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Characterization of the epigenetic modifications in human uterine leiomyomas and evaluation of their potential as therapeutic targets *in vitro*

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“De nada sirve toda la geografía, trigonometría y aritmética si no aprendes a pensar por ti mismo. Y en ningún colegio te enseñan eso. No está en el programa.” – Marina, Carlos Ruiz Zafón.

“All the geography, trigonometry, and arithmetic in the world are useless, unless you learn to think for yourself. No school teaches you that. It's not on the curriculum.” — Marina, Carlos Ruiz Zafón

A todas las personas que me enseñaron a pensar.
En especial a mi madre, Isabel, y mi padre, José Luis.

To all the people who have taught me to think.
Especially my mother, Isabel, and my father, José Luis.

La **Dra. Hortensia Ferrero Cháfer**, Doctora en Biotecnología, Investigadora Miguel Servet del Instituto de Salud Carlos III, Investigadora en Fundación IVI - Instituto de Investigación Sanitaria La Fe, en el área de investigación Fertilidad y Medicina Materno-Infantil, Medicina Reproductiva.

CERTIFICA:

Que el trabajo de investigación titulado: "**Characterization of the epigenetic modifications in human uterine leiomyomas and evaluation of their potential as therapeutic targets in vitro**" ha sido realizado íntegramente por María Cristina Carbajo García bajo mi dirección. Dicha memoria está concluida y reúne todos los requisitos para su presentación y defensa como TESIS DOCTORAL ante un tribunal.

Y para que así conste a los efectos oportunos, firmo la presente certificación en Valencia a 27 de octubre de 2022.

Fdo. Dra. Hortensia Ferrero Cháfer

El **Profesor Antonio Pellicer Martínez**, Catedrático en Ginecología, Doctor en Medicina y Cirugía, Profesor titular del Departamento de Pediatría, Obstetricia y Ginecología de la Facultad de Medicina de la Universidad de Valencia, fundador del Instituto Valenciano de Infertilidad y CEO de IVIRMA global.

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Fdo. Dr. Antonio Pellicer Martínez

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"Yo soy yo y mis circunstancias" - Ortega y Gasset

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ABSTRACT

Uterine leiomyomas or fibroids (UL) are the most common benign tumors in women of reproductive age. Despite the high prevalence, and the large amount of management options, there is no effective treatment for UL size reduction minimally invasive and without long-term side effects. Its tumor pathology remains unclear, which hampers the development of safe and effective treatments. Therefore, the identification of molecular mechanisms involved in UL pathogenesis could allow the development of more efficient treatments. Epigenetic modifications are involved in tumor development, and they can be reversed by chemical agents. This possibility opens insights into new therapeutic options for patients with UL. Considering this, the main objective of our study is to describe the epigenetic mechanisms, regarding DNA methylation and histone modification, involved in UL development, which could be a new therapeutic target to treat UL. Our results revealed a higher DNA methylation in UL compared to adjacent myometrium (MM). This aberrant methylation regulates the expression of genes involved in tumor development and maintenance, causing hypomethylation/upregulation of oncogenes and hypermethylation/downregulation of tumor suppressor genes. Consequently, several important processes in UL physiology are altered such as proliferation, invasion, extracellular vesicles, metabolism, deposition of extracellular matrix (ECM), and Wnt/β-catenin pathway. In addition, DNA methyltransferases inhibition by 5-aza-CdR treatment inhibits cell proliferation, ECM formation, and Wnt/β-catenin signaling pathway in HULP cells in vitro. These results suggest that DNA methylation inhibition could offer a new therapeutic option to treat patients with UL. We also found an altered acetylation in Lysine 27 of Histone 3 (H3K27ac), causing a hyperacetylation/upregulation of oncogenes and hypoacetylation/downregulation of tumor suppressor genes in UL, which are related to the immune system, angiogenesis, invasion, altered metabolism, aberrant deposit of ECM, TGFβ and Wnt/β-catenin pathway dysregulation. The reversal of histone deacetylation through SAHA treatment induces the reduction of ECM deposition, decreases cell cycle progression and cell proliferation in UL cells in vitro, which may induce inhibition of UL growth. These findings highlight that inhibition of HDACs through SAHA may be a viable therapeutic target to treat UL. Based on the results obtained in this thesis, we conclude that epigenetic mechanisms, through DNA methylation and histone modifications as acetylation of histone 3, are key trigger of UL pathogenesis. In addition, targeting enzymes that catalyze these epigenetic changes is a possible therapeutic approach to treat patients with UL.

RESUMEN

INTRODUCCIÓN

1. ÚTERO: ANATOMÍA Y FUNCIÓN

1.1. Anatomía del útero

El aparato genital femenino está compuesto por los genitales externos, que son el perineo y la vulva, y los órganos internos, que son los ovarios, las trompas de Falopio, el útero y la vagina. El útero es el principal órgano reproductor femenino. Es una estructura muscular de paredes gruesas situada en la línea media de la cavidad pélvica abdominal. Tiene cuatro partes: el cuello uterino, el istmo, el cuerpo y el fundus. El cuello uterino es una estructura fibromuscular cuya función principal es el paso de la sangre menstrual y los espermatozoides y conecta el cuerpo uterino con el lumen vaginal. El cuerpo uterino es el segmento más grande y se conecta al cuello uterino a través del istmo. El útero está conectado con el ovario a través de las trompas de Falopio, situadas entre el fundus y el cuerpo, las cuales se unen al ovario mediante fimbrias.

El cuerpo uterino contiene tres capas:

- **Endometrio**. Es la capa mucosa más interna que recubre la cavidad uterina. Su grosor y la estructura varían en función de la estimulación hormonal. Se divide en una capa funcional, que se desecha durante la menstruación, y otra basal, que se conserva y regenera la capa funcional.
- **Miometrio**. Es la capa muscular y media. El miometrio (*Myometrium*; MM) es la capa más gruesa (12-15 mm) y la más vascularizada del útero. Está compuesto por células musculares lisas incrustadas en una matriz extracelular (*ExtraCellular Matrix:* ECM) rica principalmente en colágeno. Durante el embarazo, las células musculares se dividen (hiperplasia) y se agrandan (hipertrofia), para que el útero pueda crecer mientras el endometrio nutre el embarazo.
- **Perimetrio.** El perimetrio es la capa serosa más externa. Cubre el cuerpo uterino y el fondo del útero.

1.2. Función uterina

El útero es responsable de muchas funciones en los procesos de implantación, gestación, menstruación y parto. Responde a la producción de hormonas femeninas, creando cambios para permitir la implantación de un óvulo fecundado, o la menstruación cuando no se produce el embarazo. El útero funciona alimentando el óvulo fecundado, que pasa por la trompa de

Falopio. El MM se expande rápidamente con el desarrollo del embarazo para dar cabida al feto en desarrollo. Por último, al final del embarazo, tiene una función contráctil para el parto.

1.3. La matriz extracelular

La ECM es una red tridimensional de macromoléculas que proporciona soporte estructural y bioquímico a las células miometriales. Está implicada en la adhesión, la comunicación celular y la diferenciación. Los principales componentes de la ECM son colágeno, proteoglicanos, elastina, integrinas, y fibronectina. Los cambios que sufre la ECM están controlados por las metaloproteinasas de la matriz (*Matrix MetalloProteinases*; MMP), enzimas con actividad proteolítica que están parcialmente reguladas por los inhibidores tisulares de las MMP (*Tissue Inhibitors of MetalloProteinases;* TIMP). La expresión de estas enzimas está influida por las citocinas proinflamatorias, las hormonas y los factores de crecimiento, como el factor de crecimiento transformante β (TGFβ). Un desequilibrio entre las MMPs y los TIMPs puede promover condiciones patológicas como quistes ováricos, endometriosis o miomas uterinos (*Uterine Leiomyoma;* UL).

2. MIOMAS UTERINOS

2.1. Descripción

Los UL, también llamados fibromas uterinos (*Uterine Fibroids;* UF), son tumores benignos dependientes de estrógenos que surgen de las células musculares lisas localizadas en la capa miometrial del útero. Se caracterizan por una distribución desordenada de células musculares lisas y fibroblastos combinados en una gran cantidad de ECM.

2.2. Clasificación

La clasificación de los UL está basada en su localización en las capas del útero. En base a ella, los UL submucosos son aquellos que deforman el endometrio o protruyen la cavidad uterina. Los UL subserosos, protruyen desde la capa más externa del útero (la serosa), dando lugar a úteros con forma irregular. Finalmente, los UL que crecen entre la pared uterina se denominan intramurales. La mayoría de los UL son una combinación de varios de estos tipos.

2.3. Epidemiología y síntomas

2.3.1. Epidemiología del leiomioma uterino

Los UL son los tumores ginecológicos más prevalentes en las mujeres en edad reproductiva. La prevalencia de los UL varía entre los distintos estudios y países, desde el 4,5% hasta el 68,6%. Estas diferencias se deben al tipo de investigación, al método de diagnóstico y a la demografía

racial/étnica de la población estudiada. Son tres veces más frecuentemente diagnosticados en mujeres de ascendencia africana que en las mujeres caucásicas. Los UL afectan principalmente a las mujeres durante sus años reproductivos, siendo extremadamente raros antes de la menarquia, y retrocediendo típicamente después de la menopausia. La edad es un factor de riesgo en la patogénesis del UL, siendo más probable que se desarrolle en mujeres de entre 30 y 40 años. Otros factores de riesgo asociados son la menarquia temprana, la menopausia tardía, la obesidad, la nuliparidad, la dieta hipertensiva, el ejercicio, el tabaquismo, el alcohol, el estrés y otros factores ambientales. Además, la deficiencia de vitamina D, o hipovitaminosis D, se ha asociado a una mayor incidencia de UL.

2.3.2. Síntomas

Más del 30% de las pacientes con UL presentan síntomas de diferente gravedad que pueden ser síntomas como dolor pélvico o abdominal, dismenorrea, dispareunia, hemorragia uterina anormal, anemia, infertilidad y/o malos resultados obstétricos. Por otro lado, las pacientes con UL también pueden sufrir consecuencias psicológicas, teniendo un mayor riesgo de desarrollar mal estar emocional, depresión y ansiedad.

2.4. Tratamiento del leiomioma uterino.

2.4.1. Manejo quirúrgico

Las opciones de tratamiento quirúrgico incluyen:

- **Miomectomía:** método de preservación del útero que consiste en la extirpación del UL dejando el útero intacto.
- **Histerectomía:** extirpación de todo el útero.
- **Ablación térmica volumétrica por radiofrecuencia laparoscópica:** técnica laparoscópica ambulatoria realizada bajo guía ecográfica que utiliza una sonda electroquirúrgica, insertada dentro del UL, para inducir la miolisis coagulativa.
- **Ablación endometrial:** en este procedimiento se utilizan globos térmicos, microondas, hidrotermablación, ablación endometrial por radiofrecuencia bipolar o dispositivos de crioterapia endometrial para lograr la destrucción del endometrio.

2.4.2. Manejo no quirúrgico: Procedimientos radiológicos de intervención

El manejo no quirúrgico de las pacientes con UL tiene como objetivo principal disminuir sus síntomas. Las mujeres que rechazan la cirugía o no son buenas candidatas a ella pueden optar por otros procedimientos radiológicos de intervención, como:

- **Embolización de la arteria uterina:** método angiográfico mínimamente invasivo que utiliza microesferas de gelatina de trisacrilo o alcohol polivinílico no esférico para perturbar el suministro de sangre uterina y provocar la necrosis isquémica del UL.
- **Ablación por radiofrecuencia focalizada guiada por resonancia magnética**: también llamada ultrasonido focalizado de alta intensidad (*High-Intensity Focused Ultrasound*; HIFU), es un procedimiento que utiliza ondas de ultrasonido transabdominal para inducir la necrosis coagulativa y la regresión del UL.

2.4.3. Tratamiento no quirúrgico: Tratamiento médico

También existen tratamientos médicos para las mujeres que rechazan la cirugía o no son buenas candidatas a ella. Incluyen agentes antifibrinolíticos, antiinflamatorios no esteroideos (AINE), anticonceptivos hormonales combinados, tratamientos con sólo progesterona, moduladores selectivos de los receptores de progesterona (*Selective Progesterone Receptor Modulator;* SPRM), antiprogestágenos, inhibidores de la aromatasa y agonistas o antagonistas de la hormona liberadora de gonadotropina (*Gonadotropin-Releasing Hormone*; GnRH). Recientemente se han propuesto nuevas dianas médicas, como el extracto de té verde, la vitamina D, la cabergolina, la gestrinona y los análogos de la somatostatina.

2.4.4. Orientaciones futuras

A pesar de la elevada prevalencia del UL, no existe ningún tratamiento eficaz para reducir los UL con efectos secundarios mínimos a largo plazo. El tratamiento de referencia actual es la cirugía, pero deben considerarse tratamientos menos invasivos en función del deseo de fertilidad futura de la paciente, el deseo de preservar el útero, la posibilidad de alcanzar los objetivos del tratamiento y el estado de salud global. La identificación de los mecanismos moleculares implicados en la patogénesis del UL podría permitir el desarrollo de tratamientos nuevos y más eficaces.

2.5. Patogénesis

A pesar de su alta prevalencia, la patogénesis del UL sigue sin estar clara. Son muchos los factores que contribuyen al desarrollo del UL, entre ellos las células madre, las mutaciones genéticas, las hormonas esteroideas, la ECM, las vías de señalización Wnt/β-catenina y TGFβ, los factores de crecimiento y el medio ambiente.

2.5.1. Células madre del miometrio y formación de leiomiomas uterinos

La presencia de células madre somáticas (*Somatic Stem Cells;* SSC) en el tracto reproductivo femenino ha sido corroborada por varios estudios. Las células madre del MM (*Myometrial Stem Cells;* MSC) representan el 2% de la población celular de dicho tejido. Son fundamentales para la regeneración uterina y la remodelación de los tejidos, así como para el crecimiento tisular durante el embarazo. Además, existe una subpoblación de MSC que presenta características de células iniciadoras de tumores (*Tumor Initiating Cell*; TIC). Las alteraciones genéticas y epigenéticas, la hipoxia uterina o señalización anormal de estrógenos en las MSC pueden originar una TIC con mayor capacidad de autorrenovación y proliferación, que se expanden clonalmente dando lugar a células hijas intermedias diferenciadas. Estas células hijas pueden entonces diferenciarse en una célula de UL, generando la aparición del tumor.

2.5.2. Alteraciones genéticas

Los factores genéticos son uno de los principales procedimientos implicados en el inicio del crecimiento del tumor y en la aparición de los diferentes tipos de UL. Entre los impulsores de la formación de los UL, encontramos cambios estructurales y funcionales en el genoma de las células madre. Los principales impulsores genéticos del UL incluyen los *genes MED12, HMGA2, FH* y *COL4A5-COL4A6*, así como las anomalías del cariotipo.

2.5.3. Hormonas esteroideas

El crecimiento de los UL se ve afectado por las concentraciones de hormonas esteroideas, en concreto de estrógeno y progesterona. Ambas hormonas interactúan para promover sinérgicamente el desarrollo de los UL mediante la proliferación celular y el aumento de volumen de los componentes celulares y extracelulares.

2.5.4. Matriz extracelular

Los UL se caracterizan por anomalías cuantitativas y cualitativas en las proteínas que conforman la ECM, lo que contribuye a la expansión del mismo. El UL contiene un 50% más de ECM que su MM adyacente. La ECM puede funcionar como depósito de factores de crecimiento, citocinas, quimiocinas, mediadores de la respuesta angiogénica e inflamatoria y proteasas producidas por las células tumorales.

2.5.5. Vía Wnt/β-catenina

La vía de señalización Wnt regula la proliferación, la apoptosis, la determinación del destino celular, la polaridad, la migración durante el desarrollo y el mantenimiento de las células madre

en los adultos. La activación de la vía de señalización Wnt/β-catenina desempeña un papel importante en la patogénesis del UL. Esta vía es uno de los factores clave que controlan la transformación de las MSC en células tumorigénicas, con una división descontrolada y una síntesis activa de ECM, formando el UL.

2.5.6. Vía del TGFβ

El TGFβ es una de las citocinas más importantes asociadas al MM. Es responsable de la modulación de factores paracrinos y autocrinos de inflamación, ciclo celular y crecimiento. TGFβ está sobreexpresado en el UL en comparación con el MM adyacente estimulando el crecimiento del tumor y potenciando el metabolismo tumoral. Esta vía de señalización es directamente responsable del desarrollo del fenotipo fibrótico del UL al aumentar la deposición de la ECM y la proliferación celular.

2.5.7. Interacción paracrina entre poblaciones celulares de leiomioma

La presencia de células maduras de MM o de UL a lo largo del tejido permite la comunicación paracrina entre ellas, lo que contribuye a aumentar la proliferación de las células madre del UL y la consiguiente formación del mismo. El estrógeno, la progesterona y los factores de crecimiento son algunas de las biomoléculas incluidas en esta comunicación célula-célula. Como consecuencia, se induce la proliferación, la síntesis de ECM y la angiogénesis. La señalización Wnt/β-catenina también desempeña un papel importante en esta interacción paracrina. El tratamiento con estrógenos/progesterona induce la expresión de ligandos Wnt en las células maduras del MM. La vía Wnt también puede estimular la expresión de TGFβ, dando lugar a una producción excesiva de ECM y a un aumento de la proliferación celular.

2.5.8. Medio ambiente

Algunos factores ambientales también están implicados en la patobiología de los UL, ya que pueden aumentar o disminuir el riesgo de desarrollarlos. Entre estos factores de riesgo se encuentran la ascendencia africana, el índice de masa corporal elevado, el consumo de carne, el consumo de alcohol, la deficiencia de vitamina D, la menarquia temprana, la hipertensión y los antecedentes de enfermedad inflamatoria pélvica o infecciones vaginales. Por el contrario, los factores que disminuyen el riesgo son el uso de anticonceptivos hormonales, el parto y el consumo de verduras. Estos hallazgos sugieren que los factores epigenéticos también están implicados en la generación de UL. Los cambios epigenéticos pueden ser revertidos por agentes químicos. Esta posibilidad abre la puerta a nuevas opciones terapéuticas para las pacientes con UL.

2. EPIGENÉTICA

3.1. Definición

La epigenética se define como el estudio de los cambios en la expresión génica que no están asociados a modificaciones en la secuencia primaria del ADN. Estos cambios afectan al empaquetamiento de la cromatina. En el núcleo, el ADN genómico se envuelve alrededor de las histonas en subunidades de nucleosomas que se condensan en la cromatina. La cromatina muy condensada se conoce como heterocromatina y contiene principalmente genes inactivos. Por el contrario, la eucromatina tiene una estructura abierta y contiene genes activos. Los principales mecanismos implicados en la regulación epigenética son la metilación del ADN, la modificación de las histonas y los ARN no codificantes, que contribuyen a un complejo "código epigenético" que se superpone a la secuencia de nucleótidos para dirigir la expresión de los genes. La naturaleza dinámica y reversible de los mecanismos epigenéticos hace que estos procesos tengan relevancia terapéutica en muchas enfermedades.

3.2. La metilación del ADN

En el genoma de los eucariotas, la metilación del ADN es la marca epigenética más común y mejor caracterizada. Consiste en la adición covalente de un grupo metilo al carbono 5 del anillo de citosina tras la replicación. La mayoría de los dinucleótidos CpG se concentran en regiones denominadas islas CpG que se encuentran cerca de los sitios de inicio de la transcripción (*Transcription Start Site*; TSS) en aproximadamente el 70% de los promotores de los genes. Las enzimas que catalizan las reacciones de metilación del ADN se conocen como metiltransferasas del ADN (*DNA Methyltransferase*; DNMT). Por su parte, la desmetilación del ADN puede producirse de forma pasiva durante las sucesivas rondas de replicación o de forma activa por la acción de las desmetilasas del ADN (*Ten-Eleven Translocation*; TET). La hipermetilación de la isla CpG en la región promotora generalmente resulta en la represión de la expresión génica, mientras que la hipometilación conduce a la transcripción activa.

3.2.1. Metilación del ADN y tumorigénesis

La metilación anormal está implicada en el proceso de tumorigénesis y afecta a la expresión constitutiva de genes implicados en la regulación del ciclo celular, la reparación del ADN, la apoptosis, la diferenciación, la resistencia a los fármacos, la angiogénesis y la metástasis. Una metilación aberrante del ADN, a través de la hipometilación de oncogenes y la hipermetilación de genes supresores de tumores, se ha encontrado en varios tipos de cáncer, como el de próstata, el neuroblastoma, el colorrectal y el de mama. Asimismo, también se han descrito

alteraciones de las DNMT en los tumores. A diferencia de los eventos genéticos, los cambios epigenéticos son reversibles. Debido a esta plasticidad inherente, los mecanismos epigenéticos han sido utilizados como tratamiento antitumoral.

3.2.2. Tratamientos dirigidos a la metilación del ADN

Este tipo de terapia antitumoral epigenética tiene como objetivo alterar el estado de metilación con agentes desmetilantes. Los primeros compuestos reconocidos como inhibidores de la DNMT (DNMTi) fueron los análogos de la citosina 5-azacitidina (aza, Vidaza) y 5-aza-2'-deoxicitidina (5-aza-CdR; decitabina, Dacogen), que producen una hipometilación a dosis bajas. Estos azanucleósidos son inhibidores competitivos de las DNMT. La capacidad de los DNMTi para reactivar los genes supresores de tumores fue una de las motivaciones para el uso de estos agentes en enfermedades hematológicas. Además, estos fármacos reducen la tumorigenicidad y se dirigen a las células madre cancerosas dentro del tumor en los cánceres sólidos. En 2004 y 2006, respectivamente, se aprobaron aza y decitabina para el tratamiento de los síndromes mielodisplásicos.

3.2.3. Metilación del ADN y leiomioma uterino

La metilación del ADN desempeña un papel clave en la patogénesis del UL. Se ha descrito un estado de metilación global aberrante del ADN en UL en comparación con el MM, lo que sugiere que esta modificación epigenética puede desempeñar un papel importante en la patogénesis de los UL al alterar el perfil de expresión génica normal del MM. En este sentido, se ha descrito que la hipermetilación disminuye la expresión de genes de diferenciación en las células madre de los UL en comparación con sus células diferenciadas, induciendo el inicio del tumor. Esto subraya la importancia de la metilación del ADN en la aparición de tumores. Recientemente, George y colaboradores reportaron la existencia de diferentes perfiles de metilación del ADN y expresión génica en los diferentes subtipos genéticos de UL, como MED12 y HMGA2. En este sentido e análisis de la interacción de los perfiles de metilación del ADN y expresión génica en los UL en comparación con el MM desde un enfoque holístico podría permitirnos identificar las vías comúnmente desreguladas implicadas en el desarrollo de los UL.

3.3. Modificaciones de las histonas

Las histonas son un pequeño componente proteico básico de la cromatina que participa en la regulación de la expresión génica. Se asocian con el ADN para formar nucleosomas, que representan las unidades básicas de repetición de la cromatina. Estas modificaciones postraduccionales de las colas de las histonas incluyen la acetilación, la metilación, la

fosforilación, la ubiquitinación y la SUMOilación. La acetilación de las histonas consiste en la transferencia de un grupo acetilo del acetil coenzima A al grupo e-amino de la lisina en las colas N-terminales de las histonas. En general, la acetilación de las histonas se correlaciona con la expresión de los genes, y la desacetilación conduce a la represión transcripcional. Las enzimas que participan en la adición del grupo acetilo a las histonas son las histonas acetiltransferasas (*Histone AcetylTransferases*; HAT), mientras que las histonas desacetilasas (*Histone DeAcetylase*; HDAC) eliminan estas marcas. Las HDAC se asocian al silenciamiento de los genes, mientras que las HAT se asocian a la actividad transcripcional.

3.3.1. Modificaciones de las histonas y tumorigénesis

Las modificaciones aberrantes de las histonas, que alteran los estados de transcripción de los genes, son características comunes de las células tumorales humanas. En el cáncer de próstata, los niveles más bajos de metilación de la lisina 4 de la histona 3 (H3K4me2) y acetilación de la lisina 18 de la histona 3 (H3K18ac) se relacionaron con un mal pronóstico. Del mismo modo, un estado aberrante del perfil de acetilación de la lisina 27 de la histona 3 (H3K27ac) está implicado en varios tumores, como el cáncer gástrico, de pulmón y de ovario, lo que aumenta la proliferación, la invasión y la metástasis tumoral. Sin embargo, se sabe poco sobre el papel de H3K27ac en la patogénesis del UL. Por otra parte, los niveles de las enzimas implicadas en la acetilación de las histonas también están alterados en los tumores. Las HDACs están sobreexpresadas en cáncer de mama y de ovario, lo que resulta en una pérdida global de acetilación de las histonas y en el silenciamiento de la expresión de los genes supresores de tumores.

3.3.2. Tratamientos dirigidos a las modificaciones de las histonas

Los tratamientos dirigidos a las enzimas encargadas de las marcas de histonas están aumentando como terapia para muchas enfermedades. Los inhibidores de HDAC (HDACi) promueven la detención del ciclo celular, inducen la diferenciación y activan las vías de apoptosis en las células tumorales. El fármaco oral Vorinostat, también conocido como SAHA (*Suberoylanilide Hydroxamic Acid*) fue el primer HDACi aprobado por la Administración de Alimentos y Medicamentos de los Estados Unidos (*Food and Drug Administration;* FDA) para el tratamiento del linfoma cutáneo de células T. SAHA es un inhibidor competitivo de las HDAC de clase I y clase II que se utiliza actualmente como fármaco anticanceroso para tratar diferentes tipos de tumores. SAHA bloquea la proliferación celular y el crecimiento tumoral en el adenocarcinoma hepatoide, la leucemia mieloide y el cáncer de próstata, con poca toxicidad.

3.3.3. Modificaciones de las histonas y leiomioma uterino

Los estudios epigenéticos sobre la patogénesis del UL se han centrado principalmente en la metilación del ADN. Sin embargo, las modificaciones de las histonas también desempeñan una función importante en las alteraciones de la cromatina y, por lo tanto, hay un interés creciente en el papel de estas modificaciones en la patogénesis de los UL. La actividad de las HDAC es mayor en los UL que en el MM adyacente, lo que sugiere que la transcripción de los genes implicados en la función normal del MM, genes supresores de tumores, puede estar reprimida debido a una disminución de la acetilación de las histonas que conduce a un aumento del crecimiento y el mantenimiento de los UL. Además, publicaciones recientes han subrayado la importancia de H3K27ac, H3K4me3 y H2A.Z en los potenciadores y promotores, encontrando características diferenciales entre los subtipos de UL en función del estado de la mutación. Sin embargo, el impacto de estas marcas de histonas en la expresión génica de los UL, y por lo tanto la patogénesis de los UL, es poco conocido.

HIPÓTESIS

El estudio de las modificaciones epigenéticas en los UL podría permitirnos comprender mejor su origen y ayudarnos a describir nuevas dianas terapéuticas y posibles tratamientos para reducir el tamaño de los mismos y la gravedad de sus síntomas.

OBJETIVO

El objetivo principal de este estudio es describir los mecanismos epigenéticos, relativos a la metilación del ADN y la modificación de las histonas, implicados en el desarrollo de los UL, los cuales podrían ser nuevas dianas terapéuticas para tratarlos.

Los objetivos específicos son:

1. Determinar el patrón de metilación génica y su interacción con el patrón de expresión génica en los UL frente a los MM adyacente, así como evaluar el efecto del inhibidor de DNMT 5-AZA-2'-deoxicitidina en las células de UL sobre la expresión de los genes controladores seleccionados a partir de los resultados ómicos. MANUSCRIPTO 1.

2. Determinar si los procesos biológicos implicados en el desarrollo del UL, como la proliferación celular, apoptosis, formación de la ECM y vía Wnt/β-Catenina están regulados por la metilación del ADN, utilizando el inhibidor de DNMT 5-AZA-2'-deoxicitidina en células primarias de UL (HULP) cultivadas in vitro. MANUSCRIPTO 2.

4. Determinar el perfil de acetilación de la lisina 27 de la histona 3 (H3K27ac) y su interacción con el patrón de expresión génica en UL vs MM, así como evaluar el efecto del inhibidor de deacetilasas de histonas (SAHA) sobre la expresión de los genes controladores seleccionados a partir de los resultados ómicos. MANUSCRIPTO 3.

5. Determinar si los procesos biológicos implicados en el desarrollo del UL, como la proliferación celular, apoptosis, formación de ECM y vía del TGFβ están regulados por la acetilación de histonas, utilizando el tratamiento con SAHA en células HULP cultivadas in vitro. MANUSCRIPTO 4.

RESULTADOS

MANUSCRITO 1. Integrative analysis of the DNA methylome and transcriptome in uterine leiomyoma shows altered regulation of genes involved in metabolism, proliferation, extracellular matrix, and vesicles. doi: 10.1002/path.5920

El análisis de metilación del ADN realizado mediante la técnica "*genome-wide DNA methylation*" reveló un perfil global de metilación diferente en UL en comparación con el MM adyacente (n = 31). Además, se descubrió un mayor nivel de metilación en el UL (0,704 UL frente a 0,681 MM, valor p ajustado por FDR = 0,004). En concreto, encontramos 43.241 CpGs diferencialmente metilados (Holm < 0,05), siendo 11.335 hipometilados y 31.906 hipermetilados. El análisis de RNA-seq (n = 28) reveló 10.339 genes diferencialmente expresados (*Differential Expressed Genes;* DEGs) en UL vs MM, con 5.690 genes sobreexpresados y 4.649 infraexpresados (FDR < 0,05, log2FC > 0,5 o < 0,5).

Entre los 43.241 CpGs diferencialmente metilados (Holm < 0,05), seleccionamos 11.772 CpGs que estaban cerca o dentro de una región promotora y correlacionamos estos CpGs con los 10.339 DEGs en UL. Este análisis identificó 93 genes regulados por metilación con al menos un sitio CpG que coincidía con el filtro de hipometilación/sobreexpresión o hipermetilación/infraexpresión y un coeficiente de correlación de Spearman > 0,7 o < 0,7. Entre ellos, 22 estaban hipometilados/sobreexpresados y 71 estaban hipermetilados/infraexpresados.

El análisis de enriquecimiento funcional de estos DEGs identificó procesos biológicos significativamente desregulados en el UL relacionados con metabolismo y fisiología celular, respuesta a señales extracelulares, invasión y proliferación celular, que son vías clave en la patogénesis tumoral. Además, encontramos componentes celulares significativamente enriquecidos en el UL, que estaban principalmente relacionados con las membranas celulares, vesículas, ECM y las uniones celulares. Entre las funciones moleculares enriquecidas en el UL, la

mayoría estaban relacionadas con el metabolismo, la fisiología celular y la respuesta a señales extracelulares. Por último, el análisis de las vías KEGG reveló vías implicadas en el cáncer y la biología uterina desreguladas en los tejidos blandos.

Los resultados de la metilación del ADN se validaron mediante la pirosecuenciación y los de RNA-seq se validaron mediante qRT-PCR en los genes seleccionados (*PRL, ATP8B4, CEMIP, RIMS2, ZFPM2-AS1, EFEMP1, FBLN2, ARHGAP10 y HTATIP2*) en función de su potencial importancia en la tumorigénesis y/o la patología del UL. La inhibición de la metilación del ADN con el inhibidor de las DNMT (5-aza-CdR) en células HULP in vitro (n = 10) aumentó la expresión génica de los genes supresores de tumores descritos en UL en este estudio como hipermetilados/desregulados, tales como *EFEMP1, FBLN2, ARHGAP10,* y *HTATIP2,* los cuales fueron seleccionados para su validación.

MANUSCRITO 2. 5-aza-2'-deoxycitidine inhibits cell proliferation, extracellular matrix formation and Wnt/β-catenin pathway in human uterine leiomyomas. doi: 10.1186/s12958-021-00790-5

Se observó una mayor expresión génica de *DNMT1* y una actividad de DNMT significativamente mayor en el tejido de UL en comparación con el MM adyacente (n = 7). Se evaluó la actividad de la DNMT en las células HULP y del MM para determinar la correlación entre la actividad de la DNMT en el tejido y en las células cultivadas. In vitro, la actividad de la DNMT fue mayor en las células HULP en comparación con las células MM (n = 3) en el día 7, día 9 y día 10.

El tratamiento in vitro con 5-aza-CdR disminuyó el porcentaje de células HULP viables (n = 16) de forma dependiente de la dosis. No se observaron diferencias en la ratio BAX (proapoptótica)/ BCL2 (antiapoptótica), lo que sugiere que 5-aza-CdR no afectó a la apoptosis. Sin embargo, el tratamiento con 5-aza-CdR disminuyó la expresión de la proteína PCNA, el marcador de proliferación estándar, en las células HULP (n = 8) de forma dependiente de la dosis, mostrando que dicho tratamiento disminuye la proliferación celular.

Respecto a su efecto sobre la ECM, 5-aza-CdR disminuyó la expresión de las proteínas de ECM COLÁGENO I, FIBRONECTINA y PAI-1 de forma dependiente de la dosis.

Se analizaron los niveles de expresión de proteínas y ARNm de las dianas finales de la vía Wnt/β-catenina tras el tratamiento con 5-aza-CdR en células HULP. Los resultados revelaron una inhibición de la expresión de la proteína WISP1 y la expresión génica de *c-MYC* y *MMP7* en las células HULP tratadas con 5-aza-CdR (n = 8) en todas las dosis probadas en comparación con las células no tratadas.

MANUSCRITO 3. Deciphering the Role of Histone Modifications in Uterine Leiomyoma: Acetylation of H3K27 Regulates the Expression of Genes Involved in Proliferation, Cell Signaling, Cell Transport, Angiogenesis and Extracellular Matrix Formation. doi: 10.3390/biomedicines10061279

El perfil de acetilación de H3K27 reveló una separación entre tejido de UL en comparación con el MM adyacente (n = 21). Se observó una menor cantidad de marca H3K27ac en la región promotora de los genes de UL en comparación con el MM, lo que sugiere una hipoacetilación global del UL.

El análisis de expresión diferencial integrando los datos de RNA-seq de los estudios GSE192354 y GSE142332 reveló 922 DEGs en el UL en comparación con las muestras de MM (n = 52) con un valor p ajustado por FDR < 0,01, log2FC> 1, o < -1. En concreto, 559 estaban sobreexpresados y 363 infraexpresados. Entre estos genes, 482 (52,3%) presentaban la marca de histona H3K27ac alrededor de la región promotora. El análisis diferencial del estado de H3K27ac mostró que 82 DEGs presentaban acetilación diferencial (FDR < 0,05) en el UL en comparación con el MM, con 29 hiperacetilados/sobreexpresados y 53 hipoacetilados/infraexpresados.

El análisis de enriquecimiento funcional de estos 82 DEGs asociados a un perfil H3K27ac diferente reveló 30 procesos biológicos significativamente desregulados en el tejido UL humano frente al MM que estaban principalmente relacionados con la proliferación celular, la señalización celular y transporte celular y la angiogénesis. Además, encontramos componentes celulares enriquecidos relacionados con la ECM, tales como colágeno y región extracelular.

Para resaltar la importancia de los DEGs relevantes para el UL con estado diferencial de H3K7ac, se validaron genes seleccionados en base a su relevancia para la patogénesis del UL. El análisis de expresión génica corroboró la mayor expresión de *COL24A1, NDP*, *HOXA13* e *IGFL3* en una cohorte separada de UL en comparación con el MM adyacente. Asimismo, este análisis de expresión demostró la menor expresión de *CD40*, *DPT, GIMAP8, GPX3* e *IL15*. La inhibición de las HDACs mediante el tratamiento con SAHA en las células HULP cultivadas in vitro (n = 10) aumentó significativamente la expresión de los genes supresores de tumores hipoacetilados/infraexpresados seleccionados, *CD40* (fold-change = 6. 78, p valor = 0,001), *DPT*, *GIMAP8*, *GPX3 e IL15*.

MANUSCRITO 4. Histone deacetylase inhibition by suberoylanilide hydroxamic acid: a therapeutic approach to treat human uterine leiomyoma. doi: 10.1016/j.fertnstert.2021.10.012

Las muestras de tejido de UL mostraron un aumento estadísticamente significativo de la expresión de los genes *HDAC1, HDAC3 y HDAC6* (n = 10), así como una actividad de las HDAC significativamente mayor que el MM adyacente (n = 6). In vitro, la actividad de las HDAC tendía a aumentar en las células HULP en comparación con las células MM (n = 5), lo que corrobora que el aumento de la actividad HDAC observado en los tejidos no se ve modificado por las condiciones in vitro. Además, evaluamos el efecto del tratamiento con SAHA sobre la actividad HDAC in vitro en células HULP y células MM adyacente del mismo paciente. La actividad HDAC tendió a disminuir en las células HULP tras el tratamiento con SAHA, mientras que esta tendencia no se observó en las células de MM. El tratamiento con SAHA disminuyó significativamente el porcentaje de células HULP viables (n = 10) de forma dependiente de la dosis. En cambio, el número de células de MM viables (n = 10) no se vio alterado tras el tratamiento con SAHA, lo que sugiere que este fármaco parece tener un mayor efecto sobre las células HULP que sobre las células de MM.

En lo que respecta a la apoptosis, no se observaron diferencias significativas en la relación BAX (proapoptótico)/BCL2(antiapoptótico), lo que sugiere que SAHA no afectó a la apoptosis. Sin embargo, el tratamiento con SAHA disminuyó significativamente la expresión de la proteína PCNA, marcador de proliferación, en las células HULP (n = 10) de forma dependiente de la dosis, mostrando una disminución de la proliferación celular con SAHA. También estudiamos la expresión génica de los marcadores del ciclo celular *C-MYC y CCND1*, lo que reveló una disminución significativa de ambos en las células HULP (n = 10) tras el tratamiento con SAHA.

Respecto a la ECM, la expresión de las proteínas FIBRONECTINA y COLLAGEN I disminuyó de manera significativa con el tratamiento SAHA. Para evaluar el efecto del SAHA sobre la vía de señalización del TGFβ, se analizó la expresión de los genes *TGFβ3* y *MMP9, los cuales están* implicados en esta vía. Se redujo la expresión de *TGFβ3* y *MMP9* en las células HULP (n = 10) tratadas con SAHA.

DISCUSIÓN

El UL es una enfermedad ginecológica con gran influencia en la salud de la mujer, siendo una de las principales causas de infertilidad. Su patogénesis no está clara y no existe un tratamiento no invasivo eficaz para las mujeres que sufren UL. Por este motivo, el objetivo principal de esta tesis

es definir mecanismos epigenéticos implicados en el desarrollo del UL y nuevas dianas terapéuticas para tratarlos.

En primer lugar, nos propusimos describir en profundidad la interacción entre la **metilación del ADN y el perfil de expresión génica** de los UL en comparación con el MM adyacente. En este estudio revelamos un estado de hipermetilación global de los UL en comparación con el MM, así como un comportamiento transcriptómico diferente, mostrando una sólida correlación entre la metilación del ADN y la expresión génica en estas pacientes caucásicas. Estos resultados están en concordancia con estudios previos que describen una expresión génica diferencial en el UL, así como la metilación aberrante del ADN en el cáncer, y en el UL. A continuación, identificamos 93 DEGs anormalmente metilados en el UL en comparación con el MM, con 22 genes hipometilados/sobreexpresados y 71 hipermetilados/infraexpresados.

Además, describimos que procesos biológicos tales como metabolismo y la fisiología celular, la respuesta a las señales extracelulares, la invasión y la proliferación celular están desregulados debido a la metilación aberrante del ADN en los UL. Las células transformadas adaptan su metabolismo para apoyar el inicio y la progresión del tumor, lo que explica la alteración de este proceso en el UL. Además, la respuesta desregulada a las señales de muerte celular, proliferación incontrolada e invasión, son procesos asociados a cáncer. En este estudio, demostramos que estos reguladores clave de la fisiología tumoral están bajo la influencia epigenética en el UL. Además, se desregularon componentes celulares como las membranas, las vesículas, la ECM y las uniones celulares. La excesiva síntesis y deposición de ECM es la principal causa del crecimiento del UL, lo que demuestra que la desregulación de la ECM por la metilación del ADN es una característica importante en este tumor. Además, las células cancerosas producen más exosomas que las células normales, y estas vesículas extracelulares están implicadas en el desarrollo del cáncer, la metástasis y la inmunidad antitumoral, lo que explicaría la desregulación de vesículas observada en los UL.

Para analizar más a fondo las nuevas dianas moleculares controladas por la metilación del ADN en el UL, estudiamos los principales genes hipometilados/sobreexpresados. Destacamos genes como *TFAP2C, PRL, ATP8B4, CEMIP y ZFPM2-AS1,* que son oncogenes implicados en la angiogénesis, la vía de señalización Hippo, la proliferación, la invasión y la vía Wnt/β-catenina, mecanismos moleculares clave para el inicio y la progresión del UL. También examinamos los principales genes hipermetilados/infraexpresados y seleccionamos *EFEMP1, FBLN2, ARHGAP10 y HTATIP2*, genes supresores de tumores, implicados en la invasión y la acumulación de la ECM, angiogénesis, metabolismo y la vía Wnt/β-catenina. La inhibición de las DNMTs con 5-aza-CdR

incrementó los supresores tumorales *EFEMP1, HTATIP2, FBLN2 y ARHGAP10*, lo que apoya que la metilación del ADN está implicada en la expresión de los genes supresores de tumores que desencadenan el desarrollo del UL.

Teniendo en cuenta la reversibilidad de los cambios epigenéticos, los inhibidores dirigidos a estos procesos son estrategias prometedoras contra el cáncer. Por esta razón, centramos nuestro estudio en la **reversión de la metilación del ADN mediante el inhibidor de DNMT 5-aza-CdR** como una nueva opción de tratamiento para el UL.

En primer lugar, corroboramos que hay una mayor expresión de *DNMT1* y actividad de DNMT en tejido de UL en comparación con el MM, apoyando el uso de inhibidores de DNMT para revertir la metilación aberrante del ADN en los UL. Este aumento se mantiene en condiciones de cultivo celular a lo largo del tiempo, lo que confirma la reproducibilidad del modelo in vitro. Otros autores describieron un aumento de la expresión del ARNm de *DNMT1* y *DNMT3a* en muestras de UL en comparación con el MM, respaldando nuestros resultados.

En este estudio, descubrimos que el tratamiento con 5-aza-CdR en las células HULP inhibía el número de células viables en cultivo, y esta disminución se debía a una reducción de la proliferación celular. Estos hallazgos demostraron el efecto antiproliferativo de 5-aza-CdR en las células HULP descrito previamente en diferentes tumores. El crecimiento de las células HULP no sólo se debe a la proliferación celular, sino también a una excesiva síntesis y deposición de ECM. Nuestros resultados mostraron que el tratamiento con 5-aza-CdR disminuyó significativamente la expresión de proteínas de la ECM, tales como COLÁGENO I, FIBRONECTINA y PAI-1. Estos resultados sugieren un importante papel de 5-aza-CdR en la regulación de las proteínas fibróticas clave implicadas en la expansión de la UL. Por lo tanto, 5-aza-CdR redujo la expansión de la ECM y la proliferación celular en las células HULP, lo que contrarresta el crecimiento del UL, ofreciendo un prometedor candidato para tratar este tumor.

La vía Wnt/β-catenina es un regulador clave de la proliferación celular y la producción de ECM que está desregulada en varios tipos de cáncer y de UL. Para dilucidar si 5-aza-CdR tiene efectos sobre esta vía, evaluamos los niveles de expresión de proteínas o ARNm de las dianas finales de la vía Wnt/β-catenina. Encontramos que tanto la expresión del gen *c-MYC* como la expresión de la proteína WISP1 estaban disminuidas en las células HULP tratadas. Esto sugiere que la inhibición de DNMT por 5-aza-CdR podría impedir la proliferación celular a través de la inhibición de la vía Wnt/β-catenina. Por último, estudiamos *MMP7* porque es una metaloproteasa implicada en la fisiología de la ECM, la transición epitelial-mesenquimal y la invasión tumoral que está sobreexpresada en algunos tipos de cáncer. Observamos una disminución de la

expresión génica de *MMP7* tras el tratamiento con 5-aza-CdR en las células HULP, lo que sugiere una menor capacidad invasiva de las células tratadas con HULP a través de la inhibición de la vía Wnt/β-catenina.

Con estos resultados, sugerimos que la metilación del ADN está implicada en la regulación de la vía Wnt/β-catenina y, en consecuencia, en la proliferación celular y la formación de la ECM en las células HULP, y su reversión mediante el inhibidor de DNMTs 5-aza-CdR podría reducir la proliferación celular aberrante y la formación de ECM característica de los UL, sugiriendo 5-aza-CdR como tratamiento para reducir el crecimiento de los UL.

Los estudios epigenéticos sobre los UL se han centrado principalmente en la metilación del ADN. Sin embargo, las modificaciones de las histonas también desempeñan una función importante en la expresión génica. En este sentido, el estado aberrante del **perfil de acetilación de la lisina 27 de la histona 3** (H3K27ac) está implicado en varios tumores como el gástrico, el de pulmón y el de ovario. Por esta razón, estudiamos el papel de la modificación H3K27ac sobre la expresión génica en UL.

En este análisis encontramos una hipoacetilación global de H3K27 en las regiones promotoras de UL en comparación con el MM adyacente, la cual podría llevar a una desregulación de la expresión génica del UL que podría tener un papel importante en su patogénesis. Describimos 82 DEGs que presentan un estado diferencial de H3K27ac en el UL en comparación con el MM, con 29 hiperacetilados/sobreexpresados y 53 hipoacetilados/infraexpresados. Estos genes presentan múltiples funciones, siendo efectores clave del desarrollo y mantenimiento del tumor. Encontramos hiperacetilación/sobreexpresión de oncogenes tales como *NDP, HOXA13, COL24A1, e IGFL3*, los cuales están implicados en la invasión, angiogénesis, vía Wnt/β-catenina y TFGβ. Además, describimos la hipoacetilación/infraexpresión de genes supresores de tumores como *CD40, GIMAP8, IL15, GPX3 y DPT*, con funciones antiangiogénicas y proinmunes, metabolismo, ECM, proliferación celular y la invasión por Wnt/β-catenina, y la vía del TGFβ.

El análisis de enriquecimiento funcional de los 82 DEGs regulados por H3K27ac demostró su implicación en la proliferación celular, la señalización celular, el transporte celular y la angiogénesis. Los UL se caracterizan por una proliferación celular incontrolada, una de las principales características de la biología tumoral. Además, la comunicación y la señalización celular están alteradas en los tumores, contribuyendo a la respuesta aberrante a las señales extracelulares y potenciando el desarrollo tumoral característico de este tipo de enfermedades. El inicio de la angiogénesis tumoral es un sello distintivo del cáncer y un requisito para la progresión del tumor. Las células malignas requieren oxígeno y nutrientes para sobrevivir y

proliferar, necesitando la proximidad de los vasos sanguíneos para acceder al sistema de circulación sanguínea. Por lo tanto, la vascularización aberrante que se encuentra en el UL puede ser desencadenada por un cambio en las marcas de histonas como H3K27ac. También encontramos componentes celulares significativamente enriquecidos en el UL, que estaban relacionados con una alteración de la formación de la ECM, tales como la región extracelular y el colágeno.

Por último, inhibimos la desacetilación de las histonas in vitro en las células del HULP mediante la inhibición de las HDACs con SAHA. Nuestro estudio demostró que este tratamiento con SAHA aumenta la expresión de genes hipoacetilados/infraexpresados supresores tumorales, *CD40, GIMAP8, IL15, GPX3 y DPT*, en las células HULP in vitro, los cuales fueron descritos en el estudio de integración de H3K27ac y expresión génica. Estos hallazgos sugieren que la alteración de H3K27ac en los UL disminuye genes supresores de tumores, llevando a la desregulación de las vías implicadas en la patogénesis de los UL, como la proliferación celular, la señalización y el transporte celular, la angiogénesis o la formación de ECM; y la reversión de la acetilación de las histonas mediante SAHA podría ser un enfoque terapéutico prometedor para tratar los UL.

Basándonos en la aplicabilidad de esta terapia, centramos nuestro estudio en la **inhibición de las HDACs por SAHA** como una nueva estrategia terapéutica para el UL. En primer lugar, corroboramos que existe una mayor expresión génica de *HDAC1, HDAC3 y HDAC6*, así como una mayor actividad de HDAC en el tejido del UL comparado con el MM adyacente. Posteriormente, estudiamos la actividad de la HDAC en cultivo celular y confirmamos que la actividad de la HDAC tendía a aumentar en las células HULP en comparación con las células MM. Estos resultados sugieren que el entorno in vitro no modifica el estado de la HDAC observado en el tejido.

Tras el tratamiento con SAHA, la actividad de las HDAC tendía a disminuir en las células HULP, pero no en las células MM. Además, el porcentaje de células viables disminuyó significativamente tras el tratamiento con SAHA en las células HULP, pero no en las células MM. Estos resultados indican que el tratamiento con SAHA es más eficaz en las células HULP que en las células de MM, lo que demuestra su potencial como tratamiento de UL, siendo el MM adyacente menos afectado.

Además, el tratamiento de las células HULP con SAHA disminuyó la proliferación celular. Este efecto antiproliferativo se ha descrito previamente en otros tumores. Los genes reguladores del ciclo celular, *CCND1* y *C-MYC,* también disminuyeron su expresión tras el tratamiento con SAHA, sugiriendo que la disminución de la proliferación celular se produce mediante la detención del ciclo celular, la cual ha sido previamente demostrada en cáncer. La apoptosis no aumentó tras

el tratamiento, lo que demuestra la ausencia de toxicidad de SAHA en las células HULP. Estos resultados colectivamente indican que SAHA podría reducir el crecimiento del UL al disminuir la proliferación celular y detener el ciclo celular.

Respecto a la ECM, en este estudio demostramos que SAHA disminuyó significativamente las proteínas asociadas a la ECM, como la FIBRONECTINA y el COLÁGENO I, lo que sugiere un papel importante de la acetilación de las histonas en la regulación de la formación de la ECM.

La vía de señalización TGFβ está sobreexpresada en el UL y es directamente responsable del desarrollo del fenotipo fibrótico del UL al aumentar la deposición de ECM y la proliferación celular. En estudios anteriores en cáncer, la acetilación se ha relacionado con la vía de señalización TGFβ. Nuestro estudio mostró que el tratamiento con SAHA disminuye la expresión génica de *TGFβ3* y *MMP9*, dianas finales de la vía de señalización TGFβ. Por lo tanto, sugerimos que la inhibición de las HDACs mediante el tratamiento con SAHA altera la funcionalidad de la vía TGFβ, lo que conduce a una reducción de la deposición de ECM.

En general, proponemos que la reducción de la deposición de ECM y la disminución de la progresión del ciclo celular y la proliferación celular generada por la reversión de la desacetilación de histonas con el tratamiento con SAHA induce la inhibición del crecimiento del UL. Estos resultados ponen de manifiesto que la inhibición de las HDAC puede ser una diana terapéutica viable para tratar el UL.

Basándonos en los resultados obtenidos en esta tesis, concluimos que los mecanismos epigenéticos, a través de la metilación del ADN y modificaciones de las histonas tales como la acetilación de la histona 3, son un desencadenante clave de la patogénesis del UL. Además, dirigirse a las enzimas que catalizan estos cambios epigenéticos es un posible enfoque terapéutico para tratar a las pacientes con UL.

CONCLUSIONES

Las principales conclusiones extraídas de esta tesis son:

- El UL presenta un estado de hipermetilación global en comparación con el MM adyacente. Esta metilación aberrante regula la expresión de genes implicados en el desarrollo y mantenimiento del tumor, provocando la hipometilación/sobreexpresión de oncogenes y la hipermetilación/infraexpresión de genes supresores de tumores. En consecuencia, se alteran procesos importantes en la fisiología de los UL, como la proliferación celular, la invasión, las vesículas extracelulares, el metabolismo, la deposición de la ECM y la vía Wnt/β-catenina.

- La inhibición de las metiltransferasas del ADN (DNMTs) mediante el tratamiento con 5-aza-CdR revierte la expresión génica de los genes supresores de tumores hipermetilados/infraexpresados, sugiriendo que esta reversión podría inhibir procesos clave en el desarrollo de los UL.

- La reversión de la metilación del ADN mediante el tratamiento con 5-aza-CdR inhibe la proliferación celular, la formación de ECM y la vía de señalización Wnt/β-catenina en las células HULP in vitro. Estos resultados sugieren que la inhibición de la metilación del ADN mediante el inhibidor de la DNMT 5-aza-CdR podría ofrecer una nueva opción terapéutica para tratar a las pacientes con UL.

- La acetilación aberrante en la lisina 27 de la histona 3 regula la expresión de los genes implicados en la patogénesis del UL, provocando una hiperacetilación/sobreexpresión de oncogenes y una hipoacetilación/infraexpresión de genes supresores de tumores en el UL, que están relacionados con el sistema inmunitario, la angiogénesis, la invasión, la alteración del metabolismo, el depósito de ECM y la desregulación de las vías Wnt/β-catenina y TGFβ.

- La inhibición de las HDACs mediante el tratamiento con SAHA revierte la expresión génica de los genes supresores tumorales hipoacetilados/infraexpresados, sugiriendo que esta reversión podría inhibir procesos clave en el desarrollo del UL.

- La reversión de la desacetilación de las histonas mediante el tratamiento con SAHA reduce la deposición de ECM, disminuye la progresión del ciclo celular y la proliferación celular, así como inhibe la vía de señalización TGFβ en las células HULP in vitro, lo que podría inducir la inhibición del crecimiento del UL. Estos resultados sugieren que la inhibición de las HDAC a través del SAHA puede ser una diana terapéutica viable para tratar el UL.

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ABBREVIATIONS

5-aza-CdR	5-aza-2’-deoxycytidine
5-caC	5-carboxylcytosine
5-fC	5-formylcytosine
5-hmC	5-hydroxymethylcytosine
5-mC	5-methylcytosine
ABCA8	ATP binding cassette subfamily a member 8
ACVR1	Activin A receptor type I
AO	Amine oxidase
APC	Adenomatous polyposis coli
ARHGAP10	Rho GTPase activating protein 10
ATP8B4	ATPase phospholipid transporting 8B4
AUB	Abnormal uterine bleeding
BAX	BCL2 associated-X
BCL2	B-cell lymphoma-2
BMI	Body mass index
CaMKII	Calmodulin-dependent protein kinase II
CAPN6	Calpain 6
CCND1	Cyclin D1
CD40	B Cell Surface Antigen CD40
CEMIP	Cell Migration Inducing Hyaluronidase 1
CK1	Casein kinase 1
c-MYC	MYC proto-oncogene
COL24A1	Collagen Type XXIV Alpha 1 Chain
COL4A5	Collagen type IV α5
COL4A6	Collagen type IV α6
COL-I	Collagen I

DCX	Doublecortin
DEG	Differentially expressed gene
DKK	Dickkopf Wnt signaling pathway inhibitor 1
DNMT	DNA methyltransferase
DPT	Dermatopontin
ECM	Extracellular matrix
EFEMP1	EGF containing fibulin extracellular matrix protein 1
ER	Estrogen receptor
FAD	Flavin adenine dinucleotide
FBLN2	Fibulin 2
FDA	Food and drug administration
FGF	Fibroblast growth factor
FH	Fumarate hydratase
FIGO	Federation of Gynecology and Obstetrics
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
GIMAP8	GTPase, IMAP family member 8
GNAT	Gcn5-related N-acetyltransferases
GPX3	Glutathione peroxidase 3
GnRH	Gonadotropin-releasing hormone
GSK-3	Glycogen synthase kinase 3
GO	Gene Ontology
H3K4me3	Trimethylation mark of histone 3 at the 4th lysine
H3K27ac	Acetylation mark of histone 3 at the 27^{th} lysine
HAT	Histone acetylase
HDAC	Histone deacetylase

HIFU	High-intensity focused ultrasound
HMGA2	High mobility AT-hook 2
HOXA13	Homeobox A13
HOXB4	Homeobox B4
HTATIP2	HIV-1 Tat interactive protein 2
HULP	Human uterine leiomyoma primary
IGF-1	Insulin-like growth factor 1
IGFL3	IGF Like Family Member 3
IL	Interleukins
IL15	Interleukin 15
IP	Infundibulopelvic
IP3	Inositol 1,4,5-triphosphate
IRS4	Insulin receptor substrate 4
IUD	Intrauterine device
KAT	Lysine acetyltransferases
KCNMA1-AS1	Potassium calcium-activated channel subfamily M alpha 1 antisense RNA 1
KEGG	Kyoto encyclopedia of genes and genomes
KLF11	Kuppel like factor 11
KLHDC8A	Kelch domain containing 8a
KMT	Lysine methyltransferase
KRT19	Keratin 19
LEF	Lymphoid enhancer factor
LINC01160	Long intergenic non-protein coding RNA 1160
LSD1	Lysine-specific demethylase 1
MAO	Monoamine oxidase
MAOI	Monoamine oxidase inhibitor
MED12	Mediator complex subunit 12

MM	Myometrium
MMA	Monomethyl arginine
MMP	Matrix metalloproteinase
MRgFUS	Magnetic resonance-guided focused ultrasound surgery
MSC	Myometrial stem cell
NDP	Norrin cystine knot growth factor
NPTX2	Neuronal pentraxin 2
NSAIDs	Nonsteroidal anti-inflammatory drugs
PAI-1	Plasminogen activator inhibitor-1
PCA	Principal component analysis
PCNA	Proliferating cell nuclear antigen
PDGF	Platelet-derived growth factors
PLAC9	Placenta Associated 9
PR	Progesterone receptor
PRL	Prolactin
PRMT	Arginine methyltransferase
qRT-PCR	Quantitative-real time polymerase chain reaction
RIMS2	Regulating synaptic membrane exocytosis 2
RNA	Ribonucleic acid
RFVTA	Radiofrequency volumetric thermal ablation
SAMe	S-adenosyl methionine
SATB2	Special AT-rich sequence-binding protein
SAHA	Suberoylanilide hydroxamic acid
SERMs	Selective estrogen receptor modulators
sFRP	Secreted Fzd-related proteins
SHOX2	Short stature homeobox 2

SPRMs	Selective progesterone receptor modulators
SSC	Somatic stem cells
ST8SIA2	ST8 Alpha-N-acetyl-neuraminide alpha-2,8-sialyltransferase 2
TCF	T cell factor
TDG	Thymine DNA glycosylase
TET	Ten-eleven-translocation
TFAP2C	Transcription factor ap-2 gamma
TGFβ	Transforming growth factor β
TIMPs	Tissue inhibitors of MMPs
TNFα	Tumor necrosis factor-alpha
UAE	Uterine artery embolization
UL	Uterine leiomyoma
UF	Uterine fibroids
UPA	Ulipristal acetate
VEGF	Vascular endothelial growth factor
WIF	Wnt inhibitory factor
WISP1	WNT1 Inducible Signaling Pathway Protein 1
Wnt	Wingless-type
ZFPM2-AS1	Zinc Finger Protein, FOG Family Member 2 Antisense RNA 1

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I. INTRODUCTION

"To find any solution, you have to know where you are concerning the problem" – Antonia Scott (Reina Roja), Juan Gómez Jurado

I. INTRODUCTION

1 UTERUS: ANATOMY AND FUNCTION

1.1 Uterine anatomy

The female genital system is composed of external and internal organs. The external genitals include the perineum and vulva, while the internal organs include the ovaries, fallopian tubes, uterus, and vagina (Ramírez-González *et al.*, 2016) (Figure 1).

The uterus, commonly referred to as the womb, is the primary female reproductive organ. It is a thick-walled muscular structure located in the midline of the abdominal pelvic cavity. The uterus measures approximately 8 cm in length, 4 cm in width, and 5 cm in depth, in its normal, healthy, non-pregnant state. The uterus lies posterior to the bladder and anterior to the rectum (Gasner and A, 2021).

The uterus has four parts: the cervix, isthmus, corpus (body), and fundus (Figure 1). The cervix is a fibromuscular structure of approximately 2 cm in length, whose main functions are to mediate the expulsion of menstrual blood and entry of spermatozoa, and act as a barrier that protects the growing fetus from microbial infection once pregnancy is achieved. On the upper extremity, the cervix connects to the corpus (the largest segment of the uterus) via the isthmus, while on the lower extremity, it joins the uterine body to the vaginal lumen (Cooke *et al.*, 2013; Roach and Andreotti, 2017). The uterine or Fallopian tubes, bilaterally positioned between the uterine fundus and body, curve towards to each ovary, and each have an infundibulum at the distal portion, that attaches to the ovary through fimbriae to receive ovulated oocytes. The round ligament attaches the uterus to the abdominal wall for support, and includes the artery of Sampson. On the other hand, the broad ligament attaches the lateral portion of the uterus with each fallopian tube and ovary, and contains the uterine artery, cardinal arteries, and ureter. The ovarian ligaments maintain the position of the ovaries bilateral to the uterus, while the infundibulopelvic (IP) ligaments attach them to the abdominal wall and regulate vascularization through the ovarian artery and vein. Meanwhile, the uterine vascularization is mainly provided by the uterine artery, with some collateral supply from the ovarian artery. Finally, the uterus is innervated sympathetically and parasympathetically, through the hypogastric nerve and pelvic splanchnic nerves, respectively (Ameer *et al.*, 2021; Gasner and A, 2021).

