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Beyond Dosage Compensation: Xist and Tsix Orchestrate Fibrosis Through TGF-β Signalling in Systemic Sclerosis

Dipanjan Bhattacharjee
Institute of Postgraduate Medical Education and Research (IPGME&R) / SSKM Hospital
Sanchaita Misra
Institute of Postgraduate Medical Education and Research (IPGME&R) / SSKM Hospital
Sudipta Chatterjee
Institute of Postgraduate Medical Education and Research (IPGME&R) / SSKM Hospital
Pradyot Sinhamahapatra
sinhamahapatra@yahoo.com
Institute of Postgraduate Medical Education and Research (IPGME&R) / SSKM Hospital
Madhurima Basak
Institute of Postgraduate Medical Education and Research (IPGME&R), SSKM Hospital
Suchandrima Ghosh
Institute of Postgraduate Medical Education and Research (IPGME&R) / SSKM Hospital
Ayindrila Saha
Institute of Postgraduate Medical Education and Research (IPGME&R) / SSKM Hospital
Sulagna Chatterjee
Institute of Postgraduate Medical Education and Research (IPGME&R) / SSKM Hospital
Avik Sarkar
Institute of Postgraduate Medical Education and Research (IPGME&R) / SSKM Hospital
Abhishek Saha
Institute of Postgraduate Medical Education and Research (IPGME&R) / SSKM Hospital
Nitai Pada Bhattacharya
Biomedical genomic Centre
Soma Banerjee
Institute of Postgraduate Medical Education and Research (IPGME&R), SSKM Hospital
Parasar Ghosh
Institute of Postgraduate Medical Education and Research (IPGME&R) / SSKM Hospital
Alakendu Ghosh
Institute of Postgraduate Medical Education and Research (IPGME&R) / SSKM Hospital

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Abstract

Background:
Systemic sclerosis (SSc) is a complex autoimmune disorder characterized by progressive fibrosis, primarily driven by TGF-β signalling. While long non-coding RNAs (lncRNAs) such as Xist and Tsix are traditionally associated with X-chromosome inactivation, their role in fibrotic regulation remains poorly understood.

Objective:
This study aimed to investigate the regulatory role of Xist and Tsix in the fibrotic pathophysiology of SSc and their interaction with TGF-β signalling.

Methods:
Expression profiles of Xist and Tsix were analyzed in SSc patients and healthy controls. Fibrocyte proportions and PBMC-derived fibroblast transformation were assessed through in vitro experiments, including serum induction and TGF-β stimulation. Knockdown studies were performed to evaluate their regulatory influence on fibrotic markers and TGF-β expression.

Results:
SSc patients exhibited tissue- and gender-independent upregulation of both Xist and Tsix, contrasting with the physiological gender-based expression patterns observed in healthy controls (HCs). Increased fibrocyte percentages and enhanced fibroblast transdifferentiation and activation were observed *in vitro* serum stimulation to HC PBMC and LX2 cell. Both lncRNAs showed strong positive correlations with fibrotic markers and demonstrated synergistic overexpression during fibroblast transdifferentiation and activation. TGF-β stimulation further amplified Xist and Tsix expression, while their knockdown resulted in reduced TGF-β levels, indicating a positive regulatory coordination in the Xist–Tsix–TGFβ axis to modulate the fibrotic pathophysiology in SSc.

Conclusion:
Our study has demonstrated the collaborative association of Xist and Tsix, upstream regulators of fibrosis in SSc in the TGF-β mediated canonical pathway of fibrosis. Xist and Tsix could be promising biomarkers for disease activity. Moreover, targeting the Xist and Tsix may provide a more selective and potentially safer therapeutic alternative, despite inhibition of TGF-β. Further studies are required to elucidate their precise molecular mechanisms and translational or clinical applicability.

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].

TGF-β 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 TGF-β and TSIX in the regulation of the fibrotic pathogenesis of scleroderma. According to this study, overexpressed TGF-β might induce Tsix transcription, which stabilises col-I mRNA, thereby promoting fibrosis. They did not find any alteration or regulative association of XIST with the SSc pathogenesis.[20]

This study aimed to explore the potential interplay between Xist and Tsix in driving the constitutive upregulation of fibrotic markers, with particular focus on their role in cellular transformation and activation in systemic sclerosis.

