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Splicing isoforms associated with TGFβ-induced myofibroblast activation

Opeoluwa Alli-Oke¹, Danny Bergeron², Fiona Kessai^{1,5,6}, Philippe Thibault^{1,7}, Mathieu Durand² and Jean-Philippe Brosseau^{1,3,4*}

Abstract

Background Myofibroblast differentiation is a key process in developmental biology and involved in numerous physiopathology. The gene expression program orchestrating fibroblast to myofibroblast differentiation, as well as its recapitulation by TGFβ stimulation in vitro, is relatively well characterized. Intriguingly, it is known that the splicing isoform EDA+FN1 is a marker and driver of myofibroblast differentiation, but the alternative splicing landscape of myofibroblast is unknown.

Results Here, we performed a high-throughput transcriptomic approach by RNA-Seq in a primary skin fibroblast line and uncover more than 250 splicing isoforms associated with TGFβ-induced myofibroblasts using two different bioinformatic pipelines. This splicing profile highlights a distinct layer of regulation when compared to the global gene expression profile of myofibroblasts. A 5 alternative splicing event (ASE) signature [*ACTN1*-19 A/19B; *COL5A1*-64 A/64B; *COL6A3* exon 4; *FLNA* exon 30 and *TPM1*-6a/6b] was further validated by ddPCR and AS-PCR and retrieved in publicly available RNA-Seq datasets describing other TGFβ-stimulated lung and skin fibroblasts. Surprisingly, TGFβ does not induce an EDA+FN1 splicing shift, although it stimulates global fibronectin expression.

Conclusions Thus, we conclude that the 5 ASEs signature may be used as putative universal myofibroblast markers and be of functional significance to myofibroblast formation and biology.

Keywords Alternative splicing, Myofibroblast, TGFβ, RNAseq, GM05386

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Introduction

Following injury, fibroblasts follow a developmental program to differentiate into myofibroblasts. This way, they acquire enhanced contractile properties through the assembly of the characteristic stress fibers and additional extracellular matrix (ECM) secretion capacity necessary to regenerate normal tissue function [1]. One of the hallmarks of myofibroblast is the expression of alpha-smooth muscle actin (α -SMA). α -SMA overexpression not only serves to identify myofibroblasts but also drives their formation [2].

Injury-induced myofibroblast differentiation can be recapitulated in vitro by stimulating fibroblasts with the tumor growth factor beta (TGFβ), the master regulator



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of myofibroblast differentiation. The signaling cascade downstream of TGF β activating the gene expression program driving the myofibroblasts state is incomplete. Canonical signal downstream of TGF β receptors involved a phosphorylation cascade culminating in the translocation of a transcriptional complex binding SMAD-responsive elements, driving the expression of α -SMA, among others [3]. Some genes have been identified to drive myofibroblast activation, either by overexpressing or silencing them. An example is periostin (POSTN), a non-structural matricellular protein that has been studied to drive this developmental program [4]. In addition, the collagen family is another group of genes that has been well associated with myofibroblast activation. In fact, one of the universal markers for myofibroblast activation is the increase in the production of collagen types I and III [5].

Another major ECM protein is fibronectin (FN1). Despite the wide study of various genes linked with myofibroblast activation, only fibronectin extra domain A (EDA+FN1) has been identified as a spliced variant that drives the myofibroblast program [6]. Intriguingly, no other splicing isoforms have been associated with myofibroblast activation. Alternative splicing is a powerful process producing many protein isoforms from a single

gene by selectively including or excluding certain exons of its mature RNA. Numerous examples have illustrated the functional impact of alternative splicing in the literature [7]. In this study, we hypothesized that there is more than the EDA+FN1 isoform that is associated with myofibroblast differentiation.

Results

TGF β -drive myofibroblast differentiation in primary skin fibroblasts

To confirm that TGF β stimulation differentiates the primary skin fibroblast into myofibroblast; an immunofluorescence assay examining the α -SMA expression was performed. In comparison to untreated fibroblasts; formation of stress fibres was witnessed in the TGF β -treated condition (Fig. 1A). Quantification of the α -SMA resulting fluorescence signal confirm a significant increase in the TGF β -treated samples (Fig. 1B). Untreated fibroblasts are sensitive to the stiffness of their environment and do express basal level of α -SMA upon culturing on plastic dish [8] but the characteristic stress fibers and morphology changes associated with myofibroblast formation were only observed in the TGF β -treated condition. Moreover, we monitored the increase in the mRNA

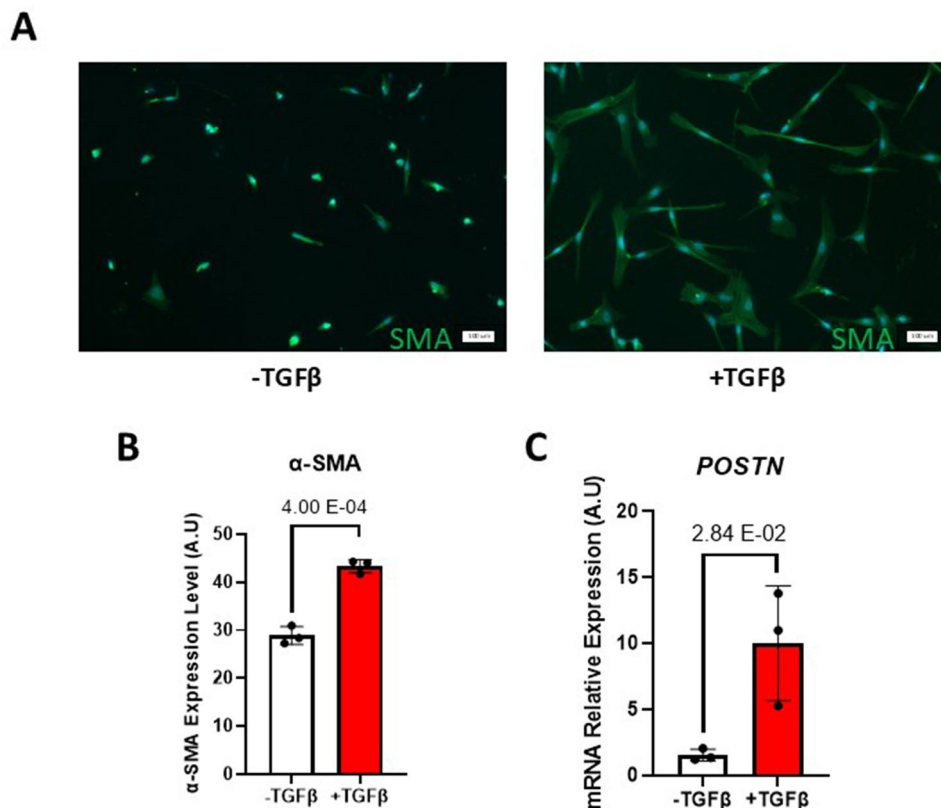


Fig. 1 TGF β -induced differentiation of primary skin fibroblasts to myofibroblast. **(A)** Immunofluorescence images showing α -SMA stress fibers (Green) indicative of myofibroblast after two days of TGF β stimulation merged to DAPI (Blue). The scale bar equals 100 μ m **(B)** Bar graph representing the quantification of α -SMA immunofluorescence signal from (Fig. 1A) as arbitrary units (A.U.) **(C)** Bar graph representing the relative mRNA expression of the myofibroblast marker *POSTN* by qRT-PCR. Errors bars are shown as mean $\pm$ standard error of the mean

expression of *POSTN* as an additional surrogate for myofibroblast formation by real-time quantitative PCR (qRT-PCR). As expected, we observed a significant increase in *POSTN* expression with samples stimulated with TGF β compared with untreated samples (Fig. 1C).

