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Long Non-Coding RNA XIST and TSIX Modulate TGF-β-Mediated Cellular Transdifferentiation and Fibrosis in Systemic Sclerosis

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Abstract

Background

Systemic sclerosis (SSc) is a complex autoimmune disease characterized by immune dysregulation, microvascular injury, and progressive fibrosis, with transforming growth factor-β (TGF-β) serving as a central driver of fibrotic responses. Although the long non-coding RNAs (lncRNAs) XIST and TSIX are recognized as important epigenetic regulators, their contribution to TGF-β-mediated fibrosis in SSc remains poorly defined.

Method

Tissue- and gender based XIST and TSIX expression was evaluated in SSc patients and healthy controls (HCs). Functional analyses were performed *in-vitro*, using Differential serum induction (Autologous or Patients' serum) and Synthetic TGF-β stimulation on PBMCs from healthy individuals. siRNA-based knockdown analyses were performed to evaluate their regulatory influence on fibrotic markers and TGF-β expression.

Result

Both lncRNAs were significantly upregulated in patients with SSc, regardless of tissue type or sex, in contrast to the physiologically female-biased expression of XIST observed in healthy controls. Exposure of healthy PBMCs to SSc serum promoted fibrocyte transdifferentiation and increased the expression of fibrosis-associated markers. XIST and TSIX expression positively correlated with profibrotic genes and was further induced following TGF-β stimulation. Conversely, silencing either lncRNA reduced TGF-β expression together with downstream fibrotic mediators, supporting their involvement in TGF-β-associated profibrotic signalling.

Conclusion

XIST and TSIX are the upstream regulators of TGF-β and are also associated with the immune-fibrotic pathophysiological axis of systemic sclerosis, highlighting their potential as biomarkers of disease activity. Targeting them may offer a selective, safer alternative to direct TGF-β inhibition. Further mechanistic studies are required to define their precise roles in SSc pathogenesis.

INTRODUCTION

Systemic sclerosis (SSc) is a complex autoimmune connective tissue disease characterized by immune dysregulation, vasculopathy, and progressive fibrosis of the skin and internal organs. It typically manifests during the early decades of life and is more prevalent in females [1–3]. Persistent activation of fibroblasts into myofibroblasts results in excessive extracellular matrix (ECM) deposition and irreversible tissue remodelling, ultimately leading to fibrosis, the hallmark feature of SSc. Despite advances in understanding immune-mediated mechanisms, the molecular regulators that link genetic susceptibility, epigenetic control, and fibroblast dysfunction remain incompletely understood [1, 4, 5].

Long non-coding RNAs (lncRNAs), which have been identified as a class of RNA transcripts longer than 200 nucleotides, have emerged as critical regulators of gene expression in health and disease, especially in female breast and ovarian cancer [6–10]. lncRNAs have been identified to orchestrate cellular differentiation, immune signalling, and tissue remodelling by modulating transcriptional, post-transcriptional, and epigenetic processes [11]. Increasing evidence implicates dysregulated lncRNAs in fibrotic disorders, such as SSc, where they shape the cellular and molecular landscape of fibrosis [12].

XIST (X Inactive Specific Transcript) and TSIX (TSIX Transcript, XIST Antisense RNA) are mutually antagonistic long non-coding RNAs (lncRNAs) recognized for their roles in X chromosome inactivation [13–15]. In SSc, there is evidence of skewed X chromosome inactivation [12, 16–18].

TGFβ is a pro-fibrotic cytokine considered the key modulator of fibrosis, and it is found to be elevated in patients with SSc [19]. A study has shown the association between TGFβ and TSIX in the regulation of the fibrotic pathogenesis of scleroderma. According to this study, overexpressed TGFβ might induce TSIX transcription, which stabilises col-I mRNA, thereby promoting fibrosis. They did not find any alterations or regulatory associations of XIST with SSc pathogenesis.[20]

In accordance with this background, this study aimed to investigate the association of XIST and TSIX in the regulation of fibrosis in SSc and to elucidate their interaction with TGFβ signalling in the modulation of fibrosis with special reference to fibrocyte transdifferentiation and activation.

MATERIALS AND METHODS

Recruitment of study participants and sample collection:

A total of twenty-eight patients (n = 28) diagnosed with diffuse cutaneous systemic sclerosis (dcSSc) with SSc [fulfilling American College of Rheumatology (ACR) criteria for SSc, 2013] were recruited from the outpatient department (OPD) of the Department of Clinical Immunology and Rheumatology, Institute of Postgraduate Medical Education and Research (IPGME&R), Kolkata, India [21, 22]. Age and sex-matched healthy individuals (n = 25) were recruited as the control group (HC). The following were included for the recruitment of study participants: Age 18–55 years. Individuals with prior use of antibiotics, antivirals, or allergy drugs in the month before or during recruitment were excluded from the study. Current or past history of tobacco usage, history of diabetes, hypertension, renal impairment, and pregnancy were excluded from the study. Additionally, genetic predisposition to any rheumatic disease was an exclusion criterion for the HC. This study was approved by the institutional ethical committee of IPGME&R (No: IPGME&R/IEC/2019/386). Written informed consent was taken from each participant.

Peripheral blood (10 ml) was collected from each study participant, including HCs. The collected blood from each participant was equally distributed into an ethylene diamine tetra acetic acid (EDTA) vial and a clot vial. Skin punch biopsies were done on 7 individuals of the recruited patient group. Skin tissues from five healthy individuals fulfilling the mentioned criteria were collected from the Department of Plastic Surgery, IPGME&R, Kolkata, India.

Extraction of total RNA:

Total RNA was extracted from the collected skin tissues, blood, and/or cultured cells for subsequent experiments. Tissue samples collected from both of the study groups were homogenized in 1 ml of TRIzol (Thermo Fisher Scientific, MA, USA) using a homogenizer, and after that, the RNA was extracted based on the TRIzol-chloroform method [22]. Following the protocol, the air-dried RNA was then dissolved in RNase-free water and stored at -80° C for downstream transcriptional experiments.

Collected blood (500 µl) was incubated at room temperature with RBC lysis buffer at a 1:10 ratio for 10 mins and washed twice with 1X PBS. Following this washing step, the RNA was isolated as mentioned above. The same protocol was followed to isolate the RNA from the cultured cells after the cells were harvested and washed twice with 1X PBS.

Quantitative Real-Time PCR (qRT-PCR) to analyse gene expression:

The extracted RNA was quantified spectrophotometrically. For qRT-PCR, about 500 nanograms (ng) of total RNA was treated with DNase I (Roche Diagnostics, Mannheim, Germany) and the first-strand cDNA was synthesized following the manufacturer's protocol of Prime Script 1st strand cDNA Synthesis Kit (Cat. #6110A, TAKARA BIO INC.) by using oligo-DT primers. The expression of genes: XIST, TSIX, Col-I, Col-III, TGFβ, αSMA, and Vimentin, was quantified using SYBR green (Roche Diagnostics, Mannheim, Germany) using respective sets of forward and reverse primers. For relative quantification of the gene expression, 18S ribosomal RNA was used as an internal control (IC). Each reaction was run in duplicate for statistical validation. The cycle threshold (Ct) values for each lncRNA were compared to the internal control, 18S rRNA, and the means of these differences were used to represent the data of gene expression from real-time PCR. The change in expression was defined as the Ct_{lncRNA}- $Ct_{18S\ rRNA}$, and denoted by delta Ct (dCt). To calculate the fold change in expression, all the dCt values were transformed into 2^{-dct}. The derived numerical values were normalized by multiplying them by 10^2 or 10^3.

