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Fibulin-2 is a key mediator of the pro-neurogenic effect of TGF-beta1 on adult neural stem cells☆

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ABSTRACT

Transforming growth factor beta 1 (TGF-beta1), an anti-inflammatory cytokine, has been shown to have pro-neurogenic effects on adult Neural Stem Cells (aNSC) from the dentate gyrus and in vivo models. Here, we expanded the observation of the pro-neurogenic effect of TGF-beta1 on aNSC from the subventricular zone (SVZ) of adult rats and performed a functional genomic analysis to identify candidate genes to mediate its effect. 10 candidate genes were identified by microarray analysis and further validated by qRT-PCR. Of these, Fibulin-2 was increased 477-fold and its inhibition by siRNA blocks TGF-beta1 pro-neurogenic effect. Curiously, Fibulin-2 was not expressed by aNSC but by a GFAP-positive population in the culture, suggesting an indirect mechanism of action. TGF-beta1 also induced Fibulin-2 in the SVZ in vivo. Interestingly, 5 out of the 10 candidate genes identified are known to interact with integrins, paving the way for exploring their functional role in adult neurogenesis.
In conclusion, we have identified 10 genes with putative pro-neurogenic effects, 5 of them related to integrins and provided proof that Fibulin-2 is a major mediator of the pro-neurogenic effects of TGF-beta1. These data should contribute to further exploring the molecular mechanism of adult neurogenesis of the genes identified and the involvement of the integrin pathway on adult neurogenesis.

1. Introduction

The neurogenic niche is fundamental to determining the fate of aNSC. Several studies have shown that constitutive or induced pro- and anti-inflammatory signals are part of this niche and can modulate adult neurogenesis (Ekdahl et al., 2003; Monje et al., 2003; Graciarena et al., 2010; Mathieu et al., 2010a,b). TGF-beta1, an anti-inflammatory cytokine, has been shown to have pro-neurogenic effects in a variety of in vivo and in vitro models (Battista et al., 2006; Graciarena et al., 2010, 2013; Mathieu et al., 2010a,b). As for most cytokines, the pro-neurogenic effect of TGF-beta1 seems to be context-dependent (Miguez et al., 2013). We have also previously shown that TGF-beta1 exerts its pro-neurogenic effect on aNSC via the activation of the Smad2/3 signal transduction pathway (Graciarena et al., 2013). However, no study has attempted to clarify which genes are involved in the pro-neurogenic effect of TGF-beta or whether this effect can be observed in other types of aNSC.

In an attempt to elucidate these issues, in this work we have observed that TGF-beta1 is also pro-neurogenic on cultured primary aNSC from the SVZ of adult Wistar rats and performed a functional genomic analysis on these cells. After a differential expression analysis, 10 candidate genes were selected and all validated by qRT-PCR from independent samples. Of these, the specific functional inhibition of Fibulin-2 leads to a blockade of the pro-neurogenic effect of TGF-beta1, indicating a fundamental role of this molecule in this effect. Surprisingly, Fibulin-2 was not induced in typical Tuj1+ (Beta-III-Tubulin+) cells, but rather in GFAP+ cells, suggesting an indirect effect of this molecule.

2. Methods

2.1. Animals

Adult Wistar rats (8–10 weeks old) were housed under controlled temperature (22 ± 2 °C), with a 12-h cycle period with food and water ad libitum. All animal procedures were performed according to

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1044-7431/

Table 1
Primer sequences used for qRT-PCR.

Gene	Forward sequence	Reverse sequence	Amplicon
GAPDH	ACAACTCCCTCAAGATTGTCAGCA	TTCTGAGTGGCAGTGATGGCAT	136 bp
Beta Actin	TCTGTGTGGATTGGTGGCTCTA	CTGCTTGCTGATCCACATCTG	69 bp
B2MG	TCTTTCTGGTGCTTGTCTC	AGTGTGAGCCAGGATGTAG	242 bp
CTGF	AGCGCACAGTGACAGAACGCA	GCTGGCTTCAGCCTCACCGA	213 bp
Pmepa1	GGAATGCCGGAGCCACAGGTC	TGGGGGCTCCTCCCCATCAG	162 bp
IL33	TCCCATGGTGCAGTCAGAAGTCCT	GCAAGCAGACAGGCAGCAGAGA	181 bp
Gldn	TCAACAGCACCCAGCCAGCG	GCCACATGCACAAGAGCCCGT	157 bp
Tagln	CGACCAAGCCTTTTCTGCCTCAACA	TGGAGGGCGGGTTCTCAGGC	280 bp
Tnfrsf11b	ACTGCCACCAGGAGTCCAGTGTT	GGGTTGTTGAACGTTGGGGGCA	191 bp
Fbln2	ATGGCGAGTGCACCGACGTG	CACGCCTGCAGACCAGTGGG	261 bp
Postn	AGAGACCCGGGAAGAACGCATCA	TCGCCTTCTAGACCCTTGAACCCTT	163 bp
Spp	TCGATGTCCCTGACGGCCGAG	TGGCTGGTCTTCCCGTTGCTG	217 bp
Crlf1	ACGACCAGTGGCGTGCTTGG	TAGCCGGCAGGACCTCTCGC	106 bp
GLAST	GAAGCCATCATGAGATTGGTA	CACTGTATACATGGCAAGCTG	132 bp
Nestin	GCGCTCGGGAGTGTCGCTTA	GCTTCCACAGCCAGCTGGAACTT	148 bp
BIII tubulin	TATCTTCGGTCAGAGTGGTGCTGGC	AGCCCTGCAGGCAGTCACAAT	128 bp
DCX	GGGGATTGTGTACGCTGTTT	CGACCAGTTGGGATTGACAT	244 bp
MAP2	CAAACGTCATTACTTTACAACTTGA	CAGCTGCCTCTGTGAGTGAG	122 bp

the regulations of the National Institutes of Health, USA. Animal experiments were approved by the CICUAL of the Institute Leloir Foundation.

