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Authors
Chuang, Tsai-Der
Quintanilla, Derek
Boos, Drake
et al.

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Tryptophan Catabolism is Dysregulated in Leiomyomas

Tsai-Der Chuang, Ph.D., **Derek Quintanilla, BS.**, **Drake Boos, BS.**, **Omid Khorram, M.D., Ph.D.**

Department of Ob/Gyn Harbor-UCLA Medical Center and The Lundquist Institute, Torrance, CA

Abstract

Objective: To determine the expression and functional roles of indoleamine 2,3-dioxygenase (IDO1) and tryptophan 2,3-dioxygenase (TDO2) in leiomyoma.

Design: Experimental study

Setting: Academic research laboratory

Patients: Women undergoing hysterectomy for leiomyoma.

Intervention: Blockade of IDO1 and TDO2.

Main Outcome Measure: Expression of IDO1 and TDO2 in leiomyoma and the effects of their inhibitors on extracellular matrix (ECM).

Results: Leiomyoma as compared with matched myometrium expressed significantly higher levels of IDO1 and TDO2 mRNA (60.3%, 35/58 pairs; 98.3%, 57/58 pairs, respectively) and protein (54%, 27/50 pairs; 92%, 46/50 pairs, respectively), and kyneurenine (KYN; 78.3%, 36/46 pairs), a marker of enzyme activity. The expression of TDO2 but not IDO1 mRNA was significantly higher in fibroids from African American (AA) as compared with Caucasian and Hispanic patients. TDO2 but not IDO1 protein and mRNA levels were more abundant in fibroids bearing the MED12 mutation as compared with wild type leiomyomas. Treatment of LSMC (leiomyoma smooth muscle cells) and MSMC (myometrial smooth muscle cells) spheroids with the TDO2 inhibitor, 680C91 but not the IDO1 inhibitor, Epacodostat significantly repressed cell proliferation and the expression of collagen type I (COL1A1) and type III (COL3A1) in a dose-dependent manner; these effects were more pronounced in LSMC as compared with MSMC spheroids.

Conclusion: These results underscore the physiological significance of tryptophan degradation pathway in the pathogenesis of leiomyomas and the potential utility of anti-TDO2 drugs for treatment of leiomyomas.

Capsule:

Tryptophan catabolism plays a role in leiomyoma pathogenesis through upregulation of two key enzymes IDO1 and TDO2

Address all Correspondence to: Omid Khorram, M.D., Ph.D., Department of Ob/Gyn, Harbor-UCLA Medical Center, 1124 W. Carson St., Box 467, Torrance, CA. 90502, okhorram@lundquist.org.

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Keywords

Leiomyoma; tryptophan; indoleamine 2,3-dioxygenase (IDO1); tryptophan 2,3-dioxygenase (TDO2); MED12 mutation

Introduction

Leiomyomas are benign uterine tumors with fibrotic characteristic. Although their etiology remains unknown, leiomyoma develop in ~70% women during reproductive years with a higher prevalence and symptom severity in African Americans (AA) (1–3). Risk factors for fibroids are race, age, premenopausal status, hypertension, positive family history and time since last birth (3).

Although ovarian steroids are known to be key regulators of leiomyoma growth, altered expression of many protein-coding genes (1, 4, 5) as well as genetic heterogeneity associated with chromosomal re-arrangements and mutation in a number of genes such as Mediator Complex Subunit 12 gene (MED12), fumarate hydratase (FH), High Mobility Group AT-Hook 2 (HMGA2) have also been associated with their development and growth progression (1). Recent studies have demonstrated that somatic MED12 mutations in exon 2 occurs at a frequency of up to 80% and have a functional role in leiomyoma pathogenesis (6–8) potentially through activation of Wnt/β-catenin pathway (9–11). MED12 is a component of Mediator complex which functions as a transcription coactivator by transmitting signals from transcription factors to RNA polymerase II (8). Our laboratory has focused on the role of non-coding RNAs in fibroid pathogenesis and as such have identified a host of differentially expressed small non-coding RNAs (12) and long-coding RNAs (13) using next generation sequencing. In the course of our sequencing studies we identified a markedly aberrant overexpression of the rate limiting tryptophan (Trp) degradation enzymes, indoleamine 2,3-dioxygenase (IDO1) and tryptophan 2,3-dioxygenase (TDO2) in fibroid tumors which led to the current study.

Trp is an essential amino acid which is not only essential for protein synthesis and serotonin but also serves as a precursor of many important metabolites following its degradation which can occur through 4 pathways (14). Of these four pathways, the kynurenine degradation pathway which exists mainly in the liver holds the greatest importance accounting up to 95% of Trp degradation (14). The first step in the degradation of Trp is catalyzed by action of either TDO2 in the liver or IDO1 extra-hepatically to N-formylkynurenine which is then hydrolyzed to kynurenine by NFK formamidase (15). TDO2 is primarily localized in the liver although two variants have been identified in the mouse brain where it plays a role in development and in brain tumors (14). IDO1 is ubiquitously distributed while a second isoform of IDO, IDO2 has lower catalytic activity, is expressed in kidney, liver, brain, and reproductive tract and has immunoregulatory function (14). The objective of this study was to characterize the expression and regulation of Trp catabolic enzymes in fibroids and their relevance to regulation of fibroid cell proliferation and aberrantly expressed genes such as collagen type I (COL1A1) and type III (COL3A1). We hypothesized that Trp catabolic pathway plays a key role in the pathogenesis of fibroid tumors.

