Subtype-Specific Tumor-Associated Fibroblasts Contribute to the Pathogenesis of Uterine Leiomyoma

Xin Wu, Vanida A. Serna, Justin Thomas, Wenan Qiang, Michael L. Blumenfeld, and Takeshi Kurita

Abstract

Recent genomic studies have identified subtypes of uterine leiomyoma (LM) with distinctive genetic alterations. Here, we report the elucidation of the biological characteristics of the two most prevalent uterine leiomyoma subtypes, MED12-mutant (MED12-LM) and HMGA2-overexpressing (HMGA2-LM) uterine leiomyomas. Because each tumor carries only one genetic alteration, both subtypes are considered to be monoclonal. Approximately 90% of cells in HMGA2-uterine leiomyoma were smooth muscle cells (SMC) with HMGA2 overexpression. In contrast, MED12-LM consisted of similar numbers of SMC and non-SMC, which were mostly tumor-associated fibroblasts (TAF). Paradoxically, TAF carried no mutations in MED12, suggesting an interaction between SMC and TAF to coordinate their growth. The higher amount of extracellular matrix in MED12-LM than HMGA2-LM was partially due to the high concentration of collagen-producing TAF. SMC growth in a xenograft assay was driven by progesterone in both uterine leiomyoma subtypes. In contrast, TAF in MED12-LM proliferated in response to estradiol, whereas progesterone had no effect. The high concentration of estrogen-responsive TAF in MED12-LM explains the inconsistent discoveries between in vivo and in vitro studies on the mitogenic effect of estrogen and raises questions regarding the accuracy of previous studies utilizing MED12-LM cell culture. In addition, the differential effects of estradiol and progesterone on these uterine leiomyoma subtypes emphasize the importance of subtypes and genotype in designing nonsurgical therapeutic strategies for uterine leiomyoma. Cancer Res; 77(24); 6891–901. ©2017 ACR.
**Materials and Methods**

**Collection of human uterine leiomyoma and myometrium tissues**

The acquisition and research use of surgical specimens were approved by the Institutional Review Board of the Ohio State University and Northwestern University, and were conducted in accordance with the Belmont Report. Uterine leiomyoma and myometrium samples were obtained from hysterectomy or myomectomy patients ages 28 to 52 years with prior written informed consent and delivered to research personnel within 5 hours of surgical removal. Should the tumor exceed 5 cm in diameter, only the tissues from the peripheral regions of tumors, the distribution of SMCs showed substantial heterogeneity within tissue sections. To avoid selection bias toward the areas with CNN1-staining, the entire area of CNN1-stained sections (~250–100 mm²) were scanned, and the areas with different cellularities were imaged proportionally to the original tissue. The morphometric analyses were blindly performed by designated researchers: The morphometric analyses were blindly performed by designated researchers: The vascular cells were manually excluded from each image, and the areas for morphometric analyses were manually selected to include SMCs or non-SMCs only. In SMC concentration analysis, at least 615 total cells from 3–6 view fields at 40× magnification were counted blindly for each uterine leiomyoma or myometrium. The nuclear size (area) was measured in the blue channel (nuclear staining) of the same field using "Analyze particle" function of ImageJ (NIH). The MKI67 labeling indices of SMCs and non-SMCs in PDTXs were determined by counting positive and negative cells in double IF-stained sections for CNN1 and non-SMCs. The proportion of smooth muscle cells (SMC) and non-SMC in myometrium and uterine leiomyomas was determined by counting the nuclei of SMCs (CNN1-positive) and non-SMCs (CNN1-negative) in IF-stained sections for CNN1 (green) with Bisbenzimide H 33258 nuclear staining. Although uterine leiomyoma tissues were collected from peripheral regions of tumors, the distribution of SMCs showed substantial heterogeneity within tissue sections. To avoid selection bias toward the areas with CNN1-staining, the entire area of CNN1-stained sections (~25–100 mm²) were scanned, and the areas with different cellularities were imaged proportionally to the original tissue. The morphometric analyses were blindly performed by designated researchers: The vascular cells were manually excluded from each image, and the areas for morphometric analyses were manually selected to include SMCs or non-SMCs only. In SMC concentration analysis, at least 615 total cells from 3–6 view fields at 40× magnification were counted blindly for each uterine leiomyoma or myometrium. The nuclear size (area) was measured in the blue channel (nuclear staining) of the same field using "Analyze particle" function of ImageJ (NIH). The MKI67 labeling indices of SMCs and non-SMCs in PDTXs were determined by counting positive and negative cells in double IF-stained sections for CNN1 and anti-MKI67.

