Splicing machinery dysregulation drives glioblastoma development/aggressiveness: oncogenic role of SRSF3

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Glioblastomas remain the deadliest brain tumour, with a dismal ~12–16-month survival from diagnosis. Therefore, identification of new diagnostic, prognostic and therapeutic tools to tackle glioblastomas is urgently needed. Emerging evidence indicates that the cellular machinery controlling the splicing process (spliceosome) is altered in tumours, leading to oncogenic splicing events associated with tumour progression and aggressiveness. Here, we identify for the first time a profound dysregulation in the expression of relevant spliceosome components and splicing factors (at mRNA and protein levels) in well characterized cohorts of human high-grade astrocytomas, mostly glioblastomas, compared to healthy brain control samples, being SRSF3, RBM22, PTBP1 and RBM3 able to perfectly discriminate between tumours and control samples, and between proneural-like or mesenchymal-like tumours versus control samples from different mouse models with gliomas. Results were confirmed in four additional and independent human cohorts. Silencing of SRSF3, RBM22, PTBP1 and RBM3 decreased aggressiveness parameters in vitro (e.g. proliferation, migration, tumoursphere-formation, etc.) and induced apoptosis, especially SRSF3. Remarkably, SRSF3 was correlated with patient survival and relevant tumour markers, and its silencing in vivo drastically decreased tumour development and progression, likely through a molecular/cellular mechanism involving PDGFRB and associated oncogenic signalling pathways (PI3K-AKT/ERK), which may also involve the distinct alteration of alternative splicing events of specific transcription factors controlling PDGFRB (i.e. TP73).

Altogether, our results demonstrate a drastic splicing machinery-associated molecular dysregulation in glioblastomas, which could potentially be considered as a source of novel diagnostic and prognostic biomarkers as well as therapeutic targets for glioblastomas. Remarkably, SRSF3 is directly associated with glioblastoma development, progression, aggressiveness and patient survival and represents a novel potential therapeutic target to tackle this devastating pathology.

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Astrocytomas are a subtype of malignant gliomas characterized by rapid growth and high diffusion through the brain (Louis et al., 2016). They are stratified from low to high aggressiveness (WHO criteria) (Louis et al., 2016) with low-grade astrocytomas (grades I and II) and high-grade astrocytomas (HGA; grades III and IV), and grade IV [glioblastoma multiforme (GBM)] being the most aggressive and one of the most common malignant cancers in the brain (14.7% of total) and the CNS (Ostrom et al., 2018). Currently, HGA glioblastomas are detected by diagnostic imaging, with surgery as the first line of treatment, combined with chemotherapy and/or radiotherapy, depending on various clinical factors (type and degree of tumour, location and size, patient’s age and sex, etc.). However, about two-thirds of patients with HGA, mainly glioblastomas, do not have a survival rate >2 years after diagnosis (Ozdemir-Kaynak et al., 2018).

Tumour pathologies, including HGAs, are known to share atypical presence, alteration or loss of relevant components of key molecular machineries regulating cell physiology, including the splicing machinery, which governs the splicing mechanism, that adequately processes the pre-mRNA to obtain mature mRNA (Chen and Manley, 2009; Pelechano et al., 2013). Splicing is an intricate process, tightly regulated and carried out by the spliceosome (Yan et al., 2019), a macromolecular machinery organized as a complex of RNA-protein multi-subunits, which act in a coordinated and sequential manner to develop its action in a highly dynamic way. Specifically, the spliceosome consists of a main core composed of five small nuclear ribonucleoproteins (SNRNPs), which is assisted by a large number of auxiliary core proteins and the so-called splicing factors (Jurica and Moore, 2003), which cooperate in the precise recognition of a multitude of target introns (Chen and Manley, 2009). In mammals there are two types of spliceosomes, the main or major spliceosome, also known as U2-dependent, and the minor, U12-dependent spliceosome (Chen and Manley, 2009; Matera and Wang, 2014).

Alternative splicing is not infrequent, as it is the mechanisms processing >90% of the human coding genes, and thereby provides an essential source of biological versatility. However, it is also a vulnerable regulatory point. Thus, growing evidence indicates that defects in the splicing process are frequent, leading to the appearance of altered spliceosome components, splicing factors and/or aberrant splicing variants (generated by alternative splicing), which are associated with the development, progression and aggressiveness of various types of cancer, such as lung, breast, prostate, colon, brain and pancreas (Stratton et al., 2009; Cordoba-Chacon et al., 2011; Guo et al., 2015; Kozlovski et al., 2017; Barbagallo et al., 2018; Song et al., 2019; Jimenez-Vacas et al., 2020). In fact, the study of the expression profile of splicing isoforms (‘splicing signature’) has been shown in some tumours to provide a more useful tool than the analysis of the genetic/omic profile (‘transcriptome signature’) to provide an accurate classification of tumours/patients in terms of diagnosis and treatment selection (Feero et al., 2010). Therefore, a better understanding of the regulation of splicing in normal and pathological tissues may help to identify novel diagnostic and prognostic biomarkers and therapeutic tools to target tumour pathologies.

However, to the best of our knowledge, no studies have reported hitherto a comprehensive analysis to ascertain whether the splicing machinery, i.e. the spliceosome and its auxiliary splicing factors, is altered in HGAs/glioblastomas. These data could provide new valuable avenues to develop novel strategies to tackle these terrible tumours. Thus, in this study we aimed to determine—for the first time—the expression profile of a representative set of spliceosome components and splicing factors and their relationship with key clinical and molecular features of glioblastoma samples/patients, as well as to determine the putative functional role and therapeutic potential of the most relevant components identified within this cellular machinery in this devastating disease.

Materials and methods

Reagents

Unless otherwise indicated, reagents and products were purchased from Sigma-Aldrich. Platelet-derived growth factor D (PDGF-D) (R&D systems, #1159-SB-025) was administered in vitro at 20 ng/ml based on a dose response experiment in glioblastoma cell lines (data not shown).
Patients and samples

Fresh HGAs/glioblastoma samples (AIII/IV; n = 29; Cohort-1) were obtained by intracranial surgery from patients previously diagnosed with HGA/glioblastomas. Control brain tissue samples (CTs; n = 16) were obtained from healthy donors [autopsy; four different brain areas (Brocca, Wernicke, cingulate and medial)] (Supplementary Table 1A). All samples were histologically studied by expert anatomopathologists to confirm as HGA/glioblastoma and control samples. Portions of each sample were rapidly frozen in liquid nitrogen and stored at −80°C until extraction for total RNA (see below) or formalin-fixed paraffin embedded (FFPE) for immunohistochemistry (IHC) analysis (see below). A validation cohort consisting of additional HGA/glioblastoma (AIII/IV; n = 46; Cohort-2) and control (n = 3; obtained by lobectomy surgery) samples were similarly obtained and analysed (Supplementary Table 1B). Demographic and clinical characteristics of both cohorts were collected to carry out clinical correlations. This study was approved by the Reina Sofia University Hospital Ethics Committee and was conducted in accordance with the principles of the Declaration of Helsinki. Written informed consent was obtained from all individuals included in the study.