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 Post Graduate 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 [23]. 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 the 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 [24]. 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.

PBMC isolated from the healthy individuals were cultured following the same protocol mentioned above. To identify the association among TGF-β, 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) [25]. To avoid biasness, 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 done with 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 the 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 direction. BD Cytofix- cytoplasm

was added to fix the cells, and then the cells were pelleted down and dissolved within 100µl 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 by using GraphPad Prism v 5.0 (GraphPad Software Inc., CA, USA). The distribution of the data was analysed by the Shapiro-Wilk test. Mann-Whitney test and paired t-test were done to compare nonparametric and parametric data, respectively. Unpaired t-tests with Welch's correction were done to compare normally and non-normally distributed data. The data showing $p < 0.05$ were considered statistically significant for every analysis. Correlation of any two parameters was done using Spearman’s correlation coefficient.

RESULTS

Baseline characteristics:

The mean ages of patients (n = 28): 35.30 ± 9.46 years (mean ± SD), HCs (36.37 ± 10.85, n = 25) did not show a significant difference (*p = 0.01*). The male-to-female ratio of HC (20:5) and the patient groups (22: 6) didn’t show any significant difference. Among the total patient groups, 96.4% of patients were SCL70 positive. Detailed demographical data and the status of autoantibody formation are tabulated in **Table 1.**

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) **(**Fig. 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) **(**Fig. 1B and 1C**).** Tsix and Xist showed a moderate-to-strong positive correlation both in HCs (*r = 0.5, p = 0.007*) as well as in patients (*r = 0.9, p < 0.0001*) **(**Fig. 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, no discernible variations were observed in Xist and Tsix expression between male and female (*p = 0.4 for both*) **(**Fig. 1F and 1G**).** Gel electrophoresis data showed significant overexpression of both Xist and Tsix in patients compared to healthy controls **(**Fig. 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 **(**Fig. 2A-2B**)** than of HCs **(**Fig. 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 **(**Fig. 2C**)** than in the HCs **(**Fig. 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 TGF-β (*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 HC and patients. **(**Fig. 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*) **(**Fig. 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*) **(**Fig. 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*) **(**Fig. 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*) **(**Fig. 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*) **(**Fig. 3E**)**. Vimentin didn't show any significant difference between the serum-treated or untreated group **(**Fig. 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). Significant difference was also observed between the Xist expression of the untreated and the HC serum-treated group **(**Fig. 3G**)**. A significant change in the Tsix expression was observed irrespective of 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 significant elevated expression of Tsix (*p = 0.01*) **(**Fig. 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 = 0.00*3 respectively). No significant differences were observed for Col-I expression between the HC serum treated and untreated group (*p = 0.7*) **(**Fig. 3I**)**. The expression of TGFβ showed elevation after treatment with from healthy serum (*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 **(**Fig. 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 the treatment with patient serum **(**Fig. 3K**)**.

TGFβ-mediated induction of fibrosis and its association with Xist and Tsix

FACS analysis showed a higher percentage of transdifferentiated fibroblast $CD90^{+}$/$Col\text{-}I^{+}$ was observed after stimulation with TGFβ to the PBMCs isolated from HCs compared to the unstimulated group *(p < 0.0001*) **(**Fig. 4A and 4B**).** Microscopic data also demonstrated significant increased expression of COL-I and CD90. Microscopic data showed elevated expression of COL-I and CD90 upon stimulation with TGFβ to the cultured HC PBMC compared to the unstimulated HC PBMCs *(p < 0.0001 for both*) **(**Fig. 4C, 4D and 4E, **respectively).** Upon stimulation with TGFβ, there was significant upregulation of αSMA (*p < 0.0001*) and CD90 *(p < 0.0001*) **(**Fig. 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) **(**Fig. 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 **(**Fig. 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). (Fig. 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 **(**Fig. 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*) **(**Fig. 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 **(**Fig. 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 **(**Fig. 5I**)**.