Material and Methods

Tissue Collection and Primary Cell Isolation

Portions of uterine leiomyomas and paired myometrium were obtained from patients (N=58) not on hormonal treatments for at least 3 months prior to surgery at Harbor-UCLA Medical Center with prior approval from the Institutional Review Board (#036247) at the Lundquist Institute at Harbor-UCLA Medical Center. Informed consent was obtained from all the patients participating in the study prior to surgery. The paired tissues were obtained from Caucasians (N=12 with 3 pairs kindly provided by Dr. Al Hendy at University of Chicago), African Americans (N=25) and Hispanics (N=21). Race/ethnicity data was obtained from the electronic medical records which are based on self-report. The Hispanic group was entirely composed of White Hispanics. The mean age of patients was 45 ± 4.8 years with a range of 35–54 years. The menstrual cycle phase was determined by histologic analysis of hematoxylin and eosin stained endometrial sections (16) with 27 specimens being identified as in the proliferative phase and 14 specimens in the secretory phase. The MED12 mutation status was determined by PCR amplification and Sanger sequencing. Of the specimens sequenced, 40 fibroids had the MED12 mutations (68.9%) with no mutations in the myometrium. Missense mutations in MED12 exon 2 were the most frequent alteration (85%), followed by in-frame insertion-deletion type mutations (15%). The missense mutations in exon 2 included c.130G>C (p.Gly44Arg) (11.8 %), c.130G>A (p.Gly44Ser) (23.5 %), c.130G>T (p.Gly44Cys) (8.8 %), c.131G>C (p.Gly44Ala) (2.9 %), c.131G>A (p.Gly44Asp) (35.3 %), and c.131G>T (p.Gly44Val) (17.6 %). The leiomyomas used in this study ranged in size from 2 to 5 cm in diameter and were intramural. The tissues were either snap frozen and stored in liquid nitrogen for further analysis or used for isolation of MSMC and LSMC as previously described (17, 18). Briefly, LSMC were cultured in DMEM supplemented with 10% fetal bovine serum until reaching confluence with a change of media every 2–3 days. Cells at passages p1 to p3 were used for all experiments. Cell culture experiments were performed at least three times using MSMC and LSMC obtained from different patients. Overall, 8 LSMC and 10 MSMC were used for the in vitro experiments. All supplies for the isolation and cell culture were purchased from Sigma-Aldrich (St. Louis, MO), Invitrogen (Carlsbad, CA) and Fisher Scientific (Atlanta, GA).

RNA Isolation and qPCR Analysis

Total RNA was isolated from leiomyoma and matched myometrium using Trizol (Thermo Scientific, Waltham, MA) and RNA concentration and integrity was determined using a Nanodrop 2000c spectrophotometer (Thermo Scientific) and Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA) according to manufacturer's protocols as previously described (12). Subsequently, RNA sample of 1 μg each was reverse transcribed using random primers for IDO1 and TDO2. Quantitative PCR was carried out using SYBR gene expression master mixes (Applied Biosystems, Carlsbad, CA). Reactions were incubated for 10 min at 95°C followed by 40 cycles for 15 seconds at 95°C and 1 min at 60°C. mRNA levels were quantified using the Invitrogen StepOne System and normalized to FBXW2 (19). All reactions were run in triplicate and relative expression was determined using the comparative cycle threshold method ($2^{-\Delta\Delta Cq}$), as

recommended by the supplier (Applied Biosystems). Abundance values was expressed as fold change compared to the corresponding control group. The primer sequences in the 5'–3' direction used for IDO1 detection are sense, CCCTTCAAGTGTTTCACCAAATC and antisense, GTCTTCCCAGAACCCTTCATAC, and for TDO2 detection are sense, GCGATCAACTGTGAGTGATAGG and antisense, GGTTGGGTTCATCTTCGGTATC. For FBXW2 detection are sense, CCTCGTCTCTAAACAGTGGAATAA and antisense, GCGTCCTGAACAGAATCATCTA.

MED12 Mutation Analysis

Genomic DNA from leiomyomas and paired myometrial specimens was extracted from 100 mg of freshly frozen tissue using Tissue Genomic DNA Miniprep Kit (Bioland Scientific LLC, Paramount, CA), according to the manufacturer's protocol. PCR amplification and Sanger sequencing (Laragen Inc. Culver City, CA) was performed to investigate the MED12 exon 2 mutations using the primer sequences in the 5'–3' direction: sense, GCCCTTTCACCTTGTTCCTT and antisense, TGTCCCTATAAGTCTTCCCAACC. PCR products were sequenced using Big Dye Terminator v.3.1 sequencing chemistry and the sequences were analyzed with the Software ChromasPro 2.1.8 and compared with the MED12 reference sequence (NG_012808 and NM_005120).

Measurement of Kynurenine

Kynurenine concentration in paired leiomyoma and myometrium homogenates (N=46) was measured in duplicate using the Human Kynurenine ELISA kit (MBS766153; MyBioSource, San Diego, CA) according to the manufacturer's instructions. Absorbance of each plate was measured spectrophotometrically at a wavelength of 450 nm and the concentration was determined by comparing the optical density value of samples to the standard curve.

Immunoblotting

Total protein isolated from paired tissue samples and MSMC and LSMC spheroids following treatment conditions was subjected to immunoblotting as previously described (20, 21). Briefly, samples were suspended in RIPA buffer containing 1 mM EDTA and EGTA (Boston BioProducts, Ashland, MA) supplemented with 1 mM PMSF and a complete protease inhibitor mixture (Roche Diagnostics, Indianapolis, IN), sonicated, and centrifuged at 4°C for 10 min at 14,000 rpm. The concentration of protein was determined using the BCA™ Protein Assay Kit (Thermo Scientific Pierce, Rockford, IL). Equal aliquots (thirty micrograms) of total protein for each sample was denatured with SDS-PAGE sample buffer and separated by electrophoresis on a SDS polyacrylamide gel. After transferring the samples to a nitrocellulose membrane, the membrane was blocked with TBS-Tween + 5% milk and probed with the following primary antibodies: IDO1 (Cell Signaling Technology, Danvers, MA), TDO2, COL3A1 (Proteintech Group, Inc, Chicago, Illinois) and COL1A1 (Fitzgerald Industries Intl, Acton, MA). The membranes were washed with TBS containing 0.1%Tween-20 wash buffer after each antibody incubation cycle. SuperSignal West Pico Chemiluminescent Substrate™ (Thermo Scientific Pierce) was used for detection, and photographic emulsion was used to identify the protein bands, which were subsequently quantified by densitometry. The membranes were also stripped and

probed with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody (Santa Cruz Biotechnology, Santa Cruz, CA) serving as the loading control. The densities of the specific protein bands were determined using image J program (http://imagej.nih.gov/ij/), normalized to GAPDH or a band obtained from staining the membrane with Ponceau S. Results were expressed as means ± SEM as a ratio relative to the control group designated as 1.

