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Platelet-Derived Growth Factor C Is Upregulated in Human Uterine Fibroids and Regulates Uterine Smooth Muscle Cell Growth¹

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ABSTRACT

Leiomyomata uteri (i.e., uterine fibroids) are benign tumors arising from the abnormal growth of uterine smooth muscle cells (SMCs). We showed here that the expression of platelet-derived growth factor C (PDGFC) is higher in approximately 80% of uterine fibroids than in adjacent myometrial tissues examined. Increased expression of PDGFC is also observed in fibroid-derived SMCs (fSMCs) relative to myometrial-derived SMCs (mSMCs). Recombinant bioactive PDGFC homodimer stimulates the growth of fSMCs and mSMCs in ex vivo cultures and prolongs the survival of fSMCs in Matrigel plugs implemented subcutaneously in immunocompromised mice. The knockdown of PDGFC receptor-alpha (PDGFRα) through lentiviral-mediated RNA interference reduces the growth of fSMCs and mSMCs in ex vivo cultures and in Matrigel implants. Furthermore, two small molecule inhibitors of the PDGFR tyrosine kinase (i.e., imatinib and dasatinib) exerted negative effects on fSMC and mSMC growth in ex vivo cultures, albeit at concentrations that cannot be achieved in vivo. These results suggest that the PDGFC/PDGFRα signaling module plays an important role in fSMC and mSMC growth, and that the upregulation of PDGFC expression may contribute to the clonal expansion of fSMCs in the development of uterine fibroids.

dasatinib, imatinib, lentivirus, PDGF receptor, shRNA

INTRODUCTION

Uterine fibroids are the most common benign tumors among women of reproductive age. The cumulative incidence of fibroids by age 50 yr is more than 80% for black women and nearly 70% for white women [1]. Although uterine fibroids rarely progress to malignancy, they can cause a variety of symptoms that require clinical intervention. In fact, uterine fibroids are the primary indication for hysterectomy, accounting for more than 200,000 hysterectomies annually in the United States [2]. Fibroids are clonal tumors, each derived from the growth of a single uterine smooth muscle cell (USMC) [3]. Clinical observations suggest that the fibrotic transformation and clonal expansion of fibroid SMCs (fSMCs) is a common event in otherwise healthy women; however, the mechanisms underlying this transformation and the subsequent clonal expansion are poorly understood [4].

The clonal expansion of fSMCs is likely to be driven by mitogenic pathways downstream of receptor tyrosine kinases (RTKs), because RTK-dependent signaling is a common mechanism in the stimulation of cell proliferation [5]. Therefore, inhibition of RTKs that play a critical role in the proliferation of USMCs may interfere with the growth of fibroid tumors. To identify RTKs that are involved in fibroid growth, we constructed a custom microarray to question the expression of RTKs and their ligands in fibroid and surrounding myometrial tissues collected from 42 patients undergoing hysterectomy for symptomatic fibroids. This focused expression profiling study has led to the molecular classification of patient samples into three groups according to the tissue RNA levels of two genes, CYR61 and EFNA4, which encode extracellular ligands that regulate RTKs and non-receptor tyrosine kinases. The three subgroups defined by our study are: 1) comparable levels of CYR61 and EFNA4 RNA in fibroids and adjacent myometrial tissues; 2) CYR61 RNA downregulated and EFNA4 RNA upregulated in fibroids; and 3) CYR61 RNA downregulated but EFNA4 RNA not upregulated in fibroids. This result suggests that the ligand regulators of protein tyrosine kinase (TK) activities, rather than the expression of protein TKs per se, are misexpressed in fibroid tissues. In addition to CYR61 and EFNA4, we found that the PDGFC gene expression is also upregulated in fibroid tissues across the three groups of patient samples.