Transient transfection and knockdown analysis of XIST and TSIX

To study the regulatory association of XIST and TSIX with the fibrotic parameters, the knockdown assay was performed (for XIST and TSIX) in the LX-2 human hepatic stellate cell line (SCC064, Sigma-Aldrich, Germany). Small interfering RNA (siRNAs) (10 nM) were prepared (Sigma-Aldrich, Germany) from the three different targets of the gene to ensure knockdown possibilities. LX-2 cells were

plated (with DMEM,10% FBS), followed by incubation at 37°C in 5% CO_2, and after a couple of days, siRNAs were mixed with Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA, USA). Control (scrambled) siRNA was used as a negative control or placebo control (Santa Cruz Biotechnology). After the transfection, cells were incubated for 96 h following the manufacturer's protocol. Cells were harvested, and RNA was isolated for further analysis as described earlier.

PBMC isolation and cell culture:

PBMCs were isolated from EDTA-anticoagulated blood of participants using HiSep TM (Himedia, India, LS001) by gradient centrifugation at 2500g for 30 mins. Isolated PBMCs were cultured in Dulbecco's Modified Eagle Medium (DMEM, Sigma Aldrich, Germany No: D1152) with 10% Foetal Bovine Serum (FBS, Himedia Laboratories, Cat no RM10434) and 1% antibiotic-antimycotic (Himedia Laboratories, cat no A001A) and were maintained at 37°C in 5% CO_2. After a couple of days, nonadherent cells were removed by gentle aspiration, fresh media were added, and the culture was maintained for another 14 days, according to the protocol of Bucala et al, 1994 [23]. During this procedure, the morphological changes in cells were observed, and after 14 days, the cells were harvested, and the comparison of fibrotic markers between the 1st and the 14th day was done.

PBMCs isolated from HCs were cultured following the same protocol mentioned above. To identify the association among TGFβ, XIST, and TSIX, the healthy PBMCs were treated additionally with or without serum (autologous serum of the respective healthy individual, serum isolated from the patient) and TGFβ (10ng/ml) [24]. To reduce experimental biases, before harvesting, the cells were cultured in fresh media for three days without any serum or TGFβ treatment. Following this, the cell supernatant was collected, and the cells were isolated for further experiments.

The cellular transformation and activation were measured by immunocytochemistry. Transcriptional and translational expression of fibrotic genes (tgfβ, col-I, and col-III) was also assessed using the isolated RNA from the harvested cells and the collected cell supernatant by ELISA.

The associations of XIST and TSIX with fibrotic molecules were validated on LX-2 cells using the same procedure of differential serum administration as described above.

Characterisation of Fibrocytes Based on Cellular Transformation and Activation:

The percentage of fibrocytes and their trans-differentiation into fibroblasts and their activation status were measured in PBMC and cultured cells. Peripheral blood (collected in an EDTA vial) was taken into a fresh microcentrifuge tube with BD FACS lysing solution (BD Pharming, US) at a ratio of 1:100 and incubated for 15 minutes. After RBC lysis, the remaining cells were washed twice with PBS and finally dissolved in 100 µl of PBS. The cells were incubated for 1 hour with the surface antibodies against CD34 (cat no: 348057), CD45 (cat no: 560777), α-SMA (Cat No: NBP2-34522APC), CD14 (Cat no: 562698), CD90 (Cat No: NBP2-47755PCP), as per the manufacturer's directions. BD Cytofix-Cytoperm was added to fix the cells, and then the cells were pelleted and dissolved in 100 µl of 1x BD-wash buffer. Following this, a 30-minute incubation was done with an antibody against collagen-I (cat no: FCMAB412F). Cells were spun down and resuspended in 400 µl PBS. For the cultured cells, after harvesting the cells, the same procedures were followed except for the RBC lysis. The percentage of fibrocytes and their activation into myofibroblasts was analysed by FACS.

Measurement of Cytokines and Cell-adhesion Molecules by ELISA:

Expressions of the regulatory molecules of fibrosis (TGF β, Col-I, and Col-III) were measured from both serum and differentially treated healthy cells with or without serum or TGFβ. To separate serum, the collected blood (within the clot vial) was spun down at 3000 X g for 7 minutes. Cell supernatants were also spun similarly to avoid cellular contamination. Following this, an ELISA (KINESIS Dx, Los Angeles) was done to determine the concentrations of the molecules according to the manufacturer's protocol.

Statistical Analysis:

Statistical analyses were performed using GraphPad Prism version 5.0 (GraphPad Software Inc., San Diego, CA, USA). The distribution of data (normality) was assessed using the Shapiro–Wilk test. Based on data distribution, comparisons between groups were

performed using the Mann–Whitney *U* test or Student's *t*-test, with Welch's correction applied to unpaired *t*-tests when equality of variances was not assumed. Correlations between two variables were evaluated using Spearman's rank correlation coefficient (*r*). Strength of correlation was assessed based on the correlation coefficient, which was interpreted as follows: *r* = 0.00–0.19 (very weak), 0.20–0.39 (weak), 0.40–0.59 (moderate), 0.60–0.79 (strong), and 0.80–1.00 (very strong).[25] Positive and negative values of *r* indicated direct and inverse associations between the parameters, respectively. All tests were two-sided, and a *P* value ≤ 0.05 was considered statistically significant.

RESULTS

Baseline characteristics:

The mean age of patients (n = 28): 35.30 ± 9.46 years (mean ± SD); HCs (36.37 ± 10.85, n = 25) did not differ significantly (p = 0.9). The male-to-female ratio of HC (20:5) and the patient groups (22: 6) didn't show any significant difference. Across all patient groups, 96.4% were SCL70-positive. Detailed demographical data and the status of autoantibody formation are tabulated in Table 1.

Table 1
Detailed demography of the recruited participants

Variables	Healthy (n = 25)	SSc Patients (n = 28)	P value
Age (Years, mean ± SD)	35.3 ± 9.4	35.6 ± 10.5	NS
Sex (female/male)	20: 5	22: 6	NS
Disease duration (Years, mean ± SD)	-	2.9 ± 2.3	NS
Treatment duration (Years, mean ± SD)	-	-	NS
mRDss (mean ± SD)	-	23.4 ± 9.0	NS
Drugs received	-	-	
SCL 70	-	n = 28	
NOR 90	-	n = 5	
Centromere A	-	n = 2	
Centromere B	-	n = 4	
Th/TO	-	n = 10	
PM-SCL 75	-	n = 3	
PM-SCL 100	-	n = 1	
RNA POL-III (RP-11)	-	n = 5	
RNA POL-III (RP-155)	-	n = 4	
RO 52	-	n = 5	
Ku	-	n = 4	
Ribosomal–P–protein (RIB)	-	n = 1	
Fibrillarin	-	n = 1	
PDGFR	-	-	

Relative expression pattern and Comparative association of XIST and TSIX

In comparison to HCs, increased expression of XIST and TSIX was observed in the skin tissue from SSc patients (p = 0.01 and p = 0.04, respectively) **(Figure_1A).** Similar results were observed regarding the expression of XIST and TSIX in PBMC (p = 0.001 and p = 0.005, respectively) and plasma (p = 0.0009 and p = 0.01, respectively) **(Figure_1B and 1C).** TSIX and XIST showed a moderate-to-

strong positive correlation in both HCs (r = 0.5, p = 0.007) and patients (r = 0.9, p < 0.0001) **(Figure_1D and 1E).** The data on XIST and TSIX expression were identical irrespective of cell/tissue type; the downstream experiments were conducted solely using PBMCs.