**Isolation of uterine leiomyoma cells**

The isolation of uterine leiomyoma cells was performed as previously described (18). Briefly, uterine leiomyoma tissues were cut into pieces (~<9 mm³), washed twice with Dulbecco PBS containing 1x Gibco Antibiotic–Antimycotic (Thermo Fisher Scientific), and digested in Hanks Balanced Salt solution containing 1.5 mg/mL collagenase Type I (Sigma-Aldrich), 83.3 g/mL DNase I (Sigma-Aldrich), and 1× Gibco Antibiotic–Antimycotic at 37°C for 5 hours. After filtration through a 100 μm Falcon cell strainer (BD Falcon), residual erythrocytes in cell suspensions were lysed with Red Blood Cell Lysis Buffer (00-4300-54, eBioscience) for 15 minutes at room temperature.

**FACS analysis**

**Materials and Methods**

Freshly isolated uterine leiomyoma cells were further dispersed into single cells by incubating with 0.1% trypsin for 15 minutes at 37°C. Singly dispersed cells were stained with the Live/Dead AxyProbes® Near-infrared Stain Kit (Thermo Fisher Scientific), fixed with 4% paraformaldehyde for 10 minutes and incubated with Flow-Cytometry Permeabilization/Wash Buffer I (R&D systems) for 10 minutes. The permeabilized uterine leiomyoma cells were then incubated with anti-collagen I (1:100 LS-B342) and anti-collagen III (1:200 LS-B693) IgGs together or with normal rabbit IgG (negative control, 1:100, sc-2027, Santa Cruz Biotechnology) for 30 minutes on ice, followed by incubation with...
rearrangements of HMGA2, are associated with larger tumor selection of smaller uterine leiomyomas, which have greater HMGA overexpression was lower (Fig. S1). This incidence of HMGA overexpression was lower between HMG2-LMs and MED12-LMs: In HMG2-LMs, fibroblasts were concentrated in perivascular connective tissues (Fig. 1B, white dotted lines), whereas in MED12-LMs small clusters of SMCs were embedded in fibroblast-rich extra-cellular matrix (ECM; Fig. 1B and C). As the cellular compositions of MED12-LMs and HMG2-LMs appeared to be different, the percentage of SMCs and non-SMCs, excluding vascular cells, was determined in different uterine leiomyoma subtypes. Although myometrium consisted of 61.2% ± 14.9% of C11-positive SMCs, HMG2-LMs consisted of 91.5% ± 5.6% SMCs (Fig. 1D), suggesting that the overexpression of HMG2 drives the overgrowth of SMCs leading to uterine leiomyomas. Indeed, immunostaining confirmed that HMG2 was overexpressed only in SMCs (Fig. 1E). On the other hand, the SMC concentration of MED12-LMs (60.1% ± 10.6%) was not significantly different from that of myometrium (Fig. 1D). The SMC concentration in MED12-LMs widely ranged from 31.4% to 76.7%. Because the analysis of SMCs concentration can be biased by the size and morphology of nuclei, we assessed the correlation between SMC concentration and nuclear size ratio between SMCs and fibroblasts in 6 MED12-LMs (Fig. 1F). Although the ratio of nuclear sizes in SMCs and fibroblasts was also variable among MED12-LMs, there was no correlation with SMC concentration. Thus the diverse SMC concentrations in MED12-LMs are not an artifact due to different nuclear sizes.