DISCUSSION

Xist and Tsix have long been acknowledged for their pivotal role in regulating dosage compensation or 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, individuals diagnosed with SSc exhibited elevated Tsix and Xist expression in both their skin and blood samples (including PBMCs and plasma). This observation suggests a potential involvement of these two molecules in the pathogenesis of SSc. Furthermore, our findings revealed a significant positive correlation between the expression levels of these two long non-coding RNAs (lncRNAs). However, no such association between Xist and Tsix was found in previous research [13]. Notably, X chromosome inactivation is a fundamental regulatory process ensuring dosage compensation in females. This process is primarily governed by the lncRNA Xist, with Tsix identified as its antagonist within this regulatory framework [16]. Similar to previous reports, our study found that in HC, females have elevated Xist expression compared to males, whereas Tsix did not show any gender-biased differences, which might be due to the regulatory feature of dosage

compensation in mammalian females.[13,14] In contrast to this, concomitant elevation of Xist and Tsix was observed irrespective of gender within the patients with SSc. The normal gender-based regulation of Xist and Tsix appears to be disrupted in SSc and shows abnormal co-expression. Moreover, both Xist and Tsix showed an inverse expression pattern contrasting HC. No significant difference regarding the expression of Xist between the male and female SSc patients, and a comparative elevation of Tsix in males compared to the female patients reflects a compensatory response to this imbalance. Overall, this non-gender biased concomitant alterations of both Xist and Tsix suggest that Xist–Tsix dysregulation could contribute to the underlying disease progression in SSc.

Recent studies have highlighted Tsix as a regulator of fibrosis, particularly through its role in the stabilization of collagen mRNA, while Xist has not been previously defined for such mechanisms.[20,26] Contrary to early studies, however, our findings reveal a significant association of both Xist and Tsix with fibrotic markers, which might have an indication that Xist, alongside Tsix, may actively contribute to the fibrotic process in SSc which suggests a previously underappreciated regulatory association of these molecules in modulating fibrosis.

In our investigation, we found that the percentage of fibrocytes was significantly higher in SSc patients than in HCs. The in vitro experiment with PBMCs, isolated from both HCs and patients, revealed morphological transformation from fibrocytes to fibroblasts, accompanied by a marked upregulation of fibrotic markers along with Xist and Tsix. The concurrent overexpression and positive correlation of these lncRNAs with fibrotic molecules strongly suggest that this “twin lncRNA” axis may play a pivotal role in regulating the fibrotic processes underlying SSc.

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 isolated from HC. Moreover, upon differential serum induction to the HC PBMCs exhibited synergistic overexpression of Xist and Tsix alongside fibrotic markers, highlighting their potential involvement in fibroblast transdifferentiation and activation.

Additionally, TGF-β has been identified as a key regulator of scleroderma. Recent studies have identified this as influencing fibrosis through epigenetic mechanisms.[27] While earlier studies have linked Tsix with TGF-β–mediated collagen regulation, the role of Xist has remained unclear and somewhat contradictory. [20,28] However, in our study, TGF-β stimulation resulted in clear fibroblast activation along with a simultaneous increase in both Xist and Tsix. Thus, it is clear from this data that Tsix and Xist, together with TGF-β signalling, synchronise with fibroblast activation, suggesting a pointing toward a more active and coordinated role in driving fibrosis in SSc.

The knockdown studies of Xist and Tsix also revealed their transcriptional connection with the potential regulatory molecules of fibroblast transdifferentiation activation and fibrosis. Our study also demonstrated that TGFβ, as the master regulator of fibrosis, gets downregulated in response to both of them. So, Xist and Tsix are positive regulators of TGFβ, and have a combinatory effect 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 traditional regulation of dosage compensation. These lncRNAs are functionally linked to key fibrotic events, including fibrocyte transdifferentiation, fibroblast activation, and upregulation of profibrotic markers. Notably, their positive regulatory influence on TGF-β positions them as upstream modulators of the fibrotic cascade and suggests them as “masters of master regulator” in the context of Xist–Tsix–TGF-β pathological axis, which may serve as a critical regulatory checkpoint for disease prognosis. Moreover, in physiological processes the pleiotropic roles of TGF-β includes growth, immune regulation, and tissue homeostasis. Studies suggested therapeutically targeting TGF-β will be associated with undesirable adverse effects and may alters the physiology.[29] In this context, targeting Xist and Tsix may offer a more selective and potentially safer strategy to modulate fibrosis. Furthermore, their consistent association with fibrotic markers and cellular transformation highlights their potential utility as diagnostic or prognostic biomarkers for disease activity and progression in SSc.

