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Posgrado en Biología Experimental**

GDF11 represses the STAT3 signaling pathway, decreasing the aggressiveness of cells derived from human hepatocellular carcinoma.

TESIS

Que para obtener el grado de

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PRESENTA

Melissa Sánchez Rodríguez
Matricula: 2241800020
CVU: 1248040
Correo: msr171199@gmail.com

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Vocal: Dra. Elsy Soraya Salas Silva
Universidad Hanyang. Seúl, Corea del Sur



Vocal: Dra. Monserrat Gerardo Ramírez
Universidad de Virginia

Tutorial Committee



Codirector: Verónica Souza Arroyo, Ph.D.
Universidad Autónoma Metropolitana Unidad Iztapalapa,
Depto. Ciencias de la Salud, DCBS
SNI: Nivel I
veso@xanum.uam.mx



Codirector: Arturo Simoni-Nieves, Ph.D.
Weizmann Institute of Science. Rehovot, Israel
SNI: Nivel I
arturo.simoni-nieves@weizmann.ac.il



Advisor: Monserrat Gerardo-Ramírez, Ph.D.
University of Virginia, Charlottesville, VA, USA
Pqw5dz@virginia.edu

Dedicatoria

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Resumen

La enfermedad hepática asociada a disfunción metabólica (MASLD, antes MAFLD) durante las últimas décadas ha aumentado debido al estilo de vida que seguimos, al aumento de la vida sedentaria, a una dieta de ultraprocesados, grasas y azúcares, además de factores genéticos (Hannou, Haslam, McKeown, & Herman, 2018; Rinella, Lazarus, et al., 2023). Esta enfermedad afecta al 32% de la población mundial (Teng et al., 2023) y se estima que el 50% de la población en México la padece, lo que la convierte en una de las principales causas de trasplante hepático (Bernal-Reyes et al., 2019; Fraile, Palliyil, Barelle, Porter, & Kovaleva, 2021).

La progresión de la enfermedad va desde MASLD hasta un hígado con esteatohepatitis (MASH), a fibrosis, cirrosis y culmina en un carcinoma hepatocelular (HCC) (Dominguez-Perez et al., 2019). El HCC representa el 90% de los tumores hepáticos primarios y constituye una de las principales causas de mortalidad hepática (Asafo-Agyei & Samant, 2024).

El HCC derivado directamente del MASH puede saltarse algunos estadios de la enfermedad, como la fibrosis y la cirrosis, lo que sugiere mecanismos tumorales distintos. Es importante recalcar que, según los datos, entre 20-25% de la población desarrollará MASH y se estima que hasta el 56% lo hará durante la próxima década (X. Wang, Zhang, & Dong, 2025).

Se ha observado que las dietas ricas en lípidos (sobre todo en colesterol) tienen un gran impacto en las vías oncogénicas, como la de STAT3. Un perfil transcriptómico enriquecido en esta vía se asocia con una mayor agresividad tumoral y un peor pronóstico (Simoni-Nieves et al., 2021).

Existen algunos inhibidores aprobados por la FDA conocidos como jakinibs (inhibidores de las JAK quinisas), los cuales inhiben principalmente a JAK2; sin embargo, estos inhibidores aún no son una terapia prometedora porque apenas reducen la activación de STAT3 en HCC, pues esta activación puede ser por vías alternativas, por lo que se propone una inhibición dual de ambas fosforilaciones de STAT3, como una alternativa más prometedora (José M. Serra López-Matencio, 2018; Jr, 2005).

El factor de crecimiento y diferenciación 11 (GDF11) pertenece a la súper familia del factor de crecimiento transformante beta (TGF- β), la cual consta de factores de crecimiento que regulan la proliferación y diferenciación de muchos tipos celulares, siendo fundamental en la organogénesis temprana, sin embargo, conforme el organismo va dejando atrás las etapas embrionarias disminuye el contenido de dicha proteína,

manteniendo una mayor expresión en el hipocampo, mientras que en el hígado se encuentran los niveles más bajos (Simoni-Nieves et al., 2019).

El GDF11 es una posible terapia; diversos estudios han mostrado su actividad antitumoral en diversos tumores, principalmente en HCC y leucemia, pues reduce la agresividad de las células, como la proliferación y la migración celular, además de modular el metabolismo de lípidos, glicólisis y la función mitocondrial. Estos cambios persisten incluso días después de retirar el tratamiento (Gerardo-Ramirez et al., 2019; Hernandez et al., 2021; Sánchez-Rodríguez. et al., 2025; Simoni-Nieves et al., 2019).

Al verse afectadas tantas rutas metabólicas, se sugiere que la vía de señalización de STAT3 es el blanco clave del tratamiento con GDF11. STAT3 (Signal Transducer and Activator of Transcription 3) es un factor de transcripción que regula varios procesos celulares, siendo el regulador central del metabolismo lipídico y del colesterol, pues la vía JAK2-STAT3 incrementa la expresión de SREBP2, lo que conduce a una mayor expresión de una enzima limitante en la síntesis del colesterol como HMGCR, lo que una clara activación aberrante se vincula con la acumulación de grasa en el hígado (Tse, Warner, Farook, & Cronin, 2020; Zhao et al., 2018).

Hipótesis

GDF11 inhibe la vía de señalización de STAT3 en células de carcinoma hepatocelular humano (HCC), reduciendo sustancialmente la agresividad tumoral a través de mecanismos moleculares complejos. Al interferir con cascadas de señalización oncogénicas críticas, GDF11 modula el comportamiento celular, transformando el fenotipo maligno de las células de HCC.

GDF11 media sus efectos antitumorales suprimiendo de manera integral el potencial proliferativo, las capacidades migratorias y las características invasivas. Esta supresión ocurre mediante modificaciones transcripcionales y post-transcripcionales de moléculas clave de señalización, alterando finalmente el entorno pro-tumorigénico modulado por varias vías activadas, como las SMAD, MAPK, AKT y, sobre todo, por la vía de STAT3.

La intervención molecular de GDF11 va más allá de los mecanismos celulares directos, pudiendo influir en la dinámica del microambiente tumoral, las respuestas inflamatorias y la reprogramación metabólica. Al dirigirse a múltiples procesos oncogénicos de manera simultánea, GDF11 surge como un regulador molecular prometedor con un potencial significativo para tratar la progresión del carcinoma hepatocelular.

Pregunta de investigación

¿El GDF11 tiene un papel en la represión de la vía de señalización de STAT3 disminuyendo la agresividad de las células derivadas de HCC?

Objetivo general

Evaluar el mecanismo molecular por el que el GDF11 modula la vía de señalización de STAT3 en células de HCC bajo condiciones que asemejan la enfermedad (MASLD) y caracterizar los efectos subsecuentes en la agresividad y el metabolismo lipídico celular.

Objetivos particulares

- Evaluar el perfil transcriptómico de la línea celular Huh7 derivada de HCC en respuesta al tratamiento de corta duración con GDF11.
- Evaluar el mecanismo por el cual el GDF11 reprime la vía de señalización de STAT3 en un modelo celular con sobrecarga lipídica.
- Desarrollar e implementar por ingeniería genética un sistema de inhibición de STAT3 para comparar los efectos del modelo con GDF11.
- Evaluar el impacto de la represión de STAT3 mediada por el GDF11 en la agresividad de las células derivadas de HCC.

Resultados

Desregulación de la vía oncogénica STAT3 bajo el tratamiento con GDF11

Como se puede observar en la figura 4 (A), obtuvimos una excelente secuenciación de ARN en células Huh7 tratadas con GDF11 (50 ng/ml) durante 12 horas. Por lo tanto, en la figura 4 (B), que es un mapa de calor, observamos 1450 genes expresados diferencialmente, donde también se puede observar la expresión diferencial entre los dos grupos. Además, en la figura 4 (C) podemos analizar las diferentes vías de señalización que se desregulan con el tratamiento, la mayoría de las cuales están relacionadas con la vía oncogénica STAT3 (entre ellas, la vía mTOR, HGF, apoptosis, etc.).

Posteriormente, se creó un gráfico de volcán a partir de los datos obtenidos en la figura 5 (A), mostrando genes sobreexpresados (en naranja) y regulados a la baja (en azul).

La vía STAT3 está mayormente desregulada bajo el tratamiento con GDF11. De manera similar, la figura 5 (B) mostró que la mayoría de las proteínas blanco de la vía de señalización STAT3 están desreguladas. Específicamente, los genes involucrados en la proliferación (como MYC), metástasis (WNT5A), regulación del ciclo celular, apoptosis (MCL-1) y transición epitelio-mesénquima (β -catenina) fueron afectados, todos ellos regulados a la baja, mientras que se observó sobreexpresión de algunos supresores tumorales y reguladores negativos de STAT3.

Usando el software Integrity Pathway Analysis (IPA), confirmamos que las vías afectadas por el tratamiento con GDF11 están relacionadas con STAT3, como la fosforilación oxidativa, el metabolismo de esfingolípidos, el transporte de electrones, la señalización de sirtuinas, la fibrosis, las integrinas, los mecanismos moleculares del cáncer, la señalización de TGF- β . Con la ayuda del software IPA, analizamos la desregulación de algunas proteínas involucradas en la vía de señalización STAT3, particularmente PIAS (Protein Inhibitors of Activated STATs) y algunas otras fosfatasa como PP2A, por lo que los datos sugieren que la disminución en la activación de STAT3 podría deberse a una de estas fosfatasas.

En la figura 8 (A) también mostramos cómo las células tratadas con GDF11 se correlacionan con pacientes con HCC con un mejor pronóstico (clúster 2), mientras que las células Huh-7 no tratadas se agrupan con pacientes con un peor pronóstico (clúster 1). En la figura 8 (B), el clúster 1 muestra una mejor supervivencia, menor promoción de la invasión y una respuesta más tardía a TGF- β .

En la figura 9 (A) podemos ver un histograma que muestra el clúster 2 (células tratadas con GDF11 con un mejor pronóstico, con un menor estadio de invasión, así como una respuesta temprana a TGF- β). Por otro lado, en la figura 9 (B) se realizó un mapa de calor con varias firmas moleculares de las cuales podemos observar que las células tratadas con GDF11 desactivan las vías de señalización MET, WNT y TGF- β tardía.

Señalización basal de STAT3

En la figura 10 (A) se puede observar la técnica de Western blot en células Huh7 tratadas con GDF11 por tiempos cortos. Los datos no sugieren una diferencia significativa en la activación de la vía de señalización basal de STAT3. Se realizó un curso temporal para determinar el momento en que IL-6 activa la vía de señalización de STAT3, donde los datos sugieren que la activación comienza a los 5 minutos. En la figura 10 (B) se pueden apreciar las densitometrías correspondientes.

La Figura 11 (A) muestra nuevamente el análisis por Western blot, esta vez a lo largo de un curso de tiempo extendido. Los datos sugieren que el tratamiento con GDF11 a tiempos prolongados disminuye la fosforilación basal de la proteína STAT3, particularmente en la fosforilación de serina, mientras que, en la fosforilación de tirosina, el tratamiento con GDF11 disminuye después de 3 horas. La Figura 11 (B) muestra las densitometrías correspondientes.

Posteriormente, se realizaron ensayos de PCR, en los cuales tomamos un tiempo de 12 horas con GDF11 para analizar los ARNm de los blancos de STAT3, como MCL1 y β -CATENINA. Como se observó en la secuenciación, estos ARNm se ven afectados por el tratamiento y, como podemos ver en la figura 12 (A), MCL1 disminuye con el tratamiento.

Medios esteatogénicos

Como hemos observado, las dietas altas en grasas (HF) elevan la vía de señalización STAT3, como vimos en los tejidos de ratones tratados con una dieta HF con DEN durante 3 y 8 meses. Como se muestra en la figura 13, la dieta HF con DEN durante tres meses aumenta la activación de esta vía de señalización, ya que es evidente el contenido de ambas fosforilaciones en la proteína.

Por lo tanto, en la figura 14 realizamos un co-tratamiento, en el cual se administró HGF como activador de la vía, mientras se agregaba también el tratamiento con GDF11 durante 12 horas, porque habíamos observado que era el tiempo estimado que tardaba GDF11 en disminuir la activación de esa vía. Al no observar un efecto claro en la activación de la vía de STAT3 con el tratamiento con HGF, decidimos optar por un medio esteatogénico llamado medio MASL, el cual aumenta el contenido de ácidos grasos y colesterol, como podemos ver en la figura 15. El tratamiento con este medio proporciona agresividad a las células, ya que promueve un aumento en la proliferación de estas células.

Para verificar que las células realmente estaban cargando ácidos grasos y colesterol, en la figura 16 se realizó una tinción con Bodipy. Los datos sugieren un mayor contenido de colesterol en las células tratadas con ese medio. La visualización de los lípidos teñidos se realizó utilizando microscopía de fluorescencia con filtros de longitud de onda de excitación/emisión de 493/503 nm. Los lípidos neutros se observaron como inclusiones verdes brillante distribuidas en el citoplasma.

Una vez que se estandarizó el medio MASL, comenzamos los ensayos de migración, donde el medio estatogénico y el tratamiento con GDF11 se pusieron a competir. En la

figura 17, podemos ver que después de 48 horas con el medio MASL, la herida ya se había cerrado, mientras que el co-tratamiento no permite que la herida se cierre, resultando en que el medio esteatogénico proporciona agresividad a las células al aumentar la migración celular, mientras que el tratamiento con GDF11, a pesar de estar con el medio MASL, disminuye la agresividad, ya que reduce la migración celular. Posteriormente, para evaluar la viabilidad celular de los tratamientos anteriores, en la figura 18 se utilizó cristal violeta, donde se observó una disminución de la viabilidad en las células tratadas con GDF11 y una ligera disminución en el co-tratamiento, mientras que el tratamiento con el medio MASL aumentó la viabilidad, al igual que las células tratadas con IL-6.

Sistema de inhibición de la función de STAT3

Por lo tanto, para determinar si es la vía de señalización STAT3 la que está proporcionando agresividad al tumor, decidimos llevar a cabo un tratamiento simulado con GDF11, inhibiendo la fosforilación de STAT3 con el dominio SH utilizando un vector como suponemos que lo hace el tratamiento con GDF11. La transducción se realiza en diferentes líneas celulares, utilizando HEK293 como control, Huh7 como una de las más importantes, y también Hep3B y HepG2, como se muestra en la Figura 19.

Como podemos observar en la Figura 20, se realizó un Western Blot para determinar el contenido de ambas fosforilaciones de STAT3 en células Huh7 con el sistema de silenciamiento. Como se puede ver, hay una diferencia en el contenido de la proteína STAT3 (NT vs SH) que se debe únicamente al uso del vector (rompiendo el bucle de retroalimentación positiva de la misma vía de señalización de STAT3). Asimismo, la diferencia en el contenido de proteína entre las tres muestras silenciadas en comparación con el vector vacío es evidente. Con base en esto, queda claro que hay un mayor impacto en la disminución del contenido de fosforilación en tirosina de STAT3, así como del contenido total de la proteína. Utilizamos el tratamiento con sorafenib como control positivo para la disminución del contenido proteico.