The similar concentration of SMCs and fibroblasts in MED12-LMs implied that these two populations arose from the same progenitor population. Indeed, Holdsworth-Carson and colleagues (20) previously proposed that fibroblasts and SMCs in uterine leiomyomas arose from a same clone, based on a human androgen receptor assay. However, the sensitivity and accuracy of X-chromosome inactivation assay is limited, as it only determine the presence of 1 or 2 gene silencing patterns in the tissue. Furthermore, because silencing of the X-chromosome occurs during embryogenesis, the pattern must be shared by all cells in tissues derived from a progenitor cell (21). Hence, although fibroblasts and SMCs in uterine leiomyomas may be derived from the same Müllerian duct mesenchymal cell, MED12 mutations may occur exclusively in one population after their lineages are segregated. We therefore determined MED12 genotypes in SMCs and non-SMCs of MED12-LMs. MED12-LMs with a missense mutation in exon 2 of MED12 were digested into single cells, and 3 fractions were collected, based on the expression levels of C11 (C11–, C11+ and C11++). C11 preferentially stains tumor SMCs over vascular SMCs (Fig. 2A). Hence, C11– and C11++ fractions should mostly consist of fibroblasts and uterine leiomyoma SMCs respectively, whereas C11+ should be a mixture of fibroblasts, vascular cells, and uterine leiomyoma SMCs. Each MED12-LM demonstrated a unique population distribution for the C11 signal levels (Fig. 2B). Nevertheless, MED12 mutations were totally absent in non-SMCs, whereas C11– and C11++ fractions contained MED12-mutant cells.

Because C11 and ACTA2 can be detected in uterine soft-tissue tumors of non-SMC origin, we assessed the expression patterns of cellular composition of MED12-LMs and HMG2-LMs is distinctive

Using HMG2 IHC in 230 uterine leiomyoma samples, we identified 11 HMG2-LMs (4.8%; Fig. 1A and Supplementary Fig. S1). This incidence of HMG2 overexpression was lower than that previously reported (5, 8, 9). One of the factors contributing to the lower incidence of HMG2-LMs was our selection of smaller uterine leiomyomas, which have greater cell viability for PDX studies than larger uterine leiomyomas; however, karyotype abnormalities, including those leading to rearrangements of HMG2, are associated with larger tumor size (19).
Figure 1.
Two most prevalent subtypes of human uterine leiomyoma show distinctive cellular compositions. A, Histological characteristics of uterine leiomyoma subtypes. Representative images for HMGA2 IHC and hematoxylin and eosin (H&E) of HMGA2-LM and MED12-LM subtypes. Scale bar, 100 μm. B, Tissue distribution of SMCs and fibroblasts in HMGA2-LM and MED12-LM tissues. CNN1 (green) stains SMCs, whereas VIM (red) stains both SMCs and fibroblasts. In the HMGA2-LM tissue, perivascular connective tissues devoid of tumor SMCs are marked with dotted lines. Scale bar, 100 μm. C, Cellular and nuclear appearance of SMCs and TAFs. The nuclei of SMCs (white arrows) and fibroblasts (yellow arrows) are indicated by arrows. Scale bar, 25 μm. D, Box-plot for the concentration of SMCs in myometriums (MM) and uterine leiomyomas. The MED12 mutations are listed in Supplementary Table S1. Statistical significance by ANOVA, ***, P < 0.001; ns, not significant, P > 0.05. E, HMGA2 is overexpressed in SMCs but not in the nuclei of TAFs arrow. Scale bar, 50 μm. F, Relative nuclear area of TAFs (blue box) and SMCs (red box) in MED12-LMs. The ratio between average nuclear sizes of SMCs and TAFs (SMC/TFA) is indicated below the boxplot. There was no clear association between nuclear size-ratio, mutation type, and SMC concentration. Statistical significance by ANOVA, ***, P < 0.001; **, P < 0.01; ns, not significant, P > 0.05.
two additional SMC markers, desmin (DES) and caldesmon (CALD1), which are more specific for SM tumors (22). Vascular SMCs expressed DES and CNN1 at noticeably lower levels compared with non-vascular SMCs. Meanwhile, tumor SMCs in MED12-LMs expressed all four SMC markers (N = 10; Fig. 2A), indicating that the CNN1-positive population in MED12-LMs represents homogenous SMCs. Although MED12 was expressed in both SMCs and fibroblasts, the expression level was higher in the SMCs than fibroblasts (Fig. 2C), suggesting the critical roles of MED12 in SMCs.