Platelet-derived growth factors (PDGFs) play crucial roles in the regulation of a wide range of biological processes, including cell proliferation, survival, migration, angiogenesis, tissue remodeling, and organogenesis (e.g., the development of axial skeleton, palate, teeth, and the cardiovascular system) [6, 7]. The PDGFC gene family consists of four members: PDGFA, PDGFB, PDGFC, and PDGFD, which exert their biological functions by binding to and activating two receptor TKs (PDGF receptor alpha [PDGFRα] and PDGFRβ). PDGFC contains an N-terminal CUB domain, a hinge region, and a C-terminal growth factor domain, which has to be released by proteolysis to bind to PDGFRα [8, 9]. The proteolytically processed homodimer (PDGFCC) binds to PDGFRα/β homodimers with high affinity but fails to interact with the PDGFRβ/β homodimers [8]. Among normal human tissues, PDGFC RNA is highly expressed in the heart, the pancreas, the liver, and the kidney [9], and it has been linked to fibrosis and tumorigenesis in various organs [10–12].

In this study, we examined the expression of the four members of the PDGF family (PDGFA, PDGFB, PDGFC, and PDGFD) and their TK receptors (PDGFRα and PDGFRβ) in fibroid and adjacent myometrial tissues. We also established a panel of primary SMC cultures from the fibroid and myometrial tissues of 31 patients and determined their growth response to PDGFCC in cultures and in Matrigel plugs grown subcutaneously in immunocompromised mice. The results of
this study establish PDGFCC as a mitogen for USMCs, thus suggesting that the upregulation of PDGF expression in fibroid tissues may promote the clonal expansion of transformed USMCs in the formation of fibroids.

MATERIALS AND METHODS

Materials

CodeLink slides were purchased from Amersham. Dulbecco modified Eagle medium (DMEM) and penicillin-streptomycin (PS) were purchased from Cellgro (Mediatech Inc.). Collagenase II, fetal bovine serum (FBS), puromycin, polybrene, and protease cocktail were purchased from Sigma. MicroRNA-adapter short hairpin RNA (shRNA/mir) target sets designed for PDGFRA and PDGFRB lentiviral vector were purchased from Open Biosystems (Thermo Scientific). Transfection reagent (GeneTrans) and plasmid maxi kit was purchased from Bioanalytic (San Diego, CA). Bicinchoninic acid protein assay kit was purchased from Pierce, and Agilent Low Input cRNA Amplification Kit was purchased from Biomiga (San Diego, CA). Bicinchoninic acid protein assay kit was purchased from Bioanalytic. Anti-PDGFRA (c-20) antibody and goat anti-rabbit horseradish peroxidase (HRP)-conjugated secondary antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-GAPDH was purchased from Chemicon International. Anti-ACTA2 antibody was purchased from Abcam. Alexa Fluor 488 donkey anti-rabbit immunoglobulin G (IgG), 594 donkey anti-goat IgG, and 594 donkey anti-mouse IgG were purchased from Invitrogen. Dako universal LSAB kit (Dako k0679) was purchased from Dako. Cell Counting Kit 8 (CCK-8) was purchased from Dojindo (Kumamoto, Japan). Bioactive recombinant human PDGFCC protein was purchased from R&D Systems. RAG2+/γ−/− (double knockout of IgH and γc) mice were purchased from the Jackson Laboratory. The manufacturer instructions. Polymerase chain reaction (PCR) reactions, and 31 sets of RNA were extracted from frozen fibroid tissues and primary myometrial tissues and was converted into cDNA labeled with either Cy5 or Cy3 with the Agilent Low Input cRNA Amplification Kit following the manufacturer’s instructions. After hybridization, the slides were scanned with an Axon Instruments GenePix 4000B scanner, and the signals were quantified and extracted with the GenePix Pro 5.0 software (Axon Instruments). The fluorescence signals were normalized with the quantile normalization algorithm (R-package DNAmer. J. Cabrera, Rutgers University. www.rmetric.sacks. edu/~cabrera/DNAMR/) and fitted with a linear mixture-effect model (provided by R-package DNAMR. J. Cabrera, Rutgers University. www.rmetric.sacks. edu/~cabrera/DNAMR/) and fitted with a linear mixture-effect model (provided by R-package DNAMR. J. Cabrera, Rutgers University. www.rmetric.sacks. edu/~cabrera/DNAMR/) and fitted with a linear mixture-effect model (provided by R-package DNAMR. J. Cabrera, Rutgers University. www.rmetric.sacks. edu/~cabrera/DNAMR/). The ratios of normalized signals were derived from pairs of fibroid vs. myometrium, hierarchically clustered and displayed with the Genesis program [14].