The uterine body is composed of three layers (Figure 1):

- The **endometrium;** the innermost mucosal layer that covers the uterine cavity. The endometrium's thickness and structure vary based on hormonal stimulation. This layer is subdivided into a functional and basal layer. The functional layer is shed with menstruation, while the basal layer, adjacent to the myometrium, is preserved, and used to regenerate a fresh functional layer, for each menstrual cycle.
- The **myometrium;** the muscular and middle layer. The myometrium is the thickest (12-15 mm) and the most vascularized layer of the uterus. It is composed of smooth muscle cells embedded in a collagen-rich extracellular matrix (ECM). During pregnancy, the muscle cells divide (hyperplasia) and enlarge (hypertrophy), so that the uterus can grow with the developing fetus.
- The **perimetrium;** the outermost serosal layer. It covers the uterine body and fundus (Coad J, 2011; Rogers, 2011; Ramírez-González *et al.*, 2016).

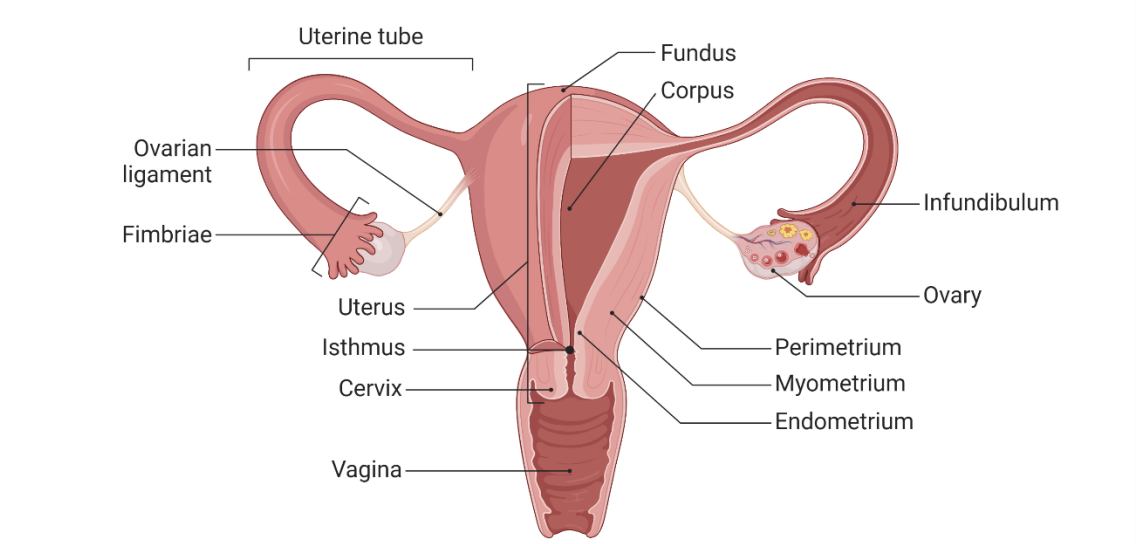


Figure 1. Anatomy of the female reproductive system*. The internal organs/tissues are represented in this figure. The vagina is located at the inferior portion of the female reproductive tract, connecting with the cervix. The uterus is divided into four parts: the fundus, isthmus, corpus (body), and cervix. The uterine wall is composed of three main layers: the endometrium, myometrium, and perimetrium. The uterine or Fallopian tubes, positioned between the uterine fundus and body, curve towards each ovary, and at their distal end, the infundibulum attaches to the ovary through fimbriae. Created with BioRender.com*

1.2 Uterine function

The uterus is responsible for many functions in the processes of menstruation, implantation, gestation, and labor. In response to female hormones (e.g., estrogen, progesterone), it undergoes dynamic changes that allow implantation of a fertilized oocyte, or menstruation when pregnancy does not occur. The uterus nourishes the fertilized oocyte, supporting early embryo development, as it passes through the fallopian tube towards the body of the uterus. After implantation in the endometrium, the embryo receives nourishment from the local blood vessels that were exclusively developed for this purpose during the proliferative phase of the menstrual cycle. As pregnancy progresses and the embryo grows and matures, the uterus rapidly expands to accommodate the developing fetus.

Throughout a woman's reproductive life, the uterus undertakes pregnancy-induced expansion mostly provided by the myometrium, which is composed of myometrial cells. The two main functions of the myometrium are to protect the fetus from the tensions and stress generated during pregnancy (by growing and stretching), and facilitate childbirth (through muscle contraction, which is mediated by endocrine, paracrine, and mechanical pathways) (Shynlova *et al.*, 2009) (Ameer *et al.*, 2021; Gasner and A, 2021). Remarkably, by the end of a pregnancy, the uterus increases up to 1000-fold in volume and 20-fold in weight (Maruyama *et al.*, 2013; Ono *et al.*, 2013).

Further, the myometrium undergoes different phases during pregnancy. In the early proliferative phase, there is an increase in myocyte quantity (hyperplasia) and size (hypertrophy), the interstitial matrix is synthesized, and focal adhesions are remodelled. This is followed by a positive regulation of contractile proteins and downregulation of myometrial inhibitory pathways. As a result, contractile proteins accumulate, and uterotonic agonists are synthesized, leading to contractions. After delivery, transcriptomic profiles associated with apoptosis, wound repair, and tissue regeneration are enhanced, favoring the uterus' return to its normal condition, a process known as uterine involution (Shynlova *et al.*, 2006, 2009; Maruyama *et al.*, 2013).

1.3 The extracellular matrix

The ECM is an essential part of the uterine myometrium. Its three-dimensional network of extracellular macromolecules provides structural and biochemical support to surrounding cells, playing key functions in cell adhesion, communication, and differentiation. The components of

ECM are synthesized by resident cells, and secreted into the surroundings via exocytosis (Basu, 2016).

The main components of the ECM include:

- **Collagen fibrils** and interspersed smooth muscle cells, which are responsible for the mechanical strength of uterine tissue (Leppert *et al.*, 2014);
- **Proteoglycans** (such as decorin, hyaluron, or versican), these glycoproteins consist of a protein core with covalently-attached glycosaminoglycans that attract cations and bind water, allowing the tissue to adapt to pressure changes;
- **Elastin**, a hydrophobic protein that provides elasticity to uterine tissues, allowing them to stretch and recoil back to their original state after delivery (Leppert *et al.*, 2014; Basu, 2016)
- **Integrins**, which are transmembrane receptors, formed by two subunits (α and β) that contain cytoplasmic, transmembrane, and extracellular components, that mediate signals between cells and their ECM, and act as mechanosensors, participating in important biological processes, such as cell adhesion, signaling, and survival, or ECM organization (Leppert *et al.*, 2014);
- **Fibronectin**, that organizes matrix assembly, by binding collagen, integrins, and other ECM molecules (Leppert *et al.*, 2014), and participates in processes involving ECM remodelling or assembly (i.e., cell migration, adhesion, growth, differentiation, and fibrosis) (Islam *et al.*, 2018).

The changes that the uterine ECM undergoes are mediated by the matrix metalloproteinase (MMP) system. MMPs are enzymes with proteolytic activity, and are partly regulated by tissue inhibitors of MMPs (TIMPs). The expression of MMPs and TIMPs is influenced by pro-inflammatory cytokines, hormones, and growth factors such as transforming growth factor β (TGFβ). An improper balance between MMPs and TIMPs can promote pathological conditions, such as ovarian cysts, endometriosis, or uterine fibroids (Curry and Osteen, 2003).

2 UTERINE LEIOMYOMAS

2.1 Pathophysiology

Uterine leiomyomas (UL), also known as uterine fibroids (UF), are benign estrogen-dependent tumors arising from the smooth muscle cells located in the myometrial layer of the uterus**.** This condition is characterized by a modest cell proliferation, disordered distribution of smooth muscle cells and fibroblasts, and an excessive deposition of disorganized ECM (Parker, 2007).

2.2 Classification

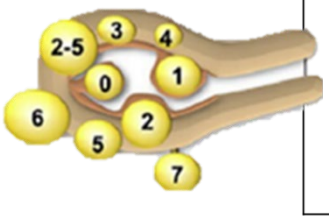
Approximately two-thirds of women have multiple myomas of various sizes (Giuliani *et al.*, 2020). In general, these leiomyomas are classified according to their location in the uterine layers (Stewart *et al.*, 2016):

- **Submucosal leiomyomas** invade the endometrial cavity, disturbing endometrial function, and, therefore, causing more fertility issues.
- **Intramural leiomyomas** are restricted to the myometrial layer (i.e., located within the uterine wall).
- **Subserosal leiomyomas** extend beyond the myometrium, towards the outer layer of the uterus (serosa), altering the shape of the uterus.

Nevertheless, since most UFs present overlapping characteristics of these subtypes, alternative classification is required.

Although numerous UL classifications can be found in the literature, the most recent and widely adopted is the 2011 International Federation of Gynecology and Obstetrics (FIGO) classification (Munro *et al.*, 2011), which describes eight classes of UFs, and accounts for their location and degree of intramural/intracavitary extension (Figure 2), as follows:

- Type 0: intracavitary lesions are attached to the endometrium by a narrow stalk (pedunculated).
- Type 1: submucosal leiomyomas with a portion of the lesion found to be <50% intramural.
- Type 2: submucosal leiomyomas with a portion of the lesion found to be >50% intramural.
- Type 3: intramural leiomyomas that remain in contact with the endometrium.
- Type 4: intramural leiomyomas that are entirely within the myometrium, with no extension to the endometrial surface or the serosa.
- Types 5: subserosal leiomyomas with a portion of the lesion found to be >50% intramural.
- Type 6: subserosal leiomyomas with a portion of the lesion found to be <50% intramural.
- Type 7: subserosal leiomyomas that are attached to the serosa by a stalk (pedunculated).
- Type 8: ULs that do not involve the myometrium, such as cervical lesions (Munro *et al.*, 2011; Stewart *et al.*, 2016).



Submucosal	0	Pedunculated intracavitary
	1	<50% intramural
	2	>50% intramural
O- Other	3	Contacts endometrium; 100% intramural
	4	Intramural
	5	Subserosal ≥50% intramural
	6	Subserosal <50% intramural
	7	Subserosal pedunculated
	8	Other (specify e.g. cervical, parasitic)

Hibrid leiomyomas (impact both endometrium and serosa)	Two numbers are listed separated by a hypen. By convention, the first refers to the relationship with the endometrium while the second refers to the relationship to the serosa. One example is below.	
	2-5	Submucosal and subserosal, each with less than half of the diameter in the endometrial and peritoneal cavities, respectively.

Figure 2. International Federation of Gynecology and Obstetrics (FIGO) subclassification system (PALM-COEIN) for leiomyomas. *FIGO established an 8-point numerical score to describe the location of ULs. Figure adapted from Munro et al. 2011.*

2.3 Epidemiology and symptoms

2.3.1. Epidemiology of uterine leiomyomas

ULs are the most prevalent gynecological tumors found in women of reproductive age. Their prevalence is presumably underestimated by epidemiologic studies, because they focus mainly on symptomatic women (who represent a minority of affected patients), leaving behind a large cohort of women who are asymptomatic or underreported their symptoms (Bulun, 2013; Marsh *et al.*, 2013). The reported prevalence of fibroids varies enormously among different studies and countries, ranging from 4.5% to 68.6%. These differences are due to the type of study, method of diagnosis, and racial/ethnic demographics of the study cohort (Stewart *et al.*, 2017). Indeed, more than 80% of women of African ancestry, and almost 70% of Caucasian women, are diagnosed with ULs in the United States, however, the prevalence of fibroids in other racial/ethnic groups such as Asian and Hispanic women remains unclear (Giuliani *et al.*, 2020).

There are remarkable **racial differences** in the prevalence and presentation of fibroids. ULs are more common, larger, numerous, often presented by younger patients, and diagnosed in women of African descent three times more frequently than Caucasian women (de la Cruz and Buchanan, 2017; Giuliani *et al.*, 2020). This disparity has been attributed to dissimilarities in socioeconomic status, access to healthcare, genetics, and environmental exposures (Styer and Rueda, 2016).

Age is also a risk factor in UL pathogenesis. Due to their hormone-dependent nature, fibroids mainly manifest during reproductive years (particularly between the ages of 30 and 40) (Bulun, 2013; Stewart *et al.*, 2016) – they are extremely rare before menarche, and typically regress after menopause. Other associated risk factors include early menarche, late menopause, obesity, nulliparity, hypertension diet, exercise, smoking, alcohol, stress, environmental factors (Bulun, 2013; Giuliani *et al.*, 2020), and Vitamin D deficiency (Baird *et al.*, 2013; Sabry *et al.*, 2013; Halder and Al-Hendy, 2016; Ciebiera *et al.*, 2021).

The elevated prevalence of fibroids has a profound impact on healthcare costs worldwide. The annual costs related to UL management is estimated to be approximately $34.4 billion in the United States, $348 million in Germany, $120 million in France, and $86 million in England (Soliman *et al.*, 2015), supporting the relevance of UL as a disease of major socioeconomic concern.

2.3.1 Symptoms

The complications caused by ULs depend on the tumor location, size, multiplicity, patient's desire to remain fertile, among other factors. Approximately 70% of patients affected by UL remain asymptomatic, with the majority being diagnosed during radiologic procedures conducted for other indications. The other 30% of these patients present physical symptoms, in a spectrum of disease severity (Bulun, 2013; Giuliani *et al.*, 2020), including:

- Pelvic or abdominal pain, resulting from the morphological changes of the UL in the uterus, which exert greater pressure on adjacent organs (Su *et al.*, 2012);
- Dysmenorrhea and abnormal uterine bleeding (AUB), which are associated with increased uterine vascularity and endometrial surface area, impaired uterine contractibility, endometrial ulceration, or compression of the venous plexus in the myometrium (Sinai Talaulikar, 2018);
- Anemia, as a consequence of excessive blood loss during menstruation;
- Sexual dysfunction or dyspareunia, due to the increased pressure on other organs;
- Bladder or bowel symptoms (urinary frequency or constipation, respectively);
- Infertility and poor obstetrical outcomes, such as the increased risk of ectopic gravidity, recurrent abortion, preterm labor, cesarean delivery, antepartum bleeding, fetal malpresentation, and growth restriction (Parker, 2007; Stewart *et al.*, 2017; Giuliani *et al.*, 2020). Apart from anatomic or functional changes in the uterine cavity or myometrium, these conditions may also be caused by the molecular

changes that alter the adjacent endometrium, which can lead to excessive uterine bleeding or impair blastocyst implantation (Galliano *et al.*, 2015).

On the other hand, patients with ULs may also experience psychological and/or emotional distress, depression, and anxiety, as a result of their symptoms affecting their well-being, lifestyle, and ultimately, quality of life (Ghant *et al.*, 2015).

2.4 Management of uterine leiomyomas

At present, the therapeutic strategies for patients with ULs include pharmacological treatments, interventional radiology and surgical procedures (Figure 3), that are designed to alleviate UL-associated symptoms by controlling AUB, shrinking or eliminating the fibroids.

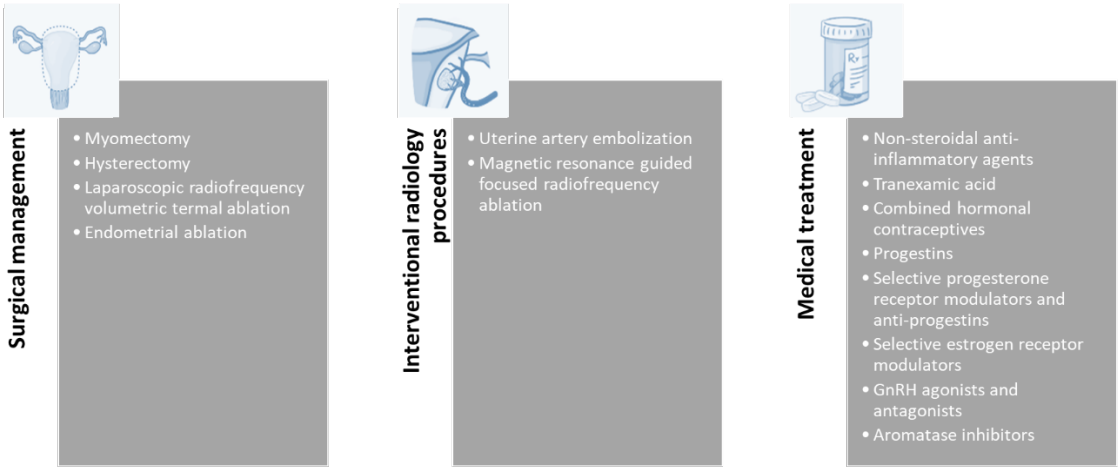


Figure 3. Pharmacological, radiological, and surgical treatment options for symptomatic ULs.

2.4.1 Surgical procedures

ULs can be managed surgically, by myomectomy, hysterectomy, laparoscopic radiofrequency volumetric thermal ablation, and endometrial ablation.

- Myomectomy

Myomectomy is a uterine-sparing surgery, that involves excising the UL while leaving the uterus intact. This procedure can be performed via hysteroscopy, laparoscopy (conventional or robotically-assisted), or laparotomy, based on the quantity, size, and location of UL(s). This option is principally offered to patients who wish to preserve their fertility and/or uterus. Despite temporarily reducing uterine volume, and improving symptoms in up to 80% of women,

the risk of recurrence is 27% after removal of a single fibroid, or greater than 50% in the case of multiple fibroids (Kotani *et al.*, 2018). Only 1%–5% of myomectomies present complications, with the most common being a high intraprocedural blood loss (Bhave Chittawar *et al.*, 2014).

- Hysterectomy

Hysterectomy is the surgical removal of the entire uterus, by vaginal, laparoscopic, robotic, or open approaches. Remarkably, the majority of women who undergo this procedure report a significant improvement in quality of life and symptoms, as early as 3 months after surgery (Pitter *et al.*, 2014). Overall, hysterectomies are associated with relatively few complications, a 0.4% incidence of major complications, and a low rate of reoperations for adhesions, pelvic prolapse, or fistulas (Pitter *et al.*, 2014). Despite the advances in medical and uterine-preserving treatments in the past years, and socioeconomic burden of this surgery, hysterectomy remains the procedure most offered by clinicians and elected by women with symptomatic fibroids, who have no desire to conceive or are in perimenopause (40-50 years of age). Indeed, approximately one-third of all the hysterectomies performed worldwide are for indications related to uterine fibroids (Donnez and Dolmans, 2016a; Giuliani *et al.*, 2020).

- Laparoscopic radiofrequency volumetric thermal ablation

Radiofrequency volumetric thermal ablation (RFVTA) is a laparoscopic outpatient technique, performed under ultrasound guidance, where an electrosurgical probe is inserted within the fibroid to induce coagulative myolysis. Its efficacy has been demonstrated, by an improvement in symptom severity, and up to 77% reduction in fibroid volume, within six months. Further, RFVTA is associated with a low rate of re-intervention (11%), shorter hospital stays, and lower blood loss compared to laparoscopic myomectomies (Havryliuk *et al.*, 2017). However, few centers offer this technique, and there is limited evidence of subsequent pregnancy and long-term safety.

- Endometrial ablation

Endometrial ablation is a procedure that destroys the innermost lining of the uterus (the endometrium), using thermal balloons, microwave, hydrothermablation, bipolar radiofrequency endometrial ablation, or endometrial cryotherapy devices. Some contraindications of this technique are the desire to preserve fertility, active pelvic infections, endometrial cancer, and uterine congenital anomalies (Munro, 2018). Therefore, good candidates for this procedure are perimenopausal women who have completed childbearing.

2.4.2 Non-surgical management: Interventional radiology procedures

Non-surgical management of UL patients principally aims to alleviate associated symptoms. Women who decline surgery, or are poor surgical candidates, may opt for alternative interventional radiology procedures, such as uterine artery embolization or magnetic resonance-guided focused radiofrequency ablation. Both these techniques are effective, minimally-invasive treatments for UL patients, with short hospital stays, quicker recovery period, and fewer surgical complications.

- Uterine artery embolization

Uterine artery embolization (UAE) is a minimally-invasive angiographic method that uses tris-acryl gelatin microspheres, or non-spherical polyvinyl alcohol, to disrupt uterine blood supply, and ultimately induce ischemic necrosis of the UL mass. Women undergoing UAE reported an average of 42% fibroid shrinkage at 3 months, shorter menstrual duration, and improvement of symptoms. Further, UAE is a shorter procedure that allows patients to recover and resume their normal activities quicker than with surgery. Disadvantages of this technique are that it affects the whole uterus, and thus it may permanently compromise uterine and ovarian function (Lee and Yu, 2016), and has a higher 5-year recurrence rate (20%) than myomectomies, resulting in more women requiring additional interventions (de Bruijn *et al.*, 2016).

- Magnetic resonance-guided focused radiofrequency ablation

Magnetic resonance-guided focused radiofrequency ablation (MRgFUS), also called high-intensity focused ultrasound (HIFU), is a procedure that employs transabdominal ultrasound waves to induce coagulative necrosis and regression of fibroids. At six-month follow-up, approximately 71% of women who receive this treatment reported an improvement in symptoms. The main risks associated with HIFU are reversible pelvic neuropathy and local skin burns. Notably, HIFU is not conventionally used for UL therapy because the rates of re-intervention are higher (30.5%) than with myomectomies or UAE (Sandberg *et al.*, 2018). Further research is also needed to verify pregnancy outcomes following HIFU.

2.4.3 Non-surgical management: Medical treatments

Pharmacological treatments available for women who decline surgery or are poor surgical candidates include anti-fibrinolytic agents, non-steroidal anti-inflammatory drugs (NSAIDs), combined hormonal contraceptives, progesterone-based treatments, selective progesterone receptor modulators (SPRMs), anti-progestins, selective estrogen receptor modulators (SERMs),

aromatase inhibitors, and gonadotropin-releasing hormone (GnRH) agonists or antagonists. Emerging alternatives also including green tea extract, vitamin D, cabergoline, gestrinone, and somatostatin analogs (Sohn *et al.*, 2018).

- Non-steroidal anti-inflammatory agents

First-line medical management of symptoms caused by fibroids, such as AUB and dysmenorrhea, includes the use of NSAIDs. Clinicians often prescribe ibuprofen or naproxen, because they are inexpensive, widely-available, and produce limited adverse events, despite their inefficiency to reduce menstrual bleeding. Of note, NSAIDs do not alter UL size or uterine physiology, increasing the probability of UL progression, severity of the symptoms, and further treatment (Lethaby *et al.*, 2013; Sohn *et al.*, 2018).

- Tranexamic acid

Women presenting with AUB have overactive endometrial fibrinolysis during menstruation (Chwalisz and Taylor, 2017). Tranexamic acid is a synthetic lysine derivative, that works as an anti-fibrinolytic agent and promoter of blood clot formation. By preventing fibrin degradation of the plasminogen lysine receptor site, it favors coagulation, reducing menstrual blood flow and improving AUB-associated symptoms (Sohn *et al.*, 2018). Tranexamic acid causes gastrointestinal- and musculoskeletal-related side effects, and is contraindicated for patients with color blindness, active bleeding, a history of intravascular clotting, or hypersensitivity to the medication (Lukes *et al.*, 2010).

- Combined hormonal contraceptives

Contraceptives combining estrogen and progesterone prevent endometrial thickening, and thus decrease the quantity of endometrial shedding during the menstrual cycle. They are standard care for AUB, especially in women with ULs, but they do not reduce UL size. Hormonal contraceptives are easily accessible, inexpensive, and can be used in the form of pills, vaginal devices, or transdermal patches, on a cyclical or continuous basis. However, side effects of these contraceptives include nausea and headaches (Moroni *et al.*, 2015).

- Progestins

Conventional progesterone-based treatments for AUB include oral progestins (e.g., norethindrone acetate, medroxyprogesterone acetate, or megestrol) and progesterone-releasing intrauterine devices (IUDs). These methods repress the estrogen-stimulated growth of

the endometrium, decreasing blood loss during menstruation. The most frequently reported side effects of these treatments are gastrointestinal reactions, irregular bleeding, acne, breast tenderness, and mood changes (Vannuccini *et al.*, 2018).

Notably, **levonorgestrel-releasing IUDs** act locally on the endometrium, enhancing endometrial absorption and reducing systemic side effects. The amenorrhea, and enhance menorrhagia and anemia in up to 50–60% of patients with AUB between 6–12 months of use, including patients with ULs. Nevertheless, women with UL are reported to have higher expulsion rates of this device (Jiang *et al.*, 2014).

- Selective progesterone receptor modulators and anti-progestins

SPRMs and anti-progestins act through peripheral progesterone receptors, inducing apoptosis, inhibiting fibroid cell proliferation, and thinning the endometrial lining. In particular, **ulipristal acetate (UPA)** shrinks fibroids by 25–50%, and reduces uterine bleeding by greater than 90% (Donnez *et al.*, 2012; Donnez and Dolmans, 2016a). These treatments are contraindicated for patients with severe asthma and liver impairment, or morphological changes in the endometrium (i.e., large cystic glands) or stroma (e.g., fibroblast and vasculature) (Whitaker *et al.*, 2017). For these reasons, it is recommended the use of UPA for women in whom surgery is not an option and, as preoperative treatment during a maximum of 3 months. The efficacy of other SPRMs, such as **mifepristone** (which reduces AUB, but does not efficiently shrink ULs), **asoprisnil, vilaprisan,** and **telapristone acetate**, is currently being investigated to treat ULs (Murji *et al.*, 2017). Further, the long-term safety of SPRMs is being evaluated, due to the increased risk of endometrial hyperplasia/cancer from unopposed estrogen signaling.

- Selective estrogen receptor modulators

Tamoxifen and Raloxifene are popular nonsteroidal SERMs, employed as tissue-specific estrogen receptor (ER) agonists/antagonists. Although **Tamoxifen** decreases blood loss and pain intensity in patients with ULs, clinicians weight these benefits against its side effects, including hot flashes, dizziness, endometrial changes, and the development of ovarian cysts (Sadan *et al.*, 2001; Sohn *et al.*, 2018). Alternatively, **Raloxifene** shrinks leiomyomas and only causes mild side effects, such as increased appetite, weight gain, gastralgia, and hot flashes (Deng *et al.*, 2012).

- GnRH agonists and antagonists

After an initial stimulation of gonadotropin release (known as the flare effect), **GnRH agonists** downregulate the pituitary production of gonadotropins and gonadal steroids, inhibiting further fibroid growth. For example, **leuprolide acetate** is typically used in preoperative treatment of uterine fibroids. Although GnRH agonists produce amenorrhea in 98% of women, and have shrunken fibroids by 35–65% within 3 months of treatment, prolonged treatment (over 6 months) induces a menopausal-like hypoestrogenic state associated with hot flashes, vaginal dryness, decreased libido, sleep disturbances, bone loss, and unattainable pregnancy (Giuliani *et al.*, 2020). The use of GnRH agonists is therefore limited to three to six months, at most, after which the ULs rapidly return to pretreatment size within six months (Stewart, 2001).

On the other hand, **GnRH antagonists,** like **cetrorelix** and **ganirelix acetate,** provide an immediate clinical response, without flare effects. However, since they are more expensive, and require daily injections (due to their shorter half-life), they are not frequently used. Nevertheless, the efficacy of a promising new oral GnRH antagonist (**elagolix**) has been demonstrated, in terms of reduced menstrual bleeding and uterine volume (Archer *et al.*, 2017).

- Aromatase inhibitors

Aromatase inhibitors also induce a hypoestrogenic state, by preventing the conversion of androgens to estrogens, which results in the thinning of the endometrial lining and reduced menstrual bleeding. Specifically, letrozole and anastrozole shrink fibroids by 40-50%, improve dysmenorrhea, menorrhagia, and duration of menses (Song *et al.*, 2013). Despite these drugs being tolerated better than GnRH agonists (with fewer anti-estrogenic adverse effects in the short-term), at present, there is insufficient evidence to support their broad and long-term use.

2.4.4 Current state of the field and future directions

Notwithstanding the high prevalence of UL, there are currently no medical approaches, interventional radiology strategies, or surgical procedures that can simultaneously reduce fibroids permanently and produce minimal side effects. The current gold standard treatment for ULs is neoadjuvant treatment followed by debulking surgery. However, less invasive treatments should be considered, based on the patient's desire for future fertility and/or to preserve her uterus, the possibility of achieving the desired clinical outcomes, and overall health. Available therapeutic strategies aim to alleviate UL-associated symptoms by shrinking fibroids, controlling AUB, and/or definitively curing the fibroids, rather than targeting mechanisms involved in UL

initiation and progression. Further, pharmacological options are only temporary solutions [in most cases, leiomyomas return to their initial size 6 months after the treatment is over (Manyonda *et al.*, 2004)], and often produce menopausal symptoms and osteoporosis. Thus, the identification of molecular mechanisms involved in UL pathogenesis will deepen understanding of the disease, facilitating the development of alternative strategies that optimize UL reduction and produce no long-term side effects that can compromise quality of life.

2.5 Pathogenesis

Like most tumors, ULs appear to have a multifactorial etiology. Contributing risk factors include tumor-initiating cells (TICs), abnormal steroid hormone signalling, the ECM), aberrant wingless-type MMTV integration site family (Wnt)/β-catenin and TGFβ signaling pathways, an excess of growth factors, and the environment.

2.5.1 Stem cells from myometrium and uterine leiomyoma formation

The presence of somatic stem cells (SSCs) in the female reproductive tract has been corroborated by several studies. Indeed, myometrial stem cells (MSCs) represent 2% of the undifferentiated myometrial cell population. They are critical for uterine regeneration and tissue remodelling. During pregnancy, the mechanical stretching of the myometrium results in hypoxia, which stimulates growth of SSCs and MSC proliferation, that in turn, facilitates uterine growth with their high capacity for self-renewal (Maruyama *et al.*, 2013; Mas *et al.*, 2014). Notably, aberrant methylation and estrogen signaling have also been proposed as possible causes of this tumorigenic transformation (Ono *et al.*, 2012).

A subpopulation of MSCs exhibit key characteristics of tumor-initiating cells (TICs). These TICs maintain an undifferentiated state, have a high capacity for cell colony formation under hypoxic conditions *in vitro,* and are associated with impaired proliferation and cell migration (Mas *et al.*, 2012; Ono *et al.*, 2012). Under certain *in vitro* conditions, TICs were differentiated into adipocytes, osteocytes, and chondrocytes, while in xenograft animal models they formed myometrial tissue *in vivo* (Santamaria *et al.*, 2018). It is postulated that genetic and epigenetic modifications in MSCs mediate their transformation into TICs, which have an increased capacity for self-renewal, proliferation, and UL initiation (Ono *et al.*, 2012). As TICs clonally expand, they are giving rise to intermediately differentiated daughter cells, that in turn, may differentiate into a differentiated leiomyoma cell that begins forming a UL (Figure 4). As this original tumor progresses, it accumulates mutations, generating subclones of daughter cells with a selective

growth advantage and potential for clonal expansion, that may lead to intrauterine metastases and formation of multiple leiomyomas (Bulun *et al.*, 2015).

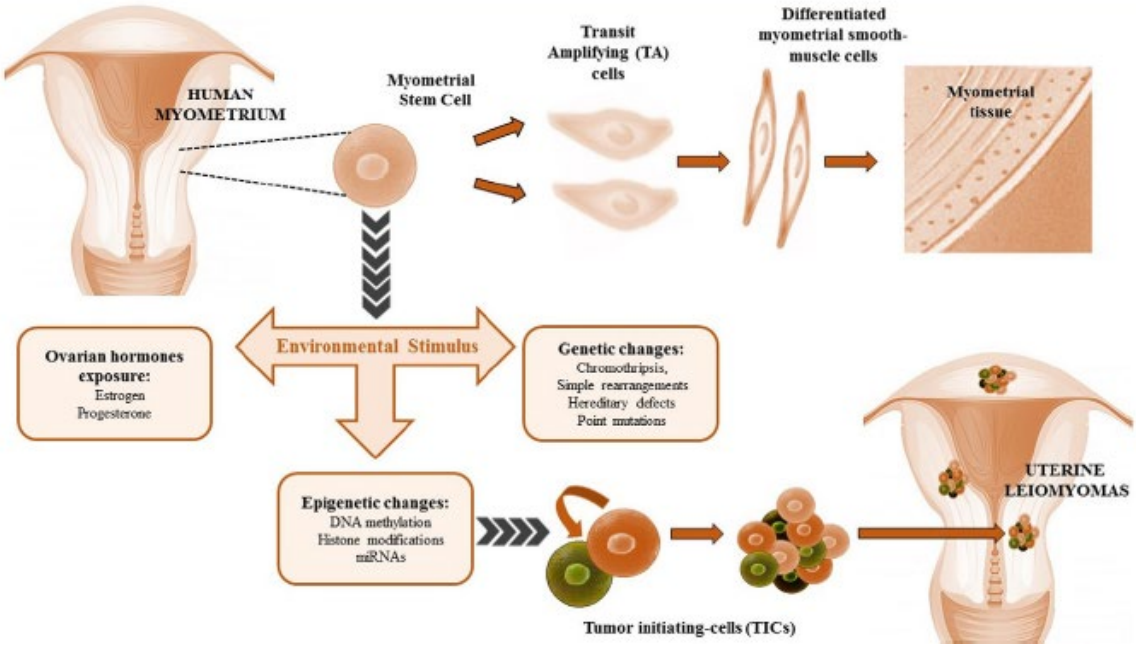


Figure 4. Uterine leiomyoma initiation based on tumor-initiating cells in the myometrial niche. *Under normal circumstances, myometrial stem cells can self-renew and differentiate into new myometrial smooth muscle cells that extend or repair myometrial tissue during pregnancy or post-partum recovery, respectively. However, certain environmental insults can cause excessive/aberrant ovarian hormone exposure, or genetic/epigenetic modifications that alter the transcriptome and function of the myometrial stem cells, consequently, transforming them into tumor-initiating cells. In turn, proliferation of the tumor-initiating cells leads to the development of a benign tumor, known as a UL. Obtained from Santamaria et al. 2018.*

2.5.2 Genetic modifications

Uterine leiomyomas are benign heterogeneous tumors, with different molecular mechanisms triggering their development. Known drivers of UL initiation include mutations in the mediator complex subunit 12 (*MED12),* high mobility group AT-hook2 *(HMGA2),* fumarate hydratase *(FH)*, and collagen type IV alpha 5 and 6 (*COL4A5/6)* genes, and karyotype abnormalities that cause structural and functional changes in the stem cell genome (Mehine *et al.*, 2014).

- **MED12:** Approximately, 70–75% of patients with ULs present somatic mutations of the *MED12* gene (Mäkinen *et al.*, 2011; Osinovskaya *et al.*, 2016) that encodes a subunit of an RNA polymerase II regulator complex (Bulun, 2013). MED12 mutation alters signaling

pathways involved in stem cell renewal, cell proliferation and fibrosis, such as Wnt/β-Catenin and TGFβ pathways (Huang *et al.*, 2012; Markowski *et al.*, 2012). A common *MED12* variant (produced by a gain-of-function mutation) drove fibroid formation and genomic instability in mice, suggesting a causative role of *MED12* mutations in fibroids. Alternatively, overexpression of wild-type *MED12* promoted the proliferation of leiomyoma cells.

- **HMGA2:** Cytogenetic rearrangements of chromosome 12q15 are present in 40-50% of leiomyomas. Among these, 20% exhibit a rearrangement at the breakpoint region 12q14–q15, upregulating *HMGA2* (Baranov *et al.*, 2019). The *HMGA2* gene encodes a non-histone chromatin protein that regulates transcriptional processes affecting growth, differentiation, apoptosis, and cellular transformation (Galindo *et al.*, 2018). Specifically, *HMGA2* encodes three DNA-binding domains that recognize AT-rich domains in nuclear DNA (referred to as AT hooks), and is implicated in the assembly of protein complexes that regulate gene transcription.

- **FH:** The *FH* gene encodes an enzyme in the tricarboxylic acid cycle that is associated with the development of hereditary leiomyomatosis. *FH* is a classic tumor suppressor gene, and as such, its biallelic inactivation increased cell survival, growth, and metastases, stimulating UL progression (Arenas Valencia *et al.*, 2018). Notably, 10.5% of UL patients present this biallelic loss of *FH* (Lehtonen *et al.*, 2004).

- **COL4A5/6:** The *COL4A5/6* genes are mapped on the long arm of the X chromosome, and specific deletions of these genes are associated with diffuse leiomyomatosis. Indeed, almost 4% of patients with UL present with *COL4A5/6* deletion (Mehine *et al.*, 2016), which leads to the overexpression of insulin receptor substrate 4 (IRS4), that in turn, amplifies cell proliferation via increased insulin-like growth factor 1 (IGF-1) signaling (Mehine *et al.*, 2014, 2016).

- **Karyotype abnormalities:** Approximately 25-40% of patients with UL present chromosome abnormalities [e.g., translocations, deletions, or more complex rearrangements of chromosome fragments, within one, or between several different chromosomes (known as chromothripsis)] (Styer and Rueda, 2016; Koltsova *et al.*, 2019). Cytogenetic rearrangements characteristic of UL include chromosomes 12 and 14 translocation, trisomy 12, deletions on chromosomes 3, 7, or 1, as well as rearrangements of the short arm of chromosome 6 and chromosome 1, 3, 10, 13, and

X. In addition, various submicroscopic genomic changes are frequently found in UL cells (Bowden *et al.*, 2009).

2.5.3 Steroid hormones

Typically, ULs develop only after menarche and regress after menopause, due to the higher concentration of steroid hormones, particularly estrogen and progesterone, during reproductive years. Estrogen signaling begins with direct binding to the intranuclear estrogen receptor alpha (ER-α) and beta (ER-β) transcription factors, that induce the expression of growth factors and/or activate mitogenic signaling pathways. For example, estrogen significantly downregulated p53 protein expression in leiomyoma cells *in vitro*, suggesting that it may stimulate leiomyoma development in part by suppressing normal p53 functions. Furthermore, estrogen signaling can alter the expression of numerous oncogenes, including c-fos, c-jun, connexin 43, progesterone receptor (PR), IGF-1, insulin-like growth factor receptors (IGFRs), insulin-like growth factor binding protein 5 (IGFBP5), A-myb, and MKP-1 (Swartz *et al.*, 2005; Islam *et al.*, 2013). On the other hand, progesterone also plays a critical role in UL pathogenesis, by similarly contributing to cell growth in primary cultures of human UL cells (Ishikawa *et al.*, 2010). Indeed, the two predominant isoforms of PR, PRA and PRB, are ligand-activated transcription factors. PRB activates progesterone-responsive genes, whereas PRA represses PRB transcriptional activity (Sant'Anna *et al.*, 2017). A recent study found that ULs overexpress both progesterone receptors with respect to the adjacent myometrium (MM) (Sant'Anna *et al.*, 2017). Interestingly, both sex hormones interact to synergically promote UL development through cell proliferation and volumetric increases in cellular and extracellular components. Specifically, estrogen promotes PR expression and supports progesterone signaling, maintaining the volume or facilitating expansion of human ULs (Ishikawa *et al.*, 2010).

2.5.4 Extracellular matrix

Quantitative and qualitative abnormalities in ECM proteins (especially collagen, fibronectin, laminin, and sulfated proteoglycans) promote UL progression (Figure 5) (Leppert *et al.*, 2004). Indeed, ULs contain 50% more ECM than the MM, which may act as a reservoir of growth factors, cytokines, chemokines, angiogenic and inflammatory response mediators, and proteases produced by the tumor cells (Malik *et al.*, 2010; Islam *et al.*, 2018). As the tumor grows, the volume of ECM gradually increases, generating environmental stimuli that affect local cells (Figure 5). Communication through cell surface integrins or other transmembrane mediators induces actin polymerization, which activates conserved signaling pathways that mediate diverse cellular responses (i.e., p38MAPK/ERK), impairs gene expression of TGFβ, activin A

receptor, type I (ACVR1), platelet-derived growth factor (PDGF), and tumor necrosis factor-alpha (TNFα), that regulates the metabolism of steroid hormones. Due to the substantial influence of the ECM in UL progression, drugs that effectively control its size (e.g., Fasudil) are emerging as promising therapeutic alternatives (Islam *et al.*, 2018; Baranov *et al.*, 2019).

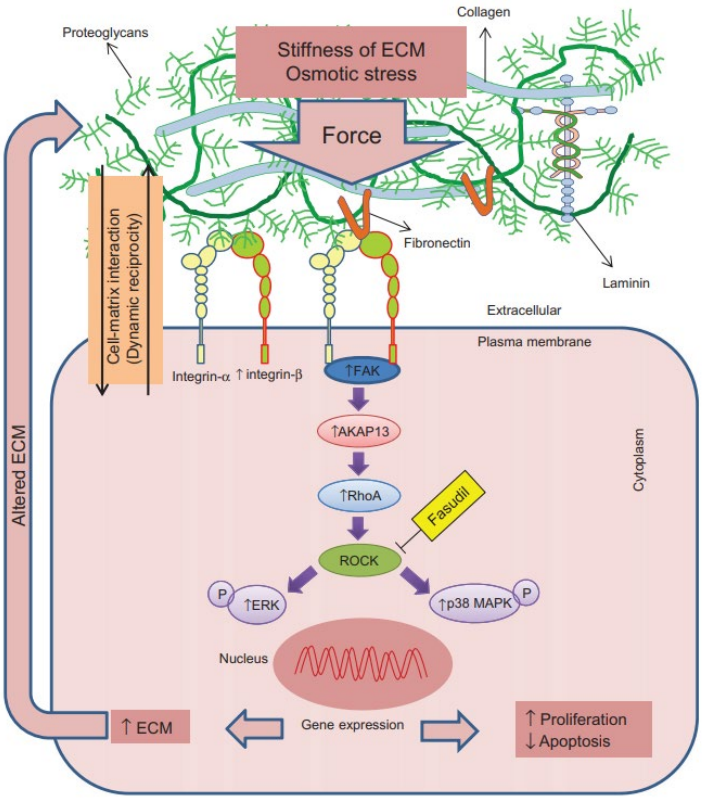


Figure 5. Mechanotransduction of uterine leiomyoma cells. *Uterine leiomyomas present an excessive deposition of stiff extracellular matrix (ECM), with a high density of proteoglycans, collagen, laminin, and fibronectin. The mechanical forces exerted by the overabundance of these proteins, or osmotic stress, activates intracellular signaling cascades through the transmembrane integrin receptors. Signaling through FAK initiates actin polymerization, and downstream AKAP13-RhoA-ROCK interactions to activate the ERK/p38 MAPK-signaling cascade. Ultimately, this pathway results in gene expression changes that alter cell proliferation, decrease apoptosis, and upregulate the synthesis of other ECM proteins. The mechanism of action of Fasudil (indicated by a yellow square), a selective ROCK inhibitor, is illustrated. Image obtained from Islam et al. 2018.*

2.5.5 Wnt/β-catenin pathway

The Wnt signaling pathway is one of the most evolutionarily-conserved cellular responses. It orchestrates several cellular functions, such as proliferation, apoptosis, cell fate determination, polarity, migration during development, and stem cell maintenance in adults (Gruber *et al.*, 2016; El Sabeh *et al.*, 2021). This pathway is deregulated in several cancers (Clevers and Nusse,

2012; Shang *et al.*, 2017; Zhan *et al.*, 2017) and UL (Ono *et al.*, 2013; Borahay *et al.*, 2015). Activation of the Wnt/β-catenin signaling pathway plays an important role in the pathogenesis of ULs, by mediating the transformation of MSCs into TICs that have uncontrolled division and synthesis of ECM components (Mas *et al.*, 2014).

Generally, secreted Wnt proteins are ligands that bind to specific membrane receptors which activate intracellular signaling pathways in target cells. Briefly, Wnt binding to its cognate transmembrane receptor activates disheveled (Dsh or Dvl), which, in turn, modulates the canonical (β-catenin dependent) and non-canonical (β-catenin-independent) Wnt signaling cascades (Komiya and Habas, 2008) (Figure 6).

In canonical Wnt signaling (Figure 6A), a stable β-catenin detaches from a multimeric destruction complex, that contains adenomatous polyposis coli (APC), scaffolding proteins (Axin), casein kinase 1 (CK1), and glycogen synthase kinase 3 (GSK-3). In the presence of the Wnt ligand, its receptor gets activated and phosphorylates GSK-3β, preventing the formation of this degradation complex. In this case, β-catenin is free to translocate into the nucleus, and associate with the T cell factor/lymphoid enhancer factor (TCF/LEF) complex on the promoter region of several target genes (e.g., *c-MYC, CCND1, WISP1,* or *MMP9*) to co-activate their transcription. The resulting proteins are involved in proliferation, cell cycle regulation, survival, migration, lineage commitment, and differentiation. Alternatively, in the absence of the Wnt ligand, β-catenin is phosphorylated and inactivated by the multimeric complex, leading to its ubiquitination and subsequent proteasomal degradation (MacDonald *et al.*, 2009; El Sabeh *et al.*, 2021).

There are several non-canonical Wnt pathways that do not involve β-catenin or its co-receptor LRP5 (Figure 6B). The non-canonical planar cell polarity pathway is implicated in tissue organization during embryogenesis and adult tissue homeostasis. This pathway begins with Wnt binding to Frizzled (FZD) receptors and co-receptors such as Ryk, receptor tyrosine-kinase like orphan receptor 2 (ROR2), or NRH. The recruitment and activation of Dvl then activates Rho family GTPases and c-Jun-N-terminal kinase (JNK) (van Amerongen, 2012). In the case of the non-canonical Wnt/Ca2+ pathway, binding of Wnt to the FZD receptor activates phospholipase C (PLC), which results in the formation of inositol 1,4,5-triphosphate (IP3). In turn, IP3 interacts with the calcium channels on the endoplasmic reticulum, mobilizing intracellular calcium. The rise in calcium activates the calcium-calmodulin-dependent protein kinase II (CaMKII) (De, 2011). Ultimately, the calcium and PLC pathways activate several regulatory proteins, including NF-KB, CREB, and NFAT.

Finally, upstream of Wnt/β-catenin signaling are extracellular Wnt antagonists, including the Wnt inhibitory factors (WIFs), Dkk family, and secreted Fzd-related proteins (sFRP) (Komiya and Habas, 2008).

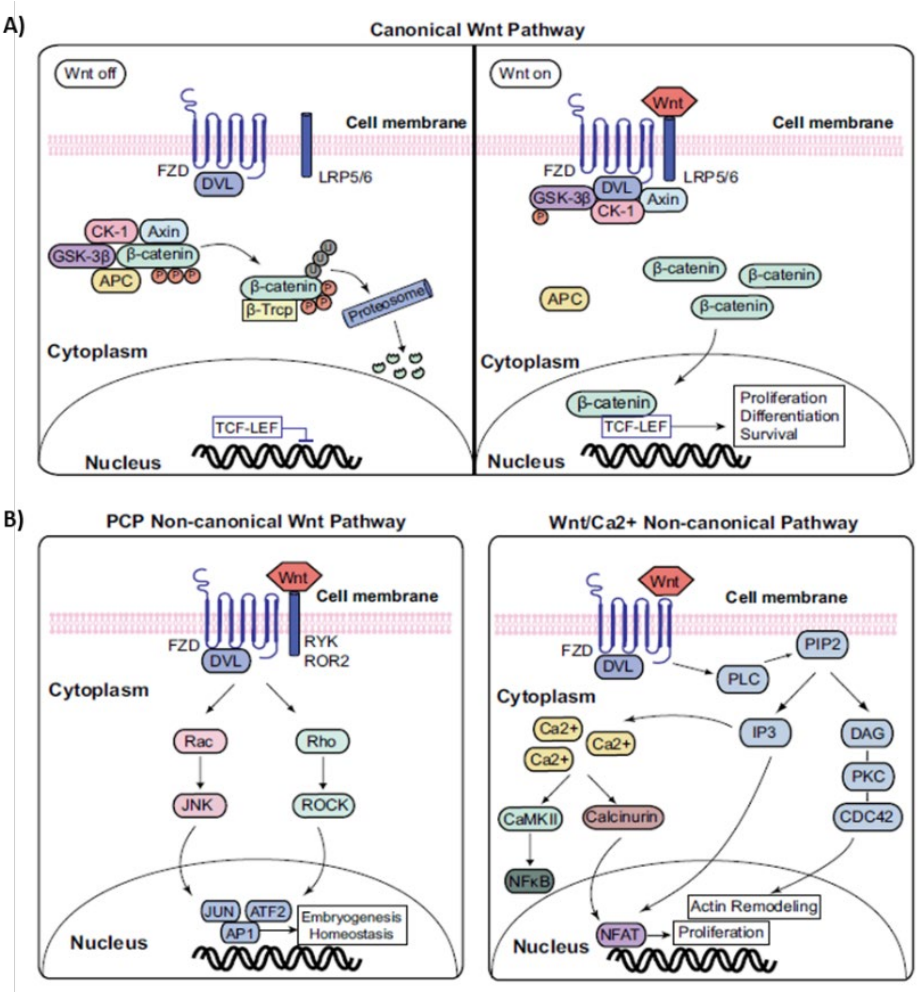


Figure 6. Canonical and non-canonical Wnt signaling pathways. *A) Canonical Wnt Pathway. B) Non-canonical pathways. Abbreviation: Wnt– Wingless–Type MMTV Integration Site Family; FZD– frizzled; LRP– low–density lipoprotein receptor–related protein; Dvl– disheveled; GSK3– glycogen synthase kinase 3; CK1– casein kinase 1; TCF/LEF– T–cell factor/lymphoid enhancer factor; RYK– Receptor Like Tyrosine Kinase; ROR– receptor tyrosine kinase–like orphan receptor; RAC– Rho family of GTPases; JNK– c–Jun N-terminal kinases; AP1– Activator protein 1; ATF2– Activating Transcription Factor 2; ROCK– Rho–associated protein kinase; RhoA– Ras homolog family member A; CaMKII– Calcium/calmodulin–dependent protein kinase type II alpha chain; PLC– Phospholipase C; PIP2– Phosphatidylinositol 4,5–bisphosphate; IP3– inositol 1,4,5–trisphosphate; NFAT– nuclear factor of activated T–cells; NF–κB– nuclear factor–κB, DAG– diacylglycerol; PKC– Protein kinase C; CDC42– Cell division control protein 42. Image obtained from El Sabeh et al. 2021.*

2.5.6 TGFβ pathway

The TGFβ superfamily modulates paracrine and autocrine factors of inflammation, cell cycle, and growth. TGFβ is one of the most distinguished cytokines associated with the myometrium, because it is a potent chemoattractant for macrophages and fibroblasts, mitotic inhibitor, apoptotic mediator, and also affects the development of the ECM (Ciebiera *et al.*, 2017).

Aberrant TGFβ has been related to abnormal or uncontrolled fibrosis (e.g., myocarditis), nephropathy, bowel inflammatory diseases, and found directly responsible for the development of the UL fibrotic phenotype, by increasing ECM deposition and cell proliferation (Lee and Nowak, 2001; Lewis *et al.*, 2019; Corachán *et al.*, 2021). Further, different TGFβ isoforms have been associated with UL pathophysiology. Specifically, the TGFβ3 isoform was expressed almost fivefold in UL tissue compared to healthy MM (Sozen and Arici, 2002; Borahay *et al.*, 2015; Ciebiera *et al.*, 2017), likely stimulating tumorigenesis and enhancing tumor metabolism.

2.5.7 Paracrine communication between leiomyoma cell populations

The paracrine stimulation of UL cells leads to enhanced tumor growth as a consequence of aberrant cell proliferation, ECM deposition, and decreased apoptosis (Bulun, 2013). Within the UL, mature myometrial cells (synthesizing estrogen, progesterone, and growth factors) communicate with leiomyoma cells in a paracrine manner to encourages the proliferation of leiomyoma stem cells and UL progression (Ono *et al.*, 2012). However, growth factors produced locally by smooth muscle cells and fibroblasts [e.g., epidermal growth factor (EGF), heparin-binding EGF, PDGF, IGF-1, TGFα/β, vascular endothelial growth factor (VEGF), acidic fibroblast growth factor (FGF), and basic FGF (bFGF)] also contribute to the proliferation, ECM synthesis, and angiogenesis, required for leiomyoma growth (Sozen and Arici, 2002). Similarly, several interleukins (ILs; i.e., IL-1, IL-6, and IL-11) have been identified in myometrial pathophysiology (Islam *et al.*, 2013).

Wnt/β-catenin signaling also plays an important role in this paracrine interaction between cells. In UL, estrogen/progesterone treatment induces the expression of Wnt ligands in mature myometrial cells. As previously described herein, Wnt ligands induce nuclear translocation of β-catenin in leiomyoma stem cells, leading to TCF-regulated transcription of target genes that favor stem cell proliferation (Ono *et al.*, 2013). The Wnt pathway can also stimulate the expression of TGFβ, resulting in excessive ECM production and increased cell proliferation (Ciebiera *et al.*, 2017).

2.5.8 Environment

Other environmental risk factors contributing to UL pathogenesis include African descent, high body mass index (BMI), meat consumption, alcohol consumption, Vitamin D deficiency, early menarche, hypertension, and a history of pelvic inflammatory disease or vaginal infections. On the other hand, factors that reduce the risk include using hormonal contraception, giving birth, and consuming green vegetables (Faerstein *et al.*, 2001; Giuliani *et al.*, 2020). These findings suggest that epigenetic factors are also involved in UL progression, making it an epimutation-related disease. While genetic mutations are difficult to reverse, some epigenetic changes can easily be reversed with chemical agents, providing a plethora of possibilities for new therapeutic options for patients with UL.

3 EPIGENETICS

3.1 Definition

Epigenetics is defined as the study of heritable changes in gene expression not associated with modifications in the primary DNA sequence. Epigenetic alterations affect secondary interactions, namely packaging of chromatin, playing a crucial role in the regulation of gene expression (Navarro *et al.*, 2012b). Normally, gene expression patterns are controlled by the three-dimensional architecture of chromatin, and the action of multiprotein complexes, especially RNA polymerase, which transcribes DNA into RNA. In the nucleus, genomic DNA is wrapped around histones, into nucleosome subunits, that are in turn, condensed into chromatin. Highly condensed chromatin that is inaccessible by transcriptional machinery is known as heterochromatin and contains mostly inactive genes. Alternatively, euchromatin has an open structure and contains active genes (Bennett and Licht, 2018). Epigenetic changes are heritable through cell division (Turner, 2007). They play an important role during embryogenesis, X-chromosome silencing, cellular proliferation and differentiation, in disease states, as well as stem cell developmental potential and cell fate. Notably, altered epigenetic mechanisms in progenitor, differentiating, or terminal cells promote carcinogenesis (Jones and Baylin, 2007; Turner, 2007; Meissner, 2010).

The main mechanisms involved in epigenetic regulation are DNA methylation, histone modification, and non-coding RNAs, which contribute to a complex “epigenetic code” that superposes the nucleotide sequence to direct gene expression (Figure 7) (Turner, 2007). In this regard, epigenetic regulation is a dynamic and reversible process. Proteins that carry out epigenetic modifications are considered “writers”, “readers”, and “erasers”. Epigenetic “writers” catalyze the addition of epigenetic marks onto DNA or histones, “readers” recognize

or are recruited to a specific epigenetic mark, and “erasers” remove epigenetic marks. Epigenetic modifications may be affected by the environment, making them important mechanisms in complex multifactorial diseases. The dynamic and reversible nature of epigenetic mechanisms makes these processes of therapeutic relevance in many diseases, including cancer (Bennett and Licht, 2018).

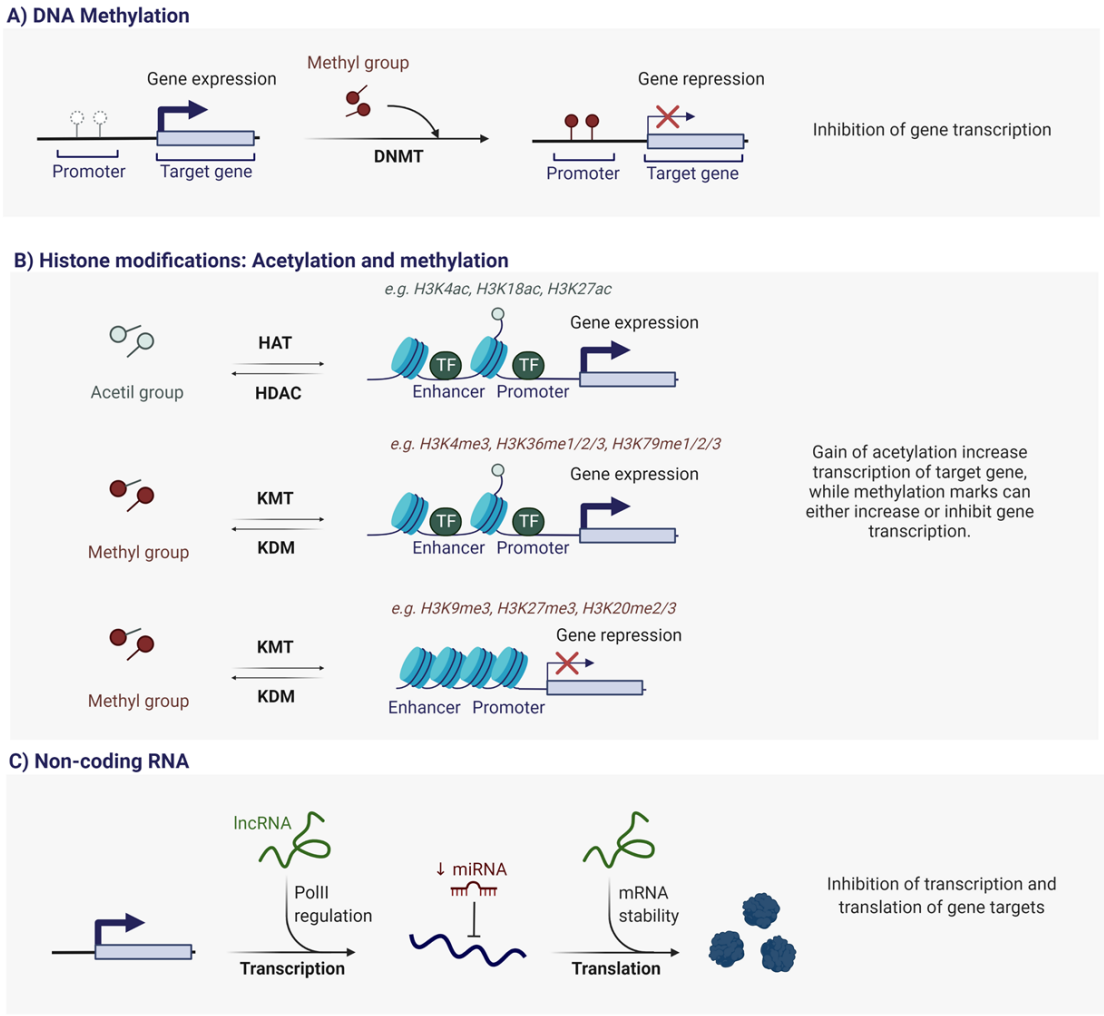


Figure 7. Mechanisms of epigenetic regulation. A) Addition of methyl groups to the CpG islands in promoter regions leads to inhibition of gene transcription. B) Histone modifications can exert different effects on chromatin structure. Acetylation opens the chromatin allowing the union of transcription factors and, therefore, gene expression. Specific methylation of lysine 4 on histone 3 also opens the chromatin promoting transcription of target genes. C) Non-coding RNAs alter transcription and translation of genes through interaction with the transcription complex or direct union with the transcript. In general, non-coding RNAs lead to the repression of these processes. DNMT, DNA methyltransferases; HAT, histone acetylase; HDAC, histone deacetylase; KMT, lysine methyltransferase; KDM, lysine demethylase. Created with BioRender.com

3.2 DNA methylation

In the eukaryotic genome, DNA methylation is the most common and well-characterized epigenetic mark. It ensures proper regulation of gene expression under physiological conditions, but it also has an important role in genetic imprinting and interplay with histone modifications (Cedar and Bergman, 2009). DNA methylation consists of the covalent addition of a methyl group to the 5-carbon of the cytosine ring following replication (Lopez *et al.*, 2016). The majority of CpG dinucleotides are concentrated within CpG-rich DNA regions, termed CpG islands, that are located near transcription start sites, at approximately 70% of gene promoters. Specifically, methylation of a promoter CpG island triggers the binding of proteins that condense the chromatin and make the promoter region inaccessible to transcription factors. This process blocks transcription initiation and, thereby, represses gene expression (Saxonov *et al.*, 2006; Jin *et al.*, 2019). The creation of DNA methylation patterns during embryogenesis establishes the compartmentalization of the genome into transcriptionally active and inactive domains. DNA methylation is fundamental for the correct expression of imprinted genes, chromosomal dosage compensation (X-chromosome inactivation), and tissue-specific gene expression (Jones and Baylin, 2007; Meissner, 2010; Lopez *et al.*, 2016).

The enzymes which catalyze DNA methylation reactions are known as DNA methyltransferases (DNMTs). They transfer a methyl group, from the donor S-adenosyl methionine (SAMe) to the 5-position of the pyrimidinic ring. There are three main types of these enzymes: DNMT1, DNMT3A, and DNMT3B. DNMT1 maintains DNA methylation patterns during DNA replication, allowing the propagation and conservation of the DNA methylation patterns through future generations. DNMT3A and DNMT3B establish new methylation patterns and are therefore called *de novo* methyltransferases (Figure 8) (Lopez *et al.*, 2016; Yang *et al.*, 2016a; Laganà *et al.*, 2017).