Además, en la Figura 21, evaluamos la viabilidad de las células tratadas con el vector utilizando violeta de cristal. Como podemos observar, las células con silenciamiento y tratamiento con sorafenib muestran una disminución de la viabilidad de aproximadamente un 50%, afectando la viabilidad celular, lo cual hemos observado en diferentes líneas celulares como Huh7 y Hep3B. Las células HEK 293 se utilizaron como control positivo para la transducción.

Para corroborar lo que observamos con la tinción de violeta de cristal, se realizó un análisis de citometría de flujo, en la Figura 22, que mostró una mayor sensibilidad al tratamiento con sorafenib, confirmando que tanto la disminución de la viabilidad celular como el aumento de la sensibilidad al tratamiento se deben a la inhibición de STAT3.

Conclusión

Como se ha observado, la relación de STAT3 en múltiples vías de señalización promueve la supervivencia, así como la proliferación, migración y otras respuestas celulares que hacen que los tumores sean agresivos, por lo que el uso de tratamiento con GDF11 podría resultar en una excelente terapia, ya que el factor apunta a varias proteínas que pertenecen a la vía de señalización de STAT3, disminuyendo su actividad, y, por lo tanto, disminuyendo la agresividad del tumor. El evidente aumento en la fosforilación de tirosina en una dieta esteatogénica como el alta en grasas con DEN y la consecuente disminución en la misma fosforilación en células Huh7 muestra que el tratamiento con GDF11 es una estrategia ideal para reducir la agresividad en células tumorales, como se menciona a lo largo del texto, la inhibición del activador de la vía clásica es menos tóxica que la inhibición de serina. El tratamiento con GDF11 sigue siendo un prometedor supresor tumoral, ya que afecta múltiples vías pro-tumorales sin impactar la viabilidad celular, al mismo tiempo que mitiga la agresividad de las células tumorales, como la migración celular. El uso de ingeniería genética para generar un péptido inhibidor de STAT3 resalta la dependencia de las líneas celulares tumorales de esta vía, la cual les confiere agresividad que se reduce con el tratamiento con GDF11, lo que asumimos que disminuye esta vía de señalización.

Introduction

During the last decades, society has been affected by various diseases that are becoming increasingly common, while in the past years, they were very little known. In this case, the lifestyle (a sedentary lifestyle), the poorly balanced diet (due to the incredible increase in the consumption of ultra-processed foods, fats, and sugars, among others), and genetic factors can trigger the development of steatotic liver disease related to metabolism or MASLD (previously known as nonalcoholic fatty liver disease, NAFLD) (Hannou et al., 2018; Rinella, Lazarus, et al., 2023).

Epidemiology: Metabolically-dysfunction-associated steatotic liver disease (MASLD) is a disease with accelerated incidence growth in recent years, and it is estimated that around 32% of the world's population suffers from this disease, with a higher incidence in men (Teng et al., 2023). In Mexico, it is estimated that around 50% of the population suffers from it (Bernal-Reyes et al., 2019), and it is currently the main cause of liver transplantation (Fraile et al., 2021).

It is estimated that around 20-25% of the population will be affected by metabolic dysfunction-associated steatohepatitis (MASH), and data suggest that 56% of the population will suffer from it in the next decade, while the rate of obesity-associated metabolic diseases, including type 2 diabetes, insulin resistance, and fatty liver diseases, is also increasing. Therefore, MASH is considered a factor in the incidence of liver cancer. The development of HCC directly derived from MASH can arise in the absence of fibrosis, suggesting different mechanisms of tumorigenesis and microenvironment conditions (X. Wang et al., 2025).

Pathophysiology: Metabolically dysregulated steatotic liver disease (MASLD) encompasses a spectrum of liver disorders characterized by the accumulation of intracellular fat. The metabolic alteration that generates insulin resistance is due to the activation of lipogenesis, as it promotes the release of free fatty acids to the liver, where they are converted into triglycerides (which favor hepatic steatosis) and decrease fatty acid oxidation (Dua et al., 2025). The accumulation of hepatic fat (among many things) can cause lipotoxicity and oxidative stress, since in an environment with excess free fatty acids, reactive oxygen species (ROS) are generated, as fatty acids interfere with the electron transport chain in the mitochondria (one of the organelles where their metabolism takes place), which reduces ATP production and increases electron leakage, raising ROS above the liver's antioxidant capacity, known as oxidative stress, which

leads to damage to lipids, proteins, and DNA. It is worth noting that once mitochondrial function is damaged, it becomes a vicious cycle of cellular damage.

In addition, protein stress is generated due to the alteration of endoplasmic reticulum homeostasis, which contributes to apoptosis and mitochondrial dysfunction (Arruda et al., 2024; Steinberg, Valvano, De Nardo, & Watt, 2025). Due to chronic oxidative stress, signaling pathways such as NF- κ B and JNK are activated, which promote the release of proinflammatory cytokines (such as IL-6), favoring progression to fibrosis (Svobodova, Horni, Velecka, & Bousova, 2025).

On the other hand, intestinal dysbiosis increases the permeability of the intestinal barrier, which allows endotoxins (such as LPS) to enter the portal circulation, in addition to promoting lipogenesis, deregulating bile acid metabolism, generating an inflammatory microenvironment (carried out by LPS that activate Toll-like receptors in Kupffer cells) that accelerates disease progression (Tamaki et al., 2026; Zhou, Zhu, Bing, Wang, & Zhao, 2026).

Although there are also genetic variants in the PNPLA3, TM6SF2, and MBOAT7 genes that increase susceptibility to the disease, especially in the Mexican population (Arreola Cruz et al., 2025). For example, the PNPLA3 gene encodes a lipase called “patatin-like phospholipase domain-containing 3” that is involved in triglyceride metabolism in hepatocytes. The I148M mutation reduces triglyceride breakdown, and this mutation is associated with steatosis, inflammation, and fibrosis. It is considered the major genetic determinant associated with MASLD, as it is independent of obesity or insulin resistance (Kim et al., 2026; Paolini et al., 2026).

On the other hand, the TM6SF2 gene encodes a protein called “transmembrane 6 superfamily member 2” responsible for the secretion of VLDL lipoproteins from the liver. The E167K mutation promotes triglyceride accumulation, making it a genetic predisposition factor for MASLD (Arreola Cruz et al., 2025).

Finally, the MBOAT7 gene encodes an enzyme with the name 'membrane-bound O-acyltransferase domain-containing 7' which remodels membrane phospholipids. The variant alters the remodeling by the composition of phosphatidylinositol, favoring inflammation and progression towards fibrosis (Arreola Cruz et al., 2025).

Clinical spectrum: MASLD encompasses a spectrum of liver disorders characterized by the accumulation of intracellular fat, progressing to MASH. If left untreated, these conditions can advance to liver fibrosis, cirrhosis, and, ultimately, hepatocellular

carcinoma (HCC), which is one of the most aggressive forms of liver cancer (Dominguez-Perez et al., 2019) since it exhibits distinct molecular and immune characteristics compared to HCC of other etiologies, affecting men and women equally (X. Wang et al., 2025). HCC accounts for approximately 90% of primary liver tumors and is a leading cause of liver-related mortality (Asafo-Agyei & Samant, 2024).

Diagnosis: Regarding the diagnosis of MASLD, there are imaging methods, serum biomarkers, and, in some cases, liver biopsy. Among the imaging methods is ultrasound to detect moderate to severe steatosis, FibroScan, which is an elastography that allows measurement of liver stiffness and fat content, magnetic resonance imaging, which is the most reliable non-invasive standard to date that quantifies the percentage of liver fat, and finally, computed tomography, which is the least used due to radiation exposure (Osta et al., 2025).

On the other hand, among the most commonly used serum biomarkers are alanine aminotransferase (ALT) and aspartate aminotransferase (AST), both of which reflect liver damage; a ratio greater than 1 can suggest fibrosis; however, they are not reliable markers. Additionally, there is the FIB-4 index, which has better performance for fibrosis, as it uses 4 different variables, such as age, AST, ALT, and platelets, in a formula like the one shown in Figure 1 (Diaz et al., 2025; European Association for the Study of the Liver. Electronic address, Clinical Practice Guideline, Chair, representative, & Panel, 2022; Rinella, Neuschwander-Tetri, et al., 2023; Shah et al., 2009; Sterling et al., 2006).

$$\text{FIB-4} = \frac{\text{Age (years)} \times \text{AST (U/L)}}{\text{Platelet Count (10}^9\text{/L)} \times \sqrt{\text{ALT (U/L)}}}$$

Figure 1. Evaluation and Staging of Liver Fibrosis. Taken from: Core Concepts - Evaluation and Staging of Liver Fibrosis - Evaluation, Staging, and Monitoring of Chronic Hepatitis C - Hepatitis C Online

The JAK/STAT3 Pathway as a Molecular Bridge Between MASLD and HCC: In recent years, it has been reported that the accumulation of lipids (mainly cholesterol) is a leading

cause of serious diseases, such as HCC, since tumors from lipid-enriched diets exhibit a profound change in the expression of genes that favor cell survival and proliferation while decreasing tumor suppressor signaling pathways (Simoni-Nieves et al., 2021). High-lipid diets generate inflammation that enhances the activation of highly oncogenic pathways such as Janus kinase (Jak)- Signal Transducer and Activator of Transcription 3 (STAT3).

STAT3 structure: STAT3 is conform by 5 important domains for its gene expression. The first is the N-terminal domain (NTD), which allows oligomerization and interaction with other factors, enabling the stability of the STAT3 dimer. The second domain is for DNA binding (DBD), located in the central region. It is responsible for recognizing specific sequences called GAS (gamma-activated sites), which allow the regulation of genes involved in processes such as proliferation, apoptosis, inflammation, and cell differentiation (Haan et al., 1999). The third domain is the linker and coil-coil binding domain, whose main function is to connect the DBD with the rest of the protein, allowing interactions with cofactors and regulatory proteins. The fourth domain is the SH2 domain (Src Homology 2), which is located in the C-terminal region and is responsible for recognizing and subsequently binding to phosphotyrosine residues. This domain is essential for STAT3 dimerization, which, consequently, allows its translocation to the nucleus and the activation of its target genes. It is important to highlight that mutations in this site are associated with multiple diseases, emphasizing cancer (Haan et al., 1999). The last domain is the transactivation domain (TAD), which is responsible for enhancing transcriptional activity by recruiting coactivators and regulatory proteins (such as CBP/p300), being key to integrating other signaling pathways (such as MAPK, AKT) (Kasembeli, Rodas, & Tweardy, 2025).

Activation pathways: For the STAT3 activation pathways, we can divide them into “JAK-dependent and JAK-independent.” The dependent pathway (also known as canonical) uses cytokines (such as IL-6, IL-10, IL-22) that bind to their membrane receptor, causing a conformational change that activates the signaling cascade through JAKs associated with the receptor, phosphorylating STAT3 at Tyr705. Meanwhile, the independent pathway (or non-canonical) can occur via EGFR (Epidermal Growth Factor Receptor) or VEGFR (Vascular Endothelial Growth Factor Receptor), which, when their ligand binds, allows the activation of their receptor and, in turn, the activation of other tyrosine kinases (such as Src). Finally, there is crosstalk with other pathways, such as AKT, MAPK, and NF-κB, which can enhance phosphorylation at Ser727 (Ma et al., 2020; Rad, Dodd,

Thomas, Upadhyaya, & Tee, 2015; Wei-Hsin Lin, 2021; Yu, Lee, Herrmann, Buettner, & Jove, 2014; Zhang, Wang, Jove, & Vande Woude, 2002; Zhao, Qi, & Yu, 2021).

It is important to clarify the differences between the phosphorylations of STAT3. The first of them is on tyrosine (Tyr705), which is essential for the dimerization of STAT3 and occurs in the SH2 domain, activating the transcription of target genes. Subsequently, both dimers expose a nuclear localization sequence (NLS), which allows their active transport to the nucleus driven by importins (for example, importin- α 5), where they can interact with cofactors and regulatory proteins for gene transcription. Aberrant activity is related to oncogenesis, promoting resistance to apoptosis and tumor progression (Junhao Yang 2020).

On the other hand, phosphorylation on serine (Ser727), which occurs in the TAD, modulates STAT3 transcriptional activity (Wei-Hsin Lin, 2021), enhancing or regulating gene expression through the binding of coactivators (such as CBP/p300, which helps to stabilize the interaction) (Hua Yu, 2014). Overactivation promotes angiogenesis, invasion, and metastasis (Stenckova, Nenutil, Vojtesek, & Coates, 2022).

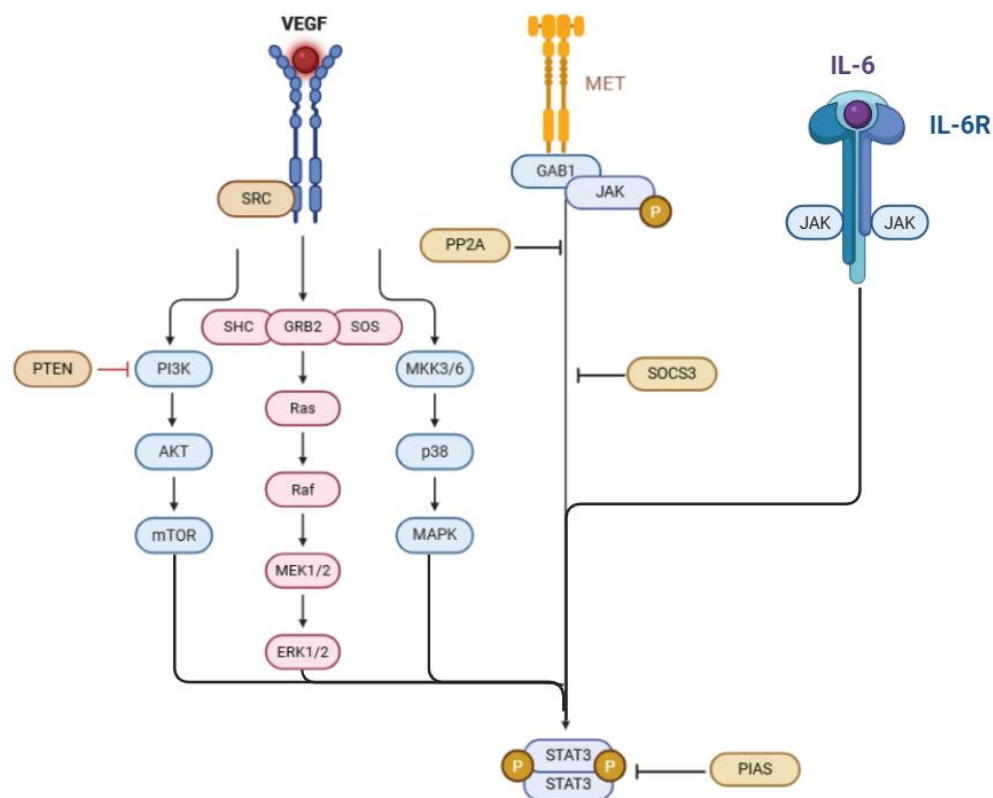


Figure 2: Canonical and Convergent Pathways of STAT3 Activation. A schematic representation of the canonical STAT3 activation pathway. The figure details the intracellular signaling networks downstream of three key receptors, tyrosine kinase (VEGFR, and c-MET) and the cytokine receptor IL-6R, demonstrating how their synergistic crosstalk culminates in the phosphorylation and activation of STAT3. Created by Biorender.

