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

Guangli Suo, Yong Jiang, Bryan Cowan, and Jean Y.J. Wang

ABSTRACT

Leiomyomata uteri (i.e., uterine fibroids) are benign tumors arising from the abnormal growth of uterine smooth muscle cells (SMCs). We show 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 implanted subcutaneously in immunocompromised mice. The knockdown of PDGF 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, shRNAmir

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 PDGF 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 PDGFA/A homodimers with high affinity but fails to interact with the PDGFRB/B 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 PDGFFC as a mitogen for USMCs, thus suggesting that the upregulation of PDGFC 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-adapted short hairpin RNA (shRNA/mir) target sets designed for PDGFFA and PDGFFB lentiviral vector were purchased from Open Biosystems (Thermo Scientific). Transfection reagent (GeneTrans) and plasmid maxi kit was purchased from BioCode (San Diego, CA). Bicinchoninic acid protein assay kit was purchased from Pierce, and Agilent Low Input cRNA Amplification Kit was purchased from Agilent. The RNAeasy Mini Kit and RNA-Free DNase Set were purchased from Qiagen. SuperScript II kit and PAGE gel were purchased from Invitrogen, and SYBR Green PCR Master Mix was purchased from Applied Biosystems. Anti-PDGFFA (c-20) antibody and goat anti-rabbit horseradish peroxidase (HRP)-conjugated secondary antibody were purchased from Invitrogen, and SYBR Green PCR Master Mix was purchased from Agilent. The RNAeasy Mini Kit and RNA-Free DNase Set were purchased from BioCode. Alexa Fluor 448 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 PDGFFC protein was purchased from R&D Systems. RAG2-γc−/− (double knockout of Rag2 and Il2rg; the two alleles are Rag2tm1Sorree and Il2rg tm1Rko) female mice (13–9 wk old were kind gifts from Dr. Catriona Jamieson at the Moores Cancer Center (University of California, San Diego [UCSD], La Jolla, CA). Matrigel was purchased from BD Biosciences, and D-luciferin was purchased from Caliper (University of California, San Diego [UCSD, La Jolla, CA). Matrigel was purchased from BD Biosciences, and D-luciferin was purchased from Caliper LifeSciences.

Methods

Sample collection. The fibroid and adjacent myometrial tissues were collected from 76 patients (mean age, 42.9 yr; range, 35–52 yr) undergoing hysterectomy. The University of Mississippi Institute Regulation Board approved the sample collections with patient consent. Tissues were explanted from the fibroids and the adjacent myometrium randomly and without any preference. The samples were either frozen in liquid nitrogen for RNA retrieval and/or immersed in DMEM for cell retrieval. Of the 76 sets of samples, 42 sets of RNA were analyzed by the custom microarrays. 76 sets of RNA were analyzed by quantitative real-time RT-PCR (qRT-PCR) reactions, and 31 sets of tissues were converted into primary SMC cultures.

Custom oligonucleotide array hybridization. The custom microarrays were constructed by covalent binding of selected 60-mer oligonucleotides to the activated CodeLink slides. Total RNA was extracted from fibroid and adjacent myometrial tissues and was converted into cRNA 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 DNAarray. J. Cabrera, Rutgers University, http://www.rci.rutgers.edu/~cabra/DNAarray/) and fitted with a linear mixture-effect model (provided by R-package nlme. Jose Pinheiro, Douglas Bates, Saikat DebRoy, Deepayan Sarkar, and the R Core team 2008. NLME: Linear and Nonlinear Mixed Effects Models. R-package version 3.1-90). 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. The digested tissues were cultured in a humidified 5% CO2 incubator at 37°C without any disturbance until they became attached the bottom of the plate. Fresh media were given 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 transfected for qRT-PCR, lentiviral transduction, and proliferation assays between passages two and five.

Lentivirus-mediated RNA interference targeting PDGFR. Five shRNA/mir target sets designed for PDGFR 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 pGFP 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, HEK293 cells were seeded in the 24-well plates 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 fluorescent 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.

RNA extraction and qRT-PCR. Total RNA from tissues and primary cultures of USMCs were extracted using the RNAeasy Mini Kit according to the manufacturer’s instructions. Contaminating genomic DNA was removed on the columns using the RNA-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. First-strand cDNA (1.0 µg) was amplified in 2 µl of first-strand cdNA (1:10 dilution, 1.25 µl of SYBR Green PCR Master Mix, and 0.5 µM of each forward and reverse primers was performed for 40 amplification cycles in an ABI PRISM 7700 Sequence Detection System (Applied Biosystems). Cycling conditions were 50°C for 2 min, 40°C for 10 min, 95°C for 15 sec, and 60°C for 1 min. All reactions were run in triplicates. The primers for the PCR amplification of the following genes (PDGFA, PDGFB, PDGFC, PDGFD, PDGFR, and PDGFRB) were designed using the Primer Express software (version 2.0; please see Supplemental Table S1). The relative expression levels of genes were expressed in arbitrary units, where the Ct value of the gene of interest was normalized to that determined for GAPDH RNA, a housekeeping gene, to correct for differences in concentrations of the cDNA templates.