On the other hand, DNA demethylation can occur passively, during successive rounds of replication, or actively, via the action of DNA demethylases. Enzymatic DNA demethylation consists of the removal or modification of 5-methylcytosine (5-mC) with the regeneration of unmodified cytosine (Bhutani *et al.*, 2011; Shen *et al.*, 2014). DNA demethylation is catalyzed by the ten-eleven-translocation (TET) enzymes, which convert 5-mC to 5-hydroxymethylcytosine (5-hmC), 5-formylcytosine (5-fC), and 5-carboxylcytosine (5-caC) in a gradual manner (Figure 8). Finally, 5-fC and 5-caC may subsequently be converted to unmethylated cytosine, by thymine DNA glycosylase (TDG), via the base excision repair pathway, ultimately leading to DNA demethylation and gene activation (Ito *et al.*, 2011) (Figure 8).

Overall, these enzymes all contribute to the establishment of the methylation pattern that control the cell's gene expression. The hypermethylation of CpG islands in the promoter region of each gene generally results in repression of expression, while hypomethylation leads to active transcription. Aberrant DNA methylation has been reported in many diseases, including cancers (Jones and Baylin, 2007).

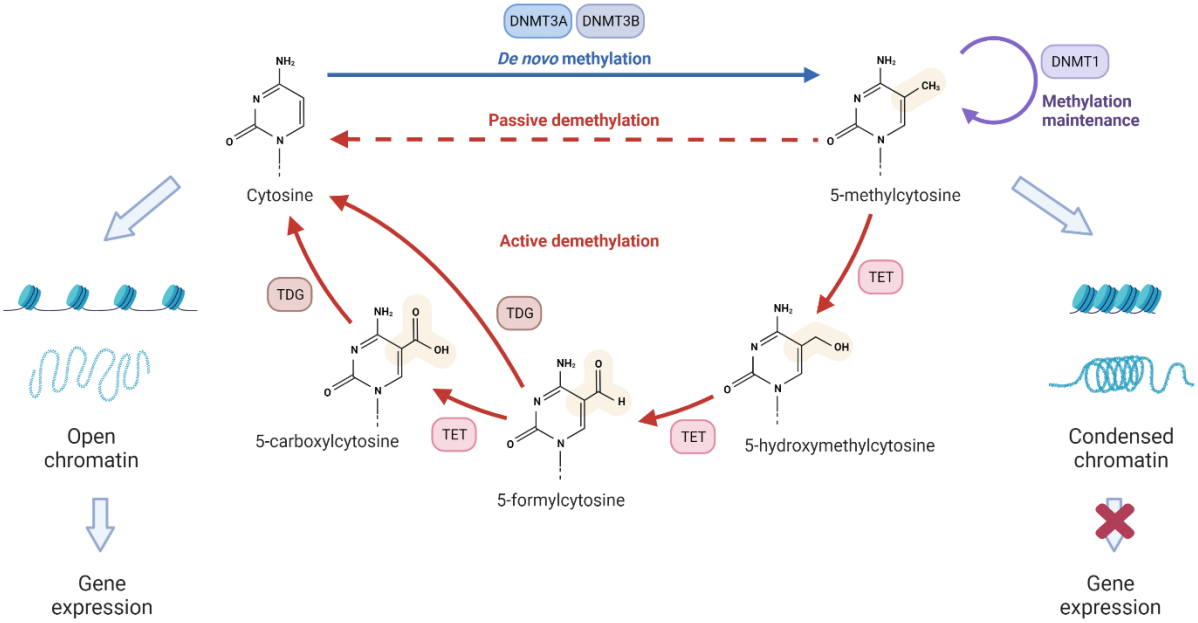


Figure 8. DNA methylation and demethylation pathways. *DNA methyltransferases (DNMTs) catalyze the methylation of the pyrimidinic ring of cytosines, closing the chromatin conformation, and thereby, repressing transcription. Demethylation can occur passively, through rounds of DNA replication, or actively, via enzymatic action. Ten-eleven-translocation (TET) enzymes oxidize 5-methylcytosine (5-mC) to generate 5-hydroxymethylcytosine (5-hmC), which in turn is oxidized to produce 5-formylcytosine (5-fC) and 5-carboxylcytosine (5-caC). These two products can be excised from DNA during the base excision repair process, by thymine DNA glycosylase (TDG). The resulting abasic site can be used to regenerate unmodified cytosine, and ultimately, create an open chromatin structure, where the transcription machinery can access and function. Created with BioRender.com*

3.2.1 DNA methylation and tumorigenesis

Mutations, amplifications, deletions, and rearrangement of genes that can dysregulate epigenetic regulation pathways occur frequently in cancer (Lawrence *et al.*, 2014). Driver mutations in epigenetic regulators can extensively alter DNA methylation, dysregulating chromatin structure and disrupting normal patterns of gene expression. Specifically, changes in both global and individual gene methylation patterns are often involved in the pathogenesis of human tumors, and methylation patterns have been used to distinguish tumor subtypes.

Both DNA hypermethylation and hypomethylation are associated with tumorigenesis. The tumorigenic effect of DNA methylation is mostly associated with the formation of a repressive chromatin structure on promoter regions. In this context, hypermethylation of specific CpG-rich regions that silence tumor suppressor genes has been reported frequently in malignant transformation. On the contrary, loss of DNA methylation can restore transcriptional activity and amplify the expression of oncogenes that promote tumor formation. This abnormal methylation pattern impairs the constitutive expression of genes involved in cell cycle regulation, DNA repair, apoptosis, differentiation, drug resistance, angiogenesis, and metastasis (Jones and Baylin, 2007; Turner, 2007; Timp and Feinberg, 2013).

Aberrant DNA methylation, through either oncogene hypomethylation or tumor suppressor gene hypermethylation, has been observed in several cancer types, including prostate, colorectal, breast, and neuroblastoma (Long *et al.*, 2021; Ruscito *et al.*, 2021; Wang *et al.*, 2021b). Accordingly, mutations in DNA methyltransferases have also been described in tumors. For example, recurrent DNMT3A loss-of-function mutations were reported in acute myelogenous leukemia and were associated with subtle losses of DNA methylation (Russler-Germain *et al.*, 2014).

Unlike genetic mutations, epigenetic changes are reversible. Because of this inherent plasticity, characterizing how instabilities in epigenetic mechanisms lead to malignant transformation may provide a basis for developing new cancer treatments that target these mechanisms.

3.2.2 Treatments targeting DNA methylation

Given the aforementioned evidence of aberrant DNA methylation patterns in tumor pathobiology, emerging medical treatments are aiming to stabilize the methylation status with demethylation agents. Initially, cytosine analogs [e.g., 5-azacytidine (Azacitadine; Vidaza®) and 5-aza-2-deoxycytidine (Decitabine; Dacogen®)] were used as DNMT inhibitors to slightly reduce hypomethylation. The azanucleosides acted by competitively inhibiting DNMTs - substituting nitrogen for carbon at the C-5 position of the pyrimidine ring, and incorporating into DNA to irreversibly mark DNMT1 for degradation (Bennett and Licht, 2018). While Decitabine is generally incorporated into DNA, 80–90% of Azacitadine is incorporated into RNA (suggesting its antineoplastic function is mediated by incorporation into both DNA and RNA) (Bennett and Licht, 2018). The ability of DNMT inhibitors to reactivate tumor suppressor genes was promising for their use in hematologic and other malignancies (Tsai *et al.*, 2012). Furthermore, these drugs reduce tumorigenicity and target cancer stem cells within solid tumors (Li *et al.*, 2018).

In 2004 and 2006, respectively, Azacitadine and Decitabine were approved for the treatment of myelodysplastic syndromes. Treatment with Decitabine led to the formation of DNA-DNMT adducts and consequent double-stranded DNA breaks, resulting in G2 cell cycle arrest. Moreover, DNMTs have been found in complexes with histone-modifying enzymes, and a global increase in histone H3 and H4 acetylation was observed after treatment with Azacitadine (Bennett and Licht, 2018).

3.2.3 DNA methylation in uterine leiomyomas

DNA methylation may participate in UL pathogenesis by modifying the normal myometrial gene expression profile. The first evidence of the dysregulation of DNA methylation in UL was provided by Dr. Bulun's group (Navarro *et al.*, 2014). They demonstrated an increase in the 5-hmC content of UL tissue compared to the MM, produced by upregulation of the TET protein 1 (TET1) and TET protein 3 (TET3) enzymes, which ultimately enhanced the proliferation of human ULs. Additionally, a differential expression of DNMTs and consequent aberrant DNA methylation was observed in UL compared with normal MM tissue (Li *et al.*, 2003; Yamagata *et al.*, 2009).

An aberrant DNA methylation status of X-chromosome-related genes has also been observed in UL. First, Sato *et al.* 2014 demonstrated a higher incidence of aberrant DNA hypomethylation on the X chromosome when compared to the whole genome in UL (Sato *et al.*, 2014). While more recently, Asada *et al.* 2018 reported that *ERα* gene expression is hypomethylated at seven CpG sites in the distal promoter region in UL compared to normal MM. This hypomethylation of the ERα distal promoter region was then validated with high ERα mRNA expression (Asada *et al.*, 2008).

Further analyses that characterize the UL methylome through genome-wide DNA methylation have highlighted the impact of methylation in UL initiation and progression. Aberrant global DNA methylation statuses has been described in UL compared to the MM, and have been postulated to underlie the altered mRNA expression profile (Yamagata *et al.*, 2009; Navarro *et al.*, 2012b; Maekawa *et al.*, 2013). In this context, different studies have evaluated DNA methylation in UL, focusing on stem cell population or mutation status, to further characterize its implication in UL development. Remarkably, hypermethylation has been found to initiate tumors by repressing differentiation genes in UL stem cells, in comparison to differentiated UL cells (Liu *et al.*, 2020). Further, differential DNA methylation and gene expression profiles were used to distinguish genetic subtypes of UL, such as *MED12* and *HMGA2* (George *et al.*, 2019).

Based on these findings, the holistic analysis of the interaction of DNA methylation and gene expression profiles in UL, compared to MM, could facilitate identification of the commonly dysregulated pathways involved in UL development.

3.3 Histone modifications

Histones are small basic proteic components of chromatin that are involved in the regulation of gene expression (Cedar and Bergman, 2009). They consist of a globular domain with a flexible and charged NH2-terminus (known as the histone tail protruding from the nucleosome). DNA winds around histones to form nucleosomes, which represent the basic repeating units of chromatin. In each nucleosome, roughly two superhelical turns of 146 base pairs of DNA wrap around an octamer of core histone proteins (formed by four histone partners: a H3–H4 tetramer and two H2A–H2B dimers) (Mai *et al.*, 2005).

Histone modification is another epigenetic mechanism implicated in the post-transcriptional regulation of gene expression. These modifications, occurring on distinct amino acid residues at the N-terminal tail of the globular domains of core histones, include acetylation, methylation, phosphorylation, ubiquitination, and SUMOylation (Gibney and Nolan, 2010; Yang *et al.*, 2016a). Varying combinations of these histone modifications comprise a histone code, that directs biological processes via the recruitment of specific chromatin-associated proteins, leading to distinct gene expression patterns (Bártová *et al.*, 2008).

One of the best-characterized epigenetic modifications are histone acetylation patterns. Histone acetylation consists of the transfer of an acetyl group, from acetyl coenzyme A to the lysine e-amino group on the N-terminal tails of histones. In general, histone acetylation is correlated with gene expression, and deacetylation leads to transcriptional repression (Verdone *et al.*, 2005). It is considered a central switch that allows interconversion between permissive and repressive chromatin structures and domains. Within the nucleosome, positively charged hypoacetylated histones are tightly bound to the phosphate backbone of DNA, maintaining chromatin in a transcriptionally silent state. Acetylation neutralizes the positive charge on histones, disrupting higher-order structures in chromatin, thereby enhancing access of transcription factors, transcriptional regulatory complexes, and RNA polymerases to promoter regions of DNA. Histone deacetylation restores a positive charge on lysine residues of core histones, allowing chromatin to condense into a tightly supercoiled, transcriptionally silent conformation (Mai *et al.*, 2005).

The enzymes participating in the addition of acetyl group to histones are histone acetyltransferases (HATs), while histone deacetylases (HDACs) remove these marks (Verdone *et al.*, 2005; Seto and Yoshida, 2014). HDAC is associated with gene silencing, whereas HAT is associated with transcriptional activity (Figure 9). On the one hand, there are four different classes of HDACs based on their sequence similarities: class I proteins (HDAC1, HDAC2, HDAC3, and HDAC8), class II proteins (HDAC4, HDAC5, HDAC6, HDAC7, HDAC9, and HDAC10), class III proteins or sirtuins (SIRT1, SIRT2, SIRT3, SIRT4, SIRT5, SIRT6, and SIRT7), and class IV protein (HDAC11). On the other hand, HATs are classified as lysine (K) acetyltransferases (KATs). Type B HATs (KAT1 and KAT4) are cytoplasmic enzymes that modify free newly synthesized histones, so they may be shuttled across the nuclear membrane and integrated into newly synthesized DNA (Wapenaar and Dekker, 2016). Alternatively, Type A HATs are mainly nuclear enzymes responsible for the acetylation of histones and non-histone proteins in the nucleus. Based on their sequence homology, most nuclear HATs can be assigned to families: the GNAT (Gcn5-related N-acetyltransferases) family consisting of KAT2A and KAT2B; the largest MYST family (named after its members MOZ, YBF2/SAS3, SAS2, and TIP60) consisting of KAT5, 6A and 6B, 7, and 8; the p300/CBP family consisting of KAT3A and 3B. Other HATs that additionally have acetyltransferase functions are classified as transcriptional (e.g., KAT4 and KAT12) and steroid receptor co-activators (e.g., KAT13A-D) (Verdone *et al.*, 2005; Seto and Yoshida, 2014).

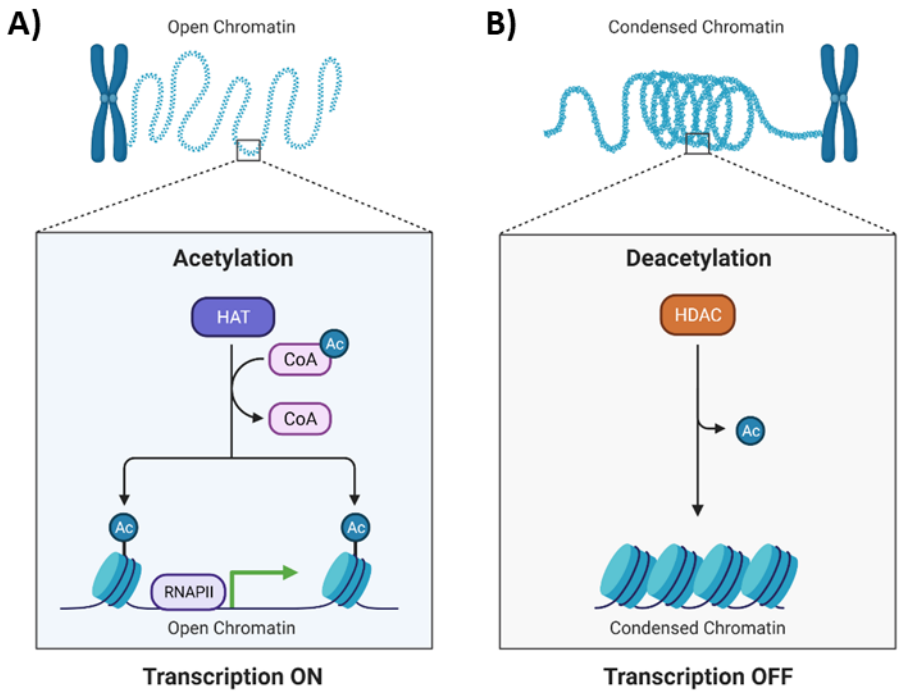


Figure 9. Histone acetylation. *A) Histone acetyltransferases transfer the acetyl group from Coenzyme A to the histones, maintaining open chromatin and permitting gene transcription by RNA polymerase II*

(RNAPII). B) Histone deacetylases (HDAC) remove the acetyl group from chromatin, maintaining chromatin in a condensed status and impeding gene transcription. Created with BioRender.com.

3.3.1 Histone modifications and tumorigenesis

Aberrant histone modifications that alter gene transcription states are common hallmarks of human tumor cells. Studies on leukemias have provided paradigmatic examples for the functional implications of genetic and epigenetic alterations in cancer development (Mehdipour *et al.*, 2015). These studies underline the possibility of reversing disease-associated aberrant epigenetic states by targeting the catalytic activities of chromatin remodelling enzymes. Thus, these enzymes are attractive targets for therapeutic intervention in cancer. Precisely, the abnormal status of a specific lysine within a determined histone has been associated with tumor development. For example, in prostate cancer, lower levels of the dimethylation of histone H3 on lysine K4 (H3K4me2) and the acetylation of histone H3 on lysine K18 (H3K18ac) were correlated with a poor prognosis. Similarly, aberrant acetylation of histone H3 on lysine K27 (H3K27ac) profiles were identified in gastric, lung, and ovarian cancers (Ma and Zheng, 2021; Wang *et al.*, 2021a; Zhang *et al.*, 2021), and associated with enhanced proliferation, invasion, and tumor metastasis. Currently, there is limited evidence about the role of H3K27ac in UL pathogenesis.

Since HDACs are involved in multiple biologic processes, such as cell cycle progression, cell differentiation and survival, their dyregulation unbalances histone acetylation and also leads to the development of different tumors (Gallinari *et al.*, 2007). Many reports demonstrate that HDACs are overexpressed in cancers, resulting in a global loss of histone acetylation and silencing of tumor suppressor genes. HDACs have been linked to as breast and ovarian cancer development (Hervouet *et al.*, 2015; Bitler *et al.*, 2017), and regulation of the tumor suppressor gene *KLF11*, which is relevant to uterine diseases (Zheng *et al.*, 2014).

3.3.2 Anticancer treatments based on histone deacetylase inhibitors

Treatments targeting the enzymes responsible for maintaining genomic stability through histone modifications are emerging as therapeutic alternatives for many diseases. The overexpression of HDAC and subsequent silencing of tumor suppressor genes may be a common transcriptional regulation mechanism favoring tumor onset and development (Marks, 2007; Wang *et al.*, 2013; Freese *et al.*, 2019). HDAC inhibitors work through many mechanisms to promote cell cycle arrest, induce differentiation, and activate apoptosis pathways in tumor cells, and are therefore being explored as anticancer agents. *In vivo*, HDAC inhibitors may also

promote tumor regression via activation of the host immune response and inhibition of angiogenesis.

In vitro, treatment with HDAC inhibitors resulted in an accumulation of acetylated histones (i.e., H4, H3, H2A, and H2B) in both normal and tumor cells, however the tumor cells appeared to be much more sensitive to the growth inhibition and pro-apoptotic stimuli than normal cells (Yang *et al.*, 2016a), suggesting they may selectively target cancer cells without damaging the surrounding healthy tissue. Moreover, Numerous studies have demonstrated the therapeutic potential of HDAC inhibitors in different cancer cell lines and animal models of tumors. Specifically, *in vitro* and i*n vivo*, HDAC inhibitors have blocked the activity of partially purified HDACs *in vitro* and *in vivo*, reversing cell transformation and inhibiting cell proliferation. In fact, they have effectively induced cell cycle arrest, terminal differentiation, and/or apoptosis in a wide range of malignant cell types, including solid tumor and hematological neoplastic cell lines (Kawano *et al.*, 2011; Estella *et al.*, 2012; Falkenberg and Johnstone, 2014; Wei *et al.*, 2018). In corroboration, they inhibit cancer growth in various tumor-bearing models, some of them currently in clinical trials. Notably, in clinical studies evaluating the efficacy of epigenetic treatment, the HDAC inhibitors were administered alone, or in combination with conventional anticancer therapies (usually chemotherapy, but occasionally with radiotherapy) to boost their effects (Ree *et al.*, 2010; Nervi *et al.*, 2015).

In 2006, suberoylanilide hydroxamic acid (commonly known as SAHA; sold as the oral drug Vorinostat) was the first HDAC inhibitor approved by the U.S. Food and Drug Administration (FDA) for the treatment of cutaneous T cell lymphoma, in patients who have progressive, persistent, or recurrent disease following the failure of two systemic therapies (Mann *et al.*, 2007). In 2009, Romidepsin (also known as Depsipeptide) was also approved for the same indications. Similarly, in 2015, the FDA also approved Panobinostat in combination with Bortezomib and dexamethasone for the treatment of patients with multiple myeloma (San-Miguel *et al.*, 2014), and Belinostat for the treatment of patients with peripheral T-cell lymphoma (O'Connor *et al.*, 2015).

SAHA is a competitive inhibitor of class I and class II HDACs (Marks and Breslow, 2007) that is used as an anticancer treatment for different types of tumors (Kawano *et al.*, 2011; Wei *et al.*, 2018). One of the well-characterized molecular effects of SAHA is the overaccumulation of acetylated core histones (resulting from HDAC inhibition) that blocks cell proliferation and tumor growth in hepatoid adenocarcinoma (Kyaw *et al.*, 2019), myeloid leukemia (Abou Najem *et al.*, 2019), and prostate cancer (Butler *et al.*, 2000), with little toxicity to surrounding tissue.

3.3.3 Histone modifications as targets for uterine leiomyoma treatment

Thus far, epigenomic studies aiming to characterize UL pathogenesis have mainly focused on DNA methylation, however, the role of histone modifications in UL pathogenesis is starting to be explored. In particular, the hyperactive HDACs in ULs suggest that normal gene transcription in the MM may be repressed by the shortage of histone acetylation (that leads to an increase in the growth and maintenance of UL) (Yang *et al.*, 2016a; Sant'Anna *et al.*, 2017). This was corroborated by Wei *et al.,* who reported an overexpression of HDAC6 in the cytoplasm of smooth muscle cells, and poor expression of HDAC6 in the adjacent MM, and concluded that HDAC6 regulated ERα expression in ULs (Wei *et al.*, 2011). Other histone alterations such as H3K27ac, H3K4me3, and H2A.Z in enhancers and promotors, have significantly been associated to ULs and aided in distinguishing different subtypes of ULs (Moyo *et al.*, 2020; Berta *et al.*, 2021; Leistico *et al.*, 2021). Nevertheless, the complexities and long-term implications of these histone marks on UL gene expression, and thereby UL pathogenesis, remain poorly understood.

II. HYPOTESIS

“The scientist is not a person who gives the right answers, but the one who asks the right questions”- Claude Levi-Strauss.

II. HYPOTHESIS

The study of epigenetic modifications in UL, driven by changes in DNA methylation or histone modifications (in particular, acetylation), could provide a better understanding of UL etiology, reveal novel therapeutic targets, and facilitate the development alternative strategies to shrink ULs and alleviate symptoms.

III. OBJECTIVES

"When it is obvious that the goals cannot be reached, don't adjust the goals, adjust the action steps"- Confucio

III. OBJECTIVES

The main objective of this PhD dissertation is to characterize the epigenetic mechanisms (i.e., DNA methylation and histone modification) involved in UL development, to identify possible new therapeutic targets for patients with ULs.

The specific objectives are to:

1. Characterize the gene methylation pattern and altered transcriptomic profile in UL compared to MM through the genomic methylation and RNA sequencing analysis, and evaluate the effect of the DNMT inhibitor 5-AZA-2'-deoxycytidine on selected driver genes in leiomyoma cells. **MANUSCRIPT 1.**
2. Elucidate if DNA methylation regulates the biological processes involved in UL progression (i.e., proliferation, apoptosis, excess extracellular matrix deposition, and Wnt/β-Catenin signaling), by treating primary uterine leiomyoma cells with the DNMT inhibitor 5-AZA-2'-deoxycytidine *in vitro*. **MANUSCRIPT 2.**
3. Evaluate expression of the acetylated Lysine 27 of Histone 3 (H3K27ac) and association with the UL transcriptome, in UL vs MM through Chip-Seq and RNA sequencing analysis, in addition to evaluating the effect of the HDAC inhibitor SAHA on the expression of selected driver genes. **MANUSCRIPT 3.**
4. Determine whether histone acetylation regulates the biological processes involved in UL development (i.e., proliferation, apoptosis, excess extracellular matrix deposition, and TGFβ signaling), by treating using primary uterine leiomyoma cells with the HDAC inhibitor SAHA *in vitro*. **MANUSCRIPT 4.**

IV. MANUSCRIPTS

“Life is and will ever remain an equation incapable of solution, but it contains certain known factors.”- Nikola Tesla

IV. MANUSCRIPTS

MANUSCRIPT 1. Carbajo-García MC, Corachán A, Juárez-Barber E, Monleón J, Payá V, Trelis A, Quiñonero A, Pellicer A, Ferrero H. Integrative analysis of the DNA methylome and transcriptome in uterine leiomyoma shows altered regulation of genes involved in metabolism, proliferation, extracellular matrix, and vesicles. J Pathol. 2022 Aug;257(5):663-673. doi: 10.1002/path.5920. Epub 2022 Jun 13. PMID: 35472162.

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ORIGINAL ARTICLE

Integrative analysis of the DNA methylome and transcriptome in uterine leiomyoma shows altered regulation of genes involved in metabolism, proliferation, extracellular matrix, and vesicles

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Abstract

Uterine leiomyomas (ULs) are the most common benign tumors in women of reproductive age. Despite the high prevalence, tumor pathology remains unclear, which hampers the development of safe and effective treatments. Epigenetic mechanisms appear to be involved in UL development, particularly via DNA methylation that regulates gene expression. We aimed to determine the relationship between DNA methylation and gene expression in UL compared with adjacent myometrium (MM) to identify molecular mechanisms involved in UL formation that are under epigenetic control. Our results showed a different DNA methylation profile between UL and MM, leading to hypermethylation of UL, and a different global transcriptome profile. Integration of DNA methylation and whole-transcriptome RNA-sequencing data identified 93 genes regulated by methylation, with 22 hypomethylated/upregulated and 71 hypermethylated/downregulated. Functional enrichment analysis showed dysregulated biological processes and molecular functions involved in metabolism and cell physiology, response to extracellular signals, invasion, and proliferation, as well as pathways related to uterine biology and cancer. Cellular components such as cell membranes, vesicles, extracellular matrix, and cell junctions were dysregulated in UL. In addition, we found hypomethylation/upregulation of oncogenes (*PRL*, *ATP8B4*, *CEMIP*, *ZPMS2-AS1*, *RIMS2*, *TFAP2C*) and hypermethylation/downregulation of tumor suppressor genes (*EFEMP1*, *FBLN2*, *ARHGAP10*, *HTATIP2*), which are related to proliferation, invasion, altered metabolism, deposition of extracellular matrix, and Wnt/β-catenin pathway dysregulation. This confirms that key processes of UL development are under DNA methylation control. Finally, inhibition of DNA methyltransferases by 5-aza-2'-deoxycitidine increased the expression of hypermethylated/downregulated genes in UL cells *in vitro*. In conclusion, gene regulation by DNA methylation is implicated in UL pathogenesis, and reversion of this methylation could offer a therapeutic option for UL.

Keywords: DNA methylation; gene expression; uterine leiomyoma; 5-aza-2'-deoxycytidine

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No conflicts of interest were declared.

Introduction

Uterine leiomyomas (ULs), also known as fibroids, are benign estrogen-dependent monoclonal tumors of smooth muscle cells arising in the myometrium (MM). They are the most common gynecological tumor, affecting 80% of women of African American descent and 70% of European descent by 50 years of age [1]. UL cause severe symptoms in 25% of patients, such as heavy menstrual bleeding, pelvic pain, and reproductive dysfunction [2,3]. Surgery, myomectomy, and hysterectomy are the main treatment options. Alternative treatments based on induction of gonadal suppression have been developed, such as gonadotrophin-releasing hormone (GnRH) analogs [4]. These treatments reduce UL size and associated symptoms, but they should be administered intermittently due to their hypoestrogenic side effects [5–8]. UL size recovers after treatment, leaving a gap in long-term treatments for UL with minimal side effects. Hysterectomy is the only treatment that eliminates the risk of future recurrence, although it is not a choice for patients who wish to preserve fertility [8]. Further understanding of the mechanisms that lead to UL could help to palliate the lack of

efficient therapy [9]. Therefore, identification of the molecular mechanisms involved in UL pathogenesis may allow the development of new treatments.

UL pathogenesis remains incompletely understood, but there are four established molecular subtypes of UL based on mutations of different genetic drivers: *HMGA2* rearrangements, *MED12* mutations, biallelic inactivation of *FH*, and deletions affecting *COL4A5* and *COL4A6* [10]. Race, diet, age, body mass index, and parity are also risk factors for UL [11], suggesting involvement of epigenetic mechanisms. Epigenetic effects encompass changes in gene expression not caused by changes in the DNA sequence but from processes such as DNA methylation, histone modification, and non-coding RNAs [12]. These modifications are inherited somatically and, unlike genetic mutations, are reversible, which make them potential therapeutic targets. DNA methylation consists of the addition of a methyl group to cytosine residues in CpG sites by specific DNA methyltransferases (DNMTs) [13]. Generally, hypermethylation of a promoter CpG island represses gene expression, while hypomethylation activates gene expression [13]. Altered DNA methylation patterns are implicated in prostate, colorectal, and breast cancers [14–17]. Aberrant DNA methylation patterns also occur in UL compared with MM [18–20]. In addition, studies have evaluated DNA methylation in UL focusing on stem cell populations [21] or mutation status [22].

Further analysis of the interaction between DNA methylation and gene expression profiles of UL compared with adjacent MM from a holistic approach could help to identify common deregulated pathways in UL development. Thus, we aimed to define this relationship using advanced genome-wide DNA methylation technology and RNA-sequencing (RNA-seq) in Caucasian women using matched UL and adjacent MM samples. The same patient samples were used for both analyses to identify gene expression changes under epigenetic control. Our findings provide insight into the molecular pathways involved in UL pathogenesis, which may guide new therapeutic approaches focused on reversing epigenetic modifications.

Materials and methods

Ethics statement

This study was approved by the Clinical Ethics Committee at Hospital Universitario y Politécnico La Fe (Spain) (2018/0097), and all participants provided informed consent.

Sample collection

Human UL ($n = 31$) and adjacent MM ($n = 31$) matched samples taken from the same patient were collected from Caucasian premenopausal women aged 31–48 years who had not received any hormonal treatment for the previous 3 months and who were undergoing myomectomy or hysterectomy due to symptomatic UL at Hospital Universitario y Politécnico La Fe (Spain). Additional samples from different women ($n = 11$) were collected following the same criteria for validation experiments (supplementary material, Table S1).

Epigenomic studies

Genomic DNA was extracted from UL and adjacent MM frozen tissues ($n = 31$) using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany). Purified DNA (500 ng) was processed using the EZ-96 DNA Methylation Kit (Zymo Research, Irvine, CA, USA) following the manufacturer's recommendations. Bisulfite-converted DNA was processed as previously described [21]. Epigenomic studies were performed with the Infinium MethylationEPIC BeadChips 850K array (Illumina, San Diego, CA, USA) for analysis of over 850 000 CpG sites that are the main methylation targets [22]. The methylation score for each CpG was represented as a β value according to the fluorescence intensity ratio. β values range between 0 (non-methylated) and 1 (completely methylated). Raw data (IDAT files) were normalized using functional normalization as implemented in the R-package minfi (version 1.22.1) [23].

Prefiltering and statistical analysis of DNA methylation data

Every β value in the EPIC array was accompanied by a detection *P* value that represents confidence. Probes and sample filtering involved a two-step process that removed unreliable β values with high detection $p > 0.01$ and CpGs associated with SNPs. After filtering, the remaining 861 483 CpGs were considered valid. A Wilcoxon test for paired samples was performed for each CpG β value. *P* values were adjusted using false discovery rate (FDR) and Holm correction.

Library construction and RNA-seq

Total RNA from UL and adjacent MM tissues ($n = 30$) was extracted with TRIzol (Thermo Fisher Scientific, Waltham, MA, USA) and quantified using TapeStation 4200 (Agilent, Santa Clara, CA, USA). cDNA synthesis and library generation were performed using the Illumina TruSeq RNA Library Prep Kit v2 according to the manufacturer's instructions. DNA pools generated were quantified using the Qubit DNA HS Assay Kit (Thermo Fisher Scientific). Libraries were sequenced on an Illumina NovaSeq 600 with a paired-end design.

RNA-seq: bioinformatic analysis

Sequencing reads were aligned against human genome reference hg38 using STAR_2.5.2 mapping software with default settings [24]. To quantify gene expression, uniquely mapping reads were submitted to featureCounts [25] to obtain the count matrix. Genome reference annotation was extracted from Gencode 24

(Ensembl version 84). Sequencing yielded an average of 30 million reads per sample. All raw sequencing data are available through NCBI's Gene Expression Omnibus (GEO) under GEO Series accession number GSE192354. A total of 60 RNA-seq data libraries were processed and subjected to statistical analysis within the R/Bioconductor (version 3.5.0) [26] computing environment. Quality control of DNA libraries was assessed based on FastQC software. Sequences that did not match quality control standards were erased by Trim Galore! software [27]. rRNA sequences were filtered out with SortMeRNA software [28]. Trimmed reads were mapped to *Homo sapiens* genome assembly GRCh38 (hg38) using STAR software for alignment. Principal component analysis (PCA) was performed to check concordance of DNA libraries. Two libraries (MM4 and MM20) resulted in very different PCA values (supplementary material, Figure S1) and quality control metrics, and were not considered satisfactory and were therefore filtered out from downstream processing to avoid biased results. Library replicates were considered satisfactory and filtered out from consequent analysis. Clustering was performed using Euclidean distances and the complete agglomeration method. Differential gene expression analysis with R package DESeq2 (version 1.30.1) was performed for UL compared with MM ($n = 28$). The default workflow for DESeq2 was applied, as described elsewhere [29]. Briefly, input data were unnormalized raw counts matrix, with the DESeq2 model internally correcting for library size differences. Normalization was performed with the median-of-ratios method previously described and used in DESeq2. The standard workflow was used for differential expression analysis using a negative binomial generalized linear model and estimation of false discovery rate by multiple testing correction (Benjamini–Hochberg). Genes were considered relevant when adjusted $p < 0.05$ and $\log_2$ fold-change was greater than 1.5 or less than −1.5.

Correlation of DNA methylation and gene expression

Results were integrated by selecting the CpGs nearby or inside a promoter region (defined as ±1.5 kb of the transcription start site) with a threshold Holm-corrected $p < 0.05$ from DNA methylation analysis and correlated with differentially expressed genes (DEGs) from RNA-seq by computing Spearman correlation coefficients greater than 0.7 or less than −0.7. Genes that fulfilled the hypomethylation/upregulation or hypermethylation/downregulation relationship after integration were selected. Functional enrichment analysis of these genes was performed with ShinyGO (version 0.61) [30].

Validation

Validation of DNA methylation and gene expression levels was performed in samples from different women with UL ($n = 11$). DNA methylation levels at the CpG sites in promoter regions of the selected genes were validated by pyrosequencing in this separate cohort of UL versus MM. PCR primers for each selected gene were designed using Qiagen PyroMark assay design software (version 2.0.01.15) to ensure methylation-independent amplification (supplementary material, Table S2). Pyrosequencing and DNA methylation quantification were performed in a Qiagen PyroMark Q24 system (version 2.0.6). The average DNA methylation value of all samples was obtained from CpG dinucleotides from each sequence analyzed, with one valid CpG per primer. Controls to assess correct DNA bisulfite conversion and sequencing were included in each run to ensure fidelity of the measurements.

To validate gene expression, cDNA was synthesized from total RNA using a PrimeScript RT Reagent Kit (Takara, Kusatsu, Japan) and qPCR was performed with a StepOnePlus Real-Time PCR System (Applied Biosystems, Waltham, MA, USA) and PowerUp SYBR Green (Thermo Fisher Scientific). Gene expression levels were normalized to *GAPDH* and quantified by the ΔΔCt method. Primers were designed using PrimerQuest Tool (Integrated DNA Technologies, Coralville, IA, USA) (supplementary material, Table S2).

5-Aza-2'-deoxycytidine (5-aza-CdR) treatment and gene expression in human UL primary cells

Human UL primary (HULP) cells obtained from individual patients ($n = 10$) were isolated from UL samples as previously described [31]. To evaluate the effect of 5-aza-CdR on the selected downregulated and hypermethylated genes, HULP cells were treated with 0 μM (control) or 10 μM 5-aza-CdR (Abcam, Cambridge, UK) for 72 h, with the medium replaced every 24 h. Total RNA from HULP cells was obtained using a Qiagen RNeasy Mini Kit, and cDNA was synthesized using a PrimeScript RT Kit (Takara, Shiga, Japan). Expression of selected genes in HULP cells treated with or without 5-aza-CdR was evaluated by qPCR, as described above.

Statistical analysis

Analyses of omics data were performed using R (version 3.5.1; R Foundation for Statistical Computing, Vienna, Austria). Graphics were made using the R core package and gplots [32], ggplot2 [33], RColorBrewer [34], and GraphPad Prism 8.0 (GraphPad Software Inc, San Diego, CA, USA). Gene expression and pyrosequencing validation analyses were carried out using GraphPad Prism 8.0, applying Student's *t*-test or the Wilcoxon test. $p < 0.05$ was considered statistically significant.

Results

Global DNA methylome profile in UL compared with adjacent MM

To determine the overall genome DNA methylation profile in UL compared with adjacent MM tissues, we performed exploratory analysis of all β values. PCA and a heatmap revealed clear separation between UL and

adjacent MM (Figure 1A,B). Further analysis of methylation status showed a higher median methylation level in UL compared with adjacent MM (0.704 UL versus 0.681 MM, FDR-adjusted $p = 0.004$) (Figure 1C). Differential methylation analysis was also performed for each CpG. Using a Holm-adjusted P value cut-off of 0.05, we found 43 241 differentially methylated CpGs (11 335 hypomethylated and 31 906 hypermethylated) (supplementary material, Table S3). These results indicate a gain of global methylation in UL compared with adjacent MM.

Global transcriptome profile in UL compared with adjacent MM

We performed an exploratory analysis to assess the role of UL in gene expression. There was a clear effect of tumorigenic phenotype on global transcriptome behavior in UL tissue compared with adjacent MM after clustering the 56 tissues (28 UL and 28 MM) using PCA (Figure 1D), as well as using a heatmap (Figure 1E), where the expression of most genes clearly discriminated both populations. RNA-seq of the 28 patients showed 10 339 DEGs between UL tissue compared with adjacent MM, with 5690 upregulated and 4649 downregulated genes (FDR-adjusted $p < 0.05$, $\log_2$ fold-change > 0.5 or < 0.5) (supplementary material, Table S4).

Abnormally methylated and differentially expressed genes in UL

To further analyze the expression of genes controlled by DNA methylation and their function, we integrated the results from RNA-seq and genome-wide DNA methylation analyses. Among the 43 241 differentially methylated CpGs (Holm-corrected $p < 0.05$), we selected 11 772 CpGs that were near or inside a promoter region and correlated these CpGs with the 10 339 DEGs. Subsequently, we selected genes with a Spearman correlation coefficient greater than 0.7 or less than −0.7 (supplementary material, Figure S2). We identified a total of 93 genes regulated by methylation with at least one CpG site matching the hypomethylation/upregulation or hypermethylation/downregulation filter (supplementary material, Table S5), of which 22 were hypomethylated/upregulated and 71 were hypermethylated/downregulated (Figure 2A). Heatmaps of the top 20 hypermethylated/downregulated and top 20 hypomethylated/upregulated genes in UL compared with MM are shown in Figure 2B,C.

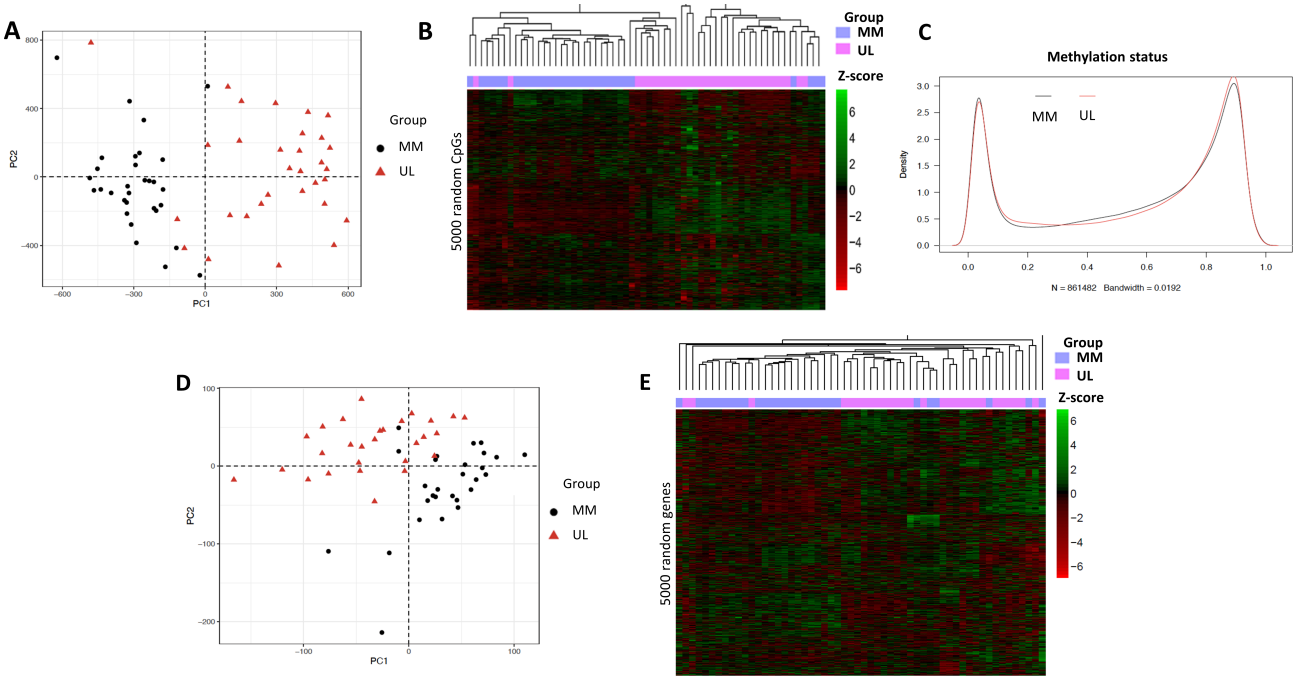

Figure 1. DNA methylome and global transcriptome behavior in uterine leiomyoma compared with adjacent myometrium tissues. (A) Principal component analysis (PCA) of the global DNA methylome in uterine leiomyoma (UL; red) and adjacent myometrium (MM; black) ($n = 30$ per group). (B) Heatmap based on methylation of 5000 random CpGs (y axis) from β-value regression analysis for differential methylation among all tissues (x axis) after unsupervised clustering of UL (pink) and MM (purple) ($n = 30$ per group). Z-scores calculated from β values are represented. Color scale ranges from red for lower methylation to green for higher methylation levels. (C) Distribution of mean β values in UL (red) and MM (black) samples ($n = 30$ per group), representing global methylation status. (D) PCA of the global transcriptome in UL (red) and MM (black) ($n = 28$ per group). Dimensions represent percent variances. (E) Heatmap based on transcriptomics of 5000 random genes (y axis) of all samples (x axis) from UL (pink) and MM (purple) ($n = 28$ per group). Z-scores calculated from normalized counts are represented. Color scale ranges from red for lower expression to green for higher expression levels. FDR-adjusted $p < 0.05$, $\log_2$ fold-change > 0.5 or < 0.5; $p < 0.001$.

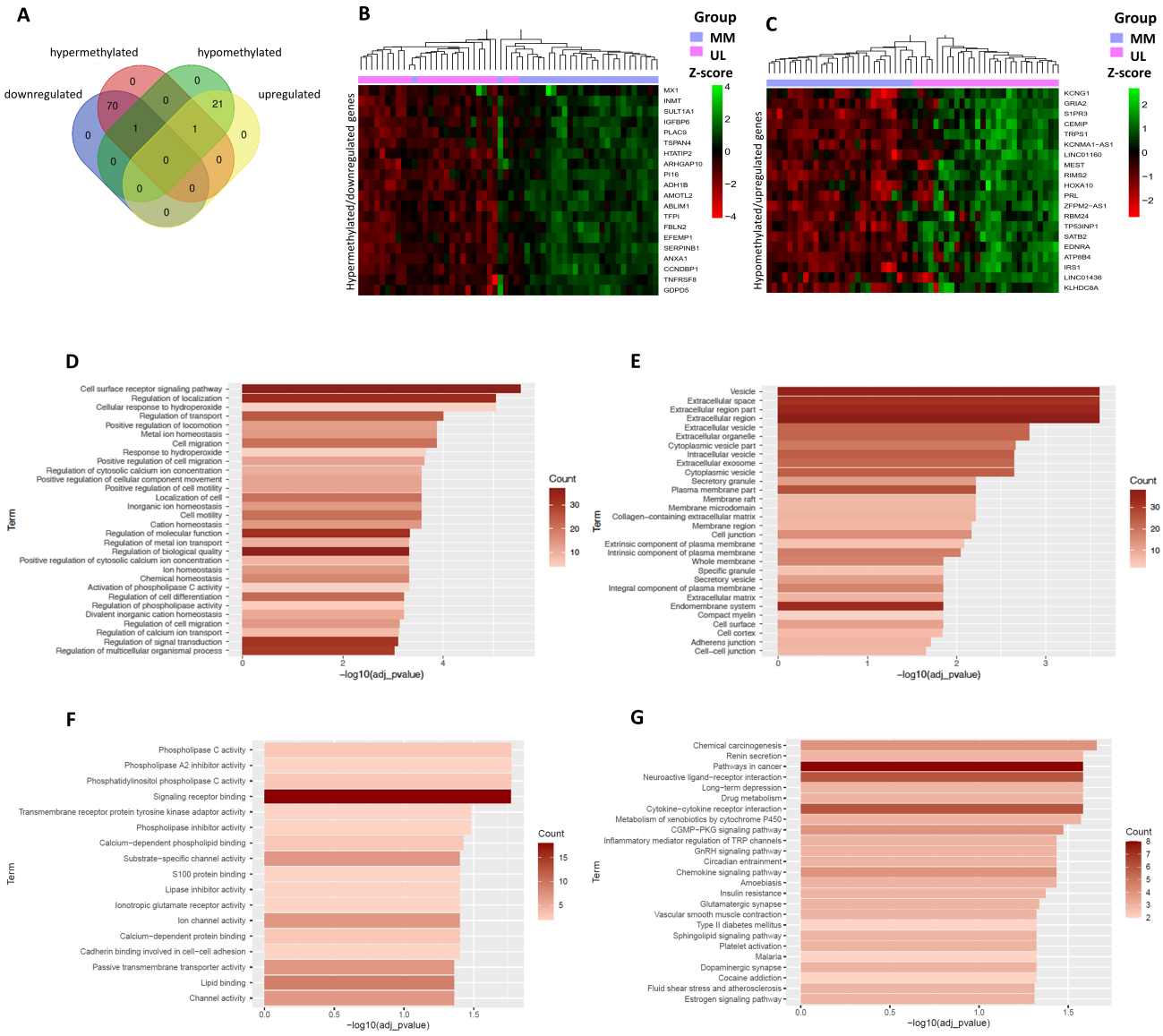

Figure 2. Identification of aberrantly methylated and differentially expressed genes and functional enrichment analysis. (A) Venn diagrams show overlap of differentially expressed genes (DEGs) and differentially methylated genes. (B, C) Heatmap of the top 20 significant hypermethylated/downregulated genes (*y* axis) (B) and hypomethylated/upregulated genes (*y* axis) (C) after Spearman correlation (coefficient > 0.7 or < −0.7) of CpGs nearby/inside promoter regions and DEGs. Z-scores calculated from normalized counts are represented. Color scale ranges from red for lower expression to green for higher expression levels. Samples (*x* axis) are represented as pink for uterine leiomyoma (UL) or purple for adjacent myometrium (MM). (D–G) Most significant biological processes (D), cellular components (E), molecular functions (F), and KEGG pathways (G) obtained after functional enrichment analysis of all selected aberrantly methylated genes and DEGs in UL versus MM tissues. The total number of genes from the gene set that are involved in each function is referred to as counts.

Functional implications of genes controlled by aberrant DNA methylation in UL

We applied functional enrichment analysis of the 93 selected genes to describe functions deregulated in UL due to aberrant DNA methylation. This analysis identified 30 biological processes significantly deregulated in human UL (Figure 2D), within functional groups such as metabolism and cell physiology, response to extracellular signals, invasion, and proliferation (supplementary material, Table S6), which are key pathways in tumor pathogenesis. In addition, we found 30 cellular components significantly enriched in UL (Figure 2E), which were mainly related to cell membranes, vesicles, extracellular matrix, and cell junctions (supplementary material, Table S7). Among the molecular functions enriched in UL (Figure 2F), most were associated with metabolism, cell physiology, and response to extracellular signals (supplementary material, Table S8). Finally, KEGG pathway analysis (Figure 2G) revealed pathways involved in cancer and uterine biology dysregulated in UL (supplementary material, Table S9).

Validation of results from integrative analysis

To validate the results from RNA-seq and DNA methylation analysis, genes were selected based on their potential significance in tumorigenesis and/or UL pathology

among the top 20 hypomethylated/upregulated genes (*PRL*, *ATP8B4*, *CEMIP*, and *ZPMS2-AS1*) and the top 20 hypermethylated/downregulated genes (*EFEMP1*, *FBLN2*, *ARHGAP10*, and *HTATIP2*). In relation to DNA methylation, pyrosequencing results corroborated the lower levels of DNA methylation in CpG islands from *PRL* (70.9% MM versus 32.7% UL, $p < 0.001$), *ATP8B4* (70.5% versus 31.7%, $p = 0.001$), *CEMIP* (30.6% versus 11.2%, $p = 0.001$), and *ZPMS2-AS1* (92.3% versus 71.5%, $p = 0.0016$) (Figure 3A–D), as well as higher levels of *EFEMP1* (2.7% MM versus 38.04% UL, $p = 0.001$), *FBLN2* (90.6% versus 96.81%, $p = 0.0066$), *ARHGAP10* (16.8% versus 52.4%, $p < 0.001$), and *HTATIP2* (18.2% versus 62.8%, $p < 0.001$) (Figure 3E–H) in UL compared with adjacent MM.

Gene expression analysis by RT-qPCR corroborated upregulation of *PRL* (fold-change = 658.8, $p = 0.0039$), *ATP8B4* (fold-change = 16.23, $p = 0.0039$), *CEMIP* (fold-change = 34.7, $p = 0.002$), *ZPMS2-AS1* (fold-change = 44.43, $p = 0.0059$), *RIMS2* (fold-change = 24.57, $p = 0.002$), and *TFAP2C* (fold-change = 86.59, $p = 0.002$) in a separate cohort of UL compared with adjacent MM (Figure 4A–F). Similarly, RT-qPCR validated downregulation of *EFEMP1* (fold-change = 0.1, $p = 0.002$), *FBLN2* (fold-change = 0.48, $p = 0.002$), *ARHGAP10* (fold-change = 0.84, $p = 0.27$), and *HTATIP2* (fold-change = 0.79, $p = 0.16$) (Figure 4G–J).

Inhibiting DNA methylation upregulates expression of hypermethylated/downregulated genes *in vitro*

To evaluate the role of DNMT in methylation-dependent regulation of gene expression, we inhibited DNMT in HULP cells using 5-aza-CdR and evaluated the expression of genes hypermethylated and downregulated in UL, previously selected for their potential significance in UL pathology. Treatment with 5-aza-CdR upregulated *EFEMP1* (fold-change = 2.88 ± 2.49, $p = 0.0098$), *FBLN2* (fold-change = 1.73 ± 1.37, $p = 0.0137$), *ARHGAP10* (fold-change = 17.76 ± 26.21, $p = 0.0195$), and *HTATIP2* (fold-change = 1.57 ± 0.72, $p = 0.0371$) (Figure 5A–D).

Discussion

We performed a paired study analyzing DNA methylation and gene expression in Caucasian women using matched UL and MM samples extracted from the same patient for both analyses, and observed good correlation

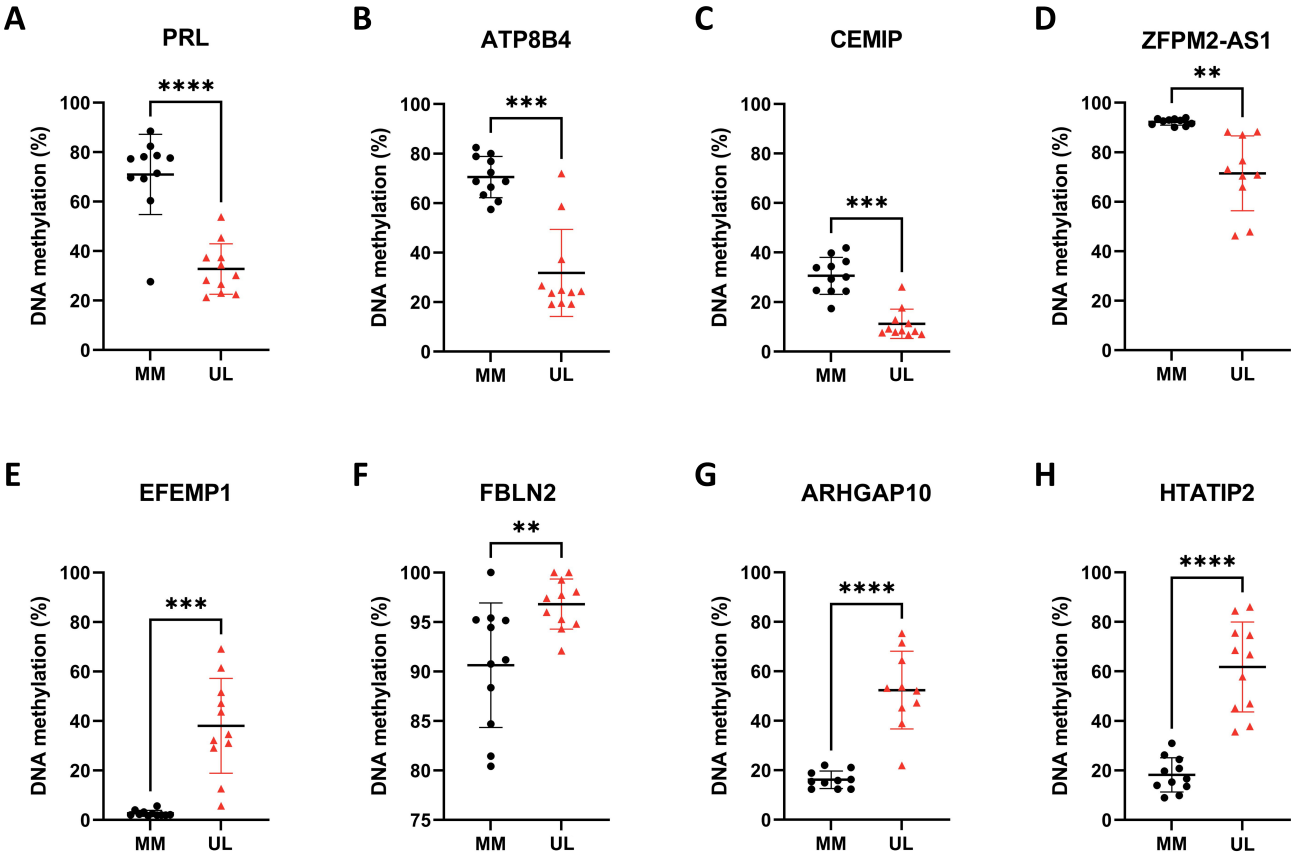

Figure 3. Validation of DNA methylation. (A–H) DNA methylation levels in CpG islands of *PRL* (A), *ATP8B4* (B), *CEMIP* (C), *ZFPM2-AS1* (D), *EFEMP1* (E), *FBLN2* (F), *ARHGAP10* (G), and *HTATIP2* (H) in the validation set of uterine leiomyoma (UL) compared with adjacent myometrium (MM) ($n = 11$). DNA methylation was evaluated by pyrosequencing and is expressed as percentage. $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

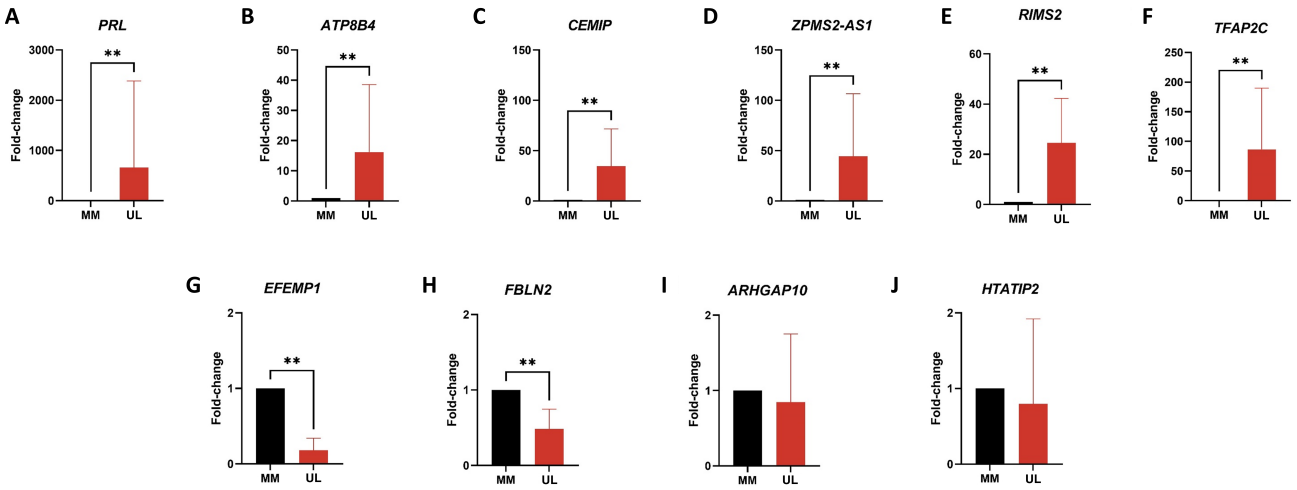

Figure 4. Validation of RNA-seq results. (A–J) RT-qPCR expression levels of *PRL* (A), *ATP8B4* (B), *CEMIP* (C), *ZPMS2-AS1* (D), *RIMS2* (E), *TFAP2C* (F), *EFEMP1* (G), *FBLN2* (H), *ARHGAP10* (I), and *HTATIP2* (J) in the validation set of uterine leiomyoma (UL) compared with adjacent myometrium (MM) ($n = 11$). $^{**}p < 0.01$.

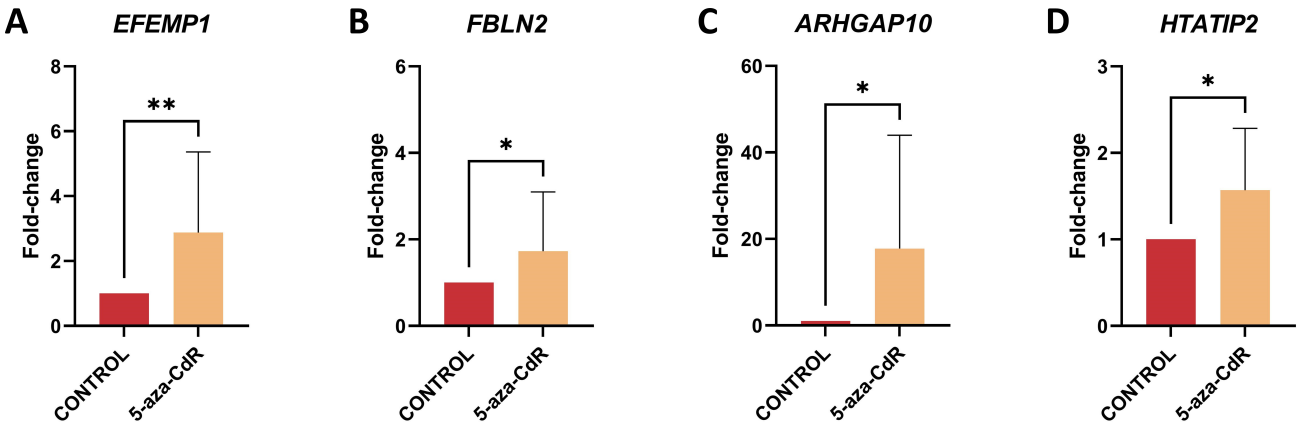

Figure 5. Gene expression analysis in human uterine leiomyoma primary cells after 5-aza-CdR treatment. (A–D) RT-qPCR expression levels of hypermethylated/downregulated genes *EFEMP1* (A), *FBLN2* (B), *ARHGAP10* (C), and *HTATIP2* (D) in human uterine fibroid primary (HULP) cells treated with 0 μM (control) or 10 μM 5-aza-CdR for 72 h ($n = 11$). $^{*}p < 0.05$, $^{**}p < 0.01$.

between them. In addition, we used the most recent DNA methylation technology, which considers 850 000 CpG sites, and correlated it with RNA-seq to holistically identify important pathways and driver genes that trigger UL formation to identify therapeutically targetable mechanisms. Our results show that the global methylome and transcriptome are altered in UL compared with MM. In addition, we found that DNA methylation regulates the expression of genes involved in important biological processes for UL pathogenesis, suggesting the therapeutic potential of approaches focused on reversion of this epigenetic modification to treat patients with UL.

First, we focused on global DNA methylation and gene expression status in UL compared with adjacent MM from Caucasian women to delineate the role of this epigenetic modification in UL pathogenesis. Previous reports described differential gene expression in UL [35–38], while other studies implicate aberrant DNA methylation in cancer [14–16,39] and UL [18–20], although the studies used different techniques and studied patients with different ethnicities. In this paired study, we demonstrated a global hypermethylation status of UL compared with MM as well as different transcriptomic behavior in UL from Caucasian women, showing a robust correlation between DNA methylation and gene expression in these patients.