In a normal context, the STAT3 signaling pathway regulates normal physiological functions, such as in the immune response, the IL-6/STAT3 pathway differentiates T lymphocytes towards a Th17 phenotype, or in cellular metabolism, serine phosphorylation of STAT3 regulates the activity of the respiratory chain and ATP production. It is also involved in development and differentiation, as it is essential for stem cell pluripotency. STAT3 also promotes cell proliferation and repair, so it plays a very important role in liver regeneration and healing. Finally, STAT3 regulates apoptosis and cell survival through proapoptotic and antiapoptotic signals (Joseph Mackie 2023; Min Shi, 2025).

In cancer, this pathway exacerbates cellular processes such as proliferation, survival, invasion, immunosuppression, and the modulation of epigenetic mechanisms, among others (Yu et al., 2014). Recent research has revealed that hepatocellular carcinoma (HCC) tumors exhibiting a transcriptomic profile enriched in the Jak-STAT3 pathway are associated with a poorer prognosis and reduced patient survival. This adverse outcome is attributed to several factors, such as enhanced inflammation, increased tumor aggressiveness, and angiogenesis promotion (Simoni-Nieves et al., 2021).

Pharmacological Inhibition of the JAK/STAT3 Pathway: There are FDA-approved drugs (Food and Drug Administration) that are inhibitors of the STAT3 signaling pathway. On one hand, there are first-generation Jakinibs such as Tofacitinib, Ruxolitinib, and Baricitinib, which selectively inhibit Jak1. There are also next-generation drugs that focus on the inhibitory activity of a specific JAK, such as Decernotinib, which acts directly on JAK3, or Filgotinib, Baricitinib, Upadacitinib, Solcitinib, and Itacitinib, which have a higher affinity for JAK1, thereby inhibiting the pro-inflammatory cytokine signaling (José M. Serra López-Matencio, 2018). However, in signal transduction in solid tumors such as HCC, the signaling is low via JAK2, so these drugs do not reduce STAT3 activity (Jr, 2005).

On the other hand, the effect of inhibiting STAT3 phosphorylation has been observed, such as tyrosine phosphorylation in HeLa cells, which is beneficial for tumor cells (Brizio Moreno-Jaime, 2016), or the inhibition of serine phosphorylation, which is also beneficial for cancer cells in clear cell renal carcinoma (ccRCC) (Arévalo Bautista, 2019). Therefore, it is estimated that the best therapy would be the dual inhibition of both STAT3 phosphorylation. Currently, there are still no optimized therapies for the treatment of HCC. The therapeutic approach must be directed towards multiple targets in cancer cell biology.

Molecular Structure and Biological Functions of GDF11: Growth differentiation factor 11 (GDF11), a transforming growth factor beta (TGF- β) superfamily member. GDF11 is synthesized as a precursor protein (pro-GDF11) with a signal peptide at the N-terminal end that directs it to the endoplasmic reticulum for secretion. In the Golgi apparatus, proteases perform a proteolytic cleavage that separates the predominance of the C-terminal region (which keeps the protein in an inactive or latent state). The proteases belong to the proprotein convertase family (for example, furin or PCSK5). Finally, a covalent dimer is formed that is linked by disulfide bonds (the state of the active protein) (Sánchez-Rodríguez. et al., 2025; Simoni-Nieves et al., 2019; Taylor Marohl, 2025).

Active GDF11 binds to its Activin-type receptors (ActRIIA, ActRIIB), generating a conformational change that leads to the activation of the SMAD2/3 signaling pathway, with its consequent translocation to the nucleus and the expression of its target genes related to development, differentiation, and cellular metabolism (Simoni-Nieves et al., 2019).

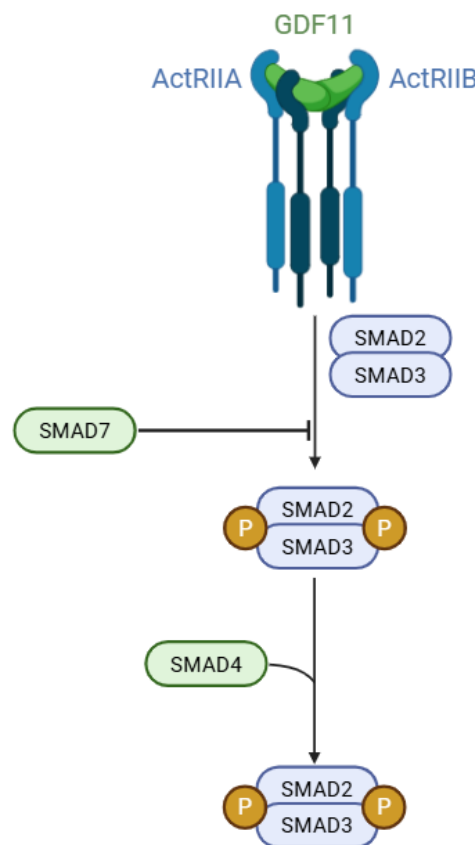


Figure 3: The ActRIIA/ActRIIB-mediated GDF11 signalling cascade. This diagram illustrates the core pathway of GDF11 signal transduction. GDF11 binds to the type II receptors ActRIIA or ActRIIB at the cell membrane. This binding facilitates the recruitment and activation of a type I receptor, leading to the phosphorylation of SMAD2 and SMAD3. These activated R-SMADs then associate with SMAD4 and translocate to the nucleus to modulate gene expression. Created by Biorender.

GDF11 displays multitask responses in many cellular systems, including differentiation, metabolism, muscle biology, and cancer. Although many reports have pointed out a series of controversies, many have been attributed to a misinterpretation of results due to the degree of cellular differentiation and poor specificity of early antibodies against GDF11 (Simoni-Nieves et al., 2019).

While some studies have suggested pro-tumoral activities for Growth Differentiation Factor 11 (GDF11), these reports may stem from methodological inconsistencies or misinterpretations of data. In contrast, our research group has consistently demonstrated the anti-tumor effects of GDF11 in human hepatocellular carcinoma (HCC) and leukemia cells (Sánchez-Rodríguez. et al., 2025). Our findings reveal that GDF11 significantly reduces cancer stem cell-like properties and overall tumor aggressiveness. Furthermore, we have observed substantial impairments in both the proliferative capacity and migratory potential of HCC cells treated with GDF11 (Gerardo-Ramirez et al., 2019). In addition to these effects on cellular behavior, GDF11 induces profound changes in cancer cell metabolism, particularly in lipid homeostasis, glycolytic pathways, and mitochondrial function and morphology. These comprehensive effects suggest that GDF11 functions as a potent tumor suppressor in HCC by modulating key signaling pathways and metabolic processes essential for cancer cell survival and progression. Our research provides a strong foundation for further investigation into GDF11 as a therapeutic target in treating hepatocellular carcinoma (Hernandez et al., 2021).

Interestingly, the primary phenotype effects are observed at 12 h under GDF11 treatment; the constant treatment with the growth factor for 72 h restates cellular changes that can persist even five days after GDF11 withdrawal (unpublished data). Remarkably, the principal and constant effect induced by GDF11 is the downregulation of signaling pathways such as AKT and STAT3, reported by a previous transcriptomic analysis (Hernandez et al., 2021), significantly impacting lipid overload and aberrant lipogenesis. STAT3 seems to be the main target of GDF11, but this issue has not been addressed nowadays.

The present project aims to identify and characterize the transcriptomic changes induced by GDF11 in liver cancer cell lines and to unveil the mechanisms that may restrain the aggressiveness of hepatocellular carcinoma (HCC), positioning GDF11 as a potential therapeutic approach in this type of cancer. In this context, one of the major challenges remains the early diagnosis of asymptomatic patients and the development of non-invasive methods to reduce the reliance on liver biopsies. Future perspectives include the use of STAT3 as a prognostic biomarker in cancer and metabolic diseases, the

design of dual therapies targeting both Tyr705 and Ser727 phosphorylation sites, and the exploration of personalized medicine strategies through combined therapeutic approaches.

Hypothesis

GDF11 inhibits the STAT3 signaling pathway in human hepatocellular carcinoma (HCC) cells, substantially reducing tumor aggressiveness through complex molecular mechanisms. By interfering with critical oncogenic signaling cascades, GDF11 modulates cellular behavior, transforming the malignant phenotype of HCC cells. GDF11 mediates its anti-tumor effects by comprehensively suppressing proliferative potential, migratory capabilities, and invasive characteristics. This suppression occurs through transcriptional and post-transcriptional modifications of key signaling molecules, ultimately disrupting the pro-tumorigenic environment modulated by activated STAT3 pathways. The molecular intervention by GDF11 extends beyond direct cellular mechanisms, potentially influencing tumor microenvironment dynamics, inflammatory responses, and metabolic reprogramming. By targeting multiple oncogenic processes simultaneously, GDF11 emerges as a promising molecular regulator with significant potential in understanding and potentially treating hepatocellular carcinoma progression.

Research question

Does GDF11 play a role in repressing STAT3 signaling and decreasing the aggressiveness of human hepatocellular carcinoma cells?

Objectives

General: To elucidate the molecular mechanisms by which Growth Differentiation Factor 11 (GDF11) modulates STAT3 signaling in hepatocellular carcinoma (HCC) cells under conditions mimicking steatotic liver disease related to metabolism and characterize the subsequent effects on tumor cell aggressiveness and lipid metabolism.

Specifics:

- Characterize the transcriptomic profile of Huh-7 hepatocellular carcinoma cells in response to short-term GDF11 treatment.

- Elucidate the mechanism by which GDF11 represses STAT3 signaling in a cellular model of lipid overload.
- Develop and implement a STAT3 genetic engineering inhibition system as a comparative model for GDF11 effects.
- Assess the impact of GDF11-mediated STAT3 repression on hepatocellular carcinoma aggressiveness.

Materials and Methods

Cell culture

Huh7, HepG2 and Hep3B cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were cultured in William's medium supplemented with 10% fetal bovine serum (FBS, Hy-Clone, Logan, UT, USA), 100 U/ml ampicillin, and 100 µg/ml streptomycin (Thermo Fisher Scientific, Waltham, MA, USA). Cells were maintained at 37 °C in a 5% CO₂ and 90% humidity atmosphere. They were plated in plastic culture flasks (Sigma-Aldrich, Saint Louis, MO, USA). Cells were treated with GDF11, as previously reported (Gerardo-Ramirez et al., 2019), at both early and late time points.

RNA-seq

RNA sequencing was performed as reported by (Hernandez et al., 2021) using the Illumina HiSeq4000 platform, and the data were deposited in the BioProject database (ID: PRJDB10804). Raw reads were filtered to remove adapter sequences, contamination, and low-quality reads. The filtered reads were mapped to the human genome reference using HISAT2 (hisat2-2.0.2-beta), followed by read summarization with featureCounts (subread-1.5.0-p1). All data analyses were conducted using the R programming language and associated packages. The output matrix from featureCounts was used as input for differential expression analysis in Bioconductor DESeq2, applying a false discovery rate (FDR) threshold of 0.5, a p-value of 0.01, and a log₂ fold change range from -1.5 to 1.5. Statistical significance was assessed using Wald test statistics. Data visualization was carried out through heatmaps and principal component analysis (PCA).

Enrichment analysis

Gene set enrichment analysis (GSEA) was performed using the GSEA software provided by the Broad Institute (<http://software.broadinstitute.org/gsea/index.jsp>). Gene sets from the MSigDB database were tested, and those with a NOM p-value < 0.05 and FDR < 0.25 were considered significantly enriched in the a priori-defined gene sets. Ingenuity Pathway Analysis (IPA, Ingenuity Systems, Inc., Redwood City, CA, USA) was used to investigate the biological pathways and networks associated with the model genes using the complete dataset. Genes, along with their log₂-fold change values, were used as input. Diseases, biological functions, and upstream regulators were considered significant if the absolute value of their activation z-score exceeded 1.5. The z-score reflected the concordance between the observed gene expression and the predicted direction of regulation.

Comparative functional genomics

Level 3 RNA-seq data (Count files) from 116 patients with HCC and 50 normal liver samples were used, and they were retrieved from The Cancer Genome Atlas (TCGA) database (<https://portal.gdc.cancer.gov/>). The data were processed as reported (Simoni-Nieves et al., 2021).

Western blotting

The protein content was assessed by Western blot, as previously reported (Gerardo-Ramirez et al., 2019). PVDF membranes were blotted with specific antibodies. Horseradish peroxidase-conjugated secondary antibodies were used according to the corresponding primary antibodies. Blots were exposed using the Super Signal West Pico Chemiluminescent substrate (Pierce Biotechnology, USA). The signal was detected using the Gel Logic 2500 imaging system (Kodak, Rochester, NY, USA).

Antibody	Brand	# cat	Dilution
pSTAT3 (Ser727)	Cell signaling	9134	1:1000
pSTAT3 (Tyr705)	Cell signaling	9145	1:1000
STAT3	Cell signaling	9139	1:1000
Actin	Cell signaling	4970	1:1000

Table 1. Antibodies used for Western blot.

Real-time quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR)

The analysis of gene expression was conducted as previously reported (Gerardo-Ramirez et al., 2019). One μg of total RNA was reverse transcribed in a 20 μl reaction volume using the SuperScript First-Strand Synthesis Kit (Invitrogen Corp.), following the manufacturer's instructions. qRT-PCR analysis was performed using a CFX96 Touch thermal cycler (Bio-Rad) in a 96-well reaction plate. Each 10 μl PCR reaction contained 5 μl of 2 $\times$ SYBR Green PCR Master Mix (Bio-Rad), 200 nM of each primer, and 1 μl of cDNA template. Reactions were incubated for 10 minutes at 95 °C, followed by 40 cycles of 30 seconds at 95 °C and 60 seconds at the specific primer annealing temperature. The expression level of ribosomal protein S18 (rs18) was used as the reference gene. Relative gene expression levels were calculated using the $2(-\Delta\Delta\text{Ct})$ method.

Animal model

Mice (C57BL/6 strain) were used and randomly divided into 4 groups, which include (1) mice fed with regular chow diet (CW); (2) mice fed with chow diet and injected with a single dose of N-nitrosodiethylamine (DEN, 10 µg/kg, i.p.) (CW/DEN); (3) mice fed with High Fat diet (HF) for three months; (4) mice fed with Western diet and injected with a single dose of DEN (W/DEN). As reported (Simoni-Nieves et al., 2021), animals received diet and water ad libitum. After 3 months, mice were euthanized under Avertin (2-2-2 tribromoethanol) anesthesia. Liver tissue was collected for subsequent western blot analysis. All animals were maintained in specific pathogen-free housing and cared for in accordance with the NIH Guide for Care and Use of Laboratory Animals and Universidad Autónoma Metropolitana guidelines for the use of animals.

Diet composition, in terms of main components are as follows: CW diet (fat 5%, cholesterol 0.01%, carbohydrates 23.30% and protein 17.10%); High Fat diet (fat 21%, cholesterol 1.0%, carbohydrates 33.00% and protein 17.50%).

