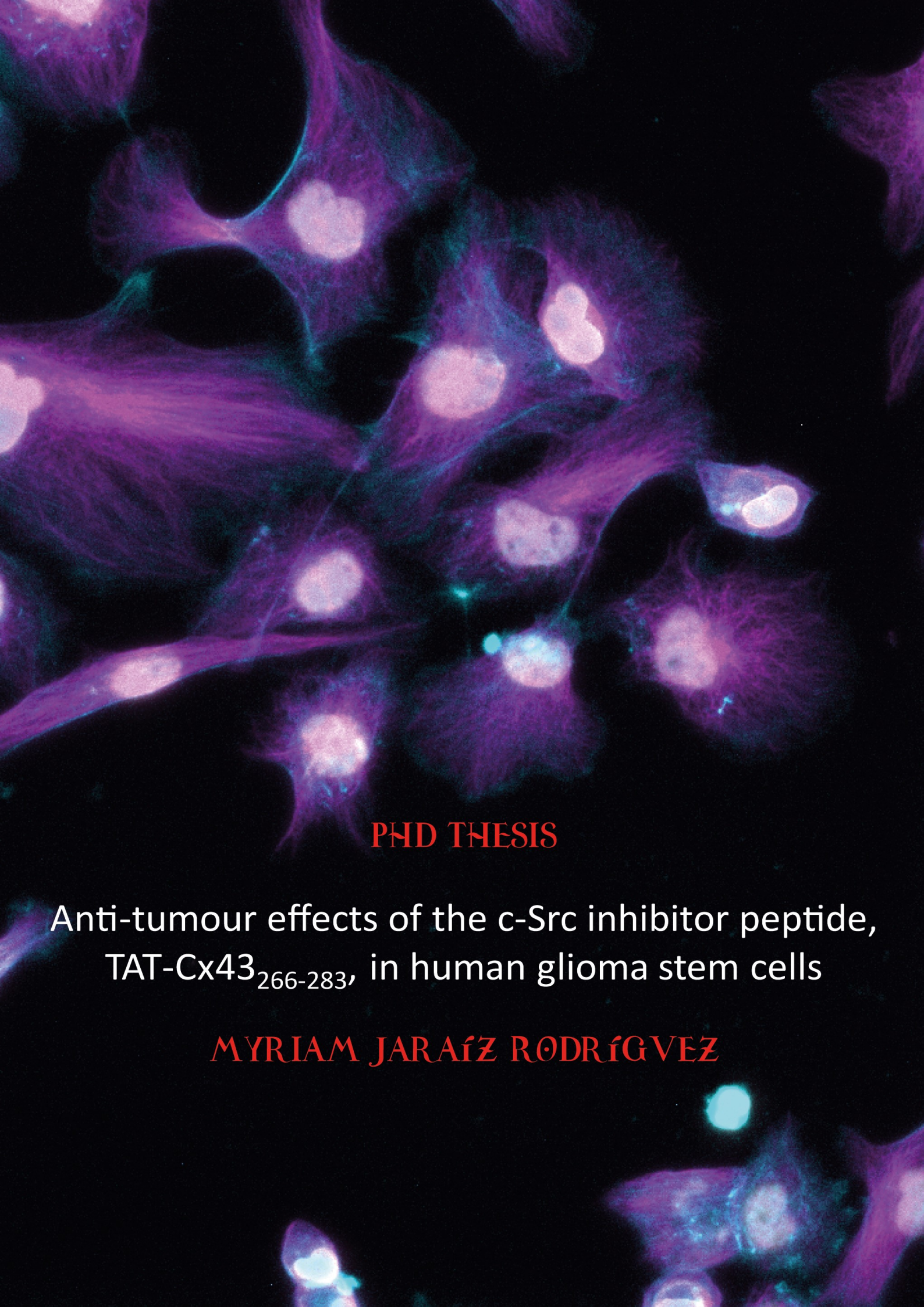

PHD THESIS

Anti-tumour effects of the c-Src inhibitor peptide, TAT-Cx43$_{266-283}$, in human glioma stem cells

MYRIAM JARAÍZ RODRÍGUEZ

VNiVERSiDAD Đ SALAMANCA

Departamento de Bioquímica y
Biología molecular

PhD THESIS SUMMARY

Anti-tumour effects of the c-Src inhibitor peptide TAT-Cx43$_{266-283}$ in human glioma stem cells