Primary culture of USMCs. Fibroid and myometrial tissues (wet weight, 1 g) were minced into 1-mm3 pieces, placed in 2 ml of PBS containing 0.4% collagenase II, and then incubated at 37°C for 3–5 h. The digested tissue mixtures were washed with 10 ml of DMEM containing 10% (vol/vol) FBS and 1% PS, and the supernatant was discarded after 5 min of centrifugation at 1000 × g. The pellets were resuspended in 10 ml of DMEM (containing 10% FBS and 1% PS) and plated on plates. The digested tissues were cultured in a humified 5% CO2 incubator at 37°C without any disturbance until they became attached at the bottom of the plate. Fresh media were added every 3 days, and a monolayer of SMCs was established after 1 wk. Confluent monolayers were dissociated with trypsin and split into fresh dishes. On average, the plates were split once every week. The SMCs were characterized by immunofluorescence staining with anti-ACTA2 antibody. The primary cultures of USMCs were infected for qRT-PCR, lentiviral transduction, and proliferation assays between passages two and five.

Lentivirus-mediated RNA interference targeting PDGFRA. Five shRNA/mir target sets designed for PDGFRA were inserted in the pGIPZ lentiviral vector, in which the TurboGFP and the puromycin resistance gene plus shRNA/mir are expressed in a single transcript, allowing for the green fluorescent protein (GFP) marking and the puromycin selection of shRNA/mir expressing cells. The plasmids were prepared with plasmid maxi kit and transfected in HEK293FT cells (Invitrogen) with the transfection reagent (GeneTrans) to produce lentiviral particles. Briefly, HEK293FT cells were cultured in DMEM medium supplemented with 10% FBS and 1× PS. Cultures with 80% confluent cells in 15-cm dishes were cotransfected with the lentiviral plasmid (20 μg; shRNA/mir plasmid or pGIPZ vector plasmid), the lentiviral packaging plasmids pRSV-Rev (5 μg) and pMDL/pRRE (10 μg), and the vesicular stomatitis virus G glycoprotein expression vector pMD2G (6 μg). The transfection method was performed according to the manufacturer’s instructions (Biomiga). The culture supernatants were collected at 2 days after transfection and filtered through a 0.45-μm filter (Millipore). The filtrate was concentrated by centrifugation for 2 h at 25 000 rpm (in an SW-28 rotor; Beckman) and resuspended in PBS. The lentivirus titer determination was performed according to the manufacturer’s instructions (Open Biosystems). Briefly, 2 × 105 HEK293 cells were seeded in the 24-well plate and cultured for 1 day. The cells were then infected by serial dilutions of concentrated viral stocks for 1 day. After culturing for 4 days in fresh medium supplemented with puromycin (1 μg/ml), the positive GFP-expressing colonies were counted under fluorescence microscopy. The transducing units per milliliter (TU/ml) was determined by total GFP-positive colonies induced by 1 ml of viral stock. For a typical preparation, the viral titer was from approximately 107 to 108 TU/ml.

The primary cultures of USMCs at 80% confluency were infected with the concentrated lentiviral stocks at 20–50 multiplicity of infection (MOI) in the manufacturer’s instructions. Contaminating genomic DNA was removed on the columns using the RNase-Free DNase Set. Total RNA (2 μg) was reverse transcribed in a 50-μl reaction system using the SuperScript II kit according to the manufacturer’s instructions. qRT-PCR reactions were performed using the DAKO LSAB+ System-HRP kit according to the manufacturer’s instructions. Briefly, endogenous peroxidase activity was determined by total GFP-positive colonies induced by 1 ml of viral stock. For a typical preparation, the viral titer was from approximately 107 to 108 TU/ml. The primary cultures of USMCs at 80% confluency were infected with the concentrated lentiviral stocks at 20–50 multiplicity of infection (MOI) in the presence of 8 μg/ml polybrene for 24 h and then changed to DMEM medium supplemented with puromycin (1 μg/ml) for 6–7 days to select for cells stably infected with the lentivirus. The infected USMCs were cultured in fresh medium for 5 days after puromycin selection and before qRT-PCR and immunoblotting experiments to determine the efficiency of PDGFRA knockdown.