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 slices were incubated at 4°C for 18 h. Antibodies and dilutions used were PDGFA (1:150) and PDGFC (1:150). After rinsing with wash buffer (Tris-buffered saline with Tween) and aspiration, the specimens were covered with biotinylated link buffer (biotinylated anti-rabbit immunoglobulin for PDGFA, and anti-goat immunoglobulin for PDGFC; 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 1 min. The sections were counterstained with hematoxylin, rinsed with distilled water three times, and then mounted for microscopic evaluation. The sections stained with second antibody only were the control.
Immunofluorescence. Cultured SMCs (ISMCs or myometrial SMCs [mSMCs]) were plated onto coverslips and grown at 37°C overnight. Cells were fixed in 4% paraformaldehyde for 10 min, permeabilized in 0.25% Triton X-100 PBS for 5 min, and blocked in 5% BSA for 30 min. The coverslips were incubated with the primary antibodies (1:200 dilution of anti-PDGFRα rabbit polyclonal antibody and 1:200 dilution of anti-PDGFC goat polyclonal antibody) in a moist chamber at 4°C for 18 h. The coverslips were washed three times with PBS and then incubated with secondary antibodies (Alexa Fluor 488 donkey anti-rabbit IgG [H+L]; 1:300 dilution and Alexa Fluor 594 donkey anti-goat IgG [H+L]; 1:300 dilution) for 2 h on the Immunoplate in the dark. To examine the expression of ACTA2 in ISMCs or mSMCs, the coverslips were stained with monoclonal ACTA2 antibody (1A4) and subsequently incubated with Alexa Fluor 594 donkey anti-mouse IgG (H+L) at 1:300 dilution. To stain the nuclei, the coverslips were incubated with 0.1 μg/ml Hoechst 33258 for 5 min and rinsed with PBS three times. The coverslips were then mounted, and the fluorescent signals were captured with a CCD camera (Nikon).

Immunoblotting. The mSMCs and ISMCs (either uninfected, infected with pGIPZ vector lentivirus, or infected with pGIPZ-shRNA against PDGFRα) were lysed in lysis buffer (20 mM Tris [pH 7.4], 150 mM NaCl, 10% glycerol, 0.5% Nonidet P-40, 1% protease cocktail, 0.5 mM EDTA) and incubated for 20 min on ice. The lysates were cleared by centrifugation at 21,000 × g for 15 min at 4°C, and the supernatants were quantitated using the bicinchoninic acid protein assay kit. Whole-cell lysates (40 μg) were fractionated on 4%–20% gradient SDS-PAGE gels and transferred to nitrocellulose membranes. The membranes were incubated with anti-PDGFRα (c-20) antibody, followed by goat anti-rabbit HRP-conjugated secondary antibody, and were visualized by chemiluminescence. Stripped membranes were reprobed with anti-GAPDH followed by goat anti-mouse IgG-HRP as loading control.

Cell proliferation. Cell proliferation was measured by the CCK-8 kit according to the manufacturer’s instructions. Briefly, USMCs were seeded in 96-well plates (5000 cells/well) in DMEM containing 10% FCS for 2 h and then washed twice with PBS and incubated for 2 h in serum-free DMEM, followed by incubation in serum-free medium with or without the supplement of recombinant bioactive mature human PDGFCC protein (100 ng/ml) at 37°C, 5% CO2. The CCK assay was performed at time 0 and at 4, 12, 24, 36, and 48 h after the addition (or not) of PDGFC. The absorbance at 450 nm was converted to the numbers of viable cells according to a standard curve constructed in parallel by seeding different numbers of cells in wells right before the time of CCK assays.

Growth inhibition by TK inhibitors. To determine the growth inhibitory effects of TK inhibitors imatinib and dasatinib, USMCs were incubated with varying concentrations of the drugs (0–100 μM for imatinib; 0–10 μM for dasatinib) in 96-well plates (5000 cells/well). Prior to drug addition, cells were cultured for 12 h in DMEM medium with 10% FBS and then shifted to medium with either 10% or 0.5% FBS during drug treatment for a total period of 96 h, with fresh drug-supplemented medium replaced every 24 h. For the majority of USMCs, the growth rates were reduced by 5% in the medium with 0.5% FBS, and the IC50 values were not significantly altered by the FBS concentrations. At the end of 96 h, 10 μl of CCK-8 solution was added to each well and cells, and the number of viable cells was determined as described above. The IC50 was defined as the concentration of imatinib or dasatinib resulting in a 50% decrease in the level of USMC viability compared with untreated control.