Myriam Jaraíz Rodríguez

INSTITUTO DE NEUROCIENCIAS CASTILLA Y LEÓN

IBSAL Instituto de Investigación Biomédica de Salamanca

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Esta tesis corresponde a un compendio de trabajos previamente publicados o aceptados para publicación:

Capítulo 1: González-Sánchez A, Jaraíz-Rodríguez M, Domínguez-Prieto M, Herrero-González S, Medina JM, Tabernero A. Connexin43 recruits PTEN and Csk to inhibit c-Src activity in glioma cells and astrocytes. Oncotarget 2016; e-pub ahead of print 6 July 2016; doi: 10.18632/oncotarget.10454
Instituto de Neurociencias de Castilla y León (INCYL), Departamento de Bioquímica y Biología Molecular, Universidad de Salamanca, C/ Pintor Fernando Gallego 1, 37007 Salamanca, Spain. Tel.: 34 - 923 - 29 45 00 ext 5311.

Capítulo 2: Myriam Jaraíz-Rodríguez, Ana Gonzalez-Sanchez, Laura Garcia-Vicente, Jose M Medina and Arantxa Tabernero. "Biotinylated cell-penetrating peptides to study intracellular protein-protein interactions". JOVE, en prensa.
Instituto de Neurociencias de Castilla y León (INCYL), Departamento de Bioquímica y Biología Molecular, Universidad de Salamanca, C/ Pintor Fernando Gallego 1, 37007 Salamanca, Spain. Tel.: 34 - 923 - 29 45 00 ext 5311.

Capítulo 3: Jaraíz-Rodríguez M[1], Tabernero MD[2,3], González-Tablas M[2,3], Otero A[4], Orfao A[3], Medina Jose M[1] and Tabernero A[1]. "A short region of connexin43 reduces human glioma stem cell migration, invasion and survival through Src, PTEN and FAK". Stem Cell Reports (2017), http://dx.doi.org/10.1016/j.stemcr.2017.06.007, en prensa.

[1]Instituto de Neurociencias de Castilla y León (INCYL). Departamento de Bioquímica y Biología Molecular, Universidad de Salamanca, Salamanca, Spain
[2]Instituto de Estudios de Ciencias de la Salud de Castilla y León (IECSCYL), Instituto de Investigación Biomedicina de Salamanca (IBSAL). Salamanca, Spain
[3]Centre for Cancer Research (CIC-IBMCC; CSIC/USAL; IBSAL), Departamento de Medicina Universidad de Salamanca, Salamanca, Spain
[4]Neurosurgery Service, Hospital Universitario de Salamanca and IBSAL, Salamanca, Spain

Las cartas de aceptación de los capítulos 2 y 3 se adjuntan a continuación.

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MYRIAM JARAÍZ RODRÍGUEZ <myriraiz@usal.es>

Your JoVE Submission JoVE56457R1

1 mensaje

Aaron Kolski-Andreaco <em@editorialmanager.com> 20 de junio de 2017, 13:40
Responder a: Aaron Kolski-Andreaco <aaronk.andreaco@jove.com>
Para: myriraiz@usal.es

You are being carbon copied ("cc:'d") on an e-mail "To" "Arantxa Tabernero" ataber@usal.es
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Dear Dr. Tabernero,

Thank you for your submission to JoVE.

I am pleased to tell you that your manuscript, JoVE56457R1 "Biotinylated cell-penetrating peptides to study intracellular protein-protein interactions," has been accepted. This paper is now considered to be In-Press and your abstract can be viewed on our JoVE In-Press Section within a few days. Please download the current version of the manuscript attached here and save it for your records. Note that this version may have been modified and should be used for any future revisions.

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MYRIAM JARAÍZ RODRÍGUEZ <myriraiz@usal.es>

Fwd: Your article STEM-CELL-REPORTS-D-17-00073R2 (Tabernero) publishing in Stem Cell Reports

1 mensaje

Arantxa Tabernero <ataber@usal.es> 14 de junio de 2017, 12:49
Para: Myriam Master <myriraiz@usal.es>

Inicio del mensaje reenviado:

De: "Stem Cell Reports Editorial" <em@editorialmanager.com>
Asunto: Your article STEM-CELL-REPORTS-D-17-00073R2 (Tabernero) publishing in Stem Cell Reports
Fecha: 14 de junio de 2017, 12:20:42 CEST
Para: "Arantxa Tabernero" <ataber@usal.es>
Responder a: "Stem Cell Reports Editorial" <stemcellreports@isscr.org>