Despite these promising insights, the precise molecular mechanisms underlying the interaction of TGF-β with Xist and Tsix remain to be elucidated, which will provide a clear idea to understand their therapeutic potential and to translate it into clinical applications. Overall, this study will open a new avenue in SSc research, positioning Xist and Tsix not merely as epigenetic regulators but as clinically relevant targets which will help to redefine current approaches to the diagnosis and treatment of fibrosis.

STATEMENT AND DECLARATION

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**

Raw data is available to the authors. Will be shared if required.

4. **Competing interests:** Nothing to declare.

5. **Funding, grant/award info:**

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6. **Authors' contributions**

All authors contributed to the study conception, study design and helped to pursue the study. D. Bhattacharjee wrote the initial draft of the work, and other contributors provided feedback on the succeeding iterations. Each author reviewed and gave their approval to the finished work.

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Table

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	-	-	
		-	

Figures

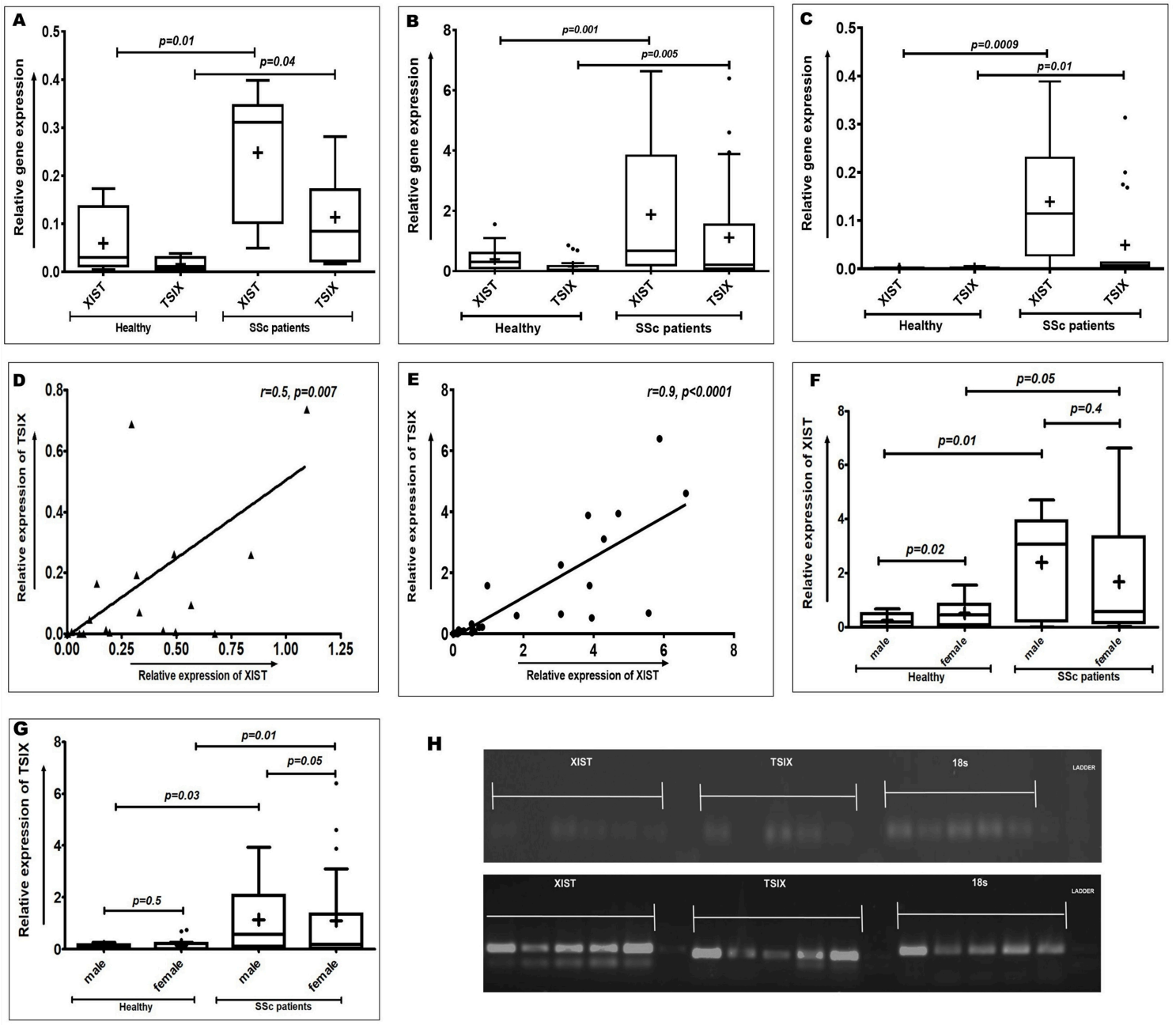


Figure 1

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 basis 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.

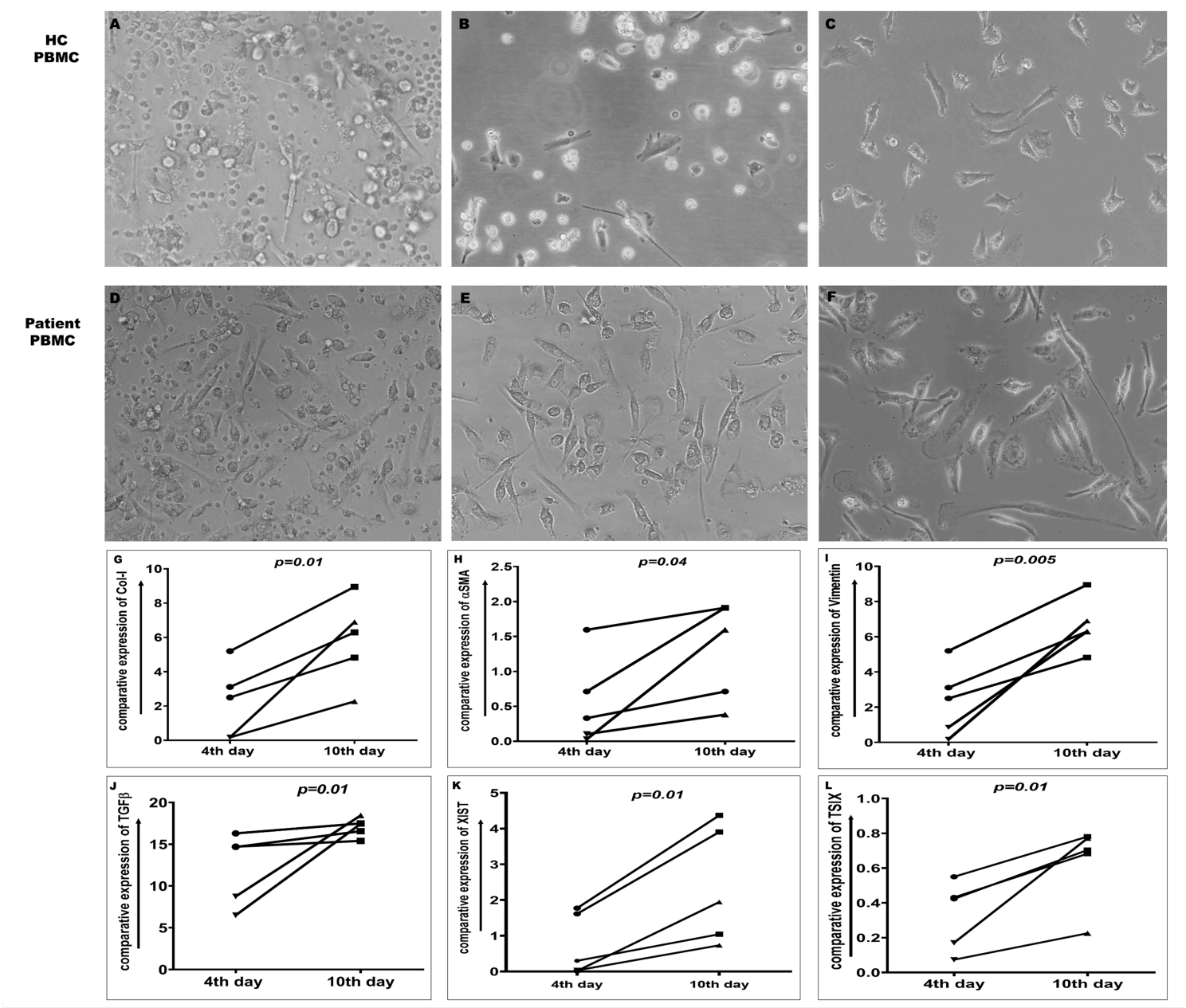


Figure 2

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 (●).

