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Tranilast Induces MiR-200c Expression through Blockade of RelA/p65 Activity in Leiomyoma Smooth Muscle Cells

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Abstract

Objective: To determine the mechanism by which tranilast induces miR-200c expression in leiomyoma smooth muscle cells (LSMC).

DESIGN: Experimental study

SETTING: Academic research laboratory

PATIENT(S): Women undergoing hysterectomy for leiomyoma.

Intervention: Blockade of RelA/p65.

MAIN OUTCOME MEASURE(S): The effects of tranilast and blockade of RelA/p65 on miR-200c expression.

Results: Tranilast, an inflammation inhibitor dose-dependently induced miR-200c in LSMC and myometrium smooth muscle cells (MSMC) with a more profound effect in LSMC as compared with MSMC. The treatment of LSMC with Bay 117082, an inhibitor of I κ B phosphorylation, further enhanced miR-200c induction by tranilast. The knockdown of RelA/p65 by siRNA also induced miR-200c expression in LSMC. Although tranilast had no effect on total RelA/p65 protein levels in LSMC, it significantly induced RelA/p65 phosphorylation at S536, while reducing its activity as well as its nuclear translocation. ChIP assay indicated that tranilast reduces the binding ability of RelA/p65 to miR-200c promoter resulting in miR-200c induction. Tranilast also inhibited interleukin 8 (IL8) expression in LSMC. The induction of miR-200c by tranilast in part mediates the inhibitory effect of tranilast on the expression of IL8 and cyclin-dependent Kinase 2 (CDK2) in LSMC.

Conclusion: Induction of miR-200c by tranilast in LSMC is mediated through a transcriptional mechanism involving inhibition of nuclear factor kappa-B (NF- κ B) signaling pathway. These results highlight the significance of inflammation in the pathogenesis of leiomyoma and the potential utility of anti-inflammatory drugs for treatment of leiomyomas.

Capsule

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Tranilast induces miR-200c expression through blockade of the activity and nuclear translocation of RelA/p65 in leiomyoma smooth muscle cells.

Keywords

Tranilast; Leiomyoma; miR-200; NF- κ B; CDK2

Introduction

Uterine leiomyomas are the most common benign fibrotic tumors affecting 40–70% of women during their reproductive years (1, 2). In addition to surgical intervention which accounts for the most common indication for all hysterectomies performed in the United States annually (1, 2), several hormonal therapies have been used as alternative interventions for management of their growth and associated symptoms such as pelvic pressure, chronic pelvic pain, abnormal uterine bleeding as well as infertility and pregnancy complications (1, 3). Although their etiology is unknown, these tumors whose growth is dependent on ovarian steroids are characterized by increased angiogenesis, excess deposition of the extracellular matrix (ECM), inflammation as well as elevated expression of pro-fibrotic cytokines such as transforming growth factor- β 3 (TGF- β 3), Activin-A and platelet-derived growth factor (PDGF) (4–6). In addition, excess accumulation of mast cells along with increased secretory products from these cells has been considered as being pivotal in the pathogenesis of several fibrotic disorders, including leiomyomas (7).

Accumulated proteomic and genomic studies have indicated that altered expression of small non-coding RNAs, including microRNAs (miRNAs), play a key role in cellular events leading to leiomyoma development and growth (5, 8–12). More specifically, our laboratory and others have provided evidence that the expression of miR-200 and miR-29 family are suppressed in leiomyoma and their altered expression at least in part accounts for molecular mechanism underlying ECM accumulation, angiogenesis and inflammation (13–17).

Tranilast (N-3, 4-dimethoxycinnamoyl anthranilic acid) is an orally administered synthetic drug with anti-allergic properties has been used clinically for treatment of several respiratory disorders as well as keloids and hypertrophic scars (18, 19). Recent reports indicate that tranilast not only inhibits the release of inflammatory mediators from mast cells, but also inhibits collagen bio-synthesis, growth factor expression, cell growth and TGF β -induced transformation of fibroblasts into myofibroblastic phenotype (19). Subsequent studies have confirmed the anti-proliferative or anti-tumor effect of tranilast in several cells including prostate, breast, glioma, gastric, pancreatic and other tumors (18, 19). We and other groups have also demonstrated that tranilast inhibited the rate of cell proliferation and the expression of several genes functionally involved in cell cycle progression [CDK2, cyclin-dependent kinase inhibitor p21(Waf1/Cip1), p53, proliferating cell nuclear antigen (PCNA) and cyclin D1 (CCND1)], collagen type I (COL1) and collagen type III alpha 1 chain (COL3A1), profibrotic cytokines [transforming growth factor- β 3 (TGF- β 3) and Activin-A], and enzymes catalyzing epigenetic modifications [DNA Methyltransferase 1 (DNMT1) and enhancer of zeste homolog 2 (EZH2)] in isolated LSMC (16, 20, 21). Furthermore, we

reported that tranilast induced miR-29c levels through an epigenetic mechanism in LSMC (16).

