**human reproduction update**

# Epigenomic and enhancer dysregulation in uterine leiomyomas

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**BACKGROUND:** Uterine leiomyomas, also known as uterine fibroids or myomas, are the most common benign gynecological tumors and are found in women of reproductive and postmenopausal age. There is an exceptionally high prevalence of this tumor in women by the age of 50 years. Black women are particularly affected, with an increased incidence, earlier age of onset, larger and faster growing fibroids and greater severity of symptoms as compared to White women. Although advances in identifying genetic and environmental factors to delineate these fibroids have already been made, only recently has the role of epigenomics in the pathogenesis of this disease been considered.

**OBJECTIVE AND RATIONALE:** Over recent years, studies have identified multiple epigenomic aberrations that may contribute to leiomyoma development and growth. This review will focus on the most recent discoveries in three categories of epigenomic changes found in uterine fibroids, namely aberrant DNA methylation, histone tail modifications and histone variant exchange, and their translation into altered target gene architecture and transcriptional outcome. The findings demonstrating how the altered 3D shape of the enhancer can regulate gene expression from millions of base pairs away will be discussed. Additionally, translational implications of these discoveries and potential roadblocks in leiomyoma treatment will be addressed.

**SEARCH METHODS:** A comprehensive PubMed search was performed to identify published articles containing keywords relevant to the focus of the review, such as: uterine leiomyoma, uterine fibroids, epigenetic alterations, epigenomics, stem cells, chromatin modifications, extracellular matrix [ECM] organization, DNA methylation, enhancer, histone post-translational modifications and dysregulated gene expression. Articles until September 2021 were explored and evaluated to identify relevant updates in the field. Most of the articles focused on in the discussion were published between 2015 and 2021, although some key discoveries made before 2015 were included for background information and foundational purposes. We apologize to the authors whose work was not included because of space restrictions or inadvertent omission.

**OUTCOMES:** Chemical alterations to the DNA structure and of nucleosomal histones, without changing the underlying DNA sequence, have now been implicated in the phenotypic manifestation of uterine leiomyomas. Genome-wide DNA methylation analysis has revealed subsets of either suppressed or overexpressed genes accompanied by aberrant promoter methylation. 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. The dysregulated genes function in tumor suppression, apoptosis, angiogenesis, ECM formation, a variety of cancer-related signaling pathways and stem cell differentiation. Aberrant DNA methylation or histone modification is also observed in altering enhancer architecture, which leads to changes in enhancer–promoter contact strength, producing novel explanations for the overexpression of high mobility group AT-hook 2 and gene dysregulation found in mediator complex subunit 12 mutant fibroids. While many molecular mechanisms and epigenomic features have been investigated, the basis for the racial disparity observed among those in the Black population remains unclear.

**WIDER IMPLICATIONS:** A comprehensive understanding of the exact pathogenesis of uterine leiomyoma is lacking and requires attention as it can provide clues for prevention and viable non-surgical treatment. These findings will widen our knowledge of the role epigenomics plays in the mechanisms related to uterine leiomyoma development and highlight novel approaches for the prevention and identification of epigenome targets for long-term non-invasive treatment options of this significantly common disease.

**Key words:** epigenomics / uterine leiomyoma / enhancer architecture / histone modification / DNA methylation / 3D-chromatin structure / stem cells

# Introduction

Uterine leiomyomas [LMs], or fibroids, are benign tumors of the uterus. The estimated prevalence of this tumor in Black (persons who identified as having African, African American or Black ancestry) and White (persons who identified as having European, Middle Eastern or Northern African ancestry) women by the age of 50 years is above 80% and ∼ 70%, respectively (Islam et al., 2013). Fibroids are typically round in shape and their size can range from a few millimeters to over 20 cm in diameter (Williams, 2017). A pelvic exam is often sufficient in identifying the presence of these tumors; however, imaging techniques may be used to detect smaller fibroids and to determine a more precise size, quantity, location and impact on surrounding anatomical structures (Dagur et al., 2016). Ultrasonography is a rapid, cost-effective first-line imaging test used to confirm the clinical detection of LM and allows for the differentiation of LM from other gynecological diseases (Wozniak and Wozniak, 2017; Florence and Fatehi, 2021). Confident differentiation is important to ensure correct management and to prevent a misdiagnosis of adenomyosis, endometriosis, pregnancy, leiomyosarcoma [LMS], uterine carcinosarcoma or endometrial cancer since these can present with similar symptoms to LM (Fleischer et al., 1978; Florence and Fatehi, 2021). Additionally, this differentiation is important for developing an accurate prognosis as LM has been associated with a potentially increased risk of developing endometrial cancer (Lagana and Scioscia, 2020). LM usually appear as a well-defined solid, spherical hypoechoic mass, but the presence of calcification and necrosis may make determining a diagnosis difficult (Wozniak and Wozniak, 2017). This is especially true when differentiating LMS from atypical LM since their potential overlapping features may challenge the use of ultrasonography as a reliable imaging technique (Sun et al., 2019). Consequently, when ultrasound findings are not clear, MRI may be utilized as a further diagnostic tool (Wozniak and Wozniak, 2017; Sam et al., 2019; Suzuki et al., 2019).

In addition, it has been estimated that 25% of women with LM experience symptoms severe enough to seek treatment within the first year (Marsh et al., 2018). Although LMs do not cause mortality, they are associated with significant morbidity related to abnormal uterine bleeding, pelvic pressure and pain, backache and leg pains, infertility and reproductive dysfunction (Bukulmez and Doody, 2006; Carranza-Mamane et al., 2015). As a result, LMs account for a significant

number of all hysterectomies performed annually (De La Cruz and Buchanan, 2017; Stewart et al., 2017). The societal and financial costs of LM are also significant. It was reported that the annual cost of healthcare for women in the USA who suffer from this disease ranges from $5.89 to $34.37 billion; with direct costs (medications, physician visits and hospital admissions) running at $4.1–9.4 billion and indirect expenses (lost work and obstetric complications related to uterine fibroids) scaling $1.79–24.97 billion (Cardozo et al., 2012). Furthermore, in a national survey of 968 women, 28% reported missing work owing to symptoms, while 24% expressed that they believed their symptoms prevented them from reaching their career potential (Borah et al., 2013). Unfortunately, LM remains understudied despite the high prevalence among women globally and the associated negative impact on a woman's quality of life. Consequently, curative non-invasive treatment options are still lacking.

These monoclonal neoplasms originate from the smooth muscle cells of the myometrium and are complemented by an accumulation of extracellular matrix [ECM] components—a hallmark of the disease. Sex steroid hormones have been discovered to play an important role in LM development and growth (Marsh and Bulun, 2006), possibly because of its tissue of origin. Furthermore, NADPH oxidase-derived reactive oxygen species have been implicated in LM growth by serving as critical intermediates in the mitogenic signaling pathways of epidermal growth factor [EGF] and platelet-derived growth factor (Mesquita et al., 2010). The main molecular/genetic subtypes of LM include: mediator complex subunit 12 [MED12] mutations, high mobility group AT-hook 1 [HMGA1] or high mobility group AT-hook 2 [HMGA2] translocations, biallelic inactivation of fumarate hydratase [FH], collagen (COL4A5–COL4A6) deletions and mutations in genes encoding the Snf2-related CREBBP activator protein [SRCAP] complex subunits (Mehine et al., 2013; Berta et al., 2021). However, a comprehensive understanding of the exact pathogenesis of LM is lacking and requires attention as it can produce clues for future prevention and viable non-surgical treatment.

For most of history, scientists have looked at genetic and environmental factors to delineate disease, but recently, the role of epigenomics in biology and human disease has been considered. Chemical alterations to the DNA structure have now been implicated in the phenotypic manifestation of many disorders, including autoimmune diseases, various cancers and neurodegenerative disorders (Moosavi and Ardekani, 2016). Over the years, literature has identified multiple epigenomic aberrations that may contribute to LM development and growth (Karmon et al., 2014; Commandeur et al., 2015; Styer and Rueda, 2016; Yang et al., 2016; Lagana et al., 2017; Falahati et al., 2021). This review will focus on the most recent discoveries in the following categories of epigenomic changes found in LM: aberrant DNA methylation and histone tail modifications. Furthermore, these will be translated into the context of altered enhancer architecture and 3D chromatin reorganization. The findings demonstrating how this altered 3D shape of the enhancer can regulate gene expression from millions of base pairs away will be discussed. These epigenomic features of LM construct a plausible explanation for the pathways leading to altered gene expression and manifestation into the LM phenotype. The enormous potential of drugs targeting the epigenome in LM treatment and understanding will be highlighted.

# An overview of genetic mutations in uterine leiomyoma

Decades ago, researchers began identifying genetic events that play important roles in LM pathogenesis. In the recent past, with technological advances, several key genetic mutations are found to be mutually exclusive and major drivers of the distinct gene expression changes in LM. These include FH biallelic inactivation, MED 12 mutations, HMGA2 overexpression, deletion of COL4A5 and COL4A6, and mutation in SCRAP subunits (Makinen et al., 2011; Mehine et al., 2013, 2016; Berta et al., 2021).

Biallelic inactivation in the FH gene was linked to predisposition to LM along with cutaneous LM and renal cell cancer in an inherited tumor susceptibility syndrome, known as hereditary leiomyomatosis and renal cell cancer (Launonen et al., 2001; Tomlinson et al., 2002). Dominant inheritance of the mutated copy followed by alterations in the wild-type FH allele cause loss in the activity of FH, a known tumor suppressor protein and a key enzyme of the tricarboxylic acid cycle, which may promote the accumulation of fumarate, and to a lesser extent succinate, in the affected tissues (Lehtonen et al., 2004; Pollard et al., 2005). Pathways that are specifically altered in this subtype of LM are nuclear factor erythroid 2-related factor 2-dependent oxidative stress and pentose phosphate metabolic pathway (Mehine et al., 2016). This subtype is considered rarer, affecting an estimated 0.4–1.6% of all patients with LM (Popp et al., 2020). However, how FH inactivation and the metabolites fumarate and succinate contribute to LM development and progression is not clear.

With the utilization of exome sequencing, and in a breakthrough discovery, frequent mutations in exon 2 of MED12 and to some extent intron 1- exon 2 junction and exon 1 alterations were observed in LM (Makinen et al., 2011). This molecular subtype is the most common with about 70% of LMs displaying this mutation (Mehine et al., 2014). MED12 is a part of the transcriptional mediator complex involved in relaying signals between enhancer-bound transcription factors [TFs] and promoter-bound RNA polymerase II transcription assembly (Borggrefe and Yue, 2011).

In ∼ 10% of LM cases, overexpression of HMGA2 is found that is mainly contributed by rearrangements of 12q15 (Quade et al., 2003; Lagana et al., 2017). HMGA2 is a chromatin-binding non-histone protein and is pro-tumorigenic (Fusco and Fedele, 2007). In these LM, insulin-like growth factor 2 mRNA-binding protein 2 [IGF2BP2] along with PLAG1 zinc finger [PLAG1], a proto-oncogene, were among the significantly deregulated genes (Mehine et al., 2016). In the fourth subtype, where deletions of COL4A5 and COL4A6 have been found, LM had characteristic upregulation of insulin receptor substrate 4 [IRS4] (Mehine et al., 2016).

Most recently, researchers identified another subtype of LM that shows mutation in SCRAP subunits (Berta et al., 2021), discussed in more detail in section 'Histone Variants, Chromatin Remodelers and a New Subtype of Uterine LM'. As has been observed before by Mehine et al. (2016), Berta et al. found that their RNA-seq samples formed separate clusters for MED12 mutant, HMGA2 overexpressing, loss of FH, and additionally there was a separate cluster for SCRAP mutant LM based on the differential gene expression (Berta et al., 2021).

The different subtypes of LM have unique gene expression changes based on the specific mutation type, but they also show features common to all LMs, which possibly converge and lead to LM formation. Owing to the affordability of high throughput screenings and the sensitivity of next-generation sequencing [NGS], many of the epigenomic modalities, which may contribute to gene expression changes observed in subtypes of LM when compared to normal myometrium (or myometrium proximal to LMs), can now be analyzed on patient samples, which together with genetic characteristics may provide a more comprehensive understanding of LM, its subtypes and its genesis. Epigenomics may also offer attractive targets for the treatment of LM. Therefore, in this review, we have discussed the progress made so far in this specific area of research.

# Aberrant DNA methylation in uterine leiomyoma

## Background

The modification of chromatin structure, through DNA methylation, influences TFs', cofactors' and enzymes' access to DNA. One of the most common epigenomic regulation mechanisms that exist in eukaryotes is the methylation of cytosine residues in CpG dinucleotides, which are embedded with the genome. The process of DNA methylation is catalyzed by a family of DNA methyltransferases [DNMTs], which work to transfer a methyl group from a S-adenosyl methionine [SAM] donor to the 5′ position of cytosine's heterocyclic aromatic ring, resulting in a 5-methylcytosine residue [5mC] (Moore et al., 2013). The formation of 5mC during germ cell development and early embryogenesis is established via de novo DNMT subtypes DNMT3A and DNMT3B, while the maintenance of specific DNA methylation patterns over replication is mediated via DNMT1 (Chen and Li, 2004; Kato et al., 2007; Ren et al., 2018). It has been found that 70–80% of CpG sites in the genome are normally methylated with the exception of gene regulatory regions such as CpG islands [CPGIs] (Jabbari and Bernardi, 2004). CPGIs are defined as stretches of DNA consisting of a large number of 5′-cytosine-phosphate-guanosine-3′ repeats. Generally, CPGIs are rarely methylated since they often house promoter sequences necessary for TF and accessory protein binding for the transcription of housekeeping genes (Aoto et al., 2020). Although the addition of a methyl group is not associated with changes to the primary sequence of DNA, the presence of 5mC regions within CPGIs of promoters can result in the stable silencing of gene expression (Mohn et al., 2008).

Previously, DNA methylation was believed to be an irreversible epigenomic event leading to gene repression. However, an enzyme termed ten-eleven translocation protein 1 [TET1] was discovered to potentially erase DNA methylation (Tahiliani et al., 2009). It has now been shown that the family of TET enzymes—consisting of TET1, TET2 and TET3—function to catalyze the oxidation reactions of 5mC to 5-hydroxymethylcytosine [5-hmC], 5-formylcytosine and 5-carboxylcytosine (Rasmussen and Helin, 2016). These oxidation products are viewed as intermediates in the process of DNA demethylation, which converts 5mC to unmodified cytosines. The regulation of DNA methylation, via TET enzymes removing the epigenomic mark, allows for the specific hypomethylated DNA regions to colocalize with TFs and other transcriptional machinery to initiate gene transcription. However, it is interesting to note that tissue-specific DNA hypomethylation occurs more frequently in intragenic or intergenic enhancers as well as in actively transcribed gene bodies than at promoters (Kundaje et al., 2015; Ehrlich et al., 2016). The outcome of hypermethylation within the promoter region is generally a repression of gene transcription, while the result of hypomethylation is generally active transcription leading to gene expression (Smith et al., 2020).