2.2. Cell culture

aNSCs were prepared from adult rats. The bilateral SVZ was dissected, triturated and enzymatically digested in trypsin (0.017% trypsin — Invitrogen — and 0.007% EDTA in PBS buffer) for 10 min (37 °C). The cell suspension was mechanically disaggregated by pipetting, washed in PBS and then in DMEM/F12 medium. Cells were plated on plastic dishes in DMEM/F12 with B27 (Invitrogen), 20 ng/ml FGF-2 (Peprotech), Glutamine (Sigma), antibiotic/antimicotic (Gibco) and incubated in hypoxia (3% O_2, 5% CO_2, 37 °C). Cells were grown in suspension for 10–15 days (replacing the medium every 3 to 4 days) until neurospheres were formed.

For differentiation experiments, neurospheres were plated (day 0) on poly-ornithine/laminin (Sigma) coated dishes in the same suspension medium, but replacing B27 for N2 (Invitrogen), and incubated in hypoxia (48 h). Then (day 2), the medium was replaced for another containing 10 ng/ml FGF-2 with or without 20 ng/ml TGF-beta1 (Peprotech), and renovated after 48 h (day 4). 48 h later (day 6), differentiation protocol was ended. Cells were fixed for immunocytochemistry, or total RNA or protein was extracted, for qRT-PCR and microarrays or Western blot, respectively.

For siRNA silencing experiments, 100 nM anti-Fibulin-2 siRNA SmartPool or scramble siRNA SmartPool (Dharmacon) were added to the plated cells 24 h prior to each TGF-beta1 stimulus. siRNA was transfected with INTERFERin (PolyPlus), according to the manufacturer's protocol.

2.3. Injections

Animals were anesthetized with ketamine chlorhydrate (80 mg/kg) and xylazine (8 mg/kg). 10 ng human recombinant TGF-beta1 was administered in 1 μl with a 50 μm-tipped finely-drawn glass capillary, stereotactically implanted in the left SVZ (bregma, +0.7 mm; lateral, +1.4 mm; ventral, −3.4 mm) (Paxinos and Watson, 1986). As control, 1 μl 0.1% BSA in water (vehicle) was injected in the right SVZ in the same animal (bregma, +0.7 mm; lateral, −1.4 mm; ventral, −3.4 mm). Injections were infused over 4 min and kept in place for an additional 1 min before removal. Animals were euthanized 48 h post-surgery.

2.4. Histology

Animals were perfused as described in (Battista et al., 2006). Brain samples were cryoprotected in 30% sucrose, frozen in isopentane and serially sectioned in a cryostat (40 μm). SVZ sections were used for free floating immunohistochemistry.

2.5. Immunocytochemistry (IC)

IC was performed as described in (Battista et al., 2006). Primary antibodies were mouse-anti-Tuj1 (1:1000, Promega), rabbit-anti-Tuj1 (1:500, Sigma), mouse-anti-Nestin (1:1000, BD-Pharmingen), mouse-anti-GFAP (1:500, Cell Signaling) and rabbit-anti-GFAP (1:1000, Dako), mouse-anti-Map2 (1:1000, Sigma), rabbit-anti-Doublecortin (1:500, Abcam) and rabbit-anti-Fibulin-2 (1:2000). Secondary antibodies were Cy2- or Cy3-conjugated donkey-anti-mouse or donkey-anti-rabbit (1:1000, Jackson).

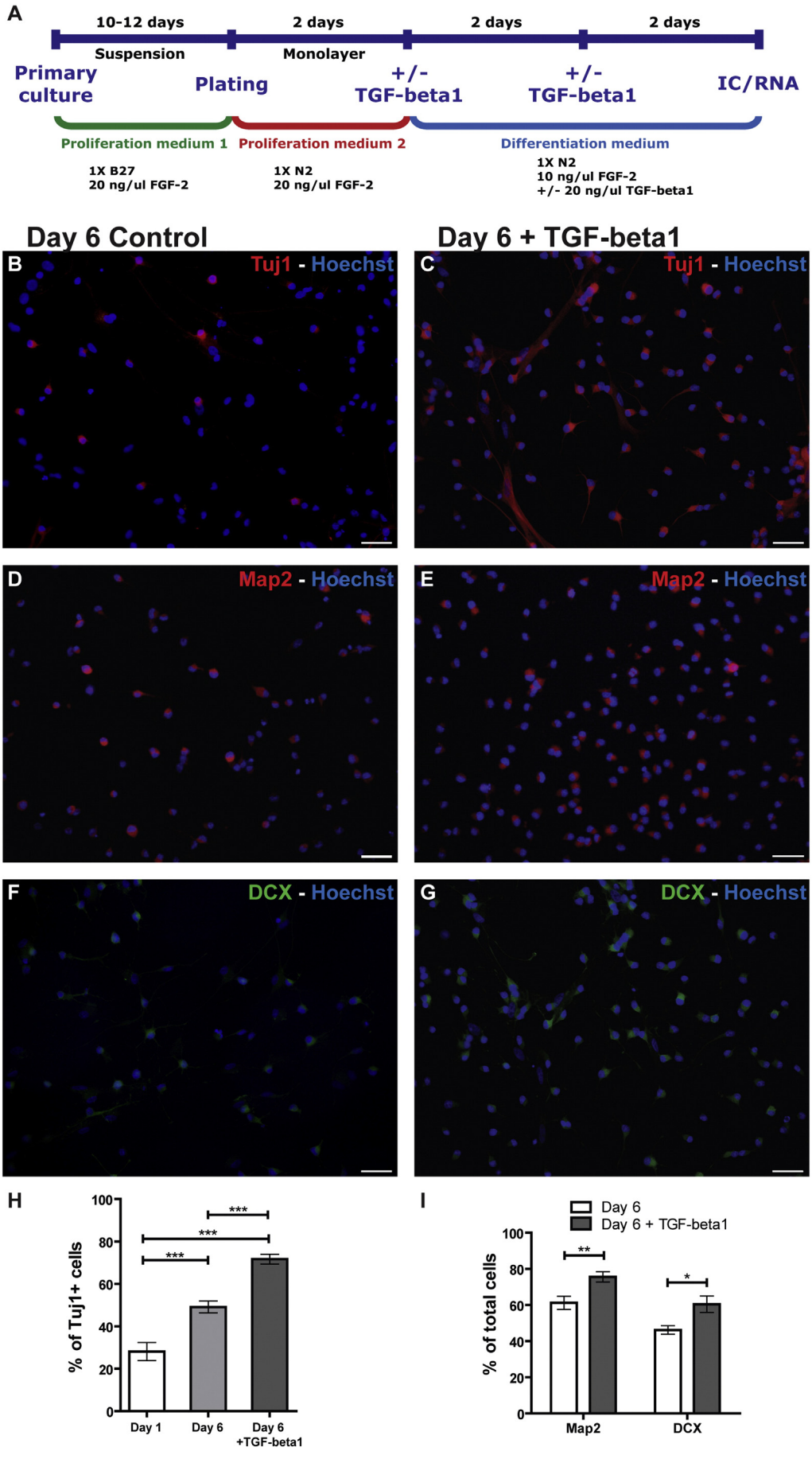
2.6. Immunohistochemistry (IHC)