Although NF- κ B is comprised of multiple subunits, RelA/p65 has received the most attention especially in studies of its phosphorylation (22). Phosphorylation of RelA/p65 causes a conformational change resulting in alteration of its protein-protein interactions, ubiquitination as well as stability (23). Among the 11 identified phosphorylation sites in RelA/p65, four sites (S205, T254, S276 and S281) were found within the N-terminal REL homology domain (RHD), two (S311 and S316) in the linker region and five (T435, S468, T505, S529 and S536) located in the C-terminal transactivation domain (TAD). Significant research has focused on S536 (22), and a number of kinases have been identified which could phosphorylate RelA/p65 at S536 including IKK α , IKK β , IKK ϵ , ribosomal subunit S6 kinase 1 (RSK1), casein kinase 1 γ 1 (CK1 γ 1) and NF- κ B activating kinase (NAK)/TANK-binding kinase 1 (TBK1) (24–27). Because RelA/p65 phosphorylation at S536 has been reported to enhance its transcriptional activity and nuclear translocation (24, 28–31), the aim of this study was to determine the effects of tranilast on RelA/p65 phosphorylation at S536.

Leiomyoma express significantly lower levels of miR-200c whose target genes include IL8, CDK2, zinc finger E-Box binding homeobox 1 (*ZEB1*), zinc finger E-Box binding homeobox 2 (*ZEB2*), vascular endothelial growth factor A (*VEGFA*), tissue inhibitor of metalloproteinases 2 (*TIMP2*), fibulin 5 (*FBLN5*) and inhibitor of nuclear factor kappa B kinase subunit beta (*IKBKB*) (17, 32, 33) all of which influence the outcome of a number of cellular events in leiomyomas. The objective of the present study was therefore to determine if tranilast which has anti-inflammatory properties alters the expression of miR-200c and secondly, to determine if the NF- κ B pathway could mediate the effects of tranilast on miR-200c.

Materials and Methods

Primary Myometrium and Leiomyoma Smooth Muscle Cells Isolation

Primary MSMC and LSMC were isolated from fresh specimens as previously described (33). Fresh tissues used for cells isolation were collected from patients not taking any hormonal medications for at least three months prior to surgery at Harbor-UCLA Medical Center with prior approval from Institutional Review Board (#036247). Briefly, MSMC and 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 or LSMC isolated from different patients as indicated in the figure legends. Overall, 5 primary MSMC and 22 primary LSMC were used in this study. All supplies for isolation and cell culture were purchased from Sigma-Aldrich (St. Louis, MO) and Fisher Scientific (Atlanta, GA).

siRNA Transfection

LSMC cultured as above and at sub-confluence were transfected with 50 nM of siRNA negative control (siNC) or siRNA against RelA/p65 (sip65; Santa Cruz Biotechnology,

Dallas, Texas) for 72–96 hours using PureFection transfection reagent (System Biosciences, Inc., Mountain View, CA) according to the manufacturer's protocol.

Loss-of-function of miR-200c

LSMC were seeded at a cell density of 5×10^4 /well in six-well plates and at sub-confluence transfected with 50 nM of anti-miR negative control (aNC) or anti-miR-200c (a-miR-200c) (Applied Biosystems, Carlsbad, CA) for 96 hours using PureFection transfection reagent (System Biosciences, Inc.) according to the manufacturer's protocol.

RNA Isolation and qRT-PCR Analysis

Total RNA was extracted from LSMC using Trizol (Thermo Fisher Scientific, Waltham, MA) and their quantity and quality was determined (ND-1000 Spectrophotometer, NanoDrop Technologies, Wilmington, DE) as previously described (11, 32, 34). Subsequently, 1 μ g RNA was reverse-transcribed using random primers for IL8 (Fisher Scientific). The miR-200c primer design and PCR conditions has been described previously (35). Quantitative RT-PCR was carried out using SYBR gene expression master mixes (Applied Biosystems). 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. The levels of mRNA and miRNA were quantified using the Invitrogen StepOne System and normalized to FBXW2 (36) and RNU6B, respectively. All reactions were run in triplicate and relative expression was determined using the comparative cycle threshold method (2^{-CT}), as recommended by the supplier (Applied Biosystems). Abundance values were expressed as fold changes compared to the corresponding control group. The primer sequences used were as follows: IL8 (sense, 5'-CTTGGCAGCCTTCCTGATTT-3'; antisense, 5'-TTCTTTAGCACTCCTTGGCAAAA-3'); and FBXW2 (sense, 5'-CCTCGTCTCTAAACAGTGAATAA-3'; antisense, 5'-GCGTCCTGAACAGAATCATCTA-3'). miR-200c (sense, 5'-AGTAATACTGCCGGTAATGA-3'; antisense, 5'-GGTCCAGTTTTTTTTTTTTTTTCCA-3'); and RNU6B (sense, 5'-ATTGGAACGATACAGAGAAGATTAG-3'; antisense, 5'-AATATGGAACGCTTCACGAAT-3').

Immunoblotting

Total protein isolated from LSMC following treatment conditions was subjected to immunoblotting as previously described (12, 37). 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 (Fifty micrograms) of total protein for each sample were denatured with SDS-PAGE sample buffer, and separated by electrophoresis on an 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: RelA/p65 (Cell Signaling Technology, Inc., Danvers, MA), p-RelA/p65 (Ser 536), Calpain, c-Jun, IL8 and CDK2 (Santa Cruz Biotechnology). 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) serving as the loading control. The densities of the specific protein bands were quantified with a scanning densitometer (Bio-Rad GS-800, Hercules, CA), and the results were expressed as means \pm SEM normalized to GAPDH.