Primary patient-derived glioblastoma cell culture

Glioblastoma samples were collected within 15 min after intracranial surgery and immediately transported to the cell culture room in sterile cold Spinner’s modified Eagle medium (S-MEM) (Gibco, #11380-037) complemented with 0.1% bovine serum albumin (BSA) (#A2153), 0.01% l-glutamine (#G7513), 1% antibiotic-antimycotic solution (Gibco, #R01510) and 2.5% HEPES (#H3537). Glioblastoma samples were dispersed into single cells within the following 30 min by a mechanic/enzymatic protocol using 2% Collagenase IV (ThermoFisher-Scientific, #17104019) and 0.15% trypsin lyophilized powder (BD, #215310). Cell viability >90% was always obtained, as determined by the trypan-blue dye exclusion method. The single cells were cultured onto poly-L-lysine (#P1524-25MG) tissue culture plates in a 10% foetal bovine serum (FBS) (#F6765) containing Dulbecco’s modified Eagle medium (DMEM) (BI, #06-1055-09-1A) complemented as S-MEM medium.

Cell lines

Glioblastoma cell lines (U-87 MG and U-118 MG) were obtained from the American Type Culture Collection (ATCC, HTB-14/#HTB-15, respectively) and cultured according to the supplier’s recommendations of passages <20. These cell lines were previously checked for mycoplasma contamination by PCR every month, as previously reported (Uphoff and Drexler, 2013).

PDGFA and PDGFB mouse models

All experimental procedures were carried out following the European Regulations for Animal Care, in accordance with guidelines and regulations, and under the approval of Ethics Committees (CEyBA) from the Spanish National Cancer Research Centre (CNIO). Samples from two glioblastoma mouse models recently reported were used (PDGFA and PDGFB models; tumour and non-tumour samples) (Bejarano et al., 2017).

Electroporated mouse models

All experimental procedures were performed according to the Cedars-Sinai Institutional Animal Care and Use Committee. Paired-end bulk RNAseq datasets from glioma mouse models generated by postnatal lateral ventricle electroporation after a plasmid DNA mix injection with driver mutations, which had been identified as driver mutations in previous models or in patient tumours (Erbb2-V664E-EGFP/Hras-G12-EGFP/Kras-G12V-EGFP) tumour versus EGFP control models) were used as previously described (Breunig et al., 2016).

RNA isolation, real-time qPCR and customized qPCR dynamic array based on microfluidic technology

Total RNA from fresh normal and tumour human and mouse tissue samples was extracted, followed by DNase treatment using the AllPrep DNA/RNA/Protein Mini Kit and the RNase-Free DNase set (Qiagen, #80004/#79254), respectively. Total RNA from glioblastoma cell lines was extracted with TRIzol® Reagent (ThermoFisher Scientific, #15596026). In all cases, total RNA concentration and purity were assessed by Nanodrop One Microvolume UV-Vis Spectrophotometer (ThermoFisher Scientific). For qPCR analyses, total RNA was retrotranscribed by using random hexamer primers and the RevertAid RT Reverse Transcription Kit (ThermoFisher Scientific, #K1691). Thermal profile and qPCR analysis to obtain absolute mRNA copy number/50 ng of sample of selected genes are reported elsewhere (Luque et al., 2013, 2015).

As recently reported (Jimenez-Vacas et al., 2019b, 2020), a qPCR dynamic array based on microfluidic technology (Fluidigm, #BMK-M-48.48) was implemented to determine the simultaneous expression of 48 transcripts in HGA/glioblastoma samples compared to control samples using the Biomark System and the Fluidigm® Real-Time PCR Analysis Software v.3.0.2 and Data Collection Software v.3.1.2 (Fluidigm). Specific primers for human and mouse transcripts including components of the major spliceosome (n = 13), minor spliceosome (n = 4), associated splicing factors (n = 28), PDGFRB pathway-related genes and three housekeeping genes were specifically designed with the Primer3 software (Supplementary Tables 2–4). To control for variations in the efficiency of the retrotranscription reaction, mRNA copy numbers of the different transcripts analysed were adjusted by a normalization factor, calculated with the expression levels of three housekeeping genes [β-actin (ACTB), hypoxanthine guanine phosphoribosyl-transferase (HPRT), glyceraldehyde 3-phosphate dehydrogenase (GAPDH); only for human samples] and peptidylprolyl isomerase-A (Cyclophilin A; only for mouse samples) (Supplementary Tables 2 and 3) and the GeNorm v.3.3 software as previously reported (Luque et al., 2015; Hormaechea-Agulla et al., 2017a; Jimenez-Vacas et al., 2019a).

Immunohistochemical analysis

IHC analysis of selected splicing factors was performed on FFPE samples obtained by intracranial surgery from patients diagnosed
with HGAs/glioblastomas (Cohort-1) and control/non-pathological samples. Specifically, rabbit polyclonal antibodies against human SRSF3 and RBM22 (#ab73891 and #ab59157, respectively), goat polyclonal antibody against human PTBP1 (#ab5642) and mouse monoclonal antibody against human RBM3 (#ab211356) were used following the manufacturer’s instructions (Abcam). Specifically, deparaffinized sections were incubated with the antibody overnight at 4°C. Then, ImmPRESS® Anti-Mouse/Rabbit IgG Peroxidase (Vector Laboratories, #MP-7500-50) was used according to the supplier’s recommendations, except for PTBP1 in which an anti-Goat IgG was added to secondary solution at a concentration of 1:500. Finally, sections were developed with 3,3′-diaminobenzidine (Envision system 2-Kit Solution DAB, ThermoFisher Scientific, #34065), contrasted with haematoxylin (#MHS128). As previously reported (Del Rio-Moreno et al., 2019), the pathologists performed histopathological analysis of the samples following a blinded protocol. In the analysis, +, ++, +++ indicate low, moderate, and high intensities of tumour region staining compared with the normal control adjacent region.

Silencing of splicing factors by specific small interfering RNA

Pre-designed and validated specific small interfering RNA (siRNA) oligos for knockdown of endogenous SRSF3, RBM22 or RBM3 (#12733, #31273, #s142991, respectively; Silencer® Select siRNAs; ThermoFisher Scientific), and for PTBP1 (1:1 from B-C sequences; #SR303857; Trilencer-27 siRNA kit; Origene) were used. Cells (n = 200 000 cells/well) were transfected with 25 nM of each siRNA individually using Lipofectamine RNAiMax® (ThermoFisher Scientific, #13778-075) according to the manufacturer’s instructions. Silencer® Select Negative Control siRNA (ThermoFisher Scientific, #4390843) was used as a scramble control. After 48 h, cells were collected for validation of the transfection (qPCR and western blot) and seeded for different functional assays (see below).

Measurements of proliferation and migration rates

As previously described, cell proliferation (Hormaechea-Agulla et al., 2017b; Vazquez-Borrego et al., 2019) and migration (Hormaechea-Agulla et al., 2017a, b; Vazquez-Borrego et al., 2019) in response to SRSF3/RBM22/PTBP1/RBM3 silencing or after PDGFDD treatment was analysed using alamarBlue® Assay (3000 cells/well for cell lines and 10 000 cells/well for primary cell cultures) (Biosource International, #BUF012B) and the wound-healing technique (150 000 U-118 MG cells/well), respectively. For the migration assay, U-118 MG cells cultured under confluence were serum-starved for 24 h to achieve cell synchronization, and the wound was then made using a 200 μl sterile pipette tip. Wells were rinsed and cells were incubated for 6 h and 24 h with supplemented medium without FBS. Wound-healing was compared with the area just after the wound was performed. Three pictures were randomly acquired along the wound per well to calculate the area by ImageJ software v.1.49 (Schneider et al., 2012).