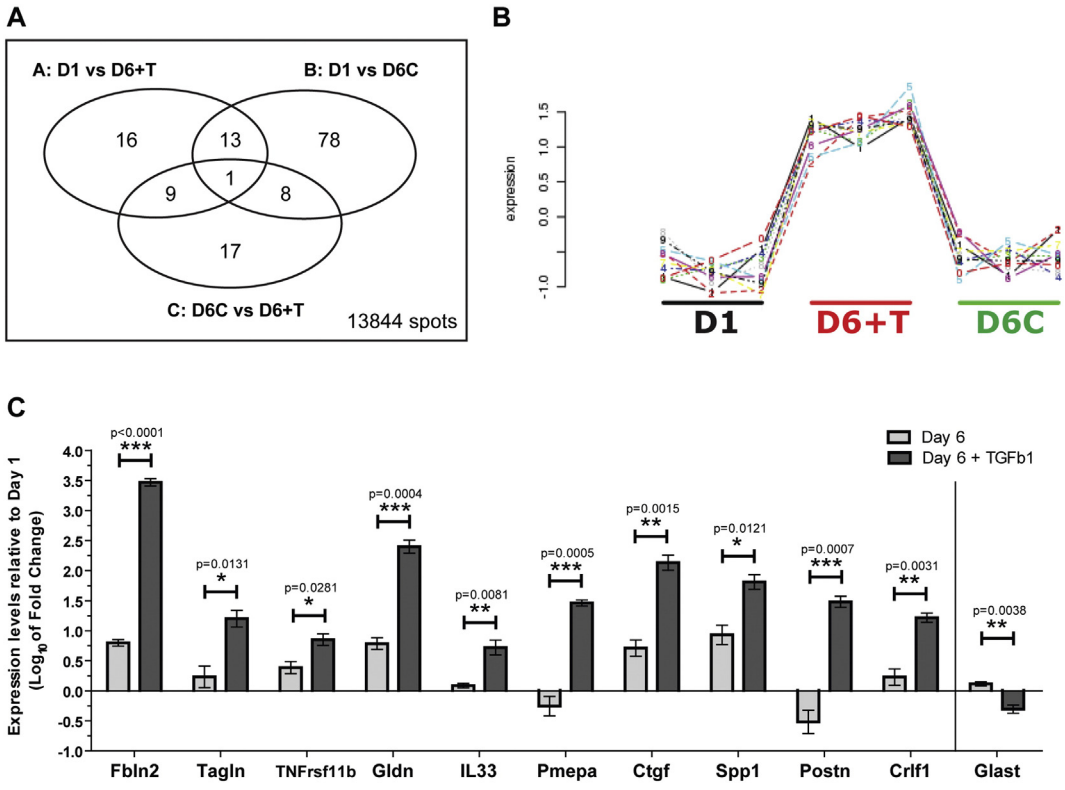
Free-floating sections were incubated in blocking buffer (1% donkey serum, 0.1% Triton in 0.1 M PB), and then incubated overnight with primary antibodies. Primary antibodies were: rabbit-anti-Fibulin-2 (1:500), goat-anti-DCX (1:100) and chicken-anti-GFAP (1:800, Neurogenomics). Then sections were incubated (2 h) with secondary antibodies: Cy3-conjugated donkey-anti-chicken (1:500; Jackson) or donkey-anti-goat (1:250; Jackson), or Cy2-conjugated donkey-anti-rabbit (1:250; Jackson). Nuclei were stained with Hoechst (5 min). Digital images were collected in a Zeiss LSM510-Meta laser scanning confocal microscope.

2.7. RNA isolation, reverse transcription, qRT-PCR

Total RNA was extracted from plated cells using RNeasy MiniKit (Qiagen) according to the manufacturer's protocol. Reverse transcription was performed with 1 μg RNA, 1 μl oligo-dT (Invitrogen) and SuperScript II reverse transcriptase (Invitrogen), according to the manufacturer's protocol.