Luciferase Reporter Assays

LSMC were seeded in six-well plates until reaching sub-confluence and transiently cotransfected with a luciferase reporter plasmid (1 μ g/well) containing nuclear factor kappa-B (NF- κ B) conserved binding sequences for NF- κ B activity detection (Signosis, Sunnyvale, CA) and pRL-TK plasmid (Promega, Madison, WI) encoding Renilla luciferase (0.2 μ g/well) as a control for differences in transfection efficiency using PureFectin transfection reagent. Firefly and Renilla luciferase activities were measured after 8 hrs of tranilast (200 μ M) treatment using the Dual-Luciferase Reporter Assay System (Promega). Firefly luciferase activity was normalized to Renilla luciferase activity and the level of induction was reported as the mean \pm SEM of three experiments performed in duplicates and compared with a ratio in cells treated with vehicle control (DMSO) set at 1.

Subcellular Fractionation

LSMC were treated with tranilast (200 μ M) for 36 hrs. Cytoplasmic and nuclear proteins were fractionated following the protocol of the subcellular protein fractionation kit (Thermal Scientific).

Chromatin Immunoprecipitation

Following treatment of LSMC and MSMC with tranilast (200 μ M) for 36h, ChIP analysis was carried out using Simple ChIP enzymatic chromatin IP kit (Cell Signaling Technology) according to the manufacturer's protocol. PCR-amplification was performed using primers designed to amplify RelA/p65 binding site on miR-200c promoter (forward: 5'-GGGATGAGGGTGGGTAAATC-3', reverse: 5'-GCCTCTGAGCCACCTTC-3'), under PCR condition of 95°C for 5 min, 35 cycles of 94°C for 30s, 55°C for 30s, and 72°C for 30s. PCR amplified products were electrophoresed on 2% agarose gel and visualized under ultraviolet illumination. Band densities were quantified through normalization to the corresponding inputs and percent input method was used to quantitate the values of the immunoprecipitated DNA.

Enzyme-linked immunosorbent assay

Collected culture conditioned media and cells lysates were centrifuged, supernatants were collected and total protein content was determined by BCA™ Protein Assay Kit (Thermo Scientific Pierce). The IL8 content in culture media was determined following the protocol of Human IL8 ELISA MAX Deluxe Set (BioLegend, San Diego, CA) with detection limit of 15.6 pg/ml and sensitivity of 8 pg/ml. The level of IL8 was reported as pg/mg of protein or as fold change compared to control experiments.

Statistical analysis

Throughout the text, all data are presented as mean \pm SEM and analyzed by PRISM software (Graph-Pad, San Diego, CA). Dataset normality was determined by the Kolmogorov–Smirnov test. Comparisons involving two groups were analyzed using unpaired Student's *t*-tests as appropriate. One-way ANOVA was used for comparisons involving multiple groups. Statistical significance was established at $P < 0.05$.

Results

This study was initiated to determine whether tranilast has any effect on miR-200c expression in leiomyoma. As such, isolated paired MSMC and LSMC were treated with tranilast and miR-200c expression was detected by qRT-PCR. In support of our previous report (17), miR-200c expression was significantly lower in LSMC as compared to MSMC and tranilast dose dependently increased the expression of miR-200c ($P < 0.05$; Fig. 1A) in both LSMC and MSMC but with a more profound effect in LSMC as compared to MSMC. Because tranilast is an anti-inflammatory agent (38), we next examined the molecular mechanism by which tranilast induces miR-200c expression in LSMC. Isolated LSMC were treated with Bay 117082, an inhibitor of I κ B phosphorylation, along with tranilast. The concentration of tranilast (200 μ M) selected was based on other studies (39–41) and dose–response experiments carried out in our previous work (16). Similar to tranilast, Bay 117082 not only induced miR-200c expression, but also enhanced the effect of tranilast on miR-200c expression in LSMC ($P < 0.05$; Fig. 1B).

Next, siRNA was used to knock down the protein expression of RelA/p65 in LSMC ($P < 0.05$; Fig. 1C). As a result of RelA/p65 knockdown the expression of miR-200c was significantly increased ($P < 0.05$; Fig. 1D), indicating that miR-200c is under regulation of RelA/p65.

Additionally, using luciferase reporter assay we demonstrated that tranilast significantly reduced RelA/p65 activity in LSMC ($P < 0.05$; Fig. 2A). Previous reports indicated that the phosphorylated RelA/p65 at serine 536 is involved in RelA/p65 activity (22). As such, to further our understanding of the molecular mechanism of tranilast action in LSMC, we assessed the levels of RelA/p65 phosphorylation at serine 536. As shown in figure 2B and 2C, treatment of LSMC with tranilast for 24 and 48 hours increased the expression of phosphorylated RelA/p65 at serine 536 ($P < 0.05$). However, tranilast had no effect on total protein abundance of RelA/p65 (Fig. 2B and 2C). Using subfractionation of cellular and nuclear protein, tranilast significantly induced RelA/p65 protein and its phosphorylation at serine 536 in the cytosolic fraction and decreased them in the nuclear fraction ($P < 0.05$; Fig. 2D and 2E). Furthermore, ChIP assay demonstrated that the binding ability of RelA/p65 to miR-200c promoter was reduced by tranilast ($P < 0.05$; Fig. 3), thus promoting the induction of miR-200c.