Normally during development, the genome undergoes complex changes in CG methylation. This is thought to contribute to development- and tissue-specific gene expression, genomic imprinting and X chromosome inactivation (Laurent et al., 2010; Moore et al., 2013; Elhamamsy, 2017; George et al., 2019). Consequently, aberrant methylation in CPGIs can manifest themselves in serious disease phenotypes. Aberrant DNA methylation has been associated with various diseases including diabetes mellitus, various neurological disorders, immunological diseases, cancers, atherosclerosis and osteoporosis (de Mello et al., 2014; Armstrong et al., 2019; Ehrlich, 2019). Altered DNA methylation has also been reported in LM, but its role in the development and growth of LM remains not entirely clear.

## Hypermethylation and hypomethylation of gene promoters

The observation of deregulated DNMTs in LM has since prompted many researchers to perform genome-wide DNA methylation studies (Yamagata et al., 2009). To investigate the role of DNA methylation events in this disease, various researchers explored the methylation profiling arrays to identify the promoter proximal sites showing aberrant methylation patterns in LM (summarized in Tables I and II). Navarro et al. (2012) compared DNA methylation and mRNA expression profiles in LM and matched adjacent normal myometrium tissues from 18 Black women. However, it is noteworthy that these results might not be specific to this ethnic group alone, as discussed later. Navarro et al. (2012) found differential promoter methylation and mRNA expression of genes believed to have tumor suppressor functions, namely, Krüppel-like factor 11 [KLF11], DLEC1 cilia and flagella associated protein [DLEC1] (previously named deleted in lung and esophageal cancer 1) and keratin 19 [KRT19]. These genes displayed more significant levels of methylated CpG dinucleotides in their promoter region, and their associated mRNA levels were lower in LM than in normal myometrium (Navarro et al., 2012). It has already been found that the silencing of tumor suppressor genes is correlated with tumor initiation in cancer and other tumor-bearing diseases (Kazanets et al., 2016; Wang et al., 2018). As such, this was the first time the repression of genes with associated tumor suppressor functions, via promoter DNA methylation, was determined in the context of LM. Another independent study identified KLF11 as being downregulated in LM tissue, and accordingly, this study provided insight into the mechanism around KLF11-regulated cell proliferation (Yin et al., 2010). Other genes, such as those involved in the retinoic acid pathway (alcohol dehydrogenase 1A [Class I], alpha polypeptide [ADH1]); in the WNT pathway (Wnt family member 2B [WNT2B]); and the developmental TFs (GATA binding protein 2 [GATA2] and Krüppel-like factor 4) (Ray, 2016; Katerndahl et al., 2021), were identified in both Black and White patient LM samples to be hypermethylated in their promoter regions and were consequently transcriptionally downregulated

**Table I** Alterations in promoter methylation leading to the downregulated expression of various genes in uterine leiomyoma.

| Deregulated gene* | Gene function | Fibroid sample type | Source |
|---|---|---|---|
| KLF11[1] | Tumor suppressor (apoptosis-related) | Tissue (n = 18 African American) Primary cells | (Navarro et al., 2012) |
| DLEC1[2] | Tumor suppressor (inhibits cell proliferation) | Tissue (n = 18 African American) Primary cells | (Navarro et al., 2012) |
| KRT19[3] | Enables structural constituent of cytoskeleton and muscle, Tumor suppressor | Tissue (n = 18 African American) Primary cells | (Navarro et al., 2012) |
| ADH1B[4] | Catalyzes the oxidation of all-trans-retinol and its derivatives (retinoic acid pathway) | Tissue (n = 12 African American, n = 12 Caucasian) Immortalized Cell Lines | (George et al., 2019) |
| WNT2B[5] | Ligand for members of the frizzled family of seven transmembrane receptors (WNT pathway) | Tissue (n = 12 African American, n = 12 Caucasian) Immortalized Cell Lines | (George et al., 2019) |
| GATA2[6] | Transcription factor for genes involved in hematopoiesis (stem cell function), Tumor suppressor | Tissue (n = 12 African American, n = 12 Caucasian) Immortalized Cell Lines | (George et al., 2019) |
| KLF4[7] | Transcription factor involved in embryonic stem cell self-renewal and maintenance, Tumor suppressor | Tissue (n = 12 African American, n = 12 Caucasian) Immortalized Cell Lines | (George et al., 2019) |
| DAPK1[8] | Tumor suppressor (apoptosis-related) | Tissue (n = 10 Japanese) | (Maekawa et al., 2013) |
| NUAK1[9] | Tumor suppressor (apoptosis-related) | Tissue (n = 10 Japanese) | (Maekawa et al., 2013) |
| EFEMP1[10] | Targets metalloproteinase pathways; modulates cell morphology/adhesion | Tissue (n = 20; unknown ethnicity) Primary cells | (Marsh et al., 2016) |

*Genes which are downregulated and have hypermethylation at their promoter in uterine leiomyoma along with their gene function and fibroid sample type used in the respective studies are summarized.
[1]Krüppel like Factor 11
[2]DLEC1 cilia and flagella associated protein
[3]keratin 19
[4]alcohol dehydrogenase 1B (class I), beta polypeptide
[5]Wnt family member 2B
[6]GATA binding protein 2
[7]Krüppel like Factor 4
[8]death associated protein kinase 1
[9]NUAK family kinase 1
[10]EGF containing fibulin extracellular matrix protein 1

**Table II** Alterations in promoter methylation leading to the upregulated expression of various genes in uterine leiomyoma.

| Upregulated gene* | Gene function | Fibroid sample type | Source |
|---|---|---|---|
| COL4A1[11] | Collagen IV biosynthesis | Tissue (n = 10 Japanese) | (Maekawa et al., 2013) |
| COL4A2[12] | Collagen IV biosynthesis | Tissue (n = 10 Japanese) | (Maekawa et al., 2013) |
| COL6A3[13] | Collagen VI biosynthesis | Tissue (n = 10 Japanese) | (Maekawa et al., 2013) |

*Genes which are upregulated and have hypomethylation at their promoter in uterine leiomyoma along with their gene function and fibroid sample type used in the respective studies are summarized.
[11]collagen type IV alpha 1 chain
[12]collagen type IV alpha 2 chain
[13]collagen type VI alpha 3 chain

(George et al., 2019). The aberrant expression of these key tumor suppressor and developmental genes may partly be involved in the pathogenesis of the benign tumors found in LM. As LM is characterized by the accumulation of ECM, determining whether ECM gene promoters also show dysregulated promoter methylation may be more helpful in better understanding this disease.

Along these lines, Maekawa et al. (2013) conducted a more comprehensive study by utilizing Illumina Infinium HumanMethylation450 Bead Chip platform in Japanese women. In their study using DNA methylome and transcriptome data, the researchers uncovered that estrogen response elements [ERE] were present in the promoter region of ∼18% of altered genes. Collagen Type IV alpha 1 chain [COL4A1], collagen Type IV alpha 2 chain [COL4A2] and collagen type VI alpha 3 chain [COL6A3] were hypomethylated and had increased gene expression in LM compared to matched or normal myometrium obtained from patients presenting with LM or cervical cancer without

LM (Maekawa *et al.*, 2013). Reduced methylation at promoters of the above-mentioned collagen genes was proposed to allow estrogen receptor alpha [ERα] to bind to its respective promoter regions and initiate an overproduction of collagen, which is the major component of the accumulated ECM (Stewart, 2001). Other genes, such as death-associated protein kinase 1 [*DAPK1*] and NUAK family kinase 1 [*NUAK1*], were hypermethylated and had decreased gene expression (Maekawa *et al.*, 2013). Increased methylation is thought to block ERα binding to these genes, and although the exact mechanism of gene inactivation remains unknown, it may play a role in inhibiting their functions of signaling apoptosis-related events (Steinmann *et al.*, 2019). This finding led the researchers to conclude that aberrant DNA methylation at the promoter of ERα target genes is responsible, at least in part, for the aberrant response to estrogen, reinforcing the previous observations on the role of estrogen in the growth of LM. Considering that ERα binds to enhancers, it will be critical to determine whether promoter distal EREs show similar methylation-sensitive properties. Additionally, causal experiments will need to be performed to verify the proposed mode of action involving ERα and DNA methylation levels in regulating the above-mentioned potential target genes.

Collagens constitute an important part of the ECM; however, a recent study pursued fibulins, which are extracellular glycoproteins. Fibulins function to modulate cell morphology, growth, adhesion and motility (Gallagher *et al.*, 2005). Studies conducted on solid malignant tumors have shown that EGF containing fibulin extracellular matrix protein 1 [*EFEMP1*], the gene that encodes fibulin-3, is downregulated and that this observation serves as a biomarker for prostate cancer and hepatocellular cancer (Nomoto *et al.*, 2010; Kim *et al.*, 2011). Furthermore, other studies have linked the loss of fibulin-3 with increased tumor angiogenesis (Sadr-Nabavi *et al.*, 2009; Hwang *et al.*, 2010; Kim *et al.*, 2011). The study performed in LM found that fibulin-3 is repressed in LM compared to normal myometrium (Marsh *et al.*, 2016). Although simple association is not sufficient to establish causality in LM genesis, the researchers also showed that treatment of primary LM cells with the DNMT inhibitor termed 5-Aza-2′-deoxycytidine [5-aza-dC] increased *EFEMP1* expression significantly compared to vehicle, partly linking DNA methylation activity to its downregulation. However, the methylation profile at or near the *EFEMP1* gene in LM compared to the normal condition needs to be validated. Various studies in both cancerous and non-cancerous conditions have shown that fibulin-3 acts by targeting metalloproteinase pathways (Klenotic *et al.*, 2004; Kim *et al.*, 2012). Further insight into the implications of fibulin-3 deregulation in LM and the mechanisms governing its altered expression may be beneficial toward its treatment.

In an effort to study the upstream regulatory genes that may play a role in LM pathogenesis, genes that were differentially methylated and altered in LM were analyzed. Ingenuity Pathway Analysis revealed the highly expressed transcription regulators, SATB homeobox 2 [*SATB2*] and neuregulin 1 [*NRG1*], as potential upstream regulatory genes. Interestingly, more than 50% of pathways perturbed in the immortalized uterine smooth muscle cell line, upon overexpression of these two genes independently, were common to altered pathways in LM (Sato *et al.*, 2019). Those pathways included the WNT/β-catenin and the transforming growth factor [TGF]-β signaling pathways known to play an important role in LM (Borahay *et al.*, 2015; Ciebiera *et al.*, 2017; Sato *et al.*, 2019). In addition, the researchers confoundingly found hypermethylation near one of their several transcription start

sites [TSS] for both *SATB2* and *NRG1* genes (Sato *et al.*, 2019). This discrepancy may be linked to the expression of variant forms from the TSS not analyzed by the DNA methylome study and/or related to other epigenomic events. Another limitation is that cell lines may not fully reflect *in vivo* conditions, such as those found in tissues. With the development of cell lines, passaging cells may cause them to lose characteristics that are found *in situ* (Kaur and Dufour, 2012). Therefore, identifying the exact mechanisms leading to higher expression of these regulatory genes in LM may expose processes crucial to its development.

It is important to mention and acknowledge that although the authors of the above-mentioned studies highlight specific genes and their associations with DNA methylation, the observations remain correlative; direct and causal experiments need to be performed to establish a mechanistic connection between promoter methylation level and gene expression changes.

## Genome-wide DNA methylation and stem cell regulation in uterine leiomyoma

Goodell *et al.* (1996) were the first ones to identify a population of cells in bone marrow that can efflux fluorescent DNA-binding dye (Hoechst 33342). This subset of the cell population was observed aside from the main population at Hoechst blue-low and Hoechst red-low corner regions in scatter plots of fluorescence-activated cell sorting and therefore termed as the side population [SP]. The SP was found to be enriched for hematopoietic stem cells (Goodell *et al.*, 1996). Afterwards, many laboratories have observed the SP in various tissues, including endometrium and myometrium, from adults and, in general, this population shows properties of somatic stem cells (Ono *et al.*, 2007; Masuda *et al.*, 2010; Cervello *et al.*, 2011). Along these lines, several studies demonstrate a SP in LM with characteristics of stem-like cells that are considered crucial for LM growth (Mas *et al.*, 2012; Ono *et al.*, 2012, 2013). Furthermore, distinct cell populations within LM tissue at different differentiation stages were identified based on the cell surface markers, CD34 and CD49b. The authors evaluated that cells with CD34+/CD49b+ had stem cell-like characteristics and were highly enriched in the side population [LSC], CD34+/CD49b– cells were intermediate [LIC] and CD34–/CD49b– cells were fully differentiated [LDC] (Yin *et al.*, 2015). DNA methylation seems to play an important role in LM, but to elucidate its role in stem cell regulation, Liu *et al.* (2020) performed Methyl Cap sequencing across all three populations of cells. Their data show that when compared to LIC and LDC, LSC harbored several thousands of differentially methylated regions [DMRs]. Most of the differential regions in LSC were hypermethylated with no drastic difference between the LIC and LDC populations. Concurrently, there was the lowest expression of two DNA demethylases in LSC, TET methylcytosine dioxygenase 1 and 3, but no significant changes in DNMTs. Key here is that the LSC population of cells, as opposed to LIC and LDC populations, harbored extensive DNA methylation in genes required for differentiation, such as estrogen receptor 1 [*ESR1*], TIMP metallopeptidase inhibitor 3 [*TIMP3*], receptor tyrosine kinase like orphan receptor 2 [*ROR2*] and myosin heavy chain 11 [*MYH11*], which suppressed their expression and the ability to develop into differentiated LM cells (Liu *et al.*, 2020). A more recent study by this group found that within the LSC population, the progesterone receptor [PR] gene contained significant

hypermethylation at several intronic regions. Treatment with 5-Azacytidine [5-Aza], a drug that inhibits DNMT, activated PR signaling and stimulated LSC differentiation. The authors suggested that the increased level of DNA methylation within *PR* and its target cistrome interfered with PR signaling and inhibited the expression of genes critical for progesterone-induced LSC differentiation (Liu *et al.*, 2021a). Interestingly, since LSC cells are deficient in steroid hormone receptors, they are possibly resistant to the hormonal therapies used to treat LM. After treatment, these cells may proliferate and differentiate, leading to tumor growth, as has been observed in breast cancer (Liu and Wicha, 2010). Expectedly, in the Liu *et al.* (2020) study, the application of 5-Aza promoted differentiation of the LSC population, raising the possibility that it may sensitize them toward hormone therapy (Liu *et al.*, 2020). When testing this drug's effect on hormonal therapy, Liu *et al.* (2021a) observed that 5-Aza treatment synergized with an anti-progestin to reduce tumor size in a xenograft mouse model, highlighting the feedback loop between progesterone signaling and DNA methylation as a potential therapeutic target that may decrease LSC stemness and tumor growth (Liu *et al.*, 2021a). However, owing to potential toxicity associated with compounds interfering with DNA methylation globally, further studies should be performed to determine success revolving around the treatment designed to target LSC and avert the formation of novel and unwanted tumors.