Fig. 1. TGF-beta1 has a pro-neurogenic effect over SVZ aNSCs. Tuj1+ neural progenitor cell (NPC) percentage increases when the NSCs are cultured as a monolayer in differentiation medium. This percentage is significantly higher after incubation with 20 ng/ml TGF-beta1. A: Timeline of NSC differentiation protocol. (B–I): Immunocytochemistry and quantitation of NPCs. Day 6 after plating (B, D, F); day 6 after plating with two TGF-beta1 stimuli (C, E, G). IHC for Tuj1+ cells (B–C), for Map2+ cells (D–E), for DCX+ cells (F–G). H: Quantitation of the NPC relative percentage (Tuj1+ cells). 1-way ANOVA, Bonferroni's post-test (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). I: Quantitation of DCX, Map2 relative percentage. Student's *t*-test (*$p < 0.05$, **$p < 0.005$). Values are mean ± SEM. Scale bar: 100 μm.


A
10-12 days
2 days
2 days
2 days
Suspension
Monolayer
Primary culture
Plating
+/- TGF-beta1
+/- TGF-beta1
IC/RNA
Proliferation medium 1
Proliferation medium 2
Differentiation medium
1X B27
20 ng/ul FGF-2
1X N2
20 ng/ul FGF-2
1X N2
10 ng/ul FGF-2
+/- 20 ng/ul TGF-beta1
Day 6 Control
Day 6 + TGF-beta1
B
Tuj1 - Hoechst
C
Tuj1 - Hoechst
D
Map2 - Hoechst
E
Map2 - Hoechst
F
DCX - Hoechst
G
DCX - Hoechst
H
% of Tuj1+ cells
100
80
60
40
20
0
***
***
***
Day 1
Day 6
Day 6 +TGF-beta1
I
Day 6
Day 6 + TGF-beta1
% of total cells
100
80
60
40
20
0
**
*
Map2
DCX



D

Gene	Full Name	Fold change*
Fbln2	Fibulin-2 isoform 1	477.34
Postn	Similar to Periostin precursor (PN) (Osteoblast specific factor 2) (OSF-2)	103.98
Pmepa1	Transmembrane, prostate androgen induced RNA	52.04
Gldn	Gliomedin	43.69
Ctgf	Connective tissue growth factor	28.37
Tagln	Transgelin (Smooth muscle protein 22-alpha) (SM22-alpha)	10.27
Crlf1	Similar to cytokine receptor like molecule 3	10.07
Spp1	Osteopontin precursor (Bone sialoprotein-1) (Secreted phosphoprotein 1)	8.14
Il33	Nuclear factor from high endothelial venules (NF-HEV) (Interleukin 33)	4.63
Tnfrsf11b	Tumor necrosis factor receptor superfamily member 11B precursor (Osteoprotegerin)	3.04

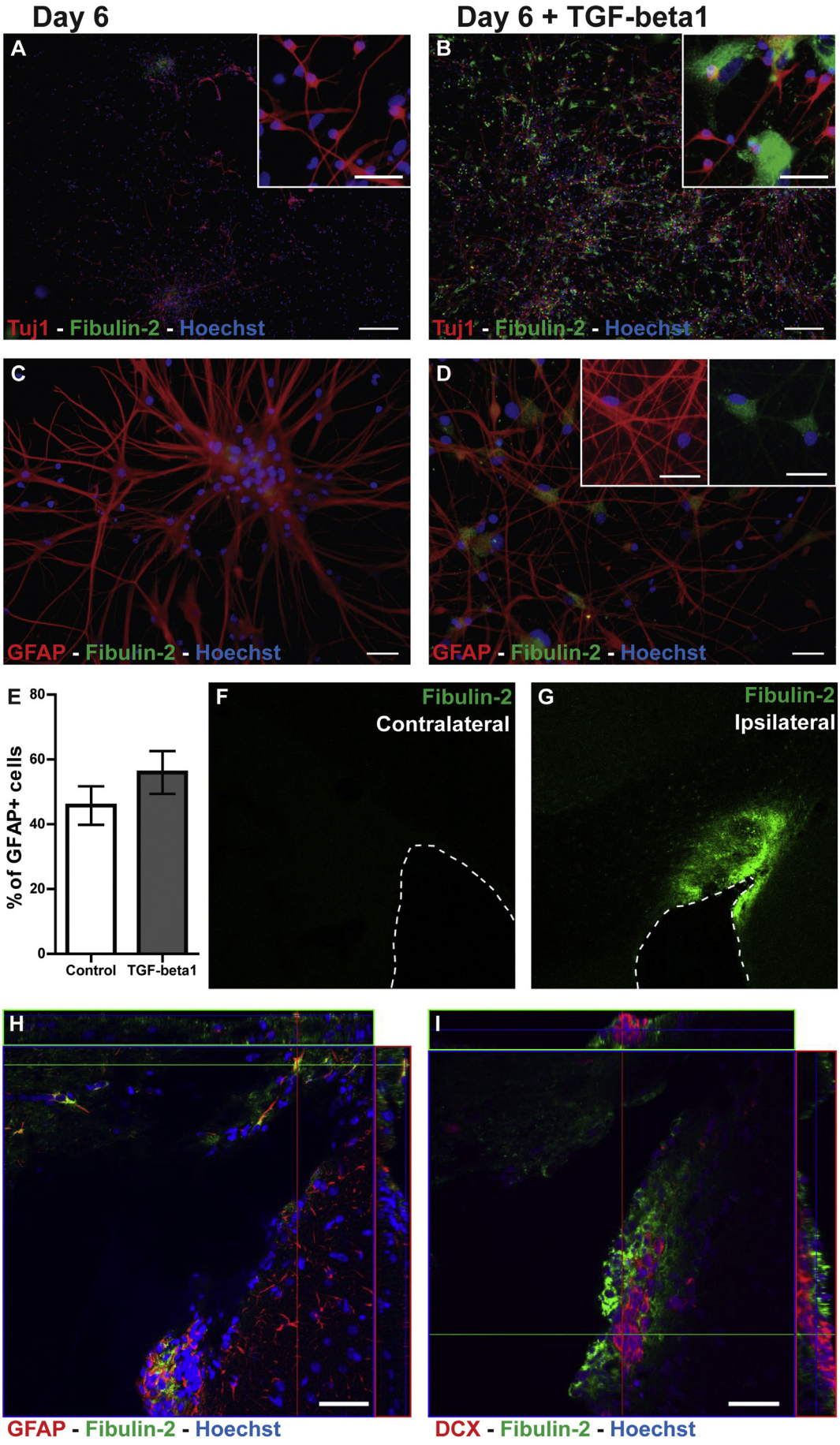
Fig. 2. Genomic analysis of TGF-beta1-induced differentiation process. A: statistical analysis showing the total number of differential expression spots in each pairwise comparison (13,844 spots analyzed). Each circle comprises all the genes with differential expression between two given experimental groups, and each intersection contains the differential genes specific for a single experimental group. Pairwise comparisons: Group A: D1 vs. D6 + T. Group B: D1 vs. D6C. Group C: D6C vs. D6 + T. B: expression profile for the 9 genes found in the A–C intersection. These genes show increased expression after TGF-beta1 stimulus, but no difference in expression between the other groups (n = 3 per group). C: Quantitation of the relative expression level of candidate genes by qRT-PCR. Results relative to D1 expression levels (log-scale). A marker of non-differentiated NSC (GLAST) shows that TGF-beta1 is not exerting a non-specific effect that may be increasing the expression level of all evaluated genes. Student's *t*-test (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). D: List of genes with differential expression after TGF-beta1 stimulus only (Osteopontin is both regulated by TGF-beta1 and the culture conditions, and it belongs to the interception between A, B and C groups). **"Fold change" represents the expression level after TGF-beta1 stimulus relative to non-stimulated group. Expression levels were measured by qRT-PCR.