Our previous work had demonstrated that IL8 is under regulation of RelA/p65 and miR-200c through targeting IKBKB in LSMC (32), therefore we next examined the effect of tranilast on IL8 expression. In support of previous published data (42), the treatment of tranilast in LSMC significantly repressed IL8 mRNA and protein expression ($P < 0.05$; Fig. 4A, 4B and

4C). Because CDK2 is also down-regulated by tranilast (16, 20) and is a direct target of miR-200c (33), in order to determine the physiological significance of miR-200c in mediating the effects of tranilast in LSMC, IL8 and CDK2 were measured following transfection of LSMC with anti-miR-200c oligonucleotides or anti-miR negative control combined with tranilast or DMSO. As shown in figure 4D to 4G, knockdown of miR-200c resulted in partial attenuation of the tranilast-induced inhibitory effect on IL8 and CDK2 expression ($P < 0.05$).

Discussion

Our results demonstrate for the first time that tranilast significantly induces miR-200c expression in MSMC and LSMC, but more profoundly in LSMC. This differential effect may be due to the presence of greater degree of inflammation in leiomyoma as compared with myometrium (5, 14). The induction of miR-200c is mediated at least in part through a mechanism involving repression of RelA/p65 nuclear translocation which results in decreased RelA/p65 activity and transcriptional regulation of miR-200c promoter. CHIP analysis further indicated that tranilast reduced the binding of RelA/p65 to miR-200c promoter. Because IL8 and CDK2 are involved in tranilast-mediated suppression of LSMC inflammation and proliferation (16, 20), as a proof of principle we selected IL8 and CDK2 for further investigation and demonstrated that the tranilast mediated inhibition of IL8 and CDK2 expression is at least in part through induction of miR-200c in LSMC.

The significance of tranilast-induced miR-200c expression relates to miR-200c regulatory function on expression of many target genes including several epithelial–mesenchymal transition (EMT)-, cell cycle-, ECM-, inflammation- and apoptosis-related genes (17, 32, 43–46). We have previously reported that the lower expression of miR-200c in leiomyoma was inversely correlated with several of its target genes expression such as *CDK2*, *ZEB1*, *ZEB2*, *VEGFA*, *TIMP2*, *FBLN5*, *IL8* and *IKBKB* (17, 32, 33). Moreover, we demonstrated that miR-200c regulates *IL8* expression indirectly through downregulation of NF- κ B signaling pathway by targeting *IKBKB*. Functionally, repression of *IL8* after gain-of-function of miR-200c in LSMC mirrors the profile in fibroids characterized by elevated *IL8* expression and lower miR-200c (17, 32, 34).

Expression profiling has demonstrated that there is aberrant expression of many miRNAs in leiomyomas as compared to myometrium (13, 17, 34, 47, 48); however, the regulatory mechanisms behind the altered expression of miRNAs in leiomyoma remain mostly unclear. Recent studies have reported that the expression of miR-200 family is under regulation by DNA methylation and histone modifications, leading to progression of glioblastoma, pancreatic adenocarcinoma and basal type of breast cancer (49, 50). Additionally, several transcription factors including ZEB1, ZEB2, krüppel-like factor 5 (KLF5), achaete-scute family BHLH transcription factor 2 (ASCL2), nanog homeobox (NANOG), octamer-binding protein 4 (Oct4), SRY-box containing gene 2 (Sox2), hepatocyte nuclear factor-1 β (HNF-1 β), specificity protein 1 (Sp1) and c-Jun have been identified as regulators of miR-200 family expression by acting directly on its promoter (51–57). To our knowledge this is the first demonstration that the transcription factor RelA/p65 also regulates miR-200c

expression and the effect of tranilast on miR-200c induction is mediated through this pathway in LSMC.

Tranilast, an inhibitor of tryptase, has been demonstrated to have various anti-proliferative and anti-inflammatory effects (19), and recently has been reported to inhibit cytokine-induced NF- κ B activity and transcriptional ability on inflammatory associated genes expression (42, 58, 59). Tryptase acting as serine proteases is stored in large amounts in mast cells and has been demonstrated to stimulate angiogenesis via several mechanisms including activation of c-Kit (CD117), high affinity IgE receptor (Fc ϵ RI) and protease-activated receptor-2 (PAR-2) (60–63). Accordingly, the correlation between mast cells, chronic inflammation and tumor development has been established (64–66). Previous studies demonstrated that mast cells are frequently abundant in leiomyomas (67–69). Additionally, another tryptase inhibitor, nafamostat mesylate, has been reported to inhibit pancreatic cancer through blockade of nuclear NF- κ B activation (70). In this regard, activation of NF- κ B signaling pathway has been identified in a variety of tumors including leiomyoma and demonstrated to play an important role in the modulation of cell proliferation, inflammatory responses and oncogenesis (5, 71, 72). Our previous work also indicated that leiomyomas express elevated levels of phosphorylated p65 at serine 536 as compared with matched myometrium (14).

