probes/genes were filtered by requiring the lowest normalized intensity values in both -DOX and +DOX samples to be >10. The normalized log₂ ratios (Cy5 sample/Cy3 control) of probes mapping to the same gene were averaged to generate independent expression estimates. We also used available microarrays from the breast cancer cell lines [21], the UNC337-patient [20], the MERGE 550-patient dataset [47] and the NKI (295 patients [48,49]). All microarray cluster analyses were displayed using Java Treeview version 1.1.3. Average-linkage hierarchical clustering was performed using Cluster v2.12 [50]. ANOVA tests for gene expression data were performed using R (<http://cran.r-project.org>).

ATF-126/Maspin cDNA gene signatures

In order to build an ATF-126 signature, we selected those genes that were significantly differentially expressed between ATF-126+DOX and ATF-126 -DOX using SAM, with <1% FDR. The resulting up-regulated gene list is shown in **Table S1**.

In order to determine the genes up-regulated with the *Maspin* cDNA, two biological replicates of the non-induced cells (-DOX) and cells induced with DOX were subjected to DNA microarray analyses, as described above. Gene expression values from the *Maspin* cDNA -DOX (N = 2) was subtracted from the *Maspin* cDNA +DOX (N = 2), and the 132 up-regulated genes are shown in **Table S5**.

MicroRNA microarrays

Total RNA was purified from the ATF-126 and the *Maspin* cDNA cells treated with either vehicle (-DOX) or DOX for 72 hours using Trizol. Small RNAs were enriched using the miRNeasy Mini Kit (SA Biosciences, Frederick, MD, USA), and cDNA was generated with the RT2 miRNA First Strand Kit (SA Biosciences, Frederick, MD, USA). Samples were subjected to MAH-102-C Micro-RNA arrays (SA Biosciences, Frederick, MD, USA), and data was normalized to the vehicle treated cells (-DOX). Expression of miR-10b, miR-1, miR-34a, miR363, and miR-124 was validated in two independent assays using hydrolysis probes (**Table S4**).

Prediction of relapse-free survival (RFS)

To determine if the 550-gene signature was represented in cancer patients from whom clinical data was available, we used two patient data sets: the NKI-295 and the MERGE-550. First, we examined the expression of the 550 genes in the MERGE-550 database and found that 322 out of the 550 genes were detected. A 2-way hierarchy cluster stratified the 322 patients into 2 groups (cluster I and cluster II; **Fig. S3**). Patients in cluster II had a significant better relapse free survival outcome (in 7 years follow-up) than patients in cluster I. Second, we repeated the analysis using the NKI dataset and found 444 genes. A 2-way hierarchy cluster stratified the 295 patients into 2 groups (cluster I and cluster II; **Fig. S4**). Here again, patients in cluster II had a significant improved relapse free survival outcome and overall survival outcome (in 18 years follow-up) relative to patients in cluster I.

Supporting Information

Figure S1 Generation of an inducible ATF expression system to monitor breast tumor and metastasis. A. Schematic representation of ATF-126 comprising the six Zinc Finger (ZF) specific DNA-binding domains and the VP64 transactivator domain. ATF-126 was targeted against a unique 18-base pair site in the *Maspin* promoter. **B.** ATF-126 induction to monitor breast tumor progression. ATF-126 was cloned into pRetroX-Tight inducible vector system. The pRetroX-Tight vector is composed of a modified tetracycline response element (TRE_{Mod}) and a minimal CMV promoter (CMV_{min}). The activator protein is a tetracycline-controlled transactivator (rtTA), which binds to the TRE_{Mod} sequences in presence of Doxocycline (DOX). Viral particles were prepared and MDA-MB-231-LUC cells (engineered with a luciferase reporter) were transduced to generate stable cell lines. ATF-126 was induced both *in vitro* and *in vivo* with the chemical inducer DOX. (TIFF)

Figure S2 Expression levels of CD44 and CD24 in a panel of breast cancer cell lines, as assessed by flow cytometry. (TIFF)

Figure S3 Gene clustering showing the 322 genes from the ATF-126-550 gene signature present in the MERGED-550 patient dataset. Two-way hierarchy cluster

stratified the 550 patients into two groups (cluster I and cluster II); patients in cluster II had a significant better relapse free survival outcome (in 7 years follow-up) than patients in cluster I. Each colored square on the upper right represents the relative mean transcript abundance (in log₂ space) with highest expression being red, average expression being black, and lowest expression being green.
(TIFF)

Figure S4 Gene clustering showing the 444 genes from the ATF-126-550 gene signature present in the NKI-295 patient dataset. Two-way hierarchy cluster stratified the 295 patients into 2 groups (cluster I and cluster II); patients in cluster II had a significant better relapse free survival outcome and overall survival outcome (in 18 years follow-up) than patients in cluster I. Each colored square on the upper right represents the relative mean transcript abundance (in log₂ space) with highest expression being red, average expression being black, and lowest expression being green.
(TIFF)

Table S1 Genes up-regulated in ATF-126 +DOX versus -DOX.
(DOC)

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Table S2 Pathway analysis of the 550-gene signature using the David database (<http://david.abcc.ncifcrf.gov/>).
(DOC)

Table S3 Antibodies used in this study.
(DOCX)

Table S4 Primers and probes used in this study.
(DOCX)

Table S5 Genes differentially regulated with the Maspin cDNA.
(DOC)

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Author Contributions

Conceived and designed the experiments: ASB PB. Performed the experiments: ASB AR HL. Analyzed the data: ASB CF. Contributed reagents/materials/analysis tools: ASB AR HL PL CF. Wrote the paper: ASB PB.

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Targeting Serous Epithelial Ovarian Cancer with Designer Zinc Finger Transcription Factors^{*[S]}

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Background: There is a need for novel targeted therapies for metastatic ovarian cancers.

Results: We reactivated the tumor suppressor *Maspin* in ovarian carcinoma cells by delivering tumor-specific nanoparticles encapsulating a chemically modified ATF-mRNA.

Conclusion: LPR nanoparticles encapsulating the ATF mRNA inhibited ovarian cancer tumor growth in a mouse model.

Significance: We report the first non-viral delivery of an ATF *in vivo* and the discovery of novel anti-metastatic targets for ovarian cancer.

Ovarian cancer is the leading cause of death among gynecological malignancies. It is detected at late stages when the disease is spread through the abdominal cavity in a condition known as peritoneal carcinomatosis. Thus, there is an urgent need to develop novel therapeutic interventions to target advanced stages of ovarian cancer. Mammary serine protease inhibitor (*Maspin*) represents an important metastasis suppressor initially identified in breast cancer. Herein we have generated a sequence-specific zinc finger artificial transcription factor (ATF) to up-regulate the *Maspin* promoter in aggressive ovarian cancer cell lines and to interrogate the therapeutic potential of *Maspin* in ovarian cancer. We found that although *Maspin* was expressed in some primary ovarian tumors, the promoter was epigenetically silenced in cell lines derived from ascites. Transduction of the ATF in MOVCAR 5009 cells derived from ascitic cultures of a TgMISIIR-Tag mouse model of ovarian cancer resulted in tumor cell growth inhibition, impaired cell invasion, and severe disruption of actin cytoskeleton. Systemic delivery of

lipid-protamine-RNA nanoparticles encapsulating a chemically modified ATF mRNA resulted in inhibition of ovarian cancer cell growth in nude mice accompanied with *Maspin* re-expression in the treated tumors. Gene expression microarrays of ATF-transduced cells revealed an exceptional specificity for the *Maspin* promoter. These analyses identified novel targets co-regulated with *Maspin* in human short-term cultures derived from ascites, such as *TSPAN12*, that could mediate the anti-metastatic phenotype of the ATF. Our work outlined the first targeted, non-viral delivery of ATFs into tumors with potential clinical applications for metastatic ovarian cancers.

Epithelial ovarian carcinoma (EOC)³ is the seventh most fatal cancer worldwide and the deadliest malignancy affecting the female reproductive organs (1). Serous ovarian carcinoma (SOC) is the most common form of EOC, comprising 30–70% of the cases (2). SOC is predominantly associated with *p53* mutations, and loss of *BRCA1/2* may also predispose to the development of the disease (3, 4). This form is mostly detected at advanced stages, when disease is widely spread and metastasized into the abdomen, in a condition known as peritoneal carcinomatosis. Late diagnosis is explained by the absence of alarming symptoms and the lack of effective screening meth-

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^[S] This article contains supplemental Tables 1–5 and Figs. 1–5.

Microarray data have been deposited in the Gene Expression Omnibus (GEO) data base under the accession numbers GSM891209, GSM891210, GSM891211, GSM891212, GSM891213, and GSM891214.

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³ The abbreviations used are: EOC, epithelial ovarian carcinoma; SOC, serous ovarian carcinoma; ATF, artificial transcription factor; mATF, murine-specific ATF; GEMM, genetically engineered mouse model; MOVCAR, mouse ovarian carcinoma; ZF, zinc finger; qRT-PCR, quantitative real time-PCR; LPR, liposome protamine RNA; AA, anisamide; EGFP, enhanced GFP; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; MB, molecular beacon; SR, sigma receptor; TSPAN12, tetraspanin 12; DSPE-PEG, 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000].

ods. The lethality of this condition is due not only to late diagnosis but also to transient response to available therapies. Thus, despite achieving optimal de-bulking with surgery and obtaining adequate response to adjuvant chemotherapy, the majority of cases will recur, and patients finally die because of resistant metastatic disease (5–7). Unfortunately, the discovery of biomarkers of metastatic progression and the development of more effective treatments for SOC has been impeded due to our limited understanding of the etiology and progression of the disease.

Genetically engineered mouse models (GEMMs) of epithelial cancers represent powerful model systems as they recapitulate the essential molecular hallmarks of disease development and progression that occur in humans (8). One of those models, the C57BL/6 TgMISIIR-TAg transgenic mouse, develops EOC with metastatic features. Mouse ovarian carcinoma (MOVCA) cell lines derived from metastatic lesions (ascites) of TgMISIIR-TAg mice recapitulate essential features of SOC, particularly the metastatic potential (9). These cells have the advantage that they can be easily manipulated *in vitro* to elucidate novel biomarkers of metastatic disease and to establish novel delivery systems for therapeutic intervention (9).