To assess the involvement of DNA methylation in gene expression and identify potential mechanisms involved in UL pathogenesis and development, we identified abnormally methylated and differentially expressed genes in UL compared with MM. We found 93 DEGs with a strong correlation between methylation status of near-to-promoter CpG islands and gene expression, with 22 hypomethylated/upregulated and 71 hypermethylated/downregulated genes, suggesting altered gene expression due to DNA methylation modifications that trigger UL development. These findings align with other studies describing DNA methylation involvement in molecular subtypes of UL and maintenance of a stem cell population in UL [40,41].

We further analyzed the biological processes and molecular functions in which these genes are involved, identifying deregulation of metabolism and cell physiology, response to extracellular signals, invasion, and proliferation in UL due to aberrant DNA methylation. Transformed cells adapt their metabolism to support tumor initiation and progression [42]. In addition, hallmarks of cancer include deregulated responses to cell death signals, uncontrolled proliferation, and invasion [43], processes also associated with UL [2,44–46]. In this study, we showed that these key regulators of tumor physiology are under epigenetic influence in UL.

Interestingly, KEGG analysis of differentially expressed and methylated genes revealed pathways related to cancer and uterine biology. Specifically, GnRH and estrogen signaling pathways were enriched in UL. DNA methylation could be associated with alteration of these hormones, which are involved in UL development and its symptomatology [47]. In addition, cellular components such as cell membranes, vesicles, extracellular matrix, and cell junctions were deregulated. Excessive synthesis and deposition of extracellular matrix is a main cause of UL growth [48,49]. Alternatively, cancer cells produce more exosomes than normal cells, and these extracellular vesicles are involved in cancer development, metastasis, and anti-tumor immunity [50–52]. Unlike cancer, the functions of vesicles in UL development have not been broadly studied. We identified *RIMS2* as a hypomethylated/upregulated gene that regulates exocytosis in neurons [53] and acrosome of spermatozoa [54], priming vesicles for release. Thus, we propose *RIMS2* as a regulator of vesicle secretion in UL, paving the way for additional studies about the interaction between extracellular vesicles and UL.

To further analyze new molecular targets involved in UL pathogenesis that are controlled by DNA methylation, we studied the top significant hypomethylated/upregulated genes. We selected *TFAP2C*, *PRL*, *ATP8B4*, *CEMIP*, and *ZPMS2-AS1* because they are oncogenes involved in key molecular mechanisms for tumor initiation and progression. *TFAP2C* is a DNA-binding transcription factor not previously linked to UL. However, it could play a role in UL cell physiology and homeostasis through angiogenesis [55] or inactivation of the Hippo signaling pathway [56]. *PRL* is a mitogenic autocrine/paracrine growth factor in human tumorigenesis [57] and is highly expressed [10] and hypomethylated in leiomyomas [58]. *CEMIP* is an oncogene that plays a crucial role in proliferation, migration [59], epithelial–mesenchymal transition, and Wnt/β-catenin and PI3K/Akt pathways [60], which are upregulated in UL [10], and our results demonstrate that DNA hypomethylation is associated with *CEMIP* upregulation. *ZFPM2-AS1* is a long non-coding RNA (lncRNA) that promotes cancer by increasing cell proliferation, tumor size, and tumor invasion [60,61]. Its upregulated expression was not associated with UL until this study. *ATP8B4* is a cation transport ATPase previously linked with cancer [59,60] and proposed as a potential prognostic marker and therapeutic target [59]. We identified hypomethylation and upregulation of *ATP8B4* in UL, suggesting that this gene is involved in UL development. Inhibition of this driver gene could decrease cell proliferation and invasion in UL cells as well as increase apoptosis, which would have a significant clinical impact for UL patients. Further studies are necessary to assess the effects of *ATP8B4* inhibition in UL.

We also studied the top significant hypermethylated/downregulated genes, selecting *EFEMP1*, *FBLN2*, *ARHGAP10*, and *HTATIP2* because of their promising roles in UL. *EFEMP1* is an extracellular matrix glycoprotein that suppresses the Wnt/β-catenin pathway in endometrial carcinoma [62]; is dysregulated in leiomyomas; and is one of the most important regulators of their pathophysiology [45,46,63]. Therefore, methylation and downregulation of *EFEMP1* could enhance the Wnt/β-catenin pathway and increase extracellular matrix formation in UL. *FBLN2* triggers invasion and metastasis through its interactions with extracellular matrix proteins in breast cancer [64]. We observed hypermethylation and downregulation of *FBLN2*, suggesting that this gene potentially drives UL development. *ARHGAP10*, a downregulated tumor suppressor in ovarian cancer [65], is involved in the Wnt pathway, cell cycle, apoptosis, and autophagic cell death [42] but was not previously associated with UL. *HTATIP2* is a tumor suppressor participating in angiogenesis, cell apoptosis, growth, angiogenesis, DNA repair, and tumor cell metabolism [66], with aberrant hypermethylation in several cancer types [67–69]. Hypermethylation and downregulation of *HTATIP2* suggest that it may induce abnormal angiogenesis, apoptosis, and cell metabolism that leads to the development of UL.

In general, our results suggest that hypomethylation/upregulation of the oncogenes involved in proliferation, invasion, and the Wnt/β-catenin pathway is important in the initiation and progression of UL, while hypermethylation/downregulation of tumor suppressor genes triggers increased proliferation, invasion, altered metabolism, and deposition of extracellular matrix that contributes to UL development. Remarkably, we found that some unknown genes were hypomethylated/upregulated (*KCNMA1-AS1*, *LINC01160*, *KLHDC8A*) or hypermethylated/downregulated (*PLAC9*) in UL versus MM. These genes are implicated in proliferation and invasion [70–73], suggesting a possible role in UL development. Further studies describing their function and relationship with tumors are necessary to clarify their roles as biomarkers, therapeutic targets, and regulators of UL development.

To corroborate that DNA hypermethylation affects the expression of tumor suppressor genes in UL, we inhibited DNMTs, enzymes that catalyze DNA methylation, *in vitro* with 5-aza-CdR treatment. Inhibition of DNA methylation upregulated the tumor suppressors *EFEMP1*, *HTATIP2*, *FBLN2*, and *ARHGAP10*, supporting the notion that epigenetic modification of DNA methylation is involved in the expression of tumor suppressor genes that trigger UL development. Because 5-aza-CdR inhibits cell proliferation, extracellular matrix

formation, and Wnt/β-catenin signaling pathway targets in HULP cells [31,41], our findings suggest that reversing DNA methylation of tumor suppressor genes could inhibit these processes. Extracellular matrix has been identified as a potential target for future therapeutics in UL [49], and we demonstrated that *EFEMP2* and *FBLN2* were associated with aberrant DNA methylation and that demethylation recovered their expression, suggesting potential therapeutic impact.

In conclusion, UL exhibits a global hypermethylation status compared with MM. This aberrant methylation alters the expression of genes involved in tumor development and maintenance, affecting important processes in UL physiology. In addition, DNMT inhibition reverts the expression of hypermethylated/downregulated tumor suppressor genes, suggesting that this reversion could inhibit key processes in UL development. Thus, DNA methylation is an important molecular mechanism implicated in UL pathogenesis, and its reversal could be a potential therapeutic option to treat UL.

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Author contributions statement

MCC-G contributed to study design, executed experiments, interpreted data, and helped to write and edit the manuscript. AC executed experiments, interpreted data, and helped to write the manuscript. EJ-B executed experiments to validate gene expression. JM, VP and AT selected and recruited patients with UL and collected samples. AQ prepared the library for RNA sequencing. AP devised and supervised the study, contributed to data interpretation, and drafted the manuscript. HF coordinated the study design, contributed to data interpretation, and edited the manuscript. All the authors reviewed the manuscript and provided critical feedback and discussion.

Data availability statement

All raw sequencing data are available through NCBI's Gene Expression Omnibus (GEO) under GEO Series accession number GSE192354 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE192354)

References

1. Baird DD, Hill MC, Schectman JM, *et al*. Vitamin D and the risk of uterine fibroids. *Epidemiology* 2013; **24:** 447–453.
2. Bulun SE. Uterine fibroids. *N Engl J Med* 2013; **369:** 1344–1355.
3. Stewart EA. Uterine fibroids. *Lancet* 2001; **357:** 293–298.
4. Donnez J, Dolmans MM. Uterine fibroid management: from the present to the future. *Hum Reprod Update* 2016; **22:** 665–686.
5. Sohn GS, Cho S, Kim YM, *et al*. Current medical treatment of uterine fibroids. *Obstet Gynecol Sci* 2018; **61:** 192–201.
6. Donnez J, Courtoy GE, Donnez O, *et al*. Ulipristal acetate for the management of large uterine fibroids associated with heavy bleeding: a review. *Reprod Biomed Online* 2018; **37:** 216–223.
7. Tristan M, Orozco LJ, Steed A, *et al*. Mifepristone for uterine fibroids. *Cochrane Database Syst Rev* 2012; **2012:** CD007687.
8. Stewart EA, Laughlin-Tommaso SK, Catherino WH, *et al*. Uterine fibroids. *Nat Rev Dis Primers* 2016; **2:** 16043.
9. El Sabeh M, Borahay M. The future of uterine fibroid management: a more preventive and personalized paradigm. *Reprod Sci* 2021; **28:** 3285–3288.
10. Mehine M, Kaasinen E, Heinonen HR, *et al*. Integrated data analysis reveals uterine leiomyoma subtypes with distinct driver pathways and biomarkers. *Proc Natl Acad Sci U S A* 2016; **113:** 1315–1320.
11. Stewart E, Cookson C, Gandolfo R, *et al*. Epidemiology of uterine fibroids: a systematic review. *BJOG* 2017; **124:** 1501–1512.
12. Peschansky VJ, Wahlestedt C. Non-coding RNAs as direct and indirect modulators of epigenetic regulation. *Epigenetics* 2014; **9:** 3–12.
13. Yang Q, Mas A, Diamond MP, *et al*. The mechanism and function of epigenetics in uterine leiomyoma development. *Reprod Sci* 2016; **23:** 163–175.
14. Pfeifer GP. Defining driver DNA methylation changes in human cancer. *Int J Mol Sci* 2018; **19:** 1166.
15. Long MD, Dhiman VK, Affronti HC, *et al*. Dynamic patterns of DNA methylation in the normal prostate epithelial differentiation program are targets of aberrant methylation in prostate cancer. *Sci Rep* 2021; **11:** 11405.
16. Wang X, Liu J, Wang D, *et al*. Epigenetically regulated gene expression profiles reveal four molecular subtypes with prognostic and therapeutic implications in colorectal cancer. *Brief Bioinform* 2021; **22:** bbaa309.
17. Ruscito I, Gasparri ML, De Marco MP, *et al*. The clinical and pathological profile of BRCA1 gene methylated breast cancer women: a meta-analysis. *Cancers (Basel)* 2021; **13:** 1391.
18. Yamagata Y, Maekawa R, Asada H, *et al*. Aberrant DNA methylation status in human uterine leiomyoma. *Mol Hum Reprod* 2009; **15:** 259–267.
19. Navarro A, Yin P, Monsivais D, *et al*. Genome-wide DNA methylation indicates silencing of tumor suppressor genes in uterine leiomyoma. *PLoS One* 2012; **7:** e33284.
20. Maekawa R, Sato S, Yamagata Y, *et al*. Genome-wide DNA methylation analysis reveals a potential mechanism for the pathogenesis and development of uterine leiomyomas. *PLoS One* 2013; **8:** e66632.
21. Sandoval J, Heyn H, Moran S, *et al*. Validation of a DNA methylation microarray for 450,000 CpG sites in the human genome. *Epigenetics* 2011; **6:** 692–702.
22. Moran S, Arribas C, Esteller M. Validation of a DNA methylation microarray for 850,000 CpG sites of the human genome enriched in enhancer sequences. *Epigenetics* 2016; **8:** 389–399.

23. Aryee MJ, Jaffe AE, Corrada-Bravo H, *et al*. Minfi: A flexible and comprehensive Bioconductor package for the analysis of Infinium DNA methylation microarrays. *Bioinformatics* 2014; **30:** 1363–1369.
24. Dobin A, Davis CA, Schlesinger F, *et al*. STAR: ultrafast universal RNA-seq aligner. *Bioinformatics* 2013; **29:** 15–21.
25. Liao Y, Smyth GK, Shi W. featureCounts: an efficient general purpose program for assigning sequence reads to genomic features. *Bioinformatics* 2014; **30:** 923–930.
26. R Core Team R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. 2020. [Accessed 23 December 2020]. https://www.R-project.org/.
27. Martin M. Cutadapt removes adapter sequences from high-throughput sequencing reads. *EMBnet J* 2011; **17:** 10–12.
28. Kopylova E, Noé L, Touzet H. SortMeRNA: fast and accurate filtering of ribosomal RNAs in metatranscriptomic data. *Bioinformatics* 2012; **28:** 3211–3217.
29. Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol* 2014; **15:** 550.
30. Ge SX, Jung D, Yao R. ShinyGO: a graphical gene-set enrichment tool for animals and plants. *Bioinformatics* 2020; **36:** 2628–2629.
31. Carbajo-García MC, Corachán A, Segura-Benitez M, *et al*. 5-aza-2'-deoxycitidine inhibits cell proliferation, extracellular matrix formation and Wnt/β-catenin pathway in human uterine leiomyomas. *Reprod Biol Endocrinol* 2021; **19:** 106.
32. Warnes GR, Bolker B, Bonebakker L, *et al*. gplots: Various R Programming Tools for Plotting Data. R package version 3.1.1. 2020. https://CRAN.R-project.org/package=gplots.
33. Wickham H. *ggplot2: Elegant Graphics for Data Analysis*. New York: Springer-Verlag; 2016.
34. Neuwirth E. RColorBrewer: ColorBrewer Palettes. R package version 1.1-2. 2014. https://CRAN.R-roject.org/package=RColorBrewer.
35. Arslan AA, Gold LI, Mittal K, *et al*. Gene expression studies provide clues to the pathogenesis of uterine leiomyoma: new evidence and a systematic review. *Hum Reprod* 2005; **20:** 852–863.
36. Anjum S, Sahar T, Nigam A, *et al*. Transcriptome analysis of mRNA in uterine leiomyoma using next-generation RNA sequencing. *Anticancer Agents Med Chem* 2019; **19:** 1703–1718.
37. Laganà AS, Vergara D, Favilli A, *et al*. Epigenetic and genetic landscape of uterine leiomyomas: a current view over a common gynecological disease. *Arch Gynecol Obstet* 2017; **296:** 855–867.
38. Styer AK, Rueda BR. The epidemiology and genetics of uterine leiomyoma. *Best Pract Res Clin Obstet Gynaecol* 2016; **34:** 3–12.
39. Klutstein M, Nejman D, Greenfield R, *et al*. DNA methylation in cancer and aging. *Cancer Res* 2016; **76:** 3446–3450.
40. George JW, Fan H, Johnson B, *et al*. Integrated epigenome, exome, and transcriptome analyses reveal molecular subtypes and homeotic transformation in uterine fibroids. *Cell Rep* 2019; **29:** 4069–4085.e6.
41. Liu S, Yin P, Xu J, *et al*. Targeting DNA methylation depletes uterine leiomyoma stem cell-enriched population by stimulating their differentiation. *Endocrinology* 2020; **161:** bqaa143.
42. Vander Heiden MG, DeBerardinis RJ. Understanding the intersections between metabolism and cancer biology. *Cell* 2017; **168:** 657–669.
43. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. *Cell* 2011; **144:** 646–674.
44. Baranov VS, Osinovskaya NS, Yarmolinskaya MI. Pathogenomics of uterine fibroids development. *Int J Mol Sci* 2019; **20:** 6151.
45. Borahay MA, Al-Hendy A, Kilic GS, *et al*. Signaling pathways in leiomyoma: understanding pathobiology and implications for therapy. *Mol Med* 2015; **21:** 242–256.
46. Ono M, Yin P, Navarro A, *et al*. Paracrine activation of WNT/β-catenin pathway in uterine leiomyoma stem cells promotes tumor growth. *Proc Natl Acad Sci U S A* 2013; **110:** 17053–17058.
47. De La Cruz MS, Buchanan EM. Uterine fibroids: diagnosis and treatment. *Am Fam Physician* 2017; **95:** 100–107.
48. Leppert PC, Baginski T, Prupas C, *et al*. Comparative ultrastructure of collagen fibrils in uterine leiomyomas and normal myometrium. *Fertil Steril* 2004; **82:** 1182–1187.
49. Islam MS, Ciavattini A, Petraglia F, *et al*. Extracellular matrix in uterine leiomyoma pathogenesis: a potential target for future therapeutics. *Hum Reprod Update* 2018; **24:** 59–85.
50. Bebelman MP, Smit MJ, Pegtel DM, *et al*. Biogenesis and function of extracellular vesicles in cancer. *Pharmacol Ther* 2018; **188:** 1–11.
51. Minciacchi VR, Freeman MR, Di Vizio D. Extracellular vesicles in cancer: exosomes, microvesicles and the emerging role of large oncosomes. *Semin Cell Dev Biol* 2015; **40:** 41–51.
52. Zhang L, Yu D. Exosomes in cancer development, metastasis, and immunity. *Biochim Biophys Acta Rev Cancer* 2019; **1871:** 455–468.
53. Kaeser PS, Deng L, Fan M, *et al*. RIM genes differentially contribute to organizing presynaptic release sites. *Proc Natl Acad Sci U S A* 2012; **109:** 11830–11835.
54. Bello OD, Zanetti MN, Mayorga LS, *et al*. RIM, Munc13, and Rab3A interplay in acrosomal exocytosis. *Exp Cell Res* 2012; **318:** 478–488.
55. Kitaya K, Yasuo T, Yamaguchi T, *et al*. Genes regulated by interferon-gamma in human uterine microvascular endothelial cells. *Int J Mol Med* 2007; **20:** 689–697.
56. Wang X, Sun D, Tai J, *et al*. TFAP2C promotes stemness and chemotherapeutic resistance in colorectal cancer via inactivating hippo signaling pathway. *J Exp Clin Cancer Res* 2018; **37:** 27.
57. Ben-Jonathan N, Liby K, McFarland M, *et al*. Prolactin as an autocrine/paracrine growth factor in human cancer. *Trends Endocrinol Metab* 2002; **13:** 245–250.
58. Sato S, Maekawa R, Yamagata Y, *et al*. Identification of uterine leiomyoma-specific marker genes based on DNA methylation and their clinical application. *Sci Rep* 2016; **6:** 30652.
59. Liu J, Yan W, Han P, *et al*. The emerging role of KIAA1199 in cancer development and therapy. *Biomed Pharmacother* 2021; **138:** 111507.
60. Kong F, Deng X, Kong X, *et al*. ZFPM2-AS1, a novel lncRNA, attenuates the p53 pathway and promotes gastric carcinogenesis by stabilizing MIF. *Oncogene* 2018; **37:** 5982–5996.
61. Yan Z, Yang Q, Xue M, *et al*. YY1-induced lncRNA ZFPM2-AS1 facilitates cell proliferation and invasion in small cell lung cancer via upregulating of TRAF4. *Cancer Cell Int* 2020; **20:** 108.
62. Yang T, Zhang H, Qiu H, *et al*. EFEMP1 is repressed by estrogen and inhibits the epithelial–mesenchymal transition via Wnt/β-catenin signaling in endometrial carcinoma. *Oncotarget* 2016; **7:** 25712–25725.
63. El Sabeh M, Saha SK, Afrin S, *et al*. Wnt/β-catenin signaling pathway in uterine leiomyoma: role in tumor biology and targeting opportunities. *Mol Cell Biochem* 2021; **476:** 3513–3536.
64. Jin W, Li QZ, Zuo YC, *et al*. Relationship between DNA methylation in key region and the differential expressions of genes in human breast tumor tissue. *DNA Cell Biol* 2019; **38:** 49–62.
65. Luo N, Guo J, Chen L, *et al*. ARHGAP10, downregulated in ovarian cancer, suppresses tumorigenicity of ovarian cancer cells. *Cell Death Dis* 2016; **7:** e2157.
66. Xu T, Jin Z, Yuan Y, *et al*. Tat-interacting protein 30 (TIP30) expression serves as a new biomarker for tumor prognosis: a systematic review and meta-analysis. *PLoS One* 2016; **11:** e0168408.
67. Nanok C, Jearanaikoon P, Proungvitaya S, *et al*. Aberrant methylation of HTATIP2 and UCHL1 as a predictive biomarker for cholangiocarcinoma. *Mol Med Rep* 2018; **17:** 4145–4153.
68. Lu B, Ma Y, Wu G, *et al*. Methylation of *Tip30* promoter is associated with poor prognosis in human hepatocellular carcinoma. *Clin Cancer Res* 2008; **14:** 7405–7412.
69. Chen X, Cao X, Dong W, *et al*. Expression of TIP30 tumor suppressor gene is down-regulated in human colorectal carcinoma. *Dig Dis Sci* 2010; **55:** 2219–2226.

70. Ma SY, Wei P, Qu F. KCNMA1-AS1 attenuates apoptosis of epithelial ovarian cancer cells and serves as a risk factor for poor prognosis of epithelial ovarian cancer. *Eur Rev Med Pharmacol Sci* 2019; **23:** 4629–4641.
71. Ai J, Tan G, Wang T, *et al*. Transcription factor STAT1 promotes the proliferation, migration and invasion of nasopharyngeal carcinoma cells by upregulating *LINC01160*. *Future Oncol* 2021; **17:** 57–69.
72. Zhu X, Chen T, Yang H, *et al*. Lactate induced up-regulation of KLHDC8A (Kelch domain-containing 8A) contributes to the proliferation, migration and apoptosis of human glioma cells. *J Cell Mol Med* 2020; **24:** 11691–11702.
73. Ouyang C, Pu YZ, Qin XH, *et al*. Placenta-specific 9, a putative secretory protein, induces G2/M arrest and inhibits the proliferation of human embryonic hepatic cells. *Biosci Rep* 2018; **38:** BSR20180820.

SUPPLEMENTARY MATERIAL ONLINE

Figure S1. Sample clustering by biological parameters

Figure S2. Experimental design

Table S1. Patient characteristics

Table S2. RT-qPCR and pyrosequencing primer sequences

Table S3. Differentially methylated CpGs between uterine leiomyoma and adjacent myometrium

Table S4. Differentially expressed genes (DEGs) between uterine leiomyoma and adjacent myometrium

Table S5. Genes selected after integration of differentially methylated and differentially expressed genes between uterine leiomyoma and adjacent myometrium

Table S6. Classification of relevant enriched biological process functional groups

Table S7. Classification of relevant enriched cellular components into global groups after functional enriched analysis

Table S8. Classification of relevant molecular functions into global groups after functional enriched analysis

Table S9. Classification of relevant KEGG pathways into global groups after functional enriched analysis

MANUSCRIPT 2. Carbajo-García MC, Corachán A, Segura-Benitez M, Monleón J, Escrig J, Faus A, Pellicer A, Cervelló I, Ferrero H. 5-aza-2'-deoxycitidine inhibits cell proliferation, extracellular matrix formation and Wnt/β-catenin pathway in human uterine leiomyomas. Reprod Biol Endocrinol. 2021 Jul 8;19(1):106. doi: 10.1186/s12958-021-00790-5. PMID: 34233687; PMCID: PMC8265104.

Reproductive Biology and Endocrinology

RESEARCH Open Access

5-aza-2′-deoxycitidine inhibits cell proliferation, extracellular matrix formation and Wnt/β-catenin pathway in human uterine leiomyomas

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Abstract

Background: Uterine leiomyoma is a benign tumor with unclear pathogenesis and inaccurate treatment. This tumor exhibits altered DNA methylation related to disease progression. DNMT inhibitors as 5-aza-2′-deoxycytidine (5-aza-CdR), have been suggested to treat tumors in which DNA methylation is altered. We aimed to evaluate whether DNA methylation reversion with 5-aza-CdR reduces cell proliferation and extracellular matrix (ECM) formation in uterine leiomyoma cells to provide a potential treatment option.

Methods: Prospective study using uterine leiomyoma and adjacent myometrium tissues and human uterine leiomyoma primary (HULP) cells ($n = 16$). In tissues, gene expression was analyzed by qRT-PCR and DNMT activity by ELISA. Effects of 5-aza-CdR treatment on HULP cells were assessed by CellTiter, western blot, and qRT-PCR.

Results: DNMT1 gene expression was higher in uterine leiomyoma vs myometrium. Similarly, DNMT activity was greater in uterine leiomyoma and HULP cells (6.5 vs 3.8 OD/h/mg; 211.3 vs 63.7 OD/h/mg, respectively). After 5-aza-CdR treatment on HULP cells, cell viability was reduced, significantly so at 10 μM (85.3%). Treatment with 10 μM 5-aza-CdR on HULP cells significantly decreased expression of proliferation marker PCNA (FC = 0.695) and of ECM proteins (COLLAGEN I FC = 0.654; PAI-1, FC = 0.654; FIBRONECTIN FC = 0.733). 5-aza-CdR treatment also decreased expression of Wnt/β-catenin pathway final targets, including WISP1 protein expression (10 μM, FC = 0.699), *c-MYC* gene expression (2 μM, FC = 0.745 and 10 μM, FC = 0.728), and *MMP7* gene expression (5 μM, FC = 0.520 and 10 μM, FC = 0.577).

Conclusions: 5-aza-CdR treatment inhibits cell proliferation, ECM formation, and Wnt/β-catenin signaling pathway targets in HULP cells, suggesting that DNA methylation inhibition is a viable therapeutic target in uterine leiomyoma.

Keywords: Uterine leiomyoma, Epigenetics, 5-aza-2′-deoxycitidine, Cell proliferation, Wnt/β-catenin pathway

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Background

Uterine leiomyomas (UL) are monoclonal benign tumors originating from smooth muscle cells located in the myometrium (MM) that affect to 25–30% of women of reproductive age [1, 2]. Prevalence of uterine leiomyomas in African American women is three times higher than Caucasian women [3, 4]. Around 30% of patients with leiomyomas present symptoms such as excessive uterine bleeding, anemia, pelvic pain, infertility, recurrent pregnancy loss, and/or preterm birth [5]. Although the gold-standard treatment for leiomyoma is surgical myomectomy or hysterectomy, other less invasive hormonal treatments, such as gonadotropin releasing-hormone agonist (aGnRH) [6] or ulipristal acetate (UPA) have been used to treat leiomyomas [7]. However, these treatments present side effects such as menopausal symptoms or hepatic damage [8], and once the treatment is stopped, leiomyomas enlarge again, in most cases recovering their initial size after 6 months [9]. For this reason, there is not an effective therapy to treat uterine leiomyoma with minimal side effects, despite being the most prevalent gynecological tumor.

Although pathogenesis of leiomyomas remains unclear, many factors have been proposed to contribute in the development of uterine leiomyomas. These factors include steroid hormones such as estrogens [10] and progesterone [11]; growth factors such as tumor growth factor beta (TGF-β), vascular endothelial growth factor (VEGF), acidic fibroblast growth factor (FGF), and basic FGF (bFGF) [12]; alterations of the Wnt/β-catenin pathway [13]; and genetic and epigenetic alterations [14]. Different subtypes of genetic mutations are described as a possible cause of leiomyoma development, including high mobility group AT-hook 2 (*HMGA2*) rearrangements, mediator complex subunit 12 (*MED12*) mutations, biallelic inactivation of fumarate hydratase (*FH*), and deletions affecting collagen type IV alpha 5 and alpha 6 (*COL4A5* and *COL4A6*) [15]. In addition, some environmental factors increase risk for developing uterine leiomyomas; these include high body mass index (BMI), meat consumption, alcohol consumption, hypertension, and vaginal infections [16–18]. These data suggest that epigenetic factors are also involved in the development of uterine leiomyoma. While genetic mutations are difficult to reverse, some epigenetic changes can be reversed, for example by chemical agents. This possibility suggests that there could be new therapeutic options for patients with UL.

DNA methylation is a widely studied epigenetic mark that consists of the addition of a methyl group to the 59-carbon of the cytosine ring through a covalent union within the CpG island after replication [19]. Specifically, methylation of a promoter CpG island triggers binding of proteins that condense the chromatin and make the promoter region inaccessible to transcription factors, blocking transcription initiation and, thereby, repressing gene expression [20]. DNA methylation is catalyzed by DNA methyltransferases (DNMT), of which there are three main types: DNMT1, DNMT3A, and DNMT3B [14]. DNMT1 maintains DNA methylation patterns during DNA replication; in contrast, DNMT3A and DNMT3B establish new methylation patterns and are therefore called de novo methyltransferases [21]. Aberrant DNA methylation, through oncogene hypomethylation and tumor suppressor gene hypermethylation, is involved in tumorigenesis of various cancer types, such as prostate cancer or neuroblastoma [22–25]. In UL, some studies reported differential expression of DNMTs and an aberrant DNA methylation compared with the adjacent MM, along with repression of tumor suppressor genes and other genes involved in cell cycle regulation, cell growth, migration, and extracellular matrix (ECM) formation [26–30]. Of particular interest is the Wnt/β-catenin pathway, which regulates proliferation, survival, migration and differentiation of many cell types and is dysregulated in numerous cancers [31–34] and uterine leiomyomas cells [7, 13, 35–37]. There is potential that aberrant DNA methylation affects this pathway in UL as it does in other tumor types, such as colorectal cancer [38]. For other tumors with altered DNA methylation that is related to disease progression, the use of DNMT inhibitors such as 5-aza-2′-deoxycytidine (5-aza-CdR) can offer effective treatment. 5-aza-CdR is a nucleoside analog that acts as a DNA demethylating agent through inhibition of DNMTs (DNMT1/3A/3B) [19], leading to changes in gene reactivation. 5-aza-CdR has been demonstrated that induce cell cycle arrest, inhibition of cell differentiation, and cell death by inhibiting post-replication methylation of DNA in human and mouse endometrium [39, 40]. Given the altered DNA methylation patterns and DNMT expression in UL, demethylation of human uterine leiomyoma cells by this DNMT inhibitor could reduce leiomyoma size, offering a new therapeutic option. However, the effect of 5-aza-CdR on UL cells is not well studied; further research is necessary to determine how this inhibitor affects the regulation of molecular mechanisms involved in leiomyoma development.

Here, we evaluated the effect of 5-aza-CdR in human uterine leiomyoma primary (HULP) cells on cell proliferation, apoptosis, ECM formation, and the Wnt/β-catenin pathway to test its potential as a new therapeutic option for leiomyoma.

Methods

Human tissue collection

Human UL and adjacent MM were collected from premenopausal women aged 31–48 years undergoing

myomectomy or hysterectomy due to symptomatic UL pathologies ($n = 16$) without any previous hormonal treatment for the last 3 months (Supplemental Table 1).

Ethical approval

This study was approved by Clinical Ethics Committee at Hospital Univeristario y Politecnico La Fe (Spain) (2018/0097), and all participants provided informed consent.

Human uterine leiomyoma primary cell isolation

UL and MM fragments were mechanically dissected into small pieces that were incubated at 37 °C with 2 mg/mL type II collagenase (Labclinics, Spain) and 1 mg/mL DNase I (Sigma-Aldrich, Saint Louis, MO) to obtain single-cell suspensions. Subsequently, cells were filtered through 50-μm polyethylene filters (Partec, Celltrics) to remove cellular clumps and undigested tissue. Isolated cells were incubated in vitro at 37 °C and 5% CO_2 in culture medium [Dulbecco's Modified Eagle Medium (DMEM)/F-12 (Gibco, Waltham, MA) with 10% fetal bovine serum (FBS) and antibiotic-antimycotic solution] for subsequent experiments of DNMT activity and 5-aza-CdR treatment.

Study of DNMT activity in vivo and in vitro

To evaluate DNMT activity over time in HULP and MM cells isolated from UL and adjacent MM tissue ($n = 3$), cells were incubated in culture medium for 7, 9, or 10 days. Subsequently, HULP and MM cells were collected, and nuclear protein extracts were isolated using EpiQuik Nuclear Extraction Kit (Epigentek, Brooklyn, NY), which was also used to obtain nuclear proteins from UL and MM tissues ($n = 7$). DNMT activity in tissues and in vitro cultured HULP and MM cells was measured with EpiQuik DNMT Activity/Inhibition ELISA Easy Kit immunoassay (Epigentek, Brooklyn, NY) using a microplate reader (Synergy HT, Bio-Tek). The ratio of methylated DNA was measured at a wavelength of 450 nm. The activity of DNMT enzymes, which is proportional to the optical density (OD) intensity measured, was calculated according to the formula provided by the manufacturer:

$$DNMT\ Activity\,(OD/h/mg) = \frac{(Sample\ OD – Blank\ OD)}{(Protein\ amount\ (\mu g) \times hour)} \times 100$$

5-aza-2′-deoxycitidine treatment

To determine the effect of 5-aza-CdR on HULP cells, cells were incubated in culture medium (day $0 = D_0$) until achieving a confluence of 70% (day $3 = D_3$). Then, they were starved in serum-free medium overnight and treated (day $4 = D_4$) with different doses of 5-aza-dC (Abcam, Cambridge, UK) ($n = 8$): 0 μM (control), 2 μM (low-dose), 5 μM 5-aza-CdR (middle-dose), 10 μM (high-dose) for 72 h (day $7 = D_7$). Medium was changed every 24 h.

Cell viability assay

HUPL cells ($n = 16$) were cultured in 96-well plate in culture medium; once 70% of confluence was achieved, cells were starved in serum-free medium overnight and treated (D_4) with 0, 2, 5, or 10 μM of the DNMT inhibitor 5-aza-CdR for 72 h (D_7). Medium was changed every 24 h. After this period, the quantity of viable cells in proliferation was measured with CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega, Madison, WI) and absorbance was measured on a microplate reader (Synergy HT, Bio-Tek) at 490 nm.

Protein extraction and western blot analysis

Proteins from HULP cells ($n = 8$) treated with/without 5-aza-CdR were extracted using radioimmunoprecipitation assay (RIPA) buffer containing protease inhibitors. Subsequently, HULP cells lysates (10 μg to 20 μg of protein) were analyzed by 12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to PVDF membranes. Western blot (WB) analysis was conducted to measure expression of ECM protein Plasminogen activator inhibitor-1 (PAI-I; sc-5297) (1:200), proliferating cell nuclear antigen (PCNA; sc-56) (1:200), Wnt1-inducible-signaling pathway protein 1 (WISP1; sc-133,126) (1:200), B-cell lymphoma-2 (BCL2; sc-7382) (1:200), and BCL2 associated-X (BAX; sc-20,067) (1:200) from Santa Cruz Biotechnology (Santa Cruz, CA). Expression of extracellular matrix proteins COLLAGEN I (COL-I;70R-CR007X) (1:1000, Fitzgerald, Acton, MA) and FIBRONECTIN (F3648) (1:2000, Sigma-Aldrich) was also evaluated. Antigen-antibody complex was detected with SuperSignal West Femto Maximun Sensitivity Substrate (ThermoFisher, Waltham, MA) and specific protein bands were visualized by chemiluminescence imaging using the LAS-3000 Imaging-System (Fujifilm, Tokyo, Japan). The intensity of each protein band was quantified with ImageJ software (National Institutes of Health, Bethesda, MD) and normalized in relation to its corresponding housekeeping protein, β-actin (1:1000; sc-47,778).

Gene expression analysis: DNMT and Wnt/β-catenin pathway

Total RNA from UL and MM tissues was extracted with TRIzol reagent (Fisher Scientific, Waltham, MA) for the study of *DNMT1* expression. Total RNA from HULP cells treated with/without 5-aza-CdR was obtained using

RNeasy Mini kit (Qiagen, Hilden, Germany) to assess expression of final targets in the Wnt/B-catenin pathway. cDNA was synthesized using PrimeScript RT reagent Kit (Takara, Japan). Quantitative real-time polymerase chain reaction (qRT-PCR) was performed with StepOnePlus System (Applied Biosystems, Foster City, CA) using PowerUp SYBR Green (Fisher Scientific, Waltham, MA). Gene expression of final targets of Wnt pathway, MYC proto-oncogene *(c-MYC)* and Matrix Metallopeptidase 7 (*MMP7),* was analyzed in HULP cells treated with and without 5-aza-CdR ($n = 8$). In addition, *DNMT1* expression was analyzed in UL and MM tissues ($n = 8$). Data were normalized with Glyceraldehyde-3-Phosphate Dehydrogenase (*GAPDH),* a housekeeping gene with minor variability. Primers were designed using Primer Quest Tool (DNA Integrated Technologies, Coralville,IA) (Supplemental Table II). ΔΔCt method was used to calculate fold change.

Statistical analysis

GraphPad Prism 8.0 was used for statistical analyses and graphic generation (San Diego, CA). Normality and logarithmic tests (Anderson-Darling, D'Agostino & Pearson, Shapiro-Wilk and Kolmogorov-Smirnov test) were performed to analyze the distribution of our results. A paired t-test was performed for DNMT activity study in UL vs MM tissue as well as HULP and MM cells. The Wilcoxon test for paired samples was performed to analyze qRT-PCR results of DNMT1 gene expression in UL tissue and adjacent MM. Repeated measures one-way ANOVA test with the Geisser-Greenhouse correction was performed for qRT-PCR analysis of multicomponent variants of *c-MYC* and *MMP7;* for WB analysis of WISP1, PCNA, and COL-I; and for cell viability assay of HULP and MM cells. Friedmann test was used to analyze BAX-BCL2 ratio, PAI-I, and FIBRONECTIN. Results of 5-aza-CdR treatment were normalized and compared with control HULP cells without treatment. Data are presented as mean ± standard deviation (SD). p-value < 0.05 was considered statistically significant.

Results

DNTM1 gene expression and DNMT activity in UL vs MM tissues

To examine whether increased global DNA methylation previously observed in UL vs MM was due to a higher expression of DNMT enzymes, *DNMT1* gene expression was analyzed by qRT-PCR in UL and MM tissues. UL tissue showed a statistically significant higher gene expression of *DNMT1* compared to MM tissue (fold change = 2.49, p-value = 0.0295) (Fig. 1A). Furthermore, DNMT activity was measured by ELISA in UL vs MM tissues to determine the correlation between gene expression and DNMT activity. Significantly higher DNMT activity was observed in UL tissue compared to adjacent

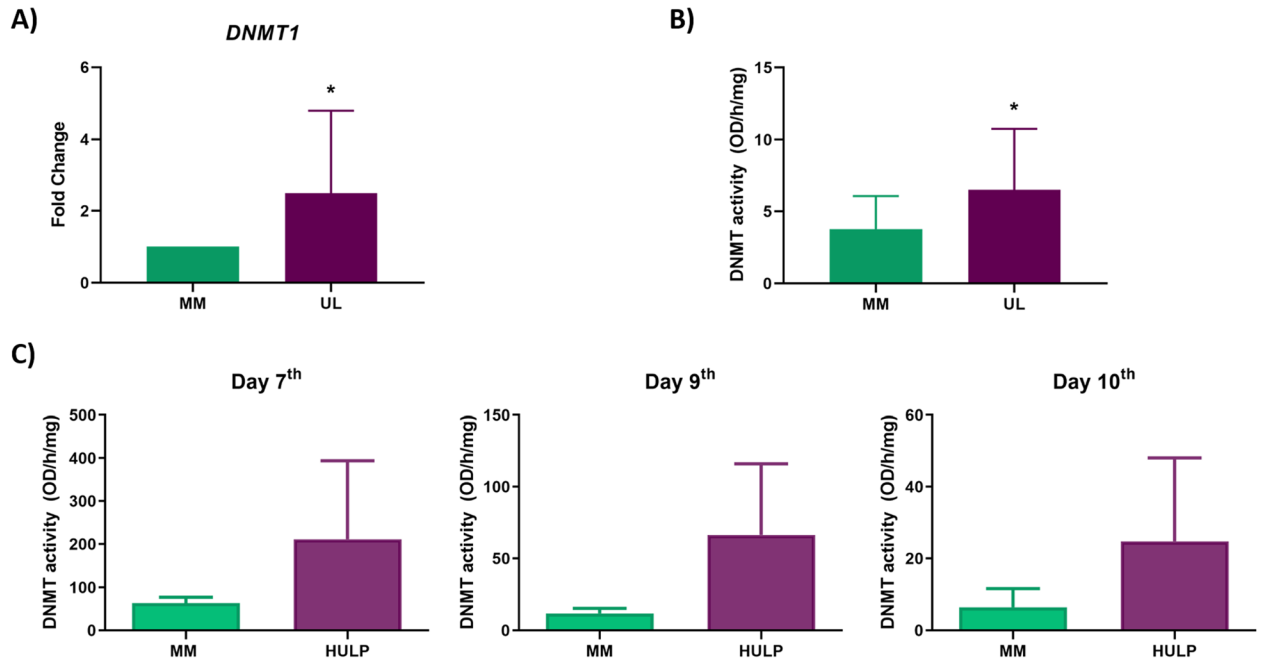

Fig. 1 DNMT status in UL compared with adjacent MM. **A** Gene expression levels of *DNMT1* in UL compared to MM tissues ($n = 14$) represented as fold change. **B** DNMT activity (OD/h/mg) of UL compared to MM tissues ($n = 7$). **C** Effects of cell culture on DNA methyltransferase (DNMT) activity of HULP and MM cells ($n = 3$) cultured for 7 days, 9 days, and 10 days. Data are expressed as the mean value ± standard error (SEM). * *p-value < 0.05*

MM tissue (6.50 vs 3.76 OD/h/mg, *p*-value = 0.026) (Fig. 1B).

DNMT activity in HULP and myometrial cells in vitro

Since epigenetic changes depend on the environment, experiments were conducted to determine the correlation between DNMT activity in vivo in UL and adjacent MM tissue and HULP and MM cells cultured in vitro. For this purpose, DNMT activity of HULP and MM cells was measured at 7, 9, and 10 days of in vitro culture. DNMT activity was greater in HULP cell compared to MM cells at day 7 (211.30 vs 63.67 OD/h/mg, *p*-value = 0.284), day 9 (66.41 vs 11.88 OD/h/mg, *p*-value = 0.217), and day 10 (24.75 vs 6.47 OD/h/mg, *p*-value = 0.337) (Fig. 1C). These results corroborated that the increased DNMT activity observed in UL compared to MM tissues is not modified in vitro by cell culture conditions (Fig. 1C).

In vitro effects of 5-aza-2′-deoxycytidine on cell proliferation in HULP cells

To demonstrate the antiproliferative effect of 5-aza-CdR on HULP cells, we measured the number of proliferating cells. Treatment with 5-aza-CdR decreased the percentage of viable HULP cells in a dose-dependent manner, being this decrease statistically significant with high-dose (10 μM) treatment in HULP cells (93.93% at 2 μM, *p*-value = 0.114; 92.15% at 5 μM, *p*-value = 0.053; 85.25% at 10 μM, *p*-value = 0.0001) compared to 0 μM (Fig. 2A).

Subsequently, to clarify whether this decrease in viable cells was due to cell death increase and/or to cell proliferation decrease, protein markers of apoptosis status and proliferation were analyzed in 5-aza-CdR-treated HULP cells by western blot. We measured BAX (proapoptotic) and BCL2 (anti-apoptotic) protein expression (Fig. 2B) and did not observe an increased apoptosis (no BCL2 upregulation or BAX downregulation). Moreover, we calculated the BAX/BCL2 ratio to determine the susceptibility to apoptosis in 5-aza-CdR-treated HULP cells compared with HULP cells without treatment (0 μM). Although the BAX/BCL2 ratio showed a trend to increase in HULP cells after treatment, no significant differences were observed with 2, 5, or 10 μM of 5-aza-CdR [fold change (FC) = 1.46, *p*-value = 0.099; FC = 1.54, *p*-value = 0.158; FC = 1.13, *p*-value = 0.099, respectively) compared to untreated cells (Fig. 2D). Therefore, 5-aza-CdR-treated HULP cells did not show a significant change in apoptosis. To further analyze the decrease in cell viability observed in 5-aza-CdR-treated HULP cells, we assessed effect of 5-aza-CdR on cell proliferation by analyzing protein expression of the gold-standard proliferation marker PCNA using western blot in 5-aza-CdR-treated and untreated HULP cells (Fig. 2C). 5-aza-CdR treatment decreased PCNA protein expression in HULP cells in a dose-dependent manner, reaching statistical

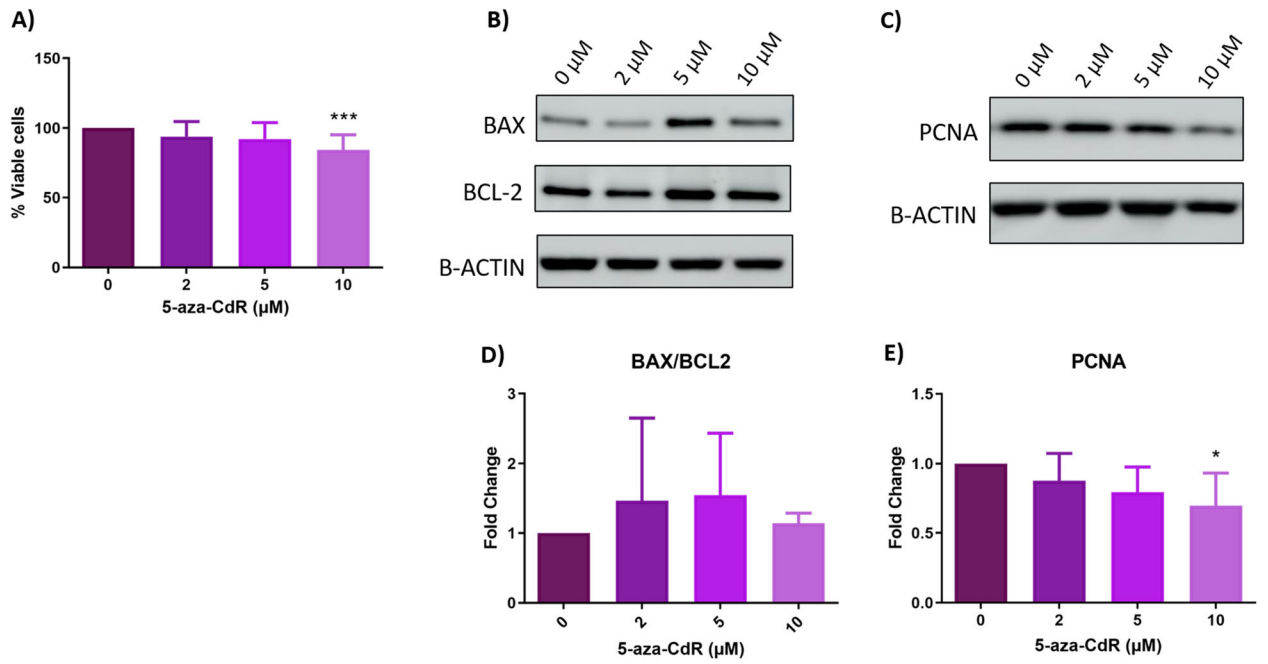

Fig. 2 Effect of 5-aza-CdR treatment on cell survival in HULP cells. **A** Percentage of viable HULP (*n* = 16) cells after 72 h of treatment with 0, 2, 5, or 10 μM of 5-aza-CdR. **B** Representative images of protein expression levels for BCL2 (23 kDa) and BAX (26 kDa) and **C** PCNA (36 kDa) analyzed in HULP (*n* = 8) after treatment with 5-aza-CdR at 0, 2, 5, or 10 μM (D-E) Means and standard deviations of normalized data of BAX/BCL2 ratio and PCNA, both represented as fold-change. * *p-value* < 0.05 *** *p-value* < 0.001

significance at the high dose (2 μM FC = 0.877, *p*-value = 0.099; 5 μM FC = 0.794, *p*-value = 0.055; 10 μM FC = 0.695, *p*-value = 0.034, respectively) (Fig. 2E).

In vitro effects of 5-aza-2′-deoxycytidine on extracellular matrix in HULP cells

To evaluate ECM status in HULP cells after 5-aza-CdR treatment, expression of proteins involved in ECM formation, such as COLLAGEN I, FIBRONECTIN, and PAI-1, was determined by western blot (Fig. 3A, B, and C, respectively). 5-aza-CdR treatment decreased COLLAGEN I expression in a dose-dependent manner, reaching statistical significance at 10 μM (FC = 0.654, *p*-value = 0.023; Fig. 3D). In addition, a tend to decrease COLLAGEN I was observed at 2 μM and 5 μM (FC = 0.6540.897, *p*-value = 0.729; FC = 0.675, *p*-value = 0.104, respectively) (Fig. 3D). Although no changes were found in FIBRONECTIN expression at 5 μM (FC = 0.826, *p*-value = 0.244), a statistically significant reduction was observed at 2 μM (FC = 0.812, *p*-value = 0.020) and 10 μM (FC = 0.733, *p*-value = 0.035) (Fig. 3E). Finally, PAI-1 expression was significantly decreased compared to control group (0 μM) after 5-aza-CdR treatment at 5 μM and 10 μM (FC = 0.865, *p*-value = 0.035; FC = 0.766, *p*-value = 0.020, respectively) (Fig. 3F). No statistically significant differences were found at 2 μM 5-aza-CdR (FC = 0.885, *p*-value = 0.244). These results showed that 5-aza-CdR inhibited ECM protein expression in HULP cells in vitro in a dose-dependent manner.

In vitro effects of 5-aza-2′-deoxycytidine on Wnt/β-catenin signaling pathway in HULP cells

To assess the effect of 5-aza-CdR treatment on Wnt/β-catenin pathway on HULP cells, we measured the protein expression of WISP1, a Wnt/β-catenin target protein, in HULP cells in presence and absence of 5-aza-CdR treatment by western blot. Results revealed an inhibition of WISP1 protein expression in HULP cells treated with 5-aza-CdR compared to control group (0 μM) in a dose-dependent manner treatment (Fig. 4A), reaching statistical significance at 10 μM (2 μM FC = 0.903, *p*-value = 0.408; 5 μM FC = 0.860, *p*-value = 0.071; 10 μM FC = 0.699, *p*-value = 0.026, respectively) (Fig. 4B).

To further analyze the regulation of Wnt/β-catenin signaling pathway by 5-aza-CdR treatment in HULP cells, gene expression levels of *c-MYC* and *MMP7*, as final targets of Wnt/β-catenin pathway, were determined in these cells by qRT-PCR. *c-MYC* was significantly downregulated in HULP cells after 5-aza-CdR treatment at all doses tested compared to HULP cells without treatment, reaching significance at 2 μM and 10 μM doses (2 μM FC = 0.745, *p*-value = 0.028; 10 μM FC = 0.728, *p*-value = 0.019, respectively) (Fig. 4C), while no significant differences were found at 5 μM (FC = 0.810, *p*-value = 0.287). Finally, a significantly decrease of *MMP7* expression was observed in HULP cells after 5-aza-CdR treatment with 5 μM and 10 μM compared to HULP without treatment (5 μM FC = 0.520, *p*-value = 0.003, 10 μM FC = 0.577, *p*-value = 0.007, respectively)

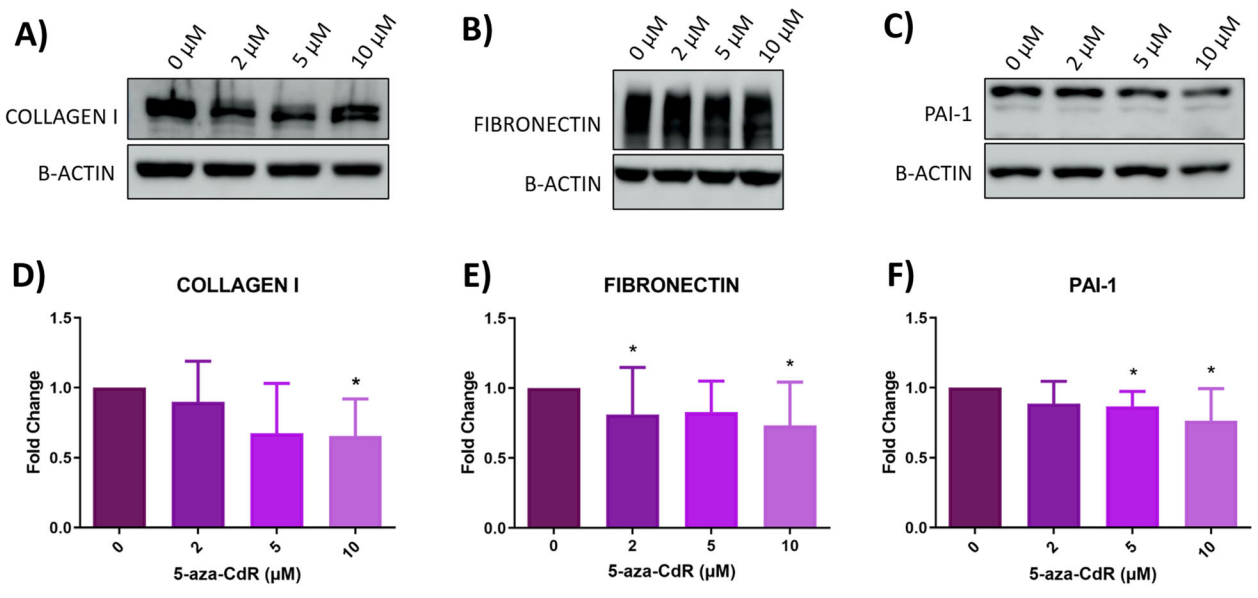

Fig. 3 Extracellular matrix evaluation in HULP cells after 5-aza-CdR treatment. Representative images of protein expression levels for **A** COLLAGEN I (140 kDa), **B** FIBRONECTIN (220 kDa), and **C** PAI-1 (50 kDa) and normalized quantitative protein expression of **D** COLLAGEN I, **E** FIBRONECTIN, and **F** PAI-1 in the HULP cells at different treatment groups with 5-aza-CdR at 0, 2, 5, or 10 μM for 72 h ($n = 8$). Data are represented as mean and deviations of fold change. * *p-value < 0.05*

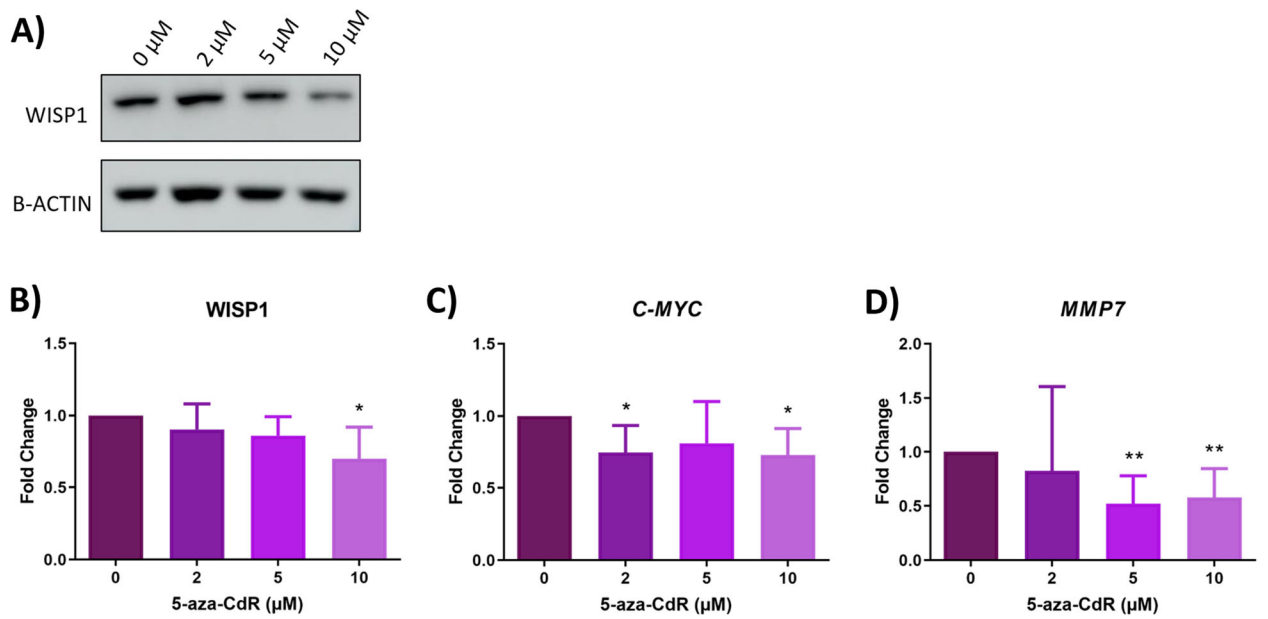

Fig. 4 Wnt/β-catenin signaling pathway analysis in HULP cells after DNMT inhibition with 5-aza-CdR treatment. **A** Representative images of protein expression for WISP1 in HULP cells after treatment with 5-aza-CdR at 0, 2, 5, or 10 μM for 72 h. **B** Normalized quantitative protein expression of WISP1 represented as mean and deviations of fold-change. Gene expression levels of **C** *c-MYC* and **D** *MMP7* in HULP cells treated for 72 h with 2, 5, or 10 μM of 5-aza-CdR compared to untreated HULP cells (0 μM), represented as fold-change. * *p-value* < *0.05* ** *p-value* < *0.01*

(Fig. 4D). No significant differences were found in *MMP7* expression at 2 μM (FC = 0.824, *p*-value = 0.860). Thus, 5-aza-CdR inhibited final targets of Wnt/β-catenin pathway in HULP cells in vitro in a dose-dependent manner.

Discussion

Our study shows that 5-aza-2′-deoxycitidine inhibits cell proliferation, ECM formation, and Wnt/β-catenin pathway in human uterine leiomyoma primary cells, suggesting DNA methyltransferases inhibitors as a new effective option for uterine leiomyoma treatment. UL is a multifactorial disease with an unclear pathogenesis and ineffective treatment [6–9]. For this reason, determining the molecular mechanisms involved in UL growth is necessary to better understand its pathogenesis, as well as define molecular targets to develop new therapeutic options against them. Factors contributing to UL pathogenesis include genetic mutation, epigenetic modifications, and several growth factors [14]. In addition, estrogen and progesterone are recognized as promoters of UL growth [14]. Accordingly, a significant growth of UL during the first trimester of pregnancy is associated with hormonal changes [41]. In this regard, epigenetic modifications could play an important role in steroid hormonal "cross-talk" for UL development and growth [14, 42]. Epigenetics is emerging as a new hallmark of tumor development with a high therapeutic application because of its potential for reversal [23, 25]. In fact, abnormal DNA methylation is found in UL compared to MM tissue, where tumor suppressor genes are hypermethylated, which contributes to the development of this tumor [14, 21, 27–30]. Based on these findings, we focused our study on DNMT methylation reversion by DNMT inhibitor 5-aza-2′-deoxycitidine as new therapeutic option for UL.

For this purpose, we first studied DNMT expression and activity of UL and MM tissue and we corroborated that there is a higher *DNMT1* expression and DNMT activity in UL compared to MM tissue. Interestingly, similar findings were reported by others, who associated the aberrant DNA methylation found in UL with an increased DNMT1 and DNMT3a mRNA expression in tumor samples compared to myometrium [26, 27]. Based on these finding, the use of DNMT inhibitors to reverse the aberrant DNA methylation found in UL could be a good therapeutic approach to treat them. Secondly, since epigenetic changes depend on the environment, we studied DNMT expression and activity in cell culture to confirm that an in vitro model maintains the epigenetic feature from origin tissue. We proved for the first time that the increased DNMT activity observed in UL tissue is maintained under cell culture conditions over time in HULP compared to MM cells, confirming the reproducibility of the in vitro model. This approach allows the study of epigenetic modifications such as DNMT methylation reversion using DNMT inhibitor.

In this regard, the use of DNMT inhibitor 5-aza-CdR is widely described in several tumor types, such as colorectal, bladder, and pancreatic cancer, demonstrating antiproliferative effects [38, 43, 44]. Uncontrolled proliferation is one of the hallmarks of cancer, and tumorigenic cells present greater proliferation and lesser apoptosis than normal cells. Accordingly, several authors showed that UL growth is due to an increased proliferation of UL cells [7, 13]. Since UL is associated with aberrant DNA methylation and increased cell proliferation, reversion of DNMT methylation by 5-aza-CdR could be an efficient treatment to reduce UL size due to its antiproliferative effects. Here, we found that 5-aza-CdR treatment of HULP cells inhibited the number of proliferating cells, and this decrease was due to a reduction in cell proliferation, as demonstrated by decreased PCNA protein expression. These findings demonstrated the antiproliferative effect in UL previously described in different tumors [38, 43, 44]. Meanwhile, apoptosis did not increase after 5-aza-CdR treatment, suggesting that this treatment would not be toxic for HULP cells. Based on our results, 5-aza-CdR would decrease cell proliferation in HULP cells, highlighting its potential as a therapeutic option to reduce UL growth.

UL growth is due not only to cell proliferation, but also to an excessive synthesis and deposition of ECM. Thus, evaluating the effect of DNMT inhibitors on ECM protein expression is necessary to assess the possible use of these inhibitors as a new therapeutic option for UL. Our results showed that 5-aza-CdR treatment significantly decreased the expression of ECM-associated proteins such as FIBRONECTIN, COLLAGEN I, and PAI-1. These findings suggested an important role of 5-aza-CdR in the regulation of key fibrotic proteins involved in UL expansion. In line with this, other studies demonstrated the success of treatments targeting ECM formation as possible therapeutic option to reduce UL grown, such as Vitamin D treatment in the Eker rat model [45], in a xenograft mouse model [46], and in human leiomyoma cells [47]. Hence, 5-aza-CdR reduced ECM expansion and cell proliferation in HULP cells, which counteracts UL growth, offering a promising candidate to treat this tumor.