## Impact of enhancer methylation on *RANKL* gene expression

With the finding of the LM stem cell population and its important function in this benign tumor growth (Yin *et al.*, 2015), researchers are now attempting to target this population specifically. Breast cancer research has identified a paracrine pathway where progesterone signals expansion and differentiation of the PR-negative mammary stem cells (Joshi *et al.*, 2010). This pathway involves the secretion of receptor activator of nuclear factor $\kappa$-B ligand [RANKL] from PR-positive mammary epithelial cells in response to progesterone. RANKL then acts on its receptor, receptor activator of nuclear factor $\kappa$-B [RANK], which is present on stem cells (Joshi *et al.*, 2010; Sigl *et al.*, 2016). As LMs are characterized as hormone-sensitive tumors, Ikhena *et al.* (2018) therefore speculated a similar phenomenon in LM stem cells and demonstrated LM growth suppression in a mouse xenograft model in the presence of RANK-Fc (a RANK/RANKL pathway inhibitor). Accordingly, compared to myometrium, the LM intermediate population [LIC] showed the highest expression of RANKL, while stem cells [LSC] exhibited high levels of RANK (Ikhena *et al.*, 2018). Moreover, *RANKL* transcription responds to progesterone signaling with increased sensitivity in LM cells (Liu *et al.*, 2019). To shed some light on the interplay between progesterone and the RANK/RANKL pathway in LM, Liu *et al.* (2019) found a distal progesterone receptor binding site [PRBS] located 87 kb upstream of the RANKL transcription start site (Liu *et al.*, 2019). This region was enriched for H3K27ac, which is an active enhancer mark indicating it as a potential enhancer region. Interestingly, Methyl Cap-Seq for robust genome-wide DNA methylation profiling revealed a DMR in the vicinity of this binding site (Brinkman *et al.*, 2010; Liu *et al.*, 2019). The hypothesis that methylation at this site prevents binding of PR and thus decreases *RANKL* expression was tested by 5-Aza treatment of LM cells. Furthermore, this site was found to be demethylated in LM tissues as compared to

normal myometrium (Liu *et al.*, 2019). The authors proposed that both the DMR and the distal PRBS compose a novel *RANKL* distal regulatory element that functions as an enhancer to regulate RANKL expression. Overall, DNA hypomethylation was suggested to be involved in recruiting and enhancing the binding of PR to the enhancer and strengthening the looping interaction between the distal PRBS and the *RANKL* promoter. A positive correlation between a mutation in *MED12* and increased binding of PR to PRBS and RANKL expression was observed, with concomitant higher affinity binding of PR to mutant *MED12* (Liu *et al.*, 2019). Altogether, this study suggests a possible relation between TF binding, histone modification and DNA methylation to the enhancer and its integration into LM phenotype. Although this comprehensive study has identified a new regulatory element for *RANKL*, the factors that determine methylation changes at this element still need to be determined as it is plausible that those factors are crucial in development of this disease. While distal PR targeting enhancers are possible regulators, clustered regularly interspaced short palindromic repeat [CRISPR]-mediated ablation of the sites will conclusively establish the role of this enhancer in PR/progestin-mediated regulation of *RANKL*. We further emphasize that causal experiments are necessary to follow-up on the regulatory roles of DNA methylation uncovered by gene-specific and global DNA methylation studies in regulating LM subtype-specific gene expression and LM genesis.

## Role of 5-hydroxy-methylcytosine

Various studies in recent years have focused on understanding 5-hmC in the context of solid and hematological tumors. The loss of 5-hmC levels has been associated with higher grade and increased metastasis in various tumors. At the same time, the reduced expression and altered function of TET enzymes were observed in these tumors (Kudo *et al.*, 2012; Yang *et al.*, 2013). In contrast to malignant tumors, benign tumors such as LM have higher levels of 5-hmC as well as upregulated TET1 and TET3 levels compared to normal myometrium. Knockdown of *TET1* or *TET3* coordinated with decreased 5-hmC levels and reduced cellular proliferation (Navarro *et al.*, 2014). Interestingly, increased expression of *TET3* is found to be linked with aberrant expression of H19 long non-coding RNA [H19 lncRNA] in LM. H19 lncRNA, by acting as a molecular sponge for miRNA let-7, regulates TET3 levels in LM, possibly explaining the increase in 5-hmC levels (Cao *et al.*, 2019). The global levels of this mark in LM are in stark contrast to malignant cancer, raising the need for further investigations to understand the role of 5-hmC in benign tumors. The ease of measuring 5-hmC in cell-free DNA and the availability of cost-effective high throughput methods for its quantitation has made this mark conducive for diagnosis and prognosis purposes (Xu and Gao, 2020). The possibility of using 5-hydroxymethylation as an epigenomic signature in detecting LMs or subtypes shall be explored in future studies.

Although 5-hmC is an intermediate in the 5mC to C conversion pathways, its role in transcriptional regulation has also been scrutinized. Previous studies have found that 5-hmC is enriched at gene bodies, promoters and TF binding sites (Nestor *et al.*, 2012). However, correlation between the presence of 5-hmC and gene expression has not yet been established and it is speculated that it is cell-type specific (Nestor *et al.*, 2012; Tan *et al.*, 2013). Researchers have shown that not only are there changes in the levels of 5-hmC in cancer cells compared to healthy cells, but also there is a change in the

distribution of this modification in stem cells from a *tet2*-mutant AML murine model (Han *et al.*, 2016; Shi *et al.*, 2017). It would be interesting to evaluate the redistribution of 5-hmC in LM and its association with gene expression, as the abundance of this modification increases in this diseased state.

# Histone modifications in uterine leiomyoma

## Background

In addition to genomic DNA methylation, post-translational modifications of histones hold importance in the epigenomic regulation of gene expression owing to their role in altering chromatin structure. Histones are defined as nucleoproteins because of their functions associated with DNA. Eukaryotes possess five canonical histone proteins: H1, H2A, H2B, H3 and H4. To create a more organized and compacted DNA, ∼147 base pairs of DNA are wrapped around a histone core (consisting of dimers of H2A, H2B, H3 and H4) to form a nucleosome (Zhou *et al.*, 2019). H1 functions to add stability to the nucleosome by binding to inter-nucleosomal space (Li and Zhu, 2015). Histones interact well with the negatively charged DNA and are themselves considered basic as they are composed largely of the positively charged amino acids—lysine and arginine. The histone proteins and DNA can form two categories of chromatin: heterochromatin and euchromatin. Heterochromatin has a condensed structure. This tight coiling, in general, prohibits access of the transcriptional machinery to DNA, thus rendering it transcriptionally silent. On the other hand, euchromatin is loosely packed, therefore it is accessible to TFs and generally transcriptionally active (Morrison and Thakur, 2021).

One mechanism capable of remodeling chromatin structure and regulating gene expression consists of the covalent post-translational modifications of histones. These modifications include methylation, acetylation, phosphorylation, ubiquitination and sumoylation among others (Castillo *et al.*, 2017). When a TF binds to DNA, it can recruit coactivator enzymes such as histone acetyltransferases [HATs]. HATs catalyze the addition of acetyl groups to lysine residues found on the N-terminal tails of histones. This event neutralizes the histone's positive charge and reduces the affinity to the adjacent nucleosome, resulting in destabilization of the nucleosome's higher-order helix (Luger *et al.*, 1997; Castillo *et al.*, 2017). This modification can be regulated via histone deacetylases [HDACs], which remove the acetyl group from histone proteins and impose a repressive effect (Wang *et al.*, 2020). Similar to histone acetylation, histone phosphorylation is generally associated with transcriptional activation. The histone tails contain serine, threonine and tyrosine residues that can be phosphorylated or dephosphorylated. Protein kinases mediate phosphorylation by transferring phosphates to these acceptor sites, while protein phosphatases mediate dephosphorylation. Phosphorylation of histones creates a repulsive force between the added negatively charged phosphate group and the negatively charged phosphates of the DNA backbone, leading to a more open chromatin structure (Banerjee and Chakravarti, 2011; Rossetto *et al.*, 2012). Other epigenomic enzymes include histone methyltransferases [HMTs], which catalyze the transfer of a methyl group from SAM to locations of the histone tails containing arginine or lysine residues, and demethylase enzymes, which work to remove the methyl groups (D'Oto *et al.*, 2016). The likely outcome of methylation varies. It has a transcriptional silencing (tri-methylation at the ninth lysine residue of the histone H3 protein [H3K9me3], tri-methylation at the 27th lysine residue of the histone H3 protein [H3K27me3]) effect, similar to the outcome of DNA methylation, but activation of gene transcription (tri-methylation at the fourth lysine residue of the histone H3 protein [H3K4me3]) has also been observed (D'Oto *et al.*, 2016; Gong and Miller, 2019). In addition, TFs, cofactors and non-histone proteins, such as β-catenin, are also targeted by HAT and HDAC enzymes.

The epigenomic studies pertaining to LM pathogenesis have mainly focused on DNA methylation; however, as histone modifications also have the potential to play an equally important function in chromatin alterations, researchers are now exploring the effect of histone modifications in this disease.

## Genome-wide histone epigenomic alterations in uterine leiomyoma

Considering that the uterus is a highly steroid-responsive tissue, it is not surprising that LM growth and development is influenced by sex steroid hormones (Marsh and Bulun, 2006). To determine the effect of environmental estrogens on LM development, various studies have been pursued. Recent work on a commonly consumed phytoestrogen, genistein, has revealed that it has both inhibitory and stimulatory effects in LM cells depending on its concentration (Di *et al.*, 2008; Di *et al.*, 2012; Castro *et al.*, 2016). At a low concentration of 1 μg/ml, genistein stimulated cell proliferation by activating the mitogen-activated protein kinase p44/42 signaling pathway [MAPK$_{p44/42}$] via ERα (Di *et al.*, 2008). Yu *et al.* (2016) showed that genistein-induced phosphorylation of MAPK$_{p44/42}$ in immortalized human uterine LM [ht-UtLM] cells increased the activation of its downstream effector mitogen- and stress-activated protein kinase 1 [MSK1], which in turn modified histone H3 to histone H3 phosphorylated at serine 10 [H3S10ph]. Colocalization of phospho-MSK1 and H3S10ph in the nuclei of ht-UtLM cells was also observed yet was abolished in the presence of MEK1 inhibitor PD98059. Genes related to cell proliferation and altered by genistein, such as inhibitor of DNA binding 1 [*ID1*] *and c-MYB*, showed enrichment of H3S10ph at their promoter regions by ChIP-qPCR assay (Yu *et al.*, 2016). This serves to indicate the possibility that genistein's induction of the MAPK$_{p44/42}$/MSK1/H3S10ph axis leads to a more open chromatin structure allowing for the transcriptional upregulation of genes involved in promoting cell proliferation in hormonally driven LM growth. Interestingly, histone phosphorylation has also been shown to play a regulatory role in androgen responsiveness of prostate cancer cells (Banerjee and Chakravarti, 2011; Kim *et al.*, 2016). In LM, steroid hormones and growth receptors crosstalk to drive its development (Borahay *et al.*, 2015). Nevertheless, it is unknown if the above-mentioned mechanism holds true for steroid hormones *in vivo* or if it is a specific effect of this phytoestrogen. Further studies using antibodies to H3S10ph are warranted to establish a genome-wide role of H3S10ph in LM biology.

## TET3 knockdown diminishes chromatin accessibility

As mentioned above, TET3 expression is elevated in LM versus matched myometrium and is regulated by the H19/let-7 axis (Navarro

*et al.*, 2014; Cao *et al.*, 2019). Likewise, downregulation of either *H19* or *TET3* leads to decreased protein levels of several LM-promoting genes: TGF-β receptor 2 [*TGFBR2*], thrombospondin 1 [*THBS1*], Rho GTPase activating protein 26 [*ARHGAP26*], secreted protein acidic and cysteine rich [*SPARC*], collagen type V alpha 2 chain [*COL5A2*], collagen type IV alpha 1 chain [*COL4A1*] and collagen type III alpha 1 chain [*COL3A1*] (Cao *et al.*, 2019). In addition, this study explored the interaction of TET3 with promoter regions of two such LM-promoting genes: the binding of TET3 at the promoter of *TGFBR2* and *THBS1* was decreased in the *TET3* knockdown cells compared to the control with concurrently increased DNA methylation. Previous findings have shown that within the promoter regions of actively transcribed genes, there is an enrichment of H3K4me3, while enrichment of H3K27me3 is associated with inactive promoters (Barski *et al.*, 2007). Consistent with this idea, ChIP analysis revealed a significant reduction in H3K4me3 and an increase in H3K27me3 at these promoters owing to *TET3* knockdown (Cao *et al.*, 2019). Thus, it may be inferred that the knockdown of *TET3* promotes a heterochromatin conformation yielding reduced DNA accessibility at the promoters of genes that enhance fibrosis and, thereby, repressed transcription. On the other hand, it may be plausible to assume that overexpression of *TET3* can create a more accessible chromatin structure, promoting transcription of the ECM component genes (*ARHGAP26, SPARC, COL5A2, COL4A1* and *COL3A1*) and TGF-β pathway genes (*TGFBR2 and THBS1*) in LM. Nevertheless, experimental data to substantiate this hypothesis and to emphasize the role of TET proteins in this disease is required.

# Hierarchical clustering and disease prediction based on DNA methylation and histone modifications

A recent critical study found differential transcriptomic profiles of LM subtypes were based on their mutation status (Mehine *et al.*, 2016). Can differential epigenomic profiles similarly segregate the LM subtypes? To answer this question in the context of DNA methylation, Sato *et al.* (2016) focused on 12 of the 120 aberrantly methylated genes by utilizing combined bisulfite restriction analysis and criteria of similar alteration in at least 70% of the LM cases. The purpose of this study was to construct a hierarchical clustering system for clinical application. A combination of 10 genes (ALX homeobox 1 [*ALX1*], cerebellin 1 precursor [*CBLN1*], corin, serine peptidase [*CORIN*], forkhead box P1 [*FOXP1*], GATA2, IgLON family member 5 [*IGLON5*], neuronal pentraxin 2 [*NPTX2*], neurotrophic receptor tyrosine kinase 2 [*NTRK2*], prolactin [*PRL*], plus STEAP4 metalloreductase [*STEAP4*]) was established to differentiate normal myometrium from LM. Furthermore, LM showed sub-clusters, but this substructure was independent of the *MED12* mutation [*MED12mt*] status. Here, the authors mainly relied on altered methylation at 10 genes for separating the different conditions. This approach could differentiate normal from the diseased state but could not identify LM subtypes (Sato *et al.*, 2016). Nevertheless, it showed that an altered epigenomic profile in addition to transcriptomic changes has the potential to segregate LM from normal myometrium. As an additional possibility, utilization of a hierarchical clustering system by a comprehensive epigenomic analysis can provide an opportunity to reveal LM subtypes. In this regard, in a key study, George *et al.* (2019) performed unsupervised clustering of 10 normal myometrium and 24 LM samples based on highly variable 1% DNA methylome identified in an Infinium Methylation EPIC array [EPIC] that includes enhancer sites. They observed the segregation of normal myometrium and LM, and further clustering of LM into *MED12mt*, overexpressed *HMGA2* [*HMGA2hi*] and overexpressed *HMGA1* [*HMGA1hi*] subtypes. Compared to normal myometrial tissues, homeobox A13 [*HOXA13*] was also identified to be hypomethylated and upregulated in LM. This overexpression correlated with homeotic transformation in the myometrium to a more cervical stroma phenotype. This observation identified a potential event that leads to the development of LM since cervical stroma and LM are both characterized by a significant amount of ECM. Additionally, it established global DNA methylation as a powerful experimental and clinical test to classify LM (George *et al.*, 2019).