Our laboratory has previously described a therapeutic approach to target tumor and metastasis suppressors in cancer cells using arrays of engineered, sequence-specific C₂H₂ zinc finger (ZF) domains (10). Each ZF is composed of a recognition α -helix that binds 3 bp of DNA with high selectivity (11). Six zinc finger (6ZF) arrays read an 18-base pair (18-bp) sequence that is potentially unique in the human genome and provide a high degree of genomic specificity and selectivity (12). Engineering binding specificity is achieved by grafting the α -helical domain of each ZF known to interact with the target DNA triplet (13). We have constructed multimodular 6ZF proteins referred as artificial transcription factors (ATFs), recognizing sequences in targeted promoters with dissociation constants in the picomolar range (10, 13–18). In an ATF, the 6ZF scaffold can be linked to a variety of protein modules to promote transcriptional activation (19–23), repression (24, 25), and more recently, epigenetic editing (26).

We have recently described an ATF (ATF-126) targeting the human mammary serine protease inhibitor (*Maspin*), a tumor and metastasis suppressor gene initially identified in breast cells (27). Although most epithelial cells express high levels of *Maspin*, the promoter is down-regulated by epigenetic mechanisms in several cancers, such as breast (28, 29) and ovarian cancer (30). Endogenous *Maspin* reactivation by ATF-126 was associated with decreased tumor growth by enhancement of apoptosis (10), cell invasion (10), and suppression of metastatic colonization in breast (18) and lung (31) cancer cells. Although most studies have been focused on breast, prostate, and lung cancer, the functional role of *Maspin* as tumor and metastasis suppressor in EOC has not been investigated. Both cytoplasmic and nuclear *Maspin* expression has been reported in some primary ovarian tumors and cell lines, with nuclear expression being a favorable prognosis factor in ovarian cancer patients (32). Decreased nuclear *Maspin* expression has been associated with tumor grade and disease progression, suggesting a role of

Maspin silencing in advanced stages of ovarian cancer, potentially in metastatic disease (32–34).

In this manuscript we took advantage of ZF technology to target the endogenous murine *Maspin* promoter in metastatic MOVCA cell lines to interrogate the functional role and therapeutic potential of *Maspin* in EOC. Delivery of engineered ZF proteins has historically been a major limitation for translational applications, most notably in cancer models. This paper reports the first non-viral delivery of ATF mRNA for future therapeutic treatment of serous epithelial ovarian cancer of advanced stage and potentially for metastatic disease. We also describe a panel of novel targets co-regulated with *Maspin*, which could be used as potential biomarkers for future diagnosis and treatment of metastatic ovarian cancer.

EXPERIMENTAL PROCEDURES

Construction of a Murine-specific ATF Recognizing the *Maspin* Promoter (mATF)—The murine-specific mATF was designed to recognize an 18-bp duplex 5'-GAAGACCTGGGTGTGGTC-3' located at –127 nucleotides upstream the first ATG triplet (translation start site) in the murine *Maspin* promoter (Fig. 1A). The specific ZF coding sequences were generated by overlapping PCR as previously described (10), and the specific α -helical sequences used for the PCR are shown in supplemental Fig. S1. The 6ZF cassette was cloned into the SfiI sites of the retroviral vector pMX-ss-IRES-GFP (10) for endogenous gene regulation studies.

Cell Lines—The T11 cell line was a generous gift from Dr. C. M. Perou (Lineberger Comprehensive Cancer Center, Chapel Hill, NC) (35). Cells derived from the TgMMTV-PyMT were kindly provided by Dr. S. Earp (Lineberger Comprehensive Cancer Center, Chapel Hill, NC). M6CCT and M6C1 breast cell lines from a C3(1)tag mammary mouse model were provided by J. E. Green (NIH, Bethesda, MD) (36). WNT1–3160, WNT1-WG4, BRCA-B1.15, and BRCA-A1.8 cell lines were generously supplied by Dr. L. Varticovski (NIH, Bethesda, MD) and were maintained under culture conditions as previously reported (37–39). MOVCA 5447, 5612, and 5009 were cultured as described (9).

Retroviral Transduction of mATF in Cancer Cell Lines—Retroviral transduction in the packaging cell line 293TGagPol (ATCC Number CRL-11654) and infection of the host cell lines was performed as described (17) using the retroviral vector pMX-IRES-GFP and the envelope protein plasmid pMDG.1. The cells were infected every 8–12 h for a total of 3 times and collected or fixed 72 h after the first infection for further experiments. The Retro-X Tet-On expression system (catalog number 632104 CloneTech, Mountain View, CA) was used to generate a MOVCA 5009 stable cell expressing the human *Maspin* cDNA (purchased from Origene, catalogue number SC303231, Rockville, MD) as described (18). To induce *Maspin* cDNA expression, cells were treated for 48 h with doxycycline 100 ng/ μ l and collected for further experiments. The 293TGagPol cells were grown in DMEM (Sigma) supplemented with 10% of fetal bovine serum (FBS) and antibiotic-antimycotic solution (Sigma).

Treatment of the MOVCAR 5009 Cell Line with Epigenetic Inhibitors—The MOVCAR 5009 cell line was treated with the 5-aza-2'-dC DNA-methyltransferase inhibitor and two histone deacetylase inhibitors, suberoylanilide hydroxamic acid and trichostatin A. Cells were treated at saturating concentrations of drugs, as determined empirically for the MOVCAR 5009 cell line: 5 μ M 5-aza-2'-dC, 10 μ M suberoylanilide hydroxamic acid, and 100 nM trichostatin A. All drugs were purchased from Sigma. Cells were plated at a density of 3×10^5 cells in 100-mm plates (Corning, NY) in DMEM media and treated with the corresponding inhibitors during 48 h. Cells were collected, and RNA was extracted with RNeasy Kit (Qiagen) and processed for quantitative real time-PCR (qRT-PCR).

qRT-PCR Assays—RNA from adherent cell cultures and tumor samples were extracted with the RNeasy kit (Qiagen) and reverse-transcribed to cDNA using the High Capacity cDNA reverse transcription kit (Applied Biosystems, Carlsbad, CA). TaqMan Fast Universal Master Mix (Applied Biosystems) and 150 ng of cDNA were used in the PCR reactions. *GAPDH* was used as an endogenous control. Primers used for qRT-PCR studies are shown in supplemental Table S4. The 7500 Software Version 2.0.5. (Applied Biosystems) was used to analyze the data. qRT-PCR was performed in duplicate wells and in three independent experiments. Statistical differences were determined by Student's *t* test considering $p \leq 0.05$ as significant (*), $p \leq 0.01$ as highly significant (**), and $p \leq 0.001$ as extremely significant (***) .

Western Blot—Total protein extract was obtained with radio-immune precipitation assay buffer (Sigma R0278). The detection was performed with the Amersham Biosciences ECL detection system (GE Healthcare). A detailed list of the antibodies used in this study, the corresponding suppliers, and the experimental dilutions are shown in supplemental Table S5.

Chromatin Immunoprecipitation (ChIP)—ChIP assay to detect the ATF/polymerase II binding to the murine *Maspin* promoter was performed as described elsewhere (40, 41). Protein-DNA complexes were pulled down with A/G beads (Santa Cruz, sc-2003, Santa Cruz, CA). The beads were washed three times with low salt buffer, three times with high salt buffer, and two times with Tris-EDTA buffer (40, 41). The beads were eluted overnight at 65 °C in elution buffer (Tris-EDTA buffer, 1% SDS, and 2 μ l of proteinase K). DNA was isolated by phenol-chloroform extraction. The immunoprecipitated DNA was amplified by PCR using the following primers flanking the mATF binding site in the *Maspin* promoter: 5'-CTGGGTGTGGTCACAGGTGAGC-3' and 5'-TCCCTTTGCTTACCTTGAGTTGC-3. The primers against the murine *GAPDH* promoter were used as controls for the PCR amplifications: 5'-TACTCGCGGCTTTACGGG-3' and 5'-TGGAACAGGGA-GGAGCAGAGAGCA-3' (42). The PCR products were loaded in an agarose gel, and the relative -fold enrichment was determined by densitometry (ImageJ, NCBI, Bethesda, MD) and normalized to the input samples.

Immunofluorescence—Cells were seeded in fibronectin-coated coverslips and fixed with 10% formalin-PBS solution at room temperature for 10 min. Cells were permeabilized with 0.5% Triton X, PBS for 15 min at 4 °C, and 4% BSA, PBS was added for overnight blocking at 4 °C. Fibronectin, formalin, and

Triton-X were purchased from Sigma. MOVCAR 5009 cells were transduced with mATF, control, or *Maspin* cDNA and stained for detection of mATF, Maspin, and actin (supplemental Table S5). For immunofluorescence detection of Maspin and tetraspanin 12 (TSPAN12) on epithelial ovarian cancer specimens, paraffin sections (obtained from the UNC Tissue Procurement Core) were deparaffinized, blocked at room temperature with 5% BSA/PBS for 2 h, and incubated with primary antibody overnight at 4 °C. The slides were next washed with 0.03% Tween 20, 1% BSA, PBS and stained with secondary antibodies at room temperature for 1 h. Details regarding the antibody sources and dilutions are specified in supplemental Table S5.

Cell Viability Assay—Cell viability was determined by Cell-Titer Glo assay (Promega, Madison, WI) as described by the manufacturer. Twenty-four hours post-transduction, 1000 cells per well were seeded in 96-well plates, and cell viability was followed every 24 h for a total of 4 days. Statistical differences were determined by Student's *t* test considering $p \leq 0.05$ as significant (*), $p \leq 0.01$ as highly significant (**), and $p \leq 0.001$ as extremely significant (***) .