A comparative study was conducted to assess gender bias in the expression of XIST and TSIX. XIST and TSIX expression was found to be considerably higher in male (p = 0.01, p = 0.03 respectively) and female (p = 0.03, p = 0.01 respectively) patients compared to the gene-specific expression of healthy males and females, respectively. HCs didn't show any gender bias in TSIX expression (p = 0.5), whereas XIST expression in HCs was higher in females than in males (p = 0.02). In patients, the expression of XIST showed no significant differences between males and females. (p = 0.4). Similar trends were observed with a borderline upregulation of TSIX in females compared to males (p = 0.05). **(Figure_1F and 1G).** Gel electrophoresis data showed significant overexpression of both XIST and TSIX in patients compared to healthy controls **(Figure_1H).**

Positive correlations were found between the expression of the regulatory molecules of fibrosis (TGFβ, Col-I, and Col-III) and both XIST (r = 0.8, p < 0.000; r = 0.7, p < 0.000;1 r = 0.7, p < 0.0001, respectively) and TSIX (r = 0.8, p < 0.0001; r = 0.7, p < 0.0001 and r = 0.6, p = 0.0002, respectively). Additionally, there was a strong positive correlation between the fibroblast activation markers vimentin and αSMA and TGFβ (r = 0.6, p = 0.0001 and r = 0.5, p < 0.0001, respectively), XIST (r = 0.5, p = 0.001 and r = 0.5, p < 0.0001, respectively), and TSIX (r = 0.6, p = 0.001 and r = 0.7, p < 0.0001, respectively).

In vitro trans-differentiation of PBMCs and their association with XIST and TSIX

A morphological alteration was observed in the PBMCs following a 10-day culture period. During the early phases of culture, the heterogeneous population of PBMCs changed into long, spindle-shaped cells, which were more common in PBMCs of patients **(Figure_2A-2B)** than of HCs **(Figure_2D-2E)**. Regardless of the treatment groups, these spindle-shaped cells later showed signs of trans-differentiation into stellate-shaped morphology. These stellate cells were more likely to transdifferentiate and proliferate predominantly in the patient **(Figure_2C)** than in the HCs **(Figure_2F)**.

Quantitative real-time data revealed significantly increased expression of the regulatory lncRNAs XIST (p = 0.01) and TSIX (p = 0.01), along with the fibrotic markers TGFβ (p = 0.01), Col-I (p = 0.01), Vimentin (p = 0.005), and αSMA (p = 0.04), on the 10th day of culture in comparison to the 4th day of culture, irrespective of HCs and patients. **(Figure_2G-2L)**.

In-vitro fibroblast activation in scleroderma with special reference to XIST and TSIX based on the differential serum induction:

XIST showed a significant upregulation in cultured PBMCs treated with serum isolated from patients, compared to the untreated and autologous serum-treated culture (p = 0.0002, p = 0.04, respectively). Treatment with autologous serum to the cultured PBMCs demonstrated significant upregulation of XIST (p = 0.02) **(Figure_3A)**. Similarly, TSIX showed a significant upregulation in the patients' serum-treated group compared to the untreated (p = 0.002) and autologous serum-treated group (p = 0.04). Moreover, TSIX also showed significant upregulation in the autologous serum-treated group compared to the untreated group (p = 0.03) **(Figure_3B)**. As a fibrotic marker, COL-I showed significant upregulation upon stimulation with autologous and patient serum, respectively (p = 0.02 and p = 0.03, respectively). No significant difference is present between the untreated and autologous serum-treated group (p = 0.1) **(Figure_3C)**. TGFβ showed significant upregulation in the HC PBMCs upon treatment with autologous serum as well as with the serum isolated from SSC patients (p = 0.01 in both of the cases). No significant difference in the expression of TGFβ was observed between the autologous and patient serum-treated group (p = 0.1) **(Figure_3D)**. HC PBMCs, upon treatment with serum isolated from patients, showed elevated expression of αSMA compared to untreated PBMCs (p = 0.03). No significant differences were observed between the differential serum-treated PBMCs (p = 0.2) **(Figure_3E)**. Vimentin didn't show any significant difference between the serum-treated or untreated group **(Figure_3F)**.

A similar result was observed regarding the expression of XIST and TSIX and the fibrotic molecules after differential serum stimulation to the LX2 cell lines. Expression of XIST is significantly higher in the LX2 cells treated with patient serum compared to the untreated and HC serum-treated group (p = 0.0009, p = 0.007, respectively). A significant difference was also observed between the XIST expression of the untreated and the HC serum-treated group **(Figure_3G)**. A significant change in the TSIX expression was observed irrespective of the treated or untreated group. LX2 cells upon stimulation with patient serum has significant increased expression compared to the untreated and HC serum-treated group (p < 0.0001 for both). LX2 cells upon stimulation with HC serum showed significantly elevated expression of TSIX (p = 0.01) **(Figure_3H)**. Data showed higher expression of Col-I in the patient serum-treated LX2 cells compared to both untreated and HC serum-treated ones (p = 0.003, p = 0.003, respectively). No significant

differences were observed for Col-I expression between the HC serum-treated and untreated group (p = 0.7) **(Figure_3I)**. The expression of TGFβ showed elevation after treatment with serum isolated from HC (p = 0.002) and patients' serum (p = 0.005) treated cells compared to the untreated subsets. No significant differences were observed (p = 0.8) between the serum-treated subsets **(Figure_3J)**. Gel images regarding the expression of the fibrotic markers along with regulatory RNAs (XIST and TSIX) confirmed the elevation in the expression of these molecules after treatment with patient serum **(Figure_3K)**.

TGFβ-mediated induction of fibrosis and its association with XIST and TSIX

FACS analysis showed a higher percentage of CD90+/Col-I+ transdifferentiated fibroblasts after TGFβ stimulation of PBMCs isolated from HCs compared to the unstimulated group (p < 0.0001) **(Figure_4A and 4B).** Microscopic data also demonstrated **a** significant increase in expression of COL-I and CD90. Microscopic data showed elevated expression of COL-I and CD90 upon stimulation with TGFβ to the cultured HC PBMCs compared to the unstimulated HC PBMCs (p < 0.0001 for both) **(Figure_4C, 4D and 4E, respectively).** Upon stimulation with TGFβ, there was significant upregulation of αSMA (p < 0.0001) and CD90 (p < 0.0001) **(Figure_4F, 4G and 4H, respectively).**

Similarly, stimulation with TGFβ also showed the elevated transcriptional expression of Col-I (p = 0.001), XIST (p < 0.0001) and TSIX (p = 0.001) **(Figure_4I, 4J and 4K, respectively).**

Correlative association of fibrotic molecules in response to the knockdown of XIST and TSIX

Comparative knockdown experiments, regardless of the siRNAs utilised (siRNA against XIST and TSIX), revealed no discernible variations in XIST and TSIX expression. XIST was significantly downregulated during siRNA knockdown against both XIST and TSIX (p = 0.001 and p = 0.01, respectively) compared to scrambled siRNA. No significant variations in XIST expression were observed following differential siRNA treatment with XIST and TSIX siRNA (p = 0.1). The expression of TSIX followed a similar trend. No matter whether siRNAs against XIST or TSIX were used in the treatment, TSIX was significantly down-regulated (p = 0.001 and p = 0.02, respectively). No difference was found (p = 0.2) in the expression of TSIX after differential treatment with XIST and TSIX siRNA **(Figure_5A)**.