Immunohistochemistry

Paired myometrium and leiomyoma were fixed with 4% paraformaldehyde in PBS, and subsequently transferred to PBS containing 30% sucrose (wt/vol) until equilibrated in cold (4°C). After fixation, 5-μm-thick paraffin sections were treated three times with Histo-Clear™ (National Diagnostics, Atlanta, GA) for 5 min, and rehydrated by a sequential ethanol wash, and then incubated in target retrieval solution (Dako, Carpinteria, CA) in a microwave for 8 min in order to retrieve the antigens. For blocking, tissues were incubated for 10 minutes with 3% solution of H2O2 followed by incubation with PBS-5% normal goat serum-0.2% Triton X–100. Tissue sections were incubated with primary antibody rabbit anti-IDO1 (dilution 1:200, Cell Signaling Technology) and anti-TDO2 (dilution 1:200, Proteintech Group, Inc) overnight at 4°C in a humidified chamber. The antigens were then visualized using biotinylated antibodies and streptavidin, conjugated with horseradish peroxidase. Control sections were incubated with the secondary antibody, with replacement of primary antibody with the dilution reagent (Dako). Diaminobenzidine (Dako) served as the substrate, and all sections were counterstained with hematoxylin and eosin. Immunostained sections were examined under a microscope (Axioskop 40; Carl Zeiss Microimaging LLC, Thornwood, NY) at 40x magnification.

Spheroid Cell Culture

Isolated MSMC and LSMC were plated in 6-well (1×10^5 cells/well) or 96-well (5×10^3 cells/well) plates which were coated with 0.5% agarose gel and incubated 48 hours for spheroids formation (22).

Cell Proliferation Assay

The MSMC and LSMC spheroids were treated with different doses of IDO1 inhibitor (Epacadostat; Cayman Chemical, Ann Arbor, MI) and TDO2 inhibitor (680C91; Sigma-Aldrich) for 48 hours. The concentrations of inhibitors used was based on other studies (23, 24) and the cell proliferation was determined using the CellTiter-Glo 3D Cell Viability Assay (Promega, Madison, WI) according to the manufacturer's protocol. The assay was performed in six replicates per condition and repeated four times using cells isolated from four different patients.

Statistical analysis

Throughout the text, all data are presented as mean ± SEM and analyzed by PRISM software (Graph-Pad, San Diego, CA). Dataset normality was determined by the Kolmogorov–Smirnoff test, Shapiro-Wilk test, D'Agostino & Pearson test and Anderson-Darling test. Data presented in Figs 1–3 were not normally distributed and therefore non-parametric tests were used for data analysis. Comparisons involving two groups were analyzed using

Wilcoxon matched-pairs signed rank test (Fig. 1) or Mann Whitney test (Fig. 2 and Fig. 3) as appropriate. Data presented in Fig. 4 were normally distributed and analyzed by one-way ANOVA. Statistical significance was established at $P<0.05$.

Results

Using paired leiomyoma and matched myometrium (N=58) the mRNA expression of IDO1, IDO2 and TDO2 in the same specimens was determined. Because of low to undetectable levels of IDO2 expression in these tissues we focused on determination of IDO1 and TDO2 expression in this study. The expression of IDO1 (60.3%, 35/58 pairs) and TDO2 (98.3%, 57/58 pairs) mRNA in fibroids as compared with myometrium was significantly higher (Fig. 1A). The degree of mRNA upregulation and the number of specimens exhibiting increased expression was significantly greater for TDO2 as compared to IDO1 (Fig. 1A). The levels of kynurenine, a marker of enzyme activity for IDO1/TDO2 was significantly higher in extracts of leiomyoma as compared with matched myometrium (Fig. 1B; 78.3%, 36/46 pairs). The IDO1 and TDO2 protein levels are shown in Fig. 1C–E. As demonstrated in this figure the IDO1 (Fig. 1C–D; 54%, 27/50 pairs) and TDO2 (Fig. 1C and 1E; 92%, 46/50 pairs) protein abundance was significantly greater in leiomyoma as compared to matched myometrium. Immunohistochemical analysis confirmed the Western blots analysis and demonstrated both IDO1 and TDO2 were localized predominantly in the smooth muscle cells with greater expression in fibroid as compared with myometrium. Both enzymes were also localized with less staining intensity in fibroblasts, lymphocytes, and vascular endothelial cells (Fig. 1F).

The expression analysis of IDO1 and TDO2 mRNA based on race/ethnicity, cycle phase and MED12 mutation status are shown in Fig. 2. The expression of TDO2 in terms of fold change (leiomyoma vs. matched myometrium) indicated significantly greater expression of TDO2 mRNA in AA (N=25) as compared with Caucasian (N=12) and Hispanic groups (N=21), with no significant race-dependent differences in IDO1 mRNA expression (Fig. 2A–B). There were no menstrual cycle phase dependent differences in the expression of IDO1 and TDO2 mRNA (Fig. 2C–D). Because somatic MED12 mutations in exon 2 occur at high frequency in leiomyomas (6, 7), we analyzed our data based on the MED12 mutation status. This analysis indicated that TDO2 mRNA but not IDO1 mRNA was expressed in greater abundance in tumors bearing the MED12 mutation (N=39) as compared with wild type tumors (N=19) (Fig. 2E–F). There were no correlations between the expression of TDO2 mRNA and different types of MED12 missense mutations in the specimens analyzed.

The protein expression of IDO1 and TDO2 was also analyzed based on race/ethnicity, menstrual cycle phase and MED12 mutation status. As shown in Fig. 3, race/ethnicity (Fig. 3A), menstrual cycle phase (Fig. 3C) and MED12 mutation status (Fig. 3E) did not influence IDO1 protein expression. There were no significant effects of race/ethnicity (Fig. 3B) or menstrual cycle phase (Fig. 3D) on TDO2 protein levels. However, in agreement with the mRNA analysis, TDO2 protein levels were significantly higher in fibroid tumors bearing the MED12 mutation as compared with wild type tumors (Fig. 3F).