Apoptosis analysis

As previously reported (Vazquez-Borrego et al., 2019), apoptosis induction in response to SRSF3/RBM22/PTBP1/RBM3 silencing in glioblastoma cell lines (5000 cells/well) were seeded for 24 h before the experiments, which were carried out following the manufacturer’s protocols.

Tumorsphere formation

This assay was carried out in SRSF3/RBM22/PTBP1/RBM3-silenced glioblastoma cell lines (100 cells/well) cultured in a Corning Costar ultra-low attachment plate (#CLS-3473) with DMEM F-12 (Gibco, #13200033) with EGF (20 ng/μl) (#SRP3027) for 10 days (refreshed every 48 h), as previously reported (Jimenez-Vacas et al., 2019a). Briefly, an inverted microscope coupled to a digital camera was used to take photographs to visualize and measure cell morphology and area in order to calculate the number of tumorspheres after 10 days of incubation. Then, tumorspheres were trypsinized to determine the number of cells per tumorsphere. In addition, glioblastoma U-87 MG and U-118 MG cell lines were similarly analysed after 5 days of incubation with PDGFDD treatment (administered from time 0) combined with SRSF3 silencing.

VEGF secretion

The VEGF Human ELISA Kit (ThermoFisher Scientific, #KHGO112) was used to quantify vascular endothelial growth factor (VEGF) secretion in response to SRSF3/RBM22/PTBP1/RBM3 silencing in glioblastoma cell lines, following the manufacturer’s instructions. Briefly, silenced cells were seeded (150 000 cells/well) and incubated for 24 h in FBS medium. Then, cells were starved in medium without FBS for 1 h and incubated under the same conditions for 4 h and 24 h. Medium was collected, centrifuged, and stored for VEGF determination.

Western blotting

To determine protein levels, cells pellets were resuspended using pre-warmed SDS-DTT sample buffer [62.5 mM Tris-HCl (#10708976001), 2% SDS (#71726), 20% glycerol (#17904), 100 mM DTT (#D0632-5G) and 0.005% bromophenol blue (#B0126)] followed by sonication for 10 s and boiling for 5 min at 95°C. Proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes (Millipore, #1704270). Membranes were blocked with 5% non-fat dry milk (#T145.3), in Tris-buffered saline/0.05% Tween-20 (#93773) and incubated with the primary antibody [anti-SRSF3, anti-RBM22, anti-PTBP1, anti-RBM3, anti-AKT (CST, #40605), anti-PAKT (CST, #92725), anti-ERK1/2 (CST, #9102S), anti-pERK (CST, #4370S), anti-PDGFRB (CST, #3162) and anti-PK3110A (CST, #4249)], and their appropriate secondary antibodies [anti-rabbit IgG-HRP (CST, #7074), anti-mouse IgG-HRP (CST, #7076) or anti-goat IgG-HRP (#A9452)]. Proteins were developed using an enhanced chemiluminescence detection system (GE Healthcare) with dyed molecular weight markers. A densitometric analysis of the bands was carried out with ImageJ software v.1.49 (Schneider et al., 2012) using total protein loading (Ponceau staining, #P3504-10G) or total protein signal (in case of AKT...
and ERK) as normalizing factor, as previously reported (Jimenez-Vacas et al., 2020).

Preclinical mouse model

A preclinical xenograft mouse model with silenced SRSF3 in vivo was developed. Specifically, 5-week-old ATHYM-Foxn1nu/nu mice (n = 5; Janvier Labs) were injected subcutaneously with 3 x 10^6 U-87 MG cells in both flanks [in 100 μl of basement membrane extract (Trevigen, #3432-010-01)]. Once the tumour was clearly measurable, each mouse received an injection with SRSF3 siRNA into one flank and a negative control siRNA (used as a scramble control) into the other flank using AteloGene® reagent (KOKEN Co, #KKN1394) to transfect the siRNA molecule into cells by local administrations. Tumour growth was monitored every 4 days using a digital caliper. Sixteen days after injection, mice were sacrificed and each tumour was dissected, fixed, and sectioned for histopathological examination following haematoxylin and eosin staining. Examination of %Ki67 and mitosis number was carried out by expert pathologists. Additional pieces from the tumour were in liquid nitrogen until RNA extraction using TRIzol reagent and protein extraction using SDS-DTT buffer, as previously reported (Jimenez-Vacas, 2019). These experiments were carried out according to the European Regulations for Animal Care under the approval of the university/regional government research ethics committees.

nCounter PanCancer signalling pathway analysis

As previously reported (Sarmento-Cabral et al., 2017), the nCounter PanCancer Pathways Panel kit (NanoString Technologies, #GXA-PATH1-12) was used and carried out at the Laboratory of Genetics at UCAIB (IMIBIC) to simultaneously examine the expression of 730 genes associated with cancer (i.e. 606 genes representing all major cancer pathways and 124 key cancer driver genes). Briefly, after analysing the quality of all samples using microelectrophoresis, RNA from four scramble-control and four SRSF3-silenced samples from the preclinical mouse model (samples with the best quality) were loaded in the plate provided in the nCounter Kit, and the experiment was run following the manufacturer’s protocol. The data were analysed using the nSolver Analysis Software v.4.0.66 from NanoString Technologies with the PanCancer Pathways Analysis Module using 36 genes as housekeeping genes. All specific target sequences and panel details are available on the manufacturer’s webpage.

Detection of splicing variants in response to SRSF3 silencing by end-point PCR

End-point PCR was developed using cDNA from glioblastoma cell lines in response to SRSF3 silencing versus scramble-control to detect different splicing variants of PDGFRB and TP73 (a PDGFRA transcription factor). Details of the end-point PCR to detect splicing events have been previously reported (Durán-Prado et al., 2009; Hormaechea-Agulla et al., 2016). Primer sequences are included in Supplementary Table 4.

Bioinformatics and statistical analysis

Several data-mining processes were carried out by bioinformatic experts (Supplementary Fig. 3A), ranging from the application of preprocessing methods to the construction of classification and clustering models for a better estimation of the relevance of specific factors. Data were evaluated for heterogeneity of variance using the Kolmogorov–Smirnov test. Statistical differences were assessed by t-test, Mann-Whitney U-test or by one-way ANOVA followed by Fisher’s correction exact test. Analysis of qPCR array results was carried out by a bioinformatic expert who used several tests, models and hierarchical cluster algorithms, which allowed us to make a fairly accurate assessment. Correlations were studied using the Pearson correlation test. All statistical analyses were performed using Prism software v.8.0 (GraphPad Software, La Jolla, CA, USA) except the clustering analyses which were performed with MetaboAnalyst Software v.4.0 (McGill University, Quebec, Canada). Paired-end bulk RNAseq data were aligned, analysed and normalized using Partek Flow® software (Partek Incorporated, St. Louis, MO, USA). For survival analysis, groups were selected based on the cut-off points determined by survminer R package or median. The computational methodology shown in Supplementary Fig. 3A was implemented in R language v.3.5. Values of P < 0.05 were considered statistically significant. Data represent median (interquartile range) or means ± standard error of mean (SEM). *P, 0.05; **P, 0.01; ***P, 0.001, significantly different from control conditions.