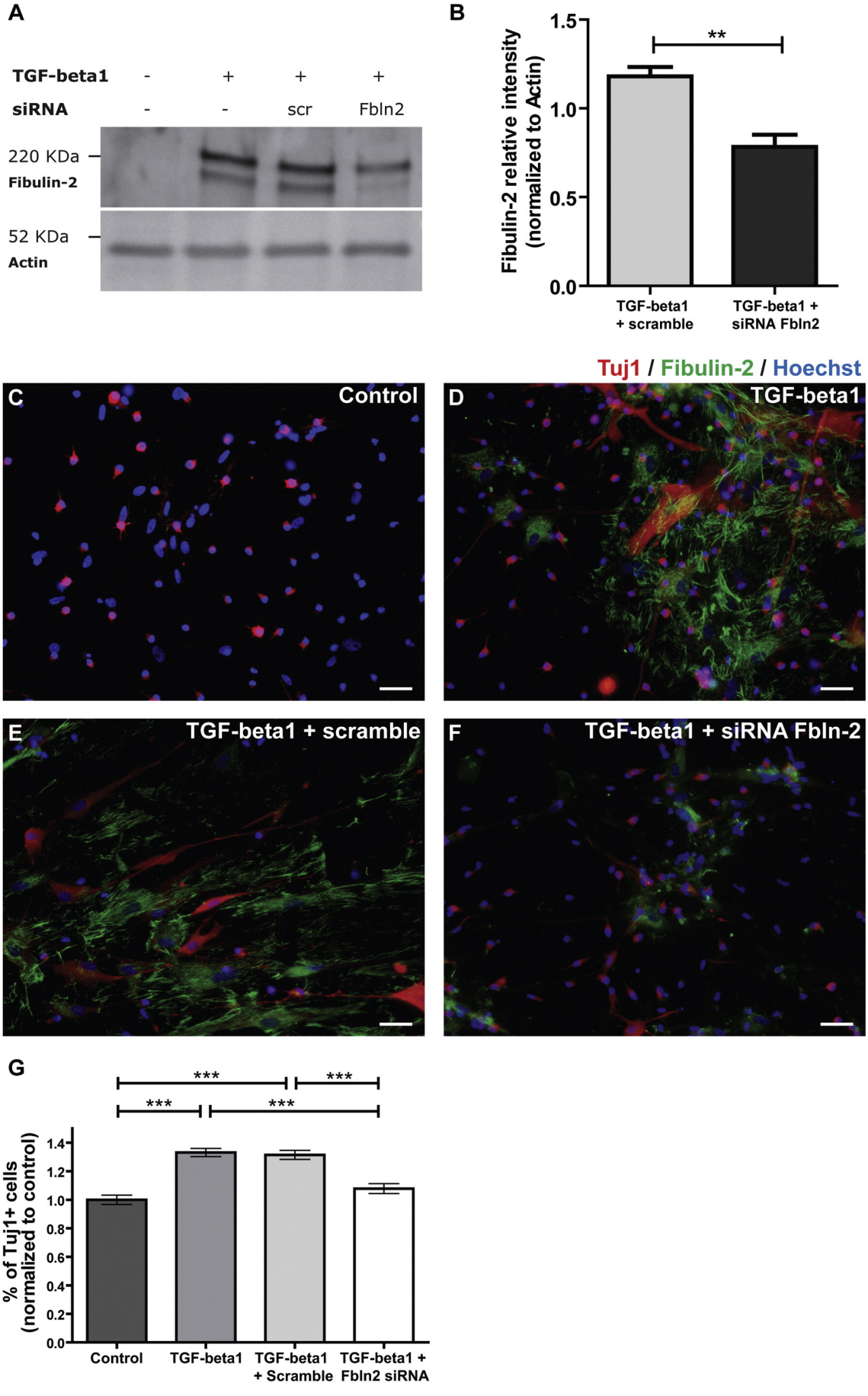
Comparative quantification was performed by qRT-PCR using SYBR-green fluorescence method and ROX as reference dye in a Stratagene MX3005p equipment. Results were analyzed with Stratagene MxPro QPCR software (Agilent). Primers are summarized in Table 1. Melting curves were performed to assess primer specificity. Additionally, PCR products were observed in 2% agarose gel electrophoresis. Samples were tested in triplicate. Three housekeeping genes (Beta2-microglobulin, Beta-Actin, GAPDH) were used to assess the expression levels using the ΔΔCt method.