Although RelA/p65 phosphorylation at S536 has been reported to enhance its transcriptional activity and nuclear translocation (24, 28–31), our results unexpectedly indicated that treatment of LSMC for 24 and 48 hours with tranilast elevated phosphorylation of RelA/p65 at serine 536. In addition, the pattern of phosphorylated RelA/p65 at serine 536 in cellular and nuclear fraction was similar to that of RelA/p65 which is accumulated in cellular fraction, but reduced in nuclear fraction in response to treatment of LSMC with tranilast. In agreement with our results, several reports suggested that S536 phosphorylation may inhibit nuclear translocation of RelA/p65, resulting in repression of NF- κ B signaling pathway (73, 74). Moreover, several studies have identified another role for S536 phosphorylation, namely promotion of proteasomal degradation of RelA/p65, thereby reducing its protein stability and transcriptional activity (25, 27). Collectively, the conditions and consequences of RelA/p65 phosphorylation at S536 are cell and physiologic condition specific.

Our data indicates that tranilast reduced NF- κ B activity (figure 2A), induced p65 phosphorylation at S536 (figure 2B) and repressed mRNA expression of IL8 (figure 4A) and CDK2 (16) in a short period of treatment, whereas the induction of miR-200c by tranilast in LSMC required a significantly longer treatment (48 hours) (figure 1A and 1B), implying that miR-200c might have an accessory role in mediating the action of tranilast in LSMC. In support of this interpretation, knockdown of miR-200c enhanced protein levels of CDK2 and secreted IL8, and partially relieved tranilast-mediated inhibition of IL8 and CDK2 expression (figure 4E, 4F and 4G). These results suggest that miR-200c mediates at least some of the effects of tranilast on several downstream target genes; however, the effects of tranilast on other miRNAs expression and target genes warrant further investigation.

In addition to tranilast, other agents such as green tea extracts, halofuginone and vitamin D have been evaluated as alternative leiomyoma therapeutic approaches based on their effects

on alteration of mast cell activation, expression of proliferation associated genes, pro-fibrotic cytokines and ECM regulation (1, 75–77). Whether these agents also exert their effects on miR-200c or other anti-fibrotic miRNAs remains to be determined.

In summary, our data provides support for direct effects of tranilast on miR-200c induction through a molecular mechanism involving altered transcriptional ability of RelA/p65 in LSMC. However, whether tranilast through this and/or other mechanisms influences the expression of other anti-fibrotic and anti-inflammatory miRNAs remains to be determined. Because the anti-fibrotic and anti-inflammatory properties of tranilast has been extensively studied under both *in vitro* and *in vivo* conditions (19), the need for an animal model is warranted to further support the potential effectiveness of tranilast for treatment of leiomyomas.

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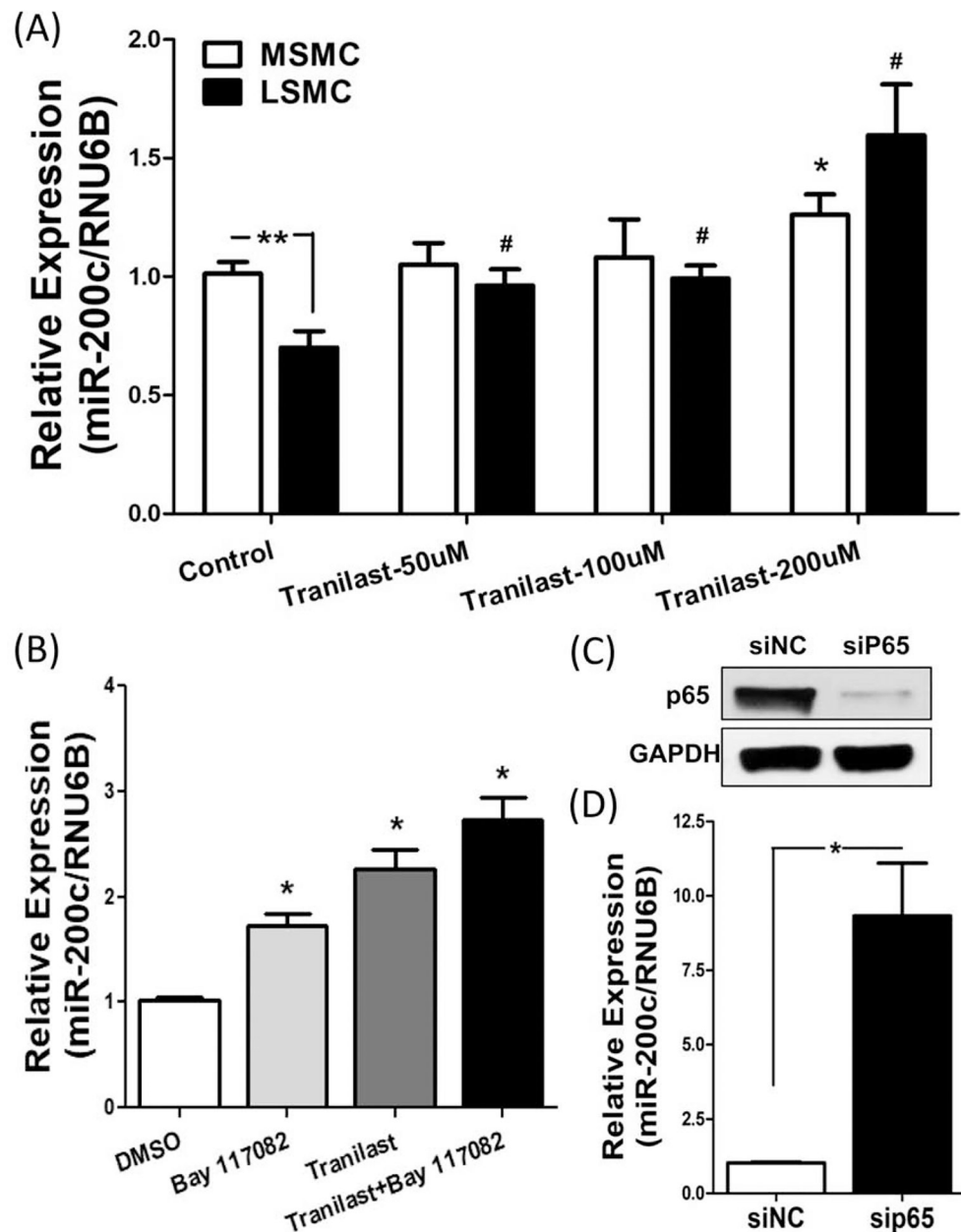