Soft Agar Assay—Cells were collected 72 h post-transduction. Colony formation was assessed using six-well plates (Corning). Five thousand cells per well were seeded on the top layer of 0.3% agar with a 0.6% agar base layer. Plates were incubated for 20 days in a 37 °C incubator with 5% CO₂. Colonies were visualized and counted after a 2-h treatment with a 0.005% crystal violet solution. Cells were seeded in triplicate, and the experiment was repeated three times. Materials were purchased from Sigma unless otherwise stated.

Tumorsphere Assay—Cells were collected 72 h post-transduction and counted. 20,000 cells were seeded in triplicate wells in 6-well plates (low attachment plates, Corning, NY) in spheroid media: HuMEC medium (Invitrogen) containing 20 ng/ml human EGF (BD Biosciences), 1 μ g/ml hydrocortisone (Stem Cell Technologies, Vancouver, Canada), insulin 5 μ g/ml (Sigma), and 1 $\times$ B27 (Invitrogen) (43). The plates were incubated at 37 °C in 5% CO₂, and tumorspheres were visualized using a cell culture Leica microscope 10 days after seeding.

Matrigel Invasion Assay—Matrigel Invasion Chambers (BD Biosciences) were used to study cell invasion as previously described (10). Briefly, cells were starved 24 h before the assay, and 1×10^5 cells were seeded in the chambers. 24 h later cells were fixed and stained with hematoxylin and eosin.

Preparation of Modified mRNA—The mATF coding sequence and enhanced green fluorescent protein (EGFP) were cloned into pcDNA 3.1 vector flanked by an untranslated 5' strong Kozak translational initiation signal and a 3' α -globin sequence for higher translation efficiency and longer half-life. The untranslated region and gene of interest was amplified by PCR with 5' primer, CTAGAGAACCCACTGCTTACTGGC-TTATCG, and 3' primer, ¹²⁰TGCGTCGACACTAGTTCTA-GACCT. The amplicons were used as templates for *in vitro* transcription. Modified mRNA was synthesized with Ambion MEGAScript T7 kit (Invitrogen). A mix of 1.6 μ g of purified amplicons, 6 mM 3'-O-Me-m⁷G(5')ppp(5')G (New England BioLabs, Ipswich, MA), 1.5 mM guanosine triphosphate, 7.5 mM adenosine triphosphate, 7.5 mM 5-methylcytidine triphos-

phate, 7.5 mM pseudouridine triphosphate, and 4 μ l of T7 enzyme were incubated at 37 °C for 4–6 h. The mRNA was purified with Ambion MEGAClear kit (Invitrogen).

Preparation of Liposome and Liposome Protamine RNA (LPR)—DOTAP (Avanti Polar Lipids, Alabaster, AL) and cholesterol (Sigma) (1:1 mol/mol) were dissolved in chloroform, and the solvent was removed under reduced pressure. The lipid film was hydrated overnight with distilled water to make the final concentration of 10 mM DOTAP and cholesterol. The liposome was sequentially extruded through 400-, 200-, 100-, and 50-nm polycarbonate membranes (Whatman, Piscataway, NJ) to form 80 ~ 100-nm unilamellar liposomes. The LPR core was prepared by mixing 146 μ l of solution A (10 μ g of mRNA) and 146 μ l of solution B (3 μ l of 2 mg/ml protamine, 12 μ l of 10 mM DOTAP/cholesterol liposome, and 131 μ l of distilled water). The resulting solution was incubated at room temperature for 10 min. The pegylation was performed by adding 4 μ l of DSPE-PEG₂₀₀₀ (Avanti Polar Lipids) (10 mg/ml) and 4 μ l of DSPE-PEG₂₀₀₀-AA (10 mg/ml) to the LPR core and incubating the mix at 50 °C for 15 min. The DSPE-PEG-AA was synthesized as previously described (44).

In Vitro Transfection of Nanoparticles in MOVCAR 5009 Cells—Ninety-six well plates were seeded with 1×10^4 cells per well. The cells were transfected with either LPR-AA-EGFP or LPR-EGFP nanoparticles equivalent to 0.5 μ g of modified mRNA encoding EGFP. Nanoparticles were added to each well in the presence of Opti-MEM medium (Invitrogen) and incubated at 37 °C in 5% CO₂. The medium was replaced with complete medium for 4 h post-transfection. The transfection efficiency was determined using a BD FACS Canto Flow Cytometer (BD Biosciences). MOVCAR 5009 cells were transfected with either LPR-AA-mATF or LPR-AA-control. The control refers to the mATF modified mRNA lacking the 5' cap nucleotide, which inhibits translation. The HA.11 antibody was applied to determine mATF expression by immunofluorescence 24 h after transfection (supplemental Table S5). Transfected cells were processed by MTT cell proliferation assays 2 days post-transfection as described by the manufacturer's instructions (Roche Applied Science). The absorbance was read at 570 nm to determine cell survival.

Molecular Beacon Design and Transfection—The software Beacon Designer (PREMIER Biosoft International, Palo Alto, CA) was employed to design a molecular beacon (MB) probe to hybridize with the mouse *Maspin* mRNA. The MB oligonucleotide sequence 5'-(Texas Red)(C6 amino)-CGCGATCTGTTTCTGATTCAGGAAATTTCTTCATGATCGCG-(3'BHQ-2)-3' was synthesized by The Midland Certified Reagent Co. (Midland, TX). LPR nanoparticles loaded with 0.2 μ g of MB and 0.8 μ g of mATF-mRNA were added into 8-well Lab-Tek Chamber Slides. Twenty-four hours post-transfection cells were examined with fluorescence microscopy observation.

Tumor Reduction Study—4–7-Week-old athymic nude mice (obtained from University of North Carolina animal models core facility) were inoculated with 1×10^7 MOVCAR 5009 cells in the flank. Mice received an intravenous injection (via tail vein) of nanoparticles equivalent to 10 μ g of mATF-mRNA/control as a palpable solid tumor was formed (on day 16). Mice received an injection every other day for five times. The

tumor sizes were monitored as an indication of therapeutic effect. Statistical analysis was undertaken using Prism 4.0 GraphPad Software. The tumors were removed and analyzed for expression of the mATF and *Maspin* reactivation by Western blot.

siRNA Transfection—Cells previously transduced with either control or mATF were collected 72 h post-transduction for the siRNA experiment. The murine *Maspin* siRNA and a nonspecific control siRNA targeting luciferase were purchased from Thermo Scientific (Waltham, MA). Transfection was performed using the manufacturer's instructions. Briefly, 1×10^5 cells were plated in 6-well plates, and DharmaFECT 4 was used to transfect the siRNA with a final concentration of 50 nM. Transfected cells were collected 48 h post-transfection and processed for qRT-PCR analysis of *Maspin* and *Tspan12* expression. Data were normalized to control-transfected samples.

Ovarian Cancer Patient-derived Samples—The normal ovary preparation SR06, ovarian primary tumors, and ascites were obtained from the Universidad Católica de Chile with approved Institutional Review Board protocols. These samples were resuspended in Trizol (Invitrogen) and processed for expression analyses. Human ascites (ASC13 to 17) were obtained from University of North Carolina hospitals with approved Institutional Review Board protocols. These cells were grown in RPMI media with 5% FBS in adherent tissue culture plates as described above. Tumor cells were passaged three times in tissue culture dishes and processed for qRT-PCR or Western blot. The epithelial origin of these cells was verified by Western blotting using anti-Pan-Cytokeratin antibodies (supplemental Fig. S4). For the qRT-PCR experiments the samples were analyzed using the GAPDH gene. Two additional endogenous controls recommended for ovarian tumor specimens (the genes glucuronidase β (GUSB) and peptidylprolyl isomerase A (PPIA) (45, 46)) were also used to validate the quantifications.

Gene Expression Microarrays—Total RNA was purified from control and mATF-transduced cells (three biological replicates) as described (18). The mRNA was amplified, labeled, and hybridized as previously described (47) using Agilent mouse 4×180 K oligo microarrays (Agilent Technologies). All microarray data have been deposited in the Gene Expression Omnibus (GEO) data base under the accession numbers: GSM891209, GSM891210, GSM891211, GSM891212, GSM891213, and GSM891214. The probes/genes were filtered by requiring the lowest normalized intensity values to be >10 in both samples and controls. The normalized log₂ ratios (Cy5 sample/Cy3 control) of probes mapping to the same gene were averaged to generate independent expression estimates. Statistical analysis of microarray for gene expression data were performed using Significance Analysis of Microarray. Gene ontology (GO) analysis was performed using EASE: the Expression Analysis Systematic Explorer (48).