Steatogenic cell culture medium

A 100 mM stock solution of palmitic acid was prepared in 100 mM NaOH and heated at 70 °C in a shaking water bath to ensure complete dissolution (*Solution A*). Fatty acid-free bovine serum albumin (BSA) was prepared at 10% (w/v) in water containing 150 mM NaCl and heated at 55 °C (*Solution B*). Solution A was added dropwise to Solution B, adjusting the concentration ratio of palmitic acid to BSA as required. The mixture was vortexed for 10 seconds and incubated at 55 °C for 10 minutes. The resulting PA/BSA complex was sterilized by filtration through a 0.45 µm pore-size membrane filter. 1×10^6 cells were seeded and the steatogenic medium was added for 48 hours, and an increase in cell density was observed.

Detection of triglycerides and cholesterol esters

For the detection of intracellular neutral lipids, such as triglycerides and cholesterol esters, the lipophilic fluorophore BODIPY™ 493/503 (Invitrogen, Cat. No. D3922) was used. The cells were cultured under standard conditions and subsequently washed with PBS to remove serum residues. A working solution of BODIPY™ was prepared at a final concentration of 1 µM by diluting the stock solution (1 mM in DMSO) in PBS. Cells were incubated with the BODIPY™ solution for 20 minutes at 37 °C in the dark. After incubation, PBS washes were performed to eliminate excess fluorophore.

Lentivirus transduction.

Plasmids p8.2, pVSV, and pLJMI-GFP were used. A pseudovirus was assembled by co-transfection of pCMV-VSV-G (plasmid #8454 from Addgene), pCMV-dR8.2dvpr (plasmid #12263 from Addgene), and pLJM1-GFP (#19319 from Addgene) or pLJM1-SH2 (inhibitor of STAT3 function) into the HEK293FT cell line using Lipofectamine 3000 (Thermo Scientific, USA). Forty-eight hours later, the supernatant containing the produced pseudoviruses was harvested and filtered (0.22 μm) to remove cellular debris or other contaminants and to transduce the cells of interest. The GFP gene was removed from the pLJMI-GFP plasmid, and the STAT3 SH2 domain was inserted. *Stb3* bacteria were transformed with the modified plasmid. Subsequently, the Huh7 cell line was transduced and cultured in medium containing puromycin. The Huh7 cell line transduced with the GFP plasmid was used as the control group, while the Huh7 cell line transduced with the STAT3 SH2 plasmid was used as the experimental group.

Wound-healing assay

We seeded 3×10^4 cells using wound-healing inserts (Ibidi, 81176, Gräfelting, Germany) as previously reported (Alejandro Escobedo-Calvario, 2025). Dishes were washed gently with phosphate-buffered saline 1 $\times$ to remove any detached cells. The healing closure was monitored, and images (100 $\times$) were taken every 12 hours up to 48 hours. The wound area (μm^2) and migration capacity (%) were analyzed using ImageJ software.

Migration and Invasion assay

Huh7 and HepG2 cells will be suspended in a medium without serum and transferred to Matrigel-uncoated or Matrigel-coated upper chambers of a Transwell insert. Medium without serum will be added to the lower chamber for 12 h and then replaced with medium containing 20% FBS for another 24-48 h, and the cells will be incubated at 37°C in 5% CO₂. The average number of cells migrated or invaded through the membrane in five fields will be photographed and counted per chamber.

Cell viability

Staining with crystal violet was used as reported (Gerardo-Ramirez et al., 2019). Flow cytometry was also performed using the BD FACSCalibur flow cytometer (Becton Dickinson, Life Sciences, Franklin Lakes, NJ, USA); the LIVE/DEAD kit (Thermo Fisher Scientific, Cat. L23101) was used as reported (Sánchez-Rodríguez. et al., 2025). The data were analyzed using FlowJoX (Version 10.X, Becton Dickinson Life Sciences). The cells were treated with Sorafenib 5 μM for 48 hours.

Statistical Analysis

Each animal and cell experiment was performed at least in triplicate using cells from a minimum of three different passages. Data were reported as the mean $\pm$ standard error of the mean (SEM). One-way analysis of variance (ANOVA) was used to compare the means among different groups. Statistical analyses were conducted using GraphPad Prism software (version 8.2.1). Differences were considered statistically significant at $p \leq 0.05$.

Results

Deregulation of the oncogenic STAT3 pathway under treatment with GDF11

As can be seen in Figure 4 (A), we obtained excellent RNA sequencing improvements in Huh7 cells treated with GDF11 (50 ng/ml) for 12 hours. Therefore, in Figure 4 (B), which is a heat map, we observed 1,450 differentially expressed genes, where differential expression between the two groups can also be observed. Furthermore, in Figure 4 (C), we analyzed the different signaling pathways that were deregulated with treatment, most of which are related to the oncogenic STAT3 pathway (among them, the mTOR pathway, HGF, apoptosis, etc)

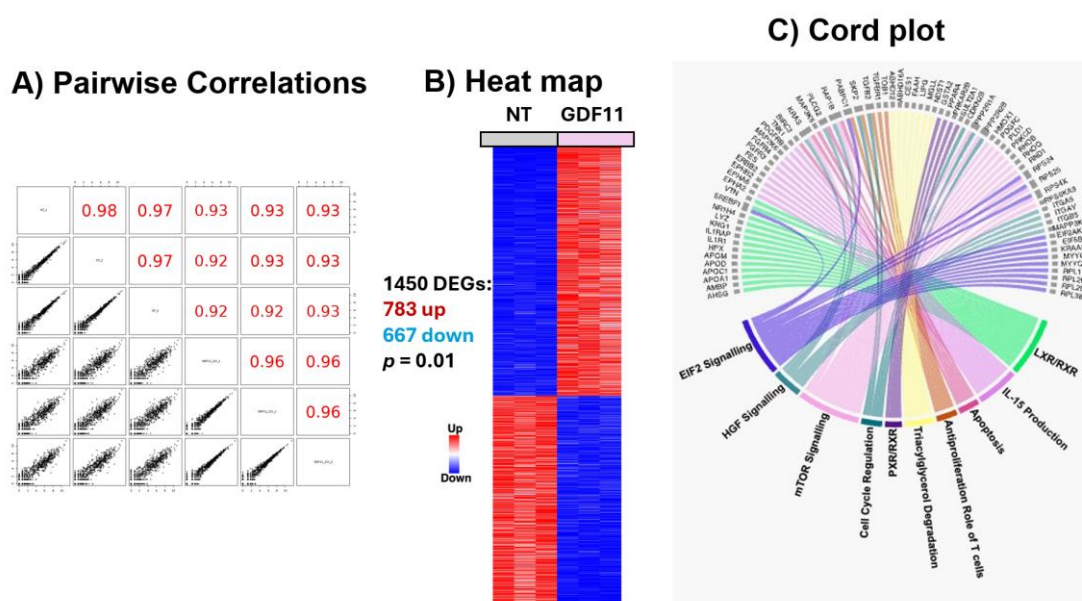


Figure 4: (A) We obtain the Pairwise correlation. (B) The heat map shows the differences in the transcriptomic profile between the two groups (Huh7 cell line, untreated and GDF11 treated for 12 hours). (C) a cord plot of the pathways deregulated by treatment.

Subsequently, a volcano plot was created from the data obtained in Figure 5 (A), showing overexpressed (orange) and downregulated (blue) genes. The STAT3 pathway is mostly deregulated under GDF11 treatment. Likewise, Figure 5 (B) showed that most target proteins in the STAT3 signaling pathway were deregulated. Specifically, genes involved in proliferation (such as MYC), metastasis (WNT5A), cell cycle regulation, apoptosis (MCL-1), and EMT (β -catenin) were downregulated, while some tumor suppressors and negative regulators of STAT3 were overexpressed.

With the help of the software, we analyzed the deregulation of some proteins involved in the STAT3 signaling pathway, particularly PIAS and some other phosphatases like PP2A, so the data suggest that the decrease in STAT3 activation could be due to one of these phosphatases.

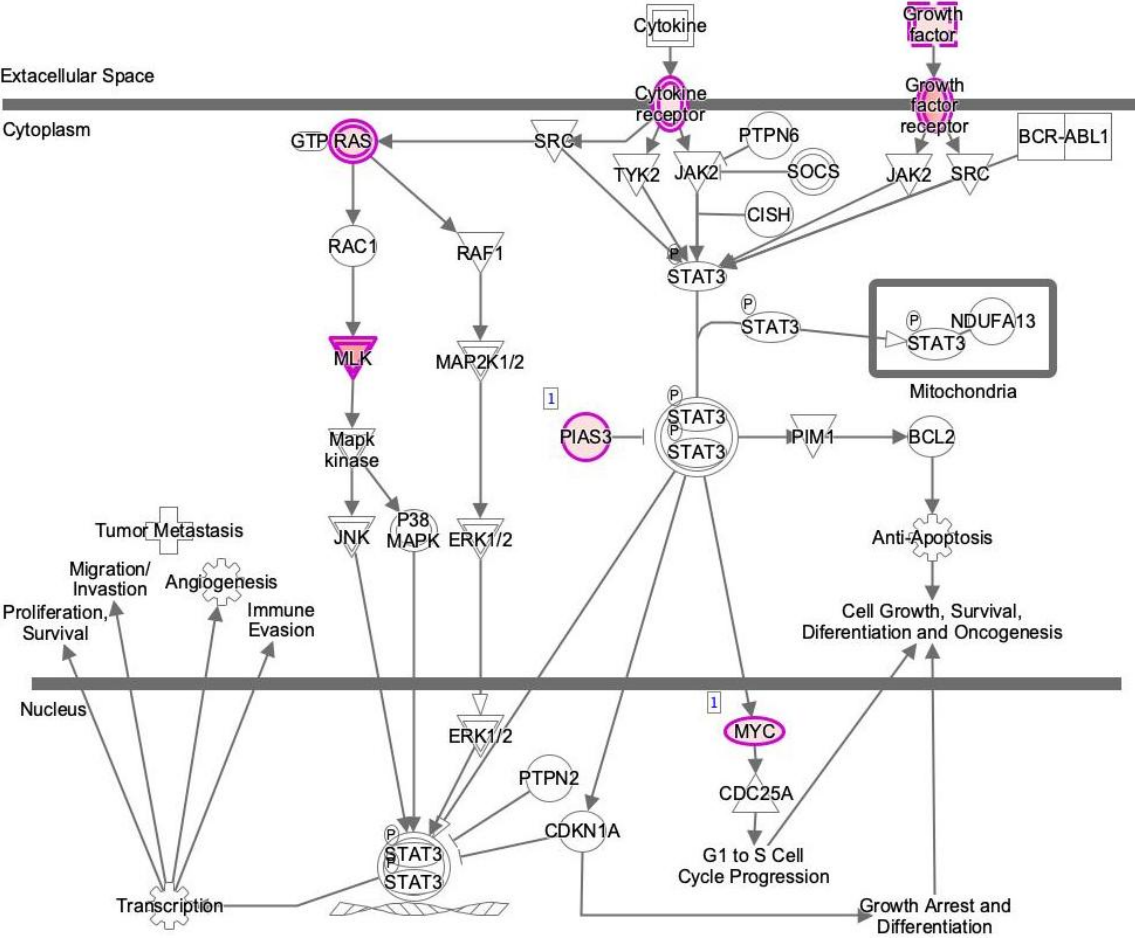
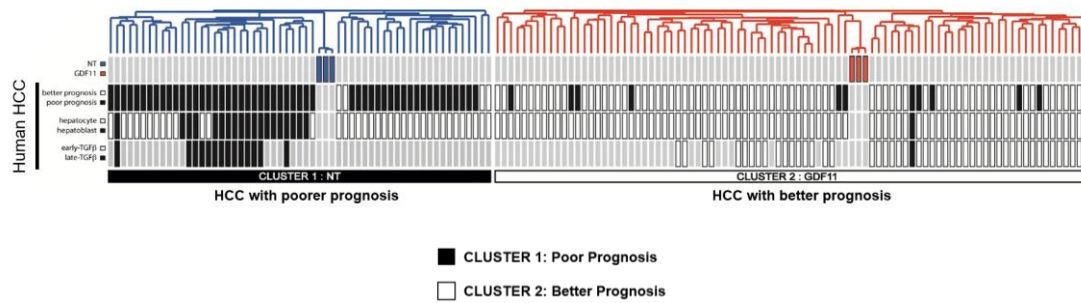


Figure 7: Deregulation of proteins involved in the STAT3 signaling pathway.

In Figure 8 (A), we also show how GDF11-treated cells correlate with HCC patients with a better prognosis (cluster 2), whereas untreated Huh7 cells cluster with patients with a worse prognosis (cluster 1). In Figure 8 (B), cluster 1 shows better survival, less invasion promotion, and a delayed TGF- β response.

A) Clustering through dendrogram



B) Kaplan Meier

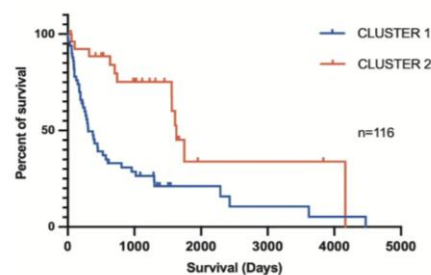


Figure 8: Transcriptional profiling and survival analysis. (A) Hierarchical clustering dendrogram comparing the transcriptomic profiles of cell samples. Untreated control cells are shown in blue, while GDF11-treated cells are shown in red. The dendrogram illustrates the degree of similarity between samples, demonstrating the transcriptional shift induced by GDF11 treatment. (B) Kaplan-Meier survival analysis based on [e.g., STAT3 pathway activation / gene signature] in a cohort of 116 patients ($n = 116$). The plot estimates survival probability over time, with statistical significance determined by the log-rank test (p -value shown).

In Figure 9 (A), we can see a histogram showing cluster 2 (cells treated with GDF11 with a better prognosis, with a lower stage of invasion, as well as an early response to TGF- β). On the other hand, in Figure 9 (B), a heat map was made with various molecular signatures from which we can observe that cells treated with GDF11 turn off the MET, WNT, and late TGF- β signaling pathways.