RNA extraction and qRT-PCR. Total RNA from tissues and primary cultures of USMCs were extracted using the RNaseasy Mini Kit according to the manufacturer’s instructions. Contaminating genomic DNA was removed on the columns using the RNase-Free DNase Set. Total RNA (2 μg) was reverse transcribed in a 50-μl reaction system using the SuperScript II kit according to the manufacturer’s instructions. qRT-PCR reactions were performed using the DAKO LSAB+ System-HRP kit according to the manufacturer’s instructions. Briefly, endogenous peroxidase activity was determined by total GFP-positive colonies induced by 1 ml of viral stock. For a typical preparation, the viral titer was from approximately 107 to 108 TU/ml. The primary cultures of USMCs at 80% confluency were infected with the concentrated lentiviral stocks at 20–50 multiplicity of infection (MOI) in the presence of 8 μg/ml polybrene for 24 h and then changed to DMEM medium supplemented with puromycin (1 μg/ml) for 6–7 days to select for cells stably infected with the lentivirus. The infected USMCs were cultured in fresh medium for 5 days after puromycin selection and before qRT-PCR and immunoblotting experiments to determine the efficiency of PDGFRA knockdown.

Immunohistochemistry. Frozen sections (5 μm) of myometrial and fibroid tissues were air dried, fixed in 10% formalin for 10 min, and washed in PBS three times each for 5 min. The immunohistochemistry reactions were performed using the DAKO LSAB+ System-HRP kit according to the manufacturer’s instructions. Briefly, endogenous peroxidase activity was blocked with 3% hydrogen peroxide for 5 min. After rinsing and aspiration, antibodies diluted in blocking solution (0.05 mol/L Tris-HCl buffer with 1% bovine serum albumin [BSA]) were applied, and the slides were incubated at 4°C for 18 h. Antibodies and dilutions used were PDGFRA (1:150) and PDGFB (1:150). After rinsing with wash buffer (Tris-bufffered saline with Tween) and aspiration, the specimens were covered with biotinylated link buffer (biotinylated anti-rabbit immunoglobulin for PDGFRA, and anti-goat immunoglobulin for PDGFB; DAKO) and incubated for 15 min. The sections were rinsed with wash buffer, incubated with streptavidin-peroxidase (Dako) for 15 min, washed, and then reacted with the substrate-chromogen (Dako) solution for 10 min. The slides were rinsed gently with Tris-buffered saline three times, counterstained with hematoxylin, rinsed with distilled water three times, and then mounted for microscopic evaluation. The sections stained with secondary antibody only were the control.
**Results**

Levels of PDGF and PDGFR RNAs in Uterine Tissues

We have examined the expression profiles of 90 human TKs and 103 ligands known to activate TKs in the fibroid and adjacent myometrial tissues from 42 patients. The microarray hybridization results suggested that the *PDGFC* RNA levels were higher in fibroid than in matching myometrial tissues in 28 of the 42 patients (Fig. 1A). However, the RNA levels of *PDGF*, *PDGFB*, and *PDGFD*, and those of the *PDGFR* receptors (*PDGFA* and *PDGFRB*) were not significantly altered between the matched fibroid and myometrial tissues (Fig. 1A). To confirm the microarray results, we applied qRT-PCR to measure the RNA levels of *PDGF*, *PDGFB*, *PDGFD*, and *PDGFR* in 49–76 pairs of fibroid and adjacent myometrial tissues. The mean level of *PDGFC* RNA in the fibroids was significantly higher than that in the myometrial tissues (P < 0.01; Fig. 1B). The ratio of *PDGFC* RNA (fibroid:adjacent myometrium) in 57 of 70 pairs was equal to or higher than 1.5-fold. By contrast, the *PDGFB* and *PDGFD* RNA levels in fibroid tissues were lower than those in myometrial tissues, and the levels of the *PDGFA* RNA were similar between matched fibroid and myometrial tissues (Fig. 1B).