To further confirm the myofibroblast state of the TGF β -induced primary skin fibroblast, a genome-wide transcriptomic analysis was performed on TGF β -treated vs. untreated fibroblasts by RNA-Seq. Initially, we performed a principal component analysis, and it indicates that biological replicates are clustering as expected (Fig. S1). We identified a total of 453 up-regulated genes associated with TGF β stimulation while 1 998 genes were downregulated out of 18 910 detected genes (Fig. 2A-B, File S1). Among the up-regulated genes, we noticed genes encoding collagens (e.g. *COL4A2*, *COL11A1*, *COL1A1*, *COL5A1*, *COL7A1*, *COL8A2*) and some involved in extracellular matrix remodelling (e.g. *MMP2*, *MMP8*, *MMP10*, *MMP14*, *LOXL2*) as expected (Fig. 2B). Of note,

the gene encoding α -SMA (*ACTA2*) is overexpressed in TGF β -induced myofibroblasts (Fig. 2C). To determine if the 453 identified upregulated genes associated to TGF β -induced fibroblast are significantly associated to biological processes related to myofibroblasts, we performed gene ontology analysis. As expected, biological processes associated to myofibroblast activation such as ECM-receptor interaction and focal adhesion were found (Fig. 2D). Similarly, further gene ontology analysis of the KEGG pathway also identified the ECM-receptor interaction pathways (Fig. S2A), while analysis of the molecular functions reveals the ECM structural component, fibronectin binding, and collagen binding (Fig. S2B). The cellular components analysis also consists mainly of ECM-related terms (Fig. S2C). Altogether, the phenotype and expression markers of TGF β -induced fibroblasts are consistent with the myofibroblast state, and hence, our TGF β stimulation procedure yields *bona fide* myofibroblasts in vitro.

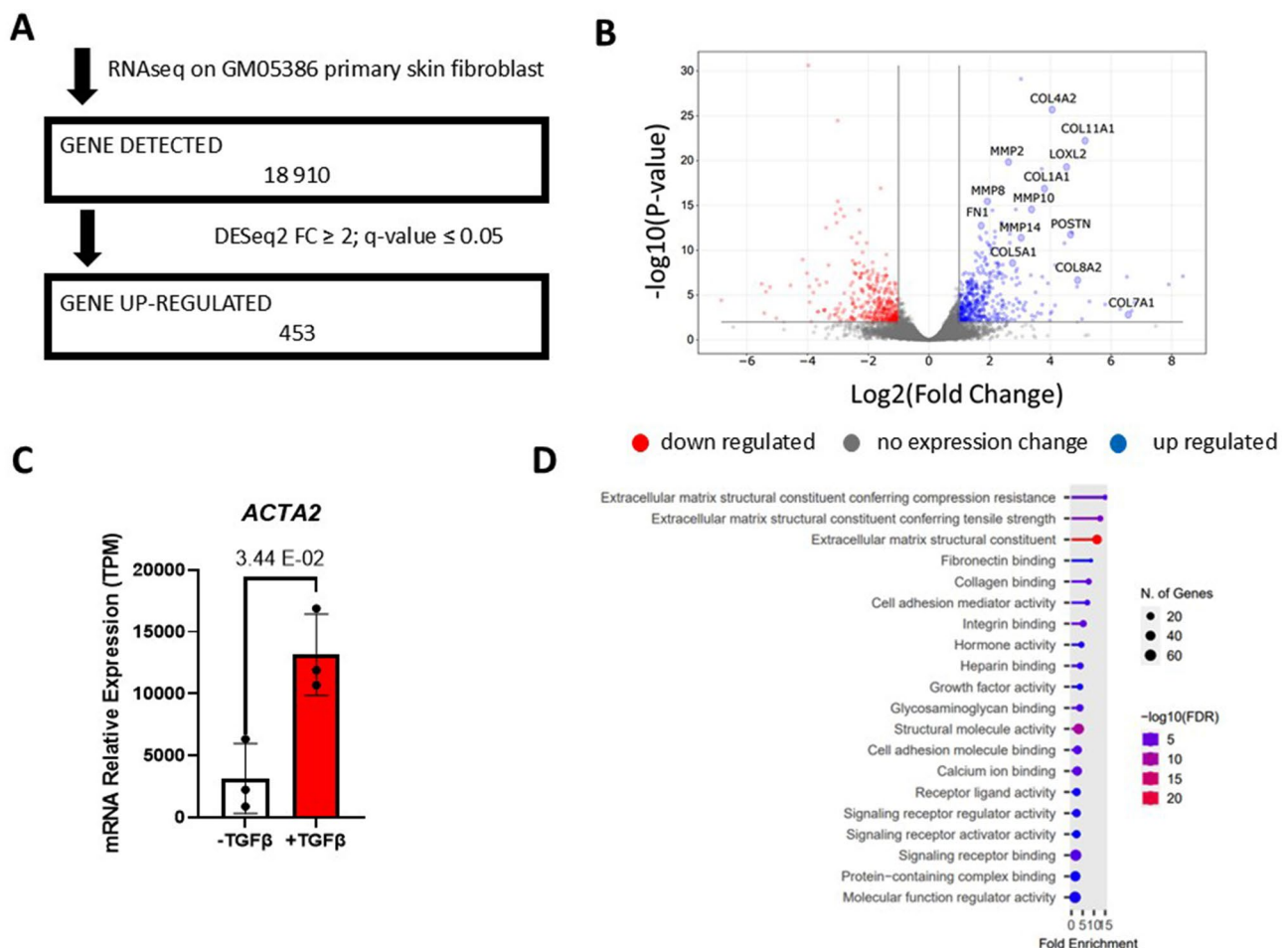


Fig. 2 Transcriptomic analysis of TGF β -treated primary skin fibroblast **(A)** RNA-Seq pipeline showing the number of genes detected, cut-off parameters and TGF β -dependent upregulated genes **(B)** Volcano plot to indicate the genes upregulated and downregulated in TGF β -induced myofibroblast **(C)** Bar graph representing the relative mRNA expression of the myofibroblast marker *ACTA2* by qRT-PCR. Errors bars are shown as mean $\pm$ standard error of the mean. **(D)** Gene ontology for biological processes associated with TGF β -induced myofibroblast activation

Transcriptome-wide splicing analysis highlights ASEs linked to TGFβ-induced myofibroblast differentiation

To identify unbiasedly any alternative splicing events (ASEs) associated with the fibroblast differentiation to myofibroblast, we re-analyzed the RNA-Seq reads we previously obtained (Fig. 2A) using two bioinformatic pipelines (Fig. 3): the RNomics UdeS pipeline [9] and rMATS [10]. Using the rMATS analysis, 24 548 isoforms were detected, and 251 isoforms were found to be significantly associated with myofibroblast (Fig. 3 – left, File S2). Out of the 30 002 ASEs detected using the RNomic UdeS pipeline, 8 ASEs were associated with myofibroblast (Fig. 3 – right, File S3). To identify the ASEs common to both analyses, a crossmatch was performed, yielding 6 ASEs that appeared in both analyses (Fig. 3, bottom). These are *ACTN1*-19 A/19B; *COL5A1*-64 A/64B; *COL6A3* exon 4; *FLNA* exon 30; *EDA*+*FN1* and *TPM1*-6a/6b (Fig. 4A). As depicted in Fig. 4A, The splicing change we observed for Col6a3 is the inclusion of exon 4 (long isoform) or exclusion of exon 4 (short

isoform). However in all cases, exon 3 is not detected. This is why it is annotated as long isoform = deltaexon 3 and short isoform deltaexon3 deltaexon 4.