The introduction of siRNAs resulted in the downregulation of the fibrotic molecules, regardless of whether XIST and TSIX were specifically knocked down. Treatment with siRNAs targeting XIST and TSIX showed downregulation of Col-I (p < 0.0001 and p = 0.002, respectively) and Col-III (p = 0.0002 and p = 0.004, respectively). (**Figure_5B and 5C**).

Significant downregulation of the fibroblast activation marker αSMA (p = 0.01 and p = 0.02, respectively) and TGFβ (p = 0.01 and p = 0.04, respectively) were observed in response to the knockdown of XIST and TSIX, respectively **(Figure_5D and 5E)**. No alteration in the expression of vimentin was found with either or the siRNA treatments (p = 0.1 and p = 0.08, respectively) **(Figure_5F)**. However, after XIST and TSIX were knocked down, there were no discernible variations in the serum concentration of Col-I and Col-III among all groups **(Figure_5G and 5H)**. Serum concentration of TGFβ successfully decreased (p = 0.01 for both), irrespective of the treatment with XIST and TSIX targeted siRNA. No significant difference was observed between the siRNA-treated groups **(Figure_5I)**.

DISCUSSION

XIST and TSIX have long been acknowledged for their canonical role in regulating dosage compensation via X chromosome inactivation in female mammals. In addition to these established functions, recent studies have unveiled their involvement in modulating the pathophysiological progression of specific diseases.[13, 14, 20] According to our investigation, elevated TSIX and XIST expression in skin and blood (PBMCs and plasma) were observed, along with a positive correlation between XIST and TSIX in SSc patients, implicating the role of both these lncRNAs in modulating SSc pathogenesis.[13] Notably, XIST is the primary driver of dosage compensation in mammalian females, mediated by X-chromosome inactivation and is antagonized by TSIX within this regulatory framework.[16] Consistent with prior reports, HCs showed higher XIST expression in females than in males, whereas TSIX demonstrated sex independent expression, likely reflecting dosage-compensation mechanisms in female mammals.[13, 14] Contrasting this, patients with SSc exhibited concomitant elevation of both XIST and TSIX irrespective of sex, indicating disruption of normal sex-regulated expression and abnormal co-expression adhering to disease context. Moreover, both lncRNAs showed an inverse expression pattern compared with HCs. We found no significant difference in XIST between male and female SSc patients, whereas TSIX was comparatively higher in males, possibly reflecting a compensatory response as an antagonistic effect against

elevated expression of XIST. Overall, the sex-independent, concomitant elevation of both XIST and TSIX highlighted their association in SSc pathophysiology.

In some recent studies, TSIX has been highlighted as a modulator of fibrosis, notably by stabilizing collagen mRNA, whereas XIST has not been previously defined in this context. [20, 26] Contrary to earlier studies, our study has demonstrated a strong positive correlation of both XIST and TSIX with fibrotic markers. Moreover, in vitro experiments revealed that fibrocyte transdifferentiation and activation were significantly higher in SSc patients compared to HCs, accompanied by marked upregulation of fibrotic markers and of XIST and TSIX. The concurrent overexpression and positive correlation of these lncRNAs with fibrotic molecules strongly suggest that both XIST, together with TSIX, actively contribute to fibrosis in SSc and reveal a previously underappreciated regulatory role of these twin regulatory RNAs.

Differential in vitro serum induction to the HC PBMCs demonstrated that patient serum more effectively drives transdifferentiation of HC PBMCs into fibroblast-like cells and promotes fibroblast activation into myofibroblasts compared to autologous serum-treated HC PBMCs. Moreover, differential serum induction also elicited synergistic overexpression of XIST and TSIX alongside fibrotic markers, highlighting their involvement in fibroblast transdifferentiation and activation.

TGFβ, as a key regulator of SSc, has been identified to influence fibrosis through epigenetic mechanisms. [19] While earlier studies linked TSIX with TGFβ–mediated collagen regulation, the role of XIST remained unclear and somewhat contradictory. [20, 27] In our study, TGFβ stimulation induced fibroblast transdifferentiation and activation. The stimulation also exhibited upregulation of both XIST and TSIX, indicating that TSIX and XIST synchronise with TGFβ signalling during fibroblast activation and highlighting their association in driving fibrosis in SSc.

The knockdown studies of XIST and TSIX also revealed their transcriptional association with the potential regulatory molecules of fibroblast transdifferentiation, activation and fibrosis. Our study demonstrated that TGFβ, as the master regulator of fibrosis, is downregulated upon knockdown of both of these lncRNAs. So, this is a suggestion that XIST and TSIX may act as upstream modulators of TGFβ signalling to drive the fibrotic cascade of SSc.

In conclusion, our study provides novel evidence that XIST and TSIX act in a coordinated manner to regulate the fibrotic pathophysiology of SSc, beyond their canonical role of dosage compensation. Each of these twin lncRNAs is functionally linked to key fibrotic events, including fibrocyte transdifferentiation, fibroblast activation, and upregulation of profibrotic markers. Collectively, the observed regulatory influence of both XIST and TSIX on TGFβ expression and downstream fibrotic mediators suggests that these lncRNAs may occupy the upstream regulators of the TGFβ-mediated fibrotic signalling network. Moreover, TGFβ has pleiotropic roles in growth, immune regulation, and tissue homeostasis. Direct therapeutic targeting of TGFβ is associated with adverse effects and can perturb normal physiology. So, targeting XIST and TSIX may therefore offer a more selective and potentially safer strategy to modulate fibrosis in SSc.

In conclusion, the present study demonstrates significant dysregulation of XIST and TSIX and also provides evidence for their involvement in fibrocyte transdifferentiation and fibroblast activation and fibrosis in SSc. The coordinated expression patterns of these lncRNAs and their influence on key fibrotic mediators highlight these lncRNAs as upstream modulators of the TGFβ-mediated fibrotic pathophysiological cascade in SSc. Furthermore, their consistent alteration in SSc highlights their potential as candidate biomarkers. Collectively, this study highlights a novel finding regarding the unique function of XIST and TSIX in the modulation of fibrosis in SSc, though further mechanistic and translational approaches are required to elucidate the exact roles of XIST and TSIX in SSc-associated fibrosis.

Declarations

1. **Ethics approval and consent to participate**:

Ethical approval information, institution(s) and number(s): This study was approved by the institutional ethical committee of IPGME&R (No: IPGME&R/IEC/2019/386).

This study was approved by the institutional ethical committee of IPGME&R (No: IPGME&R/IEC/2019/386).

Consent to participate: Written, informed consent was taken from each participant. This study was done following the Declaration of Helsinki, 1964, and its later amendments. Details of participation are mentioned in the material methods section: Recruitment of study participants and sample collection.

2. **Data sharing statement**

Consent to publish: No individual patient information or any personal description has been disclosed in the manuscript.

3. **Availability of data and materials**

The datasets generated or analysed during the current study are available with the corresponding author and can be provided upon reasonable request.