In MED12-LMs, cells positive for MC387 (granulocytes, monocytes and macrophages; Supplementary Fig. S3) and endothelial cell markers (VWF and PECAM1; Supplementary Fig. S4) did not contribute significantly to the tissue mass, indicating that fibroblasts constitute the major fraction of non-SMCs. Our results establish that MED12-LMs consist of approximately equal number of MED12 mutant SMCs and MED12 wild type non-SMCs, which are mostly fibroblasts.

Tumor-associated fibroblasts contribute ECM to the MED12-LMs
The accumulation of excess ECM is a characteristic of uterine leiomyomas (15, 18). However, HMGA2-LMs and MED12-LMs displayed striking differences in the distribution of ECM, as revealed by Masson’s trichrome staining and IHC for Type I collagen (Fig. 3A). Particularly, large pools of ECM, which contain fibroblasts but not SMCs, were frequently observed in MED12-LMs. Hence, we hypothesized that the high number of fibroblasts contributes to the accumulation of excess ECM in MED12-LMs. Indeed, the cytoplasm of both SMCs and tumor-associated fibroblasts (TAF) in MED12-LMs was stained for Type I collagen (Fig. 3B). We further analyzed the intracellular contents of collagens in...
SMCs and non-SMCs by FACS. FACS analysis for Type I and Type III collagens versus CNN1 indicated that both SMC and non-SMC population produce ECM in MED12-LMs (Fig. 3C). In addition, we assessed the regulation of procollagen I by E2 and P4 in PDTXs of MED12-LMs. Procollagen I was detected in both SMCs and TAFs in PDTXs of MED12-LMs irrespective of hormone treatment (Fig. 3D). Interestingly, procollagen signal in SMCs increased with E2 + P4 treatment, whereas procollagen I levels in fibroblasts seemed unchanged between E2 versus E2 + P4 groups. These observations indicate that ECM in MED12-LMs are produced by both MED12-mutant SMCs and non-mutant TAFs. However, the regulation of collagen synthesis by E2 and P4 appears to be different in SMCs and TAFs.

The growth regulation of uterine leiomyoma subtypes

The distinctive cellular composition and ECM contents implied fundamental differences in the growth control of MED12-LMs and HMGA2-LMs. Hence, we compared growth regulation of these uterine leiomyoma subtypes by E2 and P4 using the PDTX model (15). In PDTXs of both uterine leiomyoma subtypes, tumor volume significantly increased only when the hosts were treated with E2 + P4 (Fig. 4A and B). Likewise, SMCs had a significantly increased proliferation rate (MKI67-labeling index) in response to E2 + P4 but not to E2 or P4 only (Fig. 4C and D). Therefore, our previous findings are confirmed in both MED12-LMs and HMGA2-LMs (15, 18, 23).

In HMGA2-LM PDTXs, HMGA2 was constitutively expressed in the entire tumor tissue, and the expression pattern did not change by hormone treatments. Meanwhile, expression of PGR in HMGA2-LMs was E2 dependent (Fig. 4B).

Growth characteristics of TAFs in MED12-LMs

If MED12 mutation drives growth of SMC cell-autonomously, SMCs is expected to be the predominant cell type, as found in HMGA2-LMs. However, MED12-LMs contain a similar number of MED12-mutant SMCs and non-mutant TAFs, thus raising a question about the growth control of these two cell populations. Accordingly, we assessed the growth response of SMCs and TAFs to E2 and P4 in PDTXs of MED12-LMs. PDTXs were generated from a MED12-LM with c.131G > A missense mutation in MED12. MED12-LM PDTXs were grown in hosts supplemented with E2 + P4 for 4 weeks, and then subjected to no hormone, E2, or E2 + P4 treatment (Fig. 5A). The volume increase from 4 to 6 weeks in PDTXs of E2 + P4 groups confirmed that the uterine leiomyoma cells were actively growing (Fig. 5B). Interestingly, E2 stimulated the proliferation of SMCs and TAFs differentially: Although the MKI67-labeling index of SMCs was significantly elevated by E2 + P4 but not E2, E2 and E2 + P4 equally increased MKI67-labeling index in TAFs (Fig. 5C and D). Nevertheless, MKI67-labeling index was significantly higher in SMCs than TAFs in E2 + P4 treated MED12-LMs. Our results suggest that the diverse number of SMCs versus TAFs among MED12-LM cases reflects the history of endocrine milieu in the patient, given E2 exclusively stimulates TAFs, whereas P4 preferentially promotes growth of SMCs. In addition, the removal of P4 significantly reduced the ratio of SMCs to TAFs (Fig. 5E).
This is likely due to a reduction of cellular/nuclear volume in SMCs. Although both SMCs and TAFs expressed ESR1, the signal intensity per nucleus was always higher in SMCs than TAFs. When E2 induced PGR in PDTXs, the expression levels of PGR were also higher in SMCs than TAFs (Fig. 5F), suggesting that TAFs in MED12-LMs are less sensitive to E2 and P4 compared with MED12 mutant SMCs.