Experimental validation of RNA-Seq–identified ASEs associated with myofibroblast differentiation

Next, we proceeded by validating these 6 RNA-Seq identified ASEs (Fig. 4B). To do so, we performed digital droplet PCR (ddPCR) using isoform-specific primers and output the results as PSI values (Fig. 4C). AS-PCR was used for *FLNA* exon 30 because the short isoform was not detected by ddPCR. Impressively, 5 out of 6 ASEs were significantly associated with myofibroblast and importantly, the direction of splicing change was the same as initially observed by RNA-seq (Fig. 4B-C). Overall, we successfully identified and validated several splicing isoforms associated with myofibroblasts.

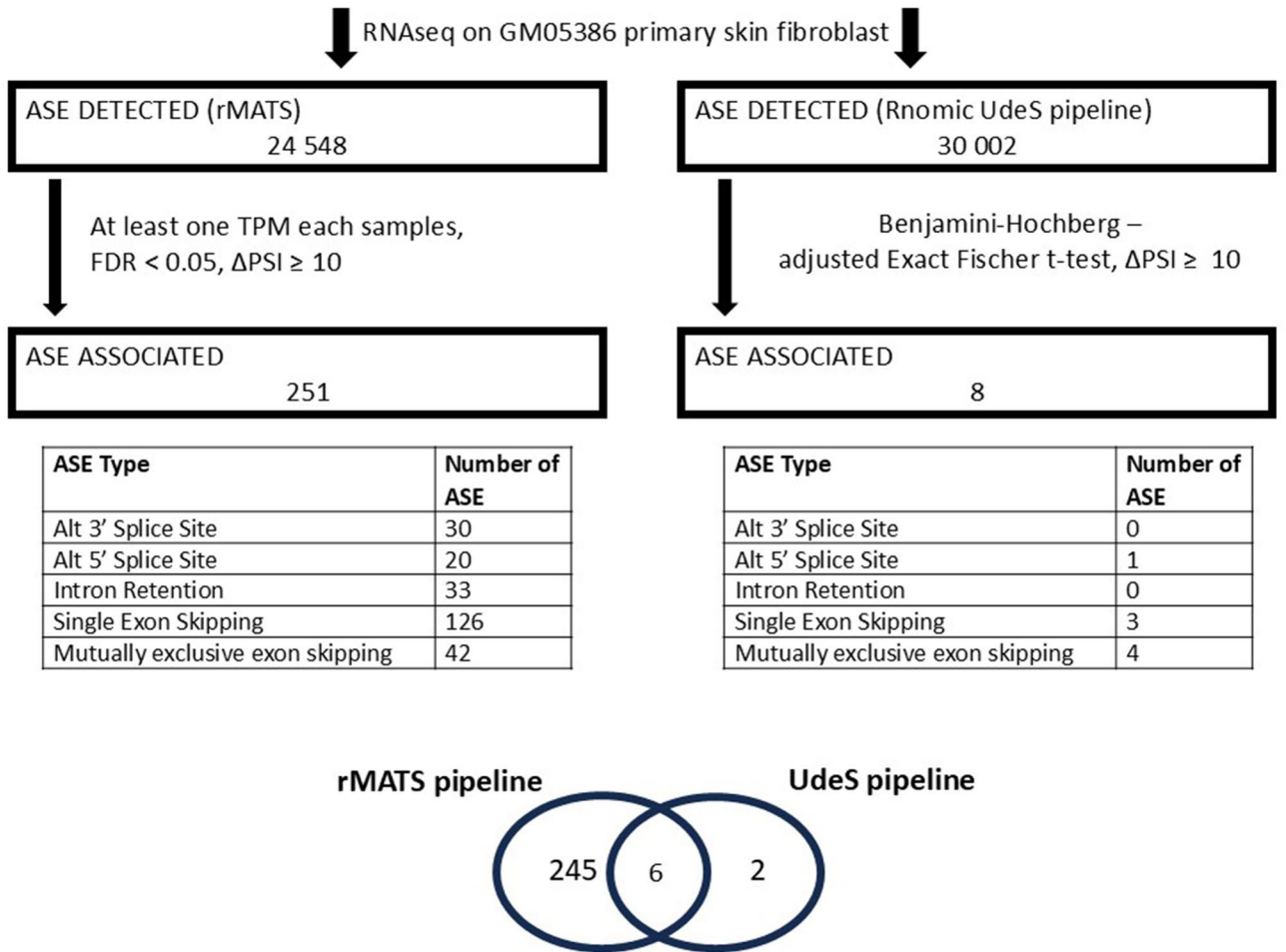


Fig. 3 Alternative splicing isoforms associated with TGFβ-induced myofibroblast. Alternative splicing events detected by RNA-Seq and associated to myofibroblast using rMATS and our in-house pipeline. The inserted tables detailed myofibroblast-associated ASE types for each analysis and the Venn diagram at the bottom indicated the 6 common myofibroblast-associated ASEs between both analyses