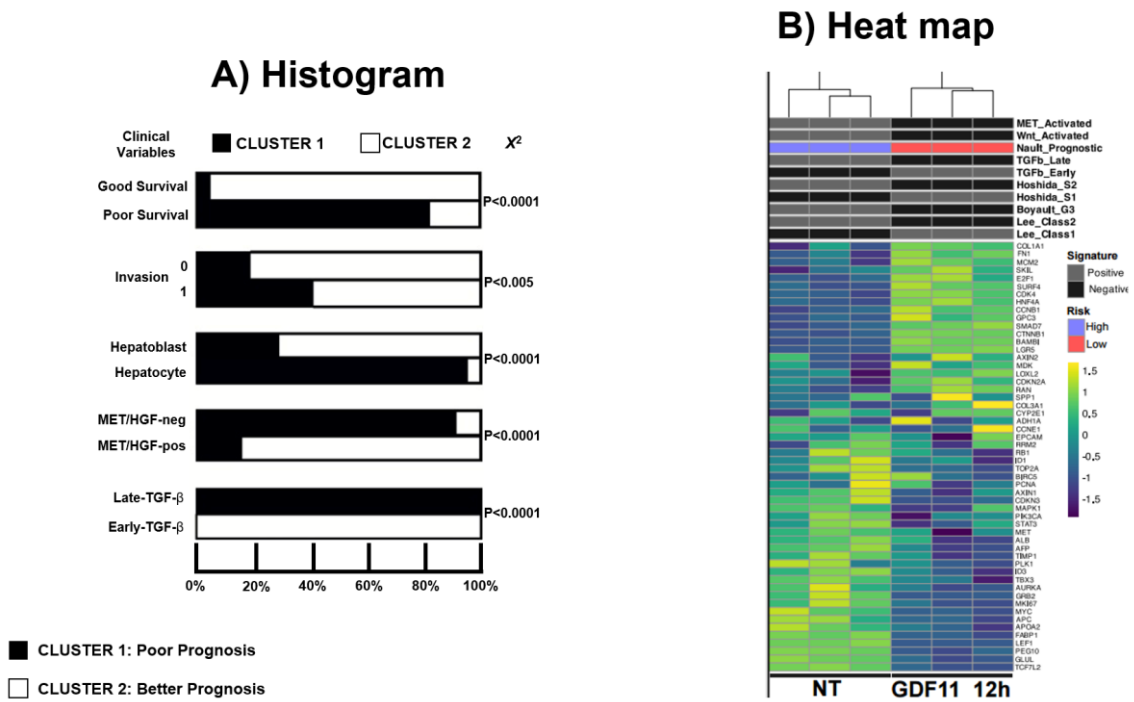


Figure 9: (A) a histogram showing cluster 1, with a poor prognosis, in the black box, while cluster 2, with a better prognosis, is shown in the white box. (B) shows a heat map showing the signatures used between untreated and GDF11-treated cells. Upregulation is shown in gray boxes, and downregulation is shown in black boxes. High gene expressions are highlighted in yellow, while low gene expressions are shown in blue.

Basal STAT3 signaling

In Figure 10 (A), the western blot is shown in Huh7 cells treated with GDF11 for short times. The data do not suggest a significant difference in the activation of the basal STAT3 signaling pathway. A time course was conducted to determine when IL-6 activates the STAT3 signaling pathway, with data suggesting activation starting at 5 minutes. In Figure 10 (B), the corresponding densitometries are shown.

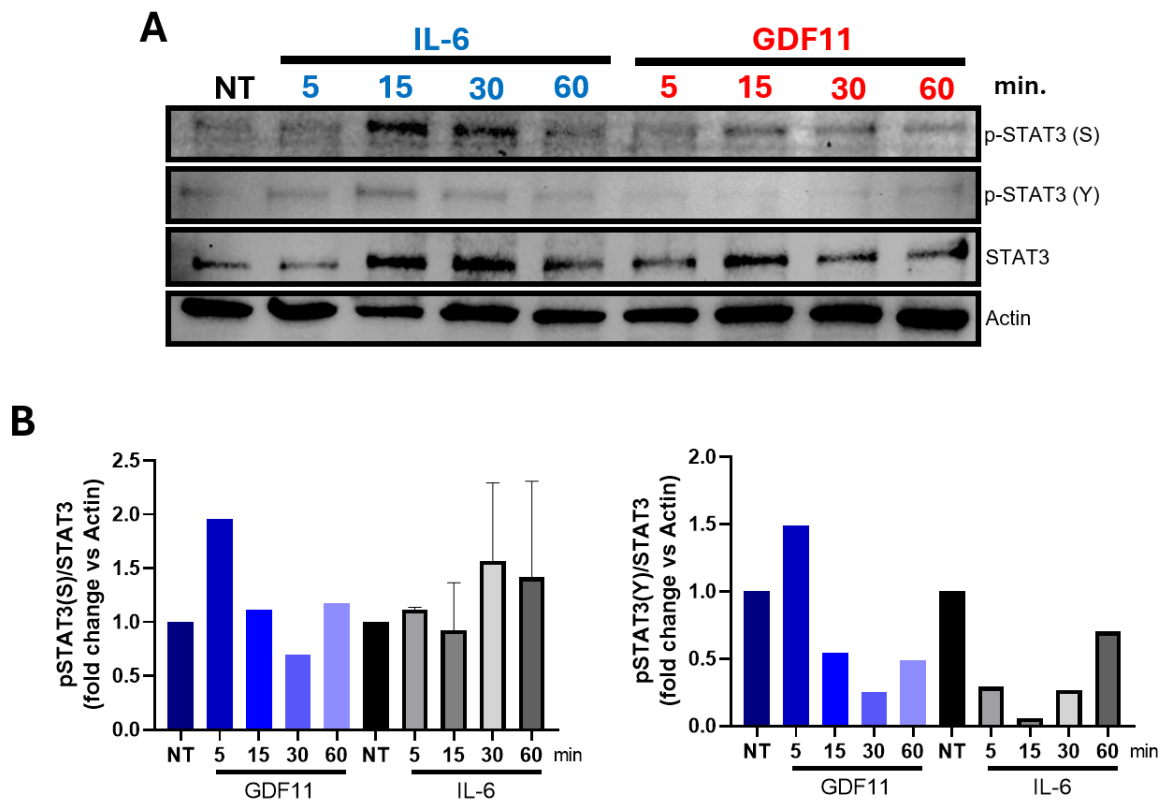


Figure 10: (A) The time course with IL-6 with GDF11 treatments at short times, and the activation of the basal STAT3 signaling pathway is observed. (B) The corresponding densitometries are shown. The left graph shows the content of serine phosphorylation of STAT3, and the right graph shows the content of tyrosine phosphorylation of STAT3. Fold change vs Actin. The blue bars represent the treatment with IL-6, and the black bars represent the treatment with GDF11. 2n

Figure 11 (A) again shows the Western blot analysis, this time over an extended time course. The data suggest that treatment with GDF11 at long times decreases the basal phosphorylation of the STAT3 protein, particularly in serine phosphorylation, while for tyrosine phosphorylation, treatment with GDF11 decreases after 3 hours. Figure 11 (B) shows the corresponding densitometries.

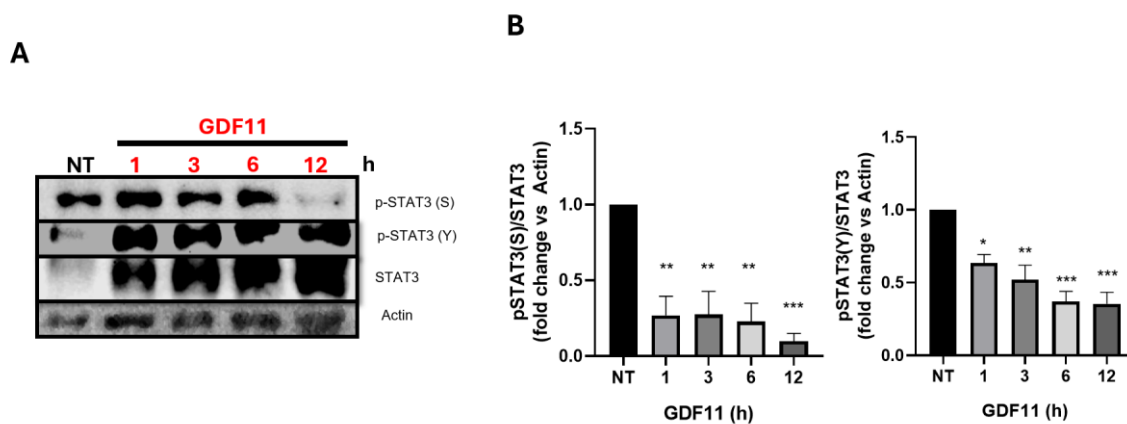


Figure 11: (A) There is a western blot of Huh7 cells with GDF11 treatment over a long time. (B) The corresponding densitometries are shown, with serine phosphorylation on the left and tyrosine phosphorylation on the right. Fold change vs Actin. 3n
 “*” p<0.01, “**” p<0.001, “***” p< 0.0001

Subsequently, PCR assays were conducted, in which we took a time of 12 hours with GDF11 to analyze the mRNAs of the STAT3 targets, such as *MCL1* and *CATENIN*. As observed in the sequencing, these mRNAs are affected by the treatment, and as we can see in Figure 12 (A), *MCL1* decreases with the treatment.

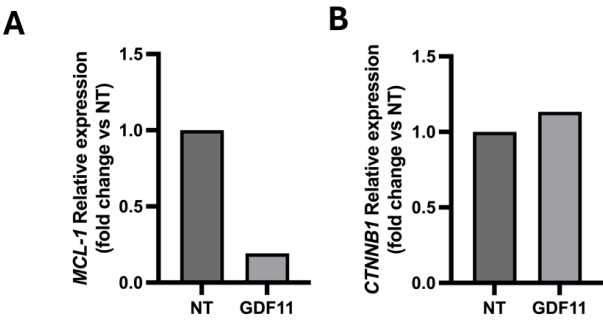
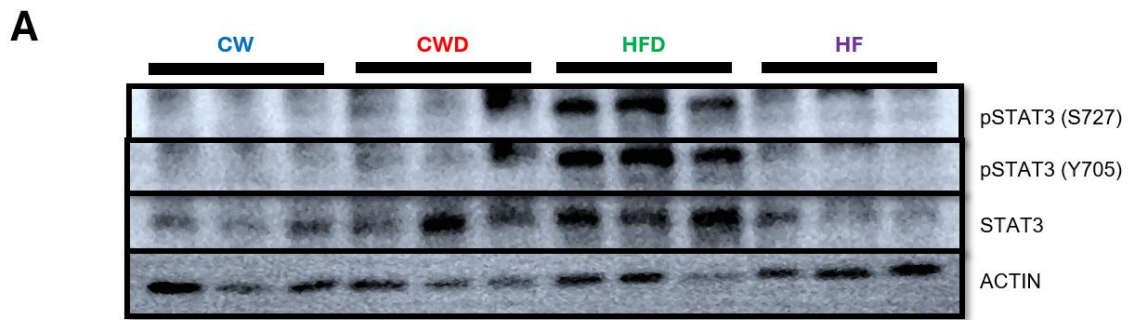


Figure 12: (A) The corresponding densitometry of *MCL1*. (B) β -*CATENIN*, from Huh7 cells treated with GDF11 for 12 hours. n1

Steatogenic medium

As we have observed, high-fat (HF) diets elevate the STAT3 signaling pathway, as we saw in the tissues of mice treated with an HF diet with DEN for 3 and 8 months. As seen in Figure 13, the HF diet with DEN for three months increases the activation of this signaling pathway, as the content of both phosphorylations in the protein is evident.



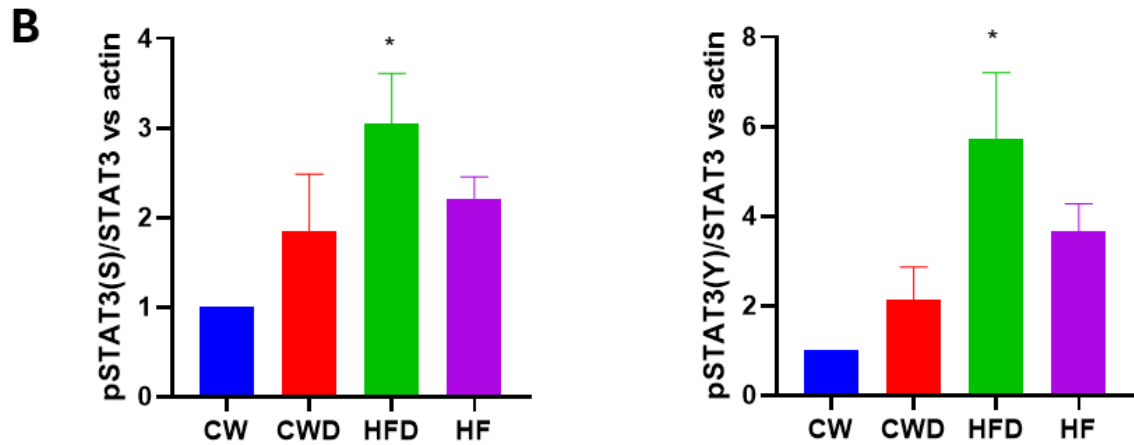


Figure 13: (A) The western blot of hepatic tissue from mice treated with a chow diet (blue), CW-DEN (red), HF-DEN (green), and HF (purple) for three months is shown. (B) shows the corresponding densitometries, with STAT3 serine phosphorylation on the left and tyrosine phosphorylation on the right. 3n

“*” p<0.01

Therefore, in Figure 14, we performed a co-treatment, in which HGF was administered as an activator of the pathway, while also adding the treatment with GDF11 for 12 hours, because we had observed that it was the estimated time it took for GDF11 to diminish the activation of that pathway.

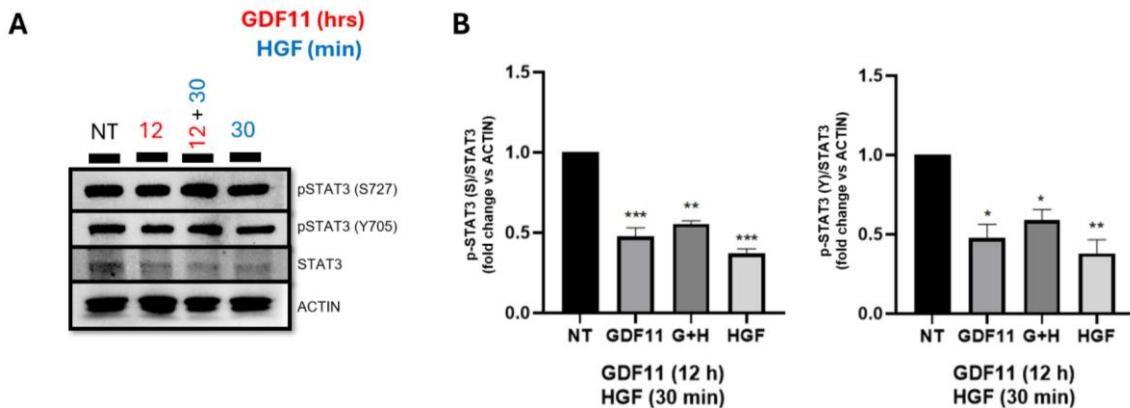


Figure 14: (A) We can observe the western blot of Huh7 cells with the co-treatment. (B) The corresponding densitometries are observed, with serine phosphorylation on the left side and tyrosine phosphorylation on the right side. 2n

“*” p<0.01, “***” p<0.001, “****” p< 0.0001

We continue with the addition of a MASL-like medium, which is a steatogenic medium, as it increases the content of fatty acids and cholesterol, as we can see in Figure 15.