Wnt/β-catenin pathway is involved in several cellular functions, being a key regulator of cell proliferation and ECM production. This pathway is dysregulated in several cancers [31–34, 48] and UL [7, 13, 35]. This pathway's implications in colorectal cancer (CRC) are well studied [49]. In CRC, the effect of 5-aza-CdR in reduction of cell self-renewal through Wnt/β-catenin pathway inhibition is demonstrated, showing the important role of epigenetic modification on Wnt/β-catenin pathway regulation and its implication in cancer development [38]. In UL, previous studies suggested that targeting Wnt/β-catenin pathway can be a promising therapeutic approach because of their aberrant activation in UL cells compared to MM cells [13, 36, 37, 50]. Due to the increased activation of Wnt/β-catenin pathway and aberrant DNA methylation found in UL, we analyzed if DNA methylation reversion by 5-aza-CdR treatment had an effect on the Wnt/β-catenin pathway in HULP cells. We assessed protein or mRNA expression of final targets of the Wnt/β-catenin pathway, such as WISP1, *c-MYC,* and *MMP7,* because of their tight association with tumor progression. We found that both *c-MYC* gene expression and WISP1 protein expression were downregulated in treated HULP cells. *c-MYC* is one of the most studied final targets of the Wnt/β-catenin pathway. It is a protooncogene involved in cell cycle progression [51] whose deregulation has been linked to an aberrant Wnt/β-catenin pathway expression in CRC [52]. WISP1 has been identified as an oncogene in several cancer types such as glioblastoma [53], CRC [54], and colon cancer [55], with involvement in tumor proliferation, migration, and poor prognosis. Inhibition of WISP1 decreases cell proliferation and invasion through increasing apoptosis and blocking cell cycle in glioblastoma and colon cancer cells [53, 55]. Therefore, the reduction of WISP1 and *c-MYC* expression observed in our study suggest that DNMT inhibition by 5-aza-CdR could impede cell proliferation via Wnt/β-catenin pathway inhibition. Finally, *MMP7* is a metalloprotease involved in ECM physiology, epithelial-mesenchymal transition, and tumor invasion that is overexpressed in some cancer types [56]. In this regard, a study in hepatocellular carcinomas demonstrated the inhibition of cell migration and invasion after diminishing MMP7 [57]. Accordingly, we observed a decrease in *MMP7* gene expression after 5-aza-CdR treatment in HULP cells, suggesting a lower invasive capacity of HULP-treated cells via Wnt/β-catenin pathway inhibition.

Based on these findings, we suggest that DNA methylation is involved in Wnt/β-catenin pathway regulation and, consequently, in cell proliferation and ECM formation in HULP cells, proposing 5-aza-CdR as a treatment to reduce uterine leiomyoma growth. Further studies are necessary to determine the role of DNA methylation on Wnt/β-catenin pathway, as well as the in vivo effect of 5-aza-CdR on UL.

Conclusions

Our study demonstrated for the first time that 5-aza-CdR reduces cell proliferation, ECM formation, and Wnt/β-catenin pathway in HULP cells in vitro. Yet, apoptosis appears unaffected. Based on our findings, we suggest DNMT inhibitors such as 5-aza-CdR could offer a new therapeutic option to treat patients with uterine leiomyoma.

Abbreviations

5-aza-CdR: 5-aza-2′-deoxycytidine.; AGnRH: Gonadotropin releasing-hormone agonist; BAX: BCL2 associated-X; BCL2: B-cell lymphoma-2; bFGF: Basic FGF; BMI: Body mass index; c-MYC: MYC proto-oncogene; COL4A5: Collagen type

IV alpha 5; COL4A6: Collagen type IV alpha 6; COL-I : Collagen I; CRC: Colorectal cancer; DNMT: DNA methyltransferase; ECM: Extracellular matrix; FBS: Fetal bovine serum; FGF: Acidic fibroblast growth factor; FH: Fumarate hydratase; GAPDH: Glyceraldehyde-3-Phosphate Dehydrogenase; HMGA2: High mobility group AT-hook 2; HULP: Human uterine leiomyoma primary; MED12: Mediator complex subunit 12; MM: Adjacent myometrium; MMP7: Matrix metalloproteinase 7; PAI-I: Plasminogen activator inhibitor-1; PCNA: Proliferating cell nuclear antigen; RIPA: Radioimmunoprecipitation assay; TGF-β: Tumor growth factor beta; UL: Uterine leiomyoma; UPA: Ulipristal acetate; VEGF: Vascular endothelial growth factor; WB: Western blot; WISP1: Wnt1-inducible-signaling pathway protein 1

Supplementary Information

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Additional file 1.

Additional file 2.

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Authors' contributions

M.C.C.-G. was involved in study design, executed experiments, and wrote and edited the manuscript. A.C. was involved in experimental execution and wrote the manuscript. M.S. analyzed results and executed QT-RT-PCR to study gene expression. J. E and J.M. were involved in selection and recruitment of UL patients and sample collection by processing UL and MM biopsies at surgery. A.F. was involved in sample collection by participating in HULP cell isolation. A.P. devised and supervised the study, contributed to data interpretation, and drafted the manuscript. I.C. and H. F coordinated the study design, contributed to data interpretation, and edited the manuscript. All authors reviewed the manuscript and provided critical feedback and discussion. The author(s) read and approved the final manuscript.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article [and its supplementary information files].

Declarations

Ethics approval and consent to participate

This study was approved by the Clinical Ethics Committee at the Hospital Univeristario y Politecnico La Fe (Spain) (2018/0097), and all participants provided informed consent.

Consent for publication

Yes.

Competing interests

The authors have no conflicts of interest to declare.

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References

1. Stewart EA. Uterine fibroids. Lancet. 2001;357:293–8 Elsevier Limited.
2. Parker WH. Etiology, symptomatology, and diagnosis of uterine myomas. Fertil Steril. 2007;87:725–36.
3. Marshall LM, Spiegelman D, Barbieri RL, Goldman MB, Manson JE, Colditz GA, et al. Variation in the incidence of uterine leiomyoma among premenopausal women by age and race. Obstet Gynecol. 1997;90:967–73.
4. Baird DD, Dunson DB, Hill MC, Cousins D, Schectman JM. High cumulative incidence of uterine leiomyoma in black and white women: ultrasound evidence. Am J Obstet Gynecol. 2003;188:100–7 Mosby Inc.
5. Gupta S, Jose J, Manyonda I. Clinical presentation of fibroids. Best Pract Res Clin Obstet Gynaecol. 2008;22:615–26.
6. Parker JD, Malik M, Catherino WH. Human myometrium and leiomyomas express gonadotropin-releasing hormone 2 and gonadotropin-releasing hormone 2 receptor. Fertil Steril. 2007;88:39–46.
7. Bulun SE. Uterine fibroids. N Engl J Med. 2013;369:1344–55.
8. Manyonda I, Sinthamoney E, Belli AM. Controversies and challenges in the modern management of uterine fibroids. BJOG An Int J Obstet Gynaecol. 2004;111:95–102.
9. Friedman AJ. Treatment of leiomyomata uteri with short-term leuprolide followed by leuprolide plus estrogen-progestin hormone replacement therapy for 2 years: a pilot study. Fertil Steril. 1989;51:526–8.
10. Maruo T, Ohara N, Wang J, Matsuo H. Sex steroidal regulation of uterine leiomyoma growth and apoptosis. Hum Reprod Update. 2004;10:207–20.
11. Patel B, Elguero S, Thakore S, Dahoud W, Bedaiwy M, Mesiano S. Role of nuclear progesterone receptor isoforms in uterine pathophysiology. Hum Reprod Update. 2015;21:155–73 Oxford University Press.
12. Sozen I, Arici A. Interactions of cytokines, growth factors, and the extracellular matrix in the cellular biology of uterine leiomyomata. Fertil Steril. 2002;78:1–12.
13. Ono M, Yin P, Navarro A, Moravek MB, Coon VJS, Druschitz SA, et al. Paracrine activation of WNT/β-catenin pathway in uterine leiomyoma stem cells promotes tumor growth. Proc Natl Acad Sci U S A. 2013;110:17053–8.
14. Laganà AS, Vergara D, Favilli A, La Rosa VL, Tinelli A, Gerli S, et al. Epigenetic and genetic landscape of uterine leiomyomas: a current view over a common gynecological disease. Arch Gynecol Obstet. 2017;296:855–67 Springer Verlag.
15. Mehine M, Kaasinen E, Heinonen HR, Mäkinen N, Kämpjärvi K, Sarvilinna N, et al. Integrated data analysis reveals uterine leiomyoma subtypes with distinct driver pathways and biomarkers. Proc Natl Acad Sci U S A. 2016;113:1315–20 National Academy of Sciences.
16. Chiaffarino F, Parazzini F, La Vecchia C, Chatenoud L, Di Cintio E, Marsico S. Diet and uterine myomas. Obstet Gynecol. 1999;94:395–8 Elsevier Inc.
17. Faerstein E, Szklo M, Rosenshein NB. Risk factors for uterine leiomyoma: a practice-based case-controls study. II. Atherogenic risk factors and potential sources of uterine irritation. Am J Epidemiol. 2001;153:11–9.
18. Faerstein E, Szklo M, Rosenshein N. Risk factors for uterine leiomyoma: a practice-based case-control study. I. African-American heritage, reproductive history, body size, and smoking. Am J Epidemiol. 2001;153:1–10.
19. Lopez M, Halby L, Arimondo PB. DNA methyltransferase inhibitors: development and applications. Adv Exp Med Biol. 2016;945:431–73 Springer New York LLC.
20. Cedar H, Bergman Y. Linking DNA methylation and histone modification: patterns and paradigms. Nat Rev Genet. 2009;10:295–304.
21. Yang Q, Mas A, Diamond MP, Al-Hendy A. The mechanism and function of epigenetics in uterine leiomyoma development. Reprod Sci. 2016;23:163–75 SAGE Publications Inc.
22. Braný D, Dvorská D, Grendár M, Ňachajová M, Szépe P, Lasabová Z, et al. Different methylation levels in the KLF4, ATF3 and DLEC1 genes in the myometrium and in corpus uteri mesenchymal tumours as assessed by MS-HRM. Pathol Res Pract. 2019;215:152465 Elsevier GmbH.
23. Klutstein M, Nejman D, Greenfield R, Cedar H. DNA methylation in cancer and aging. Cancer Res. 2016;76:3446–50 American Association for Cancer Research Inc.

24. Kobayashi Y, Absher DM, Gulzar ZG, Young SR, McKenney JK, Peehl DM, et al. DNA methylation profiling reveals novel biomarkers and important roles for DNA methyltransferases in prostate cancer. Genome Res. 2011;21: 1017–27.
25. Yang Q, Liu S, Tian Y, Hasan C, Kersey D, Salwen HR, et al. Methylation-associated silencing of the heat shock protein 47 gene in human neuroblastoma. Cancer Res. 2004;64:4531–8.
26. Li S, Chiang TC, Richard-Davis G, Barrett JC, Mclachlan JA. DNA hypomethylation and imbalanced expression of DNA methyltransferases (DNMT1, 3A, and 3B) in human uterine leiomyoma. Gynecol Oncol. 2003;90: 123–30 Academic Press Inc.
27. Yamagata Y, Maekawa R, Asada H, Taketani T, Tamura I, Tamura H, et al. Aberrant DNA methylation status in human uterine leiomyoma. Mol Hum Reprod. 2009;15:259–67.
28. Navarro A, Yin P, Monsivais D, Lin SM, Du P, Wei JJ, et al. Genome-wide DNA methylation indicates silencing of tumor suppressor genes in uterine leiomyoma. PLoS One. 2012;7:e33284.
29. Maekawa R, Sato S, Yamagata Y, Asada H, Tamura I, Lee L, et al. Genome-wide DNA methylation analysis reveals a potential mechanism for the pathogenesis and development of uterine leiomyomas. PLoS One. 2013;8: e66632.
30. Sato S, Maekawa R, Yamagata Y, Tamura I, Lee L, Okada M, et al. Identification of uterine leiomyoma-specific marker genes based on DNA methylation and their clinical application. Sci Rep. 2016;6:1 Nature Publishing Group.
31. Clevers H, Nusse R. Wnt/β-catenin signaling and disease. Cell. 2012;149: 1192–205.
32. Garg M, Maurya N. WNT/β-catenin signaling in urothelial carcinoma of bladder. World J Nephrol. 2019;8:83–94 Baishideng Publishing Group Inc.
33. Shang S, Hua F, Hu ZW. The regulation of β-catenin activity and function in cancer: therapeutic opportunities. Oncotarget. 2017;8:33972–89 Impact Journals LLC.
34. Zhan T, Rindtorff N, Boutros M. Wnt signaling in cancer. Oncogene. 2017;36: 1461–73 Nature Publishing Group.
35. Borahay MA, Al Hendy A, Kilic GS, Boehning D. Signaling pathways in leiomyoma: understanding pathobiology and implications for therapy. Mol Med. 2015;21:242–56 Uninversity of Michigan.
36. Corachán A, Ferrero H, Aguilar A, Garcia N, Monleon J, Faus A, et al. Inhibition of tumor cell proliferation in human uterine leiomyomas by vitamin D via Wnt/β-catenin pathway. Fertil Steril. 2019;111:397–407 Elsevier Inc.
37. Al-Hendy A, Diamond MP, Boyer TG, Halder SK. Vitamin D3 inhibits Wnt/β-catenin and mTOR signaling pathways in human uterine fibroid cells. J Clin Endocrinol Metab. 2016;101:1542–51 Endocrine Society.
38. Li S, Han Z, Zhao N, Zhu B, Zhang Q, Yang X, et al. Inhibition of DNMT suppresses the stemness of colorectal cancer cells through down-regulating Wnt signaling pathway. Cell Signal. 2018;47:79–87 Elsevier Inc.
39. Logan PC, Ponnampalam AP, Rahnama F, Lobie PE, Mitchell MD. The effect of DNA methylation inhibitor 5-Aza-2′-deoxycytidine on human endometrial stromal cells. Hum Reprod. 2010;25:2859–69 Oxford University Press.
40. Bin DY, Long CL, Liu XQ, Chen XM, Guo LR, Xia YY, et al. 5-Aza-2′-deoxycytidine leads to reduced embryo implantation and reduced expression of DNA methyltransferases and essential endometrial genes. PLoS One. 2012;7:e45364.
41. Vitagliano A, Noventa M, Di Spiezio SA, Saccone G, Gizzo S, Borgato S, et al. Uterine fibroid size modifications during pregnancy and puerperium: evidence from the first systematic review of literature. Arch Gynecol Obstet. 2018;297:823–35 Springer Verlag.
42. Asada H, Yamagata Y, Taketani T, Matsuoka A, Tamura H, Hattori N, et al. Potential link between estrogen receptor-α gene hypomethylation and uterine fibroid formation. Mol Hum Reprod. 2008;14:539–45.
43. Nunes SP, Henrique R, Jerónimo C, Paramio JM. DNA methylation as a therapeutic target for bladder cancer. Cells. 2020;9:1850 NLM (Medline).
44. Wong KK. DNMT1 as a therapeutic target in pancreatic cancer: mechanisms and clinical implications. Cell Oncol. 2020;43:779–92 Springer Science and Business Media B.V.
45. Halder SK, Sharan C, Al-Hendy A. 1,25-Dihydroxyvitamin D3 treatment shrinks uterine leiomyoma tumors in the Eker rat model. Biol Reprod. 2012; 86:116.
46. Corachán A, Ferrero H, Escrig J, Monleon J, Faus A, Cervelló I, et al. Long-term vitamin D treatment decreases human uterine leiomyoma size in a xenograft animal model. Fertil Steril. 2020;113:205–216.e4 Elsevier Inc.
47. Halder SK, Osteen KG, Al-Hendy A. 1,25-Dihydroxyvitamin D3 reduces extracellular matrix-associated protein expression in human uterine fibroid cells. Biol Reprod. 2013;89:150 Society for the Study of Reproduction.
48. Cheng Y-Y, Huang N-C, Chang Y-T, Sung J-M, Shen K-H, Tsai C-C, et al. Associations between arsenic in drinking water and the progression of chronic kidney disease: a nationwide study in Taiwan. J Hazard Mater. 2017; 321:432–9 Department of Environmental and Occupational Health, College of Medical, National Cheng Kung University, Tainan, Taiwan: Elsevier.
49. Deitrick J, Pruitt WM. Wnt/β catenin-mediated signaling commonly altered in colorectal cancer. Prog Mol Biol Transl Sci. 2016;144:49–68 Elsevier B.V.
50. Ali M, Shahin SM, Sabri NA, Al-Hendy A, Yang Q. Activation of β-catenin signaling and its crosstalk with estrogen and histone deacetylases in human uterine fibroids. J Clin Endocrinol Metab. 2020;105:E1517–35 Endocrine Society.
51. Zakiryanova GK, Wheeler S, Shurin MR. Oncogenes in immune cells as potential therapeutic targets. ImmunoTargets Ther. 2018;7:21–8 Dove Medical Press Ltd.
52. Rennoll S. Regulation of MYC gene expression by aberrant Wnt/β-catenin signaling in colorectal cancer. World J Biol Chem. 2015;6:290 Baishideng Publishing Group Inc.
53. Jing D, Zhang Q, Yu H, Zhao Y, Shen L. Identification of WISP1 as a novel oncogene in glioblastoma. Int J Oncol. 2017;51:1261–70 Spandidos Publications.
54. Jiang. Differential expression of the CCN family member WISP-1, WISP-2 and WISP-3 in human colorectal cancer and the prognostic implications. Int J Oncol. 2010;36:1129 Spandidos Publications.
55. Wu J, Long Z, Cai H, Du C, Liu X, Yu S, et al. High expression of WISP1 in colon cancer is associated with apoptosis, invasion and poor prognosis. Oncotarget. 2016;7:49834–47 Impact Journals LLC.
56. Scheau C, Badarau IA, Costache R, Caruntu C, Mihai GL, Didilescu AC, et al. The role of matrix metalloproteinases in the epithelial-mesenchymal transition of hepatocellular carcinoma. Anal Cell Pathol. 2019;2019:9423907 Hindawi Limited.
57. Lin Y, Liu J, Huang Y, Liu D, Zhang G, Kan H. microRNA-489 plays an anti-metastatic role in human hepatocellular carcinoma by targeting matrix metalloproteinase-7. Transl Oncol. 2017;10:211–20 Neoplasia Press, Inc.

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Deciphering the Role of Histone Modifications in Uterine Leiomyoma: Acetylation of H3K27 Regulates the Expression of Genes Involved in Proliferation, Cell Signaling, Cell Transport, Angiogenesis and Extracellular Matrix Formation

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Abstract: Uterine leiomyoma (UL) is a benign tumor arising from myometrium (MM) with a high prevalence and unclear pathology. Histone modifications are altered in tumors, particularly via histone acetylation which is correlated with gene activation. To identify if the acetylation of H3K27 is involved in UL pathogenesis and if its reversion may be a therapeutic option, we performed a prospective study integrating RNA-seq (n = 48) and CHIP-seq for H3K27ac (n = 19) in UL vs MM tissue, together with qRT-PCR of SAHA-treated UL cells (n = 10). CHIP-seq showed lower levels of H3K27ac in UL versus MM (p-value < 2.2×10^{-16}). From 922 DEGs found in UL vs. MM (FDR < 0.01), 482 presented H3K27ac. A differential acetylation (FDR < 0.05) was discovered in 82 of these genes (29 hyperacetylated/upregulated, 53 hypoacetylated/downregulated). Hyperacetylation/upregulation of oncogenes (*NDP,HOXA13,COL24A1,IGFL3*) and hypoacetylation/downregulation of tumor suppressor genes (*CD40,GIMAP8,IL15,GPX3,DPT*) altered the immune system, the metabolism, TGFβ3 and the Wnt/β-catenin pathway. Functional enrichment analysis revealed deregulation of proliferation, cell signaling, transport, angiogenesis and extracellular matrix. Inhibition of histone deacetylases by SAHA increased expression of hypoacetylated/downregulated genes in UL cells (p < 0.05). Conclusively, H3K27ac regulates genes involved in UL onset and maintenance. Histone deacetylation reversion upregulates the expression of tumor suppressor genes in UL cells, suggesting targeting histone modifications as a therapeutic approach for UL.

Keywords: histone modification; gene expression; angiogenesis; extracellular matrix; uterine leiomyoma

1. Introduction

Uterine leiomyomas (ULs) are monoclonal benign tumors arising from the myometrium (MM) that affect up to 25–30% of women of reproductive age [1,2]. Around 30% of these patients present symptoms, such as excessive uterine bleeding, infertility, or recurrent abortion [3]. Although the gold standard treatment is surgery, less invasive hormonal treatments are sometimes used to treat leiomyomas [4,5]. However, these treatments cause side effects such as menopausal symptoms or hepatic damage [6], and, once treatment is stopped, leiomyomas enlarge again [7]. For this reason, no effective therapy with minimal side effects is currently available to treat UL. The lack of efficient treatment could be because available medical options focus on the relief of symptoms and not in mechanisms impli-

cated in UL development [8]. Therefore, identification of molecular mechanisms involved in UL pathogenesis could allow the development of new and more efficient treatments.

Although UL pathogenesis remains incompletely understood, many factors contribute to its development, including steroid hormones, growth factors and genetics. ULs develop after menarche and regress after menopause [9,10]. Their growth is affected by the concentrations of steroid hormones, especially estrogen and progesterone. Estrogen and progesterone act on the tissue's mature cells and, through them, send paracrine factors to the stem cell population inducing its proliferation [11]. Therefore, UL and MM growth is dependent on these hormones. For this reason, hormonal treatments based on the inhibition of estrogen and progesterone production, such as gonadotropin releasing-hormone agonist (aGnRH), have been used for UL treatment [5]. In addition, genetic mutations have been described as a possible cause of UL development. In this regard, there are four established molecular subtypes of UL per mutations on different genetic drivers: MED12 mutations (70–75% of patients with UL [12]), HMGA2 rearrangements (20% of UL patients [13]), biallelic inactivation of FH (10.5% of UL patients [14]), and deletions affecting COL4A5 and COL4A6 (4% of UL patients [13]). However, factors such as race, diet, age, body mass index, and parity are also risk factors for UL [15], suggesting potential involvement of epigenetic mechanisms in UL development. Epigenetics include variations in the gene expression profile not caused by changes in the DNA sequence, resulting from processes such as DNA methylation, histone modification, and non-coding RNAs [16]. Genome-wide DNA methylation studies have revealed subsets of suppressed or overexpressed genes accompanied by aberrant promoter methylation [17–19], also evaluating DNA methylation in UL focusing on stem cell population [20] or mutation status [21]. Furthermore, differential promoter access resulting from altered 3D chromatin structure and histone modifications plays a role in regulating transcription of key genes thought to be involved in leiomyoma etiology [22]. These modifications are inherited somatically and are dynamic and reversible, which make them potential therapeutic targets.

Modification of histone proteins is a key epigenetic mechanism implied in the regulation of gene expression. These modifications occur at the N-terminal tail or the globular domains of core histones [23]. Epigenetic modifications of histone tails include acetylation, methylation, phosphorylation, ubiquitination, and SUMOylation. Histone acetylation is correlated with gene activation, whereas loss of acetylation (deacetylation) represses gene expression [24]. The enzymes participating in the addition of an acetyl group to histones are histone acetyltransferases (HATs), while histone deacetylases (HDACs) remove these marks [24,25]. HDACs are involved in the development of different tumors such as ovarian and breast cancer [26–28] and UL [9,23,29]. Specifically, HDAC activity was found to be higher in UL than in adjacent MM, suggesting that the transcription of genes implicated in the normal function of MM may be repressed due to a decrease in histone acetylation [9,29,30]. In addition, we previously described that inhibition of HDACs by suberoylanilide hydroxamic acid (SAHA) inhibits cell proliferation, cell cycle, extracellular matrix (ECM) formation and TGF-β3 signaling in human uterine leiomyoma primary (HULP) cells, suggesting that HDAC inhibitors may present a viable therapeutic option [29]. Aberrant status of acetylated Lysine 27 of histone 3 (H3K27ac) profile is implicated in several tumors such gastric, lung and ovarian cancers [31–33]. Since HAT/HDACs are dysregulated in UL, a holistic analysis of the interaction between gene expression and H3K27ac profiles in UL compared to MM could provide insight into key pathways and driver genes involved in UL pathogenesis that are under this histone modification control. Based on this, we aimed to further study the role of histone acetylation in UL and to identify if the histone mark H3K27ac is involved in UL development by integration of RNA-seq and CHIP-seq data. With this study, we describe the functional implications of an aberrant profile of the histone mark H3K27ac over gene expression in UL compared to adjacent MM.

2. Materials and Methods

2.1. Data/Samples Acquisition

RNA-seq data (GSE192354 and GSE142332) and the CHIP-seq data for the histone modification H3K27ac (GSE142332) were downloaded from the Gene Expression Omnibus (GEO, https://www.ncbi.nlm.nih.gov/geo/ (accessed on 10 September 2021)) of the National Center for Biotechnology Information (NCBI). In total, gene expression data of UL and adjacent MM of 31 Caucasian women (aged 31–48) was obtained from GSE192354, while RNA-seq and CHIP-seq H3K27ac data of UL and adjacent MM of 21 Caucasian/African American and Latin women (aged 41–52) were acquired from GSE142332. UL and MM tissue was obtained from women undergoing myomectomy or hysterectomy due to UL, and the origin of these tissues was confirmed through hematoxylin/eosin staining by examination of a pathologist.

2.2. CHIP-Seq Analysis

For CHIP-seq analysis of H3K27ac, the following bioinformatics analysis was performed within the R/Bioconductor (version 4.1.1) computing environment. A biomaRt package was used to bring in gene annotation data from Ensembl to R. With the data loaded into the workspace, peaks that were within the ±2 kb region from the transcription start sites (TSS) of a known human gene were defined as genes that present this modification. After centering and scaling fold-enrichment of signal values corresponding to peaks provided by CHIP-seq, Principal Component Analysis (PCA) and heatmap were performed and boxplot of H3K27ac histone mark status in UL and MM was represented with ggplot2 package. A Wilcoxon test ($p < 0.05$) was performed to test differences between H3K27ac status in UL vs. MM. Two samples were filtered out after quality analysis because of a low sequence depth.

2.3. RNA-Seq Analysis

Separate analyses were carried out for each dataset (GSE192354 and GSE142332). Raw count matrix derived from RNA-seq data libraries from GSE192354 was processed and subjected to statistical analysis within the R/Bioconductor (version 4.1.1) computing environment. PCA was performed to check concordance of DNA libraries. Differentially expressed genes (DEGs) were analysed using three different packages: DESeq2, edgeR and limma. RNA-seq data libraries from GSE142332 were analysed as previously described [34]. DEGs were obtained using DESeq2. Common DEGs between both datasets with an FDR-adjusted p-value < 0.01 and log2FC > 1 or <−1 were considered for the consequent analysis.

2.4. Correlation of H3K27ac and Gene Expression

Common DEGs resulting from RNA-seq analysis were integrated in each CHIP-seq data by selecting those for which a ChIP-seq peak was detected in the regulator region (TSS ± 2kb). A boxplot of the H3K27ac status for downregulated and upregulated genes in each group (UL and MM) was represented with a ggplot2 package. A Wilcoxon test ($p < 0.05$) was performed to test differences between H3K27ac status in the different groups. Differential peak enrichment analysis was performed using a linear model with a limma method. Peaks that were within the ±2 kb region from the TSS of the DEGs with FDR-adjusted p-value < 0.01 after limma analysis were defined as the significant differential modifications. A Venn diagram was used to identify hypoacetylated/downregulated and hyperacetylated/upregulated after H3K27ac analysis.

2.5. Functional Enrichment Analysis

Gene ontology (GO) analysis was conducted on selected genes which were hypoacetylated/downregulated and hyperacetylated/upregulated after H3K27ac analysis via Shiny Go (version 0.741) [35]. Biological processes and cellular components were considered to be statistically significant with FDR < 0.05.

2.6. Sample Collection

For gene expression validation and in vitro culture, human UL and adjacent MM (n = 10) tissue were collected from Caucasian premenopausal women aged 31–48 years without any previous hormonal treatment for the last three months and who were undergoing myomectomy or hysterectomy due to symptomatic UL at Hospital Universitario y Politécnico La Fe (Spain). This study was approved by the Clinical Ethics Committee at Hospital Universitario y Politécnico La Fe (Spain) (2018/0097), and all participants provided informed consent.

2.7. Validation of Gene Expression: qRT-PCR

Gene expression of selected DEG was validated in a distinct cohort of UL and adjacent MM (n = 10) by quantitative real-time PCR (qRT-PCR). Total RNA was extracted from tumor (UL) and normal tissues (MM) with TRIzol reagent (Fisher Scientific, Waltham, MA, USA), and complementary cDNA was synthesized employing a PrimeScript RT reagent kit (Takara, Kusatsu, Japan). Expression of genes *NDP, HOXA13, COL24A1, IGFL3, CD40, GIMAP8, IL15, GPX3* and *DPT*, was analysed by qRT-PCR with a StepOnePlus system (Applied Biosystems, Waltham, MA, USA) and PowerUp Sybr Green (ThermoFisher Scientific, Waltham, MA, USA). GAPDH gene was employed as housekeeping for gene expression normalisation. The ΔΔCt method was used to calculate fold change. Primers were designed using Primer Quest Tool (Integrated DNA Technologies, Coralville, IA, USA).

2.8. SAHA Treatment and Gene Expression Analysis in Human Uterine Leiomyoma Primary Cells

To evaluate the effect of SAHA (Abcam, Cambridge, UK) on the selected downregulated and hypoacetylated genes, HULP cells were isolated from UL tissues (n = 10) from selected women, as previously described [36] and treated with 0 μM (0.01% DMSO as a control) or 10 μM of SAHA for 48h. Then, total RNA was extracted from HULP cells using a Qiagen RNeasy Mini kit, and cDNA was synthesized using a Takara PrimeScript RT reagent kit; qRT-PCR was performed to evaluate gene expression *CD40*, *GIMAP8*, *IL15, GPX3* and *DPT* in HULP cells treated with or without SAHA, as described above.

2.9. Statistical Analysis

Omics data analysis was performed using R (version 4.1.1). Graphics were created using the R core package and packages gplots, ggplot2, as well as GraphPad Prism 8.0. Gene expression validation analysis was conducted with GraphPad Prism 8.0 employing Student's t-test or Wilcoxon test; $p < 0.05$ was considered statistically significant.

3. Results

3.1. Global H3K27ac CHIP-Seq Peak Profile in Uterine Leiomyoma Tissue Compared to Adjacent Myometrium

To determine the general H3K27 acetylation profile in human UL compared to adjacent MM tissue, an exploratory analysis of all peak signal values was performed. Principal component analysis (PCA) revealed a separation between UL and adjacent MM (Figure 1A). Clustering analysis showed common patterns in UL and MM, tending to form groups, as observed in a heatmap (Figure 1B). A boxplot of genes whose regulator region presented H3K27ac after CHIP-seq showed a lower amount of global H3K27ac peak enrichment level in UL compared to MM (p-value < 2.2×10^{-16}), suggesting a global hypoacetylation of UL (Figure 1C).

3.2. Selection of Relevant Differentially Expressed Genes

To identify relevant differentially expressed genes involved in UL development, we integrated gene expression data obtained from two different studies. First, count matrix of RNA obtained from GSE192354 was analysed using the three most widely used packages for differential expression analysis: limma, DESeq2 and edgeR. The selection of overlapping differentially expressed genes resulting from these three analyses showed

1837 DEGs significant (FDR-adjusted p-value < 0.01) and with a high difference of expression (log2FC > 1 or < −1) in UL compared to MM (Figure 2A), with 1175 upregulated and 662 downregulated. Similarly, 1998 DEGs with an FDR-adjusted p-value < 0.01, log2FC > 1 or < −1 were obtained from GSE142332 after DESeq2 analysis, with 1106 upregulated and 892 downregulated. After intersection of both outcomes, 922 genes were revealed as common DEGs in UL compared to MM samples from both datasets (Figure 2A), with 559 upregulated and 363 downregulated. These genes were considered as relevant DEGs in UL for further analysis.

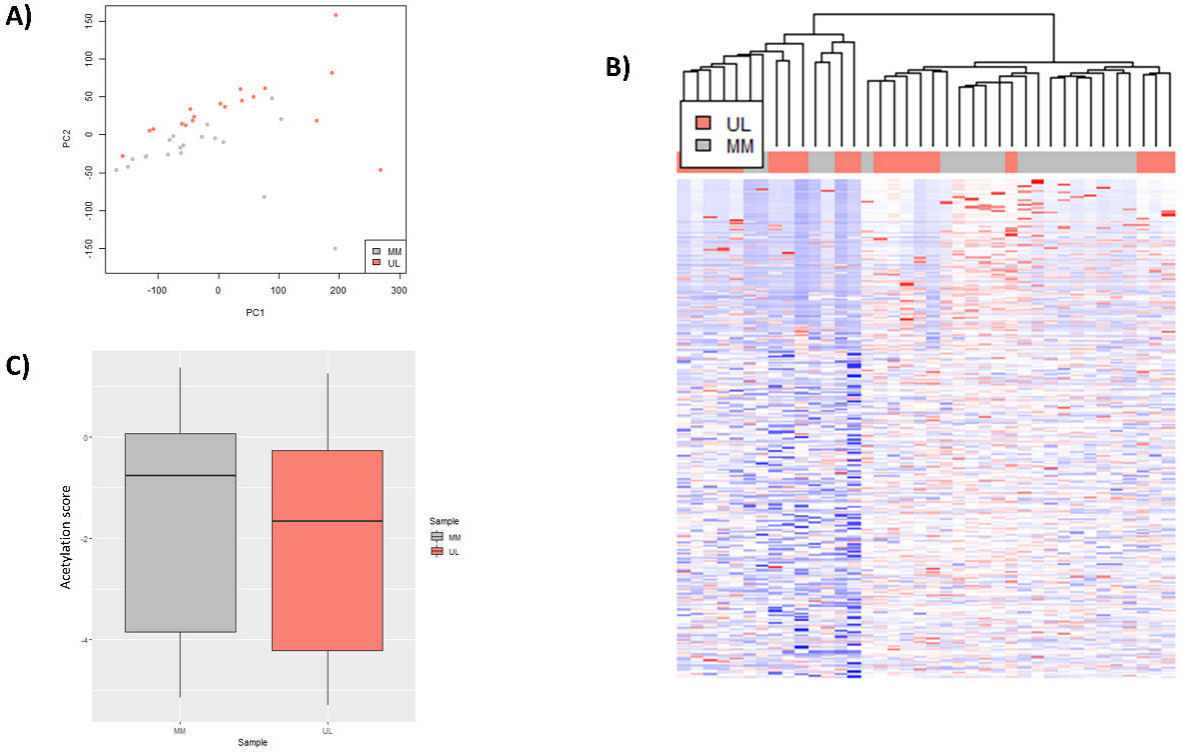

Figure 1. Global acetylation status of H3K27 in uterine leiomyoma compared to adjacent myometrium tissues from GSE142332: (**A**) principal component analysis (PCA) of global H3K27ac profile in uterine leiomyoma (UL) (pink) and adjacent myometrium (MM) (blue) (n = 19/group); (**B**) heatmap based on fold-enrichment score of genes with a CHIP-seq H3K27ac peak in TSS ±2000 bp after unsupervised clustering of UL (pink) and MM (gray) (n = 19/group); color scale ranges from red for higher normalized fold-enrichment score to blue for lower levels; (**C**) boxplot representing distribution of normalized fold-enrichment score for each peak in UL (pink) compared to adjacent MM (gray) samples (n = 19/group), representing global H3K27ac status ($p-$value < 2.2×10^{-16}).

3.3. Identification Differentially Expressed Genes with an Aberrant H3K27ac Mark in Uterine Leiomyoma Tissue Compared to Adjacent Myometrium

Next, we aimed to evaluate those genes whose change of expression was associated with a differential H3K27ac status. Among the 922 genes selected for this analysis after RNA-seq, 482 (52.3%) presented the histone mark H3K27ac around the TSS ± 2 kb. A PCA of CHIP-seq data of these genes showed a clear separation of tumor (UL) and control (MM) samples (Figure 2B), indicating a different behaviour of H3K27ac profile of relevant selected genes in UL compared to adjacent MM, as confirmed by heatmap (Figure 2C). Additionally, a boxplot of fold-enrichment score of H3K27ac peaks representing downregulated and upregulated genes demonstrated that downregulated genes presented a lower fold-enrichment score of H3K27ac peaks (p-value < 2.2×10^{-16}) in UL versus MM, while upregulated genes exhibited a higher fold-enrichment score (p-value < 2.2×10^{-16}) in UL versus MM (Figure 2D). Differential peak enrichment analysis showed that 82 DEGs

presented differential acetylation (FDR < 0.05) in UL compared to MM, with 29 hyperacetylated/upregulated and 53 hypoacetylated/downregulated (Supplemental Table S1).

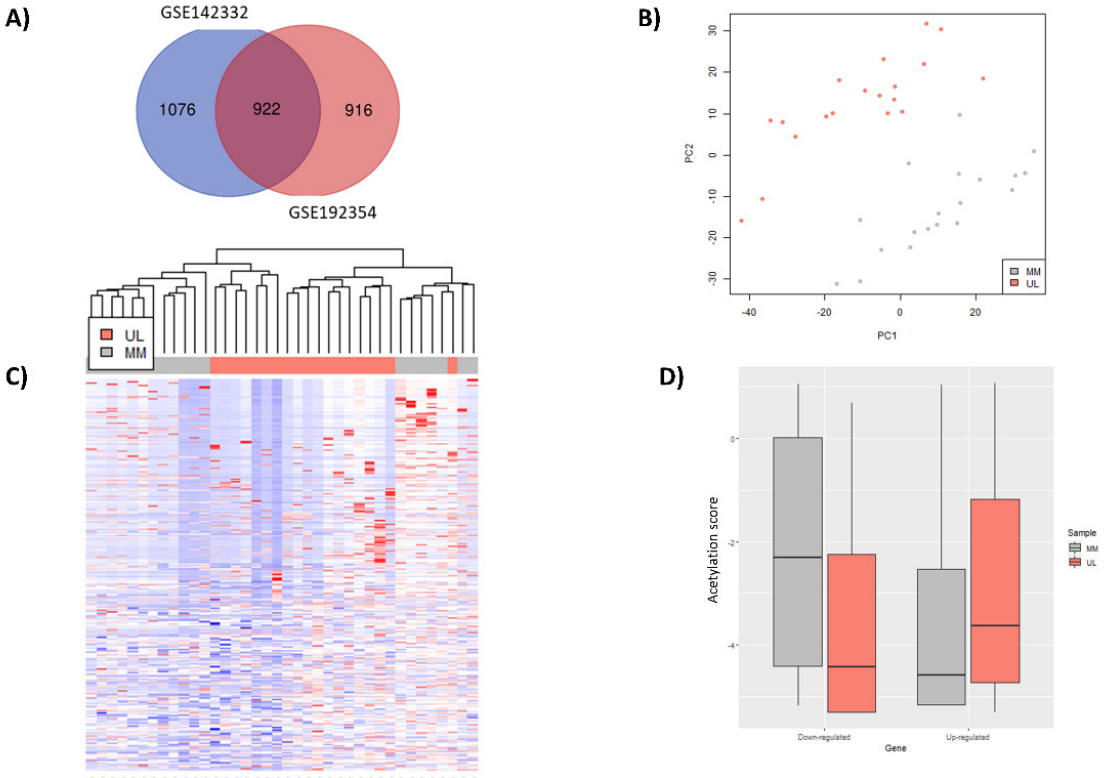

Figure 2. Identification of selected differentially expressed genes and description of their H3K27ac status in UL compared to MM tissue: (**A**) Venn diagram representing common DEGs (FDR-adjusted *p*-value < 0.01, log2FC > 1 or <−1) between GSE192354 (n = 28) and GSE142332 (n = 19) samples; (**B**) PCA of global H3K27ac profile and (**C**) Heatmap based on fold-enrichment score of 82 selected DEGs in common in both GSE192354 and GSE142332 whose promoter region presented a peak after CHIP-seq after unsupervised clustering of uterine leiomyoma (UL) (pink) and adjacent myometrium (MM) (gray) (n = 19/group); color scale ranges from red for higher normalized fold-enrichment score to blue for lower levels; (**D**) boxplot representing distribution of normalized fold-enrichment score for each peak of downregulated and upregulated genes in UL (pink) compared to adjacent MM (gray) samples (n = 19/group), representing H3K27ac status in each group of genes (p-value < 2.2×10^{-16}).

3.4. Functional Implications of Differentially Expressed Genes Associated with Aberrant H3K27 Acetylation in Uterine Leiomyoma Tissue Compared to Adjacent Myometrium

Functional enrichment analysis of 82 DEGs associated with a different H3K27ac profile revealed 30 biological processes significantly deregulated in human UL versus MM that were mainly related to cell proliferation, cell signaling and cell transport and angiogenesis, key pathways in tumor pathogenesis (Figure 3A). In addition, cellular components were found to be significantly enriched in UL, which were all related to an alteration of the extracellular matrix, one of the key features of UL (Figure 3B).

3.5. Validation of Hypoacetylated/Downregulated and Hyperacetylated/Upregulated Genes

To highlight the importance of the genes selected after integration of RNA-seq and CHIP-seq studies, gene expression of 10 genes which presented a differential H3K27ac status was validated in a different set of patients. These genes were selected as key genes of the enriched functions and based on their role in tumorogenesis after a bibliographic search among all hyperacetylated/upregulated and hypoacetylated/downregulated genes. The qRT-PCR corroborated the significant upregulation of

COL24A1 (fold-change = 15.83, $p = 0.003$), *NDP* (fold-change = 26.38, $p = 0.037$), *HOXA13* (fold-change = 1.86, $p = 0.041$) *and IGFL3* (fold-change = 13.26, $p = 0.031$) in a separate cohort of UL compared to adjacent MM (Figure 4A–D). Likewise, qRT-PCR confirmed the downregulation of *CD40* (fold-change = 0.54, $p = 0.010$), *DPT* (fold-change = 0.25, $p = 0.002$), *GIMAP8* (fold-change = 0.52, $p = 0.015$), *GPX3* (fold-change = 0.30, $p < 0.0001$) and *IL15* (fold-change = 0.36, $p = 0.005$) (Figure 4E–I).

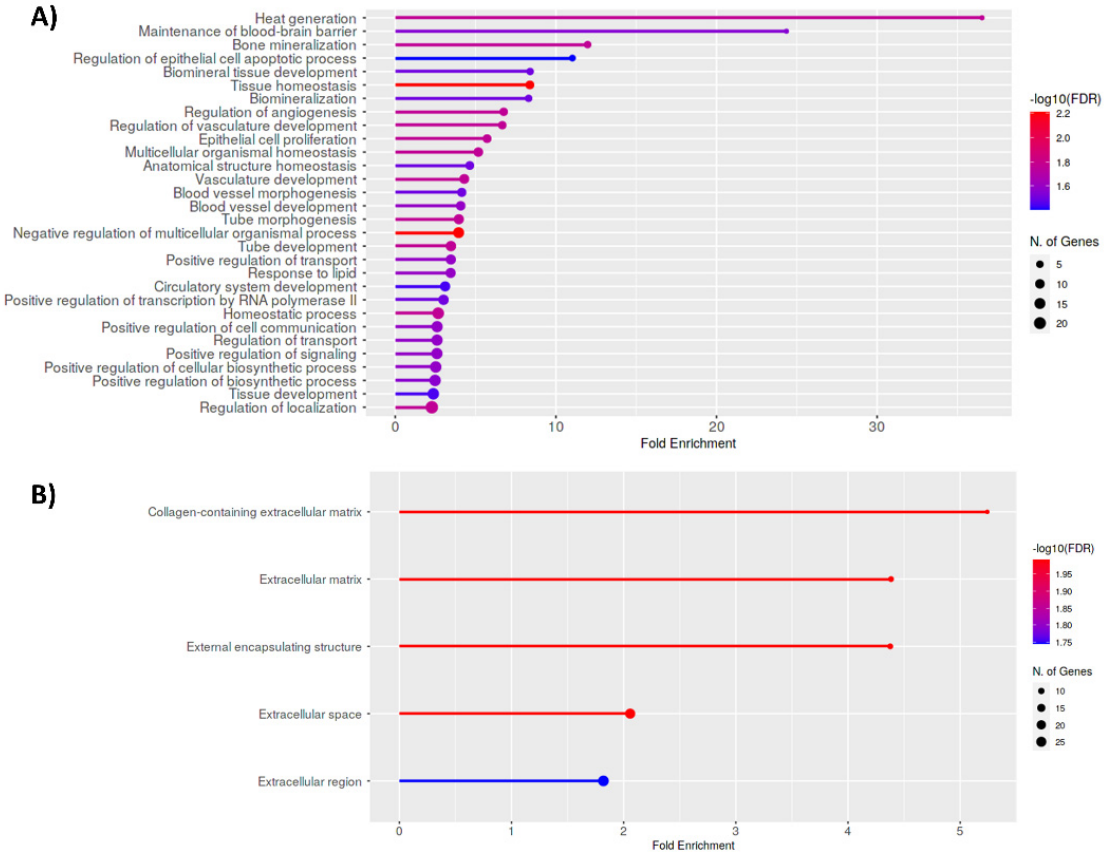

Figure 3. Functional enrichment analysis of differential expressed genes associated with aberrant H3K27ac status in UL vs. MM; most significant: (**A**) biological processes; and (**B**) cellular components obtained after functional enrichment analysis of all selected aberrantly acetylated DEGs in UL vs. MM tissues; FDR < 0.05.

3.6. Inhibiting Histone Deacetylases Reverses Expression of Hypoacetylated/Downregulated Genes in Human Uterine Leiomyoma Primary Cells In Vitro

To corroborate that histone acetylation of H3K27 is really affecting the expression of tumor suppressor genes in UL, we assessed the role of inhibition of HDACs on restoring the expression of genes controlled by H3K27ac, by inhibition of HDACs in human uterine leiomyoma primary (HULP) cells using SAHA treatment. Expression of the previously selected hypoacetylated and downregulated genes in UL was evaluated by qPCR after treatment with SAHA at 0 μM and 10 μM in HULPs. Results showed that inhibition of deacetylation by SAHA treatment significantly upregulated expression of tumor suppressor genes *CD40* (fold-change = 6.78, $p = 0.001$), *DPT* (fold-change = 1.80, $p = 0.033$), *GIMAP8* (fold-change = 30.67, $p = 0.042$), *GPX3* (fold-change = 22.15, $p = 0.001$) and *IL15* (fold-change = 2.71, $p = 0.018$) and in HULP cells (Figure 4J–N).

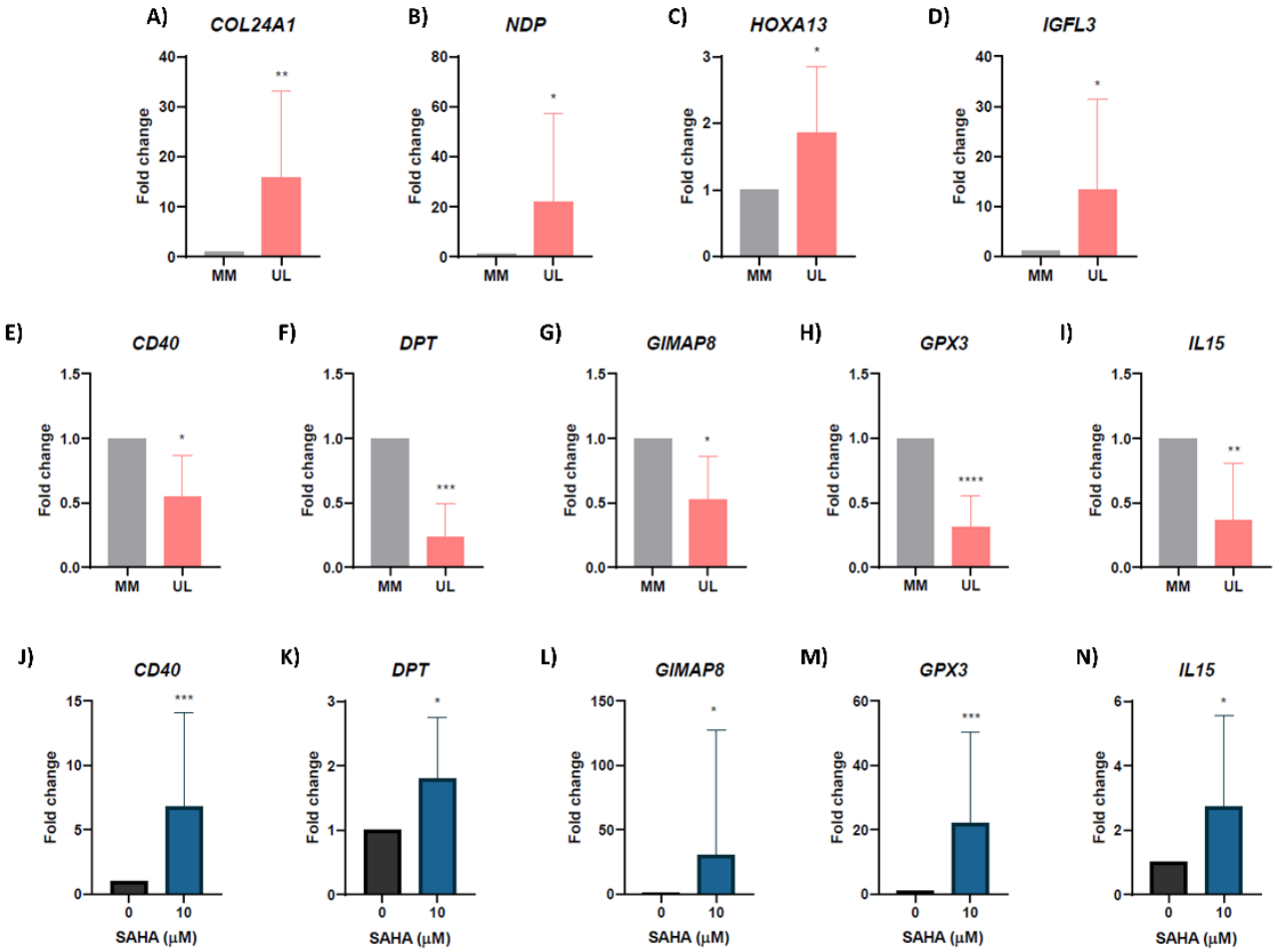

Figure 4. Validation of RNA-seq results and gene expression analysis in human uterine leiomyoma primary cells after SAHA treatment; expression levels of: (**A**) *COL24A1*; (**B**) *NDP;* (**C**) *HOXA13*; (**D**) *IGFL3*; (**E**) *CD40*; (**F**) *DPT*; (**G**) *GIMAP8*; (**H**) *GPX3;* and (**I**) *IL15* in the validation set of UL compared to adjacent MM (n = 10); gene expression levels of hypermethylated/downregulated genes: (**J**) *CD40*; (**K**) *DPT*; (**L**) *GIMAP8*; (**M**) *GPX;* and (**N**) *IL15* in human uterine fibroid primary (HULP) cells treated with 0 μM (control) or 10 μM of SAHA for 48 h (n = 10). Gene expression was analyzed by qRT-PCR, quantified by the ΔΔCt method and expressed as fold regulation. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

4. Discussion

Uterine leiomyomas are a major gynaecological disease with a great impact on women's health, being a main cause of infertility. Despite the personal and economic consequences of this tumor, its pathology remains unclear. Recently, epigenetics has emerged as a new mechanism that may be involved in UL formation [23,30]. The epigenomic studies pertaining to UL pathogenesis have mainly focused on DNA methylation [20,21,37]. However, histone modifications also have the potential to play an important function in chromatin alterations, and therefore, it is necessary to fully explore the effect of histone acetylation on the expression of genes involved in the pathogenesis of this disease. In a previous study, we demonstrated that histone deacetylase inhibitors may present a viable therapeutic option for UL [29]. Herein, we further studied the role of histone acetylation over gene expression by identifying if the modification H3K27ac, which is altered in several tumors, is involved in UL pathogenesis and supports the importance as a new therapeutic approach to treat UL patients. Our results showed that H3K27ac regulates genes implicated in key processes of UL pathogenesis such as cell proliferation, cell signaling and cell transport, angiogenesis

and ECM formation, and histone deacetylation reversion may represent a therapeutic approach to treat UL.

Post-translational modifications of histones hold importance in the epigenomic regulation of gene expression. Histone acetylation is correlated with gene expression, while deacetylation leads to repression of gene transcription [24]. After analysing the general H3K27 acetylation profile of promoter regions (TSS ± 2 kb) of human genes in UL compared to adjacent MM tissue, we found a different pattern of the histone mark H3K27ac. Specifically, a lower amount of global H3K27ac was observed in UL compared to MM, suggesting a global hypoacetylation of H3K27 in UL. The reduced H3K27 acetylation would lead to a decrease in the expression levels of genes that suppress tumor development. Accordingly, previous studies have shown that a global DNA hypermethylation is related to the downregulation of tumor suppressor genes involved in tumor development [17,21,34]. Although the role of histone modifications in UL is less understood compared to DNA methylation, recent publications have emphasized the significance of H3K27ac, H3K4me3 and H2A.Z in enhancers and promotors, finding differential features between UL subtypes based on the mutation status [22,34,38]. Herein, an altered pattern of H3K27ac in UL compared to MM regardless of their mutational subtype suggests its role in UL development and treatment.

The aberrant status of H3K27ac in UL can explain the altered chromatin structure, which aids in developing the UL-specific gene dysregulation resulting in its pathogenesis. Therefore, we selected key DEGs involved in UL development whose change of expression was associated with the histone mark H3K27ac. Among the 922 DEGs described by integration of RNA-seq analyses, 482 presented the histone mark H3K27ac around the promotor, and 82 of them exhibited a differential H3K27ac status in UL compared to MM, with 29 hyperacetylated/upregulated and 53 hypo-acetylated/downregulated.

To further analyse the new molecular targets involved in UL pathogenesis that are associated with H3K27ac, we reviewed the literature for the 82 DEGs regulated by H3K27ac and found that these genes present multiple functions, being potential key effecters of tumor development and maintenance. We found hyperacetylation/upregulation of oncogenes such as *NDP, HOXA13, COL24A1* and *IGFL3*, with *NDP* and *IGFL3* not previously related to UL. *NDP* plays a role in the regulation of angiogenesis in the colorectal region [39] and activates the Wnt/β-catenin pathway [40]. *HOXA13,* whose over-expression in UL has previously been described [21,34], is also associated with tumor size, microvascular invasion, angiogenesis, Wnt and TGFβ3 pathway in cancer [41–43]. *COL24A1,* a member of the collagen gene family, is related with vascular invasion and is proposed as a target for UL treatment [44]. Its overexpression in hepatocellular carcinoma leads to tumor and vascular invasion [45]. According to our study, it plays an intermediate role between ECM and angiogenesis in UL, being associated with a higher presence of H3K27ac in its promotor. *IGFL3* is implicated in TFGβ signaling in breast cancer [46], but was not previously linked to UL until this study. Epigenetic regulation of *IGFL3* in UL through H3K27ac could lead to dysregulation of TGFβ3 pathway in this tumor. Inhibition of any of these genes by directly targeting them or though histone acetylation/deacetylation treatment could lead to a decrease in cell proliferation, angiogenesis, ECM and other pathways involved in UL pathogenesis.

We also found hypoacetylation and downregulation of tumor suppressor genes such as *CD40, GIMAP8, IL15, GPX3* and *DPT*, with *CD40, GIMAP8 and GPX3* associated with UL for the first time in this study. *CD40* has antiangiogenic and pro-immune properties in other tumors [47,48]. We propose that *CD40* hypoacetylation and, therefore downregulation, would promote angiogenesis and hide tumor cells from the immune system. *GIMAP8* is *a* GTP-binding with a tumor suppressive role against breast cancer [49]. *IL15* contributes to excessive ECM production, tissue remodeling and leiomyoma growth [50]. It also controls migration, invasion, metabolism and angiogenesis, decreasing the number of blood vessels in prostate cancer [51]. Its hyperacetylation and consequent downregulation would contribute to an increment on UL vasculature. *GPX3* is a tumor suppressor that prevents migration and invasion through the Wnt pathway in gastric cancer [52,53]. *DPT* inhibits

cell proliferation, interacts with decorin for TGF-β binding and plays an important role in cell–matrix interactions and matrix assembly [54]. Its hypoacetylation and downregulation would disrupt the process of collagen fibrils and activate the TGF-β signaling pathway. The recovery of gene expression of these tumor-suppressor genes could stop the development of UL. All in all, the dysregulation of these genes confirms that key processes of UL development are under histone acetylation control.

The interplay of H3K27ac-gene expression and cell signaling pathways can broaden the understanding of UL development and requires more attention. Hence, functional enrichment analysis of 82 DEGs regulated by H3K27ac was performed. This analysis revealed biological processes significantly deregulated in human UL that were mainly related to cell proliferation, cell signaling and cell transport and angiogenesis processes. Uterine leiomyoma is characterized by an uncontrolled proliferation, which is also a main feature of tumors [4,55,56]. In addition, cell communication and cell signaling is altered in tumors, contributing to the aberrant response to extracellular signals and enhancing tumor development that is characteristic of this kind of disease [56–58]. Initiation of tumor angiogenesis is one hallmark of cancer and a requirement for tumor progression [56]. Malignant cells require oxygen and nutrients to survive and proliferate, needing proximity to blood vessels to access the blood circulation system. The aberrant vascularization found in UL [59] can be triggered by a change in histone marks such as H3K27ac. Different growth factors and vascular genes mediate the angiogenic process, which as demonstrated in this study is regulated by epigenetic states of genes. Accordingly, hyperacetylation/upregulation of oncogenes related with angiogenesis and vascular invasion (*COL24A1, NDP* and *HOXA13)* and hypoacetylation/downregulation of angiogenesis-tumor suppressor genes (*CD40* and *IL15)* was identified in this study. In addition, we found cellular components significantly enriched in UL, which were mainly related to an alteration of extracellular matrix formation. Excessive synthesis and deposition of ECM deposition exerts a major role in the growth and stiffness of UL, contributing to clinical symptoms, such as abnormal bleeding and abdominal pain [1,60,61]. For this reason, ECM has been considered as a crucial target for UL therapeutics [61]. Herein, we found hyperacetylation/upregulation of ECM-associated oncogenes, such as *COL24A1* and *IGFL3,* and hypoacetylation/downregulation of ECM-associated tumor suppressor genes such as *IL15* and *DPT.*

To corroborate that histone acetylation H3K27 is really affecting the expression of tumor suppressor genes in UL, we inhibited HDACs, enzymes who catalyse histone deacetylation, in vitro by SAHA in HULP cells. Inhibiting HDACs upregulated the expression of hypoacetylated and downregulated tumor suppressor genes (*CD40, GIMAP8, IL15, GPX3 and DPT*) in HULP cells in vitro. HDACs inhibitors are widely used as anticancer drugs to treat many tumors in which histone acetylation is altered, increasing the accumulation of acetylated core histones. As a consequence, SAHA blocks cell proliferation and tumor growth in tumors such as hepatoid adenocarcinoma [62], myeloid leukemia [63] and prostate cancer [64]. The impaired histone acetylation in UL shown in this study opens insights into the role of these treatments as therapeutic options to treat this disease, as it does in other tumors. We have previously demonstrated that SAHA treatment inhibits cell proliferation, cell cycle, ECM, and TGF-β3 signaling in HULP cells, suggesting that histone deacetylation may be useful to treat UL [29]. Herein, we reinforce this hypothesis by proving that reversal of histone acetylation by SAHA in HULP cells upregulated hypoacetylated/downregulated tumor suppressor genes. These results together give importance to histone acetylation as a therapeutic approach for UL patients.

Based on these findings, dysregulated pathways involved in UL pathogenesis, such as cell proliferation, cell signaling and cell transport, angiogenesis or ECM formation, could be targeted for future therapeutics through histone acetylation reversion. This study provides insight into the role of histone acetylation in UL development. Further studies focused on new treatments targeting these histone modifications will be necessary to define an effective treatment of UL without side effects.

5. Conclusions

In this study, we found hyperacetylation/upregulation of oncogenes (*NDP, HOXA13, COL24A1 and IGFL3*) and hypoacetylation/downregulation of tumor suppressor genes (*CD40, GIMAP8, IL15, GPX3 and DPT*) in UL, which are related to the immune system, angiogenesis, invasion, altered metabolism, deposit of extracellular matrix, TGFβ3 and Wnt/β-catenin pathway dysregulation. In conclusion, gene regulation by H3K27 acetylation is involved in uterine leiomyoma pathogenesis through processes such as cell proliferation, cell signaling and cell transport, angiogenesis, ECM, Wnt and TGFβ pathway, and reversal of this acetylation could offer a therapeutic option for patients with uterine leiomyomas.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/biomedicines10061279/s1, Table S1. Differential expressed genes with peaks within the ±2 kb region from the TSS of the DEGs with FDR-adjusted *p*-value < 0.01 after limma analysis.

Author Contributions: Conceptualization, M.C.C.-G.; methodology, M.C.C.-G., L.d.M.-G. and E.J.-B.; software, M.C.C.-G.; validation, L.d.M.-G. and E.J.-B.; formal analysis, M.C.C.-G.; investigation, M.C.C.-G., J.M.F. and H.F.; recruitment, J.M. and A.T.; data curation, M.C.C.-G.; writing—original draft preparation, M.C.C.-G.; writing—review and editing, J.M.F. and H.F.; visualization, M.C.C.-G.; supervision, J.M.F. and H.F.; project administration, A.P., J.M.F. and H.F.; funding acquisition, A.P., J.M.F. and H.F. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: This study was approved by the Clinical Ethics Committee at Hospital Universitario y Politécnico La Fe (Spain) (2018/0097).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The datasets analysed during the current study are available in the GEO repository, https://www.ncbi.nlm.nih.gov/geo/ (accessed on 10 September 2021).