Data availability

Microfluidic array, RNAseq, nCounter and other data used in this paper are available upon request to the corresponding author. Chinese Glioma Genome Atlas RNAseq data (CGGA) and Murat microarray data were interrogated through the GlioVis Tools (http://gliovis.bioinfo.cnio.es) (Murat et al., 2008; Bowman et al., 2017).

Results

Splicing machinery is drastically dysregulated in high-grade astrocytomas/glioblastomas

The expression levels of the components of the splicing machinery (spliceosome components and splicing factors) were analysed in HGAs/glioblastomas and control samples from a first cohort of patients (Supplementary Table 1A). An initial analysis in the four different brain areas obtained from healthy donors (control samples) revealed that the expression levels of all the splicing machinery components were completely homogenous across the different control brain areas (based on principal component analysis, PCA) (Supplementary Fig. 1A). Moreover, the individual expression level of these splicing machinery components did not vary across the four brain areas (based on qPCR analysis; data not shown). Similarly, PCA and individual mRNA
expression comparisons also revealed that expression levels of these splicing machinery components were completely homogeneous between grade III versus IV HGAs (n = 8 and 21, respectively) (Supplementary Fig. 1B and C). Based on these results, further analyses were performed combining the expression levels of the four different control brain areas of each patient (control tissues, CTs; n = 4), and both HGA samples (III and IV-glioblastoma, HGAs/glioblastomas; n = 29).

Remarkably, we found a marked dysregulation in the expression levels of multiple components of the splicing machinery (spliceosome components and splicing factors) in HGAs/glioblastomas compared to the control tissues. Specifically, there was a significant upregulation of the 11 major spliceosome components (RBM22, PRPF8, PRPF40A, TCERG1, SF3B1, SF3B1TV1, U2AF2, U2AF1, RNU5, RNU4 and RNU1) and 20 splicing factors (SNW1, TIA1, TRA2A, TRA2B, SRSF10, SRSF9, SRSF6, SRSF5, SRSF3, SRSF2, SRRM1, SND1, KHDRBS1, RBM45, RBM3, RAVER1, PTBP1, NOVA1, MAGOH and CELF1), whereas two minor spliceosome components (RNU11 and RNU12) and two splicing factors (ESRP1 and SRRM4) were significantly downregulated (Fig. 1A and Supplementary Fig. 2). Interestingly, non-supervised hierarchical analysis based on the expression pattern of all spliceosome components and splicing factors was able to perfectly separate tumour and control samples into two independent clusters (Figs 2B and 3B, respectively). Specifically, non-supervised hierarchical analysis based on the expression pattern of all spliceosome components and splicing factors was able to perfectly separate tumour and control samples into two independent clusters (Figs 2B and 3B, respectively).

Protein levels of SRSF3, RBM22, PTBP1 and RBM3 are elevated in HGA/glioblastoma samples

Consistent with the results of mRNA, IHC analyses using FFPE of all the samples from the first cohort of patients (Supplementary Table 1A) revealed that nuclear protein levels of SRSF3, PTBP1 and RBM3 were significantly elevated in HGA/glioblastoma samples compared to control samples; however, RBM22 staining was not elevated in these HGA/glioblastoma samples (Fig. 1G). Interestingly, we also had the possibility to compare the levels of these four splicing machinery components in a glioblastoma tumour tissue versus its non-tumour adjacent tissue, which confirmed that the protein levels of all of these factors, including RBM22, were clearly elevated in the glioblastoma tissue compared to the non-tumour area (Supplementary Fig. 4). In addition, low levels of cytoplasmic staining of SRSF3 were detected in HGA/glioblastoma tissues, although the score did not reach statistical significance (data not shown). Furthermore, SRSF3, RBM22, PTBP1 and RBM3 protein overexpression was corroborated in GBMs versus non-tumour samples (GTEx tissues) using the proteomic glioblastoma CPTAC dataset (Edwards et al., 2015) (Fig. 1H).

Dysregulation in splicing machinery is confirmed in different mouse models

We also studied the expression levels of the 45 splicing machinery components (the same as previously analysed in human HGAs/glioblastomas) in two mouse models with proneural-like glioblastoma (PDGFA model) and mesenchymal-like glioblastoma (PDGFB model) recently published (Ozawa et al., 2014) (Fig. 2A), as well as in electroporated glioma mouse models with constitutively active oncopgenes (Erbb2-V664E-EGFP/Hras-G12-EFPP/Kras-G12V-EGFP tumour versus EGFP-control model) as previously described (Breunig et al., 2016) (Fig. 3A).

Similar to that observed in humans, a marked dysregulation of the splicing machinery components was found in the PDGF mice and electroporated mice tumour samples (Figs 2B and 3B, respectively). Specifically, non-supervised hierarchical analysis based on the expression pattern of all these components was able to perfectly separate tumour and control samples into two independent clusters (Figs 2B and 3B). Fifteen of these components were commonly dysregulated in human and both mouse PDGF models (Fig. 2C), including the four key components (Srsf3, Rbm22, Ptbp1 and RBM3) found in the first cohort (Fig. 1A–F), the expression levels of SRSF3, RBM22, PTBP1 and RBM3 in this validation cohort again allowed the perfect discrimination between HGAs/glioblastomas and control tissues into two perfect clusters (Supplementary Fig. 3C). Based on these results, we selected SRSF3, RBM22, PTBP1 and RBM3 for further analyses.
Figure 1 Splicing machinery is drastically dysregulated in HGAs/glioblastomas. (A) Individual fold-change of the expression of all the splicing machinery components analysed in control brain tissues (CT) and HGA/glioblastoma samples. (B) Hierarchical heat map generated using the expression levels of all the spliceosome components and splicing factors determined in control brain tissues from different brain areas [Controls (Brocca, Wernicke, cingulate and medial); n = 4] and HGA/glioblastoma samples (grades AIII/AIV; n = 29). (C) Principal components analysis of the mRNA expression levels of the splicing machinery components in the same sample set. (D) VIP scores obtained from partial least (continued)
and Rbm3) previously selected in human samples. Likewise, 22 components were also commonly dysregulated in human and the electroporated mouse models, including Srsf3, Ptbp1 and Rbm3, but not Rbm22 (Fig. 3C). In fact, ROC curve analyses of these four components corroborated their capacity to significantly discriminate between tumour and control tissues showing an AUC for Srsf3, Rbm22, Ptbp1 and Rbm3 of 1, 0.78, 1 and 0.89 in the PDGFA model, of 1, 1 and 0.81 in the PDGFB-model, and of 1, 0.5, 1 and 1 in the electroporated glioma models, respectively (Figs 2D and 3D). Moreover, heat maps and PCA generated with these four components in PDGF and electroporated mouse models clearly showed that the expression pattern of just these four components is able to separate tumour and control tissues, segregating them into two perfect clusters/groups (Figs 2E and 3E).