To identify if there are subtype-specific histone modifications, Leistico *et al.* (2021) utilized chromatin immunoprecipitation followed by NGS [ChIP-seq] against 'active' histone modifications, namely H3K4me3 (promoter mark), H3K27ac (present at active promoter and enhancer) and H3K4me1 (enhancer mark) in LM and matched myometrium from 21 patients. Owing to computational challenges in analyzing heterogenous ChIP-seq datasets, the authors employed a tensor decomposition method called decomposition and classification of epigenomic tensors [DeCET]. Using DeCET, epigenomic features could distinguish myometrium from LM subtypes and, interestingly, could also classify them in > 95% of the cases analyzed (Leistico *et al.*, 2021). This model has the capacity to categorize diseased state based on a small number of datasets. Thus, it could serve as an effective diagnostic tool when combined with small-scale ChIP-seq procedures, such as CUT&RUN or ChIL-seq. Overall, this suggests that, in addition to distinct DNA methylation profiles, there are LM- and subtype-specific histone modifications (Fig. 1). The identification of HOXA13 as an important dysregulated factor by independently performed DNA methylation and histone modifications studies suggests a convergence of these two epigenomic features in LM pathogenesis (George *et al.*, 2019; Leistico *et al.*, 2021). Furthermore, enrichment of FOS, JUN at hypomethylated regions, especially in *MED12* mutant LM, corroborates the finding that activator protein-1 (AP-1) is one of the crucial factors in modifying chromatin architecture in this disease (Moyo *et al.*, 2020). Collectively, these studies established a critical role of epigenome regulation of LM. Various studies highlight the coordinated effects of DNA methylation and histone modifications in several important processes such as embryonic development, X-chromosome inactivation, etc. There is evidence suggesting the dependence of each arm on the other in different conditions (Cedar and Bergman, 2009). Consequently, a comprehensive integrative analysis with DNA methylation and histone modifications needs to be performed to understand the interplay between these two arms of epigenomic regulation in the context of this disease. In some fibrotic diseases, methyl-CpG-binding protein 2 [MeCP2] has been studied as a bridge between these two epigenomic events (discussed later). Perhaps, its role may be explored in LM.
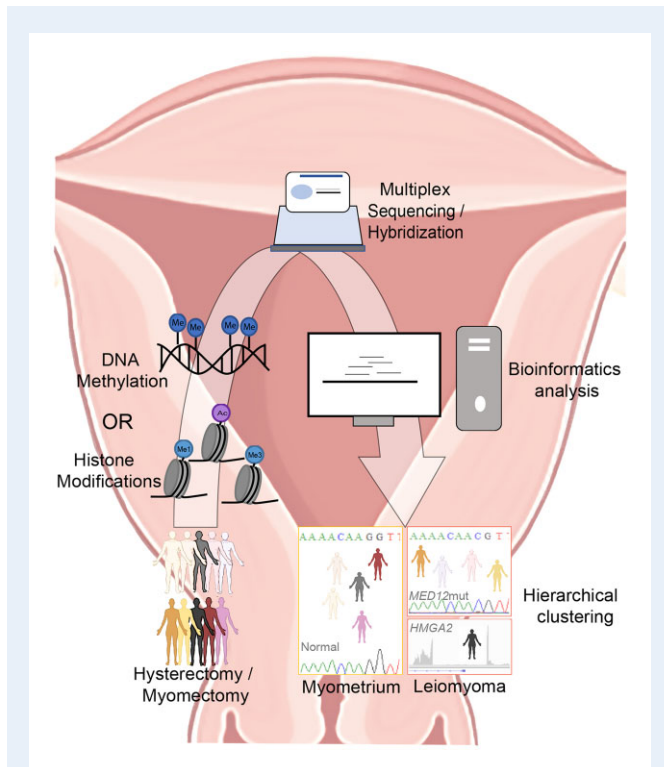
**Figure 1.** Schematic illustration of workflow employed to identify histone modification/DNA methylation patterns that discriminate uterine leiomyoma from normal myometrium. Tissue samples from patients undergoing hysterectomy or myomectomy were frozen until use. Following appropriate processing, samples were prepared either for ChIP-sequencing (histone modifications, Leistico et al., 2021) or hybridization (DNA methylation, George et al., 2019). Data were analyzed using various bioinformatic tools and unsupervised clustering was performed. Epigenomic alterations could cluster myometrium separately from uterine leiomyoma [LM] and, furthermore, LM into subtypes (MED12mut indicates mediator complex subunit 12 mutated LM subtype; HMGA2, overexpressed high mobility group AT-hook 2 LM subtype).

# Role of enhancers in uterine leiomyoma

## Background

TFs can recognize specific response elements outside of the promoter proximal regions. An enhancer is a collection of several response elements which regulates gene expression by a variety of signals and acts to amplify gene transcription levels. Like promoters, enhancers are considered cis-regulatory regions, but unlike promoters, they are not necessarily adjacent to the target gene TSS. In humans, these regions can be located as far as 2 million base pairs away, either upstream or downstream, from the affected gene (van Heyningen and Bickmore, 2013). Owing to the large distance between the promoter and enhancer regions, DNA must often be bent into a hairpin loop to form enhancer–promoter contact (Fig. 2). This is believed to occur through lineage-specific DNA-binding TFs—bound at promoters and enhancers—interacting together or recruiting the looping factors necessary to form the long-range contact.

Binding proteins, such as CCCTC-binding factor [CTCF] and cohesins, may also play a role in this interaction (Pennacchio et al., 2013).

Ample studies have recognized how alterations in promoter regions affect gene expression, but little has been explored in the context of altered enhancer landscape and resulting gene dysregulation. Recent data suggests that changes in enhancer landscape plays a key role in disease pathogenesis (Krijger and de Laat, 2016). The aberrant enhancer epigenome and changes in TF occupancy have been proposed to lead to alterations in enhancer 3D architecture, bringing about dysregulated RNA polymerase II gene transcription. Recently, this postulation has been translated into research on LM formation.

## DNA methylation landscape at enhancers in uterine leiomyoma

As discussed earlier, George et al. (2019) utilized EPIC to analyze genome-wide DNA methylation in LM and normal myometrium. One of their observations was a small shift in overall distribution of methylation at enhancer regions, from partially methylated domains to highly methylated domains in LM. While analyzing TF binding sites at differentially methylated loci located distally, they found that both MED12mt and HMGA2hi LM were enriched for enhancer of zeste 2 polycomb repressive complex 2 subunit [EZH2] and SUZ12 polycomb repressive complex 2 subunit [SUZ12] (components of Polycomb repressive complex 2 [PRC2]) binding sites in hypermethylated cytosines. Furthermore, ERα binding sites were distributed at hypomethylated sites. Interestingly, they noticed hypomethylation in a segment of the HMGA2 gene body in HMGA2 overexpressing LM compared to normal myometrial and other subtypes (George et al., 2019). In HMGA2hi LM, 12q14-15 chromosomal rearrangements are generally considered a reason for the higher expression of this gene (Ashar et al., 1995; Schoenmakers et al., 1995). Two of the cases in the study did not show the translocation at these loci using fluorescence in situ hybridization probes but still overexpressed HMGA2. The authors considered the observed hypomethylation to be responsible for this phenomenon (George et al., 2019). They located a CTCF binding site near the 3′ end distal hypomethylation locus of the HMGA2 gene to further explore it. CTCF is a TF that can bind at enhancer sites to form chromatin loops and/or attract transcriptional coactivators, repressors and RNA Polymerase II (Holwerda and de Laat, 2013). Using FANTOM5 and Chromatin Interaction Analysis by Paired-End Tag Sequencing interaction data, a CTCF looping region was observed with the HMGA2 gene within this region. DNA methylation profiles to infer accessibility of chromatin showed that this region possesses more accessibility or a more open chromatin structure in HMGA2hi LM (George et al., 2019). Although further analysis needs to be performed to confirm this observation, this alteration in chromatin structure caused by hypomethylation gives rise to the possibility of a more influential CTCF-mediated looping in HMGA2hi LM that facilitates interaction between the distal enhancer and the HMGA2 gene promoter, initiating increased gene transcription.

## Enhancer/histone epigenome dysregulation in uterine leiomyoma

The genome-wide screening of 21 patients for histone modifications via DeCET revealed that the majority (70%) of the changes in LM
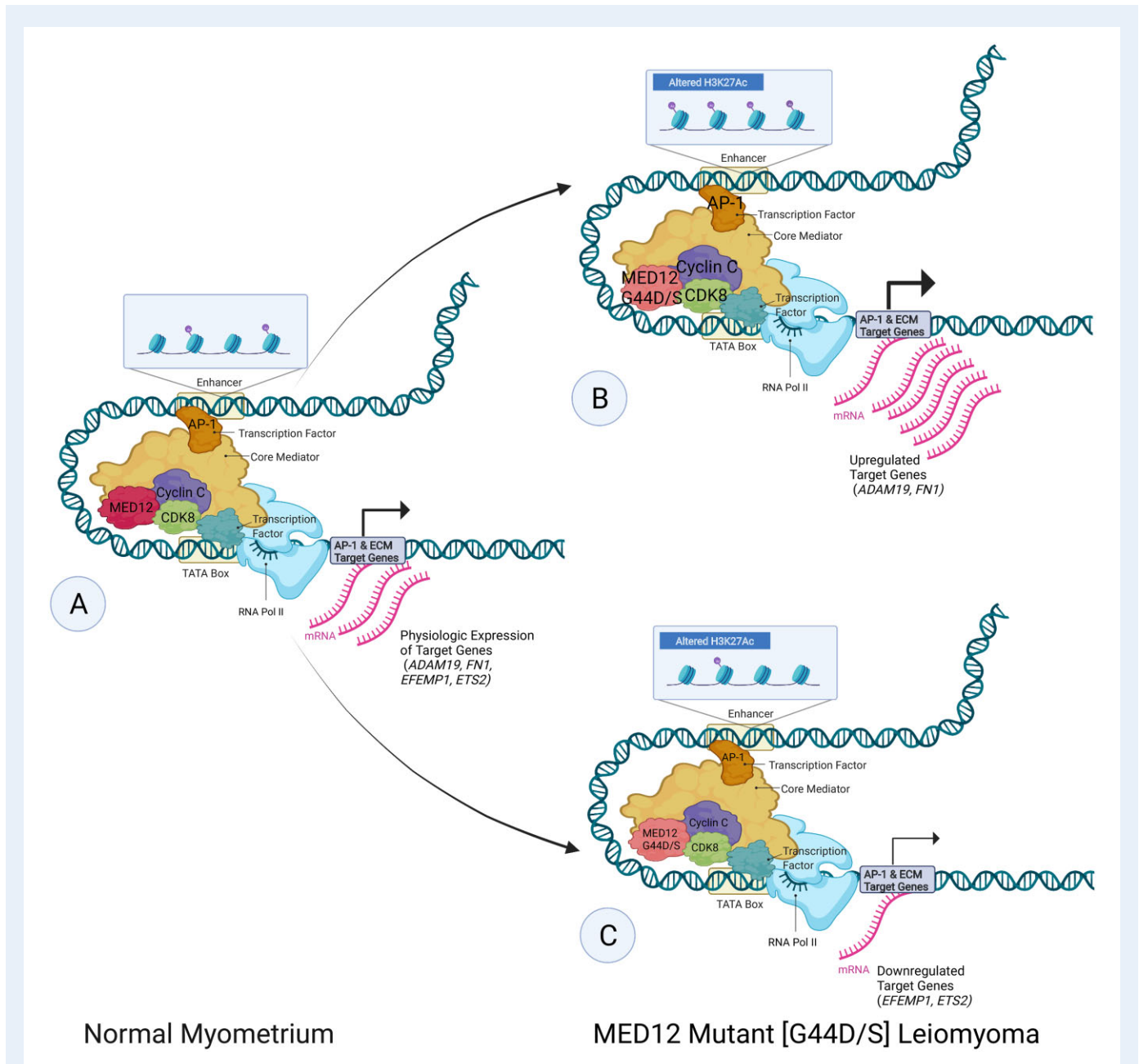
**Figure 2. Altered H3K27Ac and enhancer architecture in *MED12* mutant [G44D/S] leiomyoma.** Schematic of the proposed (Moyo *et al.*, 2020) differential activator protein 1 [AP-1] and cyclin-dependent kinase 8 [CDK8]/mediator complex subunit 12 [MED12] occupancy, altered enhancer architecture and altered acetylation of the 27th lysine of histone H3 [H3K27Ac] involved in gene dysregulation of AP-1 and extracellular matrix [ECM] target genes in the pathogenesis of G44D/S-mutated *MED12* uterine leiomyoma [LM]. Font size represents the strength of recruitment of factors (MED12, CDK8, Cyclin C, AP-1) and arrow thickness represents transcriptional output. Compared to normal myometrium (**A**), in LM, a subset of genes (*ADAM19* indicates ADAM metallopeptidase domain 19; *FN1*, fibronectin 1) shows higher CDK8/MED12 binding, increased H3K27ac signals found at the enhancer region and enriched AP-1 binding activity. The resulting stronger enhancer–promoter contact found in *MED12* mutant [G44D/S] LM leads to the activation of LM-specific gene program (**B**). Another subset of genes (*EFEMP1* indicates EGF containing fibulin extracellular matrix protein 1; *ETS2*, ETS proto-oncogene 2, transcription factor) in LM shows lower CDK8/MED12 binding, decreased H3K27ac signals found at the enhancer region and depleted AP-1 binding activity. The resulting weaker enhancer–promoter contact found in *MED12* mutant [G44D/S] LM leads to the repression of LM-specific gene program (**C**). Adapted from 'Eukaryotic Gene Regulation—Transcriptional Initiation', by BioRender.com (2020). Retrieved from https://app.biorender.com/biorender-templates

occur at distal enhancers (Leistico et al., 2021). It is similar to the observation in MED12mt LM published previously in a separate report (discussed below) (Moyo et al., 2020). The enhancer sites with increased activity in LM were located further away from promoters than those with decreased activity. Overall, these observations advocate the role of long-range interactions in the pathogenesis of LM. Leistico et al. (2021) further found that the alterations in histone modifications were mainly confined within the chromatin contact domains (annotated in HeLa cells). These contact domains may perhaps restrict alterations in the epigenome as the tumors evolve. The genes that were upregulated and within 10 kb of the sites that gained histone modification signals were enriched with gene ontology [GO] terms related to collagen and ECM, in accordance with the LM etiology. Integration of assay for transposase-accessible chromatin using sequencing [ATAC-seq] peaks with DeCET regions showed enrichment of HOXA (most notably HOXA9 and HOXA10) and serum response factor motifs in the diseased state. At the same time, compared to the diseased state, the normal condition had more nuclear receptor subfamily 3 group C member 1 [NR3C1] and E26 transformation-specific [ETS] family TF binding sites. Consistent with ATAC data, interestingly Yin et al. (2013) found that NR3C1, also known as glucocorticoid receptor, was a key downregulated nuclear receptor in LM. Furthermore, the posterior HOXA cluster showed transcriptional as well as epigenomic changes, especially in HOXA13 (Leistico et al., 2021). HOX proteins play an important role in reproductive system development and function, and are dysregulated in various tumors (Cillo et al., 2001; Daftary and Taylor, 2006). To explore the role of HOXA13 in the context of LM, Leistico et al. (2021) knocked down and overexpressed HOXA13 in primary LM and myometrial cells, respectively. The key GO terms related to genes modulated when HOXA13 expression was modified were related to ECM. As stated earlier, different arms of epigenomic regulation (i.e. DNA methylation and histone modifications) can converge, as seen in the case of HOXA13, to regulate gene expression in LM pathology (George et al., 2019; Leistico et al., 2021). Previous publications suggest that DNA methylation brings about chromatin changes by altering histone modifications (Kondo, 2009). Further knowledge of the interaction between these two mechanisms in this disease may offer better integrative mechanistic details.