The functional relevance of IDO1 and TDO2 in fibroid pathogenesis was determined by the influence of these enzymes on MSMC/LSMC spheroids cell proliferation and expression of

extracellular matrix (ECM) proteins. The effects of inhibitors of IDO1 and TDO2 on MSMC and LSMC spheroids cell proliferation as determined by CellTiter-Glo 3D Cell Viability Assay are shown in Fig. 4A–B. The IDO1 inhibitor, Epacadostat in various concentrations had no effect on MSMC and LSMC spheroids cell proliferation (Fig. 4A). In contrast, the TDO2 inhibitor, 680C91 in various concentrations significantly inhibited LSMC but not MSMC spheroid cell proliferation (Fig. 4B). The IDO1 inhibitor Epacodostat did not have any effect on the expression of ECM proteins, COL1A1 and COL3A1 (Fig. 4C). However, inhibition of TDO2 by 680C91 significantly reduced the expression of COL1A1 and COL3A1 in LSMC spheroids with no significant effect on expression of these proteins in MSMC spheroids (Fig. 4D).

Discussion

The results presented here demonstrate for the first time that the metabolism of Trp in fibroid tumors as characterized by the upregulation of the principal enzymes involved in its degradation is dysregulated. The levels of kyneurenine, a marker of IDO1/TDO2 enzyme activity as expected was higher in fibroids as compared to myometrium. The upregulation of TDO2 protein and mRNA in most specimens analyzed, the degree of its upregulation and the response to its inhibition on cell proliferation and ECM proteins as compared to IDO1 suggest a greater significance of TDO2 dysregulation relative to IDO1 in fibroid pathogenesis. The upregulation of TDO2 but not IDO1 mRNA in fibroids was dependent on race. Furthermore, TDO2 but not IDO1 mRNA and protein levels were more abundant in tumors bearing the MED12 mutation. The race and MED 12 dependent expression of TDO2 further point to the physiological relevance of TDO2 dysregulation to fibroid pathology. The pharmacologic inhibition of TDO2 but not IDO1 in MSMC and LSMC spheroids led to selective inhibition of cell proliferation and expression of COL1A1 and COL3A1 in LSMC spheroids but not MSMC spheroids cultures. Additional studies will be required to determine the effects of TDO2 and IDO1 inhibitors on other ECM components and potential clinical use of these drugs for treatment of fibroids.

The literature pertaining to the Trp metabolic pathway has so far primarily been restricted to the endometrium and mainly focused on serotonin effects which is a minor by product of Trp metabolism. The presumed source of serotonin in the uterus is from the mast cells which are abundantly found in myometrium (25). Serotonin stimulated collagenase production (26) by uterine smooth muscle via IL-1α but not IL-1β (27), and treatment of myometrial cells with serotonin inhibited collagen type I, collagen type III and fibronectin mRNA expression (28). Several studies have examined the expression of tryptophan catabolic enzymes in the endometrium. In the mouse endometrium, TDO2 was upregulated during decidualization, and both estrogen, and progesterone could induce its expression. In this same study TDO2 regulated endometrial cell proliferation, and its overexpression upregulated AhR, COX2 and VEGF in uterine stromal cells (29).

Several regulatory mechanisms have been reported to date for TDO2, including hormonal induction by glucocorticoids (GC) (30–32) and other hormones such as insulin, and glucagon (33), substrate activation by Trp, cofactor activation by heme (34), feedback inhibition by NAD(P)H (14), and more recently by NF-κB the master regulator of

inflammation (35). Previous studies by us and others have shown the relative importance of activation of inflammatory pathways (36) and NF-κB (37) in fibroids. Thus, inflammatory activation could be the mechanism driving TDO2 expression in fibroid tumors. Diet can also selectively influence the expression of these enzymes by virtue of its effect on the glucocorticoids (GC), and mice fed a high fat diet had increased hepatic expression of TDO2 and IDO2 but not IDO1 (38). Our results demonstrating a dysregulation of Trp catabolism provides a potential link between diet and its influence on fibroid development. In contrast to TDO2 which is regulated by GC, IDO1 is not inducible by GC (14), and its principal effector is IFNγ and to a lesser degree IFNα (39, 40). There is evidence for increased expression of IFNγ in fibroids (36, 41) which could be contributing to IDO1 upregulation in fibroids in select contexts such as immune activation.

A number of findings here point to a greater relevance of TDO2 overexpression relative to IDO1 in fibroid pathogenesis. These factors include 1). Universal overexpression of TDO2 mRNA/protein in the specimens analyzed, 2). A far greater fold increase in TDO2 relative to IDO1 in fibroids, 3). Race and MED12 dependence of TDO2 but not IDO1 expression in fibroids and 4). Responsiveness to the TDO2 but not IDO1 inhibitor in terms of inhibition of LSMC cell proliferation and expression of COL1A1 and COL3A1. The lack of response to IDO1 inhibitor may be because the upregulation of the enzyme occurred only in a limited number of specimens, or the degree of upregulation was not as pronounced as for TDO2, and or the possibility that the inhibitor used lacked the necessary potency. Based on our current findings targeting TDO2 inhibition rather than IDO1 holds a greater promise for therapeutic purposes, although additional studies will need to be conducted to decipher the relative significance of these enzymes.

It is well established that race is a significant risk factor for fibroid development and progression with AA as compared to Caucasians having higher prevalence, earlier onset and greater symptom severity from fibroid tumors (1). The greater expression of TDO2 mRNA in fibroids from AA patients as compared with Caucasian and Hispanic patients could be a factor contributing to the racial disparity associated with fibroids, although our findings will need confirmation in a much larger set of specimens from different racial/ethnic groups. In contrast to TDO2 mRNA, TDO2 protein levels were not significantly different among the racial groups studied. This disconnect between mRNA and protein levels could be secondary to multiple factors, such as protein stability as regulated by ubiquitin-proteosome pathway or autophagy which are independent of transcript concentrations, protein translation rates as modulated by factors such as non-coding RNAs or protein binding to regulatory elements in the transcript, and others (42). These same mechanisms could also explain our findings demonstrating a more than 93-fold average increase in TDO2 mRNA levels in fibroids in contrast to protein levels which were upregulated on an average by 5.2-fold.