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Conflicts of Interest: The authors declare no conflict of interest.

References

1. Stewart, E.A.; Laughlin-Tommaso, S.K.; Catherino, W.H.; Lalitkumar, S.; Gupta, D.; Vollenhoven, B. Uterine Fibroids. *Nat. Rev. Dis. Prim.* **2016**, *2*, 16043. [CrossRef] [PubMed]
2. Parker, W.H. Etiology, Symptomatology, and Diagnosis of Uterine Myomas. *Fertil. Steril.* **2007**, *87*, 725–736. [CrossRef] [PubMed]
3. Gupta, S.; Jose, J.; Manyonda, I. Clinical Presentation of Fibroids. *Best Pract. Res. Clin. Obstet. Gynaecol.* **2008**, *22*, 615–626. [CrossRef] [PubMed]
4. Bulun, S.E. Uterine Fibroids. *N. Engl. J. Med.* **2013**, *369*, 1344–1355. [CrossRef]
5. Parker, J.D.; Malik, M.; Catherino, W.H. Human Myometrium and Leiomyomas Express Gonadotropin-Releasing Hormone 2 and Gonadotropin-Releasing Hormone 2 Receptor. *Fertil. Steril.* **2007**, *88*, 39–46. [CrossRef]
6. Manyonda, I.; Sinthamoney, E.; Belli, A.M. Controversies and Challenges in the Modern Management of Uterine Fibroids. *BJOG An Int. J. Obstet. Gynaecol.* **2004**, *111*, 95–102. [CrossRef]
7. Friedman, A.J. Treatment of Leiomyomata Uteri with Short-Term Leuprolide Followed by Leuprolide plus Estrogen-Progestin Hormone Replacement Therapy for 2 Years: A Pilot Study. *Fertil. Steril.* **1989**, *51*, 526–528. [CrossRef]
8. El Sabeh, M.; Borahay, M. The Future of Uterine Fibroid Management: A More Preventive and Personalized Paradigm. *Reprod. Sci.* **2021**. [CrossRef]

9. Sant'Anna, G.d.S.; Brum, I.S.; Branchini, G.; Pizzolato, L.S.; Capp, E.; Corleta, H. von E. Ovarian Steroid Hormones Modulate the Expression of Progesterone Receptors and Histone Acetylation Patterns in Uterine Leiomyoma Cells. *Gynecol. Endocrinol.* **2017**, *33*, 629–633. [CrossRef]
10. Maruo, T.; Ohara, N.; Wang, J.; Matsuo, H. Sex Steroidal Regulation of Uterine Leiomyoma Growth and Apoptosis. *Hum. Reprod. Update* **2004**, *10*, 207–220. [CrossRef]
11. Ono, M.; Qiang, W.; Serna, V.A.; Yin, P.; Coon, J.S.; Navarro, A.; Monsivais, D.; Kakinuma, T.; Dyson, M.; Druschitz, S.; et al. Role of Stem Cells in Human Uterine Leiomyoma Growth. *PLoS ONE* **2012**, *7*. [CrossRef] [PubMed]
12. Mäkinen, N.; Mehine, M.; Tolvanen, J.; Kaasinen, E.; Li, Y.; Lehtonen, H.J.; Gentile, M.; Yan, J.; Enge, M.; Taipale, M.; et al. MED12, the Mediator Complex Subunit 12 Gene, Is Mutated at High Frequency in Uterine Leiomyomas. *Science* **2011**, *334*, 252–255. [CrossRef]
13. Mehine, M.; Kaasinen, E.; Heinonen, H.R.; Mäkinen, N.; Kämpjärvi, K.; Sarvilinna, N.; Aavikko, M.; Vähärautio, A.; Pasanen, A.; Bützow, R.; et al. Integrated Data Analysis Reveals Uterine Leiomyoma Subtypes with Distinct Driver Pathways and Biomarkers. *Proc. Natl. Acad. Sci. USA* **2016**, *113*, 1315–1320. [CrossRef] [PubMed]
14. Lehtonen, R.; Kiuru, M.; Vanharanta, S.; Sjöberg, J.; Aaltonen, L.M.; Aittomäki, K.; Arola, J.; Butzow, R.; Eng, C.; Husgafvel-Pursiainen, K.; et al. Biallelic Inactivation of Fumarate Hydratase (FH) Occurs in Nonsyndromic Uterine Leiomyomas but Is Rare in Other Tumors. *Am. J. Pathol.* **2004**, *164*, 17–22. [CrossRef]
15. Stewart, E.; Cookson, C.; Gandolfo, R.; Schulze-Rath, R. Epidemiology of Uterine Fibroids: A Systematic Review. *BJOG An Int. J. Obstet. Gynaecol.* **2017**, *124*, 1501–1512. [CrossRef]
16. Peschansky, V.J.; Wahlestedt, C. Non-coding RNAs as direct and indirect modulators of epigenetic regulation. *Epigenetics* **2014**, *9*, 3–12. [CrossRef]
17. Navarro, A.; Yin, P.; Monsivais, D.; Lin, S.M.; Du, P.; Wei, J.; Bulun, S.E. Genome-wide DNA methylation indicates silencing of tumor suppressor genes in uterine leiomyoma. *PLoS ONE* **2012**, *7*, e33284. [CrossRef]
18. Maekawa, R.; Sato, S.; Yamagata, Y.; Asada, H.; Tamura, I.; Lee, L.; Okada, M.; Tamura, H.; Takaki, E.; Nakai, A.; et al. Genome-Wide DNA Methylation Analysis Reveals a Potential Mechanism for the Pathogenesis and Development of Uterine Leiomyomas. *PLoS ONE* **2013**, *8*. [CrossRef]
19. Carbajo-García, M.C.; Corachán, A.; Juárez-Barber, E.; Monleón, J.; Payá, V.; Trelis, A.; Quiñonero, A.; Pellicer, A.; Ferrero, H. Integrative Analysis of the DNA Methylome and Transcriptome in Uterine Leiomyoma Shows Altered Regulation of Genes Involved in Metabolism, Proliferation, Extracellular Matrix and Vesicles. *J. Pathol.* **2022**. [CrossRef]
20. Liu, S.; Yin, P.; Xu, J.; Dotts, A.J.; Kujawa, S.A.; Coon, V.J.S.; Zhao, H.; Shilatifard, A.; Dai, Y.; E Bulun, S. Targeting DNA Methylation Depletes Uterine Leiomyoma Stem Cell–enriched Population by Stimulating Their Differentiation. *Endocrinology* **2020**, *161*. [CrossRef]
21. George, J.W.; Fan, H.; Johnson, B.; Carpenter, T.J.; Foy, K.K.; Chatterjee, A.; Patterson, A.L.; Koeman, J.; Adams, M.; Madaj, Z.B.; et al. Integrated Epigenome, Exome, and Transcriptome Analyses Reveal Molecular Subtypes and Homeotic Transformation in Uterine Fibroids. *Cell Rep.* **2019**, *29*, 4069–4085. [CrossRef] [PubMed]
22. Berta, D.G.; Kuisma, H.; Välimäki, N.; Räisänen, M.; Jäntti, M.; Pasanen, A.; Karhu, A.; Kaukomaa, J.; Taira, A.; Cajuso, T.; et al. Deficient H2A.Z Deposition Is Associated with Genesis of Uterine Leiomyoma. *Nature* **2021**, *596*, 398–403. [CrossRef]
23. Yang, Q.; Mas, A.; Diamond, M.P.; Al-Hendy, A. The Mechanism and Function of Epigenetics in Uterine Leiomyoma Development. *Reprod. Sci.* **2016**, *23*, 163–175. [CrossRef]
24. Verdone, L.; Caserta, M.; Di Mauro, E. Role of Histone Acetylation in the Control of Gene Expression. *Biochem. Cell Biol.* **2005**, *83*, 344–353. [CrossRef] [PubMed]
25. Seto, E.; Yoshida, M. Erasers of Histone Acetylation: The Histone Deacetylase Enzymes. *Cold Spring Harb. Perspect. Biol.* **2014**, *6*. [CrossRef] [PubMed]
26. Gallinari, P.; Di Marco, S.; Jones, P.; Pallaoro, M.; Steinkühler, C. HDACs, Histone Deacetylation and Gene Transcription: From Molecular Biology to Cancer Therapeutics. *Cell Res.* **2007**, *17*, 195–211. [CrossRef]
27. Bitler, B.G.; Wu, S.; Park, P.H.; Hai, Y.; Aird, K.M.; Wang, Y.; Zhai, Y.; Kossenkov, A.V.; Vara-Ailor, A.; Rauscher, F.J.; et al. ARID1A-Mutated Ovarian Cancers Depend on HDAC6 Activity. *Nat. Cell Biol.* **2017**, *19*, 962–973. [CrossRef]
28. Hervouet, E.; Claude-Taupin, A.; Gauthier, T.; Perez, V.; Fraichard, A.; Adami, P.; Despouy, G.; Monnien, F.; Algros, M.P.; Jouvenot, M.; et al. The Autophagy GABARAPL1 Gene Is Epigenetically Regulated in Breast Cancer Models. *BMC Cancer* **2015**, *15*. [CrossRef]
29. Carbajo-García, M.C.; García-Alcázar, Z.; Corachán, A.; Monleón, J.; Trelis, A.; Faus, A.; Pellicer, A.; Ferrero, H. Histone Deacetylase Inhibition by Suberoylanilide Hydroxamic Acid: A Therapeutic Approach to Treat Human Uterine Leiomyoma. *Fertil. Steril.* **2022**, *117*, 433–443. [CrossRef]
30. Laganà, A.S.; Vergara, D.; Favilli, A.; La Rosa, V.L.; Tinelli, A.; Gerli, S.; Noventa, M.; Vitagliano, A.; Triolo, O.; Rapisarda, A.M.C.; et al. Epigenetic and Genetic Landscape of Uterine Leiomyomas: A Current View over a Common Gynecological Disease. *Arch. Gynecol. Obstet.* **2017**, *296*, 855–867. [CrossRef]
31. Zhang, Y.; Liu, Z.; Yang, X.; Lu, W.; Chen, Y.; Lin, Y.; Wang, J.; Lin, S.; Yun, J.P. H3K27 Acetylation Activated-COL6A1 Promotes Osteosarcoma Lung Metastasis by Repressing STAT1 and Activating Pulmonary Cancer-Associated Fibroblasts. *Theranostics* **2021**, *11*, 1473–1492. [CrossRef] [PubMed]

32. Wang, J.; Sun, Y.; Zhang, X.; Cai, H.; Zhang, C.; Qu, H.; Liu, L.; Zhang, M.; Fu, J.; Zhang, J.; et al. Oxidative Stress Activates NORAD Expression by H3K27ac and Promotes Oxaliplatin Resistance in Gastric Cancer by Enhancing Autophagy Flux via Targeting the MiR-433-3p. *Cell Death Dis.* **2021**, *12*. [CrossRef] [PubMed]
33. Ma, Y.; Zheng, W. H3K27ac-Induced LncRNA PAXIP1-AS1 Promotes Cell Proliferation, Migration, EMT and Apoptosis in Ovarian Cancer by Targeting MiR-6744-5p/PCBP2 Axis. *J. Ovarian Res.* **2021**, *14*. [CrossRef] [PubMed]
34. Leistico, J.R.; Saini, P.; Futtner, C.R.; Hejna, M.; Omura, Y.; Soni, P.N.; Sandlesh, P.; Milad, M.; Wei, J.J.; Bulun, S.; et al. Epigenomic tensor predicts disease subtypes and reveals constrained tumor evolution. *Cell Rep.* **2021**, *34*, 108927. [CrossRef]
35. Ge, S.X.; Jung, D.; Yao, R. ShinyGO: A Graphical Gene-Set Enrichment Tool for Animals and Plants. *Bioinformatics* **2020**, *36*, 2628–2629. [CrossRef]
36. Carbajo-García, M.C.; Corachán, A.; Segura-Benitez, M.; Monleón, J.; Escrig, J.; Faus, A.; Pellicer, A.; Cervelló, I.; Ferrero, H. 5-Aza-2′-Deoxycitidine Inhibits Cell Proliferation, Extracellular Matrix Formation and Wnt/β-Catenin Pathway in Human Uterine Leiomyomas. *Reprod. Biol. Endocrinol.* **2021**, *19*. [CrossRef]
37. Sato, S.; Maekawa, R.; Tamura, I.; Shirafuta, Y.; Shinagawa, M.; Asada, H.; Taketani, T.; Tamura, H.; Sugino, N. SATB2 and NGR1: Potential Upstream Regulatory Factors in Uterine Leiomyomas. *J. Assist. Reprod. Genet.* **2019**, *36*, 2385–2397. [CrossRef]
38. Moyo, M.B.; Parker, J.B.; Chakravarti, D. Altered chromatin landscape and enhancer engagement underlie transcriptional dysregulation in MED12 mutant uterine leiomyomas. *Nat. Commun.* **2020**, *11*, 1019. [CrossRef]
39. Planutis, K.; Planutiene, M.; Holcombe, R.F. A Novel Signaling Pathway Regulates Colon Cancer Angiogenesis through Norrin. *Sci. Rep.* **2014**, *4*. [CrossRef]
40. El-Sehemy, A.; Selvadurai, H.; Ortin-Martinez, A.; Pokrajac, N.; Mamatjan, Y.; Tachibana, N.; Rowland, K.; Lee, L.; Park, N.; Aldape, K.; et al. Norrin Mediates Tumor-Promoting and -Suppressive Effects in Glioblastoma via Notch and Wnt. *J. Clin. Investig.* **2020**, *130*, 3069–3086. [CrossRef]
41. Duan, R.; Han, L.; Wang, Q.; Wei, J.; Chen, L.; Zhang, J.; Kang, C.; Wang, L. HOXA13 Is a Potential GBM Diagnostic Marker and Promotes Glioma Invasion by Activating the Wnt and TGF-β Pathways. *Oncotarget* **2015**, *6*, 27778–27793. [CrossRef] [PubMed]
42. Gu, Y.; Gu, J.; Shen, K.; Zhou, H.; Hao, J.; Li, F.; Yu, H.; Chen, Y.; Li, J.; Li, Y.; et al. HOXA13 Promotes Colon Cancer Progression through β-Catenin-Dependent WNT Pathway. *Exp. Cell Res.* **2020**, *395*, 112238. [CrossRef] [PubMed]
43. Pan, T.T.; Jia, W.D.; Yao, Q.Y.; Sun, Q.K.; Ren, W.H.; Huang, M.; Ma, J.; Li, J.S.; Ma, J.L.; Yu, J.H.; et al. Overexpression of HOXA13 as a Potential Marker for Diagnosis and Poor Prognosis of Hepatocellular Carcinoma. *Tohoku J. Exp. Med.* **2014**, *234*, 209–219. [CrossRef] [PubMed]
44. Jamaluddin, M.F.B.; Nahar, P.; Tanwar, P.S. Proteomic Characterization of the Extracellular Matrix of Human Uterine Fibroids. *Endocrinology* **2018**, *159*, 2656–2669. [CrossRef]
45. Yan, L.; Xu, F.; Dai, C. Overexpression of COL24A1 in Hepatocellular Carcinoma Predicts Poor Prognosis: A Study Based on Multiple Databases, Clinical Samples and Cell Lines. *Onco. Targets. Ther.* **2020**, *13*, 2819–2832. [CrossRef]
46. Vishnubalaji, R.; Alajez, N.M. Epigenetic Regulation of Triple Negative Breast Cancer (TNBC) by TGF-β Signaling. *Sci. Rep.* **2021**, *11*, 15410. [CrossRef]
47. Pan, P.Y.; Ma, G.; Weber, K.J.; Ozao-Choy, J.; Wang, G.; Yin, B.; Divino, C.M.; Chen, S.H. Immune Stimulatory Receptor CD40 Is Required for T-Cell Suppression and T Regulatory Cell Activation Mediated by Myeloid-Derived Suppressor Cells in Cancer. *Cancer Res.* **2010**, *70*, 99–108. [CrossRef]
48. Ragusa, S.; Prat-Luri, B.; González-Loyola, A.; Nassiri, S.; Squadrito, M.L.; Guichard, A.; Cavin, S.; Gjorevski, N.; Barras, D.; Marra, G.; et al. Antiangiogenic Immunotherapy Suppresses Desmoplastic and Chemoresistant Intestinal Tumors in Mice. *J. Clin. Investig.* **2020**, *130*, 1199–1216. [CrossRef]
49. Mégarbané, A.; Piquemal, D.; Rebillat, A.S.; Stora, S.; Pierrat, F.; Bruno, R.; Noguier, F.; Mircher, C.; Ravel, A.; Vilaire-Meunier, M.; et al. Transcriptomic Study in Women with Trisomy 21 Identifies a Possible Role of the GTPases of the Immunity-Associated Proteins (GIMAP) in the Protection of Breast Cancer. *Sci. Rep.* **2020**, *10*, 9447. [CrossRef]
50. Protic, O.; Toti, P.; Islam, M.S.; Occhini, R.; Giannubilo, S.R.; Catherino, W.H.; Cinti, S.; Petraglia, F.; Ciavattini, A.; Castellucci, M.; et al. Possible Involvement of Inflammatory/Reparative Processes in the Development of Uterine Fibroids. *Cell Tissue Res.* **2016**, *364*, 415–427. [CrossRef]
51. Rohena-Rivera, K.; Sanchez-Vazquez, M.M.; Aponte-Colon, D.A.; Forestier-Roman, I.S.; Quintero-Aguilo, M.E.; Martanez-Ferrer, M. IL-15 Regulates Migration, Invasion, Angiogenesis and Genes Associated with Lipid Metabolism and Inflammation in Prostate Cancer. *PLoS ONE* **2017**, *12*. [CrossRef]
52. Cai, M.; Sikong, Y.; Wang, Q.; Zhu, S.; Pang, F.; Cui, X. Gpx3 Prevents Migration and Invasion in Gastric Cancer by Targeting NFκB/Wnt5a/JNK Signaling. *Int. J. Clin. Exp. Pathol.* **2019**, *12*, 1194.
53. Chang, C.; Worley, B.L.; Phaëton, R.; Hempel, N. Extracellular Glutathione Peroxidase GPx3 and Its Role in Cancer. *Cancers* **2020**, *12*, 2197. [CrossRef] [PubMed]
54. Arslan, A.A.; Gold, L.I.; Mittal, K.; Suen, T.-C.; Belitskaya-Levy, I.; Tang, M.-S.; Toniolo, P. Gene expression studies provide clues to the pathogenesis of uterine leiomyoma: New evidence and a systematic review. *Hum. Reprod.* **2005**, *20*, 852–886. [CrossRef] [PubMed]
55. Ono, M.; Yin, P.; Navarro, A.; Moravek, M.B.; Coon, V.J.S.; Druschitz, S.A.; Serna, V.A.; Qiang, W.; Brooks, D.C.; Malpani, S.S.; et al. Paracrine Activation of WNT/β-Catenin Pathway in Uterine Leiomyoma Stem Cells Promotes Tumor Growth. *Proc. Natl. Acad. Sci. USA* **2013**, *110*, 17053–17058. [CrossRef] [PubMed]

56. Hanahan, D.; Weinberg, R.A. Hallmarks of cancer: The next generation. *Cell* **2011**, *144*, 646–674. [CrossRef] [PubMed]
57. Borahay, M.A.; Al Hendy, A.; Kilic, G.S.; Boehning, D. Signaling Pathways in Leiomyoma: Understanding Pathobiology and Implications for Therapy. *Mol. Med.* **2015**, *21*, 242–256. [CrossRef]
58. Baranov, V.S.; Osinovskaya, N.S.; Yarmolinskaya, M.I. Pathogenomics of Uterine Fibroids Development. *Int. J. Mol. Sci.* **2019**, *20*. [CrossRef]
59. Ciarmela, P.; Delli Carpini, G.; Greco, S.; Zannotti, A.; Montik, N.; Giannella, L.; Giuliani, L.; Grelloni, C.; Panfoli, F.; Paolucci, M.; et al. Uterine Fibroid Vascularization: From Morphological Evidence to Clinical Implications. *Reprod. Biomed. Online* **2022**, *44*, 281–294. [CrossRef]
60. Leppert, P.C.; Baginski, T.; Prupas, C.; Catherino, W.H.; Pletcher, S.; Segars, J.H. Comparative Ultrastructure of Collagen Fibrils in Uterine Leiomyomas and Normal Myometrium. *Fertil. Steril.* **2004**, *82*, 1182–1187. [CrossRef]
61. Islam, M.S.; Ciavattini, A.; Petraglia, F.; Castellucci, M.; Ciarmela, P. Extracellular Matrix in Uterine Leiomyoma Pathogenesis: A Potential Target for Future Therapeutics. *Hum. Reprod. Update* **2018**, *24*, 59–85. [CrossRef] [PubMed]
62. Kyaw, M.T.H.; Yamaguchi, Y.; Choijookhuu, N.; Yano, K.; Takagi, H.; Takahashi, N.; Oo, P.S.; Sato, K.; Hishikawa, Y. The HDAC Inhibitor, SAHA, Combined with Cisplatin Synergistically Induces Apoptosis in Alpha-Fetoprotein-Producing Hepatoid Adenocarcinoma Cells. *Acta Histochem. Cytochem.* **2019**, *52*, 1–8. [CrossRef] [PubMed]
63. Abou Najem, S.; Khawaja, G.; Hodroj, M.H.; Babikian, P.; Rizk, S. Adjuvant Epigenetic Therapy of Decitabine and Suberoylanilide Hydroxamic Acid Exerts Anti-Neoplastic Effects in Acute Myeloid Leukemia Cells. *Cells* **2019**, *8*. [CrossRef]
64. Butler, L.M.; Agus, D.B.; Scher, H.I.; Higgins, B.; Rose, A.; Cordon-Cardo, C.; Thaler, H.T.; Rifkind, R.A.; Marks, P.A.; Richon, V.M. Suberoylanilide Hydroxamic Acid, an Inhibitor of Histone Deacetylase, Suppresses the Growth of Prostate Cancer Cells in Vitro and in Vivo. *Cancer Res.* **2000**, *60*, 5165–5170. [PubMed]

MANUSCRIPT 4. Carbajo-García MC, García-Alcázar Z, Corachán A, Monleón J, Trelis A, Faus A, Pellicer A, Ferrero H. Histone deacetylase inhibition by suberoylanilide hydroxamic acid: a therapeutic approach to treat human uterine leiomyoma. Fertil Steril. 2022 Feb;117(2):433-443. doi: 10.1016/j.fertnstert.2021.10.012. Epub 2021 Nov 19. PMID: 34809976.

Histone deacetylase inhibition by suberoylanilide hydroxamic acid: a therapeutic approach to treat human uterine leiomyoma

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Objective: To evaluate the effect of inhibition of histone deacetylases (HDACs) by suberoylanilide hydroxamic acid (SAHA) treatment of human uterine leiomyoma primary (HULP) cells in vitro on cell proliferation, cell cycle, extracellular matrix (ECM) formation, and transforming growth factor $\beta 3$ (TGF-$\beta 3$) signaling.
Design: Prospective study comparing uterine leiomyoma (UL) vs. adjacent myometrium (MM) tissue and cells with or without SAHA treatment.
Setting: Hospital and university laboratories.
Patient(s): Women with UL without any hormone treatment.
Intervention(s): Myomectomy or hysterectomy surgery in women for leiomyoma disease.
Main Outcome Measure(s): HDAC activity was assessed by enzyme-linked immunosorbent assay, and gene expression was assessed by quantitative real-time polymerase chain reaction. Effects of SAHA on HULP cells were analyzed by CellTiter (Promega, Madison, Wisconsin), Western blot, and quantitative real-time polymerase chain reaction.
Result(s): The expression of HDAC genes (*HDAC1*, fold change [FC] = 1.65; *HDAC3*, FC = 2.08; *HDAC6*, FC = 2.42) and activity (0.56 vs. 0.10 optical density [OD]/h/mg) was significantly increased in UL vs. MM tissue. SAHA decreased HDAC activity in HULP cells but not in MM cells. Cell viability significantly decreased in HULP cells (81.68% at 5 μM SAHA, 73.46% at 10 μM SAHA), but not in MM cells. Proliferating cell nuclear antigen expression was significantly inhibited in SAHA-treated HULP cells (5 μM SAHA, FC = 0.556; 10 μM SAHA, FC = 0.622). Cell cycle markers, including *C-MYC* (5 μM SAHA, FC = 0.828) and *CCND1* (5 μM SAHA, FC = 0.583; 10 μM SAHA, FC = 0.482), were significantly down-regulated after SAHA treatment. SAHA significantly inhibited ECM protein expression, including FIBRONECTIN (5 μM SAHA, FC = 0.815; 10 μM SAHA, FC = 0.673) and COLLAGEN I (5 μM SAHA, FC = 0.599; 10 μM SAHA, FC = 0.635), in HULP cells. *TGFβ3* and *MMP9* gene expression was also significantly down-regulated by 10 μM SAHA (*TGFβ3*, FC = 0.596; *MMP9*, FC = 0.677).
Conclusion(s): SAHA treatment inhibits cell proliferation, cell cycle, ECM formation, and TGF-$\beta 3$ signaling in HULP cells, suggesting that histone deacetylation may be useful for treatment of UL. (Fertil Steril® 2022;117:433-43. ©2021 by American Society for Reproductive Medicine.)
El resumen está disponible en Español al final del artículo.

Key Words: Cell proliferation, extracellular matrix, SAHA, ULS-$\beta 3$ pathway, uterine leiomyoma

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Uterine leiomyomas (ULs) or fibroids are benign neoplasms of the smooth muscle cells of the uterine myometrium (MM) (1). They are the most common tumor in premenopausal women, affecting 20% to 50% of women over the age of 30 years (2–4). The incidence increases with age, and UL development occurs earlier in Black women than in White women (5, 6). ULs are associated with several symptoms, including pelvic pain, abnormal uterine bleeding, and infertility, leading to recurrent abortion and preterm labor (7, 8). Currently, hysterectomy and myomectomy are the chosen treatments for UL (9). Other, less invasive, treatments based on hormone therapy are available but cause side effects, such as premenopausal symptoms or hepatic damage, and myomas recover their initial size once treatment is stopped (10). Thus, there is no effective and noninvasive treatment for UL. Although the molecular mechanisms involved in UL development remain unclear, many factors are proposed to contribute to UL, including steroid hormones (11, 12), growth factors (13), the Wnt/β-catenin pathway (14), and genetic and epigenetic mutations (15). Epigenetic mutations can be reversed by chemical agents. Therefore, targeting epigenetic modifications could be a viable therapeutic strategy to treat UL.

Histone modification is a key component in the epigenetic regulation of genes. These modifications occur at the N-terminal tail or the globular domains of core histones and include acetylation, methylation, phosphorylation, ubiquitination, and SUMOylation (16). Histone acetylation consists of the transfer of an acetyl group from acetyl coenzyme A to the lysine e-amino group on the N-terminal tails of histones. Several studies have described altered acetylation of H3K27ac histone marks in ULs compared with the adjacent MM, suggesting that acetylation plays a significant role in the development of ULs (17–19). Generally, histone acetylation has been correlated with gene expression, and deacetylation leads to transcriptional repression (20). Histone acetyltransferases add acetyl groups to histones, whereas histone deacetylases (HDACs) erase these marks (20). There are four different classes of HDACs based on their sequence similarities: class I proteins (HDAC1, HDAC2, HDAC3, and HDAC8), class II proteins (HDAC4, HDAC5, HDAC6, HDAC7, HDAC9, and HDAC10), class III proteins or sirtuins (SIRT1, SIRT2, SIRT3, SIRT4, SIRT5, SIRT6, and SIRT7), and class IV protein (HDAC11) (21). HDACs play an important role in multiple biologic processes, such as cell cycle progression, cell differentiation, and cell survival, and are involved in, for instance, the development of different tumors (22), such as breast cancer and ovarian cancer (23, 24). Notably, HDAC activity is higher in ULs than in the adjacent MM, suggesting that the transcription of genes implicated in the normal function of MM may be repressed because of a decrease in histone acetylation, resulting in an advantage for the growth and maintenance of ULs (16, 25). Suberoylanilide hydroxamic acid (SAHA), or vorinostat, is a competitive inhibitor of class I and class II HDAC (26) that is used as an anticancer drug to treat different types of tumors (27, 28). One of the well-characterized biochemical effects of SAHA is increased accumulation of acetylated core histones caused by inhibition of HDAC activity (29, 30), which blocks cell proliferation and tumor growth in hepatoid adenocarcinoma (31), myeloid leukemia (32), and prostate cancer (33).

Therefore, if HDACs are involved in UL development through epigenetic modification of histones, the use of inhibitors such as SAHA could reduce UL size to provide a novel therapeutic option. For this purpose, we evaluated the effect of SAHA in human uterine leiomyoma primary (HULP) cells on cell proliferation, cell cycle progression, apoptosis, extracellular matrix (ECM) formation, and the transforming growth factor $\beta 3$ (TGF-$\beta 3$) signaling pathway—which is widely implicated in UL development—to test its potential as a new therapeutic option to treat leiomyoma.

MATERIALS AND METHODS

Human Tissue Collection

Samples of human UL and adjacent MM were gathered from premenopausal Caucasian women aged 31 to 48 years who were undergoing myomectomy or hysterectomy as treatment for symptomatic UL pathology (n = 15) and who had not received any hormonal treatment in the previous 3 months (Supplemental Table 1, available online). A body mass index >35 kg/m^2 and current smoking were exclusion criteria. This study was approved by the Clinical Ethics Committee at the Hospital Universitario y Politécnico La Fe (Spain) (approval no. 2018/0097), and all participants provided informed consent. The tissue samples were divided into three fragments for assessment of HDAC activity, measurement of HDAC gene expression, and isolation of single cells for in vitro study. The UL and adjacent MM cells were cultured in vitro as primary cultures from the individual patients.

Human Uterine Leiomyoma Primary Cell Isolation

The UL and MM fragments were mechanically and enzymatically processed to isolated UL and MM cells from each individual patient. The tissue was treated with 2 mg/mL of type II collagenase (Labclinics, Madrid, Spain) and 1 mg/mL of DNase I (Sigma-Aldrich, St. Louis, Missouri) to obtain single-cell suspensions. The cells were incubated in vitro at 37°C and 5% CO_2 in culture medium (Dulbecco's modified Eagle medium/F-12; GIBCO, Waltham, Massachusetts) with 10% fetal bovine serum (Fisher Scientific, Waltham, Massachusetts) and antibiotic–antimycotic solution (Fisher Scientific, Waltham, Massachusetts) to study HDAC activity and the effects of SAHA treatment.

Assessment of HDAC Activity in Tissue and HULP and MM cells

HDAC activity was evaluated in UL vs. adjacent MM tissues (n = 6) as well as in HULP and MM cells from the same individual patient (n = 5) treated with and without SAHA inhibitor. Nuclear protein extracts of tissues and cells were isolated with an EpiQuik nuclear extraction kit (Epigentek, Brooklyn, New York). HDAC activity was determined with an Epigenase HDAC activity/inhibition direct assay kit (Epigentek) and a microplate reader (Synergy HT, Bio-Tek). HDAC activity is

proportional to the amount of deacetylated products colorimetrically measured at 450 nm and was calculated according to the formula provided by the manufacturer:

$$\text{HDAC activity}(\text{optical density[OD]} / \text{min} / \text{mg}) = \frac{(\text{Sample OD} - \text{Blank OD})}{(\text{Protein amount}(\mu g) \times \text{min})} \times 1,000$$

SAHA Treatment

The effects of SAHA on HULP and MM cells were assessed in vitro by culture of isolated cells from individual patients. The cells were incubated in culture medium until they achieved a confluence of 70%. The cells (n = 15) were then starved in serum-free medium overnight and were treated with different doses of SAHA (Abcam, Cambridge, UK) dissolved in dimethyl sulfoxide (DMSO). The treatment groups included 0 μM SAHA (control), 0.01% DMSO (vehicle), 5 μM SAHA, and 10 μM SAHA for 48 hours.

Cell Viability Assay

To determine cell viability after treatment with SAHA, HULP cells and MM cells from the same individual patient (n = 10) were incubated with culture medium in a 96-well plate. After the cells reached 70% confluence, they were starved in serum-free medium overnight and treated with 0 μM SAHA, 0.01% DMSO, 5 μM SAHA, or 10 μM SAHA for 48 hours. A CellTiter 96 AQueous One Solution cell proliferation assay (Promega, Madison, Wisconsin) was used to measure the quantity of proliferating viable cells, and absorbance was measured on a microplate reader (Synergy HT, Bio-Tek) at 490 nm.

Protein Extraction and Western Blotting

A radioimmunoprecipitation assay buffer containing protease inhibitors was used to extract protein from SAHA-treated and -untreated HULP cells (n = 10). Subsequently, 20 μg of each sample was analyzed by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Western blotting was performed to determine the expression of ECM proteins, such as collagen I (COL-I; 70R-CR007X, 1:1,000; Fitzgerald, Acton, Massachusetts) and fibronectin (F3648, 1:2,000, Sigma-Aldrich), proliferation proteins, such as proliferating cell nuclear antigen (PCNA; sc-56, 1:200, Santa Cruz Biotechnology, Santa Cruz, California), and apoptosis proteins, such as B-cell lymphoma-2 (BCL2; sc-7382, 1:200, Santa Cruz Biotechnology) and BCL2-associated X (BAX; sc-20067, 1:200, Santa Cruz Biotechnology). Antigen–antibody complex was detected with a chemiluminescence detection system (Thermo Fisher), and specific protein bands were visualized by chemiluminescence imaging with an LAS-3000 Imaging System (Fujifilm). Band intensity was quantified by ImageJ software and was normalized to β-actin (1:1,000; sc-47778, Santa Cruz Biotechnology).

Analysis of Gene Expression

Total RNA was extracted from UL and MM tissues with TRIzol reagent (Fisher Scientific, Waltham, Massachusetts) and from HULP cells with an RNeasy Mini kit (Qiagen, Hilden, Germany). Quantitative real-time polymerase chain reaction analysis was conducted using a StepOnePlus system (Applied Biosystems, Foster City, California) and PowerUp SYBR Green (Fisher Scientific). *HDAC1*, *HDAC3*, and *HDAC6* gene expression was evaluated in UL and MM tissues (n = 10). *CCND1*, *MMP9*, *c-MYC*, and *TGFβ3* gene expression was assessed in SAHA-treated and -untreated HULP cells (n = 10). Primers were designed with the Primer Quest tool (DNA Integrated Technologies, Coralville, Iowa) (Supplemental Table 2). Data were normalized to the expression of *GAPDH*. The $\Delta\Delta C_t$ method was used to calculate fold change.

Statistical Analysis

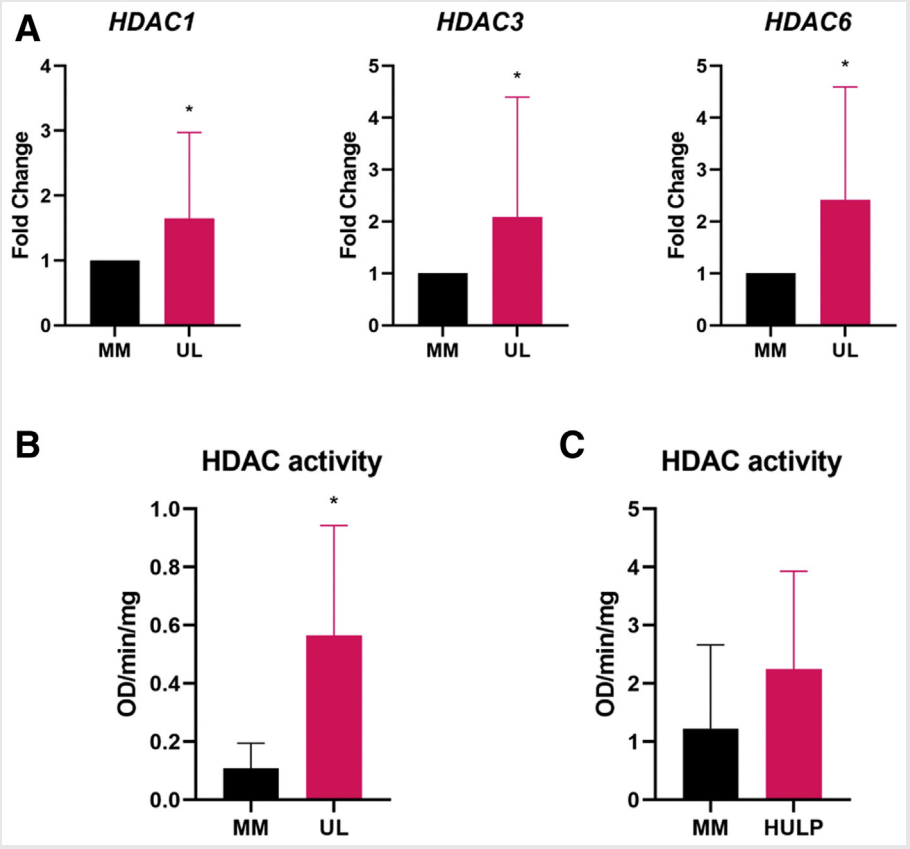
GraphPad Prism 8.0 (San Diego, California) was used for statistical analyses and generation of figures. A paired *t* test was performed to compare HDAC activity in UL vs. MM tissue and in HULP vs. MM cells. A Wilcoxon test for paired samples was performed to analyze *HDAC1*, *HDAC3*, and *HDAC6* gene expression in UL compared with adjacent MM tissues. A repeated-measures one-way analysis of variance test with a Geisser–Greenhouse correction was performed to assess cell viability and HDAC activity in HULP and MM cells after SAHA treatment, as well as *TGFβ3* and *MMP9* gene expression and PCNA, fibronectin, and collagen I protein expression. A Friedmann test was used to analyze the BAX/BCL2 ratio and *MMP9* gene expression. All SAHA treatment results were normalized and compared with control or vehicle (untreated HULP cells) groups. Data are presented as mean ± SD. A *P* value <.05 was considered to indicate statistical significance.

RESULTS

HDAC Gene Expression and HDAC Activity in UL Compared with MM Tissues

To assess whether ULs have higher expression of HDAC enzymes than adjacent MM, *HDAC1*, *HDAC3*, and *HDAC6* gene expression was compared between UL and. MM tissue samples from the same patient. UL samples displayed a statistically significant increase in *HDAC1*, *HDAC3*, and *HDAC6* gene expression with MM tissue (fold change [FC] = 1.65, *P*=.048; FC = 2.08, *P*=.042; FC = 2.42, *P*=.0413, respectively) (Fig. 1A). Global HDAC activity was measured to determine if increased gene expression of HDACs is correlated with higher activity. UL tissue exhibited significantly higher HDAC activity than MM tissue (0.56 vs. 0.10 OD/h/mg, *P*=.034) (Fig. 1B).

FIGURE 1



Histone acetylation status in UL compared with adjacent MM. (**A**) Gene expression of *HDAC1*, *HDAC3*, and *HDAC6* represented as fold change (n = 10), and (**B**) HDAC activity (OD/min/mg) in UL compared with MM tissue (n = 6). (**C**) HDAC activity (OD/min/mg) in HULP cells compared with MM cells after 7 days of cell culture (n = 5). Data are presented as the mean value ± SEM. *Significantly different between the pairs, $P<.05$. HDAC = histone deacetylase; HULP = human uterine leiomyoma primary; MM = myometrium; OD = optical density; UL = uterine leiomyoma.

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HDAC Activity of HULP and MM Cells In Vitro

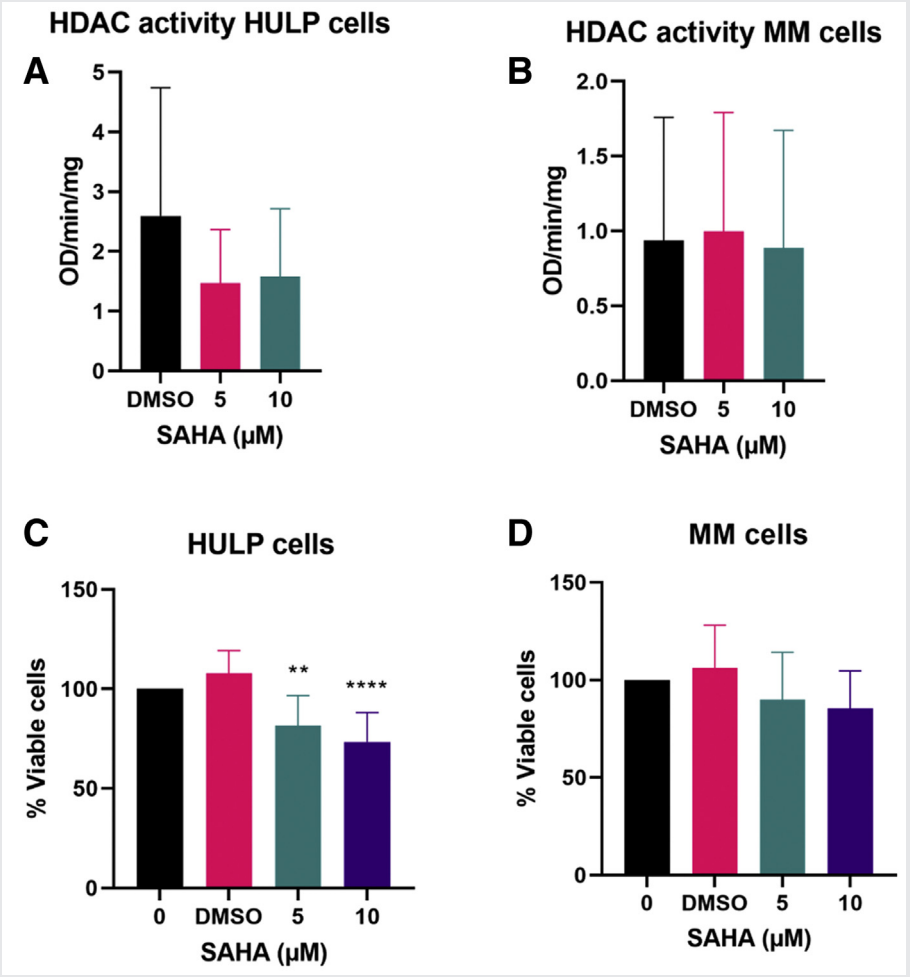
Because of the strong interaction between the environment and epigenetics, we analyzed HDAC activity in HULP compared with MM cells from the same patient after 7 days of in vitro culture to confirm that HDAC activity is not modified by the in vitro environment. The results showed that HDAC activity tended to be increased in HULP cells compared with MM cells (2.24 vs. 1.22 OD/min/mg, $P=.365$) (Fig. 1C). These results suggested that the increased HDAC activity observed in UL compared with MM tissue could not be modified in vitro by cell culture conditions, and, therefore, in vitro studies could provide a reliable framework to investigate HDACs in ULs.

In addition, we evaluated the effect of SAHA treatment on HDAC activity in vitro in HULP and MM cells from the same patient. Although HDAC activity tended to decrease in HULP cells after SAHA treatment (2.594 OD/min/mg with DMSO vs. 1.472 OD/min/mg with 5 μM SAHA [$P=.200$] and 1.575 OD/min/mg with 10 μM SAHA [$P=.422$] [Fig. 2A]), this tendency to decreased HDAC activity was not observed in MM cells treated with SAHA (0.93 OD/min/mg with DMSO vs. 0.99 OD/min/mg with 5 μM SAHA [$P=.512$] and 0.88 OD/min/mg with 10 μM SAHA [$P=.951$] [Fig. 2B]).

In Vitro Effects of SAHA on Cell Viability in HULP and MM Cells

The number of proliferating cells was determined to investigate the possible effects of SAHA on proliferation in HULP and MM cells from the same patient. Treatment with SAHA significantly decreased the percentage of viable HULP cells in a dose-dependent manner (81.68% with 5 μM SAHA [$P=.001$]; 73.46% with 10 μM SAHA [$P=.0001$] [Fig. 2C]). In contrast, the number of proliferating MM cells was not altered after SAHA treatment (89.90% with 5 μM SAHA [$P=.760$]; 85.44% with 10 μM SAHA [$P=.429$] [Fig. 2D]), suggesting that SAHA treatment has a greater effect on HULP cells than on MM cells. In addition, no effect of the treatment vehicle (DMSO) was observed in HULP and MM cells compared with untreated cells with cell culture medium only.

FIGURE 2



Effect of histone acetylation inhibition by SAHA on HDACs activity and cell viability in HULP and MM cells. HDACs activity (OD/min/mg) after SAHA treatment of HULP cells (**A**) and MM cells (**B**). Percentage of viable cells after 48 hours of treatment with 0 μM SAHA, DMSO, 5 μM SAHA, or 10 μM SAHA in HULP cells (**C**) (n = 14) and MM cells (**D**). Significantly different, ***P*<.01, ****P*<.001. DMSO = dimethyl sulfoxide; HDAC = histone deacetylase; HULP = human uterine leiomyoma primary; MM = myometrium; OD = optical density; SAHA = suberoylanilide hydroxamic acid.

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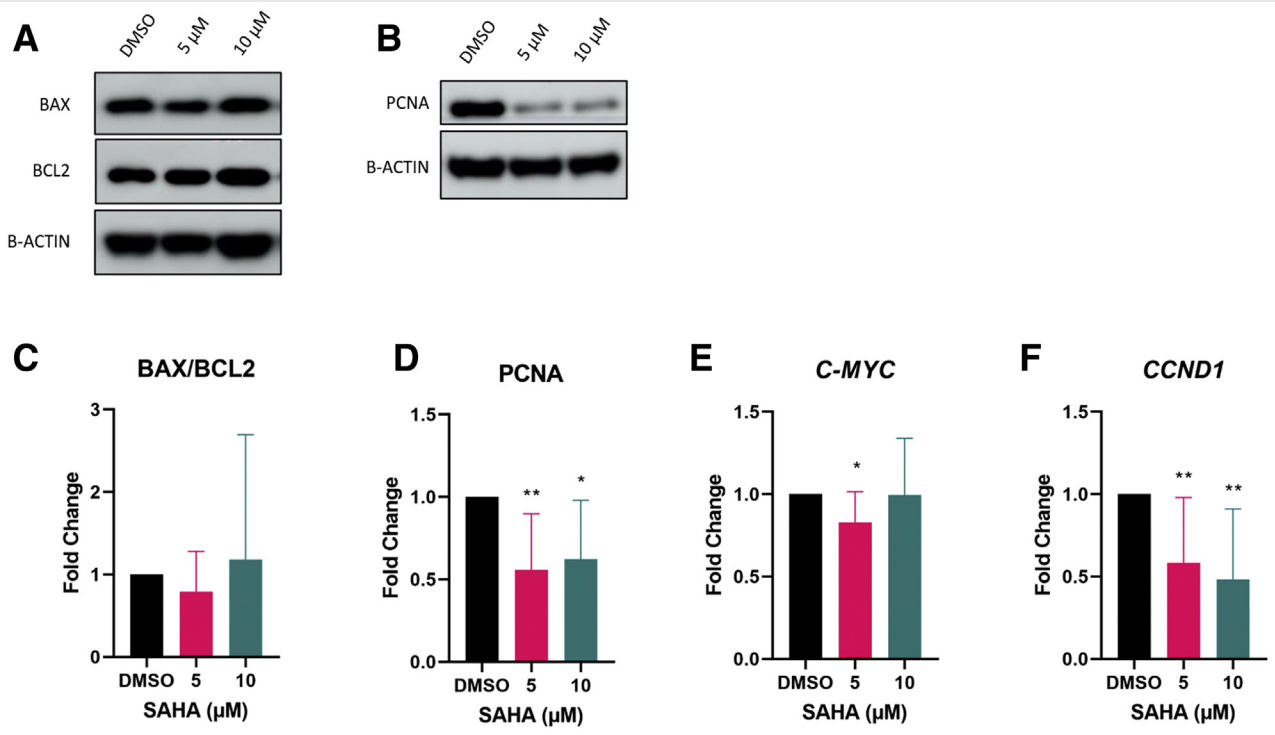
In Vitro Effects of SAHA on Apoptosis and Cell Proliferation in HULP Cells

To describe the effect of SAHA treatment on cell proliferation and apoptosis in HULP cells, we analyzed protein markers of apoptosis and proliferation by Western blot. We did not observe significant changes in BAX (proapoptotic) and BCL2 (antiapoptotic) protein expression in HULP cells treated with 5 or 10 μM SAHA compared with untreated cells (Fig. 3A), suggesting no alterations in apoptosis. Consequently, no significant differences were observed in the BAX/BCL2 ratio (5 μM SAHA, FC = 0.78 and *P*=.147; 10 μM SAHA, FC = 1.18 and *P*=.235) (Fig. 3C). We analyzed protein expression of the gold-standard proliferation marker PCNA by Western blot in SAHA-treated compared with untreated HULP cells (Fig. 3B). SAHA treatment decreased PCNA protein expression in HULP cells in a dose-dependent manner, and this decrease was statistically significant at both doses (5 μM SAHA, FC = 0.556 and *P*=.004; 10 μM SAHA, FC = 0.622 and *P*=.015) (Fig. 3D). To confirm the antiproliferative effect of SAHA treatment in HULP cells, gene expression of the cell cycle markers *C-MYC* and *CCND1* was studied by quantitative real-time polymerase chain reaction. *C-MYC* expression was significantly down-regulated in HULP cells after treatment with 5 μM SAHA (FC = 0.828 and *P*=.032) (Fig. 3E). *CCND1* expression was significantly down-regulated in HULP cells after treatment with 5 μM SAHA (FC = 0.583 and *P*=.003) and 10 μM SAHA (FC = 0.482 and *P*=.001) (Fig. 3F). Thus, SAHA inhibits gene expression of cell cycle markers in HULP cells in vitro in a dose-dependent manner.

FIGURE 3



Effect of histone acetylation inhibition by SAHA on apoptosis, cell proliferation, and cell cycle progression in HULP cells. Representative images of protein expression levels for (**A**) BAX (26 kDa), BCL2 (23 kDa), and (**B**) PCNA (36 kDa) in HULP cells (n = 10) after 48 hours of treatment with SAHA at 5 μM or 10 μM compared with cells treated with vehicle (DMSO). Quantification of normalized protein expression of the BAX/BCL2 ratio (**C**) and PCNA (**D**) represented as fold change. Gene expression levels of *C-MYC* (**E**) and *CCND1* (**F**) in HULP cells treated for 48 hours with SAHA at 5 μM or 10 μM compared with untreated HULP cells (DMSO vehicle) are represented as fold change. Significantly different, **P*<.05, ***P*<.01. DMSO = dimethyl sulfoxide; HDAC = histone deacetylase; HULP = human uterine leiomyoma primary; MM = myometrium; OD = optical density; PCNA = proliferating cell nuclear antigen; SAHA = suberoylanilide hydroxamic acid.

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In Vitro Effects of SAHA Treatment on the ECM in HULP Cells

To determine ECM status in HULP cells after SAHA treatment, we analyzed the expression of the main proteins involved in ECM formation, fibronectin and collagen I, by Western blot (Figs. 4A and B, respectively). Fibronectin expression was decreased in a dose-dependent manner, and this decrease was statistically significant at both doses (5 μM SAHA, FC = 0.815 and *P*=.010; 10 μM SAHA, FC = 0.673 and *P*=.0006) (Fig. 4C). Collagen I expression showed a statistically significant decrease in expression in a dose-dependent manner (5 μM SAHA, FC = 0.599 and *P*=.0015; 10 μM SAHA, FC = 0.635 and *P*=.008) (Fig. 4D). These results show that SAHA inhibits ECM protein expression in HULP cells in vitro in a dose-dependent manner.

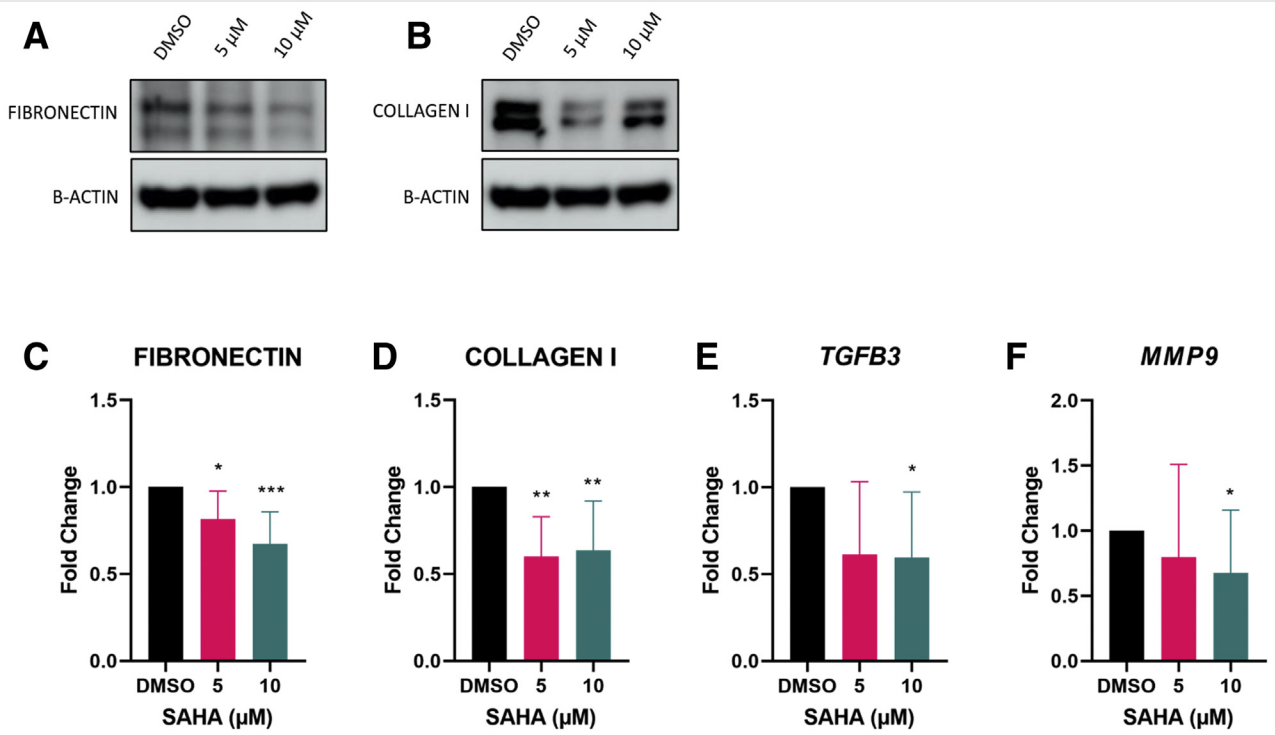
In Vitro Effects of SAHA on the TGF-β3 Signaling Pathway in HULP Cells

To evaluate the effect of SAHA treatment on the TGF-β3 signaling pathway in HULP cells, we assessed the expression of genes involved in this pathway, such as *TGFβ3* and *MMP9*, by quantitative real-time polymerase chain reaction. *TGFβ3* gene expression decreased in HULP cells after SAHA treatment (5 μM SAHA, FC = 0.611 and *P*=.060; 10 μM SAHA, FC = 0.596 and *P*=.033) (Fig. 4E). Accordingly, *MMP9* gene expression was down-regulated in treated HULP cells (5 μM SAHA, FC = 0.795 and *P*=.085; 10 μM SAHA, FC = 0.677 and *P*=.028) (Fig. 4F). These results suggest that SAHA treatment decreases the TGF-β3 signaling pathway in HULP cells.

DISCUSSION

Our study showed that SAHA inhibits cell proliferation, the cell cycle, ECM protein expression, and the TGF-β3 signaling pathway in HULP cells, which are involved in leiomyoma development, suggesting that HDACs may be a viable therapeutic target for treatment of UL. The molecular mechanisms involved in UL development remain unclear, and there is no effective and noninvasive treatment (10–14). Therefore, defining new molecular mechanisms involved in UL pathogenesis would allow us to describe new therapeutic

FIGURE 4



Effect of histone acetylation inhibition by SAHA on ECM formation in HULP cells. Representative images of protein expression levels for fibronectin (220 kDa) (**A**) and collagen I (140 kDa) (**B**) and quantitation of normalized protein expression of fibronectin (220 kDa) (**C**) and collagen I (**D**) in HULP cells treated with SAHA at 5 μM or 10 μM for 48 hours compared with control vehicle-treated HULP cells (DMSO) (n = 10). Gene expression analysis of *TGFβ3* (**E**) and *MMP9* (**F**) after SAHA treatment at 5 μM or 10 μM in HULP cells represented as fold change. Data are represented as mean and standard deviation of fold change. Significantly different, *$P<.05$, **$P<.01$, ***$P<.001$. DMSO = dimethyl sulfoxide; ECM = extracellular matrix; HULP = human uterine leiomyoma primary; SAHA = suberoylanilide hydroxamic acid.

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targets for UL. Alterations in the expression of genes that regulate epigenetic processes are frequently found as cancer drivers and may cause widespread alterations of histone posttranslational modifications, which are involved in abnormal patterns of gene expression (15, 16). Because of the inherent reversibility of epigenetic changes, inhibitors targeting these processes are promising anticancer strategies (34). By removing acetyl groups, HDACs reverse chromatin acetylation and alter the transcription of oncogenes and tumor suppressor genes (35, 36). HDAC inhibitors, such as SAHA (vorinostat, MK0683), have been emerging as promising therapies that control cancer growth and metastasis (37). On the basis of these findings, we focused on inhibition of HDACs by SAHA as a new therapeutic strategy for UL through treatment of HULP cells with those doses of SAHA tested in cells isolated from hepatocellular carcinoma (38, 39), lung cancer (28), and endometriosis (27).

For this purpose, we first studied the expression and activity of HDACs on ULs and adjacent MM tissue. It has been reported that SAHA inhibits class I and class II HDACs (40, 41). We studied HDAC1 and HDAC3 as class I HDACs and HDAC6 as a class II HDAC, because they have been proved to be important in the pathogenesis of UL (42). We confirmed that there is higher *HDAC1*, *HDAC3*, and *HDAC6* gene expression as well as higher HDAC activity in UL tissue than in adjacent MM tissue. Increased expression and activity of HDACs were reported in other studies in both breast cancer and UL (16, 25, 42), suggesting that transcription of genes involved in normal MM function may be repressed, promoting tumor growth and maintenance. On the basis of these findings, the use of HDAC inhibitors to reverse histone deacetylation could be a good therapeutic approach to treat UL. Subsequently, because of the interaction between the environment and epigenetics, we studied HDAC activity in cell culture to confirm the maintenance of this epigenetic feature in an in vitro model. HDAC activity tended to be increased in HULP cells compared with MM cells, suggesting that the in vitro environment does not modify HDAC status observed in the tissue. These results allow the study of epigenetic modifications such as inhibition of HDACs by SAHA under in vitro conditions.

To assess the effectiveness of SAHA treatment on HULP and MM cells, we studied HDAC activity after SAHA treatment in vitro. We found that HDAC activity tended to be decreased in HULP cells after SAHA treatment but not in MM cells. Accordingly, analysis of the number of

proliferating cells showed that treatment with SAHA significantly decreased the percentage of viable HULP cells but not of MM cells. These findings suggest, for the first time to our knowledge, that SAHA treatment is more effective in HULP cells than in MM cells, proving its potential as a UL treatment in which the adjacent MM will likely not be affected.

It is widely known that increased proliferation of UL cells may be involved in UL development and growth (14, 43). HDACs are an important epigenetic feature in UL development, and cell proliferation is one of the key biologic processes involved in UL development. Therefore, the reversal of histone hypoacetylation by SAHA could reduce UL size by decreasing cell proliferation. We found that treatment of HULP cells with SAHA decreased cell proliferation, as demonstrated by decreased PCNA protein expression. These findings suggest an antiproliferative effect of SAHA treatment on UL, as was previously described in other tumors (31–33). Additionally, the cell cycle regulatory genes *CCND1* and *C-MYC* were down-regulated after SAHA treatment. *CCND1* is overexpressed in UL compared with MM (44, 45), as well as in other tumor types, thus making it a negative prognostic marker of UL (46, 47). It has been reported that other HDAC inhibitors decrease UL cell growth through cell cycle arrest (44). In addition, induction of cell cycle arrest by SAHA is reported in cancer (37). Apoptosis did not increase after SAHA treatment, demonstrating the absence of SAHA toxicity in HULP cells. These results collectively indicate that HDACs are critical in the regulation of cell proliferation, and SAHA could reduce the growth of ULs by decreased cell proliferation and cell cycle arrest.

Uterine leiomyomas are characterized by excessive deposition of ECM, specifically collagen, fibronectin, and sulfated proteoglycans, causing expansion of the UL (48). ECM should be considered as a crucial target for UL therapeutics (49). Our results showed that SAHA treatment significantly decreased the expression of ECM-associated proteins, such as fibronectin and collagen I, suggesting an important role of histone acetylation in the regulation of ECM formation. Other studies have demonstrated the success of treatments targeting ECM formation to reduce UL growth, such as treatment with vitamin D (50–52). In addition, the role of histone acetylation in ECM regulation has been shown in renal tubulointerstitial fibrosis (53), in which HDAC inhibitors had an antifibrotic effect.