Matrigel implants in RAG2−/−γc−/− mice. RAG2−/−γc−/− female mice were bred and maintained on sulfamethoxazole water under specific pathogen-free conditions in the animal care facility at UCSD Moores Cancer Center. Female athymic nude mice 6–9 wk old were acquired from the UCSD Animal Care Program. All experiments on mice were conducted in accordance with the guidelines of the National Institutes of Health (Bethesda, MD) for the care and use of laboratory animals. The animal study protocol (s20013) was approved by UCSD and the Medical Experimental Animal Care Committee. The 17β-estradiol pellets (60-day release; 1.7 mg) were implanted subcutaneously on the back of the neck areas 3 days prior to the engraftment experiments. The ISMCs were infected with concentrated GFP-Luciferase lentiviral stock [15] at 50 MOI in the presence of 8 μg/ml polybrene for 12 h at 37°C. The infected ISMCs were then cultured in DMEM with 10% FBS for 36 h. To test the effect of PDGFRα knockdown on the ISMC expansion in the Matrigel implantation in the RAG2−/−γc−/− female mice, the vector and shRNA- infected ISMCs were cultured in DMEM with 10% FBS for 12 h before transplantation. To test the effect of bioactive recombinant PDGFCC, the ISMCs were cultured in serum-free DMEM for 12 h before transplantation. Subsequently, the infected ISMCs were dissociated with trypsin, collected in PBS, and then mixed with an equal volume of Matrigel at 4°C. A 0.5 × 104 ISMCs in 100 μl Matrigel with or without the supplement of recombinant bioactive mature human PDGFCC protein (100 ng/ml) were transplanted into RAG2−/−γc−/− female mice subcutaneously on both flanks. The control mice were transplanted with 200 μl of PBS-Matrigel. Whole-body bioluminescence signaling was detected using the IVIS 200 Imaging System series (Caliper) at different time points (0, 2, 4, 6, 9, 12, and 15 days), as described previously [16, 17]. Mice were anesthetized with isoflurane and injected intraperitoneally with 150 mg/kg body weight) approximately 10 min before imaging. Each mouse was subjected to four IVIS analyses; exposures of 3 min, 1 min, 20 sec, and 5 sec, every 2 or 3 days after injection of cells. Dorsal images were analyzed by using total photon flux emission (photons/sec) in the region of interest (ROI) by LivingImage Software (Xenogen). The datum in each ROI was normalized by the same ROI datum collected at first day when we injected the ISMCs. Curve diagrams were generated to evaluate the effect of PDGFCC or PDGFRα knockdown on the ISMC expansion in the Matrigel implants in RAG2−/−γc−/− female mice.

Statistical analyses. A two-tailed and paired Student t-test was used to analyze statistical differences of relative expression levels of PDGFA, PDGFB, PDGFC, PDGFRα, and PDGFRβ between myometrium and fibroid tissues or ISMCs from various numbers of patients, and it was also used to analyze statistical differences of the IC50 values for imatinib and dasatinib. A one-tailed and unpaired Student t-test and a one-way ANOVA followed by a Tukey comparison test was used to calculate the P values. To determine the IC50 values, the optical density at 450 nm (OD450) absorbance results were log-transformed and fitted a curve with nonlinear regression by Sigmoidal dose-response (variable slope) equation. The Pearson correlation test was performed to determine the correlation between the expression levels of PDGFC and PDGFA, the IC50 values, and the ratios of these values between pairs of fibroid vs. myometrial tissues and primary cell cultures. These various statistical analyses were performed using the GraphPad Prism 4 package (GraphPad Software).

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 from 28 of the 42 patients surveyed (Fig. 1A). However, the RNA levels of PDGFA, PDGFB, and PDGFD, and those of the PDGF receptors (PDGFRα and PDGFRβ) 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 PDGFA, PDGFB, PDGFD, and PDGFRA and PDGFRB 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 myometrical 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 PDGFRα RNA levels were similar between fibroid and adjacent myometrium 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. 1, D 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 PDGFRα in the tissue sections (Fig. 1G), consistent with a previously published report [18]. These results show that PDGF-C expression is upregulated in the majority of fibroid tissues, but there is not a coordinated upregulation of its receptor, PDGFRα, in fibroids.