Recent studies have demonstrated that leiomyomas bear somatic MED12 mutations in exon 2 regardless of race/ethnicity in high frequency, however the role of these mutations in the leiomyoma pathogenesis remains undetermined (6, 7). MED12 is known to regulate the function of important pathways in fibroid pathogenesis, including the Wnt/β-catenin (11), hedgehog (43) and transforming growth factor (TGF)-β receptor signaling pathways (44). Currently there are no reports exploring the effect of MED12 mutation on expression of

TDO2 in any tissue or cell type. Our findings demonstrate higher expression of TDO2 mRNA and protein in MED12 mutated leiomyomas as compared with wild type tumors. The mechanism underlying this correlation between MED12 mutation and TDO2 expression remains to be determined although it may be speculated that mutations in MED12 which are gain of function mutations could lead to recruitment of transcription factors to the promoter of TDO2 gene to induce its transcription.

The initial breakdown product of Trp by IDO1 and TDO2 is kynurenine which is a biologically active metabolite as it is an endogenous ligand of Aryl hydrocarbon receptor (AhR) (45). The upregulation of IDO1 and TDO2 in fibroids as would be expected and as indicated by our data resulted in higher levels of kynurenine in fibroids. Although it remains to be demonstrated, kynurenine would be expected to bind to AhR, translocate to the nucleus and bind to xenobiotic response elements in the promoter of target genes such as CYP1A1 and CYP1B1 thereby inducing their transcription (46, 47). Activation of AhR has pro- or anti-fibrotic effects depending on the tissue and context. In skin fibroblast kynurenine was shown to increase MMP 1 and 3 expressions in cultured dermal fibroblast by activating MAPK signaling pathway (48), and have anti-fibrotic effects in rabbit ear model (49). In this same model an AhR antagonist inhibited kynurenine-dependent regulation of CYP1A1, MMP1 and type 1 collagen (49). Additional studies will be needed to clarify the function of increased kynurenine production in fibroid progression.

In summary, we provide evidence for a marked dysregulation of Trp catabolism in fibroid tumors. Our data indicates a marked upregulation of TDO2 protein and mRNA and to a lesser degree and in a more limited number of specimens, IDO1 mRNA and protein. The expression of TDO2 but not IDO1 was race and MED12 dependent. Pharmacologic inhibition of TDO2 but not IDO1 inhibited LSMC but not MSMC spheroids cell proliferation and expression of COL1A1 and COL3A1. These results suggest that targeted inhibition of TDO2 could have therapeutic benefit for treatment of fibroids.

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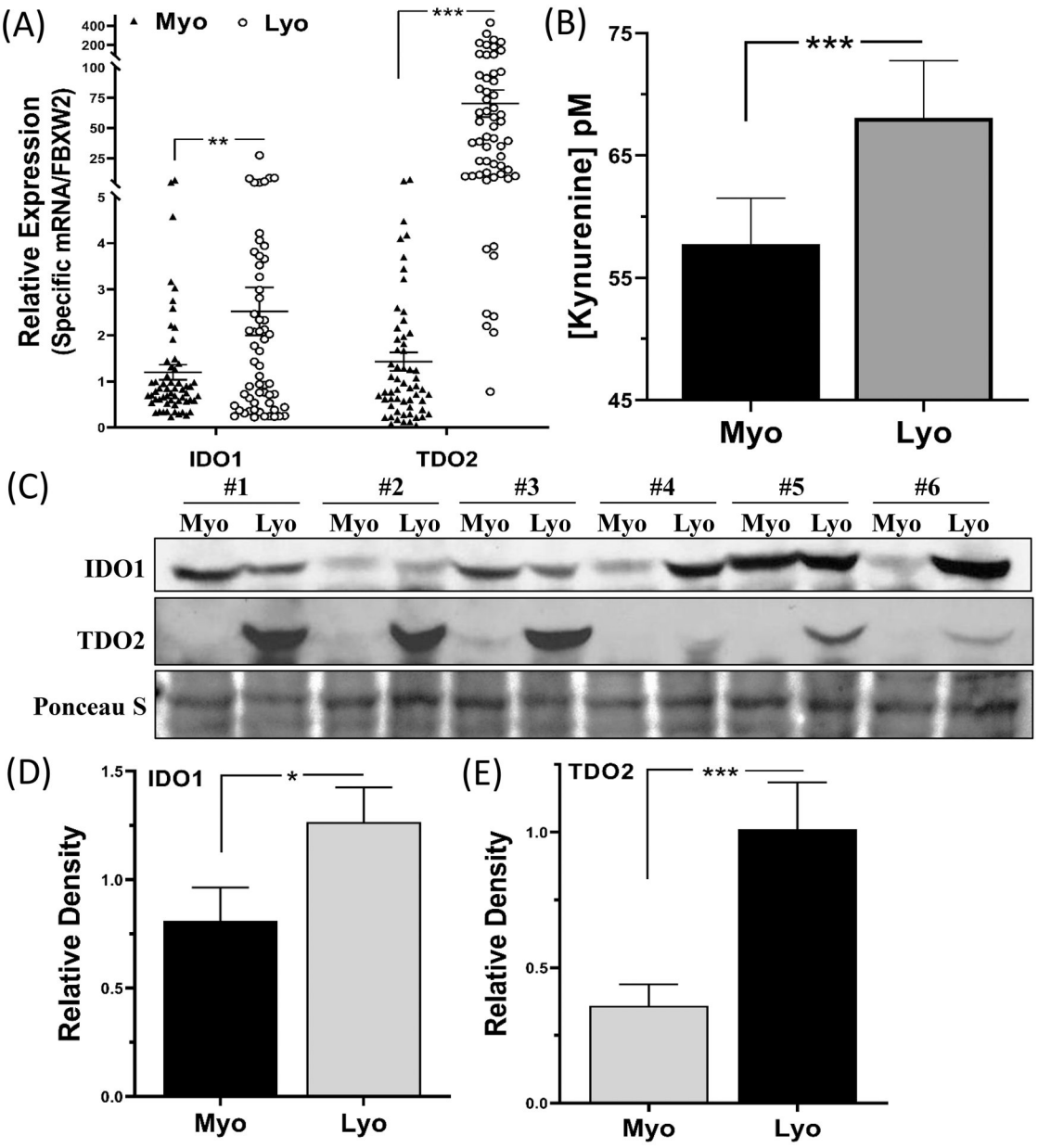