Unlike *PDGFC*, the levels of *PDGFR* RNAs were not increased in fibroid tissues. Instead, the *PDGFRB* RNA levels were slightly higher in myometrial tissues (P < 0.01) from 58 patients, whereas the *PDGFA* RNA levels were similar between fibroid and adjacent myometrial tissues from 76 patients (Fig. 1C). Pearson correlation analysis showed that there was no correlation between the levels of *PDGFC* and *PDGFA* RNAs in fibroid or adjacent myometrial tissues from 69 patients (Fig. 1D and E). To determine that the *PDGFC* RNA levels reflect the production of PDGFC proteins in the fibroid and myometrial tissues, we performed immunohistochemistry analyses of six pairs of myometrial and fibroid tissue sections and found higher levels of anti-PDGFC signals in fibroid than adjacent myometrial tissues in each pair of samples. Representative results from two pairs of tissues are
shown in Figure 1F. We also detected the expression of PDGFR in the tissue sections (Fig. 1G), consistent with a previously published report [18]. These results show that PDGFC expression is upregulated in the majority of fibroid tissues, but there is not a coordinated upregulation of its receptor, PDGFRA, in fibroids.

**PDGFC and PDGFRA Expression in Primary Cultures of USMCs**

We prepared pairs of primary cultures of fSMCs and mSMCs from 31 patient samples, and we measured the RNA levels of PDGFC and PDGFRA by qRT-PCR (Fig. 2, A and C). We found that the levels of PDGFC RNA were 1.7-fold higher in the fSMC group than in the mSMC group (P < 0.05; Fig. 2A), whereas the levels of PDGFRA RNA were similar between the two groups of cells (Fig. 2C). From the scatter plots, it is clear that the levels of PDGFC RNA were much higher in tissues than in the primary cultures (Fig. 2, A and B). Using the Pearson correlation test, we found no correlation between the levels of PDGFC RNA among the group of mSMCs (Fig. 2I) but not with the group of fSMCs (Fig. 2J). To
To determine whether PDGFCC stimulates the growth of fSMCs and mSMCs, we examined the effect of recombinant PDGFCC homodimer, composed of its C-terminal growth factor domain, on the proliferation of mSMC and fSMC cultures derived from three patients (Fig. 3, A–F). The control cultures (serum-free medium without PDGFCC) exhibited a low rate of growth during the experimental time course. The addition of PDGFCC stimulated the growth of mSMC and fSMC cultures, with the rate of proliferation significantly increased by the supplement of PDGFCC in each of the three pairs of SMC cultures tested (Fig. 3, A–F).

We also examined the expansion of fSMCs in Matrigel plugs implanted subcutaneously into immunocompromised mice (Fig. 3, G and H). We infected fSMC cultures with lentivirus carrying a GFP-Luciferase fusion gene [15], injected the infected cells with Matrigel subcutaneously into RAG2−/−/γc−/− female mice that were previously implanted with estrogen pellets, and followed the expansion of the injected cells by bioluminescence imaging for Luciferase activity (Fig. 3, G and H). In Matrigel plugs without PDGFCC, an initial expansion of Luciferase signal at Day 2 was quickly followed by a decline in bioluminescence signals (Fig. 3H). In Matrigel plugs with the recombinant bioactive PDGFCC...
supplement of PDGFCC, the initial expansion of Luciferase signals was not significantly enhanced; however, the decay of the signals was significantly slower during the next 7 days (n = 3; P < 0.05; Fig. 3H). These results show that PDGFCC promotes the survival of fSMCs as Matrigel implants in immunocompromised mice.