In addition, western blot analyses using the PDGF samples revealed that protein levels of Srsf3, Rbm22, Ptbp1 and Rbm3 were significantly elevated in glioblastoma samples compared to their control tissues in both mouse models (Srsf3 expression tended to be elevated in the PDGFB model; \( P = 0.125 \); Fig. 2F). Moreover, analysis of the RNa-seq dataset from the electroporated glioma models revealed that only Srsf3 expression was significantly higher in mesenchymal-like models in comparison with proneural-like models (Fig. 3F).

Silencing of SRSF3/RBM22/PTBP1/RBM3 expression decreases functional parameters in glioblastoma cells

Two human glioblastoma cell line models (U-87 MG and U-118 MG) and primary patient-derived glioblastoma cell cultures were used to perform functional experiments (Fig. 4A). First, a heat map and a PCA generated with the expression pattern of all spliceosome components and splicing factors (Supplementary Fig. 5A and B) and also with only SRSF3, RBM22, PTBP1 and RBM3 (Supplementary Fig. 5A) were able to perfectly separate human control tissues into independent clusters/groups from human HGA/glioblastoma tissues and glioblastoma cell lines. In addition, data from available GBM cell lines (\( n = 34 \), including U-87 MG and U-118 MG) and primary GBM lines (\( n = 12 \)) RNa-seq were also analysed (Cancer Cell Line Encyclopedia).

Dysregulation of the splicing machinery is associated with relevant clinical parameters

Low expression of SRSF3 (Fig. 5A), but not RBM22, PTBP1 and RBM3 (Supplementary Fig. 6A), was directly associated with better survival rate in glioblastoma patients from the first cohort. It should be noted that this analysis could not be implemented in our second cohort (validation cohort) as these patients were diagnosed near the end of the study. Nonetheless, we could also corroborate that SRSF3...
Figure 2 Dysregulation in the splicing machinery is also confirmed in two mouse models with proneural-like and mesenchymal-like glioblastoma. (A) Diagram of generation of mouse models of glioblastoma by overexpression of PDGFB or PDGFA in a Cdkn2a null background in Nestin-expressing cells (adapted from Bejarano et al., 2017). (B) Hierarchical heat map generated using the mRNA expression levels of spliceosome components and splicing factors in non-tumour and tumour samples from PDGFA/B-induced mouse models. (C) Venn diagram of altered splicing machinery components in PDGFA/B-induced glioblastoma mouse models and human HGA/glioblastoma samples.

(continued)
expression was directly associated with a better survival rate in glioblastoma patients using the well-characterized Murat and CGGA dataset (Fig. 5B and C), this association being with SRSF3, but not with RBM22, PTBP1 and RBM3, consistent across the different datasets (Supplementary Fig. 6B and C). In line with this association, a higher expression of SRSF3 (Supplementary Fig. 6E; CGGA dataset), but not RBM22, PTBP1 and RBM3 (data not shown), was found in human mesenchymal GBM subtype (a poor survival GBM subtype) compared to proneural GBM subtype. Consistently, we found that the difference in mouse Srsf3 expression levels was more evident, in terms of P-values, when comparing mesenchymal GBM subtype versus control samples than in the case of proneural GBM subtype versus control samples in both mouse models analysed (PDGF and electroporated mice; Supplementary Fig. 6F). Additionally, we found a significant correlation between SRSF3 (Fig. 5D), but not RBM22, PTBP1 and RBM3 (Supplementary Fig. 6D), with the %Ki67 index, a standard proliferative marker, in our cohort of patients. Likewise, a correlation between expression levels of SRSF3 and those of different key tumour progression markers (MKi67, PCNA, CDK1 and CCNB1) was also found in glioblastoma tissues (Murat and CGGA dataset), but not in the non-tumour samples (available only in Murat dataset) (Fig. 5E and F). Based on these and the previous results, SRSF3 was selected to perform further studies.

**SRSF3 silencing prevents the progression of glioblastomas in vivo**

**SRSF3 silencing in vivo** (validated at mRNA and protein levels; Supplementary Fig. 7A and B) reduced tumour volume and weight in the preclinical xenograft glioblastoma model (Fig. 6A). In fact, in vivo tumour volume across time clearly showed that glioblastoma progression was completely stopped in the SRSF3-silenced model (blue line) compared to the scramble-transfected tumours, which drastically continued their progression (green line; Fig. 6A). Moreover, the number of mitosis and %Ki67 IHC staining was significantly decreased in the SRSF3-silenced model compared to the scramble-transfected (Fig. 6B) supporting the strong anti-tumour potential of SRSF3 silencing in glioblastoma cells previously observed *in vitro* (Fig. 4) and *in vivo* (Fig. 6A).

**SRSF3 silencing alters multiple key cancer-related pathways in vivo**

SRSF3 silencing *in vivo* significantly altered the expression of 149 of 770 genes analysed with the nCounter PanCancer Pathways Panel kit compared to the scramble-transfected tumours (Fig. 6C). Specifically, the PI3K pathway was the most altered signalling pathway (i.e. 39 genes: 23 downregulated/16 upregulated), followed by the cell cycle and apoptosis pathways (i.e. 35 genes: 25 upregulated/10 downregulated; Fig. 6D). Additionally, other key signalling pathways were also altered (e.g. RAS, MAPK, Wnt, DNA-damage/repair, transcription misregulation, etc.) (Fig. 6D). Indeed, the heat maps generated with the genes involved in several of these pathways were able to perfectly discriminate between SRSF3-silenced versus scramble-transfected glioblastoma cells (Supplementary Fig. 8A).

We next used the STRING database to determine the potential functional association between the altered genes in response to SRSF3-silencing (Fig. 6E), which showed the existence of three clusters of altered genes strongly associated with the control of cell cycle/transcription/DNA replication (cluster-1: blue), PDGFR-activated pathway (cluster-2: magenta) and focal adhesion (cluster-3: green). An additional protein–protein interaction (PPI) functional association performance by Network Analyst software, revealed that the PDGFRB-activated pathway was the most relevant pathway altered (Supplementary Fig. 8B). In fact, a specific analysis of the glioma pathway using KEGG revealed a clear alteration in several genes involved in the PDGFR pathway (i.e. PDGFRB, SOS2, SHC2, PIK3CR3 and PIK3CA) (Fig. 6E), which further supported the relevance of the PDGFRB in SRSF3-silenced cells.