Figure 1.

(A) qRT-PCR analysis of miR-200c expression following treatment of paired MSMC and LSMC with different doses of tranilast for 48 hrs (N=5). (B) The relative expression of miR-200c was determined following treatment of LSMC with 200 μ M tranilast for 48 h with or without Bay 117082 (10 μ M) for the last 24 h (N=7). (C) Shows the effect of RelA/p65 knockdown through transfection of LSMC with siRNA against RelA/p65 for 96 hrs on RelA/p65 protein abundance (N=4). (D) The level of miR-200c as determined by qRT-PCR is shown following transfection of LSMC with siRNA against RelA/p65 for 96 hrs (N=4). The results are presented as mean \pm SEM of independent experiments as mentioned above using cells isolated from different patients in each set. P values (*P<0.05) are indicated by corresponding lines.

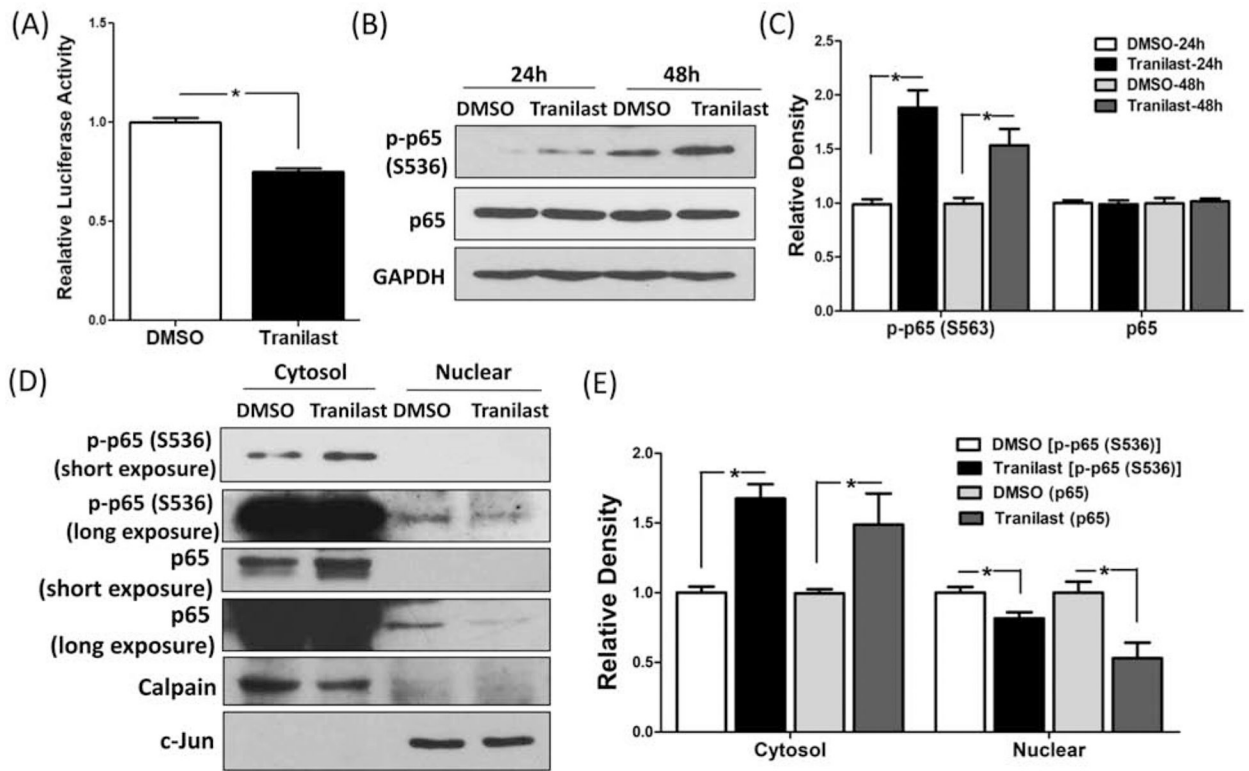


Figure 2.