A

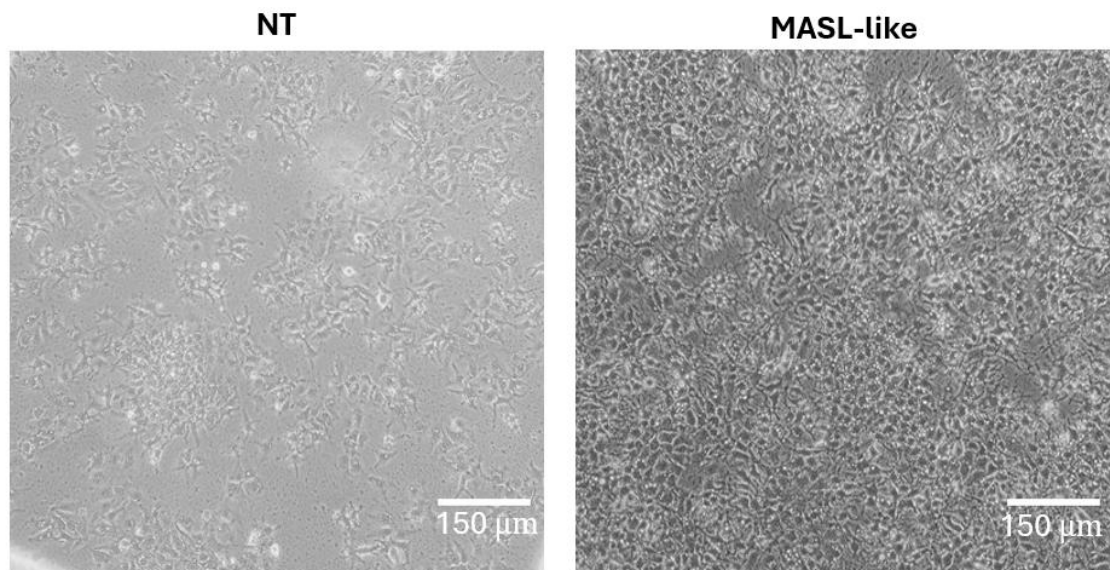
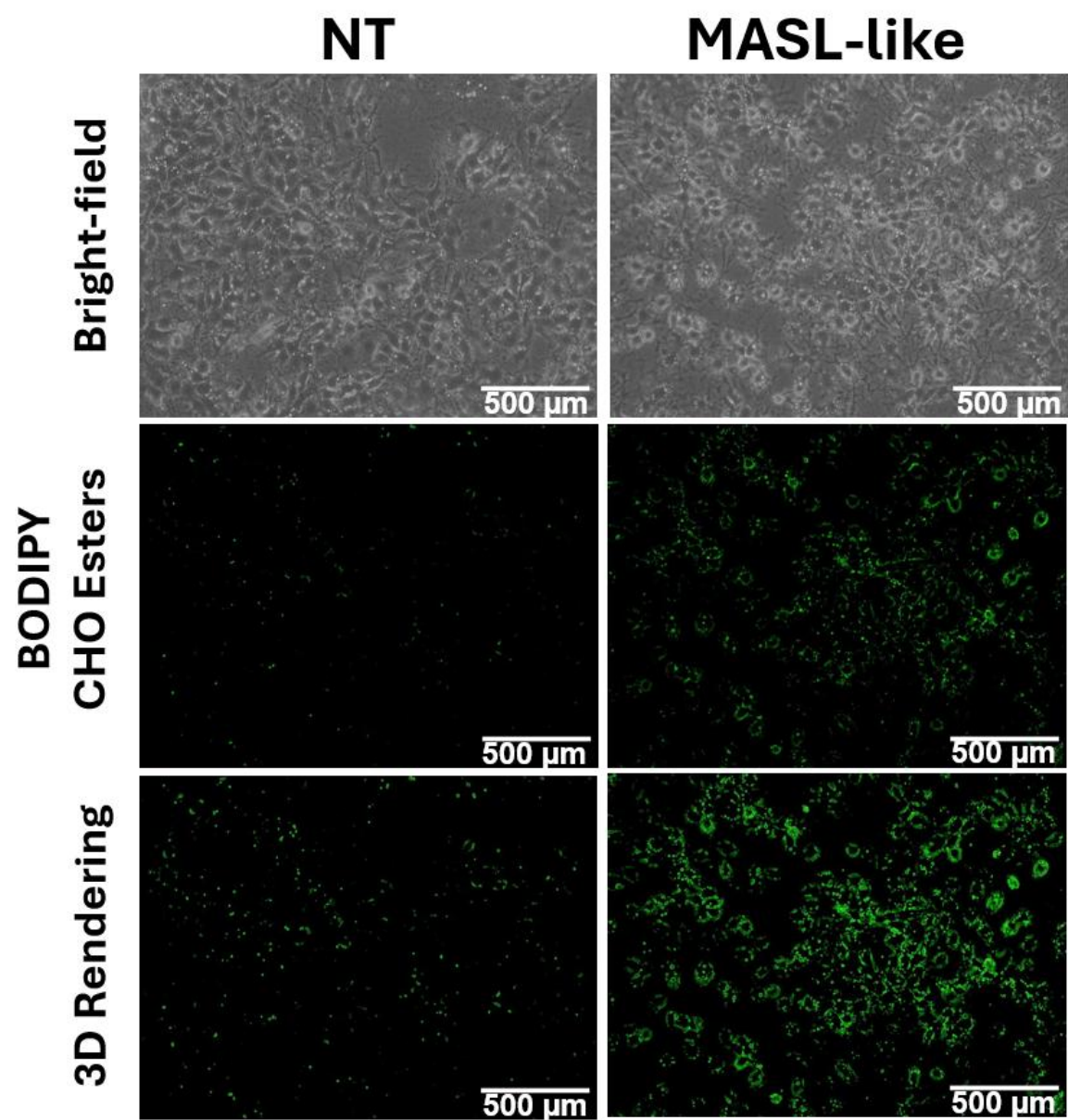


Figure 15: (A) We observe the Huh7 cells without treatment (NT). (B) The cells were treated with MASL-like medium. 2n

To verify that the cells were indeed loading fatty acids and cholesterol, in Figure 16, staining with BODIPY was performed. The data suggest a higher cholesterol content in the cells treated with that medium. The visualization of stained lipids was performed using fluorescence microscopy with filters having an excitation/emission wavelength of 493/503 nm. Neutral lipids were observed as bright green inclusions distributed in the cytoplasm.



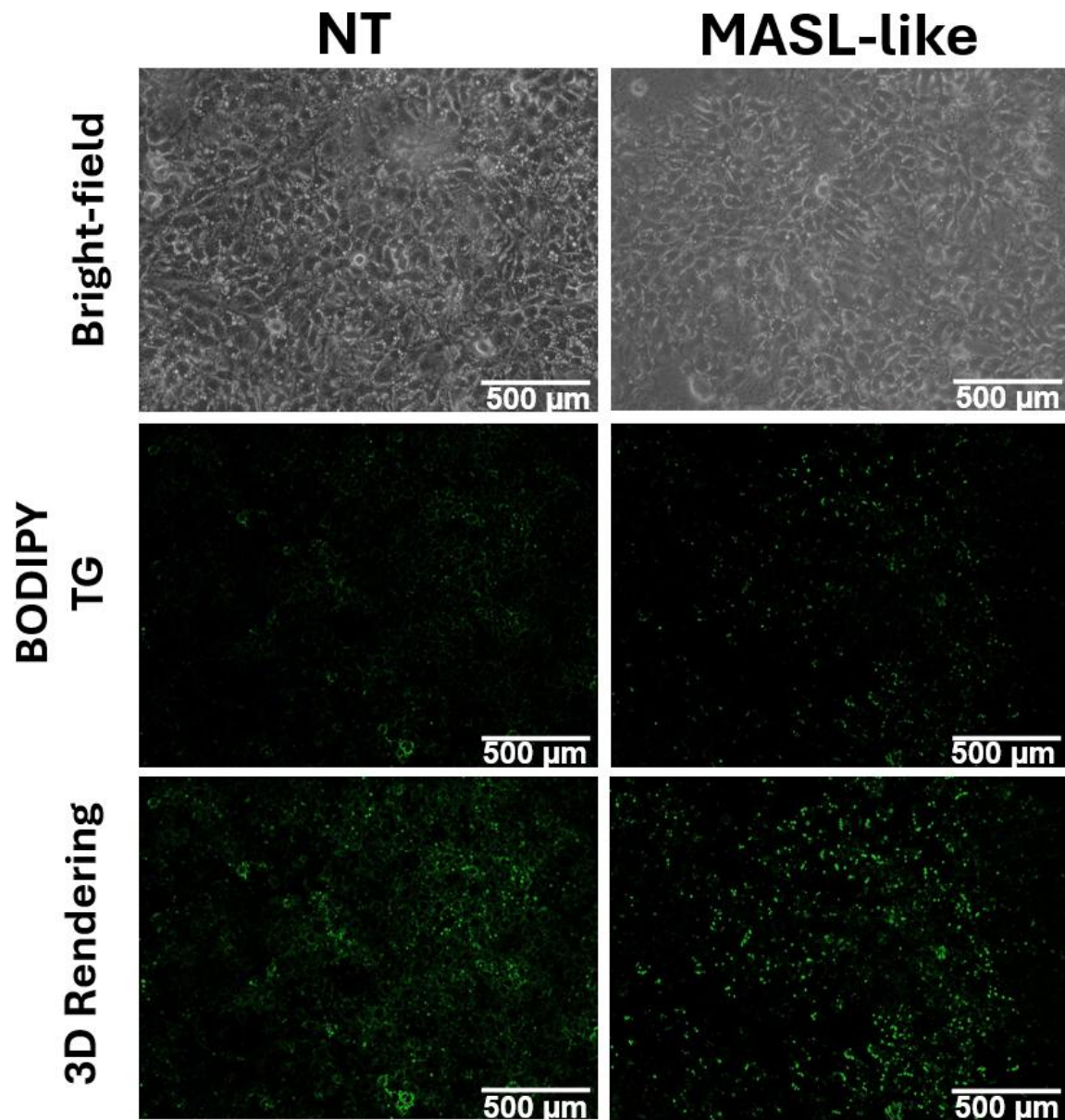


Figure 16: (A) The cholesterol esters of control Huh7 cells and those with MASL-like medium are observed with Bodipy staining. (B) The triglyceride content of the same treatments with the same staining is observed. 1n

Once the MASL-like medium was standardized, we began the migration assays, where the steatogenic medium and the GDF11 treatment were put to compete. In Figure 17, we can see that after 48 hours with the MASL-like medium, the wound had already closed, whereas the co-treatment does not allow the wound to close, resulting in the steatogenic medium providing aggressiveness to the cells by increasing cellular migration, while the GDF11 treatment, despite being with the MASL-like medium, decreases aggressiveness, as it reduces cellular migration.

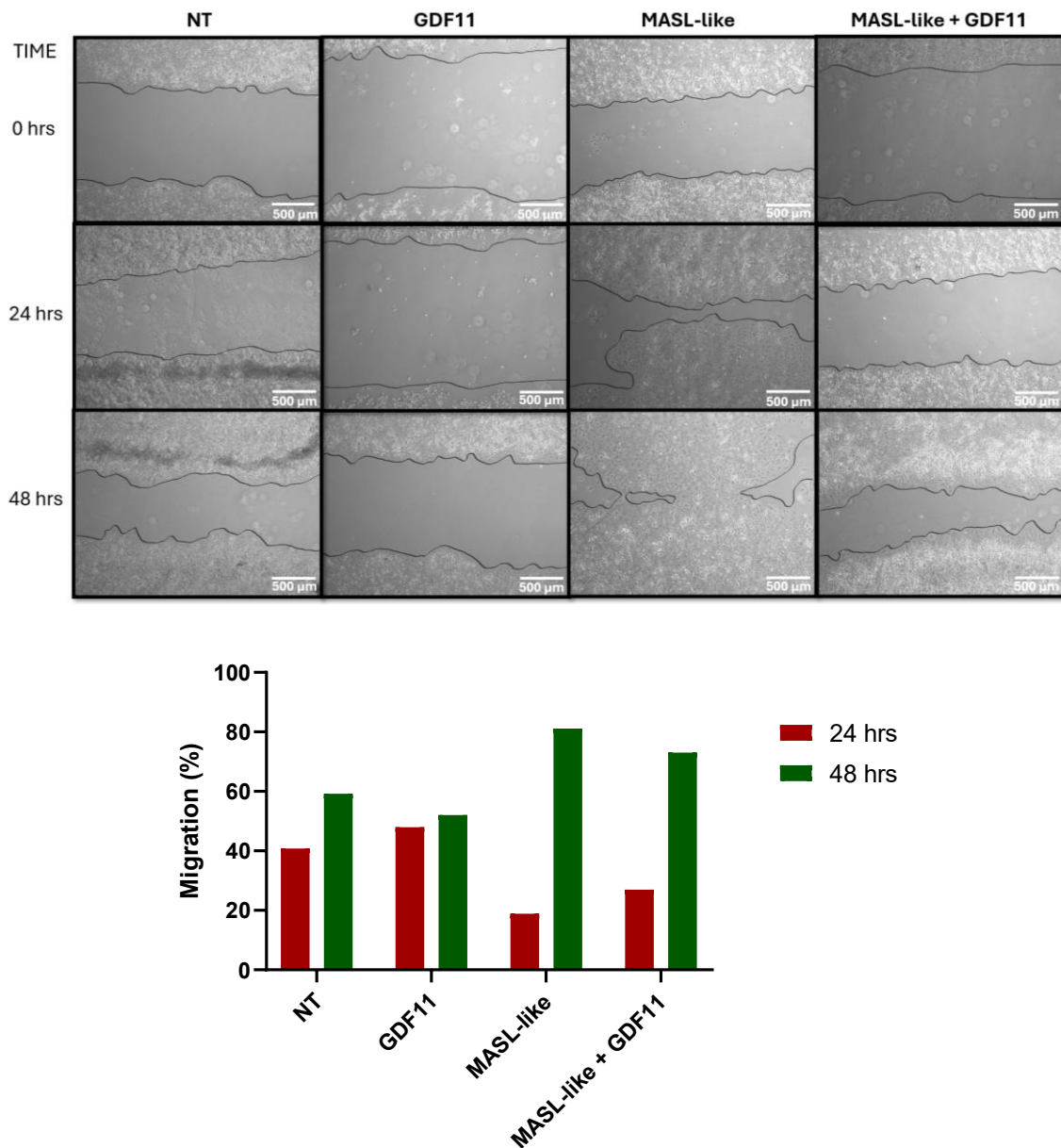


Figure 17: At the top, you can see the different treatments (NT: Not treated; GDF11: 50ng/µl; MASL-like; MASL-like + GDF11) at different times, such as 24, and 48 hours (in red, and green, respectively). At the bottom is the densitometry of the migration percentage. 1n

Subsequently, to assess the cellular viability of the previous treatments, in figure 18 crystal violet was used, where a decrease in viability was observed in cells treated with GDF11 and a slight decrease in the co-treatment, while treatment with MASL-like increased viability, as did cells treated with IL-6.