RESULTS

mATF Binds the Murine Maspin Proximal Promoter and Up-regulates Its Expression in a Panel of Cell Lines Derived from GEMMs—To up-regulate the murine *Maspin* promoter, we designed a mATF targeting an 18-bp duplex located ~127 nucleotides upstream the translation start site (Fig. 1A). This

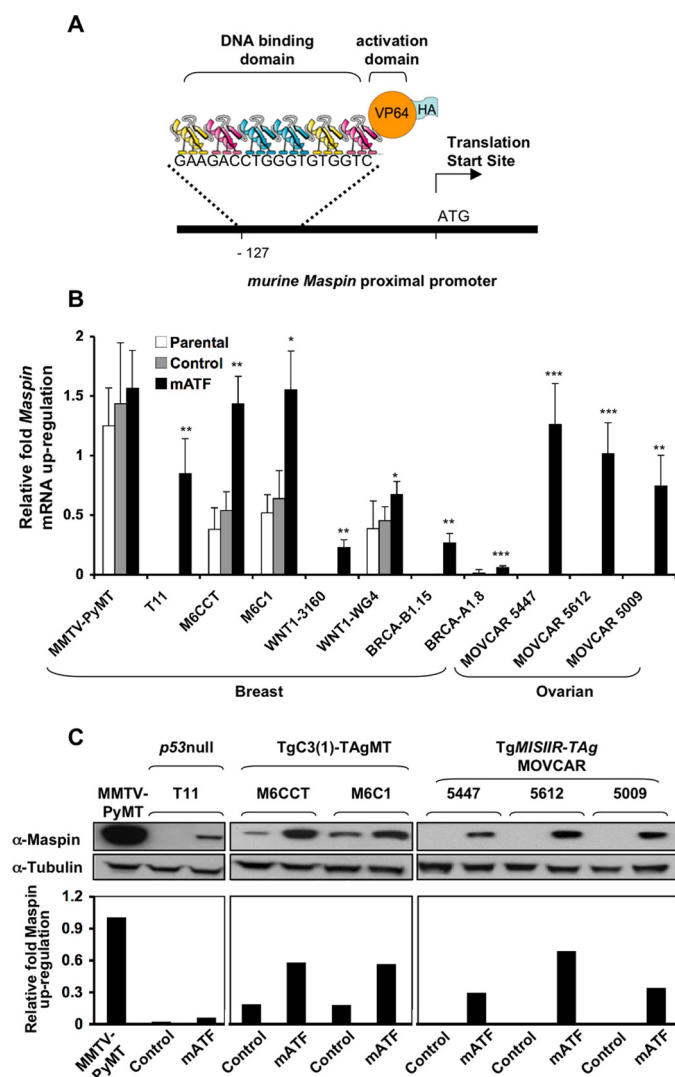


FIGURE 1. The artificial transcription factor mATF reactivates *Maspin* expression in breast and ovarian cell lines derived from GEMMs. A, shown is a schematic representation of the multidomain structure of the mATF binding the *Maspin* promoter. The DNA binding domain, composed of six ZF motifs, was designed to bind an 18-bp sequence in the murine *Maspin* proximal promoter at -127 bp upstream the translation start site. The ZF proteins were linked to the VP64 transactivator domain (1). The hemagglutinin (HA) tag was engineered at the C terminus of the ZF construct (68). B, the mATF up-regulates *Maspin* mRNA expression in breast and ovarian cancer cell lines derived from GEMMs as assessed by qRT-PCR. The mATF and empty vector (control) were retrovirally transduced in the corresponding cell lines. Relative *Maspin* up-regulation in both transduced and parental cell lines are shown in the plot. Data were normalized to the parental MMTV-PyMT cell line. Student's *t* test was used to analyze the differences in gene expression between the parental and the transfected cells (*, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$). C, shown is an immunoblot analysis of *Maspin* up-regulation in cell lines derived from GEMMs transduced either with control or mATF. A densitometric analysis of the Western blot is shown. Data were normalized to the MMTV-PyMT cell line. Transgenic mouse strains and cell lines are indicated, and sources are described under "Experimental Procedures."

specific site was chosen because an 18-bp site at the same relative position in the human promoter was successfully targeted by an artificial zinc finger factor, ATF-126 (10). Moreover, because the targeted site was not fully conserved between the murine and the human promoter, the 6ZF helices were re-engineered to regulate the murine counterpart (supplemental Fig. S1).

To have an initial assessment of the potency of the mATF in reactivating *Maspin*, we took advantage of a panel of breast and

ovarian cell lines derived from several GEMMs (Fig. 1, B and C). We delivered the mATF and an empty vector (control) using the pMX-IRES-GFP retroviral vector system (17). The mATF was able to significantly up-regulate *Maspin* in all the murine cancer cell lines examined carrying low or silenced *Maspin*. The gene expression data were normalized to the parental MMTV-PyMT breast cancer line carrying the highest expression of *Maspin* (Fig. 1B). As expected, no significant differences in gene expression were observed between the parental and control-transduced cell lines. We found that although *Maspin* was expressed in some breast cancer cell lines, the gene was silenced in all the ovarian cell lines derived from the TgMISIIR-TAg transgenic mouse. Because of their high invasive potential and their reported phenotypic resemblance to high grade EOC, we focused further experiments using the MOV5009 cell line. In this background the mATF increased *Maspin* mRNA expression by more than 70,000-fold relative to the parental line, empty vector control cells, or a control vector carrying no ZFs (VP64-SS sample, Fig. 2A). This regulation required a specific DNA binding as the delivery of a scramble library of ZF proteins linked to VP64 (67) resulted in a significant reduction (more than 3 orders of magnitude) of *Maspin* activation relative to the mATF, indicating that the specific 6ZF domains used for the construction of the protein provided sequence-selectivity (Fig. 2A). Interestingly, the reactivation of *Maspin* by the engineered protein was clearly superior to the reactivation of the endogenous gene observed when the parental cell line MOV5009 was treated with epigenetic inhibitors, such as the DNA methyltransferase inhibitor 5-aza-2'-dC (activation of 2564.9-fold over vehicle-control cells) and the histone deacetylase inhibitor trichostatin A (72.96-fold activation over vehicle control-treated cells). Unlike the human *Maspin* promoter (5), the murine counterpart was not significantly up-regulated upon exposure to the histone deacetylase inhibitor suberoylanilide hydroxamic acid. Nevertheless, the clear reactivation of *Maspin* with the methyltransferase inhibitor 5-aza-2'-dC suggested that *Maspin* was silenced by promoter methylation in the MOV5009 cell line. Our results are in agreement with previous mapping of methylated CpG islands by sodium bisulfate sequencing in human ovarian cell lines (30) and suggest that the *Maspin* promoter in metastatic ovarian cell lines is down-regulated by epigenetic mechanisms.

To demonstrate the binding of the mATF to its cognate sequence in the *Maspin* promoter, ChIP assays were performed in the MOV5009 cells (Fig. 2B). Chromatin from empty vector (control) and mATF-transduced cells was isolated, sonicated, and processed for immunoprecipitation assays using an anti-HA antibody to detect the tag engineered at the C terminus of the ZF construct (Fig. 2B). The ChIP products were amplified using primers specific for the *Maspin* promoter sequences flanking the mATF site, and the enrichment of the PCR products was evaluated by densitometry. As shown in Fig. 2B, mATF-transduced samples, but not controls, yielded the expected *Maspin*-specific 135-bp amplicon, suggesting that the mATF was physically bound to its cognate site in the *Maspin* promoter. Furthermore, when the ChIP assay was performed using an anti-RNA-polymerase II antibody, the immunoprecipitated DNA was enriched in the mATF samples relative to

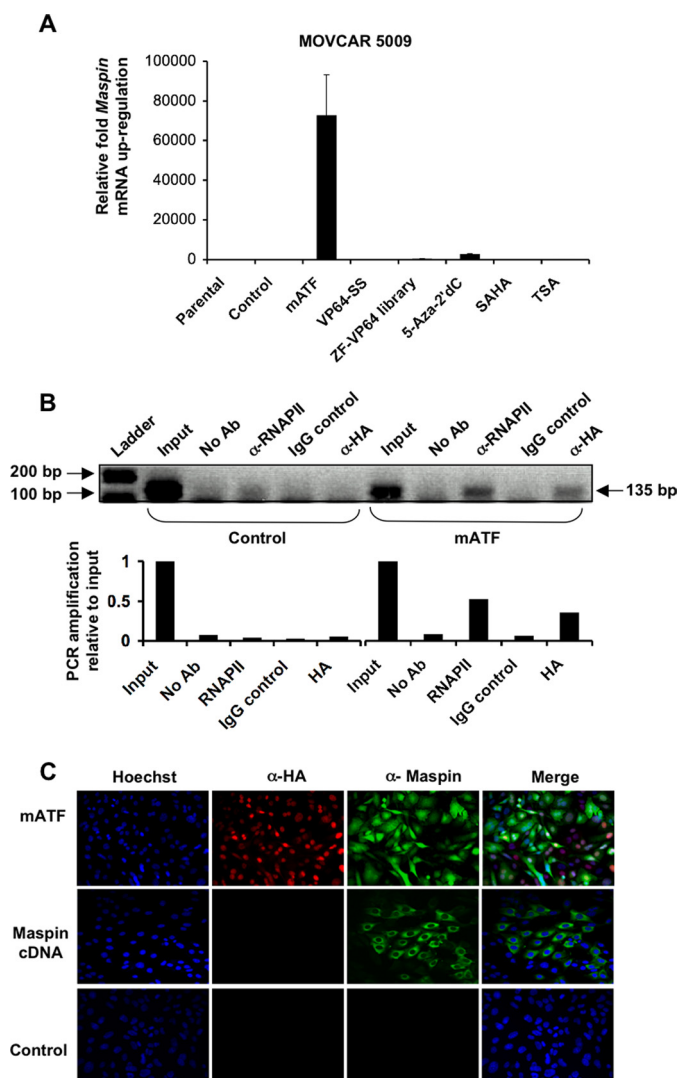


FIGURE 2. The artificial transcription factor mATF binds the *Maspin* promoter in the MOVCAR 5009 cell line and reactivates silenced *Maspin*. A, retroviral delivery of mATF specifically reactivates *Maspin*, as assessed by qRT-PCR. VP64-SS refers to cells transduced with a vector carrying the transactivator domain but lacking the 6ZF; ZF-VP64 indicates a scramble library of 3ZF domains linked to VP64 (69); Control refers to empty vector (pMX-IRES-GFP)-transduced cells. The parental cell line was treated with the epigenetic inhibitors: 5-aza-2'dC (5 μ M), suberoylanilide hydroxamic acid (10 μ M), and trichostatin A (100 nM) at saturating maximum doses. Data were normalized to the parental cell line. B, mATF physically binds its targeted site in the *Maspin* promoter. A ChIP assay was performed on the MOVCAR 5009 cell line retrovirally transfected with either control (empty vector) or mATF. α -RNA-polymerase II (RNAPII), IgG Control, and α -HA antibodies were used to immunoprecipitate the DNA-protein complexes. α -HA facilitated the immunoprecipitation of mATF. Densitometric analysis of the ChIP products loaded on agarose gels after PCR was carried out using the ImageJ software (NCBI, Bethesda, MD). Data were normalized to the signal of the input sample. Ab, antibody. C, retroviral transduction of mATF in MOVCAR 5009 cells results in reactivation of both cytoplasmic and nuclear Maspin. Immunofluorescence detected the nucleus (Hoechst 2258), the mATF expression (α -HA, red), and Maspin (green) in MOVCAR 5009 cells transduced with mATF, a Maspin cDNA-expressing retrovirus (18), and an empty retroviral vector or control.

control. This result suggests that the mATF transduction was increasing the RNA-polymerase II binding in the *Maspin* promoter, resulting in an enhanced transcriptional activity, as confirmed by qRT-PCR and Western blot (Fig. 1, B and C).