2.8. Protein extracts and Western blotting

Total protein was extracted from plated cells as described in (Zhao et al., 2004). Protein extracts were quantified using MicroBCA Kit

Fig. 3. TGF-beta1 induces Fibulin-2 expression in vitro and in vivo. (A–B) IHC for Tuj1 and Fibulin-2. Cells cultured in vitro in differentiation medium for 6 days without (A) or with (B) TGF-beta1. Fibulin-2 is expressed only after TGF-beta1 stimuli, mainly in Tuj1-negative cells. Insets: higher magnification images. (C–D) IHC for GFAP and Fibulin-2. Cells cultured in vitro in differentiation medium for 6 days without (C) or with (D) TGF-beta1. Most GFAP+ cells show Fibulin-2 expression after TGF-beta1 stimuli, as seen in the insets. E: Quantitation of GFAP+ cell relative percentage. No significant differences were found between control and treated groups. (F–G) IHC for Fibulin-2 in SVZ of animals injected in vivo with recombinant TGF-beta1, confocal images. Dashed line demarcates the ventricle. F: Contralateral hemisphere. G: Ipsilateral hemisphere. TGF-beta1 injection induces Fibulin-2 expression in the SVZ in vivo. H: IHQ of GFAP and Fibulin-2 expression in vivo, confocal image, showing partial co-localization of GFAP and Fibulin-2. I: IHQ of DCX and Fibulin 2 expression in vivo, confocal image. Fibulin-2 shows partial overlap with GFAP+ cells but not with DCX+ cells. Student's *t*-test (no significative differences found). Scale bar: A–B: 250 μm (insets: 50 μm); C–D and insets: 50 μm. F–G: 50 μm. H–I: 20 μm.


Day 6
Day 6 + TGF-beta1
A
B
Tuj1 - Fibulin-2 - Hoechst
Tuj1 - Fibulin-2 - Hoechst
C
D
GFAP - Fibulin-2 - Hoechst
GFAP - Fibulin-2 - Hoechst
E
% of GFAP+ cells
80
60
40
20
0
Control
TGF-beta1
F
Fibulin-2
Contralateral
G
Fibulin-2
Ipsilateral
H
I
GFAP - Fibulin-2 - Hoechst
DCX - Fibulin-2 - Hoechst


A
TGF-beta1 - + + +
siRNA - - scr Fbln2
220 KDa
Fibulin-2
52 KDa
Actin
B
Fibulin-2 relative intensity (normalized to Actin)
1.5
1.0
0.5
0.0
**
TGF-beta1 + scramble
TGF-beta1 + siRNA Fbln2
Tuj1 / Fibulin-2 / Hoechst
C Control
D TGF-beta1
E TGF-beta1 + scramble
F TGF-beta1 + siRNA Fbln-2
G
% of Tuj1+ cells (normalized to control)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
***
***
***
***
Control
TGF-beta1
TGF-beta1 + Scramble
TGF-beta1 + Fbln2 siRNA

(Pierce), according to the manufacturer's protocol. Western blotting was performed as described in (Leal et al., 2006), from 3–5 μg total protein extracted from the cells at the endpoint of the differentiation protocol. Primary antibodies were rabbit-anti-Beta-Actin (1:1000, Sigma) and rabbit-anti-Fibulin-2 (1:2000). Secondary antibody was HRP-conjugated goat-anti-rabbit (1:5000, Millipore).

2.9. Microarrays

RNA samples and Rat Reference RNA (Agilent, GE Healthcare) were amplified and labeled with Superscript Indirect RNA Amplification System kit (Invitrogen) and Alexa Fluor 555/647 (Invitrogen), according to the manufacturer's protocol. RNA was hybridized onto microarray slides (MI-Ready Whole-Rat Genome array, Microarrays Inc.) and scanned in an Axon GenePix 4000B scanner (Molecular Devices). Images were analyzed with GenePix Pro software. Microarray data was processed with Limma package (Smyth, 2004). Spots with saturated signal in any slide were removed from the analysis. Data was normalized by VSN normalization (Huber et al., 2002). For non-specific data filtering, spots with low signal intensity ($M < 0.25$ in at least 30% of the slides) and spots with low variability (10% of the spots with the lowest signal standard deviation across slides) were removed from the differential analysis. Pairwise contrasts between groups were made to detect differentially expressed transcripts with an associated p-value. p-Values were corrected for multiple testing using FDR method (Benjamini and Hochberg, 1995). Differentially expressed transcripts with corrected p-values lower than 0.05 and showing fold changes over 30% were informed as differentially expressed genes.

2.10. Statistical analysis

Results are presented as mean ± SEM. Comparisons were performed using unpaired two-tailed Student's *t*-test or one-way ANOVA followed by Bonferroni's post-hoc test or Tukey's test. Data were tested for normality (Shapiro–Wilks test) and variance homogeneity (Levene test) to use parametric statistical analysis. When necessary, data were transformed (logarithm) to fulfill these statistical criteria. Statistical significance level was $p < 0.05$.