4. **Competing interests:** Nothing to declare.
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6. **Authors' contributions**

- *Author 1-13. All authors contributed to the study conception, study design and helped to pursue the study.*
- *Author 1. Dipanjan Bhattacharjee wrote the initial draft of the work.*
- *Author 2-13. Other contributors provided feedback on the succeeding iterations.*
- *Author 1-13. Each author reviewed and gave their approval to the finished work.*

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References

1. Berrevoets MAH, Markhorst J, Meek IL, et al (2014) [Systemic sclerosis: a multisystem disease]. Ned Tijdschr Geneeskd 158:A7703
2. Peoples C, Medsger TA, Lucas M, et al (2016) Gender differences in systemic sclerosis: relationship to clinical features, serologic status and outcomes. J Scleroderma Relat Disord 1:204–212. https://doi.org/10.5301/jsrd.5000209
3. Gelber AC, Manno RL, Shah AA, et al (2013) Race and Association With Disease Manifestations and Mortality in Scleroderma. Medicine 92:191–205. https://doi.org/10.1097/MD.0b013e31829be125
4. Blanc GC, de Araújo BLE (2024) SYSTEMIC SCLEROSIS. In: Health in the Global Context: Challenges, Policies and Practices. Seven Editora
5. Gilbane AJ, Denton CP, Holmes AM (2013) Scleroderma pathogenesis: a pivotal role for fibroblasts as effector cells. Arthritis Res Ther 15:215. https://doi.org/10.1186/ar4230
6. Zhang T, Hu H, Yan G, et al (2019) Long Non-Coding RNA and Breast Cancer. Technol Cancer Res Treat 18:1533033819843889. https://doi.org/10.1177/1533033819843889

7. Basu S, Nadhan R, Dhanasekaran DN (2025) Long Non-Coding RNAs in Ovarian Cancer: Mechanistic Insights and Clinical Applications. Cancers (Basel) 17:472. https://doi.org/10.3390/cancers17030472
8. Rafiee A, Riazi-Rad F, Havaskary M, Nuri F (2018) Long noncoding RNAs: regulation, function and cancer. Biotechnol Genet Eng Rev 34:153–180. https://doi.org/10.1080/02648725.2018.1471566
9. Nie L, Wu H-J, Hsu J-M, et al (2012) Long non-coding RNAs: versatile master regulators of gene expression and crucial players in cancer. Am J Transl Res 4:127–50
10. He R-Z, Luo D-X, Mo Y-Y (2019) Emerging roles of lncRNAs in the post-transcriptional regulation in cancer. Genes Dis 6:6–15. https://doi.org/10.1016/j.gendis.2019.01.003
11. Mattick JS, Amaral PP, Carninci P, et al (2023) Long non-coding RNAs: definitions, functions, challenges and recommendations. Nat Rev Mol Cell Biol 24:430–447. https://doi.org/10.1038/s41580-022-00566-8
12. Luo Y, Wang Y, Wang Q, et al (2013) Systemic sclerosis: Genetics and epigenetics. J Autoimmun 41:161–167. https://doi.org/10.1016/j.jaut.2013.01.012
13. Loos F, Maduro C, Loda A, et al (2016) Xist and Tsix Transcription Dynamics Is Regulated by the X-to-Autosome Ratio and Semistable Transcriptional States. Mol Cell Biol 36:2656–2667. https://doi.org/10.1128/MCB.00183-16
14. Salama EA, Adbeltawab RE, El Tayebi HM (2020) XIST and TSIX: Novel Cancer Immune Biomarkers in PD-L1-Overexpressing Breast Cancer Patients. Front Oncol 9:. https://doi.org/10.3389/fonc.2019.01459
15. Xin Y, Sun X, Chi J, et al (2018) The TGF-β-regulated X-inactive specific transcript inhibits papillary thyroid cancer migration and invasion. Transl Cancer Res 7:958–968. https://doi.org/10.21037/tcr.2018.07.06
16. Navarro P, Pichard S, Ciaudo C, et al (2005) Tsix transcription across the Xist gene alters chromatin conformation without affecting Xist transcription: implications for X-chromosome inactivation. Genes Dev 19:1474–1484. https://doi.org/10.1101/gad.341105
17. Özbalkan Z, Bağışlar S, Kiraz S, et al (2005) Skewed X chromosome inactivation in blood cells of women with scleroderma. Arthritis Rheum 52:1564–1570. https://doi.org/10.1002/art.21026
18. Peoples C, Medsger TA, Lucas M, et al (2016) Gender differences in systemic sclerosis: relationship to clinical features, serologic status and outcomes. J Scleroderma Relat Disord 1:204–212. https://doi.org/10.5301/jsrd.5000209
19. Meng X, Nikolic-Paterson DJ, Lan HY (2016) TGF-β: the master regulator of fibrosis. Nat Rev Nephrol 12:325–338. https://doi.org/10.1038/nrneph.2016.48
20. Wang Z, Jinnin M, Nakamura K, et al (2016) Long non-coding RNA TSIX is upregulated in scleroderma dermal fibroblasts and controls collagen mRNA stabilization. Exp Dermatol 25:131–136. https://doi.org/10.1111/exd.12900
21. van den Hoogen F, Khanna D, Fransen J, et al (2013) 2013 classification criteria for systemic sclerosis: an American college of rheumatology/European league against rheumatism collaborative initiative. Ann Rheum Dis 72:1747–1755. https://doi.org/10.1136/annrheumdis-2013-204424
22. Bhattacharjee D, Mondal S, Saha A, et al (2022) Effect of vasodilator and immunosuppressive therapy on the endothelial dysfunction in patients with systemic sclerosis. Clin Exp Med 23:905–915. https://doi.org/10.1007/s10238-022-00845-w
23. Bucala R, Spiegel LA, Chesney J, et al (1994) Circulating fibrocytes define a new leukocyte subpopulation that mediates tissue repair. Mol Med 1:71–81
24. Galdo F Del, Sotgia F, de Almeida CJ, et al (2008) Decreased expression of caveolin 1 in patients with systemic sclerosis: Crucial role in the pathogenesis of tissue fibrosis. Arthritis Rheum 58:2854–2865. https://doi.org/10.1002/art.23791
25. Papageorgiou SN (2022) On correlation coefficients and their interpretation. J Orthod 49:359–361. https://doi.org/10.1177/14653125221076142
26. Manetti M, Romano E, Rosa I, et al (2017) Endothelial-to-mesenchymal transition contributes to endothelial dysfunction and dermal fibrosis in systemic sclerosis. Ann Rheum Dis 76:924–934. https://doi.org/10.1136/annrheumdis-2016-210229
27. Hu B, Zhong L, Weng Y, et al (2020) Therapeutic siRNA: state of the art. Signal Transduct Target Ther 5:101. https://doi.org/10.1038/s41392-020-0207-x