The reactivation of *Maspin* by mATF in the MOVCAR 5009 cell line was further confirmed by immunofluorescence (Fig.

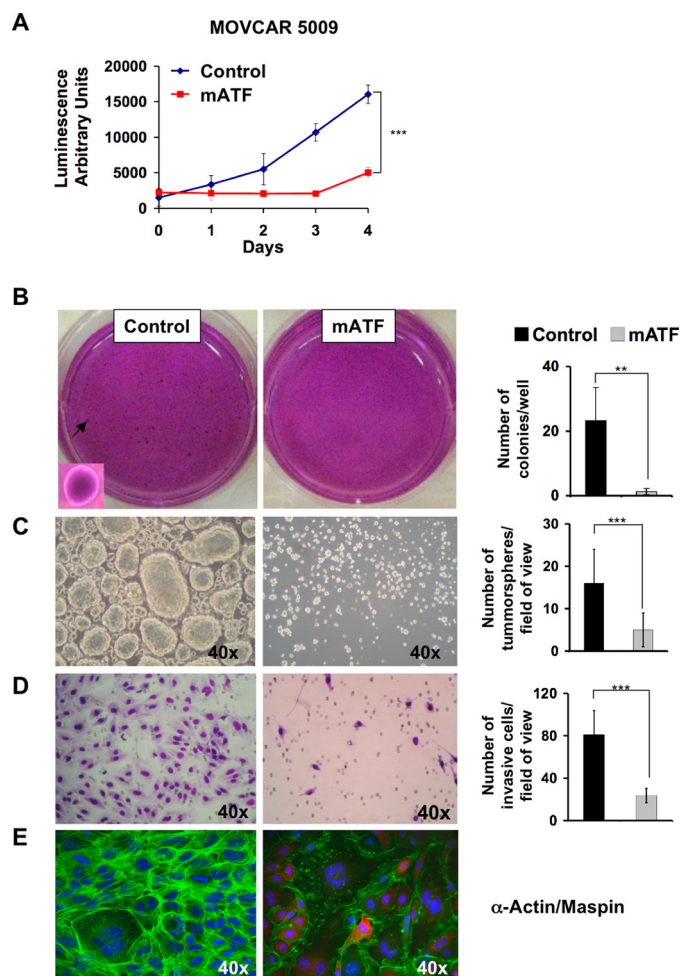


FIGURE 3. mATF expression induces a decrease in tumor cell viability and invasion and promotes cytoskeleton disruption in the MOVCAR 5009 cell line. A, retroviral delivery of mATF decreases cell viability over control-transduced cells as determined by Cell Titer Glo assay. Student's *t* test was applied to analyze the difference in tumor cell survival between the control and mATF cells, with $p < 0.001$. Three different transductions were performed, each one monitored in triplicate wells. B, mATF decreases anchorage-independent growth of MOVCAR 5009 cells. Soft agar colony formation assays were performed on control and mATF-transduced cells. The arrow in the picture (left) points to a colony amplified on the left corner. Colony quantification was performed 20 days after the seeding. Three independent experiments were carried out, and Student's *t* test was applied to analyze the data; $p = 0.0054$. C, mATF-transduced cells abolished tumor formation potential, as evaluated by tumorsphere assay formation. Cells were retrovirally transduced with either mATF or control and seeded in low-attachment culture plates with spheroid media (43). After 10 days, representative pictures of the experiment are shown outlining the abolishment of spheroid formation in mATF transduced cells. ($p = 0.0002$). D, mATF transduction in MOVCAR 5009 cells inhibits the invasion potential of the cells relative to control, as assessed by Matrigel-invasion assays (10). A representative picture of the fixed cells after 24 h of invasion is indicated. Three independent experiments were performed, and Student's *t* test was applied to analyze the data; $p = 0.0001$. E, immunofluorescence staining of actin (green) and Maspin (red) on mATF and control-transduced cells is shown. To detect actin, Alexa 488-phalloidin was applied, and anti-Maspin-Alexa 594 was used as the secondary antibody for Maspin detection. Hoechst 33258 was used to stain the nuclei (blue).

2C). In mATF-transduced cells, Maspin was found localized in the cytoplasm and in the nucleus of the tumor cells. When a *Maspin* cDNA-expressing retrovirus was used to exogenously express Maspin, only cytoplasmic Maspin was detected in the cells. The molecular basis of the differential Maspin localization observed between the mATF- and the cDNA-transduced cells is not known. A molecular hallmark of the ATFs is their ability

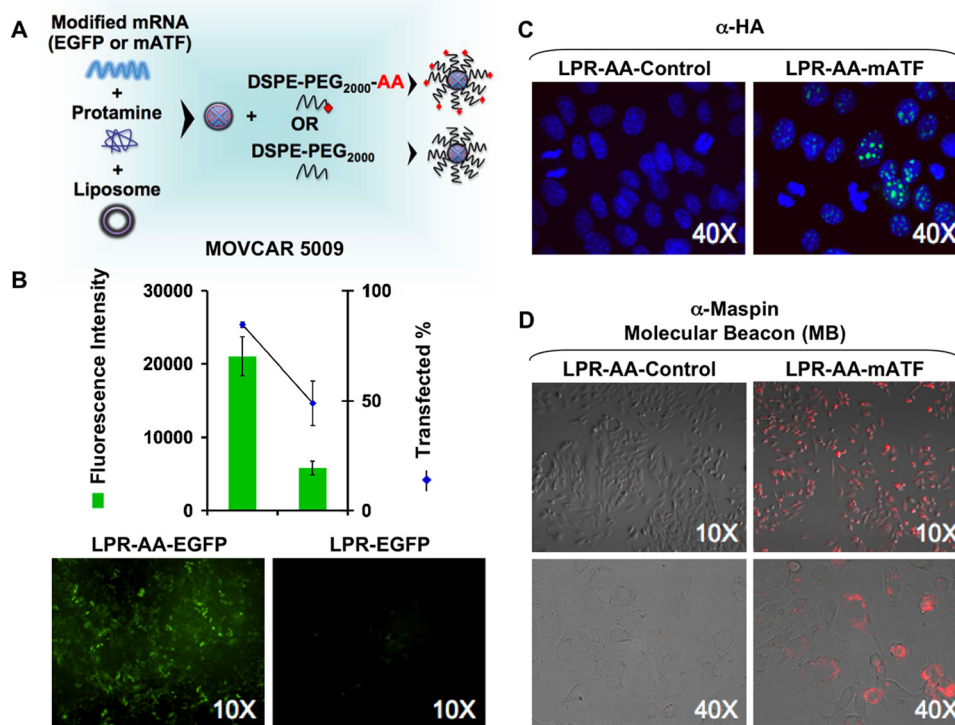


FIGURE 4. Targeted LPR-AA nanoparticles encapsulating modified mATF mRNA results in functional production of the mATF and regulation of *Maspin* in the MOVCAR 5009 cell line. *A*, shown is a schematic illustration of the generation of LPR-AA nanoparticles. The LPR nanoparticle core was prepared mixing modified mRNA (e.g. EGFP, mATF, or control, which is the mATF lacking the 5' cap and is unable to translate) plus protamine and liposome. The LPR core was pegylated by adding either DSPE-PEG₂₀₀₀-AA (targeted nanoparticle) or DSPE-PEG₂₀₀₀ (non-targeted). *B*, MOVCAR 5009 cells were transfected with either LPR-AA-EGFP (targeted) or LPR-EGFP (non-targeted) nanoparticles, and the fluorescence intensity (quantification of the total fluorescence intensity of EGFP+ events) was measured by flow cytometry 24 h post-transfection by gating the percentage % of EGFP-positive cells. Cells treated with nanoparticle controls not carrying EGFP and untreated cells were used to generate the negative gates. A representative 10 $\times$ picture of the transfected cells with LPR-AA-EGFP (targeted) or LPR-EGFP (non-targeted) nanoparticles is shown. *C*, shown is immunofluorescence detection of the mATF (α -HA, green) in MOVCAR 5009 cells. Cells were transfected with either LPR-AA-mATF or control (uncapped mATF mRNA). The cells were stained 24 h after transfection. *D*, real-time detection of *Maspin* in MOVCAR 5009 cells using an anti-*Maspin* molecular beacon (MB) is shown. Targeted nanoparticles containing either a functional mATF or an inactive control were also loaded with an anti-*Maspin* MB. Red fluorescence emission due to the hybridization of the *Maspin* transcript with the MB was detected 24 h post-transfection.

to activate the endogenous promoters (23), resulting in transcription of the physiologically relevant mRNA variants in the proper ratios, which represents a fundamental difference from the exogenous delivery of cDNAs (18).

mATF Expression Decreases Tumor Cell Viability and Inhibits Tumor Cell Invasion in MOVCAR 5009—To assess the effect of the mATF expression on the phenotype of MOVCAR 5009 cells, we first performed cell viability assays using a CellTiter Glo assay (Fig. 3*A*). The mATF-transduced cells and empty vector control were harvested 72 h post-transduction, and the cell viability was monitored every 24 h for a total of 4 days. We found that the ATF significantly decreased the tumor cell viability over time relative to control ($p < 0.001$). A significant decrease in cell viability was also observed when mATF was transduced in other MOVCAR cell lines carrying silenced *Maspin*, such as MOVCAR 5447 and 5612 (supplemental Fig. S2). Transduction of mATF also suppressed anchorage-independent growth of MOVCAR 5009 cells in soft agar colony formation assays (Fig. 3*B*). Furthermore, mATF completely suppressed the ability of MOVCAR 5009 cells to form tumorspheres in non-adherent plates, demonstrating that the engineered protein suppressed the tumorigenic properties of the cells *in vitro* (Fig. 3*C*). In addition to tumor suppression, *Maspin* re-expression in breast cancer cells has been associated

with decreased motility and invasion (10, 18, 49). Consistent with this second function of *Maspin*, we found that the mATF strongly suppressed the ability of MOVCAR 5009 cells to invade the Matrigel (Fig. 3*D*). Notably, the mATF-expressing cells underwent severe changes in cell shape, outlined by a disruption and loss of actin filaments (Fig. 3*E*).