**PDGFRB pathway inhibition as a driver of SRSF3 silencing-induced anti-glioblastoma tumour actions**

mRNA levels of the glioma pathway components PDGFRB, SOS2, SHC2, PIK3CR3 and PIK3CA were significantly downregulated in SRSF3-silenced xenografted glioblastoma cells (Fig. 7A). Similarly, these changes were also observed in U-87 MG, U-118 MG and patient-derived primary glioblastoma cell cultures (Supplementary Fig. 9A). A protein downregulation of upstream (i.e. PDGFRB and PIK3 catalytic α-subunit) components of the glioma-pathway was also found in SRSF3-silenced xenografted glioblastoma cells (Fig. 7A), and also observed (i.e. PDGFRB) in U-87 MG and...
In addition, inhibition of total protein levels of the downstream components of the PDGF-PDGFRβ pathway [i.e. AKT and ERK (classical end points of this pathway associated with cell survival, cell growth and cell proliferation)] was also observed in SRSF3-silenced xenografted glioblastoma cells (Fig. 7A) and/or SRSF3-silenced glioblastoma cells in vitro (U-87 MG and U-118 MG; Fig. 7B). Interestingly, we found
Figure 4 Silencing of SRSF3, RBM22, PTBP1 and RBM3 expression decreases functional parameters of aggressiveness in glioblastoma cell lines and primary patient-derived glioblastoma cells. (A) Diagram showing the in vitro generation of SRSF3, RBM22, PTBP1 and RBM3 knockdown glioblastoma cells by silencing with specific siRNAs. (B) Proliferation rate of SRSF3, RBM22, PTBP1 and RBM3-silenced cells compared to control scramble-transfected cells (U-87 MG and U-118 MG models and primary patient-derived glioblastoma cells; n = 3). (C) Migration rate of SRSF3, RBM22, PTBP1 and RBM3-silenced U-118 MG cells compared to control scramble-transfected cells (representative images of the migration capacity are also included; n = 3). (D) VEGF secretion of SRSF3, RBM22, PTBP1 and RBM3-silenced cells compared to control scramble-transfected cells (U-87 MG and U-118 MG models; n = 3). (E) Apoptosis levels in SRSF3, RBM22, PTBP1 and RBM3-silenced cells compared to control scramble-transfected cells (U-87 MG and U-118 MG models; n = 3). (F) Number of cells per tumorsphere and representative images of formation of tumorspheres in SRSF3, RBM22, PTBP1 and RBM3-silenced cells compared to control scramble-transfected cells on cell morphology (U-87 MG and U-118 MG models; n = 3). Data represent means ± SEM. * P < 0.05; ** P < 0.01; *** P < 0.001, significantly different from control conditions. See also Supplementary Fig. 5.
that only the phosphorylation levels of ERK, but not AKT, were significantly downregulated in SRSF3-silenced glioblastoma cells \textit{in vitro} (U-87 MG and U-118 MG; Fig. 7B). Remarkably, silencing of SRSF3 completely blocked the pro-oncogenic parameters (i.e. proliferation and number of glioblastoma stem/progenitor cells) induced in response to PDGFDD (specific ligand of PDGFRB receptor) in glioblastoma cells \textit{in vitro} (U-87 MG and U-118 MG; Fig. 7C and D). Similar results in terms of proliferation rate were also observed in patient-derived glioblastoma primary cell cultures (Fig. 7C). Supporting these results is the fact that SRSF3-silencing blocked the stimulatory effects on AKT and ERK pathways induced by PDGFDD treatment in glioblastoma cells (U-87 MG and U-118 MG) (Fig. 7E).

**Changes in the expression of TP73 splicing variants as functional link between SRSF3-PDGF-PDGFRB pathway**

Our results indicate that SRSF3 silencing does not alter the splicing of PDGFRB in GBM cell lines (Supplementary Fig. 10A) thus arguing against a direct action of this splicing factor on the receptor itself. Of note, our present results on SRSF3 silencing compare favourably with those in the Song \textit{et al.} (2019) dataset, where SRSF3 knockout also downregulated PDGFRB (Supplementary Fig. 10B), while not altering PDGFRB splicing. Thus, to explore alternative mechanisms possibly linking SRSF3 and PDGF-PDGFRB pathway, we interrogated various datasets, aiming to ascertain the potential involvement of PDGFRB-controlling transcription factors whose splicing might be influenced by SRSF3. Specifically, we analysed: (i) the Song \textit{et al.} (2019) dataset to define genes whose splicing is altered by SRSF3 knockout; (ii) ENCORI, Encyclopedia of RNA Interactomes (Ghandi \textit{et al.}, 2019), to identify mRNAs that physically interact with SRSF3; and (iii) GeneHancer database (Fishilevich \textit{et al.}, 2017), to predict transcription factors capable of regulating PDGFRB expression (Fig. 8A). Results from these analyses revealed five genes (TFDP1, CUX1, PRDM10, TEAD4 and TP73) that fulfil the three criteria and could thus represent a putative functional nexus between SRSF3 and PDGF-PDGFRB pathway (Fig. 8A). Indeed, analysis of PSI (Percent Spliced-in Index) of these five genes in the Song \textit{et al.} (2019) dataset showed that SRSF3 knockout decreased the splicing events of TFDP1,
Figure 6 SRSF3 silencing prevents the progression of glioblastomas and alters multiple key cancer related-pathways in vivo (U-87 MG-induced xenografts). (A) Diagram showing the generation of xenograft mouse models of glioblastoma by U-87 MG inoculation. SRSF3 in vivo silencing was carried out in one of the flanks of each mouse (n = 5). Average tumour volume and tumour weight of subcutaneous inoculated SRSF3 siRNA-transfected versus scramble-transfected tumours. Images of each tumour are shown. The red arrow indicates the treatment time with scramble or SRSF3-siRNA using AteloGene® reagent. (B) Mitosis number (× 10 HPF) and %KI67 IHC analysis (n = 5/group)
revealed that (Fig. 8E). Specifically, while altered the ratio of individual splicing variants with re-
Moreover, a detailed analysis of the TP73 leading to a survival rate of 12–16 months from diagnosis,
Glioblastoma is one of the most lethal human cancers, with late diagnosis and poor prognosis despite years of research,
Remarkably, increasing evidence over the last two decades has documented that the spliceosome and associated proteins are often altered in disease states, including metabolic diseases (Gahete et al., 2018; Del Rio-Moreno et al., 2019) and cancer (Dvinge et al., 2016; Jia et al., 2019; Jimenez-Vacas et al., 2019b, 2020), which augments pathobiological versatility through the generation of distinct/novel alternative splicing variants (Kozlovskii et al., 2017; Singh and Eyras, 2017). Actually, every hallmark of cancer (Stratton et al., 2009) can be associated with several examples of proto-oncogenes, tumour-suppressor genes, or other genes whose splicing is altered to produce isoforms that contribute to the transformation process (Sveen et al., 2016). In fact, recent genomic characterization of different cancers has revealed recurrent copy number changes affecting genes encoding spliceosome components and splicing factors that contribute to cancer-relevant phenotype (Dvinge et al., 2016). Here, we demonstrate for the first time a drastic dysregulation of the expression profile of the splicing machinery in a well-characterized cohort of HAGs/glioblastomas, where a representative set of spliceosome components and splicing factors was markedly altered (77% and 79%, respectively). Moreover, bioinformatics analyses defined an expression-based molecular fingerprint of these spliceosome components and splicing factors able to perfectly discriminate between HAGs/glioblastomas versus control tissues from both human and mouse, which further reinforces our notion, suggesting that HGAs/glioblastomas have a global splicing dysregulation in different species. Further clustering and hierarchical bioinformatics analyses in two independent human sample cohorts revealed that SRSF3, RBM22, PTBP1 and RBM3 were the
total ratio, it increased the TP73\(\beta\gamma\)/TP73 total ratios (Fig. 8E).