## AP-1-driven aberrant enhancer regulation in uterine leiomyoma

Mutations in MED12 have been observed in roughly 70% of patients diagnosed with LM, with the most prevalent occurring in exon 2 of the gene (Makinen et al., 2011). MED12 is a subunit of the mediator of the RNA polymerase II transcription complex and is normally involved in regulating RNA polymerase II-dependent transcription (Baranov et al., 2019).

To identify genome-wide epigenomic changes in MED12mt LM, Moyo et al. (2020) pursued NGS on frozen tissues. To keep variables to a minimum for this study, only cases with missense mutations of glycine, at the 44th codon in exon 2 of MED12, to either aspartate [G44D] or serine residue [G44S] were followed-up. The transcriptomic data revealed a clear distinction between G44D/S LM and adjacent matched myometrium (Moyo et al., 2020). Notably, H3K27ac ChIP-seq yielded features that could distinguish the diseased state from normal. As observed above, most of the differential H3K27ac signal was located distal to promoters in intergenic and intronic regions, suggesting the role of enhancers. Moreover, ∼46% of the deregulated genes did not show a significant change in H3K27ac at their promoters, which included genes such as fibronectin 1 [FN1], a disintegrin, ADAM metallopeptidase domain 19 [ADAM19] and collagen Type XII alpha 1 chain [COL12A1]. In order to define the relation between distal H3K27ac signal and transcriptomic changes, Moyo et al. (2020) performed promoter capture Hi-C (high throughout chromosome conformation capture). While capture Hi-C identifies all genomic interactions, promoter-capture HiC is more specific to determining altered enhancer interactions within promoters, thereby analyzing 'productive interactions'. About 1835 of 2715 differentially acetylated H3K27 enhancer regions, associated with differentially expressed genes, had an insignificant level of change in H3K27 acetylation in their corresponding promoters. Enhancer regions with altered contacts which showed increased acetylation status were positively correlated with increased promoter contact strength, whereas depleted acetylation had varying contact strength. Some genes involved in ECM formation that demonstrated differential gene expression related to changes in promoter contact strength include FN1, ADAM19, ETS proto-oncogene 2 transcription factor [ETS2], EFEMP1, COL6A3 and COL12A1. Furthermore, capture Hi-C showed that in LM, 672 genes displayed differential enhancer usage, wherein decreased H3K27ac levels in one enhancer for a specific gene occurred simultaneously with an increase in H3K27ac levels in another enhancer associated with the same gene (Moyo et al., 2020). A possible implication of this may allow for the alteration of strong enhancers with weak ones, and vice versa, resulting in changes in gene expression.

The mediator complex, comprising a core mediator and a CDK8 submodule, forms a stable complex that regulates transcription. Specifically, MED12 is one of the subunits of the CDK8 submodule that consists of CDK8, MED12, MED13 and cyclin C (Allen and Taatjes, 2015; Soutourina, 2018). A previous study proposed that one possible mechanism leading to the dysregulation of transcription in LM by mutant MED12 is the decreased binding affinity of cyclin C and CDK8 at active transcription sites (Turunen et al., 2014). However, Moyo et al. (2020) demonstrated that it is not a global loss of cyclin C/CDK8 binding but rather altered CDK8/MED12 binding that leads to LM-specific enhancer features. This postulation was complemented by the positive correlation observed between altered H3K27ac and changes in the CDK8 submodule chromatin occupancy in the enhancer region (Moyo et al., 2020). This modified enhancer architecture, caused by changes in chromatin from histone acetylation and altered enhancer–promoter contact, provides a potential mechanism for the gene dysregulation that occurs outside the promoter-proximal area in MED12mt LM. Moreover, understanding how the interaction of mutated MED12 with other members of the CDK8 submodule brings about changes in their DNA-binding, as observed in LM, may ultimately provide a mechanism for initiation of this disease. Again, while compelling correlative data emerges linking epigenomic changes to LM gene expression and possibly disease development and progression, in a majority of the cases, causal experiments are lacking. With the use of CRISPR-based gene editing, correlative altered enhancer and epigenome function in LM gene regulation can now be causally studied.

Additionally, it was observed that the motif of TF AP-1 was enriched at differentially acetylated enhancers (Moyo et al., 2020). The AP-1 term is used for dimeric TFs composed of subunits of JUN, FOS, activating

transcription factor or musculoaponeurotic fibrosarcoma that recognize a common motif (Bejjani *et al.*, 2019). ChIP-seq analysis of FOS and JUN uncovered a reduction in AP-1 occupancy in chromatin in LM. While it has already been reported that LM show down-regulation of *JUN* and *FOS* mRNA, the Moyo *et al.* (2020) study demonstrated that a loss of *JUN* or *FOS* gene expression led to an accompanying decrease in the binding of AP-1 at enhancer sites that contained reduced H3K27ac and therefore resulted in the dysregulation of previously identified AP-1 target genes and ECM-associated genes, among others (Lessl *et al.*, 1997; Skubitz and Skubitz, 2003; Moyo *et al.*, 2020). Strikingly, sites where AP-1 gained or lost occupancy in LM were also bound by CDK8 and MED12, with changes in their occupancy in the same direction. The simultaneous CRISPR/Cas-9-mediated depletion of JUN and FOS proteins in primary human uterine smooth muscle cells [HUtSMC] revealed differential expression of 1894 genes compared to the negative control, including those involved in ECM organization. Furthermore, changes in H3K27 acetylation patterns in AP-1-depleted cells were similar to those observed in LM, with the majority of changes occurring at promoter-distal regions (Moyo *et al.*, 2020). This resemblance in both gene expression and H3K27ac levels between LM and the AP-1-depleted cells implies that the loss of AP-1 gene expression is linked to the aberrant epigenome and dysregulation of ECM genes observed in LM. Overall, the findings propose that aberrant enhancer regulation in LM related to differential AP-1 and CDK8/MED12 occupancy, altered enhancer–promoter contact and altered H3K27 acetylation are important mechanisms in LM pathogenesis (Fig. 2). The subunits of AP-1 did not show differential H3K27ac signal in their promoters in the diseased state; differential enhancer usage was proposed to be a mechanism leading to decreased expression of these genes (Moyo *et al.*, 2020). Identification of the factors that cause the use of alternate enhancers may ultimately be beneficial in understanding the development of this disease.

Overall, it is becoming clear that enhancer dysfunction seems to be an important phenomenon in the etiology of these uterine benign tumors. Of note is that the genome-wide DNA methylation (George *et al.*, 2019), histone modifications (Leistico *et al.*, 2021) and promoter capture Hi-C (Moyo *et al.*, 2020) studies were performed in tissues, thereby enhancing the physiological relevance of these findings. Therefore, closely mapping the genes that are deregulated in LM, their distal regulatory regions and in turn regulation of those regions may offer potential therapeutic targets. More studies dissecting primary events from secondary effects may be helpful in identifying driver events leading to LM formation. Furthermore, loci-specific DNA methylation or histone modifications could provide information about deregulated genes; however, genome-wide studies give a better picture of the chromatin architecture and regulation by enhancers. Compounds currently under clinical trials for other diseases targeting the epigenome and chromatin-modifying enzymes may be proven useful in non-surgical therapeutic intervention in LM.

# Histone variants, chromatin remodelers and a new subtype of uterine leiomyoma

While altered DNA methylation and histone modifications have now been linked to LM, the role of histone variants and chromatin

remodelers is not well established. Additionally, the previously known exclusive mutations in LM do not cover genetic mutational status of all LMs, thereby necessitating more extensive studies using a larger number of human samples. A tour de force comprehensive study, using a large cohort of patient samples and state of the art genomic, epigenomic, chromatin-state and molecular analyses, now sheds new light on LM genesis, identification of the potential role of the SRCAP chromatin complex and the role of histone variant H2A.Z in LM (Berta *et al.*, 2021). This comprehensive study on 2263 fibroids from 728 women helped identify a new molecular subtype of LM, thereby advancing our knowledge of additional exclusive mutations in genes defining LM subtypes (Berta *et al.*, 2021). This new subgroup of patient samples, which represented ∼1.8% of the LM cases analyzed, harbored mutations in six out of nine genes encoding the chromatin remodeler SRCAP complex subunits, with YEATS domain containing 4 [*YEATS4*] being most frequently mutated. Epigenomic silencing of the normal allele by DNA methylation of *YEATS4* was also observed in tumors carrying heterozygous *YEATS4* mutations. As validation for their finding, the authors found that protein truncating germline mutations in *YEATS4* and another gene of this complex, zinc finger HIT-type containing 1 [*ZNHIT1*], are risk factors for the development of LM (Berta *et al.*, 2021). SRCAP is a chromatin remodeler complex that carries out histone, specifically H2A.Z, exchange/deposition (Giaimo *et al.*, 2019). H2A.Z is found at the nucleosomes flanking the promoter region, centromeres and boundaries of chromatin (Gerhold and Gasser, 2014). The functional roles of H2A.Z include DNA repair, maintaining genome integrity and controlling gene transcription (Giaimo *et al.*, 2019).

To determine if SRCAP alteration affected protein levels of H2A.Z, Berta *et al.* (2021) performed immunohistochemistry and found that SRCAP-altered LM had very weak to no staining, suggesting that the level of H2A.Z is reduced in this subtype. Myometrium, *MED12mt* LM and LM with loss of *FH* had the strongest expression, while *HMGA2* overexpressing LM had moderate expression. Moving forward, the ChIP-seq of H2A.Z revealed significant loss of H2A.Z binding not only in SRCAP-altered LM, as expected, but also surprisingly in *MED12mt* LM that displayed strong H2A.Z staining. These results suggest that additional regulatory mechanisms may be involved in H2A.Z deposition in *MED12mt* LMs. These results also suggest that dysregulated H2A.Z deposition may be a common phenomenon in all LM subtypes, which may also involve different mechanisms based on the disease subtypes. Considering that histone/nucleosome occupancy dictates open/closed chromatin states and dysregulated chromatin occupancy of H2A.Z is observed in ChIP-seq analysis, the authors performed ATAC-seq to identify open/closed chromatin states in a genome-wide manner. Their ATAC-seq analysis showed that *YEATS4*-mutated LM have a more open chromatin structure at the TSS of active or bivalent genes and these regions showed reduced H2A.Z binding (Berta *et al.*, 2021). This finding is consistent with previous observations made in other model systems (Giaimo *et al.*, 2019). Of note, bivalent genes house both activating (H3K4me3 or H3K4me1) and repressing (H3K27me3) histone marks within promoter or enhancer regions. These associated genes are expressed at low levels but poised for rapid gene activation following cellular cues (Bernhart *et al.*, 2016).

The authors then addressed the key question regarding dysregulated H2A.Z deposition and gene expression changes associated with SRCAP-altered LMs. Using RNA sequencing analysis, the authors

found that *YEATS4*-mutated LM displays upregulation of differentiation genes driven by H2A.Z sensitive bivalent promoters. These results suggest that, in SRCAP-altered LM, these gene sets are primed to be regulated based on their promoter bivalency and that rapid dysregulated differentiation may be a contributing factor in LM genesis. The authors then examined expression of genes whose promoters showed differential H2A.Z occupancy in all disease subtypes. Interestingly, the results show that for *HMGA2*, *HMGA1* and *FH* LM subtypes, decreased H2A.Z binding correlated with downregulation, while increased H2A.Z promoter occupancy correlated with upregulation of target gene expression. However, in *YEATS4*- and *MED12mt* LM subtypes, this direct correlation between H2A.Z and gene expression was not apparent. For example, the authors found that in *YEATS4*-mutated LM with H2A.Z lost in TSS, 204 genes were overexpressed while 296 genes were under expressed. GO of overexpressed genes identified morphogenetic pathways while under expressed genes failed to identify any specific biological pathways. These results suggest that the gene regulation linking H2A.Z deposition and SRCAP complex mutation is complicated and may also involve non-promoter bound H2A.Z-mediated regulation as well as H2A.Z deposition-independent functions of SRCAP complex mutations in this LM subtype. Consistent with this thought, the authors found, using H3K27ac-HiChIP-based chromatin state capture assays, that decreased H2A.Z binding outside of TSS (promoter distal/potential enhancer sites) correlates with decreased gene expression of these enhancer connected promoters (Berta *et al.*, 2021).

Additionally, Berta *et al.* (2021) found upregulation in components of canonical PRC-1 [cPRC1], which were Chromobox 2, 4 and 8 [*CBX2*, *4* and *8*]. Consistent with the findings presented in Berta *et al.*, two independent studies also found these genes to be upregulated in LMs (Mehine *et al.*, 2016; Berta *et al.*, 2021; Leistico *et al.*, 2021). cPRC1 is involved in development by bringing about epigenetic silencing of genes (Geng and Gao, 2020). Furthermore, genes coding for TFs involved in development (*HOXA13* and *SATB2*) along with steroid 5 alpha-reductase 2 [*SRD5A2*] and hydroxysteroid 17-beta dehydrogenase 6 [*HSD17B6*], genes that encode dihydrotestosterone synthesis, were upregulated in all subtypes of LM (Berta *et al.*, 2021). Leistico *et al.* (2021) had a similar observation in their RNA-seq screen (Leistico *et al.*, 2021). Again, global gene expression analysis coupled with genome-wide DNA methylation and histone modification studies identify developmental TF HOXA13 as a key dysregulated factor in LM (George *et al.*, 2019; Berta *et al.*, 2021; Leistico *et al.*, 2021). These independent studies emphasize the robustness and usefulness of genome-wide studies using tissue preparations in molecularly characterizing LMs (George *et al.*, 2019; Berta *et al.*, 2021; Leistico *et al.*, 2021).

Although researchers in the Berta *et al.* (2021) study have used many of the latest multi-omics techniques to characterize the molecular landscape of LM and found a new subtype of LM (Berta *et al.*, 2021), there are still a few questions that can open up new research areas in the future. *MED12mt* LM show high protein expression of H2A.Z but have reduced chromatin binding. What causes this alteration needs to be understood. H2A.Z occupancy in LM showed an inverse relationship with DNA methylation at binding sites and a positive correlation with chromatin accessibility; however, these associations were weak in the tumors bearing SRCAP complex mutations (Berta *et al.*, 2021). It remains elusive as to how changes in H2A.Z

binding cause formation of LM in SRCAP tumors. Berta *et al.* (2021) independently reported how the epigenetic landscape of LM changes upon H2A.Z loading deficiency, and how this epigenetic instability may be involved in LM genesis (Berta *et al.*, 2021). Similarly, altered chromatin binding and resulting changes in 3D-chromatin structure have been observed by other groups (George *et al.*, 2019; Moyo *et al.*, 2020; Leistico *et al.*, 2021). Collectively, these independent studies highlight the possibility of altered enhancer architecture being, at least partially, responsible for the pathogenesis of this disease, but causal and functional studies are largely lacking and need to be performed to move the field forward. However, it is important to note that the proneness to LM development in women together with germline mutations within SRCAP complex genes may unveil clinical implications that are of potential use therapeutically and in genetic counseling settings when advising women who are affected by LM or at risk of LM development (Ordulu, 2021).