Ref: STEM-CELL-REPORTS-D-17-00073R2, Tabernero

Dr. Arantxa Tabernero
University of Salamanca
University of Salamanca
Dpt. Biochemistry an Molecular Biology. Institute of Neuroscience Castilla y León (INCYL)
Pintor Fernando Gallego 1
Salamanca 37007
SPAIN

34923294500
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Dear Dr. Tabernero,

Congratulations on your paper "A short region of connexin43 reduces human glioma stem cell migration, invasion and survival through Src, PTEN and FAK" which has been accepted by Stem Cell Reports.
As the publisher of Stem Cell Reports, Cell Press will be happy to work with your institution to maximize media coverage of your manuscript. We encourage you to forward this e-mail with our embargo policies and practices listed below to your institutional press officer.
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Best Wishes,
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EMBARGO POLICIES AND PRACTICES

Dª **ARÁNZAZU TABERNERO URBIETA**, Catedrática de Universidad y D. **JOSÉ MARÍA MEDINA JIMÉNEZ**, Catedrático Emérito de Universidad, adscritos al Departamento de Bioquímica y Biología Molecular, de la Universidad de Salamanca.

AUTORIZAN:

La presentación de la Tesis Doctoral titulada "Anti-tumour effects of the c-Src inhibitor peptide TAT-Cx43$_{266-283}$ on human glioma stem cells", en la **modalidad de compendio de publicaciones**, realizada bajo su dirección por la Graduada en Farmacia y Licenciada en Bioquímica Dª **Myriam Jaraíz Rodríguez** en el Instituto de Neurociencias de Castilla y León y en el Departamento de Bioquímica y Biología Molecular, de la Universidad de Salamanca.

Y para que así conste firman el siguiente documento en Salamanca a 23 de Junio de 2017.

Fdo. Aránzazu Tabernero Urbieta

Fdo. José María Medina Jiménez

La Comisión Académica del Programa de Doctorado en Neurociencias de la Universidad de Salamanca, una vez examinada la documentación presentada por **Dª Myriam Jaraíz Rodríguez**, estudiante del Doctorado de Neurociencias regulado por el decreto RD 1393/2007, con DNI 70882104Z.

Informa favorablemente de la presentación de la Tesis Doctoral por la modalidad de Tesis por Compendio de Artículos/Publicaciones, tal y como se especifica en el capítulo II del Reglamento de Doctorado de la Universidad de Salamanca, sobre la elaboración y defensa de La Tesis Doctoral.

Y para que conste, a los efectos oportunos y a petición de la interesada, expido la presente en Salamanca a 23 de junio de 2017

Profa. Aránzazu Tabernero Urbieta
Directora Programa de Doctorado
en Neurociencias INCYL

Arantxa Tabernero – lab 15. Instituto de Neurociencias de Castilla y León (INCYL). *Universidad de Salamanca* -37007 Salamanca Spain -Teléf.: 923 294500 ext: 5311 -Email: ataber@usal.es

a place of mind
THE UNIVERSITY OF BRITISH COLUMBIA

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Phone 604 822 6567
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May 24, 2017

To whom it may concern:

This letter is to certify that Myriam Jaraíz Rodríguez performed research related to her Ph.D. thesis project in my laboratory in the Department of Cellular and Physiological Sciences, Life Sciences Institute, University of British Columbia. Myriam worked in my laboratory for 3 months, from June 1 to August 31, 2016. Her Ph.D. degree will be conferred by the Universidad de Salamanca.

Sincerely yours,

Christian Naus, Ph.D., FCAHS
Professor
Canada Research Chair

UNIVERSITY OF CALIFORNIA, DAVIS

Maxence Le Vasseur, PhD | mlevasseur@ucdavis.edu

June 12, 2017

To Whom It May Concern:

Although brain tumors such as glioblastoma are not as prevalent as other cancer types, their aggressiveness and high resistance to current therapies lead to a high mortality rate. Glioma stem cells (GSCs), a self-renewing population of cells with high oncogenic potential and infiltrative behavior, are particularly resistant to treatment and are associated with an increase in tumor recurrence. Understanding the molecular mechanisms controlling the behavior of GSCs could potentially lead to the development of novel therapeutic strategies in the treatment of glioblastoma and other brain tumors. In that regard, the gap junction protein Connexin 43 (Cx43) represents a putative candidate. Cx43 levels are abnormally low in GSCs and several laboratories have reported a reduction of glioma oncogenic potential upon restoration of Cx43 expression. However, prior to Ms Jaraíz Rodriguez work, the exact molecular mechanisms responsible for the anti-tumorigenic properties of Cx43 were poorly understood. Ms Jaraíz Rodriguez thesis presents novel and exciting results showing that a small region of Cx43 amino acid sequence is sufficient to recapitulate many aspects of Cx43 anti-tumorigenic properties. Importantly, Ms Jaraíz Rodriguez elegantly demonstrated that the anti-tumorigenic properties of Cx43 can be mimicked by short cell-penetrating mimetic peptides potentially amenable to therapeutic use.

Using a combination of confocal microscopy and immunoprecipitation assays, Ms Jaraíz Rodriguez showed that Cx43 recruits PTEN and Csk, hereby forming a docking platform inhibiting the proto-oncogene c-Src and reducing GSC proliferation. Ms Jaraíz Rodriguez and colleagues then demonstrated that this interaction is independent of Cx43 PDZ domain (residues 380–382) but requires Cx43 residues 266-283 and could be recapitulated using the small cell-penetrating Cx43 mimetic peptide TAT-$Cx43_{266-283}$. Treatment with TAT-$Cx43_{266-283}$

inhibited cell growth and reduced the proliferating index of human GSCs in a PTEN-dependent manner. Furthermore, Ms Jaraíz Rodriguez also showed that the TAT-Cx43$_{266-283}$-dependent inhibition of c-Src results in a decrease in focal adhesion kinase (FAK) activity and a reduction of GSC mobility and invasive properties.

Ms Jaraíz Rodriguez work is scientifically sound and of the highest standard. The various experimental approaches used in her study were carefully planned and executed. Her results and her interpretation of the results reveal a great level of scientific maturity and I firmly believe that Ms Jaraíz Rodriguez can confidently and successfully defend her thesis.

If you need any additional information, please do not hesitate to contact me by telephone or by email at mlevasseur@ucdavis.edu.

Sincerely,

Maxence Le Vasseur, PhD

Postdoctoral Researcher
Nunnari Laboratory, 214 Briggs Hall
Dept. of Molecular & Cellular Biology
University of California, Davis
One Shields Ave
Davis, CA, USA, 95616
Phone: 530-786-8413

Barts
Cancer Institute
Queen Mary University of London

London, June 8th, 2017

To whom it may concern,

Ms Jaraíz-Rodríguez thesis contains her work performed under the supervision of Professor Arantxa Tabernero Urbieta, in the laboratory 15, Instituto de Neurociencias de Castilla y León, Universidad de Salamanca, Spain.

Her thesis consisted of both technical and fundamental objectives to study the anti-tumour effects of the cell-penetrating peptide TAT-Cx43$_{266-283}$ on human glioma stem cells, which have all been appropriately addressed.

There is a large amount of work, a great focus on methods, as well as very basic interesting data in this PhD work. The PhD performed by this student is of the highest international standards, and is certainly of sufficient quality for the candidate to successfully defend her Thesis.

Sincerely,

Maruan Hijazi Vega, PhD

Postdoctoral Research Assistant

Centre for Haemato-Oncology

Barts Cancer Institute - a Cancer Research UK Centre of Excellence
Queen Mary University of London
John Vane Science Centre, Charterhouse Square, London EC1M 6BQ

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15 June 2017

To whom it may concern:

I am delighted to write this letter of support for Ms. Myriam Jaraiz Rodriguez. Glioma is an aggressive bran cancer that has no cure. The gap junction protein Cx43 has been implicated in cancer regulation. Myriam's work has strengthen the possibility of targeting Cx43 in anti-cancer therapy by continuing the original observation made by her supervisor, Professor Arantxa Tabernero Urbieta on the role of Cx43 in regulating the oncogenicity of rat C6 glioma cells. In her thesis, Myriam used human glioma stem cells as her model system to demonstrate that a peptide based on Cx43 sequence has an anti-tumor effect using in vitro and in vivo approaches. Furthermore, she is able to propose a signaling mechanism by which Cx43 can mediate its anti-cancer effect. The systematic and comprehensive approach in her research with important clinical implications for human health is the material of an excellent PhD thesis. The quality and standard of her work is outstanding. Please don't hesitate to contact me if you have any further questions.