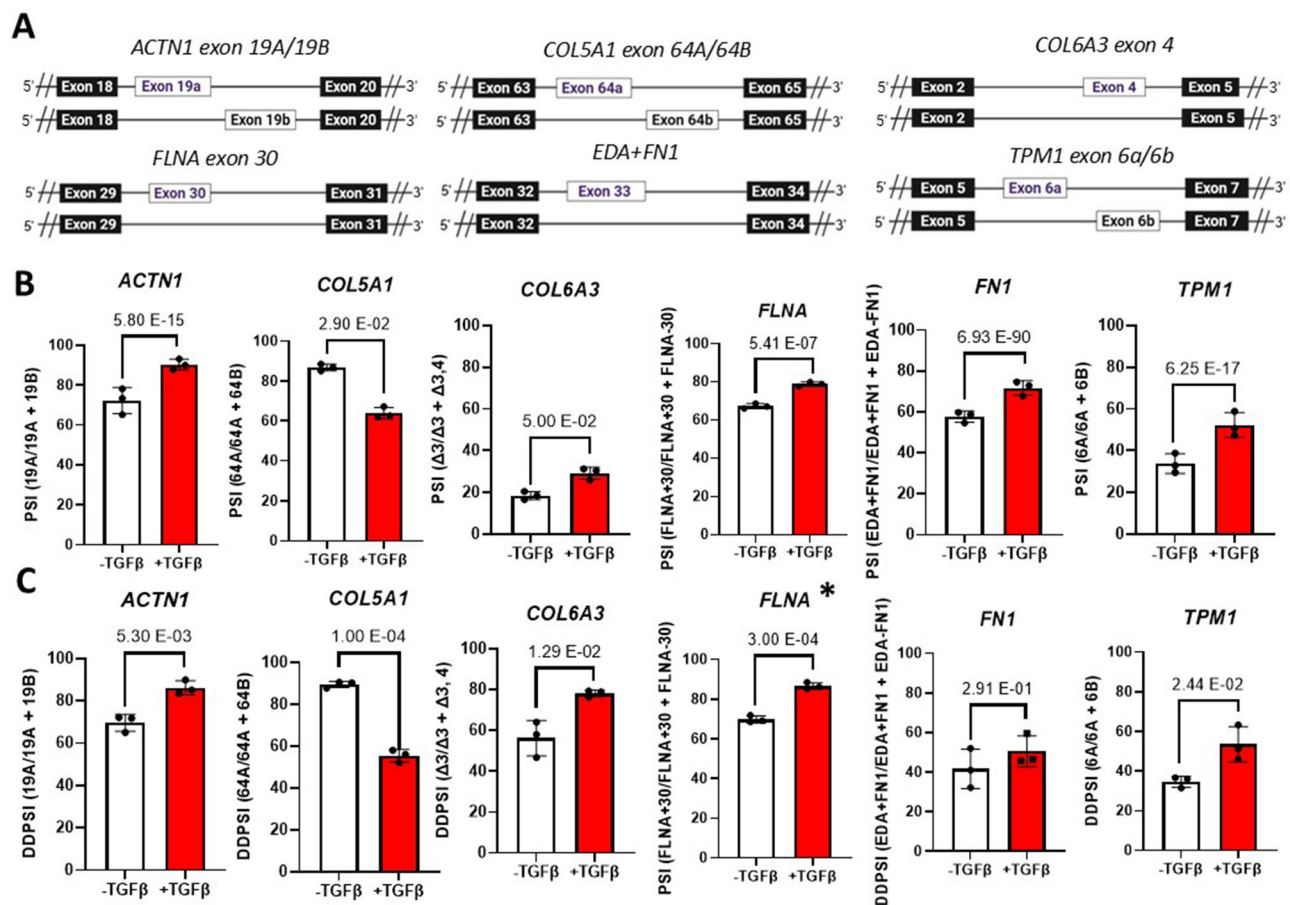


Fig. 4 Validation of ASEs associated with TGFβ-induced myofibroblast. **(A)** Schematic of 6 common myofibroblast-associated ASE **(B-C)** Bar graph representing the Splicing ratio of the 6 common myofibroblast-associated ASEs by **(B)** RNA-Seq and **(C)** ddPCR (* = AS-PCR). Errors bars are shown as mean $\pm$ standard error of the mean

Evaluation of the myofibroblast gene expression signature vs. the myofibroblast splicing isoform host gene signature

To determine if the global gene expression of the host gene of the 5 myofibroblast ASEs signature is differentially expressed in myofibroblast, we mined our DESeq2 analysis (File S1) and output bar graphs specifically for the *ACTN1*, *COL5A1*, *COL6A3*, *FLNA*, and *TPM1* (Fig. 5A). Then, we performed ddPCR using primers targeting all spliced variants of the host genes. Overall, though a trend toward a higher expression in myofibroblast was observed, none of the gene expression could be validated by ddPCR (Fig. 5B). On a broader scale, we wanted to determine the extent of overlap between the 453 myofibroblast up-regulated genes (File S1) and the host gene of the 251 myofibroblast ASEs (File S2). After careful analysis, the 251 ASEs are represented by a total of 202 host genes (File S2) and only 9 of these host genes are also up-regulated in myofibroblasts (Fig. 5C). Thus, the myofibroblast gene expression signature and the myofibroblast ASE signature are largely different.

The 5 ASEs myofibroblast signature identified is conserved across independent primary skin and lung fibroblast datasets

To confirm that the aforementioned 5 ASEs signature is not limited to the GM05386 primary skin fibroblasts we used; we searched and retrieved all possible TGFβ-induced fibroblast public RNA-Seq datasets where similar untreated controls were performed. This yield a total of three datasets: one using skin fibroblasts [11], and two using lung fibroblasts [12, 13]. Raw files were input and analyzed using our stringent in-house RNomic UdeS pipeline (File S4), and the results were output as PSI bar graphs (Fig. 6). Focusing on the 5 ASEs signature, and despite few outliers (splicing changes in *COL5A1* was not significant in one lung [12] and the skin fibroblast [11] datasets, and *COL6A3* exon 4 was not significant in the skin fibroblast dataset [11]), the results indicated that the direction of change of the 5 ASEs signature is conserved across independent groups, tissues and experimental conditions (Fig. 6). Thus, we concluded that the myofibroblast-associated splicing isoforms, especially

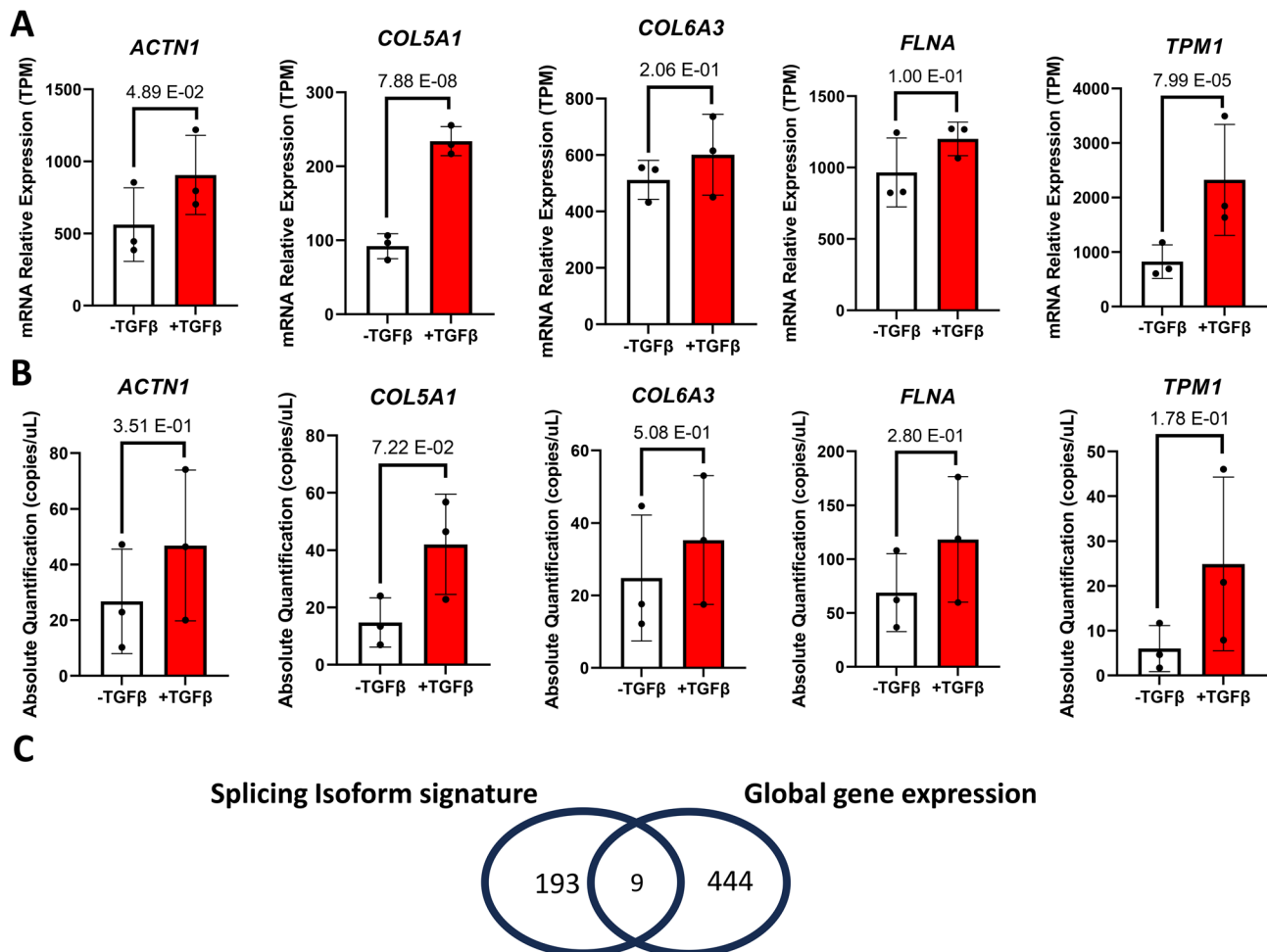


Fig. 5 Expression level of genes with myofibroblast-associated splicing isoforms. **(A-B)** Bar graph representing the global mRNA expression level of host gene of the 5 ASEs signature by **(A)** RNA-Seq and **(B)** ddPCR data **(C)** Venn diagram to indicate common genes between 251 myofibroblast ASEs from rMATS analysis (202 host genes) and the 453 upregulated genes associated with TGFβ-induced myofibroblast activation. Errors bars are shown as mean $\pm$ standard error of the mean

the 5 ASEs signature, may be used as putative universal myofibroblast markers and be of functional significance to myofibroblast formation and biology.