Figures

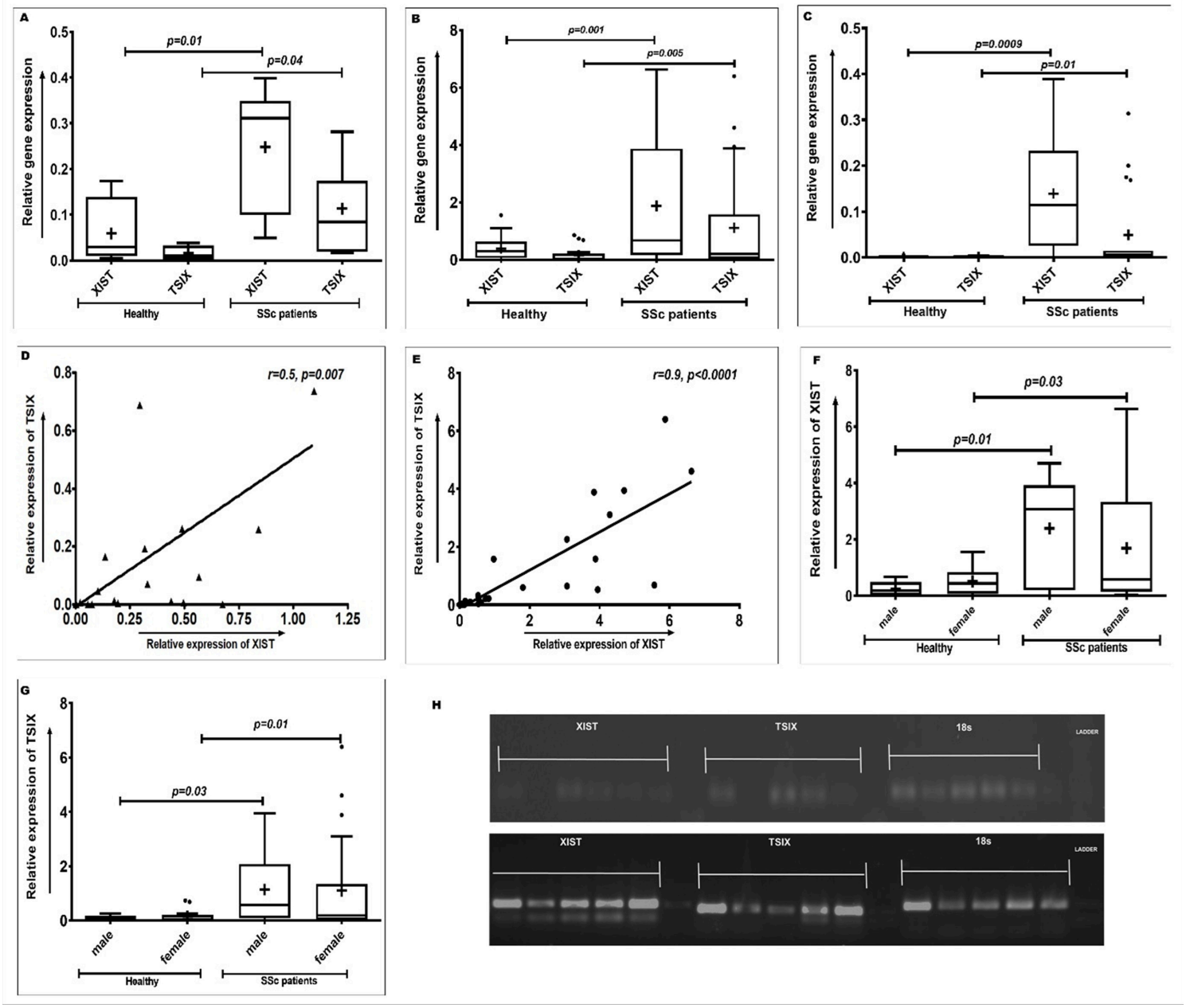


Figure 1

Relative expression of XIST and TSIX and their co-association (A-C). Comparative expression of XIST and TSIX in Skin, PBMCs, and Plasma, respectively. **(D-E)** Association of XIST and TSIX expression in healthy and patients. **(F-G)** Gender-based comparison of XIST and TSIX. **(H)** Comparative gel image regarding the transcriptional expression of XIST and TSIX and the housekeeping gene 18s between healthy and patients.

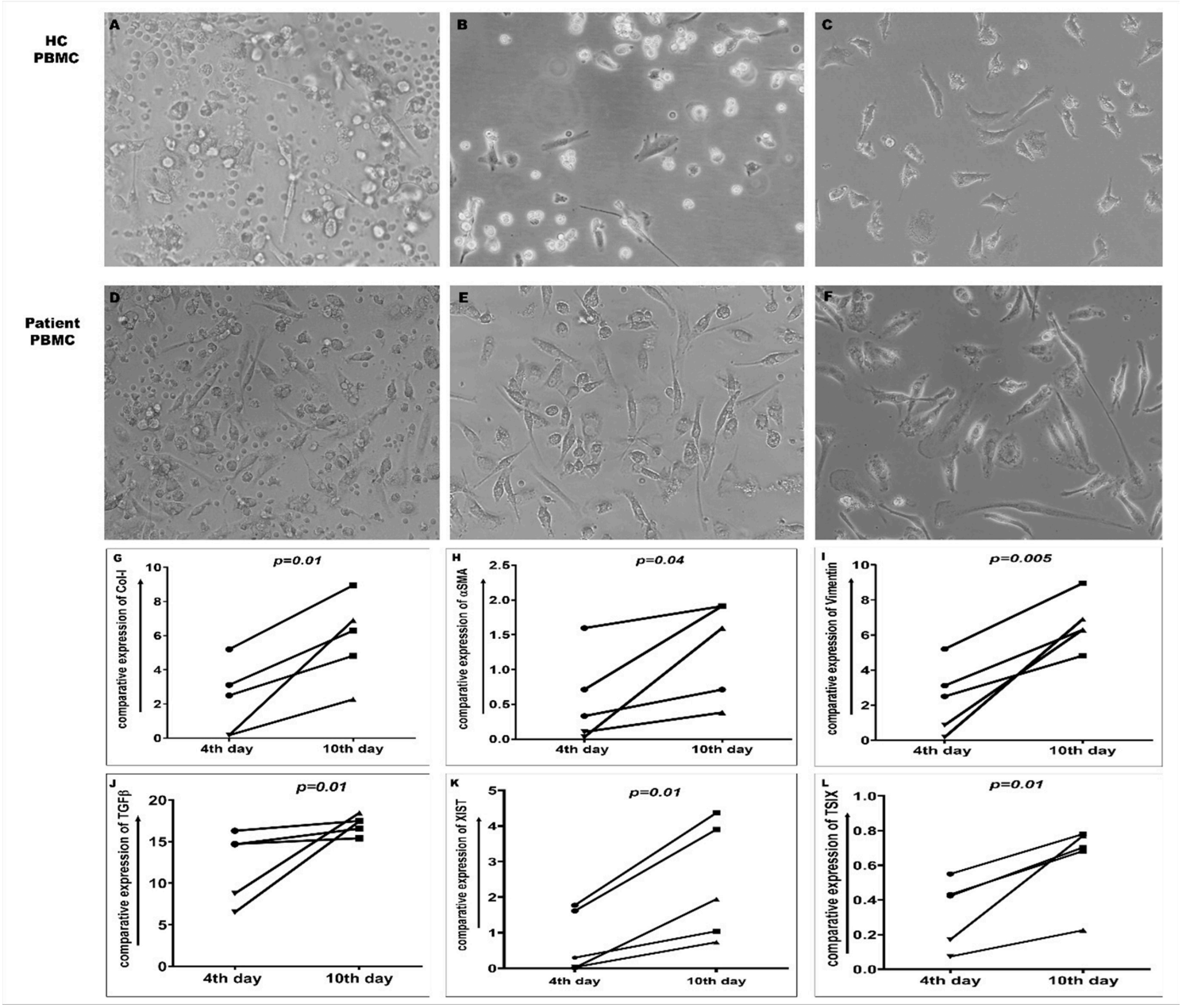


Figure 2

Fibrocyte trans-differentiation and their association with XIST and TSIX along with the fibrotic markers. (A-F) In-vitro morphological transformation of PBMCs isolated from healthy **(A-C)** and patients **(D-F)** on the 2^{nd},4^{th} and 10^{th} day of culture. Comparative graphical representation of the fibrocyte percentage (%) between healthy and patients. **(D-L)** Relative expression of Fibrotic markers along with XIST and TSIX in response to fibrocyte trans-differentiation in HCs and patients. HCs are represented as (▼) and patients are represented as (●).