Sincerely,

Wun Chey Sin (Ph.D.)
Research Associate
Email: wcsin@ubc.ca
Tel: 604-822-1737

Queda prohibido no sonreír a los problemas,

no luchar por lo que quiero,

abandonarlo todo por tener miedo,

no convertir en realidad mis sueños.

Pablo Neruda

Es preciso sacudir enérgicamente el bosque de las neuronas cerebrales adormecidas; es menester hacerlas vibrar con la emoción de lo nuevo e infundirles nobles y elevadas inquietudes.

Santiago Ramón y Cajal

A ti papá, que me enseñaste a hacer bailar mis neuronas. Sé que las tuyas habrían vibrado aún con más emoción que las mías con esta tesis.

AGRADECIMIENTOS

Al igual que en la introducción de esta tesis, voy a comenzar diciendo que el sistema nervioso central está constituido por distintos tipos de células: las neuronas, y las células gliales. De todos los tipos de células gliales, los astrocitos, llamados así por su forma de "aster", estrella, llevan a cabo toda clase de funciones para ayudar a las neuronas en su compleja tarea. Los astrocitos guían, ayudan, colaboran y apoyan a las neuronas. No me estoy llamando neurona, pero gracias a todos los que habéis sido y sois mis astrocitos día a día. Sin vosotros, la compleja tarea de realizar una tesis y, más aún, de disfrutarla, no habría sido posible.

Al laboratorio 15, creo que me habéis oído decir y más de una vez, que un laboratorio es como una casa. En este caso, además de casa, lo puedo llamar hogar gracias a todos vosotros.

A la Prof. Arantxa Tabernero, gracias por guiarme siempre, no sólo a nivel científico sino también a nivel personal. Hacemos un "dangerous tándem", y me encanta, porque nuestra retroalimentación siempre es positiva. No he podido ser más afortunada por tener la oportunidad de realizar la tesis contigo.

Al Prof. José María Medina, un auténtico honor el poder compartir ratos con un sabio como tú. No sólo por la sabiduría, sino por el humor y tantas otras cosas, menudo jefazo de laboratorio. Y sí, intentaré seguir manteniendo mi estatus de viajera…

A la Dra. Josefa M Barrientos, estoy deseando que nos toque la lotería para hacer nuestra lista inmensa de cosas. Muchísimas gracias, que las tienes y las mereces, por preocuparte tanto, por ayudarme siempre, y por supuesto por esos magníficos bombones. Y no te pienses que este piropeo es gratuito, son 5000.

A la Dra. Ana Velasco, gracias por tus ideas, por acompañarme en cada momento y, por supuesto, por escucharme siempre. Eres una súper investigadora, sólo te falta creértelo. No te canses nunca de luchar.

A Tomy, probablemente no seas consciente de lo importante que eres en el laboratorio, y no solamente por la organización y ayuda que nos proporcionas, sino por todo tu apoyo. Muchas gracias por compartir con nosotros todas las tardes de lunes.

Al Cx43 team: gracias por abrirme las puertas a este campo y por el trabajo bien hecho, yo sólo he seguido vuestro camino. Ester, gracias por iniciarme en el mundo de las células madre y por tus consejos, siempre me han venido muy bien. Ana G, parece mentira que ya haya pasado tanto tiempo desde que empezara como tu pollito, muchas gracias por instruirme en el mundo del laboratorio y gracias también por nuestros ratillos frikis.

A Maru, siempre dispuesto a ayudarme, siempre haciéndome reír, siempre enseñándome que se pueden hacer las cosas bien y por pura generosidad. Gracias.

A Marta y Sara, compañeras, amigas, mis chachis, ¿qué le puedo decir a alguien que aprovecha la oportunidad para cruzar el charco hasta el Pacífico y venirme a ver una semana? Sois lo mejor de lo mejor. Sari, mi siamesa deportiva, no me ha podido hacer más feliz tu llegada al lab y nuestras salidas infinitas. En lo único que te he echado en falta es en danza del vientre…Tu capacidad de análisis, tu paciencia y tu curiosidad te llevarán muy lejos. Gracias por caminar siempre a mi vera. Marti, he tenido 2 suertes inmensas contigo en la tesis, una compartir laboratorio contigo y dos, poder compartirlo hasta el final. Gracias por todo lo que me has enseñado, y lo que aún me enseñarás. No te puedo agradecer lo suficiente lo pendiente que estás de mí y lo mejor amiga que eres. Y por supuesto, eres la mejor compañera de karaoke del mundo.