Discussion
This study demonstrated for the first time that SMCs and TAFs are independent populations in both HMGA2-LMs and MED12-LMs, and the causal mutations are present only in the SMCs. The cellular composition of HMGA2-LMs and MED12-LMs significantly differs as HMGA2-LMs mostly consist of SMC population, whereas MED12-LMs contain equal number of SMCs and TAFs.

The concentration of SMCs in uterine leiomyomas is likely lower than the estimate in this study. We had avoided collecting uterine leiomyoma tissues from the central region of the tumor, due to necrosis and calcification; however, the central region is low in tumor cell number and high in ECM (24). In addition, we had excluded vascular cells from the SMC concentration analysis.

The high concentration of TAFs explains why MED12-mutant cells are quickly lost from the primary culture of uterine leiomyomas (25, 26); fibroblasts attach the culture dish better and grow faster than SMCs in standard culture conditions, conditions originally developed to optimize fibroblast growth (27).
Overgrowth of fibroblasts is a classic problem for the primary culturing of SMCs, including myometrium, and many techniques to repress the growth of fibroblasts in SMC culture have been proposed (20, 28–32). However, this common problem has been often overlooked in uterine leiomyoma and myometrium cell cultures because cell types are usually assessed in whole-cell lysates, in which SMC markers can be detected even when SMCs are a minor population (33, 34). In addition, ACTA2, the most commonly used SMC marker, cannot accurately detect the disappearance of SMCs from cell cultures, as ACTA2 can be induced in non-SMCs of the human uterus (35–39). Given the high initial concentration of TAFs with superior in vitro growth potential, freshly prepared primary cultures of MED12-LMs would contain more fibroblasts than SMCs, and the concentration of SMCs should further decrease, as TAFs grow faster to reach confluence. Indeed, Bloch and colleagues (26) recently demonstrated that the concentration of MED12-mutant cells gradually decreases in the primary culture of MED12-LMs even without passaging. The study

Figure 5.
Growth control of SMCs and TAFs in MED12-LM. PDTXs were prepared from a MED12-LM (MED12 c.131 G>A). Statistical significance was determined by ANOVA. A, Timeline. PDTXs were grown for 4 weeks with E2+P4 and then subjected to one of three different treatments: no hormone (no pellet), E2 or E2+P4 treatment for 2 weeks, at which point the PDTXs were harvested for analyses. B, The volume of PDTXs. * P < 0.05; ns, not significant. C, Effect of E2 and P4 on MKI67 expression in SMCs (white arrows) and TAFs (yellow arrows) within MED12-LM PDTXs. D, Proliferation rate (MKI67-labeling index) of SMCs (dark red) and TAFs (light blue). The MKI67-labeling indices were significantly higher in groups marker with "a" than "b" (P < 0.01) and in groups marked with "c" than "d" (P < 0.05). E, Effect of E2 and P4 on SMC concentrations (%) in MED12-LM PDTXs. * P < 0.05. F, Regulation of ESR1 and PGR by E2 and P4 in SMC (white arrows) and TAFs (yellow arrows) in MED12-LMs.
by Bloch and colleagues also suggested that the loss of MED12-mutant cells is partially due to the detachment of mutant cells from the culture flask, which likely reflects the weaker adherence of SMCs to the plastic surface compared to fibroblasts (40). Then, the subsequent passing further dilutes SMCs in MED12-LM cell culture (25). Conversely, the low fibroblast concentration in HMGA2-LMs explains why HMGA2-mutant cells can be maintained in vitro for multiple passages (25).