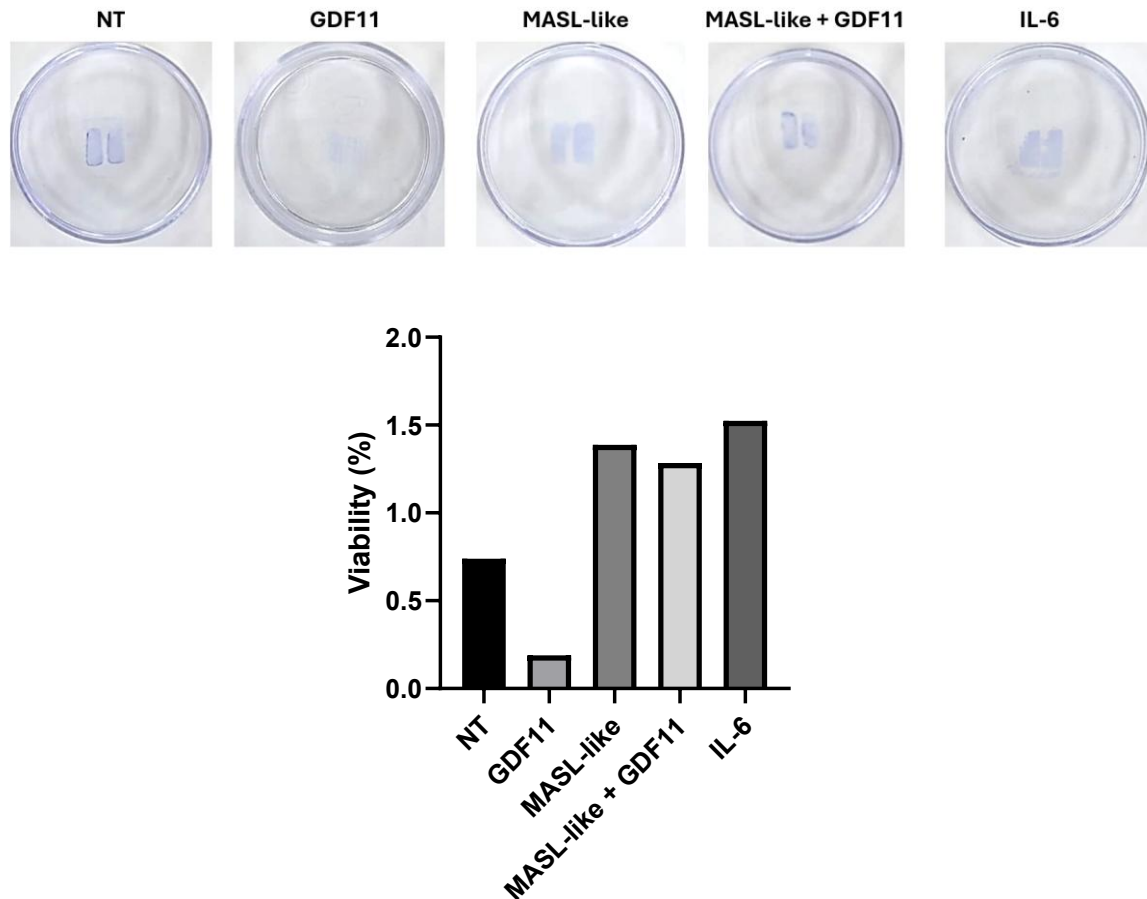


Figure 18: At the top is the violet crystal assay of the untreated cells, those treated with GDF11 (50 ng/ml), with MASL-like, the co-treatment, and the positive control, such as IL-6. At the bottom is the corresponding densitometry. 1n

Inhibitor of STAT3 function

Therefore, to determine whether it is the STAT3 signaling pathway that is providing aggressiveness to the tumor, we decided to carry out a treatment mimic with GDF11, inhibition of STAT3 phosphorylation with the SH domain using a vector as we assume the treatment with GDF11 does. The transduction is carried out in different cell lines, using HEK293 as a control, Huh7 as one of the most important, and also Hep3B and HepG2, as shown in Figure 19.

TRANSDUCTION

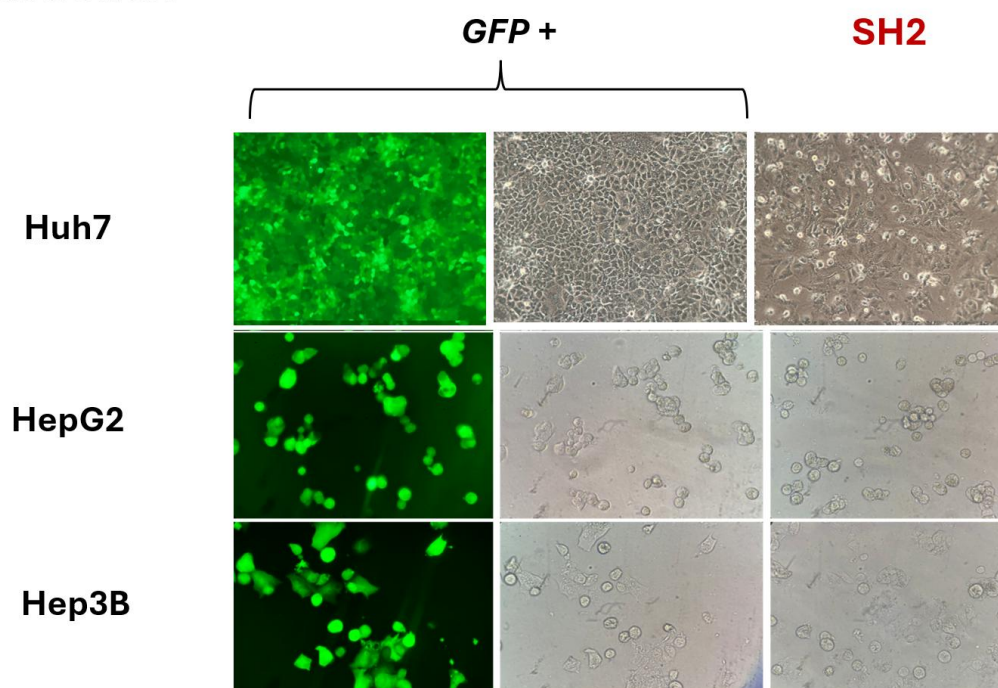


Figure 19: Evaluation of transduction efficiency in hepatocellular carcinoma cell lines. Brightfield and fluorescence microscopy images of Huh7, HepG2, and Hep3B cells following transduction with a GFP-expressing lentivirus. Efficient delivery and expression of the transgene were observed across all three lines, as indicated by the widespread green fluorescence.

As we can observe in Figure 20, a Western Blot was performed to determine the content of both phosphorylations of STAT3 in Huh7 cells with the silencing system. As can be seen, there is a difference in the content of the STAT3 protein (NT vs SH) that is solely due to the use of the vector (breaking the positive feedback loop of the same STAT3 signaling pathway). Likewise, the difference in protein content among the three silenced samples compared to the empty vector is evident. Based on this, it is clear that there is a greater impact on the decrease of STAT3 tyrosine phosphorylation content, as well as the total protein content. We used sorafenib treatment as a positive control for the decrease in protein content.

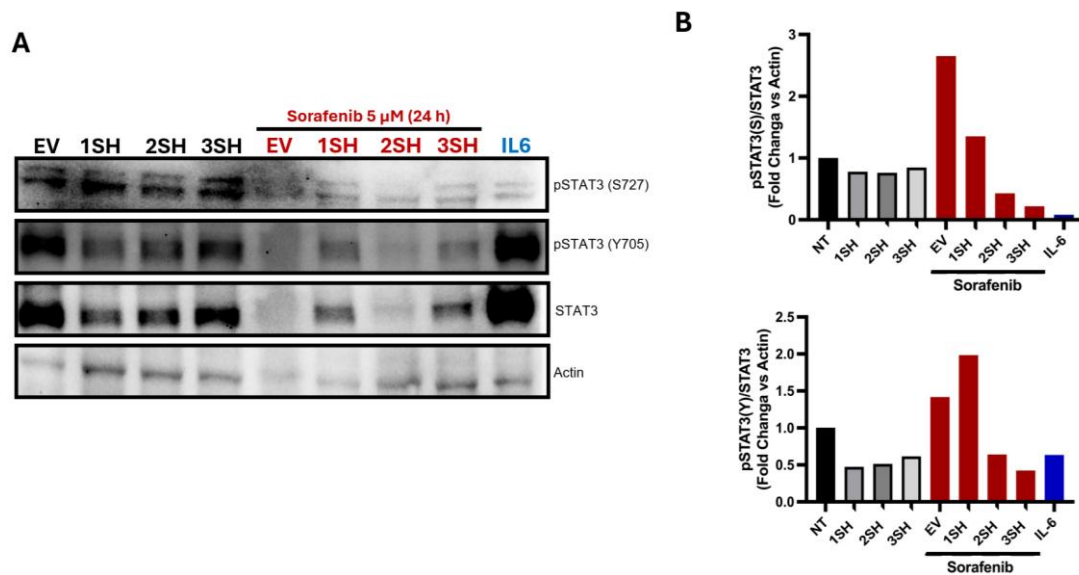
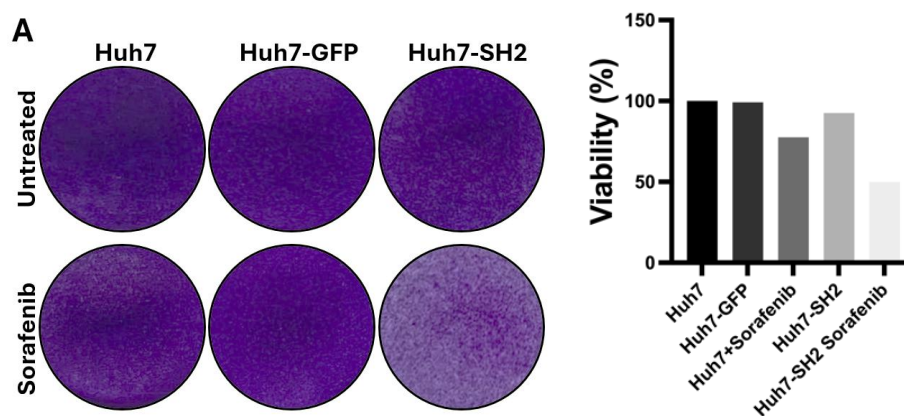


Figure 20: (A) The western blot of Huh7 cells with the vector for STAT3 silencing is observed, and as a positive control, the treatment with sorafenib for 24 hours. (B) The corresponding densitometries are observed, the graph above shows the content of serine phosphorylation of STAT3, and the graph below shows the content of tyrosine phosphorylation of STAT3. 1n

Additionally, in Figure 21, we assessed the viability of cells treated with the vector using crystal violet. As we can observe, the cells with silencing and treatment with sorafenib show a decreased viability of approximately 50%, impacting cell viability, which we have observed in different cell lines such as Huh7 and Hep3B. HEK 293 cells were used as a positive control for the transduction.



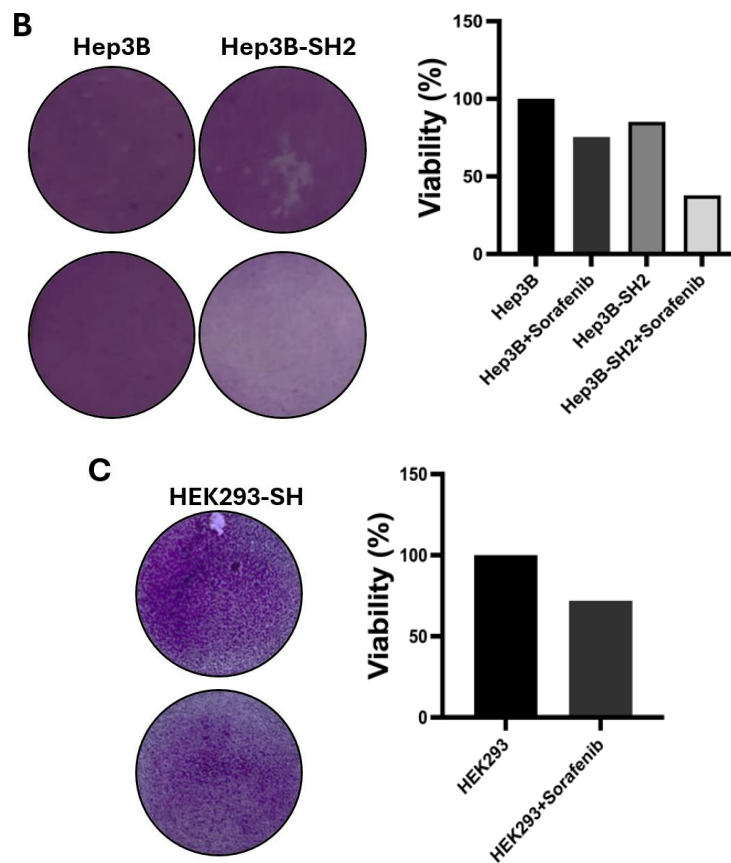


Figure 21: (A) The staining with crystal violet of Huh7 cells and those with the vector is observed. (B) The control Hep3B cells and those with the vector. (C) The control HEK 293 cells and those with the vector. At the bottom, the corresponding densitometries are shown. 1n

To corroborate what we observed with crystal violet staining, a flow cytometry analysis was performed, in Figure 22, which showed a greater sensitivity to sorafenib treatment, confirming that both the decrease in cell viability and the increase in treatment sensitivity are due to STAT3 inhibition.

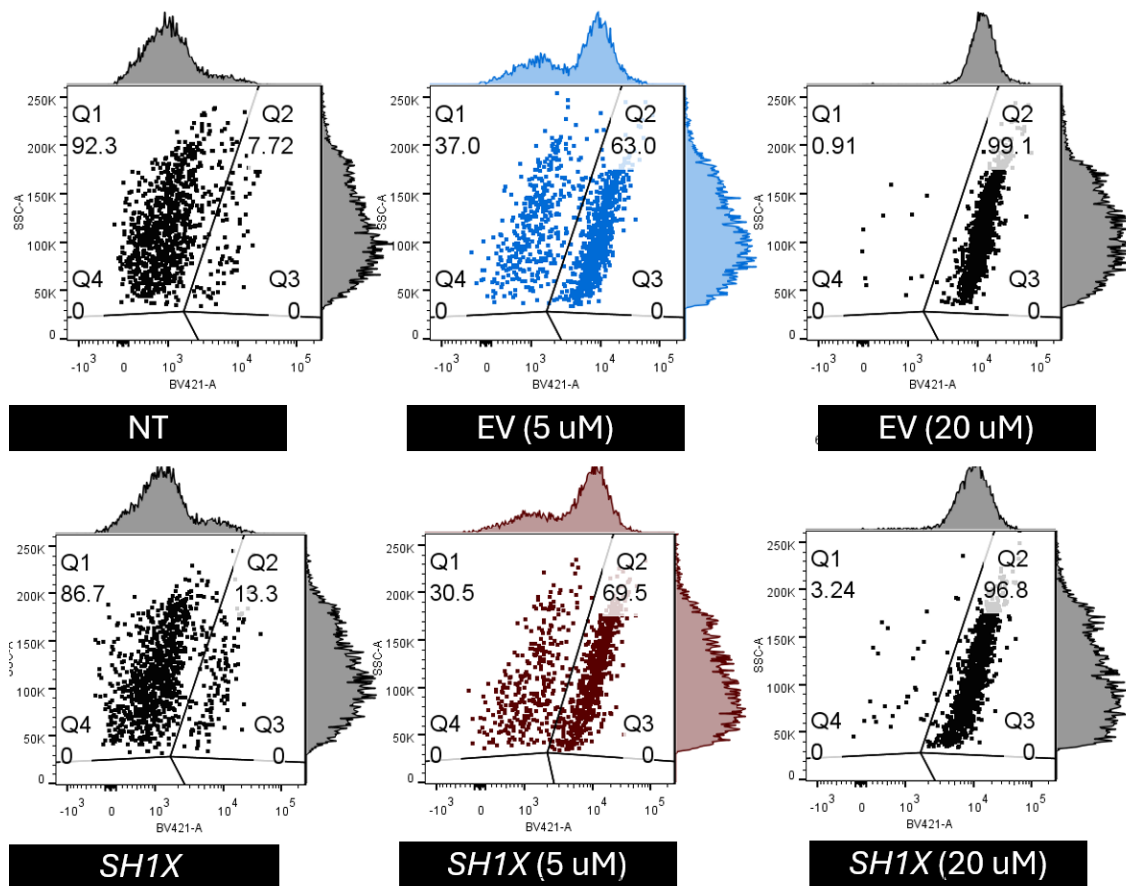


Figure 22: NT: Untreated cells; EV: cells with the empty vector treated with 5 or 20 μ M sorafenib; SH1: cells transduced with the vector treated with 5 or 20 μ M sorafenib. Treatment with 20 μ M sorafenib as a positive control led to a decrease in cell viability. 10000 events

It is worth noting that an attempt was also made to transduce the HepG2 cell line, but the cells died, demonstrating their dependence on the STAT3 signaling pathway, as data from the Human Protein Atlas show in Figure 23, that the cell line most dependent on the STAT3 signaling pathway is HepG2, followed by Hep3B, and finally, the least dependent is Huh7.