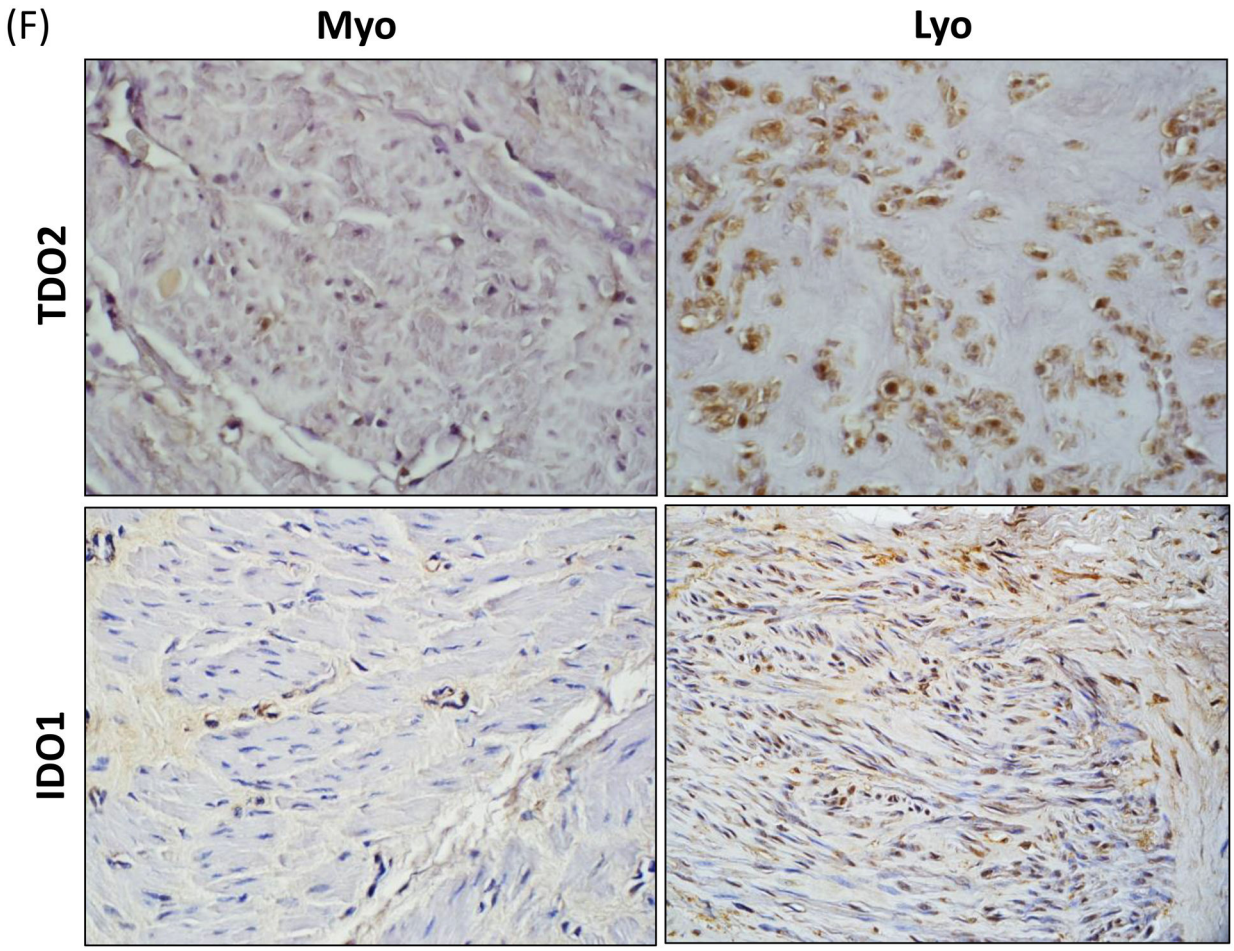

Figure 1.

(A) The expression of IDO1 and TDO2 in 58 paired myometrium (Myo) and leiomyoma (Lyo). The results in this and subsequent figures are presented as mean ± SEM with P values (*P<0.05; **P<0.01 and ***P<0.001) indicated by corresponding lines. (B) Kynurenine levels as determined by ELISA in 46 paired myometrium (Myo) and leiomyoma (Lyo). (C) Western blot analysis of IDO1 and TDO2 in paired (n=50) myometrium (Myo) and leiomyoma (Lyo) with bar graphs for IDO1 (D) and TDO2 (E) showing their relative band densities in myometrium and leiomyoma. (F) Localization of IDO1 (x400) and TDO2 (x400) in myometrium and leiomyoma as determined by immunohistochemistry.