**Discussion**

Glioblastoma is one of the most lethal human cancers, with late diagnosis and poor prognosis despite years of research, leading to a survival rate of 12–16 months from diagnosis (Singh and Eyras, 2017; Ostrom et al., 2018). As patients are often diagnosed at advanced stages, when cure is no longer possible, their quality of life is poor and worsens rapidly, while healthcare costs concomitantly increase. Indeed, despite extensive effort made in recent years to develop therapeutic approaches for tackling this pathology (Zanders et al., 2019), the current therapeutic strategies are not efficient at reducing tumour volume/growth or augmenting survival rate. This is likely due, in part, to the resistance acquired by tumours, particularly by stem cells progenitors, to current drugs (Noch et al., 2018). Thus, identification of new molecular diagnostic, prognostic and therapeutic tools to refine their detection, define tumour behaviour (from tumorigenesis and progression to metastasis mechanisms) and develop new treatments is crucial to combat this devastating pathology.

The splicing process is a highly coordinated mechanism, regulated and carried out by the spliceosome, that relies on a combination of multiple spliceosome components and splicing factors, intronic and exonic sequence elements, and temporal and spatial signalling pathways to adequately control gene expression, while increasing its complex versatility (Chen and Manley, 2009; Pelechano et al., 2013). New molecular diagnostic, prognostic and therapeutic tools to refine their detection, define tumour behaviour (from tumorigenesis and progression to metastasis mechanisms) and develop new treatments is crucial to combat this devastating pathology.
Figure 7 Inhibition of the PDGF-PDGFRB pathway as potential driver of the SRSF3 silencing-induced anti-glioblastoma tumour actions. (A) Expression levels (mRNA levels measured by nCounter PanCancer and protein levels measured by western blot) of different components of the PDGFRB activated pathways (in bold) in SRSF3 siRNA-transfected versus scramble-transfected U-87 MG-induced xenograft tumour samples (n = 4). (B) Phosphorylated protein ratios and total protein levels of AKT and ERK in SRSF3-silenced U-87 MG and U-118 MG cells compared to scramble-controls (n = 5) after 24 h of silencing. (C) Proliferation rates after treatment with PDGFDD homodimer (specific...)

(continued)
components of the splicing machinery with higher capacity to discriminate between human HGA/glioblastomas and control tissues. Importantly, these results were confirmed in two independent external in silico cohorts of human glioblastomas (Murat/CGGA) and in different mouse glioma models analysed herein (PDGF/electroporated models). Moreover, data from the CGGA dataset also revealed that SRSF3, PTBP1 and RBM3 were significantly elevated across glioma grades (IV versus III versus II) supporting the diagnostic potential of these factors.

In this study, we evidenced an overall overexpression of SRSF3, RBM22, PTBP1 and RBM3, at both mRNA and protein level, in the different human cohorts and mouse models with gliomas analysed. Notably, silencing the expression of these splicing machinery components in glioblastoma cells induced marked reductions in aggressiveness features of glioblastomas (i.e. inhibition of proliferation, migration and VEGF secretion, and increase of apoptosis). Most notably, silencing of SRSF3, RBM22, PTBP1 and RBM3 strikingly decreased the number of glioblastoma stem/progenitor cells present in each tumorsphere, a relevant functional result that may help to explore how to overcome the well-known resistance of glioblastomas to current drugs (Noch et al., 2018).

Thus, these results demonstrate that dysregulation of the splicing machinery can play a key pathophysiological role in glioblastoma cells, and some of its components (SRSF3, RBM22, PTBP1 and RBM3) could provide novel, useful tools as diagnostic biomarkers and potential therapeutic targets to tackle glioblastomas. The potential utility of SRSF3, RBM22, PTBP1 and RBM3 expression as prognostic biomarkers is further supported by the direct association found between their levels and relevant molecular features of aggressiveness (e.g. MKI67, PCNA, CDK1 and CCNB1 levels) in patients with glioblastomas but not in healthy patients, as well as between their levels and glioma grades and GBM subtypes (i.e. higher in IV versus III versus II grades, and in mesenchymal versus proneural GBM subtypes). Moreover, these observations suggested a causal link between dysregulation of these splicing machinery components and glioblastoma aggressiveness. While SRSF3, PTBP1 and RBM3, but not RBM22, have been associated with certain tumour pathologies (Guo et al., 2015; Barbagallo et al., 2018; Chen et al., 2019b; Jia et al., 2019; Melling et al., 2019; Song et al., 2019) and/or brain development (Vuong et al., 2016), to the best of our knowledge, this is the first report identifying a relevant functional role of these splicing machinery components in human glioblastomas, as well as in proneural/mesenchymal-like glioma mouse models with different prognosis.

Most importantly, this study reveals that expression of SRSF3 is directly associated with a better survival rate in HGA/glioblastoma patients, the main clinical problem in this pathology. This finding was corroborated in two external in silico cohorts of patients with glioblastomas (Murat and CGGA datasets), and is further supported by a recent study indicating that the knockout (KO) of SRSF3 extended overall survival of tumour-bearing animals (Song et al., 2019). In line with this, a higher SRSF3 expression was observed in human and mouse mesenchymal GBMs (with poor survival rate) compared to proneural GBM subtypes, which reinforced the prognostic value of SRSF3. The mechanisms underlying the link between SRSF3 and survival rate may relate to the direct association found between SRSF3 silencing and tumour progression, as well as with the noteworthy disruption in one of the main pro-oncogenic signalling pathways activated in GBM cells, i.e. the PDGFRB-glioma pathway (Liu et al., 2018). Specifically, we demonstrate here that SRSF3 is an effective target in glioblastomas in vivo, since silencing of this splicing factor effectively blocks glioblastoma progression of already established glioblastoma tumours in a preclinical mouse model of glioblastoma. In fact, SRSF3 silencing in glioblastoma in vivo markedly decreased tumour volume, tumour weight, mitosis number of glioblastoma cells and KI67 expression in all mice studied (100%), which, together with the previous in vitro data reported here using SRSF3-silencing glioblastoma cells, and with the extended overall survival observed in human studies, and in a recent study on KO-SRSF3 mice (Song et al., 2019), further demonstrate the clinicopathophysiological relevance of the strong anti-tumour role of SRSF3 silencing in glioblastomas, and its potential value as a future therapeutic target in this devastating disease. In line with our results, previous studies have also indicated that the altered expression of specific components of the splicing machinery is tightly associated with aggressive disease and poor overall survival of patients with pancreatic cancer (Qiao et al., 2019) and in colorectal cancer (Sween et al., 2011).