(A) The level of RelA/p53 activity in LSMC transfected with a luciferase reporter construct containing preserved RelA/p53 binding sites and pRL-TK plasmid as control for transfection efficiency. Cells were treated with tranilast (200 μ M) for 8 hrs. The ratio of Firefly:Renilla was determined and reported as relative luciferase activity as compared to control (DMSO) which was independently set as 1 (N=3). (B–C) Immunoblotting analysis of phosphorylated RelA/p53 at serine 536 and total RelA/p53 following treatment of LSMC with 200 μ M tranilast for 24 hrs and 48 hrs (N=3). The relative band densities are shown in (C). (D) Shows protein levels of phosphorylated RelA/p53 at serine 536 and RelA/p53 in LSMC following treatment with 200 μ M tranilast for 36 hrs (N=3). Cells were harvested and sub-fractionated into cytosol and nuclear proteins and subjected to immunoblotting analysis with Calpain and c-Jun serving as markers for the respective subcellular fractions. (E) The band density of phosphorylated RelA/p53 at serine 536 and RelA/p53 was semi-quantified and normalized to Calpain (cytosol) or c-Jun (nuclear). The results are presented as mean \pm SEM of independent experiments as mentioned above using cells isolated from different patients in each set. P values (*P < 0.05) indicated by the corresponding lines.

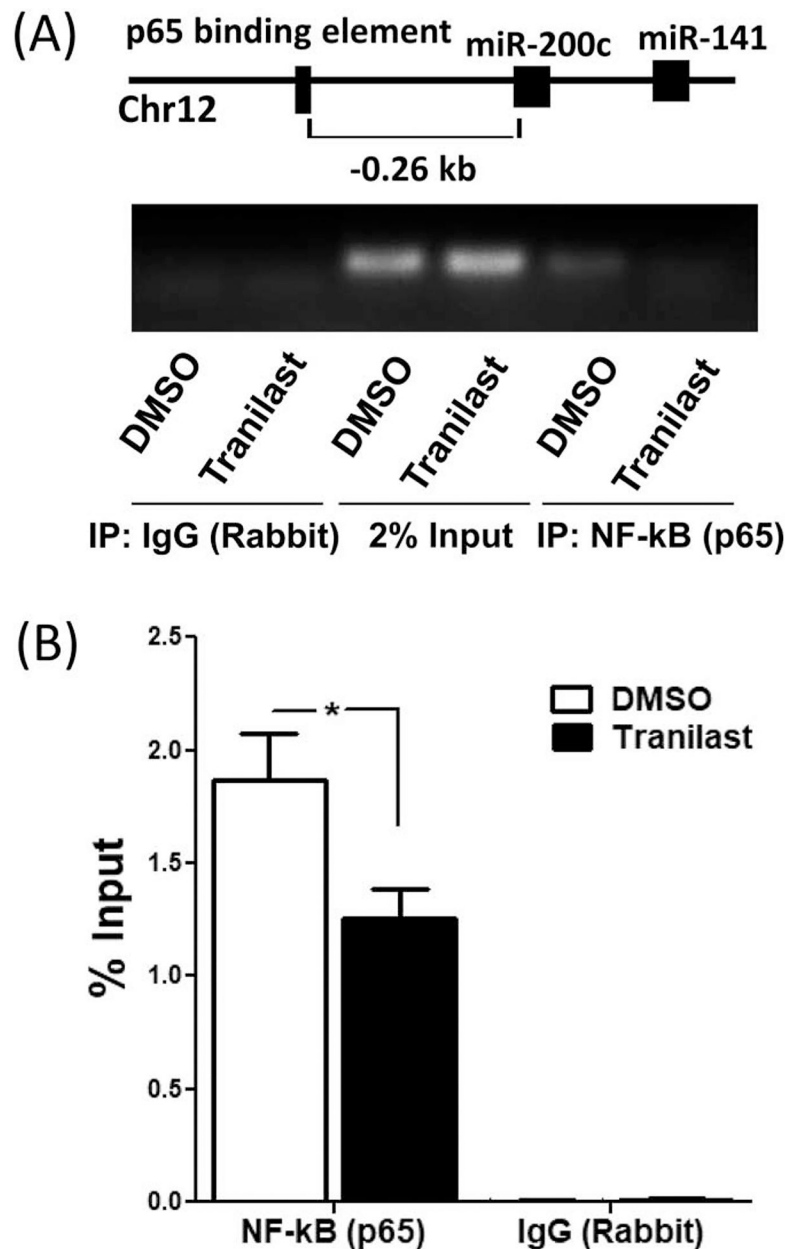


Figure 3.

(A) The graphical depiction of the miR-200c promoter with the location of RelA/p65 binding element. ChIP assay with RelA/p65 or control IgG was performed on chromatin derived from LSMC after 36 hrs incubation with tranilast (200 μ M) or vehicle DMSO (N=4). Primers specific to the depicted RelA/p65 binding element were used for PCR amplification. 2% inputs are indicated. The result is a representative from four sets of independent experiments using LSMC isolated from four patients. (B) The quantitative data is presented by percent input method as mean \pm SEM with P values (*P < 0.05) indicated by corresponding lines.