TGFβ-induced upregulation of fibronectin is independent of EDA+FN1 splicing

The EDA+FN1 splicing isoform has been well studied to drive myofibroblast formation. Surprisingly, the EDA+FN1 association with myofibroblast could not be validated by ddPCR in our model system (Fig. 4C). To further investigate the EDA+FN1 spliced isoform, we designed an immunofluorescence assay experiment where we monitored the expression of EDA+FN1 using an isoform-specific antibody. The result indicates an increase in the expression of EDA+FN1 when stimulated with TGFβ (Fig. 7A). Not surprisingly, we uncover fibronectin as one of the genes up-regulated upon TGFβ stimulation in our RNA-Seq analysis (File S1). To validate this finding, a qRT-PCR was performed, and the

global expression of fibronectin was quantified (Fig. 7B). Likewise, by examining the mRNA expression level of EDA+FN1 (EDA-containing fibronectin), we observed a similar 5-fold increase between untreated samples and treated samples (Fig. 7C). However, we also find a similar value when we evaluate the expression of the EDA-FN1 (fibronectin without EDA) isoforms of fibronectin (Fig. 7D). This suggests that the TGFβ-induced fibronectin mRNA expression increase is not due to a splicing effect but to a global transcription effect. To determine the EDA+FN1 splicing index, the quantitative percent spliced in (QPSI) of the qRT-PCR was evaluated. We observed no significant change in EDA+FN1 splicing index upon TGFβ stimulation (Fig. 7E). Similar results were obtained when AS-PCR was conducted (Fig. 7F). We concluded that TGFβ does not specifically modulate EDA+FN1 alternative splicing, at least in our model system.

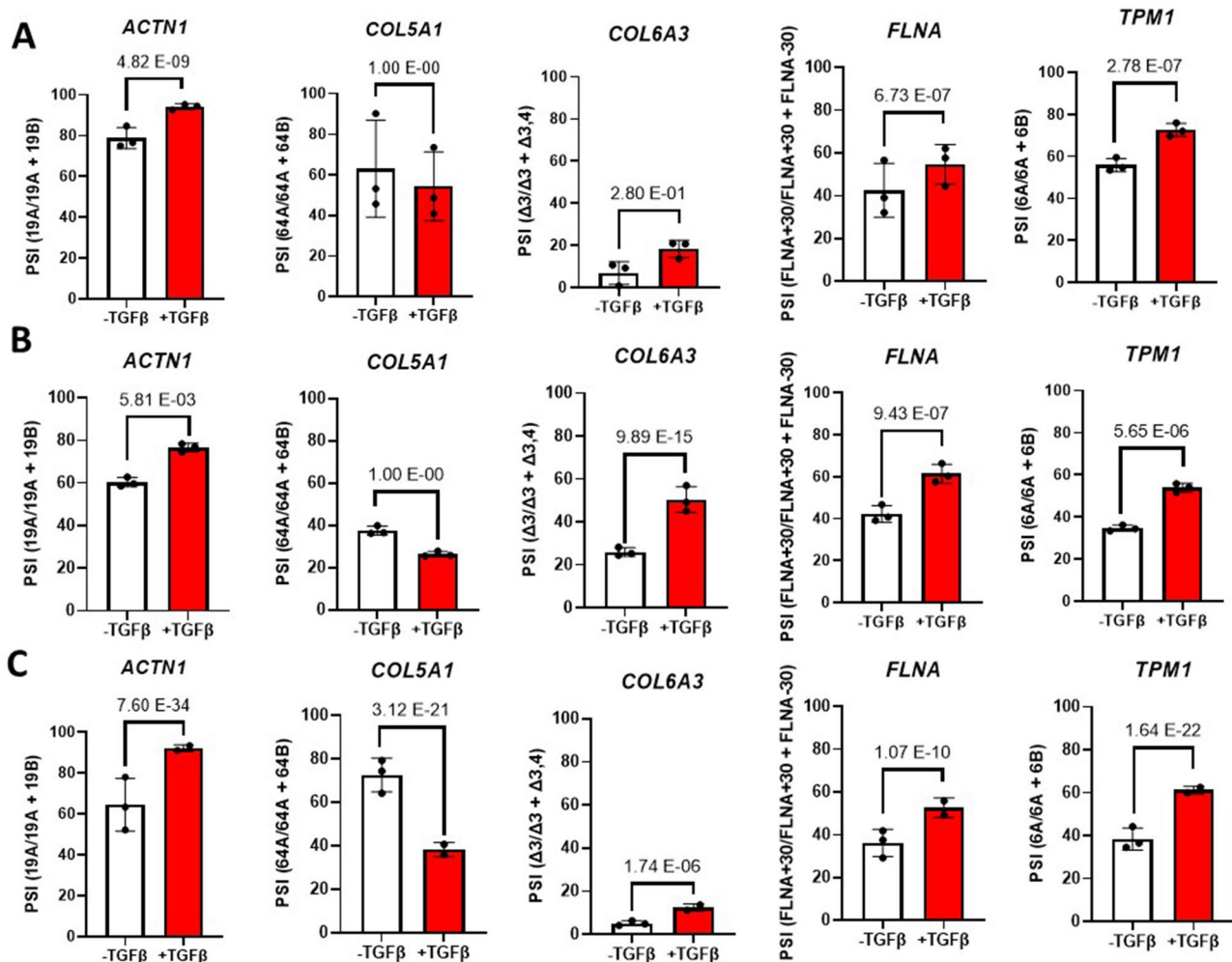


Fig. 6 Validation of the myofibroblast-associated ASEs signature in public datasets. (A–C) Bar graph representing the Splicing ratio of the 5 ASE signature by RNA-Seq from the (A) TGFβ-induced skin fibroblasts [11], the (B) TGFβ-induced WI-38 lung fibroblasts from [12] and (C) TGFβ-induced WI-38 lung fibroblasts from [13]. Errors bars are shown as mean $\pm$ standard error of the mean

To investigate whether splicing of EDA+FN1 is impacted by TGFβ stimulation in other model systems, we interrogated the splicing analysis of TGFβ-induced fibroblasts from the public datasets analyzed previously (File S4). The results are heterogeneous. While we observed a PSI increase in the Liang dataset (Fig. S3A – left), we noticed contradictory results among the datasets studying lung fibroblasts (Fig. S3B–C, left). Importantly, the global fibronectin expression is increased in all datasets as expected (Fig. S3A–C right, File S5).