The human protein atlas

STAT3 contain in liver cancer cell lines.

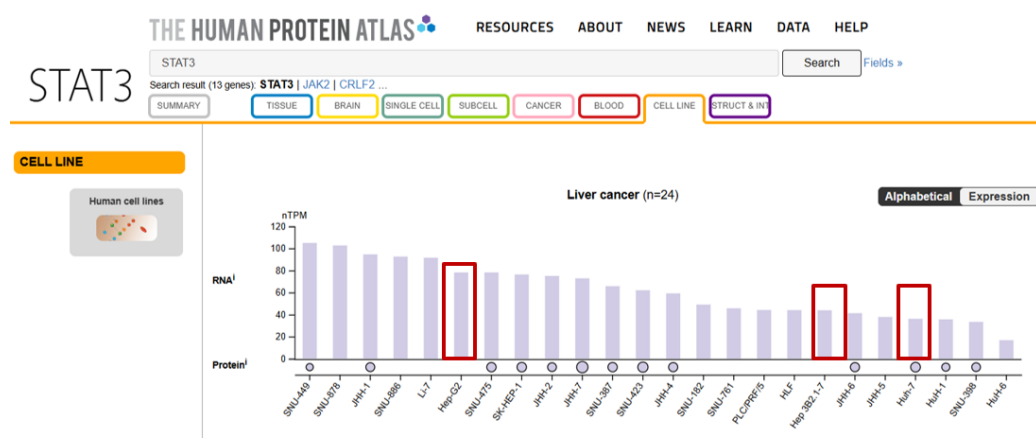


Figure 23: STAT3 expression profiles in liver cell lines from the Human Protein Atlas. Data obtained from the Human Protein Atlas database illustrating the baseline expression levels of STAT3. The analysis reveals the relative abundance of STAT3 [protein/transcript] across the indicated liver cell lines, providing context for baseline STAT3 activity before experimental manipulation.

Discussion

As can be seen in Figure 4, treatment with GDF11 in Huh7 cells for just 12 hours deregulates signaling pathways in which STAT3 is involved, such as mTOR. mTOR can also mediate STAT3 activation at serine 727, an event critical for its activation (Arevalo et al., 2023). In turn, STAT3 activation can trigger mTOR activation and stimulate cell proliferation, generating a feedback loop (Vella et al., 2020). STAT3 and mTOR are two key proteins involved in regulating multiple cellular processes, including cell growth, proliferation, differentiation, and survival (Zhou et al., 2007). It is worth noting that these interconnections between STAT3 and mTOR suggest therapeutic implications in the treatment of various diseases, including cancer (J. Wang et al., 2021).

It is also important to emphasize that most of the target proteins of the STAT3 signaling pathway that we found downregulated are very important, as they are involved in proliferation (such as MYC), metastasis (WNT5A), cell cycle regulation, apoptosis (MCL-1) and EMT (β -catenin). In figure 5, the downward deregulation of MYC is observed, STAT3 can induce MYC expression, which contributes to the proliferation, survival and growth of tumor cells, so disrupting the STAT3 pathway may also indirectly disrupt MYC signaling (Sun et al., 2022).

On the other hand, there are negative regulators of the STAT3 pathway, such as phosphatases, of which there are different types of phosphatases that regulate the transcriptional activity of STAT3 by dephosphorylating at Y705 and/or S727. The main phosphatases include PTP, PP2A, and PIAS. (X. Li, Rasul, Sharif, & Hassan, 2024). PIAS3 is a SUMO E3 ligase that blocks binding to DNA, thereby inhibiting STAT3 transactivation.

Although there is no direct inhibitor of this phosphatase, figure 7 shows that treatment with GDF11 promotes the deregulation of several genes, including the deregulation of PIAS, which could indicate the dephosphorylation of STAT3, leading to a decrease in aggressiveness in cancer cells. On the other hand, PP2A activators are under development, as this Ser/Thr phosphatase regulates serine phosphorylation, modulating the transcriptional activity of STAT3 (Johnson, Narayan, & Sharma, 2024).

In figures 8 and 9, one can observe the importance of the dual response of the TGF-beta signaling pathway, as sustained signaling generates chronic inflammation, fibrosis, and ultimately cancer, since it promotes, among many other cellular responses, extracellular matrix remodeling and immune evasion (Massague & Sheppard, 2023). Although in the literature there are various therapeutic inhibitors to block this response without affecting the initial protective response (Jing, Gao, Jing, Yang, & Liu, 2025), GDF11 continues to be a promising tumor suppressor, as it affects various pro-tumor signaling pathways.

As observed in figure 10, a decrease in tyrosine phosphorylation with GDF11 treatment at short times, which could indicate a decrease in protein dimerization and the consequent translocation to the nucleus, this phosphorylation as a classic activator (Ortega, Ondo-Mendez, & Garzon, 2017). Meanwhile, at longer treatment times, as seen in figure 11, both phosphorylations decrease, which could indicate a decrease in poor prognosis or even tumor adaptation, since (Brizio Moreno-Jaime, 2016) mention that the tyrosine phosphorylation of HeLa cells in hypoxia suggests a role in tumor adaptation. On the other hand, serine phosphorylation states that it has been associated with more aggressive phenotypes and less survival in clear cell renal carcinoma (ccRCC), even concluding a mutation in this phosphorylation that promotes a pro-tumoral phenotype, even in the absence of tyrosine phosphorylation, acting independently (Arévalo Bautista, 2019).

The relationship between STAT3 and MCL1 is crucial for oncogenesis, since the activation of STAT3 promotes the overexpression of MCL1 (Wu, Luo, & Liu, 2020), generating resistance to apoptosis, and favoring the survival and proliferation of cancer cells. In figure 12 it is observed that in Huh7 cells treated with GDF11 for 12 hours, the transcript of MCL1 decreases, suggesting a reduction in survival genes, decreasing

resistance to apoptosis. It is also important to point out that although the data are not conclusive regarding treatment with GDF11 and catenin, the latter is relevant and interacts with STAT3, since STAT3 and catenin are two important proteins that play a crucial role in various cellular processes, including development, immune response, and cancer (Lee et al., 2019). The interconnection between these two pathways promotes tumorigenesis, as it has been observed to increase the proliferation, migration, and survival of transformed cells (Liu et al., 2022).

On the other hand, a steatogenic medium can activate the STAT3 signaling pathway, as lipid accumulation generates metabolic stress, which activates inflammatory pathways, such as IL-6, which is a classic activator of STAT3 (Ibarretxe & Masana, 2021). That said, mice on a High Fat diet with DEN at 3 months, as shown in figure 13, exhibit increased protein content of both phosphorylations compared to the CW diet, indicating that the cultivation of cells derived from hepatocellular carcinoma, such as Huh7, in a steatogenic medium increases the activation of the STAT3 pathway.

The different signaling pathways that are deregulated with the treatment, of which the majority are related to the oncogenic STAT3 pathway, like the HGF pathway, mTOR, apoptosis, etc. HGF can activate STAT3 through the MET pathway, a tyrosine kinase receptor. Once activated, STAT3 can translocate to the nucleus and bind to DNA, regulating the expression of target genes to contribute to cell transformation induced by a diverse set of oncoproteins (Zhang et al., 2002).

It is important to note that STAT3 activation, which promotes HGF transcription, may be a process specific to certain cell types, such as transformed cells (Wojcik et al., 2006). In figure 14, the western blot of Huh7 cells with HGF shows that treatment with GDF11 maintains a stable decrease in the content of both phosphorylations of STAT3.

To simulate a steatogenic diet in vitro, we used the MASH-like medium as shown in figure 15, which promotes triglyceride synthesis and the formation of cholesterol esters (as seen in figure 16) that accumulate in lipid droplets, which in turn decreases beta-oxidation, favoring fat storage and increasing the activation of transcription factors that promote de novo lipogenesis like SREBP-1c and LXR (Daiana Ibarretxe, et al., 2021).

As was also seen in (Alejandro Escobedo-Calvario, 2025), in figure 17 we can observe the effect of the treatment with GDF11, which reduced migratory capacity, but what makes it even more promising is that, despite the cells being in a steatogenic medium, co-treatment with GDF11 manages to reduce the migration percentage.

As can be seen in Figure 18, a test with crystal violet was carried out to evaluate cell viability, highlighting the co-treatment of a steatogenic medium with GDF11. As reported

(Gerardo-Ramirez et al., 2019), treatment with GDF11 does not affect cell viability, but it does affect cell activity, especially in terms of tumor aggressiveness.

There are various therapeutic implications currently, for example, inhibitors that focus on tyrosine phosphorylation like Napabucasin or OPB-51602, which have high therapeutic efficacy with a moderate level of toxicity (Kyli Berkley et al., 2025).

As shown in Figure 23, a tyrosine phosphorylation inhibitor of STAT3 was designed because the SH2 domain specifically binds at that site, preventing the phosphorylation-associated function by inhibiting dimerization and signal transduction to molecular targets. It is important to clarify that the inhibition of serine phosphorylation is highly toxic, which is why current inhibitors are less developed, as they only work in tumors with altered metabolism. Despite hepatocellular carcinoma being an OXPHOS-dependent tumor, the ideal strategy is recommended to be the co-inhibition of serine phosphorylation, for example, the inhibitor YY002 (Huang Chan et al., 2024).

That said, in figure 21, the viability of the cells with the inhibitor was quantified. As we expected, it did not affect viability on its own; however, when combined with sorafenib treatment, the cells with the inhibitor became more susceptible to decreasing viability. The treatment with sorafenib inhibits tumor proliferation by blocking the Ras/Raf/MEK/ERK pathway and angiogenesis, blocking tyrosine kinase receptors such as VEGFR (vascular endothelial growth factor receptor) and PDGFR (platelet-derived growth factor receptor), prolonging the survival of patients with hepatocellular carcinoma (Lab, 2025).

Finally, in Figure 22, sorafenib treatment was used to truly verify the decrease in cell viability of the transduced cells, since the literature mentions that the Huh7 cell line has acquired or intrinsic resistance (He et al., 2025), as authors usually use between 2-5 μM of sorafenib and the cells tend to show resistance to the treatment, where viability is reduced by less than 30% (F. Li et al., 2025).

It is worth noting that there are more sensitive cell lines, for example HepG2 (figure 19). According to everything presented above, this sensitivity could be due to the dependence on the STAT3 signaling pathway, as shown in Figure 23, where the HepG2 cell line is more dependent on STAT3, followed by Hep3B, and finally Huh7, which are the least dependent.

Conclusion

The pleiotropic nature of STAT3, whereby it integrates multiple signaling pathways to promote proliferation, survival, and migration, is a key driver of tumor aggressiveness. Consequently, therapeutic strategies targeting STAT3 are highly sought after. This study identifies GDF11 as a promising candidate in this context. Our findings demonstrate that GDF11 treatment decreases the activity of several proteins in the STAT3 signaling network, thereby reducing the aggressive tumor phenotypes associated with STAT3 activation. This conclusion is supported by multiple lines of evidence. First, we observed a marked increase in STAT3 phosphorylation in livers from mice subjected to a tumor-promoting, steatogenic high-fat diet combined with DEN, confirming the pathway's activation under oncogenic conditions. Conversely, treatment with GDF11 significantly reduced this phosphorylation in Huh7 cells. This inhibitory effect on the canonical, tyrosine-phosphorylation-dependent activation pathway appears to be a key mechanism, which may offer a more specific and less toxic alternative than targeting other regulatory sites, such as serine phosphorylation.

Furthermore, the relevance of this pathway to tumor cell survival is underscored by the dependence of tumor cell lines on STAT3 signaling; genetic engineering experiments aimed at disrupting this pathway highlight its critical role in maintaining aggressive traits. The ability of GDF11 to mitigate these traits, specifically by reducing cell migration, without broadly impacting overall cell viability, reinforces its potential as a tumor suppressor. By modulating multiple pro-tumor pathways simultaneously, GDF11 offers a compelling strategy to reduce tumor aggressiveness.

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ACTA DE EXAMEN DE GRADO

No. 00271

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GDF11 represses the STAT3 signaling pathway, decreasing the aggressiveness of cells derived from human hepatocellular carcinoma.

En la Ciudad de México, se presentaron a las 16:00 horas del día 23 del mes de marzo del año 2026 en la Unidad Iztapalapa de la Universidad Autónoma Metropolitana, los suscritos miembros del jurado:

DR. BENJAMIN PEREZ AGUILAR
DRA. ELSY SORAYA SALAS SILVA
DRA. MONSERRAT GERARDO RAMIREZ
DR. MOISES VERGARA MENDOZA



MELISSA SANCHEZ RODRIGUEZ
ALUMNA

Bajo la Presidencia del primero y con carácter de Secretario el último, se reunieron para proceder al Examen de Grado cuya denominación aparece al margen, para la obtención del grado de:

MAESTRA EN BIOLOGÍA EXPERIMENTAL

DE: MELISSA SANCHEZ RODRIGUEZ

y de acuerdo con el artículo 78 fracción III del Reglamento de Estudios Superiores de la Universidad Autónoma Metropolitana, los miembros del jurado resolvieron:

Aprobar

Acto continuo, el presidente del jurado comunicó a la interesada el resultado de la evaluación y, en caso aprobatorio, le fue tomada la protesta.

REVISÓ

MTRA. ROSALIA SERRANO DE LA PAZ
DIRECTORA DE SISTEMAS ESCOLARES

DIRECTORA DE LA DIVISIÓN DE CBS



DRA. EDITH ARENAS RIOS

PRESIDENTE



DR. BENJAMIN PEREZ AGUILAR

VOCAL



DRA. ELSY SORAYA SALAS SILVA

VOCAL



DRA. MONSERRAT GERARDO RAMIREZ

SECRETARIO



DR. MOISES VERGARA MENDOZA