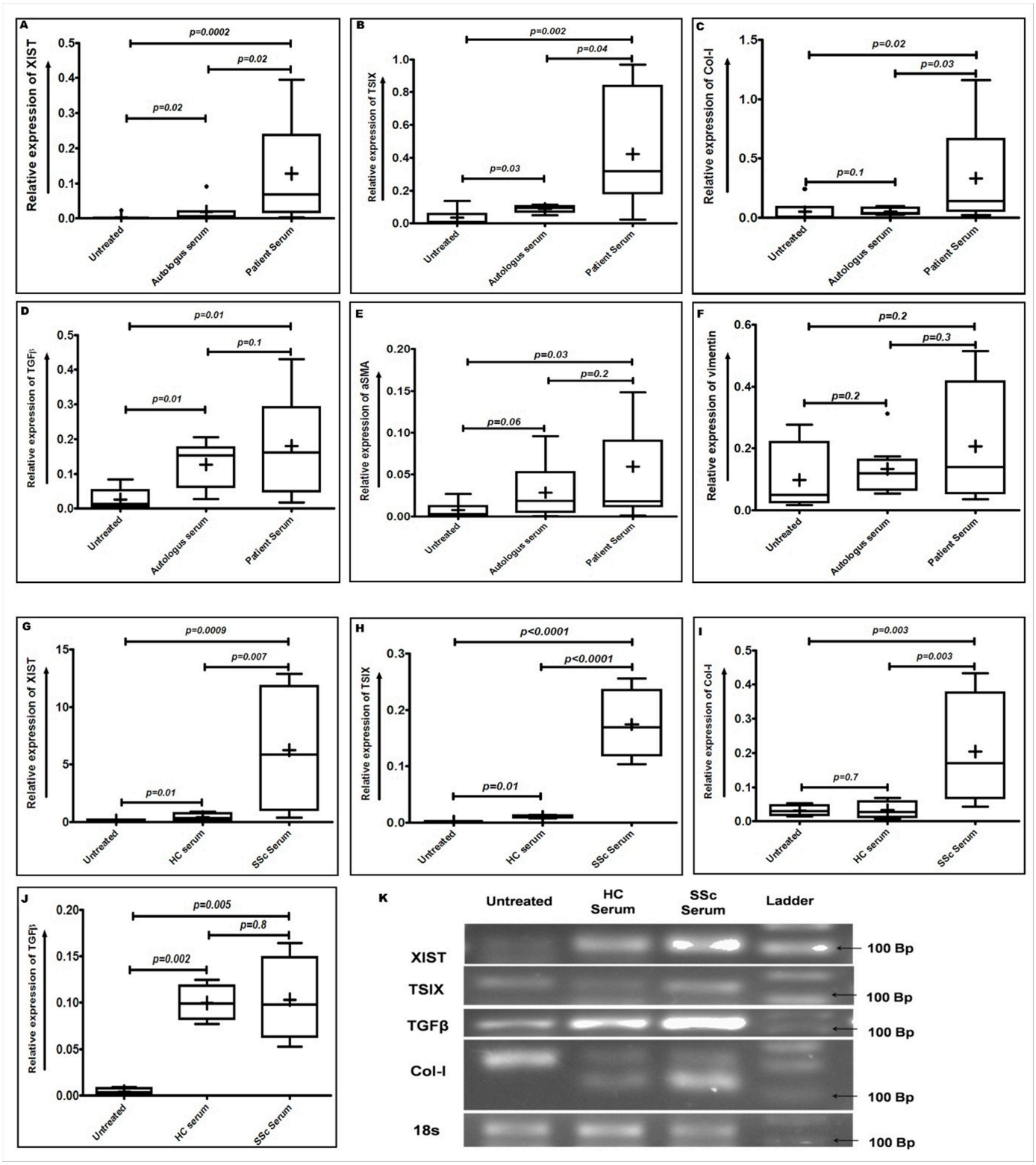


Figure 3

Relative expression of XIST and TSIX along with the fibrotic parameters upon differential serum stimulation. (A-F): Comparative expression of XIST and TSIX and fibrotic parameters after differential serum induction (autologous serum and serum isolated from patients) to the healthy PBMC culture. (**G-J):** Comparative expression of XIST and TSIX and fibrotic parameters on differential stimulation to the LX-2 cell line. (**K)** Gel