# Epigenomics in other fibrotic diseases

## DNA methylation

Like LM, many loci-specific DNA methylation events have been identified in various other fibrotic diseases. Genes that are methylated and downregulated include RAS protein activator like 1 [*RASAL1*] (tumor suppressor gene found in kidney fibrosis), peroxisome proliferator activated receptor gamma [*PPARG* or *PPARγ*] (antifibrotic gene in liver fibrosis), Fli-1 proto-oncogene, ETS TF [*FLI1*] [suppressor of collagen expression, systemic sclerosis] and Thy-1 cell surface antigen [*THY1*] (TGFβ inhibitor, idiopathic pulmonary fibrosis [IPF]), among others (Yao and Li, 2015; O'Reilly, 2017). Hypoxia in cardiac fibrosis has been understood as one of the major drivers for increased global methylation by deregulating DNMT1 and DNMT3b. The pro-fibrotic effects of hypoxia could be counteracted by a general DNMT inhibitor, 5-aza-dC. In addition, treatment with 5-azacytidine, another DNMT inhibitor and ribose analog of 5-aza-dC, attenuates folic acid-induced renal fibrosis (Yao and Li, 2015). Thus, it seems that modulators of DNA methylation can provide relief in some fibrotic diseases. However, specific DNMT inhibitors may be more beneficial by reducing the potential side effects of global DNA methylation inhibitors. In the liver, MeCP2 is considered an important regulator of fibrosis. MeCP2 binds to methylated DNA and, by interacting with HDACs, causes gene repression. MeCP2 is found to regulate *PPARγ* expression in liver fibrosis, thus leading to increased pro-fibrotic molecules in this disease. It also decreases the expression of RASAL1 in kidney fibrosis and thereby increases Ras-GTP signaling. *RASAL1* is hypermethylated in fibrotic hearts. This hypermethylation could be reversed not only by non-specific broad-spectrum demethylating agents but also, interestingly, by an endogenous antifibrotic agent, BMP7 (O'Reilly, 2017). This treatment concept may be explored even in LM to target specific DNA methylation loci to minimize side effects. As we now know from LM, enhancers play an important role in the manifestation of this disease. Potentially, similar phenomena also occur in other fibrotic diseases and, thus, more comprehensive studies should be performed exploring enhancer malfunction in these and other fibrotic diseases.

## Histone modifications

General HDAC inhibitors [HDACi] have been associated with decreasing fibrotic burden in various tissues through distinct signaling pathways, for example, in renal fibrosis via TGFβ and EGF receptor signaling, in bleomycin-induced lung fibrosis by apoptosis, and in liver fibrosis by inhibiting TGFβ and vascular endothelial growth factor pathway (Yao and Li, 2015; O'Reilly, 2017). The success of HDACi may be attributed to their dysregulated HDAC activity in fibrosis, but it seems to be highly dependent on tissue type. To develop successful treatment, the specific HDAC enzymes deregulated in each condition should be determined and treated appropriately. Not only HDACs but also HATs can also contribute to fibrosis; for example, EP300 is increased in systemic sclerosis and is regulated by TGFβ1 (O'Reilly, 2017). Therefore, a good understanding of which HDACs and HATs are aberrantly expressed during fibrosis is required for specific treatment with minimum toxic effects. In addition to acetylation, histone methylation is an important modification in regulating pro-fibrotic gene expression. Several loci-specific occurrences have been reported by different groups. These include increased H3K9me2 near the *PPARγ* promoter in liver fibrosis that leads to reduced PPARγ expression; lung fibroblasts from IPF patients show reduced H3K9me3, H3K27me3 and DNA methylation at the prostaglandin-endoperoxide synthase 2 (also referred to as *COX2*) promoter (Yao and Li, 2015; O'Reilly, 2017). In liver fibrosis, Jumonji domain containing 1A (an H3K9 demethylase) is involved in regulating fibrosis by regulating *PPARγ*; in systemic sclerosis, treatment with DZNep (a methyltransferase EZH2 inhibitor) causes more ECM formation by upregulation of Fra2 (AP-1 family member) and increases fibrotic phenotype (O'Reilly, 2017). At the *COX2* promoter in lung fibroblasts, the reduced accumulation of histone methyltransferases G9a, EZH2 and DNMTs as well as binding protein heterochromatin protein 1, Polycomb protein complex 1 and MeCP2 have been implicated in alteration of histone modifications at this site. EZH2 and ASH1 (an H3K4 methyltransferase) have also been implicated in liver fibrosis. ASH1 seems to be regulated by MeCP2, thus inducing pro-fibrotic genes (O'Reilly, 2017). This and other examples, as discussed above, link DNA methylation with histone modifications via MeCP2. The role of MeCP2 has not yet been established in LM; it may provide a good opportunity to scrutinize these two important arms of epigenetic modifications in this disease. Also, the above histone modifications studied in various fibrotic diseases suggest a diverse string of events, all leading to the fibrotic state. Thus, it is important to perform genome-wide histone modification studies to identify common and tissue-specific epigenomic events in various tissue fibrosis pathologies. In such a study, the tensor decomposition method employed for LM may be beneficial to link specific epigenome alterations of the disease.

## Racial disparities of uterine leiomyoma

LM has been found to disproportionately affect Black women, with the prevalence being two to three times greater as compared to White women (Catherino *et al.*, 2013; Eltoukhi *et al.*, 2014). Black women are particularly affected with an increased incidence, earlier age of onset, larger and faster growing fibroids and greater severity of symptoms (Marshall *et al.*, 1997; Moorman *et al.*, 2013; Bray *et al.*, 2017).

Furthermore, an online study surveying Black and White women was conducted to evaluate symptoms and impairments, concerns regarding treatment and the effect on employment and social life. It reported that Black women suffered from more severe symptoms and anemia; were more likely to miss work owing to symptoms; expressed more interference in daily and social activities; had a higher degree of impaired relationships with family, friends and significant others; and were significantly more concerned with the effects of treatment relating to fertility and a successful pregnancy. It is also noteworthy that although Black women expressed higher interest than White women in uterine-sparing procedures (Stewart *et al.*, 2013), they are still 2.4 times more likely to undergo hysterectomy, possibly owing to the increased severity (Wechter *et al.*, 2011; Richard-Davis, 2013). Overall, the increased incidence and severity of the disease in Black women has attracted many to identify the influence that ethnicity has in LM development and growth.

There are a wide range of risk factors and exposures that are posited that place Black women at an increased risk for fibroids over White women. The field of epigenomics includes gene–environment interactions that can potentially include social environments. Specifically, the idea behind social and behavioral epigenomics is that socio-cultural factors can generate trauma-associated epigenetic changes, which impact the expression of certain genes (Mulligan, 2021). It is well known that Black women experience racism that promulgates negative social and economic conditions (the social and economic determinants of health), which in turn negatively impact health and thus promote health inequities. The experience of racism and the social determinants of health and their influences on LM have been studied (Zota and VanNoy, 2021). For example, one study observed the association between increased major life discrimination events and altered DNA methylation at nine CpG sites within disease-associated genes (Barcelona de Mendoza *et al.*, 2018). Additionally, the disproportionate suffering of Black women with LM might arise from other environmental factors that induce chronic stress, such as the experiences of adverse childhood experiences [ACEs] and economic hardships, both of which are known to contribute to negative health outcomes (Hughes *et al.*, 2017; Aroke *et al.*, 2019). It has already been shown that exposure to early childhood abuse is associated with the incidence of clinically detectable LM (Boynton-Jarrett *et al.*, 2011). Not only do Black children experience a higher quantity of ACE compared to children from other races, but they are also more likely to grow up in lower socio-economic status [SES] neighborhoods (Aroke *et al.*, 2019). Needham *et al.* (2015) found that low childhood SES was associated with changes in global DNA methylation, leading to the differential expression of stress- and inflammation-related genes (Needham *et al.*, 2015). While studying the relation between psychological stress and LM, a study found that chronic stress, which Black women were determined to be more exposed to, correlates with increased hypothalamic–pituitary axis activity. This eventually leads to the increased release of sex steroid hormones that drive LM development (Qin *et al.*, 2019). Interestingly, it has also been reported that upwards mobility in Black women results in a significantly increased likelihood of racial discrimination (Colen *et al.*, 2018). While upwards mobility is thought to serve a health protective function in White women, it is thought that these same benefits are likely suppressed in racial minorities owing to the continuance of race-based unfair treatment (Colen *et al.*, 2018). As such, this suggests that the

disparity faced by racially oppressed Black women can possibly be related to socio-cultural factors, such as segregation, systemic racism, discrimination, economic hardship and other stressors, which influence the expression of LM-specific genes through imposing changes on the epigenome.

Abnormalities in vitamin D levels have already been observed in various estrogen-dependent gynecological disease pathologies, such as in endometriosis, polycystic ovary syndrome and ovarian cancer (Colonese et al., 2015). Vitamin D may be obtained through the diet, but the majority of natural vitamin D is produced by the conversion of 7-dehydrocholesterol to vitamin D3 in the skin by means of sun exposure. To form the biologically active metabolite, vitamin D3 is first hydroxylated in the liver to produce 25-hydroxyvitamin D3 (calcidiol), the major form of circulating vitamin D, which is then further hydroxylated in the kidney and other extra-renal tissues to form the active metabolite 1, 25 dihydroxyvitamin D3 (calcitriol) (Bikle, 2014). Vitamin D deficiency, or hypovitaminosis D, has also been identified as a potential LM risk factor with several studies finding an association between low serum levels of vitamin D3 and increased incidence of LM (Baird et al., 2013; Sabry et al., 2013; Ciebiera et al., 2016; Halder and Al-Hendy, 2016; Ciebiera et al., 2020; Mohammadi et al., 2020). Furthermore, Othman et al. (2018) reported that the tissue concentration of calcitriol was lower in LM compared to its adjacent myometrium; however, they did not find a significant correlation between the level of calcitriol within LM and tumor volume (Othman et al., 2018). Physiologically, vitamin D is known to play a role in regulating calcium and phosphorus metabolism, but in vitro studies of LM have suggested its role in inducing apoptosis, suppressing the action of matrix metalloproteinases, and downregulating ER and PR levels (Sharan et al., 2011; Halder et al., 2013; Al-Hendy et al., 2015). Calcitriol has also been implicated in serving to inhibit cell proliferation and deposition of ECM by reducing TGF-β3 effects in human LM cell lines (Halder et al., 2011). The authors also found that women undergoing hysterectomy for symptomatic LM had significantly higher expression of CYP24A1, a gene encoding 24-hydroxylase enzyme, compared to normal myometrium from women undergoing the same procedure for non-LM indications. Since this enzyme catalyzes the degradation of calcidiol and calcitriol, the authors indicated that the overexpression of 24-hydroxylase may be implicated in the mechanism that sustains a hypovitaminosis D state in LM (Othman et al., 2018). Interestingly, vitamin D insufficiency is roughly 10-fold more prevalent among Black women compared with White women, likely because of their darker skin pigmentation, which reduces vitamin D production from sunlight exposure (Nesby-O'Dell et al., 2002; Harris, 2006). This predisposition can provide a partial explanation for the ethnic disparity observed with regards to LM. One recent study also proposed that low vitamin D receptor [VDR] expression may be associated with the development and growth of LM (Lima et al., 2021). In this recent study, 67% of the evaluated women were of Black descent, thus it would be interesting to examine if the expression of VDR varies among races. On the contrary, in an expression profiling study of 48 nuclear receptors from 22 patients that included 36% Black women, Yin et al. (2013) reported that VDR was a hyperexpressed receptor in LM when compared with myometrium (Yin et al., 2013). Similarly, Leistico et al. (2021) found increased expression of VDR in their RNA-seq analysis from LM compared to matched myometrium that included 30% Black women. This discrepancy may be explained by the analysis of the different molecular entities that are protein (Lima et al., 2021) and RNA (Yin et al., 2013; Leistico et al., 2021). On the other hand, it may not be wrong to assume that the inconsistencies are race-related as there is differential representation of Black women in the studies. Nevertheless, many studies have further found that Vitamin D supplementation is a potentially effective, non-surgical treatment in reducing LM-related symptoms and the volume of LM (Hajhashemi et al., 2019; Davari Tanha et al., 2021; Halder et al., 2012; Suneja et al., 2021). In one such preliminary study which aimed to identify the role of vitamin D supplementation in women with concurrent hypovitaminosis D and LM, it was demonstrated that supplementation therapy with calcidiol restored normal vitamin D serum levels and stabilized the growth of LM, preventing progression of the disease and thus reducing the need for traditional surgical or other management options (Table III) (Ciavattini et al., 2016). As such, the antifibrotic effects of vitamin D should be further investigated owing to the potential use as a novel therapeutic and an explanation for the ethnic disparity seen within this disease. Similarly, studies employing 24-hydroxylase inhibitors or 24-hydroxylase-resistant calcidiol analogs may be of use to better understand the effect of augmenting vitamin D levels on LM incidence and as possible therapeutics (Othman et al., 2018). Furthermore, clinician and public health educator interventions, such as employing 25-hydroxy vitamin D tests and supplementation to those with LM or at risk of developing the disease, can lessen the burden of the disease and LM growth.

Pan et al. (2007) performed proteomic and genomic analysis on samples from Black and White women, which revealed a similar molecular environment in the two groups. Still, there was a difference in the expression level of genes and proteins involved in regulating the inflammatory response, apoptosis, cell cycle and ECM turnover between the two cohorts, potentially giving rise to the difference in LM incidence between the two ethnic groups (Pan et al., 2007).