A Rocío, se nota que eres conexina, porque has sido una más del lab desde el principio. Gracias por tu paciencia infinita con mis saltos y temblores con los ratones, y sobre todo por tu generosidad. A

Miguel, el nuevo rey del lab, tienes todos los ingredientes necesarios para quedarte y creo que todos lo estamos deseando, no se puede ser más majo. A todos mis compis del lab.15, los de mis inicios, Ángel…pollitos, Erasmus fantásticos con los que me he reído a morir, Miles, Joshua…gracias.

A mis Incylianos, sin duda, os podéis atribuir el hecho de que ahora me encante la sidra. Entramos al Incyl como compañeros y salimos como amigos. Gracias por todos esos maravillosos ratos, aunque muchos de vosotros ya no estéis aquí para repetirlos, Yan, Rodri…se os ha echado de menos en los últimos tiempos. Fer, en una carrera muy lejana, de nombre bioquímica, empezaste ya a ayudarme, cuando yo era una joven padawan. Sin duda no habría llegado hasta aquí si no me hubieras instruido a lo largo del camino. Nos unen muchas horas de célula viva, pero nos unen aún muchas más de amistad, gracias por todo amigo. Rafa, sin tu humor, el Incyl no sería el mismo, y ya eres uno más de los nuestros en el café. Ada, y Ad, mil gracias por compartir ratitos conmigo.

A la Dra. Conchi Lillo, gracias por darme esos maravillosos ratos de danza, de risas y de apoyo. Pero por supuesto también, de ayuda científica.

A todos y cada uno de los integrantes del Incyl, limpiadores, técnicos, equipo directivo, Beatriz…que ayudáis a que todo esté a punto y funcione todos los días, sois más importantes de lo que pensáis. Gracias.

A los Bioquímicos p., que lo que unen las prácticas, no lo separen los campus. Muchísimas gracias por vuestro compañerismo, y nuestros ratos juntos…¡hasta podría volver a ser voluntaria de otro congreso si fuera con vosotros! Violeta, portugueisa, muchas gracias por ser tan buena y no dudar ni un segundo en perder tu tiempo siempre que te he pedido algo. Qué de recuerdos bonitos, y qué de ellos espero que nos queden.

To my lab in Vancouver, it was incredible how well you wellcome me. To Christian Naus, I am so thankful for the opportunity you gave me in your laboratory. John, if I am able now to scruff a mouse is thanks to you. I learnt so much in those long hours in the CDM. Sin, thank you very much for guiding me and trust in me. I envy your energy, maybe it's your calcium levels… Thanks Lynne, you could not have been nicer and more caring with me. And to all the grad students, Conny, Jenni…it was very nice to share those few moments with you. Moe, thank you for helping me even when you couldn't be busier. If I told you that I keep laughing when I remember your son and Spiderman… Finally, Max, it was amazing to share lab with you. You became my friend between Western blot quantifications, confocal endless sessions and huge IPAS. Thanks for everything.

A todos mis amigos "extranjeros". Voy dejando pedacitos allá en cada rincón que piso. Aroa, mi blondita turca, cuanta complicidad en tan poco tiempo, te lo mereces todo. Lupe, mi estancia en Vancouver fue "very very good quality" gracias a ti, me dejé una auténtica amiga al otro lado del mundo. A mis amigos Erasmus: mi Carmencita, cualquiera diría que invitar a una desconocida a casa traería una amistad así, ¿verdad?, te debo mucho; María, mi malagueña guapa, gracias por lo generosa que eres siempre; a mi Nonna, sólo la Granja y Lisboa saben lo que nos queremos, espero que algún día demos la vuelta al mundo cual turistas; Paulillo, a pesar de mi supuesto ego, te reconoceré que eres mi médico favorito; Víc, qué músico más grande y qué grande amigo, qué de sonrisas me has sacado, gracias; Rivas, siempre acordándote de mí, y los que me dejo...gracias.

A Santi, si no fuera por ti, esta tesis no tendría ni números de página. Gracias por acompañarme en las horas de biblioteca que ha durado la escritura de esta tesis. Mil gracias.

A mi MDZ, porque a pesar de que la vida nos lleva por caminos distintos, estáis ahí y sé que siempre estaremos para lo que haga falta. Merys, Lau, gracias por vuestra energía y por transmitirme siempre esa vitalidad. Más aún, por escucharme y ayudarme. Pauli, si hay una constante en mi vida eres tú, mil gracias por estar siempre a mi lado.