The TGF-β3 signaling pathway is overexpressed in UL compared with the adjacent MM and is directly responsible for the development of the UL fibrotic phenotype by increasing ECM deposition and cell proliferation (54–56). Elevated levels of matrix metalloproteinase 9 (MMP-9) and total MMPs, which are targets of the TGF-β3 signaling pathway, are evident in leiomyoma (57). In previous studies of cancer, acetylation has been linked to TGF-β3 signaling (58), and TGF-β3-induced genes that regulate matrix turnover were regulated by HDACs (59). Considering that TGF-β3 has a principal role in ECM overproduction and is related to HDACs, SAHA treatment would decrease the expression of these proteins, reducing ECM deposition. Our study showed that SAHA treatment down-regulated *TGFβ3* and *MMP9* gene expression. The positive antifibrotic effect of TGF-β3 inhibition in UL treatment has been shown with the use of ulipristal acetate (55) and vitamin D (56). In addition, vitamin D effectively reduces the expression and activities of MMP-9 in cultured human uterine fibroid cells (52), as does the HDAC inhibitor trichostatin A in trophoblast cells (60). On the basis of these findings, we suggest that the TGF-β3 signaling pathway is regulated by epigenetic deacetylation and that inhibition of HDACs by SAHA treatment alters the functionality of this pathway, leading to a reduction in ECM deposition.

CONCLUSION

We suggest that the reduction of ECM deposition and the decrease in cell cycle progression and cell proliferation generated by the reversal of histone deacetylation with SAHA treatment induces inhibition of UL growth. These findings highlight that inhibition pf HDACs may be a viable therapeutic target to treat UL. Further studies are necessary to assess the potential effects of HDAC inhibition on the TGF-β3 signaling pathway as well as the in vivo effect of SAHA treatment on UL.

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REFERENCES

1. Stewart EA. Uterine fibroids. Lancet 2001;357:293–8.
2. Manyonda I, Sinthamoney E, Belli AM. Controversies and challenges in the modern management of uterine fibroids. BJOG 2004;111:95–102.
3. Parker WH. Etiology, symptomatology, and diagnosis of uterine myomas. Fertil Steril 2007;87:725–36.
4. Styer AK, Rueda BR. The epidemiology and genetics of uterine leiomyoma. Best Pract Res Clin Obstet Gynaecol 2016;34:3–12.
5. Marshall LM, Spiegelman D, Barbieri RL, Goldman MB, Manson JE, Colditz GA, et al. Variation in the incidence of uterine leiomyoma among premenopausal women by age and race. Obstet Gynecol 1997;90:967–73.
6. Baird DD, Dunson DB, Hill MC, Cousins D, Schectman JM. High cumulative incidence of uterine leiomyoma in black and white women: ultrasound evidence. Am J Obstet Gynecol 2003;188:100–7.
7. Su WH, Lee WL, Cheng MH, Yen MS, Chao KC, Wang PH. Typical and atypical clinical presentation of uterine myomas. J Chin Med Assoc 2012;75:487–93.
8. Vlahos NF, Theodoridis TD, Partsinevelos GA. Myomas and adenomyosis: impact on reproductive outcome. Biomed Res Int 2017;2017:5926470.
9. Zhang RC, Wu W, Zou Q, Zhao H. Comparison of clinical outcomes and postoperative quality of life after surgical treatment of type II submucous myoma via laparoscopy or hysteroscopy. J Int Med Res 2019;47:4126–33.
10. Friedman AJ. Treatment of leiomyomata uteri with short-term leuprolide followed by leuprolide plus estrogen-progestin hormone replacement therapy for 2 years: a pilot study. Fertil Steril 1989;51:526–8.
11. Maruo T, Ohara N, Wang J, Matsuo H. Sex steroidal regulation of uterine leiomyoma growth and apoptosis. Hum Reprod Update 2004;10:207–20.

12. Patel B, Elguero S, Thakore S, Dahoud W, Bedaiwy M, Mesiano S. Role of nuclear progesterone receptor isoforms in uterine pathophysiology. Hum Reprod Update 2015;21:155–73.
13. Sozen I, Arici A. Interactions of cytokines, growth factors, and the extracellular matrix in the cellular biology of uterine leiomyomata. Fertil Steril 2002;78:1–12.
14. Ono M, Yin P, Navarro A, Moravek MB, Coon V JS, Druschitz SA, et al. Paracrine activation of WNT/β-catenin pathway in uterine leiomyoma stem cells promotes tumor growth. Proc Natl Acad Sci U S A 2013;110:17053–8.
15. Laganà AS, Vergara D, Favilli A, La Rosa VL, Tinelli A, Gerli S, et al. Epigenetic and genetic landscape of uterine leiomyomas: a current view over a common gynecological disease. Arch Gynecol Obstet 2017;296:855–67.
16. Yang Q, Mas A, Diamond MP, Al-Hendy A. The mechanism and function of epigenetics in uterine leiomyoma development. Reprod Sci 2016;23:163–75.
17. Leistico JR, Saini P, Futtner CR, et al. Epigenomic tensor predicts disease subtypes and reveals constrained tumor evolution. Cell Rep 2021;34:108927.
18. Moyo MB, Parker JB, Chakravarti D. Altered chromatin landscape and enhancer engagement underlie transcriptional dysregulation in MED12 mutant uterine leiomyomas. Nat Commun 2020;11:1019.
19. Lin Y, Liu J, Huang Y, Liu D, Zhang G, Kan H. microRNA-489 plays an antimetastatic role in human hepatocellular carcinoma by targeting matrix metalloproteinase-7. Transl Oncol 2017;10:211–20.
20. Verdone L, Caserta M, Di Mauro E. Role of histone acetylation in the control of gene expression. Biochem Cell Biol 2005;83:344–53.
21. Seto E, Yoshida M. Erasers of histone acetylation: the histone deacetylase enzymes. Cold Spring Harb Perspect Biol 2014;6:a018713.
22. Gallinari P, Di Marco S, Jones P, Pallaoro M, Steinkühler C. HDACs, histone deacetylation and gene transcription: from molecular biology to cancer therapeutics. Cell Res 2007;17:195–211.
23. Hervouet E, Claude-Taupin A, Gauthier T, Perez V, Fraichard A, Adami P, et al. The autophagy GABARAPL1 gene is epigenetically regulated in breast cancer models. BMC Cancer 2015;15:729.
24. Bitler BG, Wu S, Park PH, Hai Y, Aird KM, Wang Y, et al. ARID1A-mutated ovarian cancers depend on HDAC6 activity. Nat Cell Biol 2017;19:962–73.
25. Sant'Anna G. dos S, Brum IS, Branchini G, Pizzolato LS, Capp E, Corleta H von E. Ovarian steroid hormones modulate the expression of progesterone receptors and histone acetylation patterns in uterine leiomyoma cells. Gynecol Endocrinol 2017;33:629–33.
26. Marks PA, Breslow R. Dimethyl sulfoxide to vorinostat: development of this histone deacetylase inhibitor as an anticancer drug. Nat Biotechnol 2007;25:84–90.
27. Kawano Y, Nasu K, Li H, Tsuno A, Abe W, Takai N, et al. Application of the histone deacetylase inhibitors for the treatment of endometriosis: histone modifications as pathogenesis and novel therapeutic target. Hum Reprod 2011;26:2486–98.
28. Wei Y, Zhou F, Lin Z, Shi L, Huang A, Liu T, et al. Antitumor effects of histone deacetylase inhibitor suberoylanilide hydroxamic acid in epidermal growth factor receptor-mutant non-small-cell lung cancer lines in vitro and in vivo. Anticancer Drugs 2018;29:262–70.
29. Richon VM, Emiliani S, Verdin E, Webb Y, Breslow R, Rifkind RA, et al. A class of hybrid polar inducers of transformed cell differentiation inhibits histone deacetylases. Proc Natl Acad Sci U S A 1998;95:3003–7.
30. Finnin MS, Donigian JR, Cohen A, Richon VM, Rifkind RA, Marks PA, et al. Structures of a histone deacetylase homologue bound to the TSA and SAHA inhibitors. Nature 1999;401:188–93.
31. Kyaw MTH, Yamaguchi Y, Choijookhuu N, Yano K, Takagi H, Takahashi N, et al. The HDAC inhibitor, SAHA, combined with cisplatin synergistically induces apoptosis in alpha-fetoprotein-producing hepatoid adenocarcinoma cells. Acta Histochem Cytochem 2019;52:1–8.
32. Abou Najem S, Khawaja G, Hodroj MH, Babikian P, Rizk S. Adjuvant epigenetic therapy of decitabine and suberoylanilide hydroxamic acid exerts antineoplastic effects in acute myeloid leukemia cells. Cells 2019;8:1480.
33. Butler LM, Agus DB, Scher HI, Higgins B, Rose A, Cordon-Cardo C, et al. Suberoylanilide hydroxamic acid, an inhibitor of histone deacetylase, suppresses the growth of prostate cancer cells in vitro and in vivo. Cancer Res 2000;60:5165–70.
34. Bennett RL, Licht JD. Targeting epigenetics in cancer. Annu Rev Pharmacol Toxicol 2018;58:187–207.
35. Li Y, Seto E. HDACs and HDAC inhibitors in cancer development and therapy. Cold Spring Harb Perspect Med 2016;6:a026831.
36. Ropero S, Esteller M. The role of histone deacetylases (HDACs) in human cancer. Mol Oncol 2007;1:19–25.
37. Natarajan U, Venkatesan T, Radhakrishnan V, Samuel S, Rasappan P, Rathinavelu A. Cell cycle arrest and cytotoxic effects of saha and rg7388 mediated through p21waf1/cip1and p27kip1in cancer cells. Medicina (Kaunas) 2019;55:30.
38. Wang YC, Yang X, Xing LH, Kong WZ. Effects of SAHA on proliferation and apoptosis of hepatocellular carcinoma cells and hepatitis B virus replication. World J Gastroenterol 2013;19:5159–64.
39. Freese K, Seitz T, Dietrich P, Lee SML, Thasler WE, Bosserhoff A, et al. Histone deacetylase expressions in hepatocellular carcinoma and functional effects of histone deacetylase inhibitors on liver cancer cells in vitro. Cancers (Basel) 2019;11:1587.
40. Marks PA. Discovery and development of SAHA as an anticancer agent. Oncogene 2007;26:1351–6.
41. Thaler F, Mercurio C. Towards selective inhibition of histone deacetylase isoforms: what has been achieved, where we are and what will be next. ChemMedChem 2014;9:523–36.
42. Wei LH, Torng PL, Hsiao SM, Jeng YM, Chen MW, Chen CA. Histone deacetylase 6 regulates estrogen receptor α in uterine leiomyoma. Reprod Sci 2011;18:755–62.
43. Bulun SE. Uterine fibroids. N Engl J Med 2013;369:1344–55.
44. Ali M, Shahin SM, Sabri NA, Al-Hendy A, Yang Q. Activation of β-catenin signaling and its crosstalk with estrogen and histone deacetylases in human uterine fibroids. J Clin Endocrinol Metab 2020;105:E1517–35.
45. Chuang T Der, Khorram O. Regulation of cell cycle regulatory proteins by microRNAs in uterine leiomyoma. Reprod Sci 2019;26:250–8.
46. Montalto FI, De Amicis F. Cyclin D1 in cancer: a molecular connection for cell cycle control, adhesion and invasion in tumor and stroma. Cells 2020;9:2648.
47. Martín-Ezquerra G, Salgado R, Toll A, Gilaberte M, Baró T, Alameda Quitllet F, et al. Multiple genetic copy number alterations in oral squamous cell carcinoma: study of MYC, TP53, CCDN1, EGFR and ERBB2 status in primary and metastatic tumours. Br J Dermatol 2010;163:1028–35.
48. Leppert PC, Baginski T, Prupas C, Catherino WH, Pletcher S, Segars JH. Comparative ultrastructure of collagen fibrils in uterine leiomyomas and normal myometrium. Fertil Steril 2004;82:1182–7.
49. Islam MS, Ciavattini A, Petraglia F, Castellucci M, Ciarmela P. Extracellular matrix in uterine leiomyoma pathogenesis: a potential target for future therapeutics. Hum Reprod Update 2018;24:59–85.
50. Halder SK, Sharan C, Al-Hendy A. 1,25-Dihydroxyvitamin D3 treatment shrinks uterine leiomyoma tumors in the Eker rat model. Biol Reprod 2012;86:116.
51. Corachán A, Ferrero H, Escrig J, Monleon J, Faus A, Cervelló I, et al. Long-term vitamin D treatment decreases human uterine leiomyoma size in a xenograft animal model. Fertil Steril 2020;113:205–16.
52. Halder SK, Osteen KG, Al-Hendy A. Vitamin D3 inhibits expression and activities of matrix metalloproteinase-2 and -9 in human uterine fibroid cells. Hum Reprod 2013;28:2407–16.
53. Nguyên-Thanh T, Kim D, Lee S, Kim W, Park SK, Kang KP. Inhibition of histone deacetylase 1 ameliorates renal tubulointerstitial fibrosis via modulation of inflammation and extracellular matrix gene transcription in mice. Int J Mol Med 2017;41:95–106.
54. Lee B-S, Nowak RA. Human leiomyoma smooth muscle cells show increased expression of transforming growth factor-β3 (TGFβ3) and altered responses to the antiproliferative effects of TGFβ 1. J Clin Endocrinol Metab 2001;86:913–20.
55. Lewis TD, Malik M, Britten J, Parikh T, Cox J, Catherino WH. Ulipristal acetate decreases active TGF-β3 and its canonical signaling in uterine leiomyoma via two novel mechanisms. Fertil Steril 2019;111:806–15.
56. Corachán A, Trejo MG, Carbajo-García MC, Monleón J, Escrig J, Faus A, et al. Vitamin D as an effective treatment in human uterine leiomyomas

independent of mediator complex subunit 12 mutation. Fertil Steril 2021; 115:512–21.
57. Inagaki N, Ung L, Otani T, Wilkinson D, Lopata A. Uterine cavity matrix metalloproteinases and cytokines in patients with leiomyoma, adenomyosis or endometrial polyp. Eur J Obstet Gynecol Reprod Biol 2003;111:197–203.
58. Suriyamurthy S, Baker D, Ten Dijke P, Iyengar PV. Epigenetic reprogramming of TGF-β signaling in breast cancer. Cancers (Basel) 2019;11:726.
59. Barter MJ, Pybus L, Litherland GJ, Rowan AD, Clark IM, Edwards DR, et al. HDAC-mediated control of ERK- and PI3K-dependent TGF-β-induced extracellular matrix-regulating genes. Matrix Biol 2010;29: 602–12.
60. Estella C, Herrer I, Atkinson SP, Quiñonero A, Martínez S, Pellicer A, et al. Inhibition of histone deacetylase activity in human endometrial stromal cells promotes extracellular matrix remodelling and limits embryo invasion. PLoS One 2012;7:e30508.

Inhibición de las histonas deacetilasas mediante el ácido hidroxámico suberoilanilida: una aproximación terapéutica para tratar los miomas uterinos humanos.

Objetivo: Evaluar el efecto de la inhibición de las histonas deacetilasas (HDACs) mediante el tratamiento con ácido hidroxámico suberoilanilida (SAHA) en células primarias de mioma humano (HULP) *in vitro* sobre la proliferación celular, ciclo celular, formación de matriz extraceluar (ECM), y señalización del factor de crecimiento transformante $\beta 3$ (TGF-$\beta 3$).

Entorno: Estudio prospectivo comparando tejido de miomas uterinos (UL) versus miometrio adyacente (MM) y células con o sin tratamiento con SAHA.

Paciente(s): Hospital y laboratorios universitarios.

Intervención(es): Mujeres con UL sin ningún tratamiento hormonal.

Principales medidas de resultado(s): La actividad de HDAC se evaluó mediante un ensayo inmunoabsorbente ligado a enzimas, y la expresión génica se evaluó mediante la reacción cuantitativa en cadena de la polimerasa a tiempo real. Los efectos de SAHA sobre las células HULP se analizaron mediante CellTiter (Promega, Madison, Wisconsin), Western Blot y reacción cuantitativa en cadena de la polimerasa a tiempo real.

Resultado(s): La expresión de los genes HDAC (*HDAC1,* tasa de cambio [FC] = 1,65; *HDAC3,* FC=2,08; *HDAC6,* FC=2,42) y actividad (0,56 vs. 0,10 densidad óptica [OD]/h/mg) fueron significativamente aumentadas en tejido de UL vs. MM. SAHA disminuyó la actividad de HDAC en las células HULP, pero no en las células de MM. La viabilidad celular disminuyó significativamente en las células HULP (81,68% a 5 μM de SAHA; 73,46% a 10 μM de SAHA), pero no en las células de MM. La expresión del antígeno nuclear de proliferación celular fue significativamente inhibida en las células HULP tratadas con SAHA (5 μM de SAHA, FC= 0,556; 10 μM de SAHA, FC= 0,622). Los marcadores de ciclo celular, incluyendo *C-MYC (*5 μM de SAHA, FC= 0,828) y *CCND1* (5 μM de SAHA, FC=0,583; 10 μM de SAHA, FC= 0,482, fueron significativamente regulados a la baja después del tratamiento con SAHA. SAHA significativamente inhibió la expresión de proteínas de ECM, incluyendo FIBRONECTINA (5 μM de SAHA, FC= 0,815; 10 μM de SAHA, FC= 0,673) y COLAGENO I (5 μM de SAHA, FC= 0,599; 10 μM de SAHA, FC= 0,635) en las células HULP. La expresión génica de *TGF-$\beta 3$* y *MMP9* fue también significativamente regulada a la baja tras el tratamiento con SAHA a 10 μM (*TGF-$\beta 3$,* FC= 0,596; *MMP9*, FC= 0,677).

Conclusión(s): El tratamiento con SAHA inhibe la proliferación celular, el ciclo celular, la formación de ECM y la señalización TGF-$\beta 3$ en las células HULP, sugiriendo que a deacetilación de histonas pueden ser útiles para tratar los miomas uterinos.

V. RESULTS

“I would give everything I know for half of what I don’t know”-
René Descartes

V. RESULTS

MANUSCRIPT 1. Carbajo-García MC, Corachán A, Juárez-Barber E, Monleón J, Payá V, Trelis A, Quiñonero A, Pellicer A, Ferrero H. Integrative analysis of the DNA methylome and transcriptome in uterine leiomyoma shows altered regulation of genes involved in metabolism, proliferation, extracellular matrix, and vesicles. J Pathol. 2022 Aug;257(5):663-673. doi: 10.1002/path.5920. Epub 2022 Jun 13. PMID: 35472162.

Global DNA methylome profile in UL compared to adjacent MM

After an exploratory analysis of all β-values to determine genome DNA methylation profile in UL compared with adjacent MM (n = 31), principal component analysis (PCA) and heatmap revealed a clear separation between UL and adjacent MM (Figure 1A and B). Analysis of methylation status showed a higher median methylation level in UL compared to adjacent MM (0.704 UL vs 0.681 MM, FDR-adjusted p-value = 0.004) (Figure 1C). Using a Holm-adjusted p-value cut-off of 0.05, we found 43,241 differentially methylated CpGs (11,335 hypomethylated and 31,906 hypermethylated).

Global transcriptome profile in UL compared to adjacent MM

An exploratory analysis of RNA-seq data was performed to assess the role of UL on gene expression. Clustering the 56 tissues (28 UL and 28 MM) using PCA (Figure 1D) and heatmap (Figure 1E) revealed a clear effect of tumorigenic phenotype on global transcriptome behaviour in UL tissue compared to adjacent MM. RNA-seq of the 28 patients showed 10,339 DEGs between UL tissue compared to adjacent MM, with 5,690 upregulated and 4,649 downregulated genes (FDR-adjusted $p < 0.05$, log2fold-change > 0.5 or < 0.5).

Abnormally methylated and differentially expressed genes in UL

To analyze the expression of genes controlled by DNA methylation and their function, we integrated the results from RNA-seq and genome-wide DNA methylation analyses. Among the 43,241 differentially methylated CpGs (Holm < 0.05), we selected 11,772 CpGs that were near or inside a promoter region and correlated these CpGs with the 10,339 DEGs. We identified a total of 93 genes regulated by methylation with at least one CpG site matching the hypomethylation/upregulation or hypermethylation/downregulation filter and a Spearman correlation coefficient > 0.7 or < 0.7. Among them, 22 were hypomethylated/upregulated and 71 were hypermethylated/downregulated (Figure 2A). Heatmaps revealed a clear separation

between the top 20 hypermethylated/downregulated genes and top 20 hypomethylated/upregulated genes in UL compared to MM (Figure 2 B, C).

Functional implications of genes controlled by aberrant D0NA methylation in UL

Functional enrichment analysis of the 93 selected genes controlled by aberrant DNA methylation identified 30 biological processes significantly deregulated in human UL (Figure 2D). These processes could be classified within functional groups such as metabolism and cell physiology, response to extracellular signals, invasion, and proliferation, which are key pathways in tumor pathogenesis. Additionally, we found 30 cellular components significantly enriched in UL (Figure 2E), which were mainly related to cell membranes, vesicles, extracellular matrix, and cell junctions. Among the molecular functions enriched in UL (Figure 2F), most were associated with metabolism, cell physiology, and response to extracellular signals. Finally, KEGG pathway analysis (Figure 2G) revealed pathways involved in cancer and uterine biology deregulated in UL.

Validation of results from integrative analysis

DNA methylation results were validated by pyrosequencing of CpG present in genes selected based on their potential significance in tumorigenesis and/or UL pathology. Pyrosequencing results corroborated the lower levels of DNA methylation in CpG islands from *PRL* (70.9% vs 32.7%, p-value < 0.001), *ATP8B4* (70.5% vs 31.7%, p-value = 0.001), *CEMIP* (30.6% vs 11.2%, p-value = 0.001), and *ZFPM2-AS1* (92.3% vs 71.5%, p-value = 0.001) (Figure 3A–D), as well as higher levels of *EFEMP1* (2.7% vs 38.04%, p-value = 0.001), *FBLN2* (90.6% vs 96.81%, p-value = 0.006), *ARHGAP10* (16.8% vs 52.4%, p-value < 0.001), and *HTATIP2* (18.2% vs 62.8%, p-value < 0.001) (Figure 3E–H) in UL compared to adjacent MM. RNA-seq results were validated in selected genes analyzing the gene expression by qRT-PCR and these results corroborated upregulation of *PRL* (fold-change = 658.8, p-value = 0.003), *ATP8B4* (fold-change = 16.23, p-value = 0.003), *CEMIP* (fold-change = 34.7, p-value=0.002), *ZFPM2-AS1* (fold-change = 44.43, p-value = 0.005) *RIMS2* (fold-change = 24.57, p-value = 0.002), and *TFAP2C (*fold-change = 86.59, p-value = 0.002) in a separate cohort of UL compared to adjacent MM (Figure 4A–F). Similarly, qRT-PCR validated downregulation of *EFEMP1* (fold-change = 0.1, p-value = 0.002), *FBLN2* (fold-change = 0.48, p-value = 0.002), *ARHGAP10* (fold-change = 0.84, p-value = 0.27), and *HTATIP2* (fold-change = 0.79, p-value = 0.16) (Figure 4G–J).

Inhibiting DNA methylation upregulates the expression of hypermethylated/downregulated genes in vitro

The inhibition of DNA methylation with the DNA methyltransferase (DNMT) inhibitor 5-Aza-2'-deoxycytidine (5-aza-CdR) upregulated the gene expression of the selected hypermethylated/downregulated genes described in UL in this study, such as *EFEMP1* (fold-change = 2.88±2.49, p-value=0.009), *FBLN2* (fold-change = 1.73±1.37, p-value = 0.013), *ARHGAP10* (fold-change = 17.76±26.21, p-value = 0.019), and *HTATIP2* (fold-change = 1.57±0.72, p-value=0.037) in primary human uterine leiomyoma primary (HULP) cells *in vitro* (n = 10) (Figure 5A–D).

MANUSCRIPT 2. Carbajo-García MC, Corachán A, Segura-Benitez M, Monleón J, Escrig J, Faus A, Pellicer A, Cervelló I, Ferrero H. 5-aza-2'-deoxycitidine inhibits cell proliferation, extracellular matrix formation and Wnt/β-catenin pathway in human uterine leiomyomas. Reprod Biol Endocrinol. 2021 Jul 8;19(1):106. doi: 10.1186/s12958-021-00790-5. PMID: 34233687; PMCID: PMC8265104.

***DNTM1* gene expression and DNMT activity in UL vs MM tissues**

To determine whether increased global DNA methylation in UL is due to a higher expression of DNMT enzymes, *DNMT1* gene expression was analyzed in UL compared to MM tissue (n = 7). UL tissue showed a statistically significant higher gene expression of *DNMT1* compared to MM tissue (fold-change = 2.49, p-value = 0.029) (Figure 1A). Accordingly, a significantly higher DNMT activity was observed in UL compared to adjacent MM tissue (6.50 vs 3.76 OD/h/mg, p-value = 0.026) (Figure 1B).

DNMT activity in HULP and myometrial cells in vitro

DNMT activity in HULP and myometrial cells was evaluated to determine the correlation between DNMT activity in tissue and cells cultured in vitro. DNMT activity was greater in HULP cell compared to MM cells (n = 3) at day 7 (211.30 vs 63.67 OD/h/mg, p-value = 0.284), day 9 (66.41 vs 11.88 OD/h/mg, p-value = 0.217), and day 10 (24.75 vs 6.47 OD/h/mg, p-value = 0.337) (Figure 1C), corroborating that increased DNMT activity observed in tissues is not modified by in vitro conditions.

In vitro effects of 5-aza-2'-deoxycytidine on cell proliferation in HULP cells

Treatment with 5-aza-CdR decreased the percentage of viable HULP cells in a dose-dependent manner, being statistically significant with high-dose (10 μM) treatment in HULP cells (n = 16) (85.25% at 10 μM, p-value = 0.0001) compared to 0 μM (Figure 2A), demonstrating its anti-proliferative effect. BAX (pro-apoptotic) and BCL2 (anti-apoptotic) protein expression were analyzed as markers of apoptosis status (Figure 2B). Although the BAX/BCL2 ratio showed a trend to increase in HULP cells (n = 8) after treatment, no significant differences were observed at any dose (Figure 2D), suggesting that 5-aza-CdR did not affect apoptosis. 5-aza-CdR treatment decreased PCNA protein expression, the gold standard proliferation marker, in HULP cells (n = 8) in a dose-dependent manner (Figure 2C), reaching statistical significance at the high dose (10 μM; fold-change = 0.695, p-value = 0.034) (Figure 2E).

In vitro effects of 5-aza-2'-deoxycytidine on extracellular matrix in HULP cells

To determine the effect of 5-aza-CdR on the expression of the main proteins involved in ECM formation, collagen I and fibronectin protein levels were evaluated. 5-aza-CdR treatment decreased collagen I protein expression in a dose-dependent manner, being significant at 10 µM (fold-change = 0.654, p-value = 0.023; Figure 3A and D). In addition, a statistically significant reduction in fibronectin expression was observed at 2 µM (fold-change = 0.812, p-value = 0.020) and 10 µM (fold-change = 0.733, p-value = 0.035) (Figure 3B and E). Finally, PAI-1 expression was significantly decreased compared to the control group (0 µM) after 5-aza-CdR treatment at 5 µM and 10 µM (fold-change = 0.865, p-value = 0.035; fold-change = 0.766, p-value = 0.020, respectively) (Figure 3C and F).

In vitro effects of 5-aza-2'-deoxycytidine on Wnt/β-catenin signaling pathway in HULP cells

The protein and mRNA expression levels of the final targets of the Wnt/β-catenin pathway were analyzed after 5-aza-CdR treatment in HULP cells. Results revealed an inhibition of WISP1 protein expression in HULP cells (n = 8) treated with 5-aza-CdR compared to the control group (0 µM) in a dose-dependent manner (Figure 4A), reaching statistical significance at 10 µM (10 µM; fold-change = 0.699, p-value = 0.026) (Figure 4B).

Furthermore, gene expression of *c-MYC* was downregulated in HULP cells (n = 8) after 5-aza-CdR treatment at all doses tested compared to untreated cells, reaching significance at 2 µM and 10 µM doses (2 µM fold-change = 0.745, p-value = 0.028; 10 µM fold-change = 0.728, p-value = 0.019, respectively) (Figure 4C). Similarly, a significant decrease in *MMP7* gene expression was observed in HULP cells after 5-aza-CdR treatment with 5 µM and 10 µM compared to HULP cells without treatment (5 µM fold-change = 0.520, p-value = 0.003, 10 µM fold-change = 0.577, p-value = 0.007, respectively) (Figure 4D).

MANUSCRIPT 3. Carbajo-García, M.C.; de Miguel-Gómez, L.; Juárez-Barber, E.; Trelis, A.; Monleón, J.; Pellicer, A.; Flanagan, J.M.; Ferrero, H. Deciphering the Role of Histone Modifications in Uterine Leiomyoma: Acetylation of H3K27 Regulates the Expression of Genes Involved in Proliferation, Cell Signaling, Cell Transport, Angiogenesis and Extracellular Matrix Formation. Biomedicines. 2022May 30;10(6):1279. doi: 10.3390/biomedicines10061279. PMID: 35740301; PMCID: PMC9219820.

Global H3K27ac CHIP-Seq peak profile in UL tissue compared to adjacent MM

An exploratory analysis of CHIP-seq data was performed to describe the H3K27ac profile in UL compared to adjacent MM tissue (n = 21). PCA revealed a separation between UL and adjacent MM (Figure 1A). Clustering analysis showed common patterns within UL different from MM, tending to segregate, as observed in a heatmap (Figure 1B). A lower amount of H3K27ac histone mark in the promoter region of all human genes was observed in UL compared to MM (p-value $< 2.2 \times 10^{-16}$), suggesting a global hypoacetylation of UL (Figure 1C).

Selection of relevant differentially expressed genes

Differential expression analysis was performed by integrating RNA-seq data from GSE192354 and GSE142332 studies. After the intersection of outcomes obtained from both datasets, 922 genes were revealed as common DEGs in UL compared to MM samples (n = 52) with an FDR-adjusted p-value < 0.01, log2fold-change > 1, or < −1 (Figure 2A). Specifically, 559 were upregulated and 363 downregulated.

Identification of differentially expressed genes with an aberrant H3K27ac mark in UL tissue compared to adjacent MM

Among the 922 genes selected for this analysis after RNA-seq, 482 (52.3%) presented the histone mark H3K27ac around the promoter region. A PCA of CHIP-seq data of these genes showed a clear separation of tumor (UL) and control (MM) samples (Figure 2B), indicating a different behaviour of the H3K27ac profile of relevant selected genes in UL compared to adjacent MM, as confirmed by heatmap (Figure 2C). Downregulated genes presented a lower fold-enrichment score of H3K27ac peaks (p-value $< 2.2 \times 10^{-16}$) in UL versus MM, while upregulated genes exhibited a higher fold-enrichment score (p-value $< 2.2 \times 10^{-16}$) in UL versus MM (Figure 2D). Differential peak enrichment analysis showed that 82 DEGs presented differential acetylation (FDR < 0.05) in UL compared to MM, with 29 hyperacetylated/upregulated and 53 hypoacetylated/downregulated.

Functional implications of differentially expressed genes associated with aberrant H3K27 acetylation in UL tissue compared to adjacent MM

Functional enrichment analysis of 82 DEGs associated with a different H3K27ac profile revealed 30 biological processes significantly deregulated in human UL versus MM that were mainly related to cell proliferation, cell signaling, and cell transport and angiogenesis (Figure 3A). In addition, cellular components were found to be significantly enriched in UL, which were all related to an alteration of the extracellular matrix (Figure 3B).

Validation of hypoacetylated/downregulated and hyperacetylated/upregulated Genes

To highlight the significance of UL-relevant DEGs with differential H3K7ac status, selected genes based on their relevance for UL pathogenesis were validated. The gene expression analysis corroborated the significant upregulation of *COL24A1* (fold-change = 15.83, p-value = 0.003), *NDP* (fold-change = 26.38, p-value = 0.037), *HOXA13* (fold-change = 1.86, p-value = 0.041) and *IGFL3* (fold-change = 13.26, p-value = 0.031) in a separate cohort of UL compared to adjacent MM (Figure 4A–D). Likewise, this expression analysis proved the downregulation of *CD40* (fold-change = 0.54, p-value = 0.010), *DPT* (fold-change = 0.25, p-value = 0.002), *GIMAP8* (fold-change = 0.52, p-value = 0.015), *GPX3* (fold-change = 0.30, p-value < 0.0001) and *IL15* (fold-change = 0.36, p-value = 0.005) (Figure 4E–I).

Inhibiting histone deacetylases reverses the expression of hypoacetylated/downregulated genes in Human Uterine Leiomyoma Primary (HULP) cells in vitro

The inhibition of histone deacetylases (HDAC) by suberoylanilide hydroxamic acid (SAHA) treatment significantly upregulated expression of the hypoacetylated/downregulated tumor suppressor genes *CD40* (fold-change = 6.78, p-value = 0.001), *DPT* (fold-change = 1.80, p-value = 0.033), *GIMAP8* (fold-change = 30.67, p-value = 0.042), *GPX3* (fold-change = 22.15, p-value = 0.001) and *IL15* (fold-change = 2.71, p-value = 0.018) in HULP cells (n = 10) (Figure 4J–N).

MANUSCRIPT 4. Carbajo-García MC, García-Alcázar Z, Corachán A, Monleón J, Trelis A, Faus A, Pellicer A, Ferrero H. Histone deacetylase inhibition by suberoylanilide hydroxamic acid: a therapeutic approach to treat human uterine leiomyoma. Fertil Steril. 2022 Feb;117(2):433-443. doi: 10.1016/j.fertnstert.2021.10.012. Epub 2021 Nov 19. PMID: 34809976.

HDAC gene expression and HDAC activity in UL compared with MM tissues

To determine whether increased global histone acetylation in UL is due to a higher expression of HDAC enzymes, *HDAC1, HDAC3* and *HDAC6* gene expression was analyzed in UL compared to MM tissue (n = 10). UL samples displayed a statistically significant increase in *HDAC1, HDAC3,* and *HDAC6* gene expression compared to MM tissue (fold-change = 1.65, p-value = 0.048; fold-change = 2.08, p-value = 0.042; fold-change = 2.42, p-value = 0.041, respectively) (Figure 1A). UL tissue exhibited significantly higher HDAC activity than MM tissue (n = 6) (0.56 vs. 0.10 OD/h/mg, p-value = 0.034) (Figure 1B).

HDAC activity of HULP and MM cells in vitro

HDAC activity in HULP and myometrial cells (n = 5) was evaluated to determine the correlation between DNMT activity in tissue and cells cultured in vitro. HDAC activity tended to be increased in HULP cells compared with MM cells (2.24 vs. 1.22 OD/min/mg, p-value = 0.365) (Figure 1C), corroborating that increased HDAC activity was observed in tissues is not modified by vitro conditions. In addition, we evaluated the effect of SAHA treatment on HDAC activity in vitro in HULP and MM cells from the same patient. HDAC activity tended to decrease in HULP cells after SAHA treatment (2.594 OD/min/mg with dimethyl sulfoxide (DMSO) vs. 1.472 OD/min/mg with 5 µM SAHA [p-value = 0.200] and 1.575 OD/min/mg with 10 µM SAHA [p-value = 0.422] [Figure 2A]), whereas this tendency was not observed in MM cells (0.93 OD/min/mg with DMSO vs. 0.99 OD/min/mg with 5 µM SAHA [p-value = 0.512] and 0.88 OD/min/mg with 10 µM SAHA [p-value = 0.951] [Figure 2B]).

In vitro effects of SAHA on cell viability in HULP and MM cells

Treatment with SAHA significantly decreased the percentage of viable HULP cells (n = 10) in a dose-dependent manner (81.68% with 5 µM SAHA [p-value = 0.001]; 73.46% with 10 µM SAHA [p-value = 0.0001] [Figure 2C]). In contrast, the number of proliferating MM cells (n = 10) was not altered after SAHA treatment (89.90% with 5 µM SAHA [p-value = 0.760]; 85.44% with 10 µM SAHA [p-value = 0.429] [Figure 2D]), suggesting that SAHA treatment has a greater effect on HULP cells than on MM cells.

In vitro effects of SAHA on apoptosis and cell proliferation in HULP cells

Protein expression of the apoptosis markers BAX (pro-apoptotic) and BCL2 (anti-apoptotic) did not show significant changes in HULP cells (n = 10) treated with SAHA compared with untreated cells (Figure 3A). Consequently, no significant differences were observed in the BAX/BCL2 ratio (Figure 3C), suggesting that SAHA did not affect apoptosis. SAHA treatment significantly decreased PCNA protein expression in HULP cells (n = 10) in a dose-dependent manner (5 µM SAHA, fold-change = 0.556 and p-value = 0.004; 10 µM SAHA, fold-change = 0.622 and p-value = 0.015) (Figure 3B and D). Gene expression of the cell cycle markers *c-MYC* and *CCND1* was studied. *C-MYC* expression was significantly downregulated in HULP cells (n = 10) after treatment with 5 µM SAHA (fold-change = 0.828 and p-value = 0.032) (Figure 3E). *CCND1* expression was significantly downregulated in HULP cells (n = 10) after treatment with 5 µM SAHA (fold-change = 0.583 and p-value = 0.003) and 10 µM SAHA (fold-change = 0.482 and p-value = 0.001) (Figure 3F).

In vitro effects of SAHA treatment on the ECM in HULP cells

To determine the effect of SAHA on the expression of the main proteins involved in ECM formation, collagen I and fibronectin protein levels were evaluated in HULP cells (n = 10). Fibronectin protein expression was significantly decreased in a dose-dependent manner at both doses with SAHA (5 µM SAHA, fold-change = 0.815 and p-value = 0.010; 10 µM SAHA, fold-change = 0.673 and p-value = 0.0006) (Figure 4C). Collagen I expression showed a statistically significant decrease in expression in a dose-dependent manner (5 µM SAHA, fold-change = 0.599 and p-value = 0.0015; 10 µM SAHA, fold-change = 0.635 and p-value = 0.008) (Figure 4D).

In vitro effects of SAHA on the TGFβ signaling pathway in HULP cells

To evaluate the effect of SAHA on the TGFβ signaling pathway, the expression of genes involved in this pathway, such as *TGFβ3* and *MMP9* was analysed in HULP cells (n = 10). *TGFβ3* gene expression decreased in HULP cells after SAHA treatment (5 µM SAHA, fold-change = 0.611 and p-value = 0.060; 10 µM SAHA, fold-change = 0.596 and p-value = 0.033) (Figure 4E). Accordingly, *MMP9* gene expression was downregulated in treated HULP cells (5 µM SAHA, fold-change = 0.795 and p-value=0.085; 10 µM SAHA, fold-change = 0.677 and p-value = 0.028) (Figure 4F).

VI. DISCUSSION

“Nothing in life is to be feared, it is only to be understood. Now is the time to understand more, so that we may fear less.” – Marie Curie.

VI. DISCUSSION

ULs are prominent gynecological neoplasms, with multifactorial etiology, that have a substantial influence on women's health, and are a leading cause of infertility. Despite the large socioeconomic impacts of these tumors, their pathogenesis remains unclear, and there is currently no effective non-invasive treatment for women affected by UL (Bulun, 2013; Donnez and Dolmans, 2016b; Giuliani *et al.*, 2020). Revealing new molecular mechanisms involved in UL development is necessary to better understand its pathophysiology, and identify new drug targets for alternative therapeutic strategies. Multiple genomic analyses have associated UL development to numerous molecular alterations and aberrant oncogene signaling. However, since race, diet, age, BMI, and parity are also contributing risk factors for UL (Stewart *et al.*, 2017), epigenetic factors may additionally mediate tumorigenesis (Yang *et al.*, 2016a; Laganà *et al.*, 2017; Mlodawska *et al.*, 2022) and are therapeutically relevant because of their potential for reversal (Kobayashi *et al.*, 2011; Klutstein *et al.*, 2016). Based on these findings, the main objective of this thesis was to characterize the epigenetic mechanisms involved in UL development (particularly DNA methylation and histone modification) and identify novel druggable targets.

We first took a holistic approach to elucidate the association between DNA methylation and gene expression profiles of UL compared to adjacent MM, and identify the commonly deregulated epigenetically-mediated molecular mechanisms involved in UL development. Our findings confirmed that both the global methylome and transcriptome are altered in ULs compared to MM. DNA methylation regulated the expression of genes involved in key biological processes for UL pathogenesis, suggesting that we could potentially reveal novel therapeutic targets in patients with UL. In our paired study, we revealed a global hypermethylation status of UL compared to MM, as well as different transcriptomic behaviour in ULs from Caucasian women, showing a robust correlation between DNA methylation and gene expression in these patients. These results agree with previously published reports that described differential gene expression in UL (Arslan *et al.*, 2005; Styer and Rueda, 2016; Laganà *et al.*, 2017; Anjum *et al.*, 2019), as well as aberrant DNA methylation in cancer (Klutstein *et al.*, 2016; Pfeifer, 2018; Long *et al.*, 2021; Wang *et al.*, 2021b) and UL (Yamagata *et al.*, 2009; Navarro *et al.*, 2012a; Maekawa *et al.*, 2013). Then, we identified the abnormally methylated DEGs in UL compared to MM. We found 93 DEGs with a strong correlation between the methylation status of CpG islands (in proximity to the gene promotor) and gene expression, with 22 hypomethylated/upregulated and 71 hypermethylated/downregulated genes. These results suggested that gene expression

altered by DNA methylation prompts UL development, These findings align with other findings describing the involvement of DNA methylation in molecular subtypes of UL and the maintenance of the UL stem cell population (George *et al.*, 2019; Liu *et al.*, 2020).

Among the biological processes and molecular functions the UL-related DEGs were involved with, we identified metabolism and cell physiology, response to extracellular signals, invasion, and proliferation to be dysregulated due to aberrant DNA methylation. Transformed cells adapt their metabolism to promote tumor initiation and progression, which supports the alteration of metabolism in UL (Vander Heiden and DeBerardinis, 2017). Further, we showed that classic hallmarks of cancer, such as dysregulated responses to cell death signals, uncontrolled proliferation, and invasion (Hanahan and Weinberg, 2011) which have previously been associated with UL (Bulun, 2013; Ono *et al.*, 2013; Borahay *et al.*, 2015; Baranov *et al.*, 2019), were controlled by epigenetic factors in patients with UL. KEGG analysis revealed pathways related to cancer and uterine biology, and more specifically, GnRH and estrogen signaling pathways, were enriched in ULs. This supports the hypothesis that DNA methylation may be associated with the alteration of these hormones, which are involved in UL progression and symptoms (de la Cruz and Buchanan, 2017). Furthermore, instabilities in DNA methylation were associated with UL-related DEGs in various cellular components such as the cell membranes, vesicles, ECM, and cell junctions, affirming that the excessive synthesis and deposition of ECM is a leading cause of UL growth (Leppert *et al.*, 2004; Islam *et al.*, 2018). On the other hand, cancer cells produce more exosomes than normal cells, and their extracellular vesicles are involved in cancer development, metastasis, and anti-tumor immunity (Minciacchi *et al.*, 2015; Bebelman *et al.*, 2018; Zhang and Yu, 2019). However, evidence of how extracellular vesicles affect UL development is limited. In this study we identified *RIMS2* among the hypomethylated/upregulated genes. Since *RIMS2* has been found to control exocytosis in neurons (Kaeser *et al.*, 2012), and the acrosome reaction of spermatozoa (Bello *et al.*, 2012), in both cases priming vesicles for release, we propose *RIMS2* plays a similar role in UL, paving the way for additional studies characterizing the function of extracellular vesicles in UL.

To reveal the UL-related genes downstream of DNA methylation, we studied the top significant hypomethylated/upregulated genes. We highlight *TFAP2C*, *PRL, ATP8B4, CEMIP,* and *ZFPM2-AS1* [which are oncogenes involved in angiogenesis (Kitaya *et al.*, 2007), Hippo signaling pathway (Wang *et al.*, 2018), proliferation (Liu *et al.*, 2021), invasion (Kong *et al.*, 2018; Yan *et al.*, 2020), and the Wnt/β-catenin pathway (Kong *et al.*, 2018; Wang *et al.*, 2018)] as key players in UL initiation and progression. Specifically, our study demonstrated that the reported upregulation of *PRL* and *CEMIP* in UL is associated with DNA hypomethylation, and novelly identified *TFAP2C*

and *ZFPMS2-AS1* as drivers of UL initiation, based on their roles in promoting angiogenesis, cell proliferation, and tumor invasion. Notably, *ATP8B4* is a cation transport ATPase, previously proposed as a potential prognostic marker and therapeutic target in cancer including colorectal, breast and ovarian cancers (Kong *et al.*, 2018; Liu *et al.*, 2021). Inhibition of this driver gene could decrease cell proliferation and invasion of UL cells, while increasing apoptosis, which could also have a significant clinical benefit, however, further studies are necessary to validate these effects of *ATP8B4* inhibition in patients with UL. We also examined the top significant hypermethylated/downregulated genes and selected *EFEMP1, FBLN2, ARHGAP10,* and *HTATIP2* because of their implication in UL progression. Among these tumor suppressor genes, *FBLN2* is involved in invasion and deposition of the ECM (Marsh *et al.*, 2016; Jin *et al.*, 2019), *HTATIP2* promotes angiogenesis and alters metabolism (Xu *et al.*, 2016), while *EFEMP1* and *ARHGAP10* enhance the Wnt/β-catenin pathway (Yang *et al.*, 2016b; Vander Heiden and DeBerardinis, 2017).

Interestingly, some of the other hypomethylated/upregulated (*KCNMA1-AS1, LINC01160, KLHDC8A*) and hypermethylated/downregulated (*PLAC9*) genes we identified were not previously associated with UL, but are also implicated in proliferation and invasion (Ouyang *et al.*, 2018; Ma *et al.*, 2019; Zhu *et al.*, 2020; Ai *et al.*, 2021), and therefore, potentially contribute to UL progression.

To confirm that DNA hypermethylation represses the expression of tumor suppressor genes that drive UL, we inhibited DNMTs *in vitro* with 5-aza-2'-deoxycytidine (5-aza-CdR) treatment. Indeed, inhibition of DNA methylation upregulated tumor suppressors *EFEMP1, HTATIP2, FBLN2,* and *ARHGAP10*. The ECM has been proposed as a potential therapeutic target for UL (Islam *et al.*, 2018). Our study revealed that *EFEMP2* and *FBLN2*, two regulators of ECM deposition, were associated with aberrant DNA methylation, and that DNA demethylation recovered their expression, suggesting its potential therapeutic impact.

The findings from this first study not only support the inherent reversibility of epigenetic changes, but also agree that targeting epigenetic processes with selective inhibitors is a promising anticancer strategy (Bennett and Licht, 2018). As such, our next study aimed to evaluate the efficacy of DNA methylation reversion (by the DNMT inhibitor 5-aza-CdR) as an alternative treatment option for patients with UL.

We first justified the use of DNMT inhibitors to reverse the aberrant DNA methylation in UL, by finding a higher *DNMT1* expression, and consequently, DNMT activity in UL compared to MM tissue. Notably, this overexpression was maintained in several cultures of our HULP cells during

10 days, confirming the reproducibility of our *in vitro* model. These findings support previous observations of increased DNMT1 and DNMT3a mRNA expression in UL samples compared to MM (Li *et al.*, 2003; Yamagata *et al.*, 2009).

Our study highlighted how 5-aza-CdR inhibited cell proliferation, ECM formation, and the Wnt/β-catenin pathway in HULP cells, setting the foundation for future preclinical assessments. Uncontrolled proliferation is one of the hallmarks of cancer and drivers of UL growth (Bulun, 2013; Ono *et al.*, 2013). The antiproliferative effect of 5-aza-CdR has previously been described in different tumors (Li *et al.*, 2018; Nunes *et al.*, 2020; Wong, 2020), and we evaluated its versatility for managing UL. We found the antiproliferative action of 5-aza-CdR in our HULP cells was mediated by reduced PCNA protein expression. Notably, 5-aza-CdR treatment did not increase apoptosis, highlighting the safety of this drug. Further, our results showed that HULP cells exposed to 5-aza-CdR significantly decreased the expression of ECM-associated proteins, such as fibronectin, collagen I, and PAI-1, which are excessively deposited during UL growth. Dysregulation of the Wnt/β-catenin pathway drives aberrant cell proliferation and ECM production in several cancers (Clevers and Nusse, 2012; Zhan *et al.*, 2017) and UL (Ono *et al.*, 2013; Borahay *et al.*, 2015). In this regard, multiple groups recently postulated that targeting the overactive Wnt/β-catenin pathway is a promising therapeutic approach (Al-Hendy *et al.*, 2016; Corachán *et al.*, 2019; Ali *et al.*, 2020). The inhibitory effect of 5-aza-CdR on the Wnt/β-catenin pathway was recently demonstrated in colorectal cancer (Deitrick and Pruitt, 2016), but remain unknown for UL. We therefore assessed the expression levels (mRNA and protein) of downstream components of the Wnt/β-catenin pathway. We found that both *c-MYC* gene expression and WISP1 protein expression were downregulated in treated HULP cells. Notably, the *c-MYC* proto-oncogene is involved in cell cycle progression (Zakiryanova *et al.*, 2018), while the WISP1 oncogene has been associated with tumor proliferation, migration, and poor prognosis in several cancer types [such as glioblastoma (Jing *et al.*, 2017), colorectal cancer and colon cancer (Davies *et al.*, 2010)]. Finally, we studied *MMP7* due to its implications with ECM, epithelial-mesenchymal transitions, and tumor invasion (Scheau *et al.*, 2019). We observed 5-aza-CdR treatment in HULP cells downregulated *MMP7*, suggesting this drug reduces the invasive capacity of UL cells via Wnt/β-catenin pathway inhibition. Taken together, our findings suggest that DNA methylation overactivates Wnt/β-catenin signaling, enhancing cell proliferation and ECM synthesis in HULP cells, but treatment with the DNMTs inhibitor 5-aza-CdR efficiently reverses methylation and suppresses these processes. Despite these promising results, further studies are necessary to elucidate the role of DNA methylation on other

components of the Wnt/β-catenin pathway, as well as the *in vivo* specificity and efficacy of 5-aza-CdR treatment for ULs.

Most studies characterizing the influence of epigenetics on UL pathogenesis have mainly focused on DNA methylation. However, histone modifications also alter chromatin structure, and therefore, it is necessary to fully explore their effect on the expression of genes involved in the pathogenesis of this disease. Among the epigenetic modifications of histones, histone acetylation promotes gene expression, while deacetylation represses gene transcription (Verdone *et al.*, 2005). Aberrant H3K27ac profiles have been associated to several cancers [e.g., gastric, lung, and ovarian (Ma and Zheng, 2021; Wang *et al.*, 2021a; Zhang *et al.*, 2021)], and we identified H3K27ac in UL pathogenesis, highlighting it as a potential therapeutic target. We additionally found that H3K27ac regulates genes implicated in key processes of UL pathogenesis (i.e., cell proliferation, cell signaling and transport, angiogenesis, and ECM formation), making histone deacetylation reversion a plausible therapeutic alternative strategy to clinically manage UL.

Notably, we found a global hypoacetylation of H3K27 in UL compared to the adjacent MM, which would repress tumor suppressor genes. Recent reports have underlined the importance of H3K27ac, H3K4me3, and H2A.Z in gene enhancers and promoter regulation, finding differential patterns between UL subtypes based on the mutation status (Moyo *et al.*, 2020; Berta *et al.*, 2021; Leistico *et al.*, 2021). Herein, an aberrant status of H3K27ac in UL (regardless of the mutational subtype) compared to MM, distinguishes its role in UL pathogenesis, aiding in developing the UL-specific gene deregulation. Therefore, we selected key DEGs involved in UL development whose change of expression was associated with the histone mark H3K27ac. Among the 922 DEGs described by integration of RNA-seq analyses, 482 presented the histone mark H3K27ac around the promoter, and 82 of them exhibited a differential H3K27ac status in UL compared to MM, with 29 hyperacetylated/upregulated and 53 hypo-acetylated/downregulated. We further reviewed the literature for the 82 DEGs regulated by H3K27ac and found that these genes were associated to multiple functions related to tumor development and maintenance. We found hyperacetylation/upregulation of oncogenes such as *NDP, HOXA13, COL24A1,* and *IGFL3*, with *NDP* and *IGFL3* not related to UL until this study. Among these genes *NDP* is involved in angiogenesis (Planutis *et al.*, 2014) and the Wnt/β-catenin pathway (El-Sehemy *et al.*, 2020), *COL24A1* is related to invasion and the ECM (Jamaluddin *et al.*, 2018), *HOXA13* is associated with invasion, angiogenesis, Wnt/β-catenin and TFGβ signaling (Duan *et al.*, 2015; Gu *et al.*, 2020), and *IGFL3 is* involved in TGFβ signaling (Vishnubalaji and Alajez, 2021). Additionally, we found hypoacetylation/downregulation of tumor suppressor

genes such as *CD40, GIMAP8, IL15, GPX3,* and *DPT,* with *CD40, GIMAP8,* and *GPX3* associated with UL for the first time in this study. Among which, *CD40* shows antiangiogenic and pro-immune properties (Pan *et al.*, 2010; Ragusa *et al.*, 2020), *IL15* controls invasion, angiogenesis and metabolism (Rohena-Rivera *et al.*, 2017) and contributes to excessive ECM production (Protic *et al.*, 2016), *GPX3* inhibits migration and invasion by Wnt/β-catenin (Cai *et al.*, 2019; Chang *et al.*, 2020), and *DPT* inhibits cell proliferation and regulates the TGFβ pathway (Arslan *et al.*, 2005). We propose that their hypoacetylation and, therefore downregulation, could promote angiogenesis and hide tumor cells from the immune system, as well as stimulate other processes that trigger UL initiation.

In general, the dysregulation of these genes confirms that key processes of UL development are controlled by histone acetylation. The interplay of H3K27ac-gene expression and cell signaling pathways can broaden the understanding of UL development and merits more attention. Therefore, we performed a functional enrichment analysis of 82 DEGs regulated by H3K27ac. This study revealed the significantly dysregulated biological processes in human UL were principally related to cell proliferation, cell signaling, cell transport, and angiogenesis. UL is characterized by uncontrolled proliferation, the main feature of tumors (Ono *et al.*, 2013; Borahay *et al.*, 2015). Furthermore, cell communication and cell signaling are altered in tumors, contributing to the aberrant response to extracellular signals and enhancing tumor development that is characteristic of cancer (Hanahan and Weinberg, 2011; Baranov *et al.*, 2019). Neoangiogenesis is another hallmark of cancer and a requirement for tumor progression (Hanahan and Weinberg, 2011). Malignant cells require oxygen and nutrients to survive and proliferate, needing proximity to blood vessels to access circulating blood. The aberrant vascularization found in UL (Ciarmela *et al.*, 2022) can be triggered by a change in histone marks such as H3K27ac. Different growth factors and vascular genes mediate the angiogenic process, which we demonstrated in this study is regulated by the epigenetic states of genes. Accordingly, our study revealed hyperacetylation/upregulation of oncogenes related to angiogenesis and vascular invasion (*COL24A1, NDP,* and *HOXA13*) and hypoacetylation/downregulation of angiogenesis-tumor suppressor genes (*CD40* and *IL15*). We also found UL-related DEGs significantly affecting cellular components related to altered ECM formation. Excessive synthesis and deposition of ECM exert a major role in the growth and stiffness of UL, contributing to clinical symptoms, such as abnormal bleeding and abdominal pain (Leppert *et al.*, 2004; Islam *et al.*, 2018). For this reason, the ECM has been considered a crucial target for UL therapeutics. Herein, we found hyperacetylation/upregulation of ECM-associated oncogenes, such as

COL24A1 and *IGFL3*, and hypoacetylation/downregulation of ECM-associated tumor suppressor genes such as *IL15* and *DPT*.

HDAC inhibitors, such as SAHA, are emerging as second-line therapy for refractory leukemia, colon, and breast cancer (Mann *et al.*, 2007; Natarajan *et al.*, 2019; Li *et al.*, 2021). Notably, SAHA selectively inhibits class I (HDAC1/3) and class II (HDAC6) HDACs (Marks, 2007; Thaler and Mercurio, 2014), and demonstrated its ability to block cell proliferation and tumor growth in hepatoid adenocarcinoma (Kyaw *et al.*, 2019), myeloid leukemia (Abou Najem *et al.*, 2019), and prostate cancer (Butler *et al.*, 2000). We treated HULP cells with SAHA to reverse the effects of H3K27ac on tumor suppressor gene expression in UL. Our study highlighted that SAHA treatment upregulated the expression of selected hypoacetylated/downregulated tumor suppressor genes (i.e., *CD40, GIMAP8, IL15, GPX3,* and *DPT*) in HULP cells *in vitro*. These findings indicated that the aberrant H3K27ac in UL represses tumor suppressor genes, deregulating pathways involved in UL pathogenesis, such as cell proliferation, cell signaling and transport, angiogenesis, and ECM formation, however the reversion of histone acetylation by HDAC inhibitor SAHA could become a promising therapeutic approach to treat UL.

Based on the clinical versatility of this therapy, we focused our next study on the inhibition of HDACs by SAHA, as a new therapeutic strategy for UL. First, we evaluated the expression and activity of HDACs in ULs and adjacent MM tissue. Our findings corroborated previous reports (Wei *et al.*, 2011; Sant'Anna *et al.*, 2017) that there is higher expression and activity of *HDAC1/3/6* in UL tissue than in adjacent MM tissue, and led us to postulate that the transcription of genes involved in normal MM function may be repressed, promoting tumor growth and maintenance. Our cell cultures validated that HDAC activity tended to be increased in HULP cells compared with MM cells, indicating that the *in vitro* environment does not modify the HDAC status observed in the tissue. After SAHA treatment, we observed HDAC activity tended to be decreased in HULP cells but not in MM cells. Accordingly, SAHA significantly decreased the percentage of viable UL cells but not MM cells, showcasing its efficacy and specificity (in terms of not affecting the adjacent MM).

As previously described herein, the increased proliferation of UL cells is involved in UL development and growth (Ono *et al.*, 2013; Borahay *et al.*, 2015). Treatment of HULP cells with SAHA reduced cell proliferation, as demonstrated by decreased PCNA protein expression, corroborating the antiproliferative effects of SAHA in other tumors (Butler *et al.*, 2000; Abou Najem *et al.*, 2019; Kyaw *et al.*, 2019). Additionally, SAHA downregulated the cell cycle regulatory genes *CCND1* and *c-MYC,* supporting recent reports of HDAC inhibitors halting UL cell

growth through cell cycle arrest (Ali *et al.*, 2020), and inducting cell cycle arrest in MCF-7 and LNCaP cancer cell lines (Natarajan *et al.*, 2019). We emphasize that SAHA treatment did not increase apoptosis of either UL or MM cells, highlighting its safety. These results collectively indicate that HDACs are critical in the regulation of cell proliferation, and treatment with SAHA could halt the growth of ULs by blocking cell proliferation and inducing cell cycle arrest.

Due to excessive deposition of ECM in UL, the ECM is emerging as a therapeutic target (Islam *et al.*, 2018). Our study proved that SAHA significantly decreased the synthesis of ECM-associated proteins (i.e., fibronectin and collagen I), distinguishing histone acetylation as a key regulator of ECM formation in UL. These antifibrotic effects were similarly described with another HDAC inhibitor (Valproic acid) in renal tubulointerstitial fibrosis (Nguyên-Thanh *et al.*, 2017). On the other hand, the TGFβ signaling pathway is overexpressed in UL and is directly responsible for the development of the UL fibrotic phenotype by increasing ECM deposition and cell proliferation (Lee and Nowak, 2001; Lewis *et al.*, 2019). In previous studies of cancer, acetylation has been linked to TGFβ signaling (Suriyamurthy *et al.*, 2019), and TGFβ-induced genes that regulate matrix turnover were regulated by HDACs (Barter *et al.*, 2010). Our study showed that SAHA treatment downregulated *TGFβ3* and *MMP-9* (the end-product of the TGFβ3 signaling pathway). Interestingly, the positive antifibrotic effect of TGFβ3 inhibition in UL treatment has also recently been shown with the use of ulipristal acetate (Lewis *et al.*, 2019) and vitamin D (Corachán *et al.*, 2021). Notably, the expression and activities of MMP-9 were effectively reduced by vitamin D in HULP cells (Halder *et al.*, 2013) and the HDAC inhibitor trichostatin A in trophoblast cells (Estella *et al.*, 2012). Taken together, these findings suggest that SAHA-mediated inhibition of HDACs disrupts the TGFβ signaling pathway, ultimately reducing ECM deposition.

Overall, our studies highlight that SAHA treatment reverses histone acetylation in HULP cells, and has the ability to reduce ECM deposition, cell cycle progression, and cell proliferation, which ultimately, have the potential to halt UL progression. These findings support our hypothesis that inhibition of HDACs is a viable therapeutic target for UL, and set the foundation for studies that further characterize the potential effects of HDAC inhibition on the TGFβ signaling pathway, as well as the *in vivo* effect and long-term safety of SAHA treatment for UL.

Based on the results obtained in this Ph.D. thesis, we conclude that epigenetic mechanisms, through DNA methylation and histone modifications (such as acetylation), are key drivers of UL pathogenesis. In addition, targeting enzymes that catalyze these epigenetic changes is a viable and alternative therapeutic strategy for clinical management of UL.