3. Results

3.1. TGF-beta1 has a pro-neurogenic effect on aNSC from the SVZ

First, we investigated whether the pro-neurogenic effect of TGF-beta1 on aNSC from the dentate gyrus could also be observed on aNSC from the SVZ. Neurospheres were generated and differentiated in vitro, and TGF-beta1 was added or not to the culture medium of these last cells 2 and 4 days after plating (Fig. 1A). Regularly, the aNSC preparation contains more than 80% nestin+ and around 30% Tuj1+ cells at the beginning of the experiment (Fig. 1H and data not shown). 6 days after plating, TGF-beta1 increased the percentage of Tuj+, DCX+ and MAP-2+ cells in the culture (Fig. 1B-G, 1H–). Importantly, the pro-neurogenic effect of TGF-beta1 after 6 days could be observed independently of the influence of time in culture (Fig. 1H–I) in all neuronal lineage markers tested.

Functional genomic analysis reveals a distinct set of genes related to the pro-neurogenic effect of TGF-beta1 on aNSC from the SVZ, including Fibulin-2

We analyzed the molecular signature of the pro-neurogenic effect of TGF-beta1 on aNSC from the SVZ by comparing the transcriptome of these cells 1 day (D1) or 6 days after plating with (D6 + T) or without (D6C) TGF-beta1. As a control, in this and following experiments, we verified that the TGF-beta1 treatment was effectively increasing NPC population in vitro by performing IHC for Tuj1 in parallel to the genomic analysis (data not shown). Differential expression analysis resulted in 142 transcripts showing differences in expression between experimental groups (Fig. 2A). Group A comprises transcripts differentially expressed between D1 and D6 + T. Group B comprises transcripts differentially expressed between D1 and D6C (genes potentially regulated by culture conditions, but not necessarily affected by TGF-beta1). Group C comprises transcripts differentially expressed between D6C and D6 + T, and consists of 35 transcripts potentially involved in the pro-neurogenic effect of TGF-beta1. In order to identify those genes differentially expressed by TGF-beta1 that were not associated with the pro-neurogenic effect of the culture conditions alone, we studied genes belonging to groups A (D1 vs D6 + T) and C (D6C vs D6 + T) simultaneously. A total of 9 genes were identified (Fig. 2A — intersection between groups A and C) and selected for further analysis. In addition, another gene (Osteopontin) was included in the analysis since it was found by comparing groups A, B and C (Fig. 2A). All 10 genes were upregulated in the D6 + T compared with D1 and D6C groups (Fig. 2B) and listed in Fig. 2D.

The differential upregulation of all 10 candidate genes was validated by qRT-PCR on independent samples (Fig. 2C). To discard technical biases in our analysis since all candidate genes were upregulated (from 3-fold to over 477-fold compared to the non-TGF-beta1 stimulated group), GLAST, an aNSC marker gene that should be downregulated due to the pro-neurogenic differentiation was also evaluated. As expected, GLAST expression was downregulated in the D6 + T samples compared to D6C samples, indicating that our analysis contained no biases towards the identification or validation of upregulated-only genes (Fig. 2C).

Of all the candidate genes identified, we decided to focus our analysis on Fibulin-2, since it showed a surprisingly robust increase (477-fold) and it is a relatively novel molecule with few functions described.

As a first approach, D6-C and D6-TGF cells were analyzed at the endpoint of the differentiation protocol for Fibulin-2 expression by IHC. Fibulin-2 expression was highly upregulated in TGF-beta1-treated cells and nearly absent in untreated controls (Fig. 3A–D). Interestingly, Fibulin-2 expression was not found in Tuj1+ cells but rather in cells with astrocytic morphology, which was confirmed by IHC (Fig. 3C–D). Fibulin-2 expression was detected in 76.5% of the GFAP+ cells after TGF-beta1 stimulus, confirming the astrocytic nature of the Fibulin-2-expressing cells. Conversely, 94.5% of the Fibulin-2 expressing cells were GFAP+ cells. The number of GFAP+ cells was not increased by TGF-beta1 treatment (Fig. 3E).

3.2. Fibulin-2 induces TGF-beta1 expression in vivo

To assess if TFG-beta1 induces Fibulin-2 in aNSC in vivo, rats were injected with recombinant TGF-beta1 in the left SVZ and contralaterally with 0.1% BSA as control. 48 h post-TGF-beta1 injection, a robust Fibulin-2 immunoreactivity was observed surrounding the injected area, whereas no Fibulin-2 expression could be detected in the contralateral side (Fig. 3F–G), similar to the results obtained in vitro.

To identify the cellular type responsible for Fibulin-2 expression, we conducted IHC for Fibulin-2 combined with GFAP (astrocytes) or DCX

Fig. 4. (A–B) Fibulin-2 silencing with specific siRNA. Western blotting. A: Western blot for Fibulin-2 (195 kDa, upper panel) and Beta-Actin (45 kDa, lower panel) as a loading control. Fibulin-2 is only detectable in the TGF-beta1-stimulated groups. B: Quantitation of the Western blot (relative to Beta-Actin and normalized to control, three independent experiments). Fibulin-2 protein expression induced by TGF-beta1 is reduced when the cells are treated with Fibulin-2 specific siRNA. 1-way ANOVA, Tukey's post-test (**$p < 0.01$). (C–G) Fibulin-2 silencing effect over NSC differentiation. IHC and quantitation of Tuj1+ neuronal progenitors. (C–F) IHC for Tuj1 and Fibulin-2. Cells cultured on differentiation medium for 6 days. C: control. D, E, F: stimulated with TGF-beta1. E: Treated with scramble siRNA. F: Treated with specific anti-Fibulin-2 siRNA. Scale bar: 50 μm. G: Quantitation of Tuj1+ cells (percentage relative to control, normalized, three independent experiments). Fibulin-2 silencing blocks the increase in Tuj1+ cell percentage induced by TGF-beta1 stimulus. 1-way ANOVA, Bonferroni's post-test (***$p < 0.0001$).