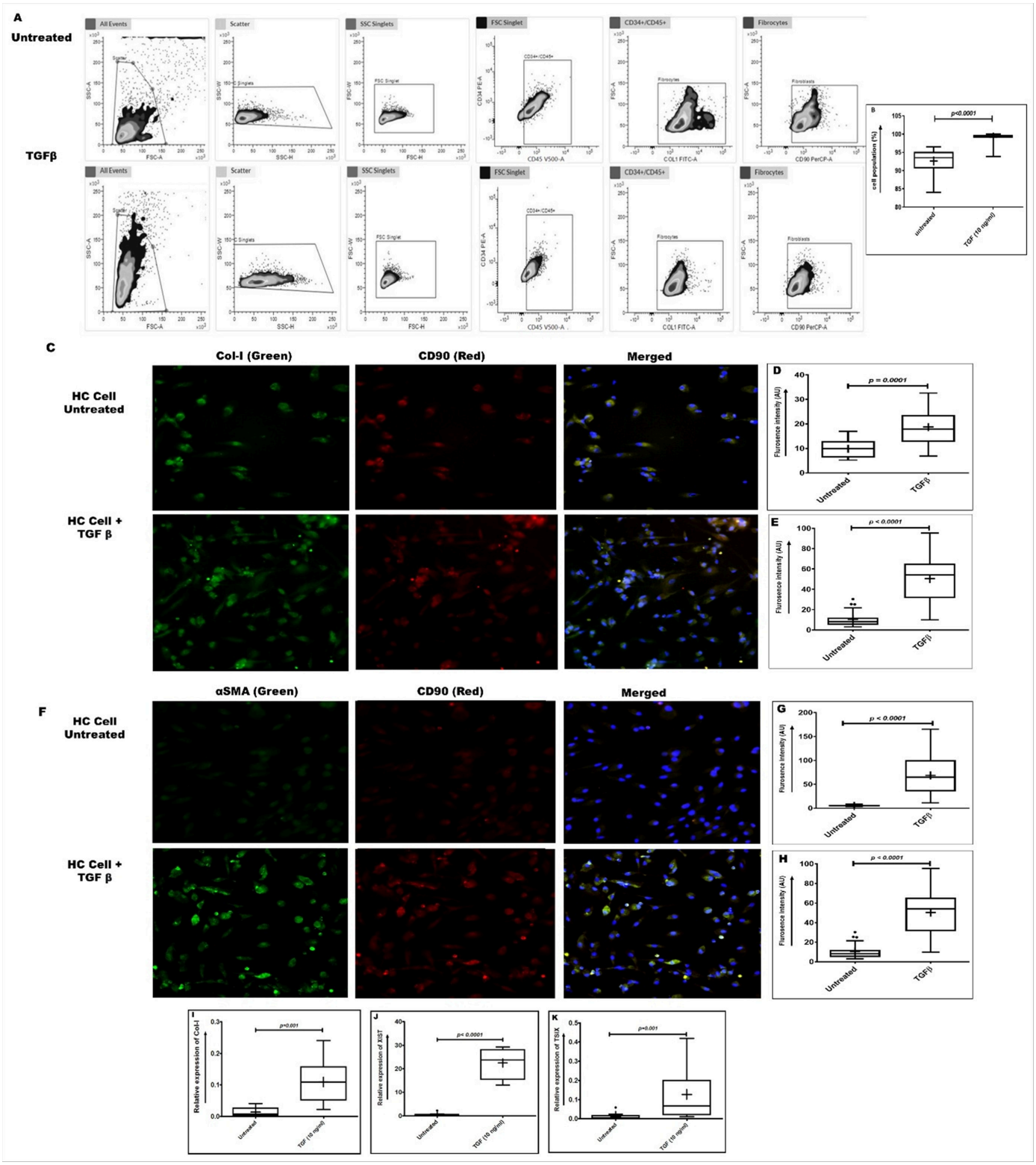


Figure 4

in-vitro assessment of fibrocyte transdifferentiation and activation and the association of TGFβ, XIST and TSIX in regulating the fibroblast activation. (A) Comparative graphical data of immunophenotyping based on in-vitro stimulation of healthy PBMCs with TGFβ (10ng/ml) and their Transdifferentiation into fibroblasts ($CD90^+/Col\text{-}I^+$) upon stimulation with TGFβ (**B)** Graphical representation of the cumulative data regarding the percentage of $CD90^+/Col\text{-}I^+$ cells**.**

(C) Microscopic data about the colocalization of fibrotic markers upon stimulation with TGFβ. Here, Col-I represents green and CD90 with red fluorescence. **(D and E)** Comparative graphical representation of the expression (fluorescence intensity) of Col-I and CD90,

respectively. **(F)** Activation of fibroblasts ($CD90^+/Col\text{-}I^+$) upon stimulation with TGFβ (10ng/ml). αSMA represents green and CD90 with red fluorescence. **(G and H)** Comparative graphical representation regarding the fluorescence intensity of CD90 and αSMA. **(I-K)** Expression of fibrotic markers Col-I, XIST, and TSIX, respectively, upon stimulation with TGFβ in healthy PBMCs.

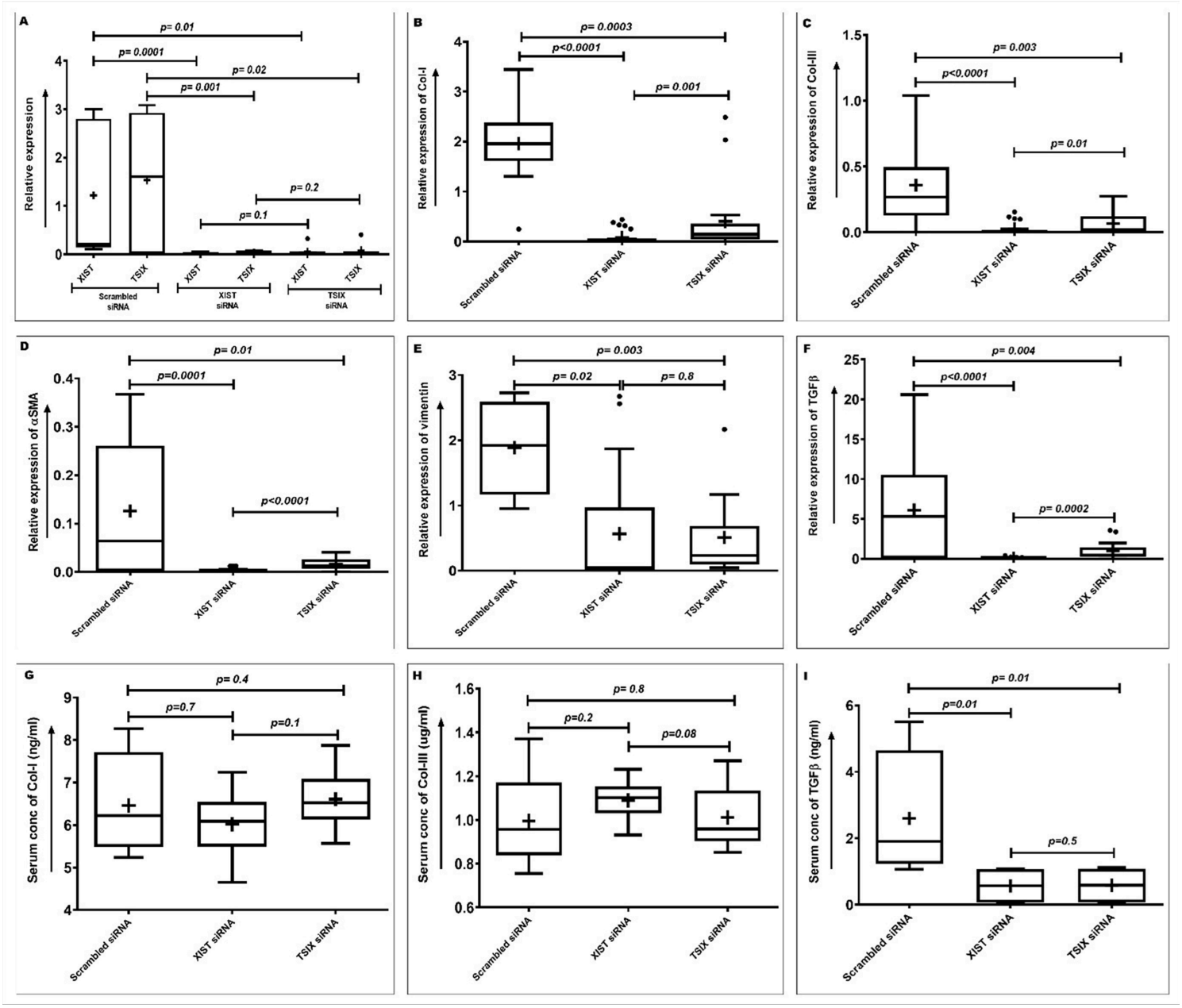


Figure 5

Interrelationship between the XIST and TSIX and the fibrotic parameters based on knockdown analysis (A) Relative expression of XIST and TSIX based on the knockdown analysis with siRNA against XIST and TSIX. **(B-F)** Comparative transcriptional expression of the fibrotic markers (Col-I, Col-III), Fibroblast activation marker (αSMA and vimentin) and the master regulator of (TGFβ) after knockdown of XIST and TSIX. **(G-I)** Comparative translational expression of fibrotic markers based on knockdown analysis with XIST and TSIX siRNA.

Supplementary Files

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- 1.AgarosegelelectrophoresisdataofPBMCfromHCregardingthetranscriptionalexpressionofXISTTSIXand18sReferencefigure1H.jpg

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- 1.AgarosegelelectrophoresisdataofXISTReferencefigure3K.jpg
- 2.AgarosegelelectrophoresisdataofTSIXReferencefigure3K.jpg
- 3.AgarosegelelectrophoresisdataofTGFReferencefigure3K.jpg
- 4.AgarosegelelectrophoresisdataofColIReferencefigure3K.jpg
- 5.Agarosegelelectrophoresisdataof18sReferencefigure3K.jpg
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