Discussion

Alternative splicing is an important process in gene expression, and its dysregulation has been implicated in driving many diseases and developmental programs [14, 15]. To investigate its role in myofibroblast formation, an RNA-Seq alternative splicing analysis was conducted in

this study, and it associates more than 250 splicing isoforms. Among those, a 5 ASEs signature [ACTN1 exons 19 A/19B; COL5A1 exons 64 A/64B; COL6A3 exon 4; FLNA exon 30 and TPM1 exons 6a/6b] that was further validated by PCR emerged. We confirmed the robustness of our findings across three publicly available transcriptomic datasets. Despite differences in experimental conditions such as fibroblast origin (lung vs. skin), time point (between 2 and 20 days), and TGFβ concentration (2 and 10 ng/mL), the consistency of the 5 ASEs signature across heterogeneous datasets highlights a core set of splicing changes. It supports their biological relevance to myofibroblast biology. Although we focused on the ASE intersecting both bioinformatic pipelines, it is very likely that several other isoforms identified in the 251 isoforms signature by rMATS actually validate by ddPCR, bonifying the 5 ASEs myofibroblast signature. The Rnomic UdeS pipeline's stringency may reduce sensitivity to

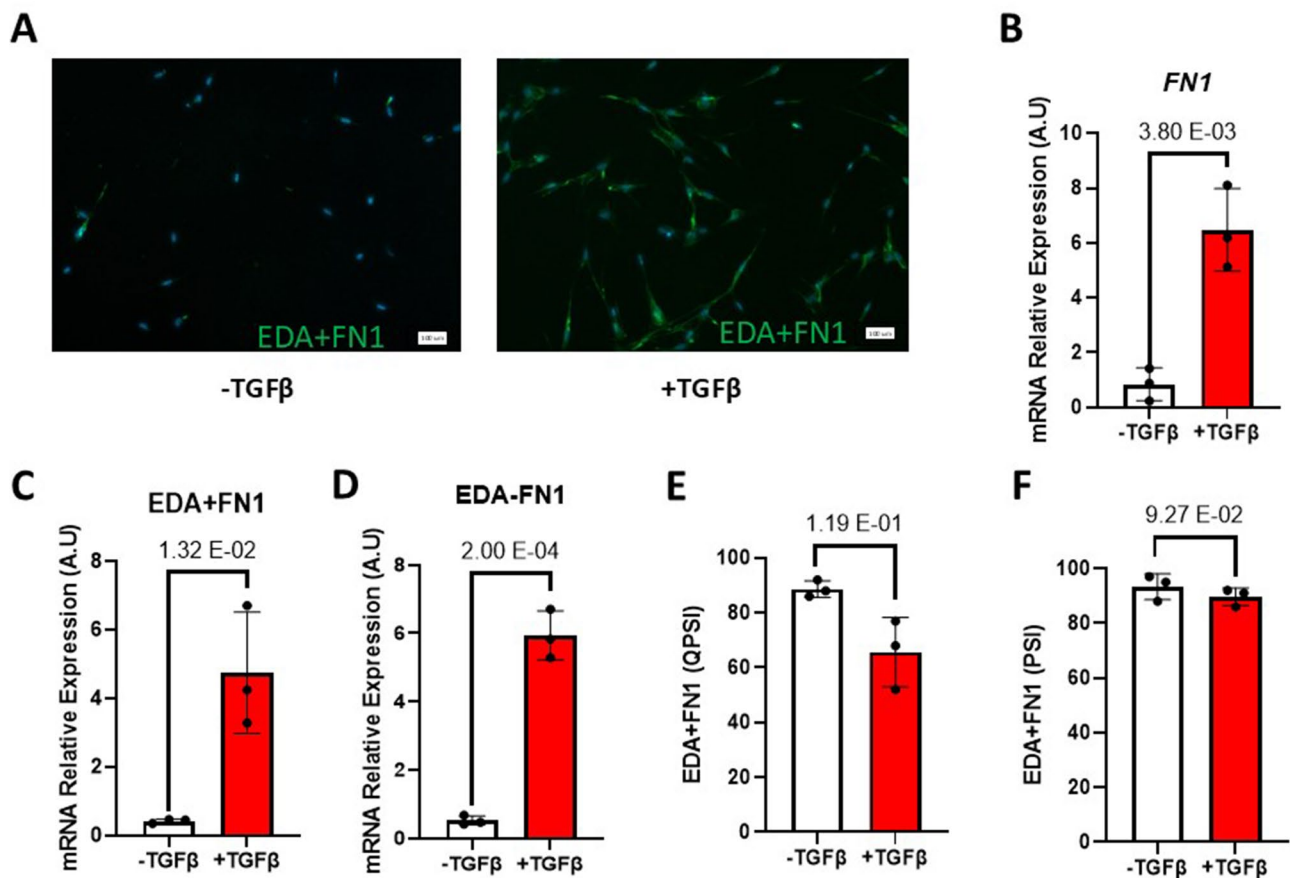


Fig. 7 TGFβ do not specifically modulates fibronectin EDA (EDA+FN1). **(A)** Immunofluorescence images showing the expression of EDA+FN1 (Green) after two days of TGFβ stimulation merged with DAPI (Blue). The scale bar equals 100 μm. **(B)** Bar graph representing the relative *FN1* mRNA expression by qRT-PCR. **(C-D)** Bar graph representing the relative **(C)** EDA+FN1 (EDA-containing fibronectin) and **(D)** EDA-FN1 (fibronectin without EDA) isoform expression by qRT-PCR **(E-F)** Bar graph representing the Splicing ratio of EDA+FN1 by **(E)** qRT-PCR and **(F)** AS-PCR. Errors bars are shown as mean $\pm$ standard error of the mean

low-abundance or low-coverage splicing events compare to rMATS, but this trade-off was intentional to maximize confidence in our final candidate list. Moreover, the use of three biological replicates coupled to the statistical stringency we applied are additional limitations that may decrease the strength of association of more subtle isoform change due to high variability between replicates.

Unexpectedly, we could not validate EDA+FN1, a known myofibroblast-associated isoform [16, 17], in our model system. Unlike the 5 ASEs signature, that show consistency across three publicly available transcriptomic datasets and despite differences in experimental conditions such as fibroblast origin (lung vs. skin), time point (between 2 and 20 days), and TGFβ concentration (2 and 10 ng/mL), EDA+FN1 contrast by showing heterogeneous results. There could be many reasons why EDA+FN1 splicing appear more context dependent including cell type-specific mechanism and time-dependency. Overall, it suggests that the promotion of

EDA+FN1 may be one of the mechanisms, among others, such as enhancing overall fibronectin expression.

Myofibroblasts are characterized by enhanced contractile properties through modulation of the actin cytoskeleton and enhanced ECM secretion capacity. Interestingly α-actinin 1 (*ACTN1*), filamin (*FLNA*) and tropomyosin 1 (*TPM1*) are all actin-binding proteins influencing the rigidity/stability of actin stress fibers whereas Collagen type V and type VI are myofibroblast-associated ECM proteins. The functional contribution of the myofibroblast-associated splicing isoforms to myofibroblast differentiation warrants further investigation. α-actinin 1 binding properties are highly regulated by calcium through its calmodulin-like domain. Calcium binding reduces conformational flexibility and impairs proper actin orientation to form a mature cytoskeleton [18]. Interestingly, the inclusion of *exon 19a* in *ACTN1* mRNA produces an α-actinin 1 isoform much more sensitive to calcium than the α-actinin 1 isoform including the exon 19b [19]. We observed an increase in inclusion

frequency of exon19a over exon19b. Filamin possesses 24 immunoglobulin-like repeat units in its C-terminal, encompassing exon 30, and this domain is critical for its ability to homodimerize. Not much is known about the functional contribution of *FLNA* exon 30 to filamin activity in general, even less about its role in the context of myofibroblast in particular. Tropomyosin 1 (*TPM1*) binds and stabilizes actin filaments essential for myofibroblast differentiation. The induction of this gene increases contractility of myofibroblasts and remodelling of tissues [20]. *TPM1* is extensively alternatively spliced at exon 1, 2, 6, and 9, creating multiple isoforms. Very recently, Wu and collaborators reported that Tpm1.6, the *TPM1* variant specifically including *exon 6b*, is induced by TGF β in renal fibroblasts [21]. Moreover, knockdown of Tpm1.6 impairs the differentiation of myofibroblasts upon TGF β stimulation [21]. It is tempting to speculate that the 5 ASE signature may also be captured and be of significance in renal fibroblast biology. Thus, suggesting that alternative splicing adds an additional layer of regulation aiming at modulating actin filament rigidity in myofibroblasts.