VII. CONCLUSIONS

"The world as we have created it is a process of our thinking. It cannot be changed without changing our thinking." - Albert Einstein

VII. CONCLUSIONS

The main conclusions drawn from this thesis are:

- Uterine leiomyoma exhibits a global hypermethylation status compared to the adjacent myometrium. This aberrant methylation regulates the expression of genes involved in tumor development and maintenance, causing hypomethylation/upregulation of oncogenes and hypermethylation/downregulation of tumor suppressor genes. The consequent alterations in cell proliferation, invasion, extracellular vesicles, metabolism, deposition of extracellular matrix, and the Wnt/β-catenin pathway, all contribute to uterine leiomyoma initiation and progression.

- Inhibiting DNA methyltransferases with 5-aza-CdR treatment reverts the gene expression of hypermethylated/downregulated tumor suppressor genes, suggesting that this reversion could inhibit key processes in UL development.

- Reversing DNA methylation through 5-aza-CdR treatment inhibits cell proliferation, extracellular matrix formation, and Wnt/β-catenin signaling pathway in primary human uterine leiomyoma cells *in vitro*, encouraging further preclinical investigation of 5-aza-CdR as a new therapeutic alternative to treat patients with uterine leiomyoma.

- Aberrant acetylation in Lysine 27 of Histone 3 (H3K27ac) causes a hyperacetylation/upregulation of oncogenes and hypoacetylation/downregulation of tumor suppressor genes that promotes uterine leiomyoma pathogenesis via dysregulated biological processes related to the immune system, angiogenesis, invasion, altered metabolism, deposition of extracellular matrix, in addition to TGFβ and Wnt/β-catenin signaling pathways.

- Targeted inhibition of histone deacetylation with SAHA reverts gene expression of hypoacetylated/downregulated tumor suppressor genes, implying the aberrant signaling pathways involved in uterine leiomyoma development are amenable to pharmacological inhibition.

- Targeted inhibition of histone deacetylation with SAHA effectively reduced extracellular matrix deposition, cell cycle progression, cell proliferation and the TGFβ signaling pathway in primary human uterine leiomyoma cells *in vitro*, demonstrating the aberrant

biological processes involved in uterine leiomyoma development are amenable to pharmacological inhibition, and highlighting that SAHA-mediated inhibition of HDACs as a promising therapeutic strategy to treat patients with uterine leiomyoma.

VIII. BIBLIOGRAPHY

“If I have seen further it is by standing on the shoulders of Giants”- Isaac Newton

VIII. BIBLIOGRAPHY

Abou Najem S, Khawaja G, Hodroj MH, Babikian P, Rizk S. Adjuvant Epigenetic Therapy of Decitabine and Suberoylanilide Hydroxamic Acid Exerts Anti-Neoplastic Effects in Acute Myeloid Leukemia Cells. *Cells* 2019;**8**:. NLM (Medline).

Ai J, Tan G, Wang T, Li W, Gao R, Song Y, Xiong S, Qing X. Transcription factor STAT1 promotes the proliferation, migration and invasion of nasopharyngeal carcinoma cells by upregulating LINC01160. *Future Oncol* 2021;**17**:57–69. Future Oncol.

Al-Hendy A, Diamond MP, Boyer TG, Halder SK. Vitamin D3 inhibits Wnt/β-catenin and mTOR signaling pathways in human uterine fibroid cells. *J Clin Endocrinol Metab* 2016;**101**:1542–1551. Endocrine Society.

Ali M, Shahin SM, Sabri NA, Al-Hendy A, Yang Q. Activation of β-Catenin signaling and its crosstalk with estrogen and histone deacetylases in human uterine fibroids. *J Clin Endocrinol Metab* 2020;**105**:E1517–E1535. Endocrine Society.

Ameer MA, Fagan SE, Sosa-Stanley JN, Peterson DC. Anatomy, Abdomen and Pelvis, Uterus. *StatPearls* 2021; StatPearls Publishing.

Amerongen R van. Alternative Wnt Pathways and Receptors. *Cold Spring Harb Perspect Biol* 2012;**4**:a007914. Cold Spring Harbor Laboratory Press.

Anjum S, Sahar T, Nigam A, Wajid S. Transcriptome Analysis of mRNA in Uterine Leiomyoma Using Next-generation RNA Sequencing. *Anticancer Agents Med Chem* 2019;**19**:1703–1718. Anticancer Agents Med Chem.

Archer DF, Stewart EA, Jain RI, Feldman RA, Lukes AS, North JD, Soliman AM, Gao J, Ng JW, Chwalisz K. Elagolix for the management of heavy menstrual bleeding associated with uterine fibroids: results from a phase 2a proof-of-concept study. *Fertil Steril* 2017;**108**:152-160.e4. Fertil Steril.

Arenas Valencia C, Lopez Kleine L, Pinzon Velasco AM, Cardona Barreto AY, Arteaga Diaz CE. Gene expression analysis in peripheral blood cells of patients with hereditary leiomyomatosis and renal cell cancer syndrome (HLRCC): identification of NRF2 pathway activation. *Fam Cancer* 2018;**17**:587–599. Fam Cancer.

Arslan AA, Gold LI, Mittal K, Suen TC, Belitskaya-Levy I, Tang MS, Toniolo P. Gene expression studies provide clues to the pathogenesis of uterine leiomyoma: new evidence and a systematic review. *Hum Reprod* 2005;**20**:852–863. Hum Reprod.

Asada H, Yamagata Y, Taketani T, Matsuoka A, Tamura H, Hattori N, Ohgane J, Hattori N, Shiota K, Sugino N. Potential link between estrogen receptor-α gene hypomethylation and uterine fibroid formation. *Mol Hum Reprod* 2008;**14**:539–545. Mol Hum Reprod.

Baird DD, Hill MC, Schectman JM, Hollis BW. Vitamin d and the risk of uterine fibroids. *Epidemiology* 2013;**24**:447–453.

Baranov VS, Osinovskaya NS, Yarmolinskaya MI. Pathogenomics of Uterine Fibroids Development. *Int J Mol Sci* 2019;**20**:. Multidisciplinary Digital Publishing Institute (MDPI).

Barter MJ, Pybus L, Litherland GJ, Rowan AD, Clark IM, Edwards DR, Cawston TE, Young DA. HDAC-mediated control of ERK- and PI3K-dependent TGF-β-induced extracellular matrix-regulating genes. *Matrix Biol* 2010;**29**:602–612. Matrix Biol.

Bártová E, Krejčí J, Harničarová A, Galiová G, Kozubek S. Histone modifications and nuclear

architecture: a review. *J Histochem Cytochem* 2008;**56**:711–721. J Histochem Cytochem.

Basu B. *Biomaterials for Musculoskeletal Regeneration: Concepts. Springer* 2016.

Bebelman M, Smit M, Pegtel D, Baglio S. Biogenesis and function of extracellular vesicles in cancer. *Pharmacol Ther* 2018;**188**:1–11. Pharmacol Ther.

Bello OD, Zanetti MN, Mayorga LS, Michaut MA. RIM, Munc13, and Rab3A interplay in acrosomal exocytosis. *Exp Cell Res* 2012;**318**:478–488. Exp Cell Res.

Bennett RL, Licht JD. Targeting Epigenetics in Cancer. *Annu Rev Pharmacol Toxicol* 2018;**58**:187–207. Annual Reviews Inc.

Berta DG, Kuisma H, Välimäki N, Räisänen M, Jäntti M, Pasanen A, Karhu A, Kaukomaa J, Taira A, Cajuso T, *et al.* Deficient H2A.Z deposition is associated with genesis of uterine leiomyoma. *Nature* 2021;**596**:398–403. Nature.

Bhave Chittawar P, Franik S, Pouwer AW, Farquhar C. Minimally invasive surgical techniques versus open myomectomy for uterine fibroids. *Cochrane database Syst Rev* 2014;**2014**:. Cochrane Database Syst Rev.

Bhutani N, Burns DM, Blau HM. DNA demethylation dynamics. *Cell* 2011;**146**:866–872. Cell.

Bitler BG, Wu S, Park PH, Hai Y, Aird KM, Wang Y, Zhai Y, Kossenkov A V., Vara-Ailor A, Rauscher FJ, *et al.* ARID1A-mutated ovarian cancers depend on HDAC6 activity. *Nat Cell Biol* 2017;**19**:962–973. Nature Publishing Group.

Borahay MA, Hendy A Al, Kilic GS, Boehning D. Signaling pathways in leiomyoma: Understanding pathobiology and implications for therapy. *Mol Med* 2015;**21**:242–256. Uninversity of Michigan.

Bowden W, Skorupski J, Kovanci E, Rajkovic A. Detection of novel copy number variants in uterine leiomyomas using high-resolution SNP arrays. *Mol Hum Reprod* 2009;**15**:563–568. Mol Hum Reprod.

Bruijn AM de, Ankum WM, Reekers JA, Birnie E, Kooij SM van der, Volkers NA, Hehenkamp WJK. Uterine artery embolization vs hysterectomy in the treatment of symptomatic uterine fibroids: 10-year outcomes from the randomized EMMY trial. *Am J Obstet Gynecol* 2016;**215**:745.e1-745.e12. Am J Obstet Gynecol.

Bulun SE. Uterine fibroids. *N Engl J Med* 2013;**369**:1344–1355.

Bulun SE, Moravek MB, Yin P, Ono M, Coon V JS, Dyson MT, Navarro A, Marsh EE, Zhao H, Maruyama T, *et al.* Uterine Leiomyoma Stem Cells: Linking Progesterone to Growth. *Semin Reprod Med* 2015;**33**:357–365. Semin Reprod Med.

Butler LM, Agus DB, Scher HI, Higgins B, Rose A, Cordon-Cardo C, Thaler HT, Rifkind RA, Marks PA, Richon VM. Suberoylanilide hydroxamic acid, an inhibitor of histone deacetylase, suppresses the growth of prostate cancer cells in vitro and in vivo. *Cancer Res* 2000;**60**:5165–5170.

Cai M, Sikong Y, Wang Q, Zhu S, Pang F, Cui X. Gpx3 prevents migration and invasion in gastric cancer by targeting NFκB/Wnt5a/JNK signaling. *Int J Clin Exp Pathol* 2019;**12**:1194. e-Century Publishing Corporation.

Cedar H, Bergman Y. Linking DNA methylation and histone modification: Patterns and paradigms. *Nat Rev Genet* 2009;**10**:295–304. Nat Rev Genet.

Chang C, Worley BL, Phaëton R, Hempel N. Extracellular Glutathione Peroxidase GPx3 and Its

Role in Cancer. *Cancers (Basel)* 2020;**12**:1–20. Cancers (Basel).

Chwalisz K, Taylor H. Current and Emerging Medical Treatments for Uterine Fibroids. *Semin Reprod Med* 2017;**35**:510–522. Semin Reprod Med.

Ciarmela P, Delli Carpini G, Greco S, Zannotti A, Montik N, Giannella L, Giuliani L, Grelloni C, Panfoli F, Paolucci M, *et al.* Uterine fibroid vascularization: from morphological evidence to clinical implications. *Reprod Biomed Online* 2022;**44**:281–294. Reprod Biomed Online.

Ciebiera M, Esfandyari S, Siblini H, Prince L, Elkafas H, Wojtyła C, Al-Hendy A, Ali M. Nutrition in Gynecological Diseases: Current Perspectives. *Nutrients* 2021;**13**:. Nutrients.

Ciebiera M, Włodarczyk M, Wrzosek M, Męczekalski B, Nowicka G, Łukaszuk K, Ciebiera M, Słabuszewska-Jóźwiak A, Jakiel G. Role of Transforming Growth Factor β in Uterine Fibroid Biology. *Int J Mol Sci* 2017;**18**:. Int J Mol Sci.

Clevers H, Nusse R. Wnt/β-catenin signaling and disease. *Cell* 2012;**149**:1192–1205. Cell.

Coad J DM. *Anatomy and physiology for midwives. Churchill Livingstone/Elsevier* 2011;

Cooke PS, Spencer TE, Bartol FF, Hayashi K. Uterine glands: development, function and experimental model systems. *Mol Hum Reprod* 2013;**19**:547–558. Mol Hum Reprod.

Corachán A, Ferrero H, Aguilar A, Garcia N, Monleon J, Faus A, Cervelló I, Pellicer A. Inhibition of tumor cell proliferation in human uterine leiomyomas by vitamin D via Wnt/β-catenin pathway. *Fertil Steril* 2019;**111**:397–407. Elsevier Inc.

Corachán A, Trejo MG, Carbajo-García MC, Monleón J, Escrig J, Faus A, Pellicer A, Cervelló I, Ferrero H. Vitamin D as an effective treatment in human uterine leiomyomas independent of mediator complex subunit 12 mutation. *Fertil Steril* 2021;**115**:512–521. Elsevier Inc.

Curry TE, Osteen KG. The matrix metalloproteinase system: changes, regulation, and impact throughout the ovarian and uterine reproductive cycle. *Endocr Rev* 2003;**24**:428–465. Endocr Rev.

Davies S, Davies M, Sanders A, Parr C, Torkington J, Jiang W. Differential expression of the CCN family member WISP-1, WISP-2 and WISP-3 in human colorectal cancer and the prognostic implications. *Int J Oncol* 2010;**36**:. Int J Oncol.

De A. Wnt/Ca2+ signaling pathway: a brief overview. *Acta Biochim Biophys Sin (Shanghai)* 2011;**43**:745–756. Oxford Academic.

Deitrick J, Pruitt WM. Wnt/β Catenin-Mediated Signaling Commonly Altered in Colorectal Cancer. *Prog Mol Biol Transl Sci* 2016;**144**:, p. 49–68. Elsevier B.V.

Deng L, Wu T, Chen XY, Xie L, Yang J. Selective estrogen receptor modulators (SERMs) for uterine leiomyomas. *Cochrane database Syst Rev* 2012;**10**:. Cochrane Database Syst Rev.

Donnez J, Dolmans M-M. Uterine fibroid management: from the present to the future. *Hum Reprod Update* 2016a;**22**:665–686.

Donnez J, Dolmans MM. Uterine fibroid management: from the present to the future. *Hum Reprod Update* 2016b;**22**:665–686. Hum Reprod Update.

Donnez J, Tatarchuk TF, Bouchard P, Puscasiu L, Zakharenko NF, Ivanova T, Ugocsai G, Mara M, Jilla MP, Bestel E, *et al.* Ulipristal acetate versus placebo for fibroid treatment before surgery. *N Engl J Med* 2012;**366**:409–420. N Engl J Med.

Duan R, Han L, Wang Q, Wei J, Chen L, Zhang J, Kang C, Wang L. HOXA13 is a potential GBM diagnostic marker and promotes glioma invasion by activating the Wnt and TGF-β pathways. *Oncotarget* 2015;**6**:27778–27793. Impact Journals LLC.

El-Sehemy A, Selvadurai H, Ortin-Martinez A, Pokrajac N, Mamatjan Y, Tachibana N, Rowland K, Lee L, Park N, Aldape K, *et al.* Norrin mediates tumor-promoting and -suppressive effects in glioblastoma via Notch and Wnt. *J Clin Invest* 2020;**130**:3069–3086. J Clin Invest.

Estella C, Herrer I, Atkinson SP, Quiñonero A, Martínez S, Pellicer A, Simón C. Inhibition of histone deacetylase activity in human endometrial stromal cells promotes extracellular matrix remodelling and limits embryo invasion. *PLoS One* 2012;**7**:. PLoS One.

Faerstein E, Szklo M, Rosenshein NB. Risk factors for uterine leiomyoma: A practice-based case-controls study. II. Atherogenic risk factors and potential sources of uterine irritation. *Am J Epidemiol* 2001;**153**:11–19. Am J Epidemiol.

Falkenberg KJ, Johnstone RW. Histone deacetylases and their inhibitors in cancer, neurological diseases and immune disorders. *Nat Rev Drug Discov* 2014;**13**:673–691. Nat Rev Drug Discov.

Freese K, Seitz T, Dietrich P, Lee S, Thasler W, Bosserhoff A, Hellerbrand C. Histone Deacetylase Expressions in Hepatocellular Carcinoma and Functional Effects of Histone Deacetylase Inhibitors on Liver Cancer Cells In Vitro. *Cancers (Basel)* 2019;**11**:. Cancers (Basel).

Galindo LJ, Hernández-Beeftink T, Salas A, Jung Y, Reyes R, Oca FM de, Hernández M, Almeida TA. HMGA2 and MED12 alterations frequently co-occur in uterine leiomyomas. *Gynecol Oncol* 2018;**150**:562–568. Gynecol Oncol.

Galliano D, Bellver J, Díaz-García C, Simón C, Pellicer A. ART and uterine pathology: how relevant is the maternal side for implantation? *Hum Reprod Update* 2015;**21**:13–38. Oxford University Press.

Gallinari P, Marco S Di, Jones P, Pallaoro M, Steinkühler C. HDACs, histone deacetylation and gene transcription: From molecular biology to cancer therapeutics. *Cell Res* 2007;**17**:195–211. Cell Res.

Gasner A, A AP. Physiology, Uterus. *StatPearls* 2021; StatPearls Publishing.

George J, Fan H, Johnson B, Carpenter T, Foy K, Chatterjee A, Patterson A, Koeman J, Adams M, Madaj Z, *et al.* Integrated Epigenome, Exome, and Transcriptome Analyses Reveal Molecular Subtypes and Homeotic Transformation in Uterine Fibroids. *Cell Rep* 2019;**29**:4069-4085.e6. Cell Rep.

Ghant MS, Sengoba KS, Recht H, Cameron KA, Lawson AK, Marsh EE. Beyond the physical: a qualitative assessment of the burden of symptomatic uterine fibroids on women's emotional and psychosocial health. *J Psychosom Res* 2015;**78**:499–503. J Psychosom Res.

Gibney ER, Nolan CM. Epigenetics and gene expression. *Heredity (Edinb)* 2010;**105**:4–13. Heredity (Edinb).

Giuliani E, As-Sanie S, Marsh EE. Epidemiology and management of uterine fibroids. *Int J Gynaecol Obstet* 2020;**149**:3–9. Int J Gynaecol Obstet.

Gruber J, Yee Z, Tolwinski NS. Developmental Drift and the Role of Wnt Signaling in Aging. *Cancers 2016, Vol 8, Page 73* 2016;**8**:73. Multidisciplinary Digital Publishing Institute.

Gu Y, Gu J, Shen K, Zhou H, Hao J, Li F, Yu H, Chen Y, Li J, Li Y, *et al.* HOXA13 promotes colon

cancer progression through β-catenin-dependent WNT pathway. *Exp Cell Res* 2020;**395**:. Exp Cell Res.

Halder S, Al-Hendy A. Hypovitaminosis D and high serum transforming growth factor beta-3: important biomarkers for uterine fibroids risk. *Fertil Steril* 2016;**106**:1648–1649. Fertil Steril.

Halder SK, Osteen KG, Al-Hendy A. Vitamin D3 inhibits expression and activities of matrix metalloproteinase-2 and -9 in human uterine fibroid cells. *Hum Reprod* 2013;**28**:2407–2416. Oxford University Press.

Hanahan D, Weinberg R. Hallmarks of cancer: the next generation. *Cell* 2011;**144**:646–674. Cell.

Havryliuk Y, Setton R, Carlow JJ, Shaktman BD. Symptomatic Fibroid Management: Systematic Review of the Literature. *JSLS J Soc Laparoendosc Surg* 2017;**21**:. JSLS.

Heiden M Vander, DeBerardinis R. Understanding the Intersections between Metabolism and Cancer Biology. *Cell* 2017;**168**:657–669. Cell.

Hervouet E, Claude-Taupin A, Gauthier T, Perez V, Fraichard A, Adami P, Despouy G, Monnien F, Algros MP, Jouvenot M, *et al.* The autophagy GABARAPL1 gene is epigenetically regulated in breast cancer models. *BMC Cancer* 2015;**15**:. BioMed Central.

Huang S, Hölzel M, Knijnenburg T, Schlicker A, Roepman P, McDermott U, Garnett M, Grernrum W, Sun C, Prahallad A, *et al.* MED12 controls the response to multiple cancer drugs through regulation of TGF-β receptor signaling. *Cell* 2012;**151**:937–950. Cell.

Ishikawa H, Ishi K, Ann Serna V, Kakazu R, Bulun SE, Kurita T. Progesterone is essential for maintenance and growth of uterine leiomyoma. *Endocrinology* 2010;**151**:2433–2442. Endocrinology.

Islam MS, Ciavattini A, Petraglia F, Castellucci M, Ciarmela P. Extracellular matrix in uterine leiomyoma pathogenesis: A potential target for future therapeutics. *Hum Reprod Update* 2018;**24**:59–85. Oxford University Press.

Islam MS, Protic O, Stortoni P, Grechi G, Lamanna P, Petraglia F, Castellucci M, Ciarmela P. Complex networks of multiple factors in the pathogenesis of uterine leiomyoma. *Fertil Steril* 2013;**100**:178–193. Fertil Steril.

Ito S, Shen L, Dai Q, Wu SC, Collins LB, Swenberg JA, He C, Zhang Y. Tet proteins can convert 5-methylcytosine to 5-formylcytosine and 5-carboxylcytosine. *Science* 2011;**333**:1300–1303. Science.

Jamaluddin MFB, Nahar P, Tanwar PS. Proteomic Characterization of the Extracellular Matrix of Human Uterine Fibroids. *Endocrinology* 2018;**159**:2656–2669. Endocrinology.

Jiang W, Shen Q, Chen M, Wang Y, Zhou Q, Zhu X. Levonorgestrel-releasing intrauterine system use in premenopausal women with symptomatic uterine leiomyoma: a systematic review. *Steroids* 2014;**86**:69–78. Steroids.

Jin W, Li Q, Zuo Y, Cao Y, Zhang L, Hou R, Su W. Relationship Between DNA Methylation in Key Region and the Differential Expressions of Genes in Human Breast Tumor Tissue. *DNA Cell Biol* 2019;**38**:49–62. DNA Cell Biol.

Jing D, Zhang Q, Yu H, Zhao Y, Shen L. Identification of WISP1 as a novel oncogene in glioblastoma. *Int J Oncol* 2017;**51**:1261–1270. Spandidos Publications.

Jones PA, Baylin SB. The epigenomics of cancer. *Cell* 2007;**128**:683–692. Cell.

Kaeser PS, Deng L, Fan M, Südhof TC. RIM genes differentially contribute to organizing presynaptic release sites. *Proc Natl Acad Sci U S A* 2012;**109**:11830–11835. Proc Natl Acad Sci U S A.

Kawano Y, Nasu K, Li H, Tsuno A, Abe W, Takai N, Narahara H. Application of the histone deacetylase inhibitors for the treatment of endometriosis: Histone modifications as pathogenesis and novel therapeutic target. *Hum Reprod* 2011;**26**:2486–2498. Oxford University Press.

Kitaya K, Yasuo T, Yamaguchi T, Fushiki S, Honjo H. Genes regulated by interferon-gamma in human uterine microvascular endothelial cells - PubMed. *Int J Mol Med* [Internet] 2007;20(5):689-97.Available from: https://pubmed.ncbi.nlm.nih.gov/17912462/.

Klutstein M, Nejman D, Greenfield R, Cedar H. DNA methylation in cancer and aging. *Cancer Res* 2016;**76**:3446–3450. American Association for Cancer Research Inc.

Kobayashi Y, Absher DM, Gulzar ZG, Young SR, McKenney JK, Peehl DM, Brooks JD, Myers RM, Sherlock G. DNA methylation profiling reveals novel biomarkers and important roles for DNA methyltransferases in prostate cancer. *Genome Res* 2011;**21**:1017–1027. Genome Res.

Koltsova AS, Pendina AA, Efimova OA, Chiryaeva OG, Kuznetzova T V., Baranov VS. On the Complexity of Mechanisms and Consequences of Chromothripsis: An Update. *Front Genet* 2019;**10**:. Front Genet.

Komiya Y, Habas R. Wnt signal transduction pathways. *Organogenesis* 2008;**4**:68–75. Organogenesis.

Kong F, Deng X, Kong X, Du Y, Li L, Zhu H, Wang Y, Xie D, Guha S, Li Z, *et al.* ZFPM2-AS1, a novel lncRNA, attenuates the p53 pathway and promotes gastric carcinogenesis by stabilizing MIF. *Oncogene* 2018;**37**:5982–5996. Oncogene.

Kotani Y, Tobiume T, Fujishima R, Shigeta M, Takaya H, Nakai H, Suzuki A, Tsuji I, Mandai M, Matsumura N. Recurrence of uterine myoma after myomectomy: Open myomectomy versus laparoscopic myomectomy. *J Obstet Gynaecol Res* 2018;**44**:298–302. J Obstet Gynaecol Res.

Kyaw MTH, Yamaguchi Y, Choijookhuu N, Yano K, Takagi H, Takahashi N, Oo PS, Sato K, Hishikawa Y. The HDAC inhibitor, SAHA, combined with cisplatin synergistically induces apoptosis in alpha-fetoprotein-producing hepatoid adenocarcinoma cells. *Acta Histochem Cytochem* 2019;**52**:1–8. Japan Society of Histochemistry and Cytochemistry.

la Cruz MSD de, Buchanan EM. Uterine Fibroids: Diagnosis and Treatment. *Am Fam Physician* 2017;**95**:100–107. Am Fam Physician.

Laganà AS, Vergara D, Favilli A, Rosa VL La, Tinelli A, Gerli S, Noventa M, Vitagliano A, Triolo O, Rapisarda AMC, *et al.* Epigenetic and genetic landscape of uterine leiomyomas: a current view over a common gynecological disease. *Arch Gynecol Obstet* 2017;**296**:855–867. Springer Verlag.

Lawrence MS, Stojanov P, Mermel CH, Robinson JT, Garraway LA, Golub TR, Meyerson M, Gabriel SB, Lander ES, Getz G. Discovery and saturation analysis of cancer genes across 21 tumour types. *Nature* 2014;**505**:495–501. Nature.

Lee B-S, Nowak RA. Human Leiomyoma Smooth Muscle Cells Show Increased Expression of

Transforming Growth Factor-β3 (TGFβ3) and Altered Responses to the Antiproliferative Effects of TGFβ 1 . *J Clin Endocrinol Metab* 2001;**86**:913–920. The Endocrine Society.

Lee BB, Yu SP. Radiofrequency Ablation of Uterine Fibroids: a Review. *Curr Obstet Gynecol Rep* 2016;**5**:318–324. Curr Obstet Gynecol Rep.

Lehtonen R, Kiuru M, Vanharanta S, Sjöberg J, Aaltonen LM, Aittomäki K, Arola J, Butzow R, Eng C, Husgafvel-Pursiainen K, *et al.* Biallelic inactivation of fumarate hydratase (FH) occurs in nonsyndromic uterine leiomyomas but is rare in other tumors. *Am J Pathol* 2004;**164**:17–22. Am J Pathol.

Leistico J, Saini P, Futtner C, Hejna M, Omura Y, Soni P, Sandlesh P, Milad M, Wei J, Bulun S, *et al.* Epigenomic tensor predicts disease subtypes and reveals constrained tumor evolution. *Cell Rep* 2021;**34**:. Cell Rep.

Leppert PC, Baginski T, Prupas C, Catherino WH, Pletcher S, Segars JH. Comparative ultrastructure of collagen fibrils in uterine leiomyomas and normal myometrium. *Fertil Steril* 2004;**82**:1182–1187. Fertil Steril.

Leppert PC, Jayes FL, Segars JH. The extracellular matrix contributes to mechanotransduction in uterine fibroids. *Obstet Gynecol Int* 2014;**2014**:1–12. Obstet Gynecol Int.

Lethaby A, Duckitt K, Farquhar C. Non-steroidal anti-inflammatory drugs for heavy menstrual bleeding. *Cochrane database Syst Rev* 2013;**2013**:. Cochrane Database Syst Rev.

Lewis TD, Malik M, Britten J, Parikh T, Cox J, Catherino WH. Ulipristal acetate decreases active TGF-β3 and its canonical signaling in uterine leiomyoma via two novel mechanisms. *Fertil Steril* 2019;**111**:806-815.e1. Elsevier Inc.

Li S, Chiang TC, Richard-Davis G, Barrett JC, Mclachlan JA. DNA hypomethylation and imbalanced expression of DNA methyltransferases (DNMT1, 3A, and 3B) in human uterine leiomyoma. *Gynecol Oncol* 2003;**90**:123–130. Academic Press Inc.

Li S, Han Z, Zhao N, Zhu B, Zhang Q, Yang X, Sheng D, Hou J, Guo S, Wei L, *et al.* Inhibition of DNMT suppresses the stemness of colorectal cancer cells through down-regulating Wnt signaling pathway. *Cell Signal* 2018;**47**:79–87. Elsevier Inc.

Li W, Wu H, Sui S, Wang Q, Xu S, Pang D. Targeting Histone Modifications in Breast Cancer: A Precise Weapon on the Way. *Front Cell Dev Biol* 2021;**9**:2463. Frontiers Media S.A.

Liu J, Yan W, Han P, Tian D. The emerging role of KIAA1199 in cancer development and therapy. *Biomed Pharmacother* 2021;**138**:. Biomed Pharmacother.

Liu S, Yin P, Xu J, Dotts A, Kujawa S, Coon V J, Zhao H, Shilatifard A, Dai Y, Bulun S. Targeting DNA Methylation Depletes Uterine Leiomyoma Stem Cell-enriched Population by Stimulating Their Differentiation. *Endocrinology* 2020;**161**:. Endocrinology.

Long MD, Dhiman VK, Affronti HC, Hu Q, Liu S, Smiraglia DJ. Dynamic patterns of DNA methylation in the normal prostate epithelial differentiation program are targets of aberrant methylation in prostate cancer. *Sci Reports 2021 111* 2021;**11**:1–9. Nature Publishing Group.

Lopez M, Halby L, Arimondo PB. DNA methyltransferase inhibitors: Development and applications. *Adv Exp Med Biol* 2016;**945**:431–473. Springer New York LLC.

Lukes AS, Moore KA, Muse KN, Gersten JK, Hecht BR, Edlund M, Richter HE, Eder SE, Attia GR, Patrick DL, *et al.* Tranexamic acid treatment for heavy menstrual bleeding: a randomized controlled trial. *Obstet Gynecol* 2010;**116**:865–875. Obstet Gynecol.

Ma SY, Wei P, Qu F. KCNMA1-AS1 attenuates apoptosis of epithelial ovarian cancer cells and serves as a risk factor for poor prognosis of epithelial ovarian cancer. *Eur Rev Med Pharmacol Sci* 2019;**23**:4629–4641. Eur Rev Med Pharmacol Sci.

Ma Y, Zheng W. H3K27ac-induced lncRNA PAXIP1-AS1 promotes cell proliferation, migration, EMT and apoptosis in ovarian cancer by targeting miR-6744-5p/PCBP2 axis. *J Ovarian Res* 2021;**14**:. J Ovarian Res.

MacDonald BT, Tamai K, He X. Wnt/β-Catenin Signaling: Components, Mechanisms, and Diseases. *Dev Cell* 2009;**17**:9–26. Elsevier.

Maekawa R, Sato S, Yamagata Y, Asada H, Tamura I, Lee L, Okada M, Tamura H, Takaki E, Nakai A, *et al.* Genome-Wide DNA Methylation Analysis Reveals a Potential Mechanism for the Pathogenesis and Development of Uterine Leiomyomas. *PLoS One* 2013;**8**:. PLoS One.

Mai A, Massa S, Rotili D, Cerbara I, Valente S, Pezzi R, Simeoni S, Ragna R. Histone deacetylation in epigenetics: an attractive target for anticancer therapy. *Med Res Rev* 2005;**25**:261–309. Med Res Rev.

Mäkinen N, Mehine M, Tolvanen J, Kaasinen E, Li Y, Lehtonen HJ, Gentile M, Yan J, Enge M, Taipale M, *et al.* MED12, the mediator complex subunit 12 gene, is mutated at high frequency in uterine leiomyomas. *Science (80-)* 2011;**334**:252–255. American Association for the Advancement of Science.

Malik M, Norian J, McCarthy-Keith D, Britten J, Catherino WH. Why leiomyomas are called fibroids: the central role of extracellular matrix in symptomatic women. *Semin Reprod Med* 2010;**28**:169–179. Semin Reprod Med.

Mann BS, Johnson JR, Cohen MH, Justice R, Pazdur R. FDA approval summary: vorinostat for treatment of advanced primary cutaneous T-cell lymphoma. *Oncologist* 2007;**12**:1247–1252. Oncologist.

Manyonda I, Sinthamoney E, Belli AM. Controversies and challenges in the modern management of uterine fibroids. *BJOG An Int J Obstet Gynaecol* 2004;**111**:95–102. BJOG.

Markowski DN, Bartnitzke S, Löning T, Drieschner N, Helmke BM, Bullerdiek J. MED12 mutations in uterine fibroids--their relationship to cytogenetic subgroups. *Int J cancer* 2012;**131**:1528–1536. Int J Cancer.

Marks P. Discovery and development of SAHA as an anticancer agent. *Oncogene* 2007;**26**:1351–1356. Oncogene.

Marks PA, Breslow R. Dimethyl sulfoxide to vorinostat: Development of this histone deacetylase inhibitor as an anticancer drug. *Nat Biotechnol* 2007;**25**:84–90. Nat Biotechnol.

Marsh E, Chibber S, Wu J, Siegersma K, Kim J, Bulun S. Epidermal growth factor-containing fibulin-like extracellular matrix protein 1 expression and regulation in uterine leiomyoma. *Fertil Steril* 2016;**105**:1070–1075. Fertil Steril.

Marsh EE, Ekpo GE, Cardozo ER, Brocks M, Dune T, Cohen LS. Racial differences in fibroid prevalence and ultrasound findings in asymptomatic young women (18-30 years old): a pilot study. *Fertil Steril* 2013;**99**:1951–1957. Fertil Steril.

Maruyama T, Miyazaki K, Masuda H, Ono M, Uchida H, Yoshimura Y. Review: Human uterine stem/progenitor cells: Implications for uterine physiology and pathology. *Placenta* 2013;**34 Suppl**: Placenta.

Mas A, Cervelló I, Gil-Sanchis C, Faus A, Ferro J, Pellicer A, Simón C. Identification and characterization of the human leiomyoma side population as putative tumor-initiating cells. *Fertil Steril* 2012;**98**:. Fertil Steril.

Mas A, Cervello I, Gil-Sanchis C, Simón C. Current understanding of somatic stem cells in leiomyoma formation. *Fertil Steril* 2014;**102**:613–620. Fertil Steril.

Mehdipour P, Santoro F, Minucci S. Epigenetic alterations in acute myeloid leukemias. *FEBS J* 2015;**282**:1786–1800. FEBS J.

Mehine M, Kaasinen E, Heinonen HR, Mäkinen N, Kämpjärvi K, Sarvilinna N, Aavikko M, Vähärautio A, Pasanen A, Bützow R, *et al.* Integrated data analysis reveals uterine leiomyoma subtypes with distinct driver pathways and biomarkers. *Proc Natl Acad Sci U S A* 2016;**113**:1315–1320. Proc Natl Acad Sci U S A.

Mehine M, Mäkinen N, Heinonen HR, Aaltonen LA, Vahteristo P. Genomics of uterine leiomyomas: insights from high-throughput sequencing. *Fertil Steril* 2014;**102**:621–629. Fertil Steril.

Meissner A. Epigenetic modifications in pluripotent and differentiated cells. *Nat Biotechnol* 2010;**28**:1079–1088. Nat Biotechnol.

Minciacchi V, Freeman M, Vizio D Di. Extracellular vesicles in cancer: exosomes, microvesicles and the emerging role of large oncosomes. *Semin Cell Dev Biol* 2015;**40**:41–51. Semin Cell Dev Biol.

Mlodawska OW, Saini P, Parker JB, Wei J-J, Bulun SE, Simon MA, Chakravarti D. Epigenomic and enhancer dysregulation in uterine leiomyomas. *Hum Reprod Update* 2022; Hum Reprod Update.

Moroni RM, Martins WP, Dias S V., Vieira CS, Ferriani RA, Nastri CO, Brito LG. Combined oral contraceptive for treatment of women with uterine fibroids and abnormal uterine bleeding: a systematic review. *Gynecol Obstet Invest* 2015;**79**:145–152. Gynecol Obstet Invest.

Moyo M, Parker J, Chakravarti D. Altered chromatin landscape and enhancer engagement underlie transcriptional dysregulation in MED12 mutant uterine leiomyomas. *Nat Commun* 2020;**11**:. Nat Commun.

Munro MG. Endometrial ablation. *Best Pract Res Clin Obstet Gynaecol* 2018;**46**:120–139. Best Pract Res Clin Obstet Gynaecol.

Munro MG, Critchley HOD, Broder MS, Fraser IS. FIGO classification system (PALM-COEIN) for causes of abnormal uterine bleeding in nongravid women of reproductive age. *Int J Gynaecol Obstet* 2011;**113**:3–13. Int J Gynaecol Obstet.

Murji A, Whitaker L, Chow TL, Sobel ML. Selective progesterone receptor modulators (SPRMs) for uterine fibroids. *Cochrane database Syst Rev* 2017;**4**:. Cochrane Database Syst Rev.

Natarajan U, Venkatesan T, Radhakrishnan V, Samuel S, Rasappan P, Rathinavelu A. Cell cycle arrest and cytotoxic effects of saha and rg7388 mediated through p21waf1/cip1and p27kip1in cancer cells. *Med* 2019;**55**:. MDPI AG.

Navarro A, Yin P, Monsivais D, Lin S, Du P, Wei, Bulun. Genome-wide DNA methylation indicates silencing of tumor suppressor genes in uterine leiomyoma. *PLoS One* 2012a;**7**:. PLoS One.

Navarro A, Yin P, Monsivais D, Lin SM, Du P, Wei JJ, Bulun SE. Genome-wide DNA methylation

indicates silencing of tumor suppressor genes in uterine leiomyoma. *PLoS One* 2012b;**7**:. PLoS One.

Navarro A, Yin P, Ono M, Monsivais D, Moravek MB, Coon JSV, Dyson MT, Wei JJ, Bulun SE. 5-Hydroxymethylcytosine promotes proliferation of human uterine leiomyoma: a biological link to a new epigenetic modification in benign tumors. *J Clin Endocrinol Metab* 2014;**99**:E2437–E2445. J Clin Endocrinol Metab.

Nervi C, Marinis E De, Codacci-Pisanelli G. Epigenetic treatment of solid tumours: a review of clinical trials. *Clin Epigenetics* 2015;**7**:. Clin Epigenetics.

Nguyên-Thanh T, Kim D, Lee S, Kim W, Park SK, Kang KP. Inhibition of histone deacetylase 1 ameliorates renal tubulointerstitial fibrosis via modulation of inflammation and extracellular matrix gene transcription in mice. *Int J Mol Med* 2017;**41**:95–106. Spandidos Publications.

Nunes SP, Henrique R, Jerónimo C, Paramio JM. DNA Methylation as a Therapeutic Target for Bladder Cancer. *Cells* 2020;**9**:. NLM (Medline).

O'Connor OA, Horwitz S, Masszi T, Hoof A Van, Brown P, Doorduijn J, Hess G, Jurczak W, Knoblauch P, Chawla S, *et al.* Belinostat in Patients With Relapsed or Refractory Peripheral T-Cell Lymphoma: Results of the Pivotal Phase II BELIEF (CLN-19) Study. *J Clin Oncol* 2015;**33**:2492–2499. J Clin Oncol.

Ono M, Qiang W, Serna VA, Yin P, Coon JS, Navarro A, Monsivais D, Kakinuma T, Dyson M, Druschitz S, *et al.* Role of stem cells in human uterine leiomyoma growth. *PLoS One* 2012;**7**:. PLoS One.

Ono M, Yin P, Navarro A, Moravek MB, Coon V JS, Druschitz SA, Serna VA, Qiang W, Brooks DC, Malpani SS, *et al.* Paracrine activation of WNT/β-catenin pathway in uterine leiomyoma stem cells promotes tumor growth. *Proc Natl Acad Sci U S A* 2013;**110**:17053–17058. Proc Natl Acad Sci U S A.

Osinovskaya NS, Malysheva O V., Shved NY, Ivashchenko TE, Sultanov IY, Efimova OA, Yarmolinskaya MI, Bezhenar VF, Baranov VS. Frequency and Spectrum of MED12 Exon 2 Mutations in Multiple Versus Solitary Uterine Leiomyomas From Russian Patients. *Int J Gynecol Pathol* 2016;**35**:509–515. Int J Gynecol Pathol.

Ouyang C, Pu YZ, Qin XH, Shen J, Liu QH, Ma L, Xue L. Placenta-specific 9, a putative secretory protein, induces G2/M arrest and inhibits the proliferation of human embryonic hepatic cells. *Biosci Rep* 2018;**38**:. Biosci Rep.

Pan PY, Ma G, Weber KJ, Ozao-Choy J, Wang G, Yin B, Divino CM, Chen SH. Immune stimulatory receptor CD40 is required for T-cell suppression and T regulatory cell activation mediated by myeloid-derived suppressor cells in cancer. *Cancer Res* 2010;**70**:99–108. Cancer Res.

Parker WH. Etiology, symptomatology, and diagnosis of uterine myomas. *Fertil Steril* 2007;**87**:725–736. Fertil Steril.

Pfeifer GP. Defining Driver DNA Methylation Changes in Human Cancer. *Int J Mol Sci 2018, Vol 19, Page 1166* 2018;**19**:1166. Multidisciplinary Digital Publishing Institute.

Pitter MC, Simmonds C, Seshadri-Kreaden U, Hubert HB. The impact of different surgical modalities for hysterectomy on satisfaction and patient reported outcomes. *Interact J Med Res* 2014;**3**:e11. Interact J Med Res.

Planutis K, Planutiene M, Holcombe RF. A novel signaling pathway regulates colon cancer

angiogenesis through Norrin. *Sci Rep* 2014;**4**:. Sci Rep.

Protic O, Toti P, Islam MS, Occhini R, Giannubilo SR, Catherino WH, Cinti S, Petraglia F, Ciavattini A, Castellucci M, *et al.* Possible involvement of inflammatory/reparative processes in the development of uterine fibroids. *Cell Tissue Res* 2016;**364**:415–427. Cell Tissue Res.

Ragusa S, Prat-Luri B, González-Loyola A, Nassiri S, Squadrito ML, Guichard A, Cavin S, Gjorevski N, Barras D, Marra G, *et al.* Antiangiogenic immunotherapy suppresses desmoplastic and chemoresistant intestinal tumors in mice. *J Clin Invest* 2020;**130**:1199–1216. J Clin Invest.

Ramírez-González JA, Vaamonde-Lemos R, Cunha-Filho JS, Varghese AC, Swanson RJ. Overview of the female reproductive system. *Exerc Hum Reprod Induc Fertil Disord Possible Ther* 2016;19–46. Springer New York.

Ree AH, Dueland S, Folkvord S, Hole KH, Seierstad T, Johansen M, Abrahamsen TW, Flatmark K. Vorinostat, a histone deacetylase inhibitor, combined with pelvic palliative radiotherapy for gastrointestinal carcinoma: the Pelvic Radiation and Vorinostat (PRAVO) phase 1 study. *Lancet Oncol* 2010;**11**:459–464. Lancet Oncol.

Roach MK, Andreotti RF. The Normal Female Pelvis. *Clin Obstet Gynecol* 2017;**60**:3–10. Clin Obstet Gynecol.

Rogers K. *The reproductive system. Br Educ Pub, Assoc with Rosen Educ Serv* 2011;

Rohena-Rivera K, Sanchez-Vazquez MM, Aponte-Colon DiA, Forestier-Roman IS, Quintero-Aguilo ME, Martanez-Ferrer M. IL-15 regulates migration, invasion, angiogenesis and genes associated with lipid metabolism and inflammation in prostate cancer. *PLoS One* 2017;**12**:. PLoS One.

Ruscito I, Gasparri ML, Marco MP De, Costanzi F, Besharat AR, Papadia A, Kuehn T, Gentilini OD, Bellati F, Caserta D. The Clinical and Pathological Profile of BRCA1 Gene Methylated Breast Cancer Women: A Meta-Analysis. *Cancers 2021, Vol 13, Page 1391* 2021;**13**:1391. Multidisciplinary Digital Publishing Institute.

Russler-Germain DA, Spencer DH, Young MA, Lamprecht TL, Miller CA, Fulton R, Meyer MR, Erdmann-Gilmore P, Townsend RR, Wilson RK, *et al.* The R882H DNMT3A mutation associated with AML dominantly inhibits wild-type DNMT3A by blocking its ability to form active tetramers. *Cancer Cell* 2014;**25**:442–454. Cancer Cell.

Sabeh M El, Saha S, Afrin S, Islam M, Borahay M. Wnt/β-catenin signaling pathway in uterine leiomyoma: role in tumor biology and targeting opportunities. *Mol Cell Biochem* 2021;**476**:3513–3536. Mol Cell Biochem.

Sabry M, Halder SK, Ait Allah ASA, Roshdy E, Rajaratnam V, Al-Hendy A. Serum vitamin D3 level inversely correlates with uterine fibroid volume in different ethnic groups: a cross-sectional observational study. *Int J Womens Health* 2013;**5**:93–100. Int J Womens Health.

Sadan O, Ginath S, Sofer D, Rotmensch S, Debby A, Glezerman M, Zakut H. The role of tamoxifen in the treatment of symptomatic uterine leiomyomata -- a pilot study. *Eur J Obstet Gynecol Reprod Biol* 2001;**96**:183–186. Eur J Obstet Gynecol Reprod Biol.

San-Miguel JF, Hungria VTM, Yoon SS, Beksac M, Dimopoulos MA, Elghandour A, Jedrzejczak WW, Günther A, Nakorn TN, Siritanaratkul N, *et al.* Panobinostat plus bortezomib and dexamethasone versus placebo plus bortezomib and dexamethasone in patients with relapsed or relapsed and refractory multiple myeloma: a multicentre, randomised, double-blind phase 3 trial. *Lancet Oncol* 2014;**15**:1195–1206. Lancet Oncol.

Sandberg EM, Tummers FHMP, Cohen SL, Haak L van den, Dekkers OM, Jansen FW. Reintervention risk and quality of life outcomes after uterine-sparing interventions for fibroids: a systematic review and meta-analysis. *Fertil Steril* 2018;**109**:698-707.e1. Fertil Steril.

Sant'Anna G dos S, Brum IS, Branchini G, Pizzolato LS, Capp E, Corleta H von E. Ovarian steroid hormones modulate the expression of progesterone receptors and histone acetylation patterns in uterine leiomyoma cells. *Gynecol Endocrinol* 2017;**33**:629–633. Taylor and Francis Ltd.

Santamaria X, Mas A, Cervelló I, Taylor H, Simon C. Uterine stem cells: from basic research to advanced cell therapies. *Hum Reprod Update* 2018;**24**:673–693. Hum Reprod Update.

Sato S, Maekawa R, Yamagata Y, Asada H, Tamura I, Lee L, Okada M, Tamura H, Sugino N. Potential mechanisms of aberrant DNA hypomethylation on the x chromosome in uterine leiomyomas. *J Reprod Dev* 2014;**60**:47–54. J Reprod Dev.

Saxonov S, Berg P, Brutlag DL. A genome-wide analysis of CpG dinucleotides in the human genome distinguishes two distinct classes of promoters. *Proc Natl Acad Sci U S A* 2006;**103**:1412–1417. Proc Natl Acad Sci U S A.

Scheau C, Badarau IA, Costache R, Caruntu C, Mihai GL, Didilescu AC, Constantin C, Neagu M. The role of matrix metalloproteinases in the epithelial-mesenchymal transition of hepatocellular carcinoma. *Anal Cell Pathol* 2019;**2019**:. Hindawi Limited.

Seto E, Yoshida M. Erasers of histone acetylation: The histone deacetylase enzymes. *Cold Spring Harb Perspect Biol* 2014;**6**:. Cold Spring Harbor Laboratory Press.

Shang S, Hua F, Hu ZW. The regulation of β-catenin activity and function in cancer: Therapeutic opportunities. *Oncotarget* 2017;**8**:33972–33989. Impact Journals LLC.

Shen L, Song CX, He C, Zhang Y. Mechanism and function of oxidative reversal of DNA and RNA methylation. *Annu Rev Biochem* 2014;**83**:585–614. Annu Rev Biochem.

Shynlova O, Oldenhof A, Borogin A, Xu Q, Mu J, Nashman N, Lye SJ. Myometrial apoptosis: activation of the caspase cascade in the pregnant rat myometrium at midgestation. *Biol Reprod* 2006;**74**:839–849. Biol Reprod.

Shynlova O, Tsui P, Jaffer S, Lye SJ. Integration of endocrine and mechanical signals in the regulation of myometrial functions during pregnancy and labour. *Eur J Obstet Gynecol Reprod Biol* 2009;**144 Suppl 1**:S2. Eur J Obstet Gynecol Reprod Biol.

Sinai Talaulikar V. Medical therapy for fibroids: An overview. *Best Pract Res Clin Obstet Gynaecol* 2018;**46**:48–56. Best Pract Res Clin Obstet Gynaecol.

Sohn GS, Cho S, Kim YM, Cho C-H, Kim M-R, Lee SR, Working Group of Society of Uterine Leiomyoma. Current medical treatment of uterine fibroids. *Obstet Gynecol Sci* 2018;**61**:192.

Soliman AM, Yang H, Du EX, Kelkar SS, Winkel C. The direct and indirect costs of uterine fibroid tumors: a systematic review of the literature between 2000 and 2013. *Am J Obstet Gynecol* 2015;**213**:141–160. Am J Obstet Gynecol.

Song H, Lu D, Navaratnam K, Shi G. Aromatase inhibitors for uterine fibroids. *Cochrane database Syst Rev* 2013;**2013**:. Cochrane Database Syst Rev.

Sozen I, Arici A. Interactions of cytokines, growth factors, and the extracellular matrix in the cellular biology of uterine leiomyomata. *Fertil Steril* 2002;**78**:1–12. Fertil Steril.

Stewart E, Cookson C, Gandolfo R, Schulze-Rath R. Epidemiology of uterine fibroids: a systematic review. *BJOG An Int J Obstet Gynaecol* 2017;**124**:1501–1512.

Stewart EA. Uterine fibroids. *Lancet (London, England)* 2001;**357**:293–298.

Stewart EA, Laughlin-Tommaso SK, Catherino WH, Lalitkumar S, Gupta D, Vollenhoven B. Uterine fibroids. *Nat Rev Dis Prim* 2016;**2**:16043. Macmillan Publishers Limited.

Styer AK, Rueda BR. The Epidemiology and Genetics of Uterine Leiomyoma. *Best Pract Res Clin Obstet Gynaecol* 2016;**34**:3–12. Bailliere Tindall Ltd.

Su WH, Lee WL, Cheng MH, Yen MS, Chao KC, Wang PH. Typical and atypical clinical presentation of uterine myomas. *J Chinese Med Assoc* 2012;**75**:487–493. J Chin Med Assoc.

Suriyamurthy S, Baker D, Dijke P Ten, Iyengar PV. Epigenetic reprogramming of TGF-β signaling in breast cancer. *Cancers (Basel)* 2019;**11**:. MDPI AG.

Swartz CD, Afshari CA, Yu L, Hall KE, Dixon D. Estrogen-induced changes in IGF-I, Myb family and MAP kinase pathway genes in human uterine leiomyoma and normal uterine smooth muscle cell lines. *Mol Hum Reprod* 2005;**11**:441–450. Mol Hum Reprod.

Thaler F, Mercurio C. Towards selective inhibition of histone deacetylase isoforms: what has been achieved, where we are and what will be next. *ChemMedChem* 2014;**9**:523–536. ChemMedChem.

Timp W, Feinberg AP. Cancer as a dysregulated epigenome allowing cellular growth advantage at the expense of the host. *Nat Rev Cancer* 2013;**13**:497–510. Nat Rev Cancer.

Tsai HC, Li H, Neste L Van, Cai Y, Robert C, Rassool F V., Shin JJ, Harbom KM, Beaty R, Pappou E, *et al.* Transient low doses of DNA-demethylating agents exert durable antitumor effects on hematological and epithelial tumor cells. *Cancer Cell* 2012;**21**:430–446. Cancer Cell.

Turner BM. Defining an epigenetic code. *Nat Cell Biol* 2007;**9**:2–6. Nat Cell Biol.

Vannuccini S, Luisi S, Tosti C, Sorbi F, Petraglia F. Role of medical therapy in the management of uterine adenomyosis. *Fertil Steril* 2018;**109**:398–405. Elsevier Inc.

Verdone L, Caserta M, Mauro E Di. Role of histone acetylation in the control of gene expression. *Biochem Cell Biol* 2005;**83**:344–353. Biochem Cell Biol.

Vishnubalaji R, Alajez NM. Epigenetic regulation of triple negative breast cancer (TNBC) by TGF-β signaling. *Sci Rep* 2021;**11**:. Sci Rep.

Wang J, Sun Y, Zhang X, Cai H, Zhang C, Qu H, Liu L, Zhang M, Fu J, Zhang J, *et al.* Oxidative stress activates NORAD expression by H3K27ac and promotes oxaliplatin resistance in gastric cancer by enhancing autophagy flux via targeting the miR-433-3p. *Cell Death Dis* 2021a;**12**:. Cell Death Dis.

Wang X, Liu J, Wang D, Feng M, Wu X. Epigenetically regulated gene expression profiles reveal four molecular subtypes with prognostic and therapeutic implications in colorectal cancer. *Brief Bioinform* 2021b;**22**:. Oxford Academic.

Wang X, Sun D, Tai J, Chen S, Yu M, Ren D, Wang L. TFAP2C promotes stemness and chemotherapeutic resistance in colorectal cancer via inactivating hippo signaling pathway. *J Exp Clin Cancer Res* 2018;**37**:. J Exp Clin Cancer Res.

Wang Y, Yang X, Xing L, Kong W. Effects of SAHA on proliferation and apoptosis of hepatocellular carcinoma cells and hepatitis B virus replication. *World J Gastroenterol*

2013;**19**:5159–5164. World J Gastroenterol.

Wapenaar H, Dekker FJ. Histone acetyltransferases: challenges in targeting bi-substrate enzymes. *Clin Epigenetics* 2016;**8**:. Clin Epigenetics.

Wei LH, Torng PL, Hsiao SM, Jeng YM, Chen MW, Chen CA. Histone deacetylase 6 regulates estrogen receptor α in uterine leiomyoma. *Reprod Sci* 2011;**18**:755–762. Reprod Sci.

Wei Y, Zhou F, Lin Z, Shi L, Huang A, Liu T, Yu D, Wu G. Antitumor effects of histone deacetylase inhibitor suberoylanilide hydroxamic acid in epidermal growth factor receptor-mutant non-small-cell lung cancer lines in vitro and in vivo. *Anticancer Drugs* 2018;**29**:262–270. Lippincott Williams and Wilkins.

Whitaker LHR, Murray AA, Matthews R, Shaw G, Williams ARW, Saunders PTK, Critchley HOD. Selective progesterone receptor modulator (SPRM) ulipristal acetate (UPA) and its effects on the human endometrium. *Hum Reprod* 2017;**32**:531–543. Hum Reprod.

Wong KK. DNMT1 as a therapeutic target in pancreatic cancer: mechanisms and clinical implications. *Cell Oncol* 2020;**43**:779–792. Springer Science and Business Media B.V.

Xu T, Jin Z, Yuan Y, Zheng H, Li C, Hou W, Guo Q, Hua B. Tat-Interacting Protein 30 (TIP30) Expression Serves as a New Biomarker for Tumor Prognosis: A Systematic Review and Meta-Analysis. *PLoS One* 2016;**11**:. PLoS One.

Yamagata Y, Maekawa R, Asada H, Taketani T, Tamura I, Tamura H, Ogane J, Hattori N, Shiota K, Sugino N. Aberrant DNA methylation status in human uterine leiomyoma. *Mol Hum Reprod* 2009;**15**:259–267. Mol Hum Reprod.

Yan Z, Yang Q, Xue M, Wang S, Hong W, Gao X. YY1-induced lncRNA ZFPM2-AS1 facilitates cell proliferation and invasion in small cell lung cancer via upregulating of TRAF4. *Cancer Cell Int* 2020;**20**:. Cancer Cell Int.

Yang Q, Mas A, Diamond MP, Al-Hendy A. The Mechanism and Function of Epigenetics in Uterine Leiomyoma Development. *Reprod Sci* 2016a;**23**:163–175. SAGE Publications Inc.

Yang T, Zhang H, Qiu H, Li B, Wang J, Du G, Ren C, Wan X. EFEMP1 is repressed by estrogen and inhibits the epithelial-mesenchymal transition via Wnt/β-catenin signaling in endometrial carcinoma. *Oncotarget* 2016b;**7**:25712–25725. Oncotarget.

Zakiryanova GK, Wheeler S, Shurin MR. Oncogenes in immune cells as potential therapeutic targets. *ImmunoTargets Ther* 2018;**Volume 7**:21–28. Dove Medical Press Ltd.

Zhan T, Rindtorff N, Boutros M. Wnt signaling in cancer. *Oncogene* 2017;**36**:1461–1473. Nature Publishing Group.

Zhang L, Yu D. Exosomes in cancer development, metastasis, and immunity. *Biochim Biophys acta Rev cancer* 2019;**1871**:455–468. Biochim Biophys Acta Rev Cancer.

Zhang Y, Liu Z, Yang X, Lu W, Chen Y, Lin Y, Wang J, Lin S, Yun JP. H3K27 acetylation activated-COL6A1 promotes osteosarcoma lung metastasis by repressing STAT1 and activating pulmonary cancer-associated fibroblasts. *Theranostics* 2021;**11**:1473–1492. Theranostics.

Zheng Y, Tabbaa ZM, Khan Z, Schoolmeester JK, El-Nashar S, Famuyide A, Keeney GL, Daftary GS. Epigenetic regulation of uterine biology by transcription factor KLF11 via posttranslational histone deacetylation of cytochrome p450 metabolic enzymes. *Endocrinology* 2014;**155**:4507–4520. Endocrinology.

Zhu X, Chen T, Yang H, Lv K. Lactate induced up-regulation of KLHDC8A (Kelch domain-

containing 8A) contributes to the proliferation, migration and apoptosis of human glioma cells. *J Cell Mol Med* 2020;**24**:11691–11702. J Cell Mol Med.

IX. ANNEX

“For most of history, Anonymous was a woman”- Virginia Woolf.

IX. ANNEX. SCIENTIFIC PRODUCTION

1. Works submitted to international conferences directly related with the present Ph.D. thesis

Poster presentation. María Cristina Carbajo-García, Lucia de Miguel-Gómez, Elena Juárez-Barber, Alexandra Trelis, Javier Monleón, Antonio Pellicer, James M. Flanagan and Hortensia Ferrero. "Targeting histone modifications: H3K27 acetylation regulates the expression of genes involved in key processes of uterine leiomyoma pathogenesis." 37th European Society of Human Reproduction and Embryology Congress, 2022, Milan (Italy)

Poster presentation. Maria Cristina Carbajo-García; Alexandra Trelis; Javier Monleón; Vicente Payá; Antonio Pellicer; James M. Flanagan; Hortensia Ferrero. "Potential Role Of Nervous System Related Functions In Uterine Leiomyoma Pathogenesis". 69th Society for Reproductive Investigation, 2022, Denver (USA).

Oral communication. Maria Cristina Carbajo García; Marina Segura-Benítez; Amparo Faus; Alexandra Trelis; Javier Monleón; Antonio Pellicer; James M. Flanagan; Hortensia Ferrero. "H3K4me3 regulates the expression of genes involved in neuronal processes, synapsis, proliferation, Wnt/βcatenin, and TGFβ pathways in uterine leiomyoma pathogenesis." 8th congress of Society of Endometriosis and Uterine Disorders, 2022, Athens (Greece).

Poster presentation. Maria Cristina Carbajo-García; Ana Corachán; Elena Juárez-Barber; Javier Monleón; Vicente Payá; Alexandra Trelis; Alicia Quiñonero; Antonio Pellicer; Hortensia Ferrero. "Correlation of Methylation Status and Gene Expression Shows Epigenetics Involvement in Key Biological Processes of Uterine Leiomyoma Development." 68th congress of Society for Reproductive Investigation, 2021, Boston (USA).

Poster presentation. Maria Cristina Carbajo-García; Ana Corachán; Marina Segura; Javier Monleón; Julia Escrig; Amparo Faus; Antonio Pellicer; Irene Cervelló; Hortensia Ferrero. "Inhibition of cell proliferation and extracellular matrix formation in human uterine leiomyomas by 5-aza-2'-deoxycytidine via Wnt/β-catenin pathway." 36th Congress of European Society of Human Reproduction and Embryology, 2021, Paris (France).

2. Works submitted to international conferences not related with the present Ph.D. thesis

Oral presentation. Marina Segura-Benítez; **Maria Cristina Carbajo-García**; Ana Corachán; Amparo Faus; Antonio Pellicer; Hortensia Ferrero. "Proteomic analysis of extracellular vesicles secreted by primary endometrial epithelial cells from fertile women reveals functions related to embryo implantation not present in an endometrial epithelial cell line." 68th congress of Society for Reproductive Investigation, 2021, Boston (USA)

3. Scientific articles not related with the present Ph.D. thesis

Corachán A, Trejo MG, **Carbajo-García MC**, Monleón J, Escrig J, Faus A, Pellicer A, Cervelló I, Ferrero H. Vitamin D as an effective treatment in human uterine leiomyomas independent of mediator complex subunit 12 mutation. Fertil Steril. 2021 Feb;115(2):512-521.

Segura-Benítez M, **Carbajo-García MC**, Corachán A, Faus A, Pellicer A, Ferrero H. Proteomic analysis of extracellular vesicles secreted by primary human epithelial endometrial cells reveals key proteins related to embryo implantation. Reprod Biol Endocrinol. 2022 Jan 3;20(1):3.