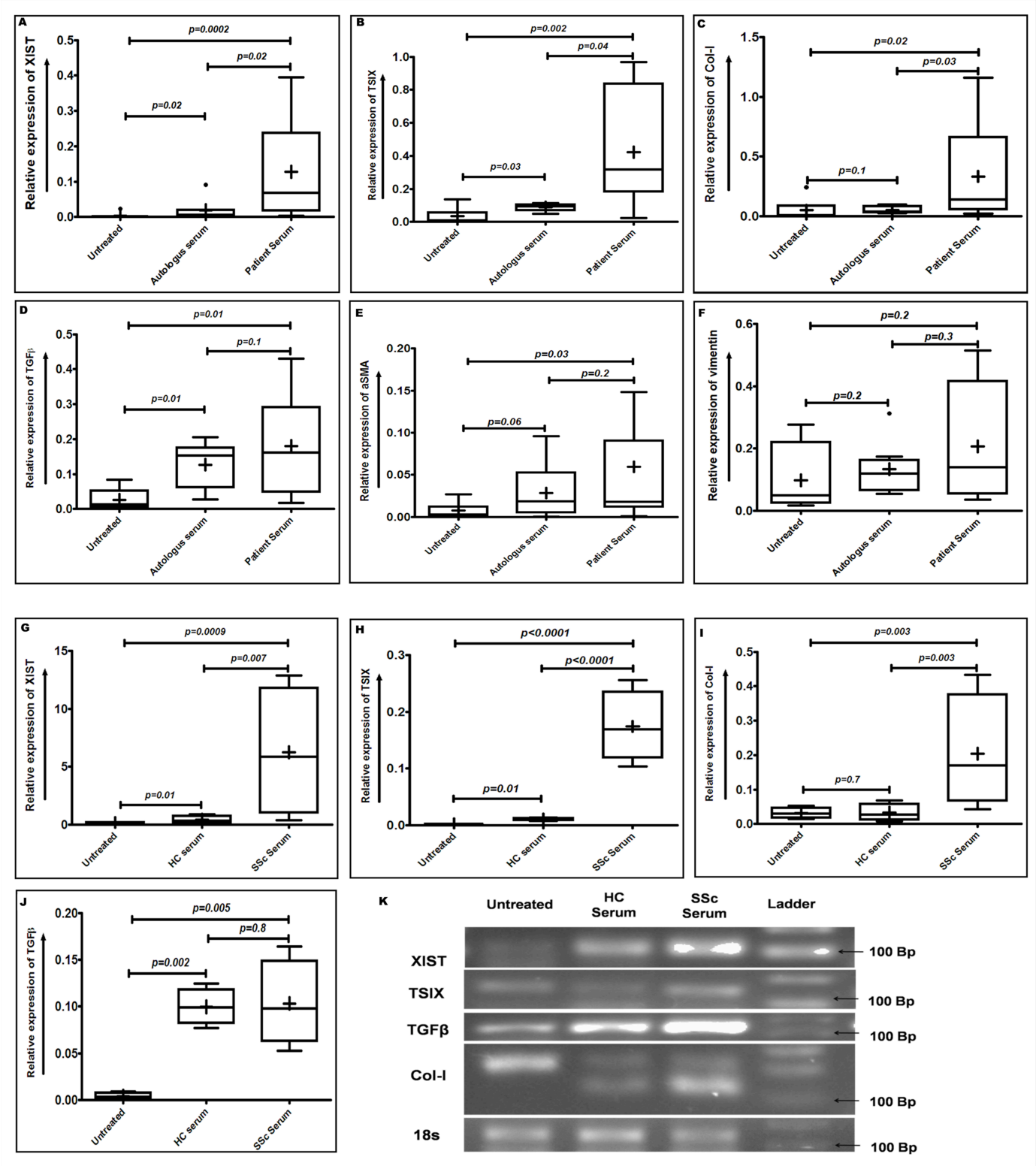


Figure 3

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 image Regarding the expression of Xist and Tsix with the fibrotic markers on stimulation with serum (isolated from healthy and patient) to the fibroblast cell line.