Collagens are an abundant and important class of structural and functional ECM proteins secreted by fibroblasts. TGF β stimulation leads mainly to Collagen type I expression, although myofibroblasts are also able to secrete Collagen types III, IV, V, and VI. Intriguingly, overexpression of the *COL5A1* exon 64a isoform promotes epithelial to mesenchymal transition [22]. This is not what our results suggest; indicating that a switch in favor of the exon 64b isoform is associated with myofibroblast differentiation. The expression of *COL6A3* was found to be specifically secreted by cancer-associated fibroblasts [23]. Of note, *COL6A3* undergoes extensive alternative splicing of exon 3, 4 and 6. Although *COL6A3* exon 4 inclusion is associated with pancreatic cancer [24], its functional contribution has not been evaluated. Overall, several evidence suggest that the 5 ASEs signature may be of functional relevance to myofibroblast biology, although further work is needed.

Conclusion

Our study reveals that alternative splicing may add an additional regulatory layer in TGF β -induced myofibroblast differentiation, distinct from global gene expression changes. Strikingly, the myofibroblast gene expression signature and the myofibroblast ASE signature are largely different with only 9 host genes overlapping. While the splicing of the canonical EDA+FN1 isoform was not modulated by TGF β stimulation, we identified and validated 5 ASEs [ACTN1-19 A/19B; *COL5A1*-64 A/64B; *COL6A3* exon 4; *FLNA* exon 30 and *TPM1*-6a/6b] consistently associated with myofibroblast activation. These findings expand current knowledge of fibroblast biology

and suggest that splicing regulation may hold functional relevance for myofibroblast biology.

Methods

Cell culture and maintenance

GM05386 primary human skin fibroblasts (Coriell Institute for Medical Research) were maintained in Alpha Modification of Eagle's medium (AMEM) containing 10% heat inactivated FBS (VWR, CA76327-086), 5% L-glutamine (Wisent, 609-065-EL), 5% penicillin-streptomycin (VWR, CA45000-652), 800 uL of amphotericin B (Wisent, 450-105-QL), 5% sodium pyruvate (Wisent, 600-110-EL), 25 uL of 40ug/mL of fibroblast growth factor β (FGF β) (Wisent, 511-126-QU), and 125 uL of 0.2% heparin (Sigma, H3393-50KU). Prior to cell seeding, the cell culture flasks were coated with 5 mL of 1ug/mL fibronectin (Sigma-Aldrich, F1141) diluted in PBS with Ca & Mg (Corning #21-030-CV) overnight at 4 °C or 4 h at 37 °C.

TGF- β differentiation

Primary skin fibroblast (GM05386) cells of 5 to 10 passage numbers were used for this experiment. The cells were seeded in 6-well per 1 mL of complete media (for RNA-Seq analysis), 96-well per 200 uL of complete media (for qRT-PCR analysis), and 8-well per 200 uL of complete media (for IF assay) plates and kept for 3 days until it reached 70 to 80% confluency. Afterwards, the supernatant was aspirated and fresh media without FBS was added followed by the addition of a 1 ug/mL TGF- β (Abeomics, 32-1763-10) stock to reach a final concentration of 10 ng/mL. Cells were kept in an incubator and harvested after 2 days.

qRT-PCR

RNA extraction was performed using Qiazol reagent as per the manufacturer recommendation, and its dosage was performed using ThermoScientific Nanodrop Lite spectrophotometer. 150 ng of total RNA was converted to cDNA using the following reagents: random primer (Sigma, 11034731001), transcriptor RT reaction buffer 5X (Roche, 21-031-CV), RNase OUT, dNTPs (Wisent, 800–410), and transcriptor reverse transcriptase (Roche, 21-031-CV) and programme: 10 min @ 25 °C, 30 min @ 55 °C, and 5 min @ 85 °C. Next, cDNA was diluted with 440 uL of RNase DNase free water and used as template for a qPCR reaction using isoform or gene specific primers and B2M as housekeeping gene (see File S6 for primer sequences) in the 2X SyBr Green mix buffer (Quantabio, 95054–02 K) under the following cycling conditions: 95 °C, 3 min; [(95 °C, 15 s, 60 °C, 30 s, 72 °C, 30 s) X 50], 72 °C, 30 s. Raw Ct values were subjected to a $2^{-\Delta\Delta C_t}$ formula to normalize them with housekeeping gene

values (B2M) and calculate the relative mRNA expression changes between non-treated and treated groups.

RNA-Seq library preparation and sequencing

RNA extraction was performed using a hybrid trizol-column protocol. Briefly, the upper aqueous phase after chloroform-trizol phase separation was then transferred and diluted with an equal volume of 70% ethanol, followed by transfer to a RNeasy mini spin column (Qiagen, 74104) as the manufacturer's recommendation. Column-bound RNA extracts were DNase treated using the RNase free DNase set (Qiagen, 79254), and their quality checked by Agilent Bioanalyzer for the RNA integrity (RIN value). Libraries were generated from 20 ng of total RNA. Adapters and PCR primers were purchased from New England BioLabs. Libraries were quantified using the KAPA Library Quantification Kits - Complete kit (Universal) (Kapa Biosystems). Average size fragment was determined using a LabChip GX (PerkinElmer) instrument. Sequencing was performed on Illumina NovaSeq by Genome Québec deposit as GEO dataset (GSE313078).

RNA-Seq differential gene expression - DESeq2 analysis

DESeq2 analysis was performed by the Plateforme RNomique / RNomics Platform from the Université de Sherbrooke. Briefly, FASTQ files from this study and from GEO datasets (GSE110021, GSE252425, GSE264038) were used as input files. Reads were trimmed using Trimomatic (V0.39 [25]), and the quality of the reads was assessed using FastQC (V0.11.9 [26]). Kallisto V0.48.0 [27], was used to align the reads to the transcriptome and to quantify the transcripts. The transcriptome of the human genome GRCh38 was created using gffread (cufflinks V2.2.1 [28]), with the Ensembl annotation and genome files (V109). Transcript abundance was combined to obtain the gene level quantification. Tximport package (V1.22.0 [29]) was used to summarize kallisto count estimates at the gene level. DESeq2 (V1.34) was subsequently used to identify Differentially Expressed Genes (DEGs) between the different conditions using the default Benjamini and Hochberg correction method and output as File S1 and S5. The functional enrichment analysis was done using ShinyGO 0.77 [30–32]. PCA analysis was performed using R software after filtering for non-expressed genes (mean TPM ≤ 1).