Our current study questions the validity of previous studies that used uterine leiomyoma cell culture as the primary research model: These studies likely analyzed the cell cultures that mostly consisted of TAFs (26). Indeed, uterine leiomyoma and myometrium cells rapidly change their gene expression profiles in vitro (41). Although the change in gene expression was attributed to the effects of artificial culture conditions, our result indicate that the overgrowth of TAFs is the primary factor that alters the gene expression of uterine leiomyoma and myometrium cells in culture. Furthermore, the rapid loss of ER1 and PGR expression in primary uterine leiomyoma cell culture (42) is undoubtedly due to the overgrowth of TAFs, which express ER1 and PGR at lower levels compared with uterine leiomyoma SMCs. Several studies have demonstrated the growth-promoting effect of estrogens on primary uterine leiomyoma cell cultures (43, 44), contradicting with results from our PDTX studies, in which E2 does not stimulate the growth of uterine leiomyoma SMCs. These conflicting observations can also be explained by the overgrowth of TAFs in uterine leiomyoma cell culture as their growth is stimulated by E2 alone.

As assessed in histology sections, MED12-mutant SMCs are several-fold larger than TAFs, particularly when they are stimulated by E2 and P4. Hence, even when uterine leiomyomas contain an equal number of fibroblasts and SMCs, SMCs should contribute several-fold higher amount of proteins and RNAs to the tumor. Accordingly, gene/protein expression profiles of the original uterine leiomyoma tissues are expected to represent the characteristics of uterine leiomyoma SMCs. On the other hand, the amount of genetic materials is equal in each cell irrespective of the type and size. Hence, epigenetic analysis of MED12-LMs requires extra caution, as tumors contain a similar number of SMCs and non-SMCs, including TAFs. For example, epigenetic signatures of MED12-LMs, such as DNA methylation patterns or histone modification patterns, should equally reflect that of SMCs and TAFs. Hence, we predict the epigenome of original MED12-LMs as whole should show more fibroblast-like patterns compared with HMGA2-LMs. As the concentrations of TAFs vary between tumor cases as well as intratumor locations, the epigenetics of MED12-LMs would require additional refining to normalize the concentration of cell types in the sample.

We have previously shown that the level of miR-29b in uterine leiomyomas was significantly lower compared with myometrium and that the downregulation of miR-29b is essential for the accumulation of ECM proteins in uterine leiomyomas (18). Because miR-29 family members are enriched in vascular SMCs compared to fibroblasts (45), it is possible that the lower levels of miR-29b in uterine leiomyomas reflect the lower ratio of SMCs to fibroblasts, which generally express lower levels of miR-29b compared with SMCs. In our previous study, collagen mRNAs and miR-29b were moderately up- and downregulated, respectively, in PDTXs with 2 weeks of E2 treatment (18). This may be due to the increase in fibroblast concentration in PDTXs by E2 treatment. Our previous study did not delineate the effects of miR-29b levels on SMCs and TAFs, given the primary cultures of uterine leiomyomas contained both cell types. To elucidate the role of miR-29b in the pathogenesis of uterine leiomyomas, the expression patterns of miR-29b in uterine leiomyoma SMCs versus TAFs should be further determined.

Because of the requirement of both E2 and P4 for the proliferation of tumor SMCs, selective progesterone receptor modulators (SPRMs) should be an effective treatment for both MED12-LMs and HMGA2-LMs. On the other hand, SPRMs may cause fibrosis of ECM-prominent MED12-LMs, as TAFs are stimulated by E2. Hence, to design non-surgical therapeutic strategies for uterine leiomyomas, it is critical to consider the uterine leiomyoma subtype.

The cell-autonomous growth-promoting effect of HMGA2 in SMCs explains the pathogenesis of HMGA2-LMs. On the other hand, the coordinated growth of SMCs and TAFs in MED12-LMs implies the presence of paracrine interactions between these two cell types. We hypothesize that E2 stimulates MED12-mutant SMCs to secrete paracrine factors that promote the growth of TAFs, and TAFs in turn support the growth of uterine leiomyoma SMCs. Several studies detected mRNAs for growth factors that are differentially expressed in paired uterine leiomyomas and myometriums (46–49). The regulation of these growth factors should be revisited in light of potential paracrine interactions between SMCs and TAFs. Our PDTX model would be an ideal platform to explore the nature of paracrine interactions and mediators.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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