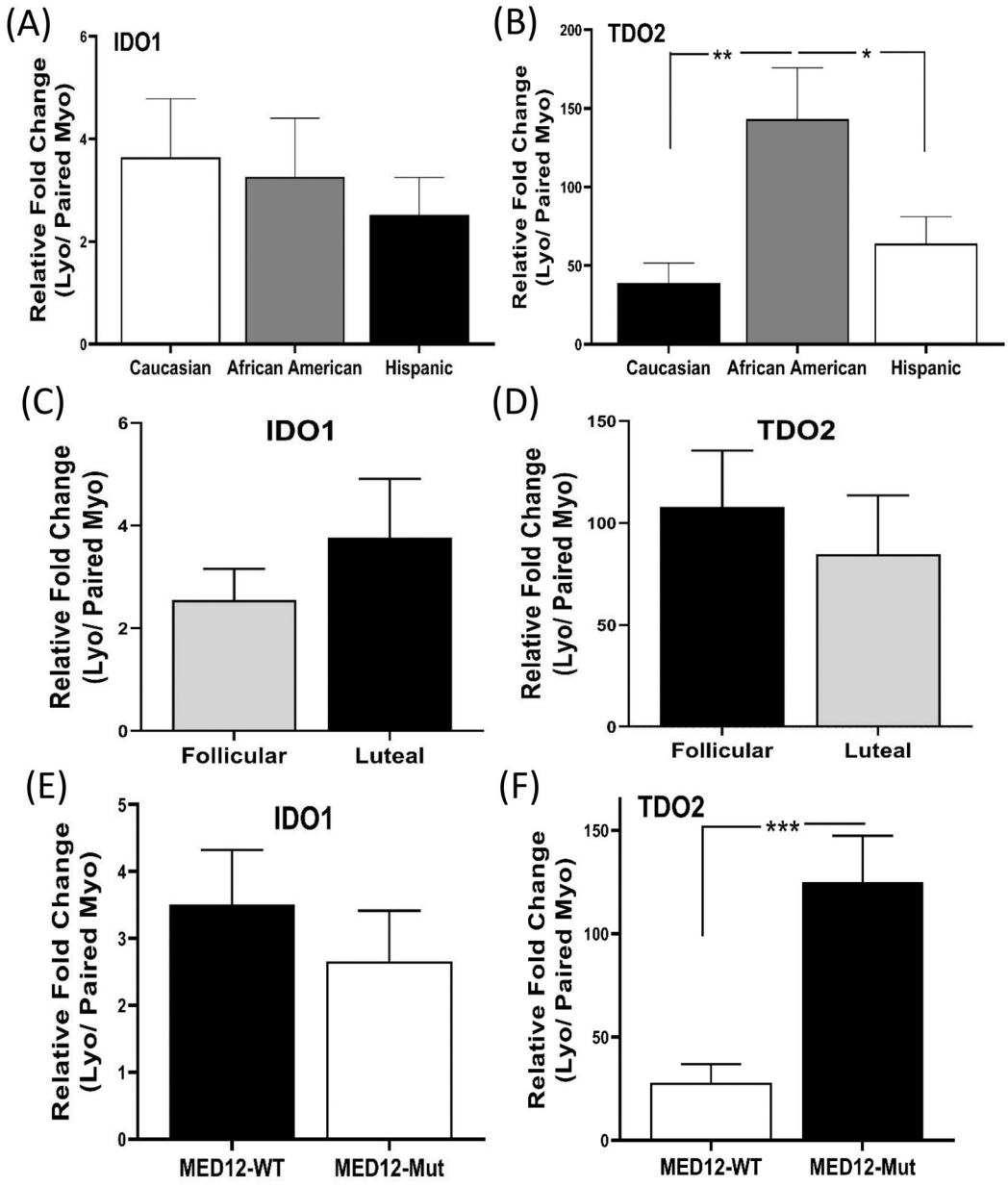

Figure 2.

Relative mRNA expression of IDO1 (A; C; E) and TDO2 (B; D; F) expressed as fold change (Lyo/paired Myo) based on race/ethnicity in Caucasian (n=12), African American (n=25) and Hispanics (n=21) (A-B); menstrual cycle phase in proliferative phase (n=27) and secretory phase (n=14) (C-D); MED12 mutation status in wild type (n=19) and MED12 bearing mutations (n=39) (E-F). The results are presented as mean ± SEM with P values (*P<0.05; **P<0.01 and ***P<0.001) indicated by corresponding lines.