RNA-Seq alternative splicing analysis - Rnomic UdeS pipeline

The RNomic alternative splicing analysis using the UdeS pipeline was performed by the Plateforme RNomique / RNomics Platform from the Université de Sherbrooke as previously done in [33], with minor modifications. Briefly, FASTQ files from this study and from GEO

datasets (GSE110021, GSE252425, GSE264038) were used as input files. Reads were aligned to the curated version 109 of the UCSC reference transcriptome (hg38), using the Bowtie 2 (V2.4.4) aligner [34]. The isoforms of the various genes were quantified for each condition in transcripts per million (TPM) using the RSEM tool (V1.3.3) [35]. From this transcript quantification, alternative splicing events were identified and quantified with percent-spliced-in (PSI) using the long form (L) and the short form (S) of each event. For each alternative splicing event (which may be a cassette-exon, mutually exclusive exons, alternative 5' and 3', etc.), a PSI value was calculated based on the ratio of the long form to the total (long + short) present in all the different isoforms containing these forms. The associated alternative splicing events were ranked using the Benjamini-Hochberg-Adjusted Exact Fisher's test and output as File S3 and S4.

RNA-Seq alternative splicing analysis - rMATS

rMATS analysis was performed by the Plateforme RNomique / RNomics Platform from the Université de Sherbrooke. Briefly, FASTQ files from this study and from GEO datasets (GSE110021, GSE252425, GSE264038) were used as input files. Reads were trimmed using Trimomatic (V0.39 [25]), and the quality of the reads was assessed using FastQC (V0.11.9 [26]). Trimmed reads were aligned to the human reference genome (GRCh38, Ensembl release 109) using STAR (v2.7.10a) with default parameters. Primary alignments were retained using Samtools (v1.16.1). Differential alternative splicing events between conditions were identified with rMATS (v4.1.2) using the STAR-aligned reads as input. rMATS results were filtered to retain events with a false discovery rate (FDR) < 0.05 , at least one TPM in each sample, and an absolute inclusion level difference greater than 0.1 (Δ PSI ≥ 10) and output as File S2.

Immunofluorescence

1×10^4 GM05386 fibroblasts were seeded in 8-well chamber slide pre-coated with poly-L-lysine (Sigma, P4707) for 30 min at room temperature. At about 70–80% confluence, the cells were further processed for TGF- β Differentiation. After the TGF- β Differentiation step, supernatant was aspirated and cells were washed with 200 μ L of 1X PBS w/o Ca & Mg (Corning #21-040-CM), and subsequently fixed with para-formaldehyde 4%, permeabilized with a 0.2% Igepal CA-630 solution, and blocked with 10% donkey serum solution. Then, cells were subsequently incubated with primary antibodies [Mouse Anti-SMA (Abnova H00000059-M02), or Mouse Anti-EDA + FN (Santa Cruz, sc-59826)] and secondary antibody [Donkey anti-Mouse AF488 (Jackson ImmunoResearch, 715-545-151)]. According to the manufacturer, the mouse anti-EDA-FN is an antibody raised

against a region of the EDA domain, and hence it targets the variant including the EDA exon. Quantification of α -SMA immunofluorescence was performed using ImageJ. Briefly, images (one field for each of the three biological replicates for each conditions totalizing 6 images for the non-treated and treated samples) were acquired using identical exposure and microscope settings at 10x magnification, were saved as 16-bit TIFF files and were uploaded into ImageJ. Then, the scale was set using a known reference distance, regions of interest (ROIs) were selected for 10 cells and the average pixel intensity value for each ROI was obtained. Finally, the mean values of each biological replicate were used for statistical analysis. Raw data can be found in File S7.

Alternative Splicing PCR (AS-PCR) and electrophoresis

cDNA (10 ng) was used as template for a PCR reaction using ASE specific primers (see File S6) and the Apex Taq RED Master Mix 2X (Genesee Scientific, 42–138) under the following cycling conditions: 94 °C, 3 min; 50 cycles of (94 °C, 30 s; 55 °C, 30 s; 72 °C, 30 s); 72 °C, 5 min. The resulting amplicons were submitted for electrophoresis analysis using PerkinElmer LabChIP GXTouch HT. AS-PCR was performed by the Plateforme RNomique / RNomics Platform from the Université de Sherbrooke.

Digital droplet PCR (ddPCR)

ddPCR was performed by the Plateforme RNomique / RNomics Platform from the Université de Sherbrooke. Briefly, cDNA (20 ng) was used as template for a PCR reaction using isoform-specific primers or global gene primers (see File S6) and the QX200 ddPCR EvaGreen Supermix 2X (Bio-Rad, #1864034). Each reaction mix (20 μ L) was converted to droplets with the Biorad QX200 droplet generator and ran under the following cycling protocol: 95 °C, 5 min; 50 cycles of (95 °C, 30 s; 59 °C, 60 s); 72 °C, 5 min; 4 °C, 5 min; 90 °C, 5 min. Finally, resulting plate was analyzed on the Biorad QX200 reader and concentration reported as copies/ μ L of the final 1x ddPCR reaction using Biorad QuantaSoft software. The copies/ μ L of three biological replicates per sample were average and plot as bar graph and displaying the data point distribution (Fig. 5B). When splicing is analyzed, then a ratio of the long isoform over the sum of the long and short isoform is calculated and called DDPSI, yielding a value between 0 and 100 (Fig. 4C) The raw data can be found in File S8.

Statistical analysis

All experimental procedures were performed with three biological replicates while analysis from public datasets are representative of at least two independent experiments. Data are represented as the mean $\pm$ standard error of the mean. RNA-Seq differential gene expression

using DESeq2 was analyzed using Benjamini and Hochberg correction method. AS analysis using the Rnomic UdeS pipeline was analysed using Benjamini-Hochberg-Adjusted Exact Fischer T-Test, and rMATS was analysed using a false discovery rate < 0.05 . IF, qRT-PCR, and ddPCR were performed with two or three technical replicates and data were analyzed for significance using unpaired t-tests with the threshold of significance set at $P \leq 0.05$ using GraphPad software (GraphPad, La Jolla, CA, USA).

Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s12860-026-00579-7>.

Supplementary Material 1: File S1. DESeq2 analysis of TGF β -induced GM05386 fibroblasts

Supplementary Material 2: File S2. rMATS analysis of TGF β -induced GM05386 fibroblasts

Supplementary Material 3: File S3. UdeS Rnomic splicing analysis of TGF β -induced GM05386 fibroblasts

Supplementary Material 4: File S4. UdeS Rnomic splicing analysis of TGF β -induced fibroblasts from public datasets

Supplementary Material 5: File S5. DESeq2 analysis of TGF β -induced fibroblasts from public datasets

Supplementary Material 6: File S6. Primers sequence for ddPCR, qRT-PCR and AS-PCR

Supplementary Material 7: File S7. Data source for SMA immunofluorescence quantification

Supplementary Material 8: File S8. Data source for ddPCR

Supplementary Material 9: Figure S1. PCA plot related to the DESeq2 analysis of TGF β -induced GM05386 fibroblasts

Supplementary Material 10: Figure S2. Gene ontology related to the DESeq2 analysis of TGF β -induced GM05386 fibroblasts

Supplementary Material 11: Figure S3. Fibronectin EDA splicing and global gene expression for, TGF β -induced fibroblasts from public datasets

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Author contributions

Writing: OA, JPB. Experiments: OA, FK. RNA-Seq analysis: DB, PT. Visualization: OA, DB, JPB. Conceptualization: JPB. Supervision: JPB, MD.

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Data availability

Raw RNAseq data is available through GEO dataset (GSE313078).

Declarations

Ethical approval

We conduct research ethically and ensure that our data collection and analysis, and authorship follow best practices and in accordance with relevant guidelines and regulations. The GM05386 is a human cell line allowed to be used for general research and there is no restriction to perform transcriptomic analysis but efforts cannot be made to establish the individual identity. Our research program and all experimental protocols involving human cells #2022–4279, including the GM05386, was reviewed and approved by an ethic committee: the Comité d'éthique de la recherche du CIUSSS de l'Estrie CHUS.

Consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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