PDGF-C and PDGFRα 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 PDGF-C and PDGFR by qRT-PCR (Fig. 2, A and C). We found that the levels of PDGF-C RNA were 1.7-fold higher in the fSMC group than in the mSMC group (P < 0.05; Fig. 2A), whereas the levels of PDGFR RNA were similar between the two groups of cells (Fig. 2C). From the scatter plots, it is clear that the levels of PDGF-C 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 PDGF-C RNA among the tissues and the corresponding primary cultures of SMCs (Fig. 2, A and B). The lack of correlation also applied to the RNA levels of PDGFR (Fig. 2, C and D). There was a significant correlation between the levels of PDGF-C and PDGFR-RNA among the group of mSMCs (Fig. 2I) but not with the group of fSMCs (Fig. 2J).
examine the expression of PDGFC in the mSMC and fSMC cultures, we performed immunofluorescence analyses. Representative results with one pair of mSMCs and fSMCs are shown in Figure 2, K–M. The mSMCs and fSMCs expressed the alpha-actin (ACTA2) gene, which is a marker of SMCs (Fig. 2L). These cells also expressed the PDGFC protein (Fig. 2M). Because serum contains PDGFs, we performed the anti-PDGFC staining using cells that were incubated in serum-free medium for 24 h prior to fixation and found that the anti-PDGFC reactivity was not diminished under serum-free conditions (Fig. 2M). Fluorescent secondary antibodies alone did not react with the mSMCs and fMSCs (Fig. 2K), demonstrating that the PDGFC and ACTA2 signals were specific to each of the two primary antibodies.

Taken together, results in Figures 1 and 2 show that PDGFC, but not PDGFRA, is upregulated in the majority of fibroid tissues examined. The increased expression of PDGFC in fibroid tissues is likely to be caused by a combination of alterations: some are SMC autonomous, whereas others may be dependent on the tissue environment. As a result, the levels of PDGFC RNA detected in the fibroid tissues are not stably maintained in the primary SMC cultures (Fig. 2F), but the PDGFC RNA levels remain higher among the fSMC than the mSMC cultures (Fig. 2A).

**Recombinant Bioactive PDGFCC Stimulates USMC Growth**

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 implemented with estrogen pellets, and followed the expansion of the infected 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
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 iSMCs 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 TGCTGTGACGTGACGACCTCTCCTCCAAAAT-GAAATAGTGACACGACAGATGTATTTCATTTGGAG-GATAGAGGGTGCCCTACTGCGCTCGGA) 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 iSMCs 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 iSMCs 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 IC\(_{50}\) 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

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**FIG. 3.** Recombinant PDGFCC stimulates the proliferation of uterine SMCs. The indicated primary SMC cultures derived from the fibroid and adjacent myometrial tissues were stimulated with (+) or without (−) recombinant, bioactive PDGFCC homodimer. Cell proliferation values shown are the means and SDs from three independent experiments with four wells of cells taken at each of the indicated time points. A, C, and E Primary culture SMCs derived from three myometrial tissues. B, D, and F Primary SMC cultures derived from the corresponding fibroid tissues. The indicated primary cell cultures infected with GFP-luciferase lentivirus were mixed with Matrigel with (+) or without (−) recombinant bioactive PDGFCC and were then transplanted subcutaneously into RAG2−/−γc−/− female mice. G Representative imaging pictures of Luciferase activity in the transplanted Matrigel plugs. H Quantitation of bioluminescence signals. The values shown are mean and SDs from three mice per experiment. *\( P < 0.05 \); **\( P < 0.01 \). p/sec/cm\(^2\)/sr, number of photons per second per square centimeter of steradian.
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, 10^3 is 10^3.
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.

With the mSMC cultures, Pearson correlation test shows that the sensitivity to imatinib and dasatinib was not concordant among the 27 primary cultures derived from myometrial tissues (I) or fibroid tissues (J). However, among the cultures of fSMCs, there was a significant correlation between the responses to imatinib and dasatinib (Fig. 5J). We also compared the ratio (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.

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indicating that fSMCs that contained higher levels of PDGFRA 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 PDGFCC 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 PDGFRA, 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 PDGFRB 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 PDGFRA 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 PDGFRA 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 PDGFRA [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 endocytose 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 fSMCs. 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 PDGFC-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 PDGFC-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 IC50 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 PDGFR 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 IC50 values observed with this large panel of primary SMCs. Although the two TK inhibitors tested may not be effective in blocking the growth of fSMCs, our results suggest that PDGFC and PDGFR are potential targets for the development of medical therapies to treat uterine fibroids.

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