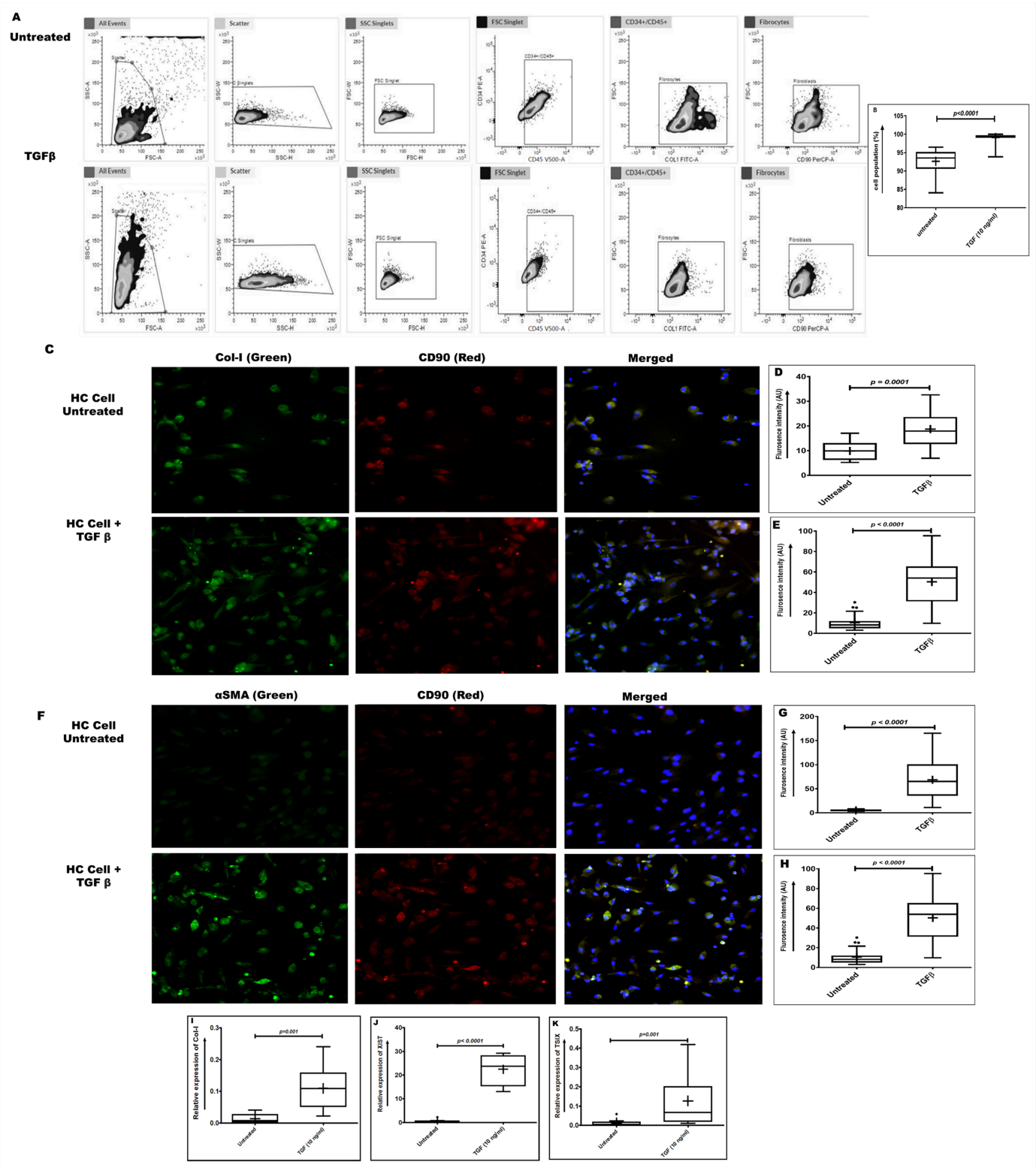


Figure 4

***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 i*n-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 the CD90 and αSMA. **(I-K)** Expression of fibrotic markers Col-I, Xist, and Tsix respectively, upon stimulation with TGFβ to the healthy PBMCs.