LPR Nanoparticles Encapsulating a Chemically Modified mATF mRNA Are Efficiently Internalized into MOVCAR 5009 Cells *In Vitro* and in Tumor Allografts—We next investigated whether the mATF could be delivered in the tumor cells using a non-viral method by delivering chemically modified messenger RNA, eliminating the possibility of undesired genomic integration due to viral vector or plasmid DNA delivery. We generated LPR nanoparticles encapsulating the mATF mRNA. LPRs are liposome-based nanocarriers capable of delivering chemically modified RNAs to the targeted tumor site with high efficiency and specificity (Ref. 76; Fig. 4*A*). The chemical ligand anisamide (AA) was grafted at distal end PEG polymer on the surface of LPR particles to target the sigma receptors (SRs), which are overexpressed in a large spectrum of human tumors and cell lines (50). In our study we employed LPR-AA nanoparticle technology to deliver the mATF to the MOVCAR 5009 cell line. The expression of SR1 in MOVCAR 5009 cells was confirmed by Western blotting (supplemental Fig. S3). To evaluate the

transfection efficiency of the LPR-AA formulation, we encapsulated an mRNA encoding EGFP, and the expression level and percentage of EGFP⁺ cells were evaluated by flow cytometry. As shown in Fig. 4B, MOVCAR 5009 cells were transfected with ~85% efficiency and, thus, with a yield comparable with retroviral or lentiviral vectors. In addition, the transfection efficiency was ligand-dependent. AA-LPR resulted in a 3-fold transgene expression reflected by fluorescence intensity and a 2-fold percent of transfected cells compared with non-targeted LPR. This implied that efficient gene delivery with high specificity was achieved as a result of enhanced receptor mediated cellular uptake. Next, we investigated whether the delivery of functional LPR-AA nanoparticles encapsulating the mATF mRNA resulted in efficient translation of the mATF and in nuclear localization of the designer protein. The expression of mATF was detected by immunohistochemistry using an anti-HA antibody to detect the C-terminal epitope tag of the protein. As shown in Fig. 4C, the functional mATF, but not an inactive control ATF mRNA without 5' cap structure, was detected in punctate structures in the nucleus of the cells, demonstrating effective translation. To investigate whether the mATF was transcriptionally competent in the cells, we co-transfected either the mATF mRNA or the uncapped ATF mRNA control, with a molecular beacon designed to specifically hybridize with *Maspin* mRNA. In the presence of untranslated control mRNA, no *Maspin* mRNA transcript was detected by the molecular beacon in the cells. However, when active mATF mRNA was included in the nanoparticle, an activation of endogenous *Maspin* mRNA was detected by the molecular beacon probe that allowed the real-time imaging of *Maspin* mRNA transcripts with a perinuclear localization (Fig. 4D). Overall, these studies demonstrated the delivery of our designer ZF protein into nanoparticles to target the ovarian cancer cells under study and effective mATF translation leading ultimately to *Maspin* re-expression.

At last, the ability of mATF-loaded nanoparticles to induce phenotypical changes in the cell line was evaluated *in vitro* (Fig. 5A). We found that LPR-AA-mATF nanoparticles significantly decreased survival of the MOVCAR 5009 cells by ~40% relative to control-loaded nanoparticles, as determined by a cell viability MTT assay (Fig. 5A). To assess the efficiency of LPR-AA nanoparticles to target MOVCAR 5009 cells *in vivo*, LPR-AA-mATF and control nanoparticles were delivered via intravenous injections into mice bearing MOVCAR 5009 subcutaneous allografts. Nanoparticle injections were performed via tail vein every 2 days with a total of 5 treatments. 24 days post-injection, a significant inhibition of tumor growth was detected on LPR-AA-mATF-injected animals relative to control ($p = 0.0220$) or vehicle-treated animals ($p = 0.0253$) (Fig. 5B). Tumor samples were processed by Western blot to demonstrate effective expression of the mATF in the tumors and concomitant *Maspin* regulation (Fig. 5C). In summary, these studies demonstrated that nanoparticle delivery of mATF mRNA led to a significant therapeutic effect inhibiting tumor cell growth of MOVCAR 5009 tumor allografts.

Genome-wide Changes in Gene Expression by mATF Delivery in MOVCAR 5009 Cells—To address the specificity of mATF in the MOVCAR 5009 cells, we performed gene expression

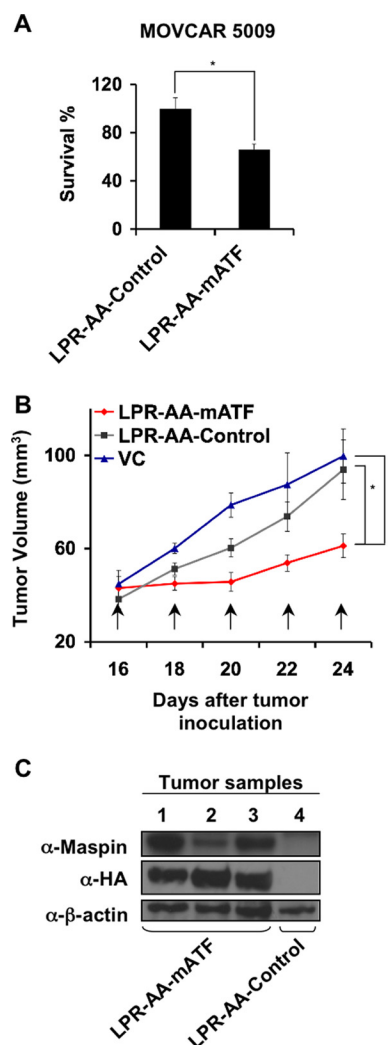


FIGURE 5. Transfection of LPR-AA-mATF nanoparticles decreases the proliferation of MOVCAR 5009 cells *in vitro* and inhibits tumor growth *in vivo*. A, delivery of LPR-AA-mATF nanoparticles decreases survival of MOVCAR 5009 cells relative to control-treated targeted nanoparticles. Cell survival was determined by MTT cell proliferation assay on cells transfected with either LPR-AA-mATF or control. Student's *t* test was applied to analyze the difference on survival between mATF and control-transfected cells; $p = 0.0002$. Data represent the averages and S.E. of three independent experiments, each one done in triplicate wells. B, LPR-AA-mATF nanoparticles inhibit the growth of MOVCAR 5009 cells in subcutaneous allografts in nude mice. MOVCAR 5009 cells were inoculated in nude mice to induce subcutaneous tumors, and nanoparticles were injected intravenously at the time points indicated in the graph (arrows). Student's *t* test was applied to analyze the difference between control ($n = 9$) and mATF treatment ($n = 9$; $p = 0.0220$) and between vehicle and mATF ($p = 0.0253$). C, mATF and *Maspin* expression were detected on the treated tumors by Western blot. An anti-HA antibody was used to detect the ATF by Western blot.

microarrays using Agilent mouse 4×180 K oligo microarrays. Cells were transduced in three independent biological replicates with either an empty retroviral vector (control) or with a mATF-expressing retrovirus. Significantly differentially expressed targets were analyzed using a two-class, unpaired SAM, with a false discovery rate $<5\%$. Supplemental Tables S1 and S2 contain the list of genes significantly up-regulated (505 genes) and down-regulated (383 genes), respectively. A gene ontology (GO) analysis of the gene sets revealed gene pathways involved in nuclear signaling, signal transduction by small GTPases, cell motility, and adhesion (supplemental Table S3).

TABLE 1

Genes differentially regulated by mATF in the MOVCAR 5009 cell line

Name	Alias	-Fold change ^a	Function
Up-regulated genes			
Tetraspanin12	TM4SF12 TSPAN12	42.17	Member of the tetraspanin family of cell-surface proteins mediating signal transduction. (70)
Mammary serine protease inhibitor	Maspin SERPINB5	31.56	Tumor and metastasis suppressor gene. (27)
Calcium and integrin binding family member 2	CIB2 KIP2	30.79	A related family member, CIB1, regulates cell migration. (71)
Potassium large conductance calcium-activated channel, subfamily M, β member 4	KCNMB4 h β 4	29.07	Auxiliary β subunit on MaxiK channels, increases calcium sensitivity. (72)
Potassium channel tetramerisation domain containing 12	KCTD12 PFETIN	26.15	Positive prognostic biomarker in gastrointestinal tumors. (73)
Down-regulated genes			
Nidogen1	NID1 Enactin	0.0617	Suggested to play a role in cell interactions with the extracellular matrix. (74)
CCAAT/enhancer-binding protein δ	CEBPD CELF	0.1266	Up-regulated in breast (75) and lung (76) cancers.
Wingless-related MMTV integration site 10a	WNT10A OODD	0.1171	Activator on the Wnt- β -catenin-TCF signaling pathway (77).

^a Relative to control (empty vector).