**Effect of PDGFRA Knockdown on the Proliferation of USMCs**

Given the knowledge that PDGFC binds PDGFRA and does not interact with PDGFRB homodimers [9], we examined the role of PDGFRA in the proliferation of fSMCs and mSMCs. We employed lentivirus-mediated RNA interference to knock down PDGFRA expression in two pairs of mSMC and fSMC cultures derived from two patient samples (P11 and P31; Fig. 4). By testing five different microRNA-adapted shRNAmir target sets designed for PDGFRA in the human glioblastoma cell line U118, we identified one target set (Open Biosystems clone ID V2LHS 58978, with the shRNA sequence TGCTGTTGACAGTGAGCGACCTCTATCCTTCCAAATGAAATAGTGAAGCCACAGATGTATTTCATTTGGAAGGATAGAGGGTGCCTACTGCCTCGGA) that could decrease PDGFRA RNA level by approximately 75% (data not shown). We then used this shRNAmir to knock down PDGFRA in mSMCs and fSMCs. As shown in Figure 4, A–C, cells infected with lentivirus carrying the shRNAmir cassette contained lower levels of the PDGFRA RNA (Fig. 4A) and the PDGFRA protein (Fig. 4B) than the uninfected controls or cells infected with lentivirus derived from the pGIPZ vector. The PDGFRA RNA was reduced by 74% in P11 mSMCs, 72% in P11 fSMCs, 48% in P31 mSMCs, and 52% in P31 fSMCs compared with uninfected or vector-infected controls (Fig. 4A), corresponding to lower levels of PDGFRA protein (Fig. 4B).

The reduction in PDGFRA protein expression was also observed by immunofluorescence staining with anti-PDGFRA of control and knocked-down cells (Fig. 4C).

We then examined the proliferation of these SMCs in cultures and found that the knockdown of PDGFRA consistently reduced the rate of proliferation in the mSMC and fSMC cultures from the two patients tested (Fig. 4, D–G). We also examined the expansion of control and PDGFRA-knocked down fSMCs in Matrigel plugs implanted in the RAG2−/−γc−/− female mice (Fig. 4, H and I). We observed the expansion of control and PDGFRA-knocked down fSMCs in the implanted Matrigel plugs during a period of 6 days (Fig. 4, H and I). However, the extent of the increase in Luciferase signals was significantly lower with PDGFRA-knocked down cells (n = 3; P < 0.05; Fig. 4I).

**Effects of TK Inhibitors on USMC Proliferation**

A number of small molecular inhibitors for protein TKs have been used successfully in the clinical setting to treat cancers. Among them, imatinib and dasatinib can inhibit the activity of PDGFRA TK [19–21]. We therefore tested the effects of imatinib and dasatinib on the proliferation of mSMCs and fSMCs. We treated 27 pairs of mSMC and fSMC cultures with varying concentrations of the drugs for a period of 96 h and employed a nonlinear regression model to calculate the IC50 value for each drug. The dose-response curves for imatinib and dasatinib on one pair of mSMC and fSMC cultures are shown in Figure 5, A, B, D, and E. As described previously, the proliferation of primary foreskin fibroblasts is not inhibited by clinically relevant doses of imatinib or
FIG. 4. Knockdown of PDGFRA reduced uterine SMC proliferation. The indicated primary cell cultures derived from two pairs of patient samples were uninfected (control [C]) or were infected with vector-lentivirus (V) or PDGFRA-shRNA-lentivirus (Sh). 

A) The levels of PDGFRA RNA determined by quantitative RT-PCR. Results shown are means and SDs from three independent measurements, a.u., arbitrary unit. 

B) Immunoblots of whole cell lysates from the indicated cell populations probed with antibodies against PDGFRA (upper) or GAPDH (lower) as a loading control. 

C) Immunofluorescence detection of PDGFA in control and knocked down (kd) cells. Bars = 40 μm; original magnification ×400. 

D-G) Cell proliferation of uninfected (control), vector-lentivirus-infected (vector), and PDGFRA-shRNA-lentivirus-infected (shRNAmir) cell populations derived from the indicated patient sample was determined. The values shown are means and SDs from three independent experiments with four wells of cells assayed per time point. The indicated primary cell cultures infected with GFP-luciferase lentivirus were mixed with Matrigel and then transplanted subcutaneously into RAG2<sup>−/−</sup>γ<sub>δ</sub><sup>−/−</sup> female mice. 