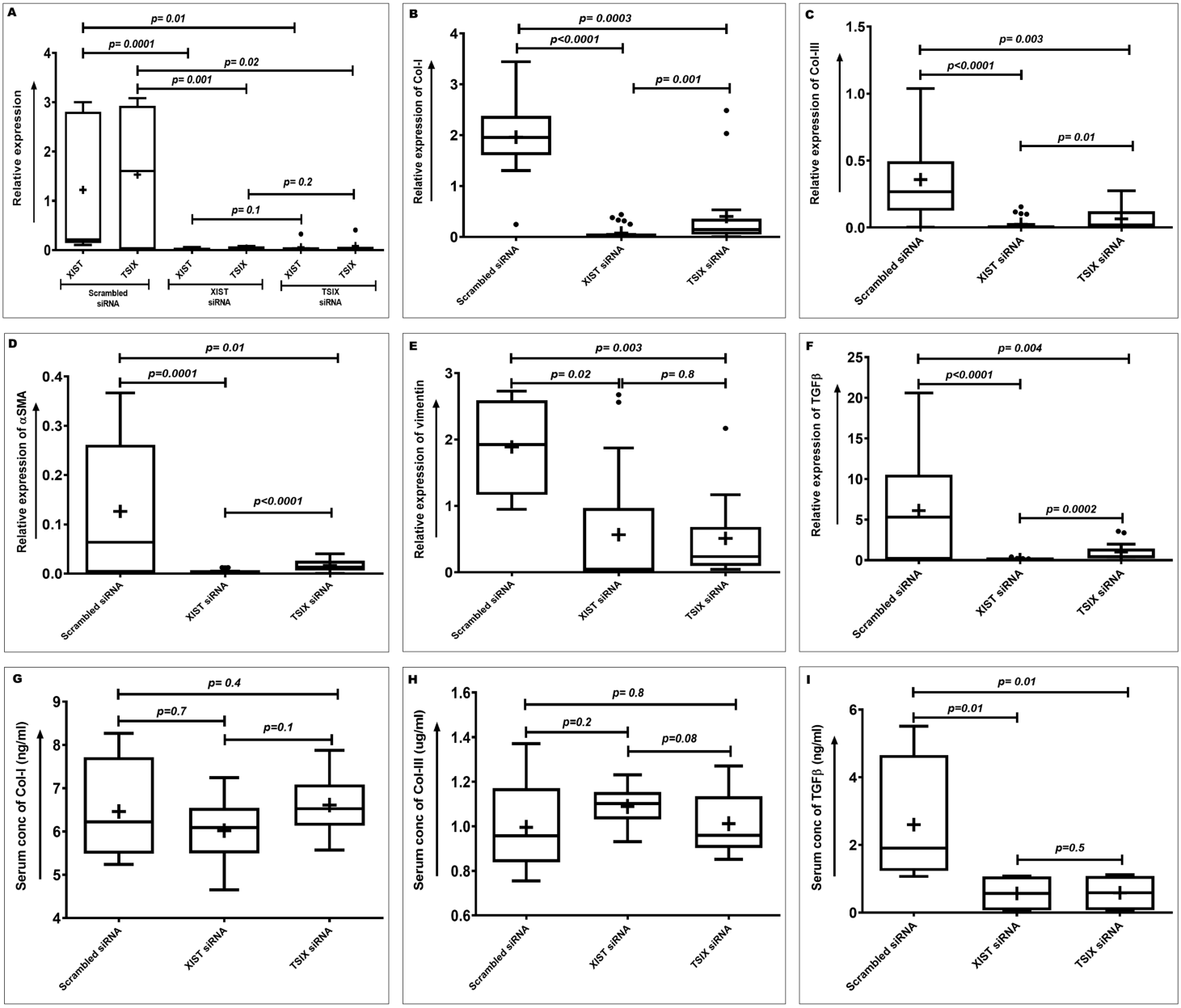


Figure 5

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 (*α*SMA 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.