Notably, the gene expression microarray datasets revealed only 3 genes, including *Maspin*, with a relative mRNA up-regulation of 30-fold or higher over empty vector control (Table 1). These findings were validated by qRT-PCR, as indicated in Fig. 6A. The strongest mATF up-regulated target was *Maspin*, outlining the high degree of potency and specificity of the mATF in MOVCAR 5009 cells. The genes Tetraspanin12 (*Tspan12*), calcium and integrin binding family member 2 (*Cib2*), potassium channel tetramerization domain containing 12 (*Kctd12*), and potassium large conductance calcium-activated channel, subfamily M, β member 4 (*Kcnmb4*) were also found up-regulated in mATF-transduced samples relative to control-transduced or parental line ($p < 0.0001$). As shown in Table 1, the genes up-regulated by mATF are membrane-associated proteins involved in signal transduction, cell adhesion and migration, and tumor suppression. The down-regulated targets (≈ 0.13 -fold relative to control) included potential oncogenes such as Wingless-related MMTV integration site 10a (*Wnt10a*) and CCAAT/enhancer binding protein δ (*Cebpd*) and regulators of cell adhesion, such as Nidogen1 (*Nid1*) (Fig. 6B). In summary, the analysis also revealed important novel targets co-regulated with *Maspin*, which could mediate the anti-metastatic and anti-tumorigenic phenotype of the ZF factor in EOC cell lines. Whether these targets represent indeed *bona fide* direct targets of the ATF or downstream genetic cascades of *Maspin* is presently not known and will require future molecular analyses, for example by zinc finger mapping in their promoter sites. However, these genome-wide data documented a high degree of potency and selectivity of mATF for its cognate *Maspin* target in MOVCAR 5009 cells.

Maspin and TSPAN12 Are Down-regulated in Short-term Cultures Derived from Human Ascites—We focused on the target TSPAN12, a member of the tetraspanin family of cell-surface proteins, some of which have been reported to mediate metastasis suppressive functions in several cancer models (51). We interrogated whether *TSPAN12* was co-regulated with *Maspin* in a panel of primary ovarian cancer tumors specimens and cell lines and whether the gene was silenced in ascites preparations from ovarian cancer patients. These samples were analyzed for *Maspin* and *TSPAN12* expression by qRT-PCR (Fig. 7A). Data were normalized to the SR06 sample, a normal total

ovary preparation. We found that *Maspin* was expressed in some ovarian primary tumors specimens and ovarian cell lines but was low or undetectable in most of the ascites samples. *TSPAN12* mRNA expression followed a similar pattern and was significantly lower in the vast majority of the ascites analyzed as compared with primary tumors and ovarian cancer cell lines. In one case examined, from which pair samples of tumor and ascites were available, the primary tumor (UC064) had significantly higher *Maspin* and *TSPAN12* mRNA expression than the ascites derived from the same patient (ASCUC064). As shown in Fig. 7D and supplemental Fig. S5, the expression of *Maspin* and *TSPAN12* in primary EOC tumors was confirmed by immunofluorescence in epithelial ovarian cancer tissue specimens, *Maspin* having a predominantly cytoplasmic distribution, whereas *TSPAN12* was localized on the plasma membrane. The down-regulation of *Maspin* and *TSPAN12* in the short-term cultures from ascites fluids was also validated by Western blotting using the OVCAR-3 tumor cell line as reference (Fig. 7B).

The above results suggest that *Maspin* and *TSPAN12* are down-regulated in metastatic cells, opening the possibility that *TSPAN12* could be a downstream target of *Maspin* in ovarian cancer. Alternatively, *TSPAN12* could be regulated as a result of mATF-dependent gene cascades, for example, by direct binding of the ATF or by indirect regulation of downstream targets. To address whether the activation of *TSPAN12* in mATF-transduced cells was dependent on *Maspin*, we challenged MOVCAR 5009 cells transduced with mATF with either a *Maspin*-specific siRNA or a control siRNA. Changes in *TSPAN12* were assessed in the transfected cells by qRT-PCR. As shown in Fig. 7C, the *Maspin* knockdown resulted in a significant down-regulation of *TSPAN12* in the MOVCAR 5009 cells, suggesting that *Maspin* could be controlling *TSPAN12* activation in these cells. In addition, we were unable to find putative binding sites of the mATF in the *TSPAN12* promoter, suggesting that *TSPAN12* is not a direct target of the ATF. Moreover, more analyses need to be done, for example, using genome-wide ZF mapping to validate the exact number and genomic location of all mATF-binding sites in the cancer cell genome. In summary, the gene expression analyses demonstrated that the ZF technology could be used for cancer inves-

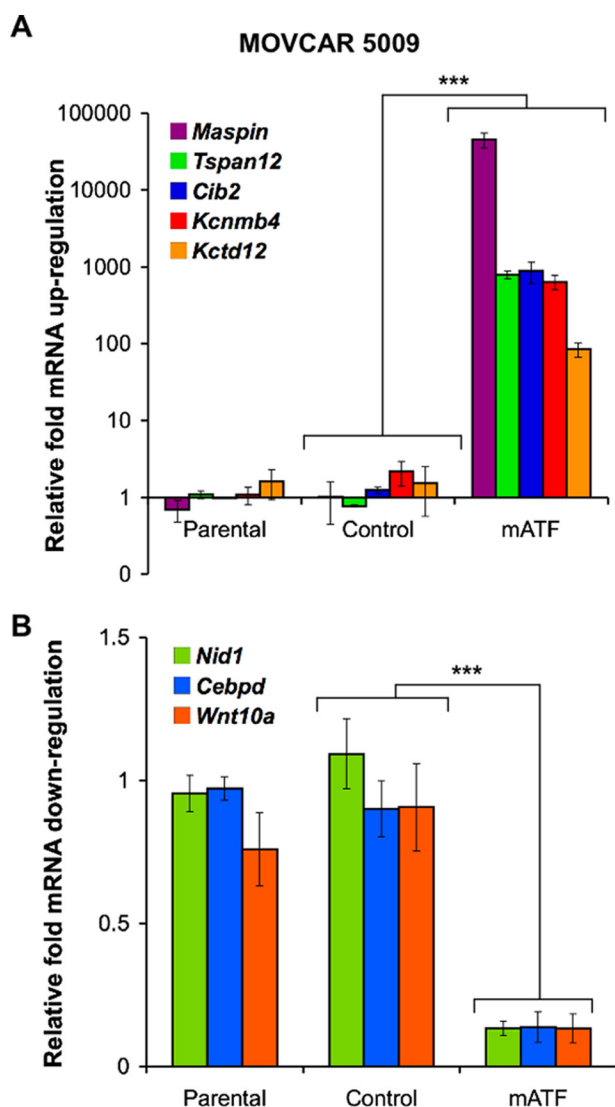


FIGURE 6. mRNA quantification of targets up- and down-regulated by mATF in MOVCAR 5009 cells. Changes in mRNA expression of genes previously identified by gene expression microarrays (supplemental Table S1 and Table S2) were validated by qRT-PCR. **A**, genes differentially up-regulated by transduction of mATF in MOVCAR 5009 cells are shown. **B**, genes down-regulated by mATF are shown. Data were normalized to the parental MOVCAR 5009 cell line and represent the mean and S.D. of at least three independent experiments. Differences in gene expression were calculated with a student *t* test; ***, $p \leq 0.0001$.

tigators to identify novel targets potentially involved in migration and metastatic progression for future diagnosis and treatment of aggressive metastatic disease.

DISCUSSION

Mammary serine protease inhibitor (*Maspin*) has emerged as an important clinical target possessing tumor and metastasis suppressive functions. Although the function of *Maspin* has been well examined in breast (52), lung (53), and prostate (54) models, its function as a tumor and metastasis suppressor in ovarian cancer has not been well investigated. In this paper we took advantage of ZF technology to interrogate the role of *Maspin* in metastatic ovarian cancer. We have described an mATF able to up-regulate endogenous *Maspin* in metastatic murine ovarian cancer cell lines derived from a TgMISRII-TAg GEMM

of serous ovarian cancer (9). The mATF had an exceptionally strong transcriptional activation in multiple murine cancer cell models derived from GEMMs, with levels of expression that were comparable with ectopic cDNA overexpression. The higher transcriptional potency of the mATF relative to the previously reported human-specific ATF (ATF-126) could be related to the configuration of the chromatin and the promoter context of the mouse promoter. Moreover, MOVCAR 5009 cells derived from ascitic fluids from the C57BL/6 TgMISRII-TAg GEMM exhibited an epigenetically silenced *Maspin*, which could be partially re-activated by DNA methyltransferase (DNMT) and histone deacetylase inhibitors. In this background the mATF was able to bind its target site in the cell and reactivated *Maspin* mRNA expression by more than 70,000-fold relative to control-transduced and parental cells. The much higher potency of the ATF in activating *Maspin* relative to chromatin remodelers could be explained by the fact that the ZFs confer locus selectivity. In contrast, epigenetic inhibitors, such as DNA methyltransferase (DNMT) and histone deacetylase inhibitors, are believed to target a large spectrum of tumor suppressors. In addition, the *Maspin*-specific ZFs are linked to the VP64 transactivator domain, which enhances transcription by recruitment of several components of the transcription machinery (55). Consistently, our ChIP analysis demonstrated an enrichment of RNA-polymerase II binding in the *Maspin* promoter upon mATF overexpression. Interestingly, the transduction of mATF elevated both cytoplasmic and nuclear forms of Maspin, whereas the exogenous cDNA overexpression up-regulated only cytosolic Maspin. Recent publications outline the importance of nuclear Maspin as being a favorable prognosis factor in breast (56), lung (57), and ovarian carcinomas (33). This result emphasizes the significance of the endogenous mechanisms of regulation of targeted promoters, which results in activation of the physiologically relevant isoform of the targeted gene. The phenotypic outcomes of *Maspin* reactivation in metastatic MOVCAR 5009 cells recapitulated the effect of the human ATF-126 in breast cancer models (10, 18). Retroviral delivery of mATF resulted in decreased cell viability, tumorigenicity, and strongly suppressed cell invasion. mATF induced profound remodeling in the actin cytoskeleton, which could have a negative impact in cell adhesion, migration, and mitosis. mATF-transduction induced the formation of multiple focal adhesions, indicating the cells were less prone to migration.