H) Representative imaging pictures of bioluminescence signals in Matrigel plugs. 

I) Quantification of bioluminescence signals. The values shown are means and SDs from three mice per experiment. *P < 0.05; **P < 0.01. In D–G, 10<sup>3</sup> is 10<sup>3</sup>. 

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Dasatinib. In our experiments, the IC_{50} of growth inhibition in human foreskin fibroblasts by imatinib was 27 nM, and that for dasatinib was 377 nM (Fig. 5, C and F). Under ex vivo culture conditions, we determined that the IC_{50} for imatinib was around 10 μM for the inhibition of mSMC and fSMC proliferation (Fig. 5G), and that for dasatinib was between 100 and 160 nM (Fig. 5H) among the 27 pairs of primary cultures tested. These IC_{50} values are also much higher than the clinically relevant dose. Consistent with these high IC_{50} values, we found that administration of imatinib or dasatinib to mice did not interfere with the transient increase in Luciferase signals in Matrigel plugs implanted in those mice (data not shown). Nevertheless, the ex vivo results show that mSMCs and fSMCs are more sensitive to these TK inhibitors than foreskin fibroblasts.

In our experiments, the IC_{50} of growth inhibition in human foreskin fibroblasts by imatinib was 27 μM, and that for dasatinib was 377 nM (Fig. 5, C and F). Under ex vivo culture conditions, we determined that the IC_{50} for imatinib was around 10 μM for the inhibition of mSMC and fSMC proliferation (Fig. 5G), and that for dasatinib was between 100 and 160 nM (Fig. 5H) among the 27 pairs of primary cultures tested. These IC_{50} values are also much higher than the clinically relevant dose. Consistent with these high IC_{50} values, we found that administration of imatinib or dasatinib to mice did not interfere with the transient increase in Luciferase signals in Matrigel plugs implanted in those mice (data not shown). Nevertheless, the ex vivo results show that mSMCs and fSMCs are more sensitive to these TK inhibitors than foreskin fibroblasts.

With the mSMC cultures, Pearson correlation test shows that the sensitivity to imatinib and dasatinib was not concordant among the 27 pairs of primary cultures derived from myometrial tissues (I) or fibroid tissues (J). K and L Correlation between the ratios (fibroid:myometrium) of IC_{50} and PDGFC RNA levels among pairs of primary smooth muscle cultures derived from 27 patient samples. K Imatinib. L Dasatinib. M and N Correlation between the ratios (fibroid:myometrium) of IC_{50} and PDGFRA RNA levels as in K and L. M Imatinib. N Dasatinib. P < 0.05 means that the correlation is statistically significant. In A–C and G, uM is μM.
indicating that FSMCs that contained higher levels of PDGFR RNA were more sensitive to the TK inhibitors.

**DISCUSSION**

Through a focused comparison of the expression profiles of protein TKs and their ligand activators, followed by quantitative real-time PCR and immunohistochemistry analyses of a large number of fibroid and myometrial tissues, we have shown that the levels of PDGFC RNA and protein are consistently upregulated in the majority of fibroid rather than adjacent myometrial tissues. This conclusion is in agreement with a previous report, where 16 pairs of fibroid and myometrial tissues were examined, and the upregulation of PDGFC RNA was observed in fibroid tissues [22]. Thus, the upregulation of PDGFC is a hallmark of uterine fibroids.

We have also shown that the levels of PDGFC RNA are higher in fibroid-derived primary SMC cultures than those derived from myometrial tissues. Interestingly, we found that the levels of PDGFC RNA in tissues were not maintained in the primary SMC cultures, suggesting that the tissue microenvironment and/or other cell types also play important roles in PDGFC expression in the fibroid tissues. Consistent with our finding, a microarray-based gene profiling study has found that ex vivo propagation of mSMCs and fSMCs causes large changes in gene expression and reduces the differences observed in the original myometrium and fibroid tissues [23]. Thus, the ex vivo cultures of USMCs are useful in studying cell-autonomous mechanisms, but they are not suitable for elucidating alterations that are dependent on inputs from the tissue microenvironment.