In an effort to identify risk alleles that differ in frequency among Black and White women, Wise et al. (2012) performed an admixture-based genome-wide scan. However, they were unable to find a specific highly differentiated locus that is responsible for the increased risk of LM in Black women (Wise et al., 2012). In a more recent study, which conducted a multi-stage genome-wide association study [GWAS] in Black women, Hellwege et al. (2017) identified several single nucleotide polymorphisms [SNPs] near cytohesin 4 [CYTH4], ER degradation enhancing alpha-mannosidase like protein 1 [EDEM1], junctophilin 1 [JPH1] and CUB and sushi multiple domains 1 [CSMD1] genes as potential loci to be associated with LM risk in Black women (Hellwege et al., 2017). However, another study was not able to validate these findings (Bray et al., 2018). In another GWAS, Valimaki et al. (2018) reported 22 genome-wide susceptibility loci among genes involved in genitourinary development and genetic stability, which when compiled into a polygenic risk score for LM association, revealed that Black women entailed the highest population-specific risk score compared to 'Other white background', 'Indian', 'Irish' and 'Northern Finland Birth Cohort' ethnicities (Valimaki et al., 2018). Various other factors, such as SNPs within genes involved in estrogen synthesis, genes related to the retinoic-acid pathway and aberrant miRNA expression levels, have since been identified as potential molecular mechanisms explaining the difference in LM development among ethnic groups but have had conflicting results when reproduced (Commandeur et al., 2015). For example, Wang et al. (2007) performed a global miRNA profile analysis and found that a subset of

**Table III** Current medical, radiological and surgical management options for uterine leiomyoma.

| | Name | Function | Limitations | Source |
|---|---|---|---|---|
| Medical management | Non-steroidal anti-inflammatory agents [NSAIDs] | Improves dysmenorrhea and menorrhagia; works by inhibiting cyclooxygenase enzyme involved in the inflammatory process | May cause gastrointestinal [GI] bleeding and ulcers | (Vane and Botting, 1998) |
| | Selective progesterone receptor modulators [SPRMs] | Reduces bleeding, pain, fibroid volume; not associated with the adverse symptoms of a hypoestrogenic state; works by inhibiting cell proliferation and inducing apoptosis | May cause liver damage; may cause progesterone receptor modulator-associated endometrial changes | (Donnez and Dolmans, 2016) |
| | Gonadotropin releasing hormone [GnRH] agonist | Reduces heavy menstrual bleeding, uterine volume and fibroid volume; works by desensitizing the GnRH receptor producing a hypogonadotropic state and thereby reducing secretion of estradiol and progesterone | Short-term use may cause adverse symptoms associated with a hypoestrogenic state; long-term use may result in reduced bone mineral density and cause histological changes in uterine leiomyoma | (Lethaby *et al.*, 2001; Donnez and Dolmans, 2016) |
| | GnRH antagonist | Provides faster symptom relief than GnRH agonists, reduces tumor volume and uterine size; works by competitively binding to GnRH receptor | Compared to GnRH agonists, GnRH antagonists are more expensive and require daily injections due to their short half life | (Lethaby *et al.*, 2001; Giuliani *et al.*, 2020) |
| | Combined estrogen-progesterone contraceptives | Reduces menorrhagia | Has no effect on decreasing tumor volume | (Marret *et al.*, 2012) |
| | Tranexamic acid | Improves abnormal uterine bleeding and symptoms by acting as an antifibrinolytic agent | May produce GI and musculoskeletal symptoms | (De La Cruz and Buchanan, 2017) |
| | Progestogen-only | Progestogen: a synthetic progesterone hormone that may be administered orally or through injections; reduces menorrhagia<br><br>Progestogen-releasing intrauterine system [IUS]: reduces menorrhagia; the device is placed inside the uterus and works by secreting progestogen to suppress endometrial lining and therefore reduce blood flow | Progestogen-associated histopathological changes may lead to misdiagnosis of leiomyosarcoma or smooth muscle tumor of uncertain malignant potential; risk of IUS expulsion; overall lack of high-quality evidence assessing its efficacy; mixed study results regarding the influence on uterine and tumor volume | (Senol *et al.*, 2015; Sohn *et al.*, 2018; Donnez, 2020; Sangkomkamhang *et al.*, 2020) |
| Interventional Radiology | Uterine Artery Embolization [UAE] | Provides symptom relief and reduces tumor volume; works by limiting blood supply to the tumor and inducing ischemic necrosis | Risk of infection; may compromise blood supply to ovaries or other organs; increased likelihood of reintervention within 2 to 5 years after initial procedure; may impact fertility; post embolization syndrome | (Gupta *et al.*, 2014; Zupi *et al.*, 2016) |
| | Magnetic resonance-guided high-intensity focused ultrasound [MRgHIFU] | Significant symptom relief and tumor shrinkage for at least 12 months; works via using a magnetic resonance imaging [MRI] device to monitor thermal ablation using ultrasonic energy to cause coagulative necrosis within fibroid tissue | May cause abdominal pain, abdominal edema, vaginal discharge, skin burn, and damage to surrounding tissue; inclusion criteria is met by only a portion of patients; can compromise fertility; higher cost due to reinterventions and the need of an MRI device; procedure time is longer compared to USgHIFU | (Gorny *et al.*, 2011; Zupi *et al.*, 2016; Wang *et al.*, 2018) |
| | Ultrasound guided-high intensity focused ultrasound [USgHIFU] | Significant symptom relief and tumor shrinkage; works by using a diagnostic ultrasound device to locate the target region to induce thermal ablation using ultrasonic energy and result in coagulative necrosis within the tissue | May cause vaginal bleeding, increased vaginal discharge, abdominal pain and edema, sciatic nerve damage, sacral injury, skin burns, and small bowel perforations.<br>The lack of real time temperature mapping (which is present in | (Wang *et al.*, 2018; Liu *et al.*, 2021b; Marinova *et al.*, 2021) |

Continued

**Table III** Continued

| | Name | Function | Limitations | Source |
|---|---|---|---|---|
| | | | MRgHIFU) limits feedback to adjust sonication power and energy and may result in incomplete or excessive ablation; risk for local reoccurrence and reintervention; can compromise fertility | |
| | Cryomyolysis | Reduces tumor volume, bleeding, pelvic pain, and urinary frequency; works by limiting primary blood supply to the tumor via cryoablation | May impact fertility; exclusion criteria limits women who can undergo this treatment; lack of histological examination of fibroids | (Zupi et al., 2016) |
| | Radiofrequency thermal ablation (Acessa) | Reduces tumor volume and provides symptom relief; works by using a thin needle to target the tumors via radiofrequency heat, causing coagulative necrosis and reabsorption of the tumor by surrounding myometrium | May result in skin burns; Possibility of reoccurrence and reintervention; limited long term data for pregnancy and pregnancy outcomes | (Lee and Yu, 2016) |
| Surgical Management | Myomectomy | Gold standard for women of reproductive age to preserve fertility and the uterus; surgical excision of fibroid via open (incision in the lower abdomen; used if fibroids are large, numerous, or deeply embedded in the uterus) or through the following minimally invasive approach procedures: Laparoscopic: The surgeon uses instruments and a lighted scope with a camera (laparoscope) to visualize and extract fibroids through small incisions in the pelvic region that provide access to the abdominal cavity Robotic: Surgeon sits at a console and guides robotic instruments to extract the fibroid; similar to laparoscopic approach except this method allows for greater dexterity, precise movements, and the ability to remove fibroids which are less accessible Hysteroscopic: used to treat smaller fibroids that grow in the uterine cavity; the surgeon inserts a small telescope (hysteroscope) into the vagina and cervix to visualize and extract the fibroid | Risk of hemorrhage; possibility of being converted to hysterectomy; risk of uterine rupture; possible scarring which can affect fertility; likelihood of reintervention within 5 to 10 years due to fibroid reoccurrence | (De La Cruz and Buchanan, 2017; Flyckt et al., 2017; Piecak and Milart, 2017) |
| | Hysterectomy | Definitive cure for fibroids; surgical procedure to remove the uterus via open or minimally invasive approach | Fertility is not preserved; vaginal cuff cellulitis; risk of infection; infected pelvic hematoma or abscess; venous thromboembolic complications | (Clarke-Pearson and Geller, 2013; De La Cruz and Buchanan, 2017) |

miRNAs, including miR-23a/b, let-7s, miR-145, miR-197, miR-411 and miR-412, were significantly different in LM of Black women compared to White women (Wang et al., 2007).

As discussed in above sections, recent multi-omics studies such as RNA sequencing (Moyo et al., 2020; Berta et al., 2021; Leistico et al., 2021), histone modifications (Berta et al., 2021; Leistico et al., 2021) and DNA methylation (George et al., 2019; Berta et al., 2021) have found differential features between LM subtypes based on the mutation status. These studies included patients with different racial backgrounds; however, the genomic and epigenomic changes found in LM

do not identify any distinctive signatures that could justify the observed ethnic disparities. As noted by Pan *et al.* (2007), possibly it is the intensity of the response to the mutational driver that leads to greater severity of this condition in Black women compared to their White counterparts (Pan *et al.*, 2007). Overall, it seems that the greater incidence and severity of LM related to the impact of ancestry of African American, African and Black descent remains unclear and may be attributable to a combination of genetic, socio-economic and environmental factors. Furthermore, more intensified studies involving a larger number of patients are clearly needed to identify and confirm the proposed molecular mechanisms and events that may explain the difference in LM incidence and LM size between ethnic groups.

# Translational values and future therapy

The available treatment options for LM form three main categories: medical management, interventional radiology and surgical management (summarized in Table III) (Vilos *et al.*, 2015; Sohn *et al.*, 2018; Giuliani *et al.*, 2020). When it comes to choosing which treatment route to undergo, various factors are considered, including the size and location of LM, the patient's age, the severity of symptoms and the desire to preserve fertility or the uterus (De La Cruz and Buchanan, 2017). Additionally, since the shrinkage of tumors and relief of symptoms is correlated with menopause, asymptomatic women or those approaching menopause may choose expectant management, or clinical surveillance, before discussing further treatment options (Vilos *et al.*, 2015).

Currently, gonadotropin-releasing hormone [GnRH] agonists and selective PR modulators [SPRMs] show the most potential as medical therapies owing to the abundance of evidence supporting their ability to shrink LM and improve symptoms (Sohn *et al.*, 2018; Lethaby *et al.*, 2001). GnRH agonists work by desensitizing GnRH receptors, resulting in a hypogonadotropic hypogonadal state (or pseudo menopause) (Ortmann *et al.*, 2002). Since LMs are steroid-responsive tissues, this hypoestrogenic state is thought to restrict the growth of the tumor (Sohn *et al.*, 2018). Unfortunately, this compound is limited to short-term therapy owing to its association with reducing bone mineral density and imposing histological changes in LM during long-term use (Palomba *et al.*, 1999). Alternatively, SPRMs are synthetic steroids that can act as agonists or antagonists on PR in progesterone-sensitive tissues, such as LM (Wagenfeld *et al.*, 2016). Some of the current SPRMs that have shown to be effective in the treatment of LM include mifepristone and ulipristal acetate [UPA]. Vilaprisan is still being investigated; meanwhile, Asoprisnil and Telapristone have been discontinued because of safety concerns (Islam *et al.*, 2020). Several clinical trials confirmed UPA's efficacy in treating LM in various races, such as the PEARL I–IV studies completed on White women in Europe (Donnez *et al.*, 2012a, 2012b, 2014, 2016), VENUS I and II studies completed on Black women in the USA (Liu *et al.*, 2018; Simon *et al.*, 2018), and a phase III randomized control trial on Japanese women in Asia (Osuga *et al.*, 2021). Although UPA had been approved in Canada and Europe, in March 2020, the Europe Medicines Agency suspended the use of UPA in treating LM until its safety is reevaluated owing to
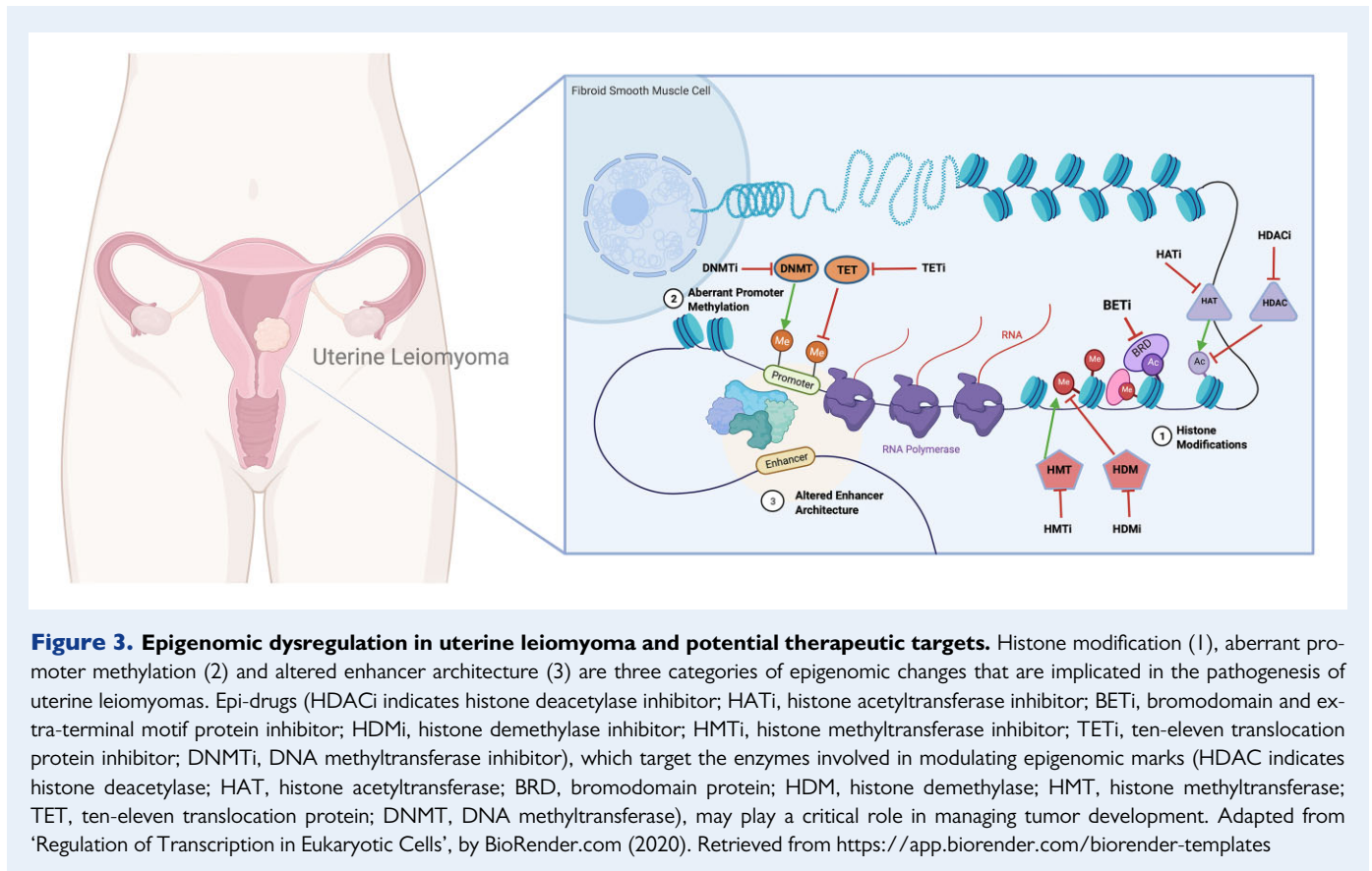
its serious risk of causing liver damage in women taking the drug (Ekanem and Talaulikar, 2021). Critics have suggested that the clinical promise of UPA has been overlooked because of its association with liver injury (Liu, 2021). They have pointed out that the risk of drug-induced liver injury is 10 out of 100 000, while the risk of death as a result of laparoscopic hysterectomy and abdominal hysterectomy of LM in the USA is 12 out of 100 000 and 32 out of 100 000, respectively (Siedhoff *et al.*, 2015; Liu, 2021). Nevertheless, there are ongoing trials that compare UPA with other drugs. The results of these trials will be of great value in determining their clinical relevance by adding to the evidence regarding their safety and undesirable effects.

Despite the possibility of reduced tumor volume and symptomatic relief provided by medical management (Sohn *et al.*, 2018), women who have severe symptoms and pain or suffer from adverse side effects often rely on surgical or radiological interventions. Unfortunately, LM removal [via methods such as minimally invasive techniques or uterine artery embolization] is not always a long-term solution as there is a risk for reoccurrence, which can require future reinterventions (De La Cruz and Buchanan, 2017). Furthermore, since many women are now delaying childbearing past the age of 35 years, uterine-sparing treatment options are highly sought (Balasch and Gratacos, 2012; Sohn *et al.*, 2018). But how can LM be treated without resorting to invasive surgical procedures that have the possibility of affecting a woman's fertility? One possibility revolves around a better understanding of the epigenome. Epigenomic alterations are unlike mutations in the sense of being potentially reversible and having great plasticity (Miranda Furtado *et al.*, 2019). Thus, understanding these events can have substantial significance when it comes to developing future mono or combinatorial non-surgical therapies.