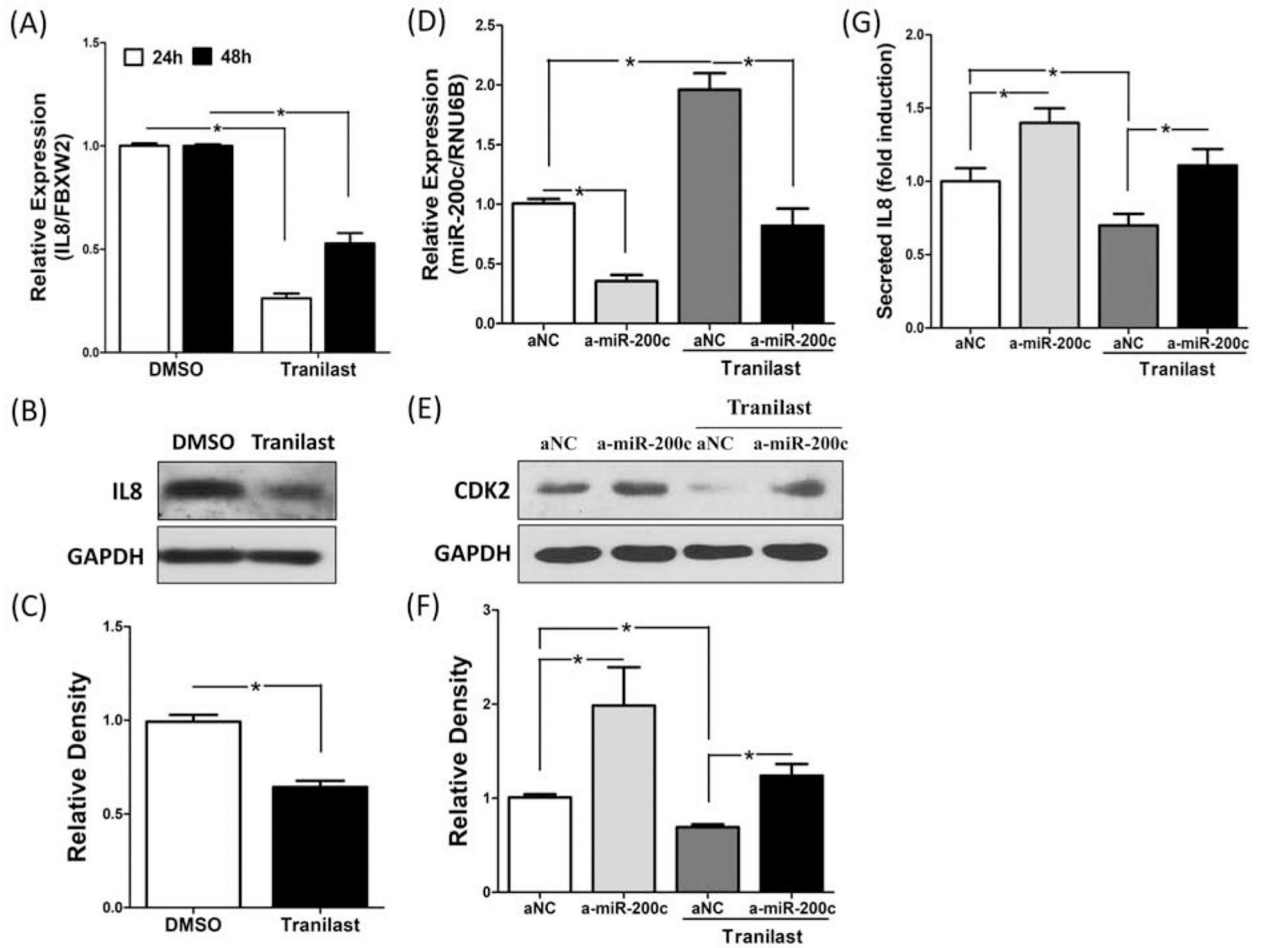


Figure 4.

(A) mRNA expression of IL8 in LSMC treated with Tranilast (200 μ M) or DMSO as control for 24h to 48h (N=6). (B) A representative immunoblotting of IL8 expression in LSMC treated with Tranilast (200 μ M) for 48h (N=5). (C) Demonstrates the relative band densities of IL8. (D) LSMC were transfected with anti-miR scrambled oligonucleotides (aNC) or anti-miR-200c oligonucleotides (a-miR-200c) for 96 hours with DMSO or tranilast (200 μ M) for the last 48 hours and the levels of miR-200c was determined (N=4). (E) A representative of gel demonstrating CDK2 expression following transfection with anti-miR scrambled oligonucleotides (aNC) or anti-miR-200c oligonucleotides (a-miR-200c) in LSMC for 96 hours with DMSO or tranilast (200 μ M) for the last 48 hours (N=4). (F) The bar plot represents the mean relative band densities of CDK2. (G) Secreted IL8 was determined from collected medium following transfection with anti-miR scrambled oligonucleotides (aNC) or anti-miR-200c oligonucleotides (amiR-200c) in LSMC for 96 hours with DMSO or tranilast (200 μ M) for the last 48 hours (N=4). The results are presented as mean \pm SEM of independent experiments using cells isolated from different patients in each set. P values (*P < 0.05) indicated by the corresponding lines.