(young neurons). Through confocal microscopy we detected an overlap between GFAP and Fibulin-2 expression in some but not all GFAP-positive cells (Fig. 3H). On the other hand, we couldn't detect co-localization between DCX and Fibulin-2 (Fig. 3I), indicating that astrocytes and cells others than neurons are expressing Fibulin-2 in response to TGF-beta1 stimulus in vivo.

3.3. Fibulin-2 is a key mediator of the pro-neurogenic effects of TGF-beta1

We sought to study the functional relevance of Fibulin-2 overexpression in the pro-neurogenic effect of TGF-beta1 by incubating aNSC from the SVZ with anti-Fibulin-2 siRNA or a scramble control before the TGF-beta1 stimulus. Western blot analysis showed a statistical reduction in Fibulin-2 expression at the endpoint of the differentiation protocol, only in cells treated with the specific siRNA (Fig. 4A–B). No Fibulin-2 expression was detected in cells not treated with TGF-beta1, confirming that Fibulin-2 expression is tightly regulated by TGF-beta1 (Fig. 4A). Importantly, siRNA-driven reduction in Fibulin-2 levels led to an inhibition of the pro-neurogenic effect of TGF-beta1 which was not observed in aNSC treated with control siRNA (Fig. 4C–G). These results indicate a key role of Fibulin-2 in mediating the pro-neurogenic effect of TGF-beta1 on NSC from the adult SVZ.

4. Discussion

In this manuscript, we attempted to clarify the molecular mechanism of the pro-neurogenic effect of TGF-beta1 on aNSC by functional genomics. In this regard, we identified and technically validated 10 candidate genes to mediate this effect. Of these, Fibulin-2, an extracellular matrix (ECM) protein, was found as a key mediator of this pro-neurogenic effect. Surprisingly, Fibulin-2 over-expression by TGF-beta1 was detected on GFAP + cells and not on newborn neurons in vitro and in vivo, suggesting an indirect effect of this molecule on aNSC differentiation.

Fibulin-2 is an ECM protein which can interact with a variety of other ECM proteins, and can be incorporated into multiple extracellular structures such as elastin/fibrillin fibers, fibronectin microfibrils, basement membranes and proteoglycan aggregates (Timpl et al., 2003). Fibulin-2 plays significant roles in embryonic development, especially at sites of epithelial–mesenchymal transition (Tsuda et al., 2001), and in tissue remodeling in adults, being induced in pathologic conditions in skin wound repair and vascular lesions (Fassler et al., 1996; Strom et al., 2006).

In addition, Fibulin-2 was induced by TGF-beta 1 in vitro and in vivo, but no constitutive expression of Fibulin-2 was observed in any model tested. Therefore, we conclude that Fibulin-2 may exert its pro-neurogenic role after TGF-beta secretion and not during basal, physiological adult neurogenesis. In addition to Fibulin-2, 4 others out of the 10 candidate genes found in this study are known to interact with integrins: CTGF, Osteopontin, Periostin and Tnfrsf11b. This observation strongly suggests that the extracellular environment plays a crucial role in determining the fate of aNSCs. Integrins are heterodimeric cell surface receptors composed of non-covalently associated subunits (Hynes, 2002). Fibulin-2 binds alphaIIb/beta3 integrin through its RGD domain with high affinity, almost as strongly as fibrinogen, the main ligand of RGD-recognizing integrins (Pfaff et al., 1995) and it has recently been found to interact with alpha3/beta1 integrin during basement membrane organization in epidermis (Longmate et al., 2014). CTGF have been found to regulate angiogenesis through integrins alphav/beta3 and alpha6/beta1 (Lau and Lam, 1999; Longmate et al., 2014). Osteopontin interacts with alphav/beta1, 3 and 5 integrins (Ross et al., 1993; Liaw et al., 1995) mediating cell attachment, cell migration, chemotaxis and intracellular signaling in various cell types. Periostin lacks a consensus RGD-binding motif, but it has been shown to interact with alphav/beta3, alphav/beta5 and alphav/beta1 integrins (Gillan et al., 2002; Ruan et al., 2009). Another candidate gene identified, Tnfrsf11b (Osteoprotegerin), is known to activate integrin/focal adhesion kinase (FAK) signaling in endothelial cells (Kobayashi-Sakamoto et al., 2008). In addition to this, the LAP peptide of inactive TGF-beta1 contains an RGD sequence (Humphries et al., 2006) and integrins alphav/beta6 and alphav/beta8 activate TGF-beta1 in vivo (Aluwihare et al., 2009). Integrins have been shown to play an important role during the early stages of neuronal differentiation, acting as receptors for different ECM molecules (Aplin et al., 1998; Milner and Campbell, 2002; Clegg et al., 2003). aNSC express integrins alpha3, alpha6, alpha7, beta1 and beta4, which bind laminins and mediate laminin-dependent migration (Flanagan et al., 2006). In conclusion, Fibulin-2 and the other 4 genes identified could be interacting with a variety of integrins to exert pro-neurogenic effects. These known interactions should stimulate further work on the functional relevance of integrins in adult neurogenesis.

Finally, it has been shown recently that Fibulin-2 might be an essential enhancer of TGF-beta1 autoinduction (Zhang et al., 2014). Fibulin-2 may be enhancing the release of TGF-beta1 from its latent complex in the ECM by competing TGF-beta1 binding sites with other ECM proteins (Ono et al., 2009). In turn, biologically active free TGF-beta1 further induces Fibulin-2 mRNA expression (Ji et al., 2007). So, Fibulin-2 may be functioning both upstream and downstream of TGF-beta1, establishing a positive feedback loop of induction.

In summary we have determined that Fibulin-2, a molecule which was previously unrelated to neurogenesis, is a key mediator of the pro-neurogenic effect of TGF-beta1, exerting its function indirectly on NSCs. The other 9 candidate molecules identified encourage further identification of other pro-neurogenic mechanisms, especially those involving integrins.

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