Our data are consistent with previous studies showing that treatment with a recombinant Maspin protein, rMaspin, resulted in increased adhesion and inhibition of cell invasion of the breast carcinoma MDA-MB-435 cell line (58). rMaspin led to changes in the cytoskeleton structure and increased the formation of focal adhesions in metastatic breast cancer cells (59). Our results with our designer protein confirm the role of *Maspin* as metastasis suppressor in metastatic ovarian cancer by modulating cell surface signaling pathways resulting in cytoskeleton remodeling.

To evaluate the specificity of the mATF in MOVCAR 5009 cells and to identify genes mediating the anti-tumor/metastatic phenotype of the protein, we performed genome-wide gene expression microarrays. These analyses revealed that only two

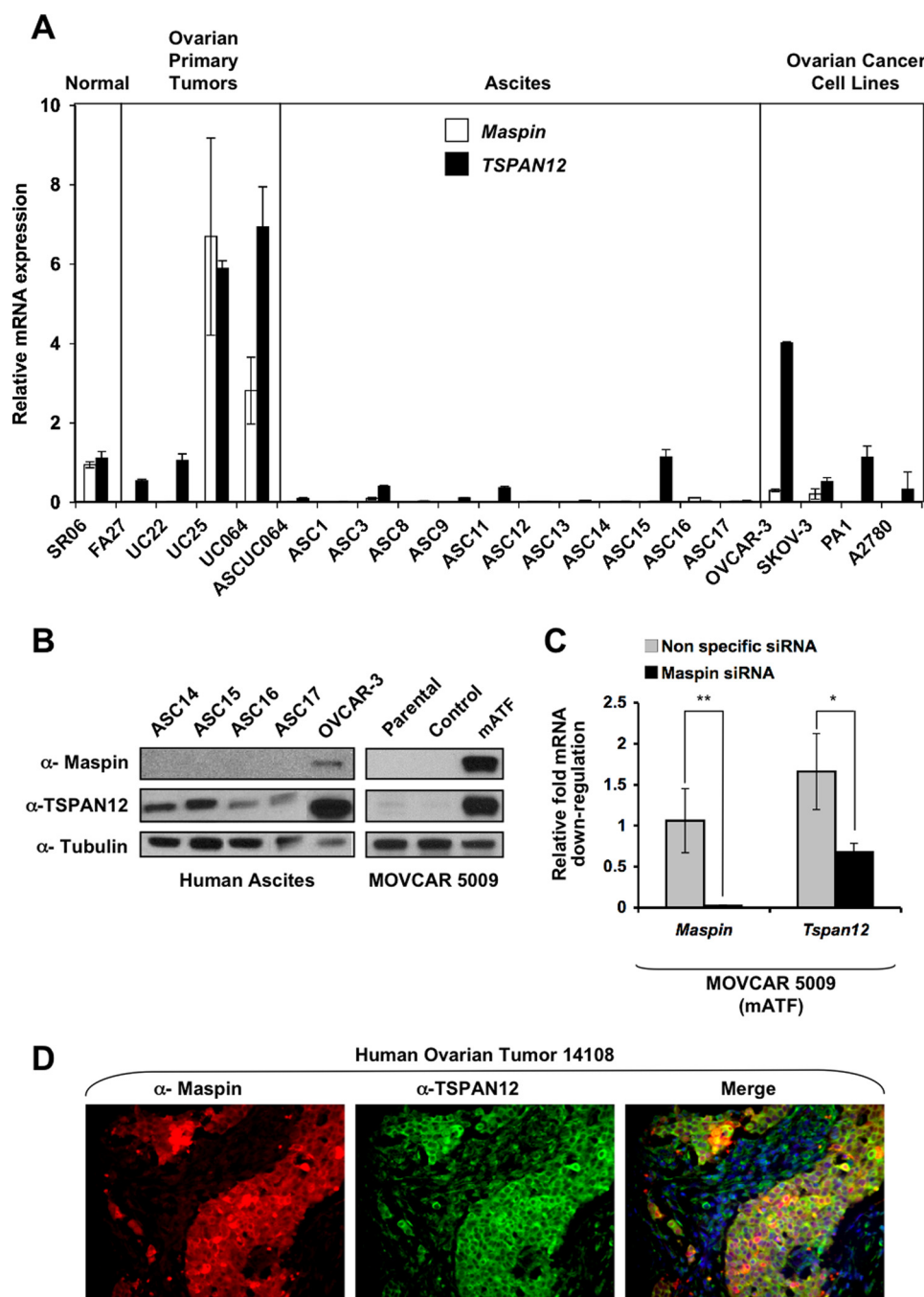


FIGURE 7. Down-regulation of *Maspin* and *TSPAN12* in short term human ascites preparations from ovarian carcinoma patients. *A*, expression of *Maspin* and *TSPAN12* in a panel of human ovarian primary tumors, ascites, and established cancer cell lines, as assessed by qRT-PCR is shown. Data were normalized to SR06, a preparation of normal ovary. UC064 and ASCUC064 represent tumor sample and ascites preparation, respectively, from the same patient. Tumor specimens, ascites preparations, and ovarian cell lines were homogenized and resuspended in Trizol for mRNA extraction and analyzed by quantitative qRT-PCR. *B*, shown is an immunoblot of *Maspin* and *TSPAN12* on protein extracts from human ascites, the human cell line OVCAR-3, and parental, control, and mATF-transduced MOVCAR 5009 cells. *C*, down-regulation of *Maspin* and *Tspan12* after *Maspin* siRNA knockdown in the mATF-transduced MOVCAR 5009 cell line as assessed by qRT-PCR is shown. Data were normalized to the cell line transfected with a control (anti-luciferase) siRNA (*, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$). *D*, shown is expression of *Maspin* and *TSPAN12* by immunofluorescence in representative sections of epithelial ovarian cancer specimens (see also supplemental Fig. S5).

targets were up-regulated together with *Maspin*, with a -fold overexpression higher than 30 relative to control-transduced cells. The subsequent qRT-PCR validation of these targets demonstrated that *Maspin* was the most regulated target of the ATF, outlining the high potency and specificity of the engineered factor.

TSPAN12 belongs to the tetraspanin family of signal transduction proteins, many of which act as tumor suppressors and regulators of metastasis (51). Tetraspanins are transmembrane proteins that physically associate with integrins to transduce signals inside the cell (60). CD9 is a tetraspanin involved in regulation of cell adhesion and motility (61). Down-regulation

of tetraspanin CD9 has been associated with metastatic behavior and poor prognosis in lung (62), breast (63), and ovarian cancers (64). CD9 down-regulates the expression of several Wnt-associated proteins in lung adenocarcinoma and fibrosarcoma cell lines (65). Interestingly, mATF delivery in MOVCAR 5009 cells led to an up-regulation of (TSPAN12) and a down-regulation of Wnt10a suggesting that modulation of Wnt signaling could be of therapeutic value for treatment of metastatic ovarian cancer. Our results identified a novel target, *TSPAN12*, that was found down-regulated together with *Maspin* in human metastatic ovarian cancer cells derived from ascites. In summary, these results demonstrate that ZF could be used to potentially discover novel molecular targets and regulators of metastasis in ovarian cancer.

Delivery of ZF proteins has been an unbearable problem for the transition of these agents into the clinic. In addition to effective delivery in pre-existing tumors, another fundamental problem is the specificity to the tumor tissue. To deliver the mATF in MOVCAR 5009 tumor allografts, we developed LPR-AA nanoparticles. The anisamide (AA) ligand coated at the surface of the particles provides specificity for the sigma receptor (SR1) (44), which is expressed at high levels in a vast spectrum of cancer cell lines (50), including the MOVCAR 5009 cells. These nanoparticles were designed with a condensed core comprising protamine and a chemically modified mATF mRNA. The delivery of modified mRNA circumvents limitations associated with traditional plasmid DNA, such as nuclear delivery of DNA through the nuclear envelope and host immunoresponse (76). *In vitro* synthesis of chemically modified RNA using ribonucleotide analogues has been shown to suppress the innate immune response by stimulating toll-like receptor and concomitantly extend the half-life of the mRNA molecule (66). Previous work has demonstrated that chemically modified mRNA can be used for delivery of reprogramming transcription factors for inducible pluripotent stem cells *in vitro* (66). Indeed, we have found that our LPR-AA nanoparticles transfected MOVCAR 5009 cells with efficiencies similar to retroviral delivery (~60%). In addition, the same LPR formulation was able to transfect some breast cancer cell lines with efficiencies close to 100% (data not shown) outlining the potential of the technology to effectively deliver ZF proteins in tumor models. The LPR nanoparticles were formulated such that high density of hydrophilic PEG was coated on the surface of the nanoparticles. The PEG coating stabilized LPR in the presence of serum proteins and reduced nonspecific uptake by reticuloendothelial system, extending the half-life in the blood circulation after systemic administration. This led to the enhanced tumor accumulation due to the enhanced permeability and retention. Notably, we demonstrated that the LPR-mATF-AA nanoparticles resulted in translation of ZF proteins in the tumor cells and in transactivation of the endogenous *Maspin*. When delivered intravenously in a MOVCAR 5009 allograft model, the LPR-mATF-AA nanoparticles significantly inhibited tumor growth, which was accompanied by *Maspin* reactivation in the tumors. These results are very encouraging and provide the first non-viral delivery of ZF proteins *in vivo* for its potential use in clinical trials to treat metastatic disease. Although our results demonstrate proof-of-concept using nude mice, the next step will

require the intravenous injection of LPRs in a syngeneic or even in the GEMM C57BL/6 TgMISRII-TAg of ovarian cancer, which develops ovarian cancer in a similar progression as in humans (9). In addition, future research will involve the intraperitoneal injection of LPRs in mice carrying abdominal metastases to assess the efficiency of the mATF to abolish pre-established metastases. Given the explosion of publications using both ZF and transcription activator-like effectors (TALEs) technologies in the past decade (particularly in the domain of gene therapy (67)), our results provide a novel non-viral delivery system of mRNA to be used for a broad spectrum of investigators working in the area of designer ZF factors.

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