Using primary SMC cultures, we have demonstrated that bioactive recombinant PDGFC stimulates the proliferation of mSMCs and fSMCs. Furthermore, reduction of the PDGFR through lentiviral-mediated RNA interference decreases the proliferation of mSMCs and fSMCs. The expression of PDGFRA in USMCs has been established previously by immunohistochemistry studies [18] and was confirmed in this study. Our results suggest that PDGFR, which is expressed in both the mSMCs and the fSMCs in the tissues and in ex vivo cultures, is likely to play an important role in uterine smooth muscle proliferation. The contribution of PDGF-B to vascular SMC proliferation is well established [24]. By analogy, PDGFRA may stimulate the proliferation of USMCs; for example, during blood clotting and the release of PDGFs from the aggregated platelets. Furthermore, our results suggest that the activated PDGFR A may also stimulate the clonal expansion of fSMCs. In this regard, the upregulation of PDGFC expression in fibroid tissues may contribute to an autocrine mitogenic pathway to drive the development of fibroids.

It is important to note that PDGFC is translated as a multidomain protein that does not bind to PDGFR with high affinity [9]. The latent PDGFC has to be processed by proteases (e.g., tissue plasminogen activator [tPA] or plasmin) to become activated for binding to PDGFR [9, 25–27]. We have detected the full-length PDGFC protein as well as varying levels of lower-molecular weight forms of PDGFC in fibroid tissue extracts by Western blotting (data not shown). However, we do not know whether the lower-molecular weight forms of PDGFC were specifically released from an activating protease or are nonspecific degradative products generated during tissue collection. We have detected higher levels of PDGFC protein in the fibroid than in adjacent myometrial tissues. Because the active PDGF/PDGFR complex is endocytozed and degraded in the lysosome, the PDGFC protein detected in the fibroid tissues is likely to be the latent form. The accumulation of latent PDGFC in fibroid tissues may provide a reservoir of a potent growth and survival factor that can be quickly activated by proteases, such as tPA and plasmin, to stimulate fibroid growth under thrombolytic conditions. Alternatively, the abnormal expression of other proteases that can cleave and activate the latent PDGFC may deregulate proliferation of iSMCs. It would therefore be of interest to search for PDGFC-processing enzymes in fibroids as a future direction to further delineate the role of PDGFC in the development of uterine fibroids.

The PDGFR signaling module plays important roles in a number of pathogenic conditions, including atherosclerosis, fibrosis, and cancer [6]. Consequently, a number of pharmacological inhibitors of the PDGFR signaling module have been developed, including 1) neutralizing antibodies against PDGFs, 2) soluble decoy receptors, 3) antibodies against PDGFR, and 4) small molecular inhibitors of the PDGFR TK [6]. In this study, we examined two TK inhibitors, imatinib and dasatinib, known to inhibit PDGFR and several other TKs [21, 28], for their effects on the proliferation of USMCs. We have found that USMCs are more sensitive to these two TK inhibitors than primary human foreskin fibroblasts. However, the IC_{50} values for the inhibition of USMC proliferation by both TK inhibitors are above the clinically relevant concentrations. Nevertheless, we did observe a significant correlation between the levels of PDGFC expression with drug sensitivities among the 54 primary SMC cultures tested, supporting the idea that the TK inhibitors target the PDGFR TK to inhibit USMC proliferation. The two TK inhibitors tested here are not specific for the PDGFR TK; in fact, they inhibit several other receptor and nonreceptor TKs [21]. Recently, imatinib also has been shown to inhibit a nonkinase target (i.e., NQO2) [29]. The promiscuity of these TK inhibitors might have contributed to the wide range of IC_{50} values observed with this large panel of primary SMCs. Although the two TK inhibitors tested may not be effective in blocking the growth of iSMCs, our results suggest that PDGFC and PDGFR are potential targets for the development of medical therapies to treat uterine fibroids.

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