To determine the signalling mechanisms underlying the anti-tumour role of SRSF3 in glioblastomas, we explored an ample range of cancer-related signalling pathways in response to SRSF3 silencing in vivo and in vitro. Our data revealed, for the first time, a striking alteration in the PI3K pathway in different SRSF3-silenced glioblastoma cell
models. This pathway has been reported to be closely associated with progression of glioblastomas and used as a target for the development of novel drugs (Massacesi et al., 2016; Mecca et al., 2018), as its inhibition or alteration causes anti-proliferative/invasive effects, and apoptosis (Mete et al., 2019; Xu et al., 2019). However, we found that the PDGFRB pathway, which is involved in widely known glioma-related PI3K pathways (currently being used as therapeutic target) (Batchelor et al., 2017), was the most relevant pathway altered in SRSF3-silenced cells, since a clear alteration in several genes involved in this pathway (i.e. PDGFRB, SOS2, SHC2, PIK3CR3 and PIK3CA; all glioma pathway components) was observed. Moreover, a protein downregulation of upstream (i.e. PDGFRB and PIK3 catalytic α-subunit) and downstream (i.e. AKT) components of the glioma pathway representing classical end points for cell survival, growth and proliferation (Brennan et al., 2013), was also found in SRSF3-silenced xenografted glioblastoma cells. Most importantly, SRSF3 silencing blocked the stimulatory effects on AKT and ERK pathways induced by PDGFDD treatment, and completely blocked key pro-oncogenic parameters (i.e. proliferation and/or number of glioblastoma stem/progenitor cells) induced in response to PDGFDD in different glioblastoma cell models (U-87/U-118 cell lines and primary GBM cells). Therefore, these data provide original, compelling evidence that SRSF3 is functionally linked to these well-known pathophysiologically relevant pro-oncogenic pathways in gliomas (PDGFRB and PI3K),

Figure 8 Changes in the expression profile of TP73 splicing variants as potential functional link between SRSF3 and PDGF-PDGFRB pathway. (A) Venn diagram of three levels of information derived from different sources: Song et al. (2019) dataset, ENCORI (Ghandi et al., 2019) and GeneHancer database (Fishilevich et al., 2017). (B) PSI (Percent Spliced-in Index) of TP73 from Song et al. (2019) dataset and diagram showing TP73 splicing variants detected and produced by a C-terminal MXE event. (C) PSI of TP73 in SRSF3 siRNA-transfected versus scramble-transfected U-87 MG and U-118 MG cells (n = 4). (D) Correlation of PDGFRB mRNA expression with PSI of TP73 in the same set of samples (n = 4). (E) Percentage of each splicing variant of TP73 identified in SRSF3 siRNA-transfected versus scramble-transfected U-87 MG and U-118 MG cells (n = 4). AS = alternative splicing, TFs = transcription factors.
which further supports the potential clinico-pathophysiological importance of the strong anti-tumour role of SRSF3 silencing in glioblastomas in vivo and in vitro.

However, SRSF3 does not appear to control PDGFRB activity by directly modulating its splicing, in that no apparent changes in PDGFRB splicing variants were found in SRSF3-silenced (this report) or in SRSF3-knockout (Song et al., 2019) GBM cells. In contrast, SRSF3 could control the PDGFRB pathway indirectly, by modulating the alternative splicing of five potential regulators of PDGFR expression such as TFDPI, CUX1, PRDM10, TEAD4 and TP73. Previously, altered TFDPI, CUX1 and PRDM10 splicing has been reported to generate variants that act as super enhancers of several oncogenes and cell cycle checkpoint genes (Rong Zeng et al., 2000; Sansregret and Nepveu, 2008; Cadieux et al., 2009; Park and Kim, 2010; Hulea and Nepveu, 2012; Ramdzan and Nepveu, 2014; Lu et al., 2016; Kaur et al., 2018; Zhang et al., 2018; Chen et al., 2019a; Zhou et al., 2020), while altered TEAD4 splicing events could result in VEGF inhibition (Appukuttan et al., 2012; Qi et al., 2016; Xu et al., 2018). Most interestingly, some splicing variants of TP73 (the only factor reported as a PDGFRB regulator from the five identified) can distinctly act as tumour suppressors, arresting the cell cycle and inducing apoptosis (Liu et al., 2004; Jancalék, 2014; Vikhrev et al., 2018). In this scenario, it is reasonable to posit that alterations in the alternative splicing of specific transcription factors (particularly TP73) induced by changes in SRSF3 expression may serve as the conduit linking SRSF3 to the PDGF-PDGFRB pathway, and thus the development/aggressiveness in glioblastoma. In support of this notion, our results indicate that SRSF3 silencing can increase the proportion of splicing variants such as TP73β and TP73γ, which have been linked to tumour suppressor roles (Liu et al., 2004) while decreasing that of the TP73α variant, which may play an oncogenic role (Jancalék, 2014; Vikhrev et al., 2018) in glioblastoma cells. Thus, modulation of TP73 MXE splicing event may serve as a putative mechanism linking SRSF3 and PDGFRB expression.

Taken together, our results unveiled new conceptual and functional avenues in glioblastomas, with potential therapeutical implications, by demonstrating for the first time a drastic dysregulation of the splicing machinery (spliceosome core and splicing factors; especially SRSF3/RBM22/FTPB1/RBM3) in HGAs/glioblastomas of different species. This is likely clinically relevant, because the dysregulation directly associates with development and aggressive features of glioblastomas. Moreover, we unveil a role of SRSF3 in crucial pathophysiological processes of glioblastomas, such as cell proliferation, migration, apoptosis, VEGF secretion and tumoursphere formation, which would underlie the relevant direct association of lower SRSF3 levels with reduced tumour progression and enhanced survival rate, observed herein in different glioblastoma human cohorts and animal models. These actions are likely mediated through the modulation of key signalling pathways [PDGFRB and PI3K] and may involve the distinct alteration of alternative splicing events of specific transcription factors controlling PDGFRB expression. Therefore, our study provides solid, convincing evidence demonstrating that SRSF3 has a functional role in the pathophysiology of glioblastomas, and invites suggestion that the development and use of SRSF3-targeting drugs could become a promising option to treat patients with this devastating pathology, offering a clinically relevant opportunity that should be tested for use in humans.

**Acknowledgement**

Special thanks to GraphicalAbstractDesign.com for assistance with graph and image design.

**Funding**

This work was funded by the Junta de Andalucía (CTS-1406, BIO-0139), the Spanish Ministry of Science, Innovation and Universities (FPU16/05059, FPU14/04290, PID2019-105564RB-I00), Instituto de Salud Carlos III, co-founded by European Union (ERDF/ESF, ‘Investing in your future’: PI16/00264, PI17/02287), Spanish Ministry of Economy and Competitiveness Projects (BFU2016-80360-R, TIN2017-83445-P) and CIBERobn. M.A.B. is funded by the Spanish Ministry of Economy and Competitiveness Projects (SAF2013-45111-R and SAF2015-72455-EXP), the Comunidad de Madrid Project (S2017/BMD-3770), the World Cancer Research (WCR) Project (16-1177), and the Fundación Botín (Spain). CIBER is an initiative of Instituto de Salud Carlos III, Spanish Ministry of Health, Social Services and Equality, Spain. J.J.B. is funded by the Samuel Oschin Comprehensive Cancer Institute (SOCCI), NIH grants (R33CA236687, and R03NS101529), American Cancer Society grant (RSG-16-217-01-TBG), and SOCCI Jack Mishkin Discovery, Prevention & Genetics, and Cancer Biology Awards.

**Competing interests**

The authors report no competing interests.

**Supplementary material**

Supplementary material is available at Brain online.

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