'Epi-drugs', or drugs that target epigenetic marks by acting on enzymes responsible for epigenomic alterations, are already an emerging area of therapy for cancer (Bennett and Licht, 2018). In the context of LM, there are various enzymes that can serve as targets for these epi-drugs. For instance, hypomethylating agents that inhibit DNMT [DNMTi] can reactivate silenced genes such as those which serve tumor suppressor functions. In another example, Carbajo-Garcia *et al.* (2021) performed DNA methylation reversion in human LM primary cells using 5-aza-dC, a DNMTi. They suggested this DNMTi as an effective treatment option owing to its ability to inhibit cell proliferation, ECM formation and the WNT/β-catenin pathway (Carbajo-Garcia *et al.*, 2021).

Alternatively, HAT and HDAC enzymes can serve as targets as well. Consistently, Moyo *et al.* (2020) and Leistico *et al.* (2021) observed extensive alterations in enhancer H3K27 acetylation, a modification catalyzed by EP300 (Moyo *et al.*, 2020; Leistico *et al.*, 2021). The enzyme EP300 serves as a coactivator of gene transcription by directly acetylating promoter-binding histones via its catalytic HAT domain. It is also the key cofactor involved in TGF-β-induced signaling associated with driving fibrogenesis (HWang *et al.*, 2020). The high expression and signaling associated with its transcriptional regulation have been observed in various fibrotic diseases such as liver (Yao *et al.*, 2018) and cardiac (Bugyei-Twum *et al.*, 2014) fibrosis. Therefore, it would be of potential use to generate potent small molecular inhibitors that target EP300's HAT domain in the hope of blocking its catalytic activity.

Furthermore, acetylated lysine residues—such as those carried out by EP300—are recognized as binding sites for bromodomain [BRD] proteins such as BRD2, BRD3 and BRD4. These proteins are

**Figure 3.** **Epigenomic dysregulation in uterine leiomyoma and potential therapeutic targets.** Histone modification (1), aberrant promoter methylation (2) and altered enhancer architecture (3) are three categories of epigenomic changes that are implicated in the pathogenesis of uterine leiomyomas. Epi-drugs (HDACi indicates histone deacetylase inhibitor; HATi, histone acetyltransferase inhibitor; BETi, bromodomain and extra-terminal motif protein inhibitor; HDMi, histone demethylase inhibitor; HMTi, histone methyltransferase inhibitor; TETi, ten-eleven translocation protein inhibitor; DNMTi, DNA methyltransferase inhibitor), which target the enzymes involved in modulating epigenomic marks (HDAC indicates histone deacetylase; HAT, histone acetyltransferase; BRD, bromodomain protein; HDM, histone demethylase; HMT, histone methyltransferase; TET, ten-eleven translocation protein; DNMT, DNA methyltransferase), may play a critical role in managing tumor development. Adapted from 'Regulation of Transcription in Eukaryotic Cells', by BioRender.com (2020). Retrieved from https://app.biorender.com/biorender-templates

members of the bromodomain and extra terminal [BET] family of proteins, which act as epigenomic readers that play a critical role in regulating transcription by recruiting additional TFs and transcriptional machinery (Borck et al., 2020). BET inhibitors have been pursued in cardiovascular clinical trials (Schooling and Zhao, 2019) and clinical development for treating cancer (Pervaiz et al., 2018). For this reason, BET inhibitors may serve as potential antifibrotic agents in the case of already acetylated lysine residues in the context of LM. On another note, enhancers play a key role in regulating the tissue-specific expression patterns of genes, so it is conceivable that changes in enhancer activity result in the enhancer malfunction that constitutes the dysregulation of LM-specific genes. Rai et al. recently reported that the acetyltransferase EP300 inhibitor prevented cardiac fibrosis in a murine model (Rai et al., 2017, 2019). It would be interesting to translate this finding into LM-based animal models to see if there is a similar antifibrotic effect. BRD4 is also found to be enriched in enhancer regions (Lee et al., 2017), so it may be plausible that BET inhibitor-induced repression of BRD4 binding may displace the mediator complex from the enhancer, preventing its interaction with transcriptional machinery (Loven et al., 2013; Shorstova et al., 2021).

On the contrary, several HDACi (vorinostat, romidepsin, belinostat and panobinostat) have also been approved by the US Food and Drug Administration to serve as treatments for various cancers by exerting antitumor effects such as cell cycle arrest, mitotic cell death, inducing apoptosis and autophagy, inhibiting angiogenesis and accumulating reactive oxygen species (Singh et al., 2018). Similarly, in human LM tissue samples, and ht-UtLM and HUtSMC cell lines, treatment using HDACi

(HDACi VIII and apicidin) showed an antiproliferative effect and induced cell cycle arrest and apoptosis (Ali et al., 2020).

Aside from their potential to serve as monotherapies, the combination of various epi-drugs with each other or with other antifibrotic therapies may be advantageous as well. In cancer cells, this combination has been hypothesized to serve as a practical approach toward sensitizing cells to given therapies and reduce the likelihood of acquired resistance to specific treatments (Morel et al., 2020). For example, in EZH2-aberrant solid tumors, BET inhibitors have been shown to re-sensitize cancer cells to EZH2 inhibitors via disrupting H3K27ac-mediated signaling (Huang et al., 2018). Therefore, compounds that target the epigenome, such as bromodomain inhibitors and inhibitors of HATs, HDACs, HMTs, HDMs, DNMTs and TET enzymes, either alone or in combination, may serve as a promising therapeutic strategy and should be investigated in the context of LM (Fig. 3). Nevertheless, the limitations of these proposed therapies, such as adverse systemic effects and limited tolerability, are a significant challenge. Can it be that low doses are sufficient to affect the hypersensitive LM cells without causing harm to distant healthy cells? Can sequential scheduling of combination therapies be more effective than concurrent administration? Will the targeted delivery of these drugs utilizing antibody–drug conjugates or liposomal particles reduce the inadvertent systemic effects (Morel et al., 2020)? These are all questions to take into consideration while seeking to develop novel epigenome-targeting drugs.

With future studies focused on histone and DNA modifying enzymes and on epigenome reader proteins, the innovative treatment options can be utilized in various in vitro assays and translated into

murine/animal-based studies, serving as models for a preliminary screen, to test the efficacy and effectiveness in targeting the epigenome in LM. Additionally, organoid and spheroid tumor models may hold an advantage over traditional cell lines in these pre-clinical assays as they are 3D units with a similar composition to the primary tissue, making them more representative of *in vivo* conditions (Gunti *et al.*, 2021). It is important to note that to aid in the usefulness of these systems for future mechanistic studies, it will be important to do a systemic analysis of the epigenome in established cell lines, primary LM and myometrium cells, spheroids and organoid models, and human tissues.

We also note that with the advent of genomics, and our ability to characterize subtypes of LM, there is great hope to develop disease subtype-specific treatments. Additionally, common dysregulated pathways in all subtypes of LM can also be targeted for future therapeutics. For example, all LM subtypes demonstrate an increase in expression of the cPRC1 that epigenetically silences genes. Therefore, the use of small molecular inhibitors targeting this epigenome regulatory complex may provide therapeutic benefits for treatment of LM. Similarly, inhibiting the enzyme HSD17B6 involved in the biosynthetic pathway for the synthesis of dihydrotestosterone, found overexpressed in all LM subtypes, might be a future therapeutic choice for LM treatment (Berta *et al.*, 2021; Ordulu, 2021). Of course, extensive functional cell- and animal-based studies need to be conducted prior to any potential human clinical trials and therapeutic use of all epigenomic drugs discussed in this review.

Overall, the ability to change the cell landscape can provide a promising non-surgical treatment of LM that affects roughly 70% of all women globally. Along these lines, continued investigation on the epigenomic dysregulation in LM is critically needed to better grasp this still poorly understood disease and for future therapeutic interventions.

# Knowledge gaps, emerging areas and concluding remarks

Aberrant epigenomic alterations are common molecular markers in tumor cells. While the field of epigenomics has advanced in other disorders and tumor-bearing diseases, its role in the context of LM etiology is still evolving. Recent studies discussed in this review have, to some extent, expanded our understanding of the role of epigenomics in LM development. This review largely focuses on how DNA methylation and histone modifications in the promoter or enhancer regions can dysregulate gene expression, which aids in developing the LM phenotype. Aside from pathways revolving around ECM production, genome-wide DNA methylation, histone modification and 3D chromatin analysis are being used to identify other biological pathways, such as those involving the HOX family of TFs or tumor suppressor proteins, that will be used in future studies to better understand this disease.

Within the context of promoter methylation status, the trend observed conveyed that hypermethylation and hypomethylation within the promoter are associated with gene silencing and activation, respectively. Thus, the genome-wide DNA methylation patterns identify LM-specific signatures that can serve as biomarkers for the identification of the disease and clinical outcomes. Furthermore, the utilization of a hierarchical clustering system can possibly provide an opportunity to

reveal information on the subtypes of LM and differentiate them from rare types of uterine smooth muscle tumors [USMT], such as LMS, LM with bizarre nuclei [LM-BN] and smooth muscle tumors of uncertain malignant potential [STUMP]. LMS are malignant USMT with unfavorable 5-year survival rates (Giuntoli *et al.*, 2003). LM-BN, previously named as atypical leiomyoma [ALM], is an intermediate grade USMT with an overall benign clinical course and occasional recurrence (Bell *et al.*, 1994). STUMP are tumors that cannot, with certainty, be histologically determined as benign or malignant (Lu and Chen, 2014; Rizzo *et al.*, 2020). Owing to some overlapping histologic and molecular features between STUMP, ALM and LMS, it is difficult to diagnose and manage these tumors (Bell *et al.*, 1994; Layfield *et al.*, 2000). Therefore, there is a need to identify genomic and/or epigenomic features that may differentiate between these tumor types. In this regard, recently, Gao *et al.* (2021) conducted whole genome copy number analysis by NGS in LMS and LM-BN (Gao *et al.*, 2021). In this study, they applied a GISTIC analysis to identify the high-resolution genomic copy number alteration [CNA] specific to LMS and LM-BN. This study demonstrates both LM-BN and LMS as being genomic unstable tumors and sharing many CNA, including loss of genomic regions of Retinoblastoma [*RB*] and tumor protein p53 [*TP53*], suggesting a genetic association for these two tumor types. Furthermore, a frequent loss of *TET2* and *DNMT1* genomic regions in LMS was observed. Conconi *et al.* (2021) performed comparative genomic hybridization array and DNA methylation studies on LMS, STUMP and LM. They identified a few genes, such as protein kinase, DNA-activated, catalytic subunit [*PRKDC*] and pumilio RNA-binding family member 2 [*PUM2*], that may have prognostic value for STUMP. Although their analysis for DNA methylation was restricted to only four samples, they found varying methylation features in the STUMP that relapsed as LMS (Conconi *et al.*, 2021). These results seem promising for the epigenomic field, and perhaps a more detailed analysis of DNA methylation as well as histone modifications can be performed on these samples to identify distinctive patterns with diagnostic/prognostic value. To overcome the limitation of sample recruitment owing to rare presentations of these tumors, formalin-fixed paraffin-embedded [FFPE] tissues may be utilized for various epigenomic modalities. Importantly, it was found that the stem cell-like population within LM is insensitive to hormonal treatments and can proliferate, leading to benign tumor growth (Conconi *et al.*, 2021). Further research is needed to establish an understanding of abnormal epigenomic regulation within these populations of cells to develop efficient therapeutic approaches.

The regulation of histone modifications in LM has historically been less understood as compared to DNA methylation, but recent publications, including those from our laboratory, have shed light on the significance this plays in disease development. The interplay of cell signaling pathways and histone modification can explain the altered 3D chromatin structure, which aids in developing the LM-specific gene dysregulation resulting in its pathogenesis. Understanding pathways or trans-regulatory elements that induce histone modification will allow for target options for suppressing the fibroid phenotype.

The application of an altered enhancer landscape and its effect on gene regulation is a new approach to a broader understanding of what drives LM development and requires more attention. The interplay of epigenomic marks, TF occupancy and enhancer chromatin structure plays a key role in transmitting information from millions of base pairs away for the regulation of gene expression. Interestingly, in mutant

*MED12* LM, modified enhancer architecture provides a novel potential mechanism of gene dysregulation that occurs outside the promoter proximal area. Whether enhancer malfunction also plays critical roles in non-*MED12* mutant LM will be a key future question to be addressed. The next challenge will be to use animal-based experiments with drugs targeting the epigenome, in combination with current drugs or alone, which will lead to better therapeutic outcomes.

With the advent of new technologies extracting pathologically relevant data at the single cell level, there are now opportunities to understand various diseases at an unparalleled depth. These high-resolution multi-omics tools have been utilized to study various fibrotic organs such as lung, liver and kidney (Henderson et al., 2020). Single-cell RNA sequencing [scRNA-seq] has revealed the presence of a subpopulation of macrophages that are pro-fibrotic and are involved in progression of lung (Misharin et al., 2017; Reyfman et al., 2019) and liver fibrosis (Ramachandran et al., 2019). In kidney fibrosis, scRNA-seq along with other modalities, namely ATAC-seq and spatial transcriptomics, led to the identification of NKD inhibitor of WNT signaling pathway 2 [NKD2], a gene that is differentially expressed in myofibroblasts and can be targeted in kidney fibrosis (Kuppe et al., 2021). The compatibility of these latest technologies with the use of preserved patient tissues, and the availability of FFPE tissues for even the rarest forms of LM, provide new avenues for research in the uterine LM field. In this context, single-cell transcriptomic analysis, as well as spatial gene expression studies, may be useful in dissecting the heterogeneity of various uterine fibroids based on genetic status and/or size. These studies may provide specific targets for the therapeutic treatment of this benign tumor with little to no effect on the normal tissues.

To reiterate, despite this condition affecting a significant number of women worldwide, LM has been an overlooked public health issue. In addition, research revolving around this disease has remained understudied compared to other non-malignant conditions (Ciavattini et al., 2013), although the National Institutes of Health and private funding agencies are now investing financial and scientific efforts to better understand this disease, which is a key for future therapies. An integrative knowledge linking genetic mutations, altered signaling and epigenomic events in the context of patients will be vital to better understand and treat this disease. In addition, using a larger number of patient samples will also be critical to better understand how the social–environmental context influences LM in Black women: particularly because environmental factors, such as experiences of childhood adverse effects, the experience of racial discrimination/racism, economic hardship and experience of redlining and residential segregation, are thought to influence the epigenome and gene expression. Now, bioinformatic programs exist that can interpret these events and analyze extensive patient data sets. The implementation of artificial intelligence-based predictions may guide us toward a better understanding of how epigenomic and enhancer dysregulation result in LM development and LM health disparity.

## Data availability

No new data were generated or analyzed in support of this research.

## Authors' roles

O.W.M. and P.S. are the co-first authors. O.W.M wrote the initial draft of the manuscript. O.W.M., P.S. and D.C. were responsible for literature search, manuscript drafting, figures, tables and critical discussions. J.B.P., J.-J.W., S.E.B. and M.A.S provided discussion and comments on the manuscript. D.C. conceived and oversaw the review. All authors read and commented on the manuscript.

## Conflict of interest

The authors declare no competing interests.

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