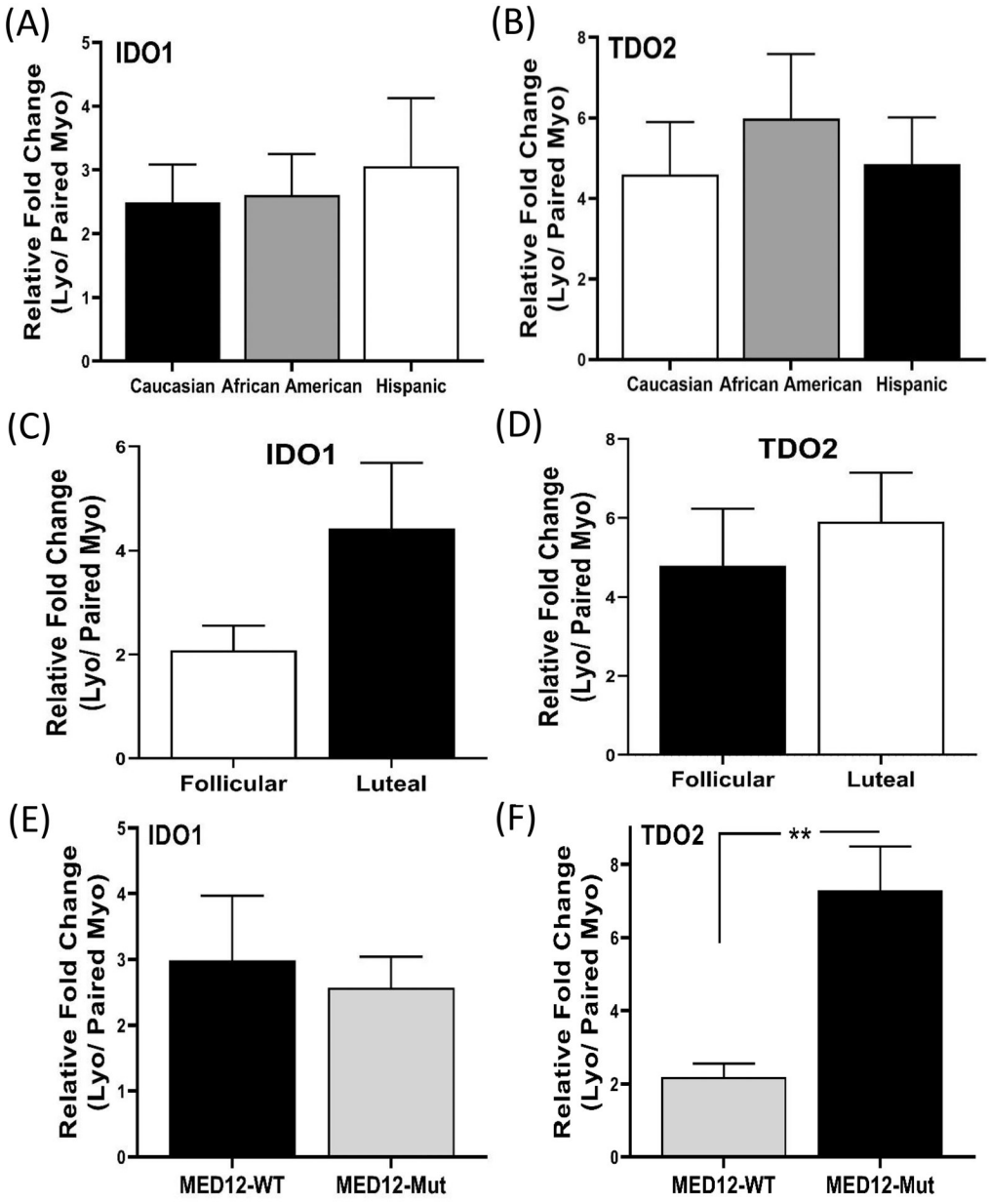

Figure 3.

Relative protein expression of IDO1 (A; C; E) and TDO2 (B; D; F) expressed as fold change (Lyo/paired Myo) based on race/ethnicity in Caucasian (n=12), African American (n=20) and Hispanics (n=18) (A-B); menstrual cycle phase in proliferative phase (n=19) and secretory phase (n=15) (C-D); MED12 mutation status in wild type (n=20) and MED12 bearing mutations (n=30) (E-F). The results are presented as mean ± SEM with P values (**P<0.01) indicated by corresponding lines.

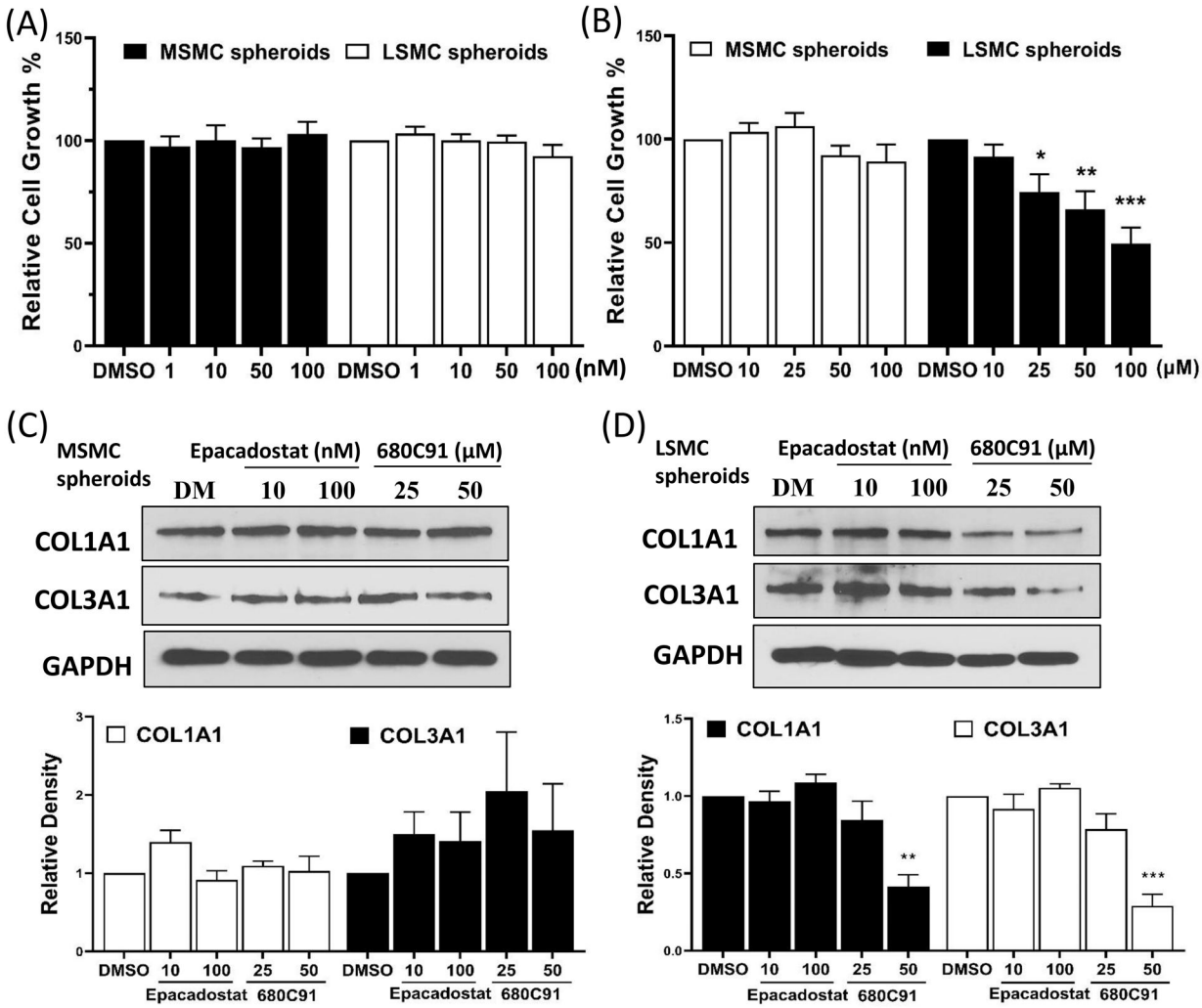

Figure 4.

The effect of IDO1 inhibitor Epacadostat (A) and TDO2 inhibitor 680C91 (B) on MSMC and LSMC spheroids cell proliferation as determined by the CellTiter-Glo 3D Cell Viability Assay (n=4). (C-D) Representative immunoblots demonstrating COL1A1 and COL3A1 expression following 48 hours treatment with different concentrations of IDO1 inhibitor (Epacadostat) and TDO2 inhibitor (680C91) in MSMC spheroids (C) and LSMC spheroids (D) with bar graphs show the relative protein band densities (n=4). The results are presented as mean ± SEM of independent experiments. *P<.05; **P<.01; ***P<.001