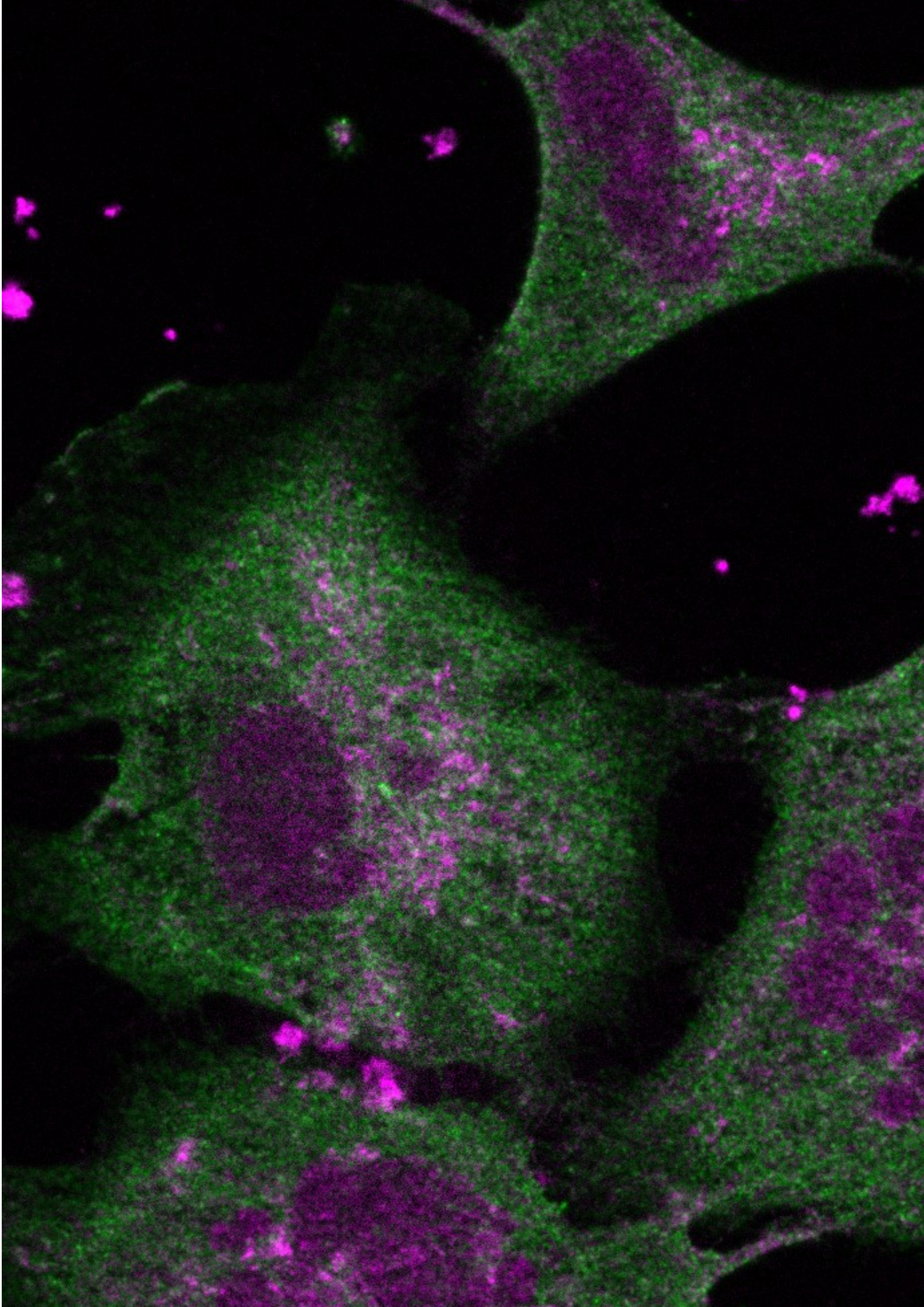
A los Jaraíz y a los Rodríguez. No os puedo nombrar uno a uno porque tendría que escribir otra tesis solo de agradecimientos. Gracias por pensar en mí y cuidar de mí como lo hacéis. Todos y cada uno de vosotros. Sois mis pilares. A mi cuñada, gracias infinitas por cuidar de mi hermano y quererlo. No me podrías hacer otro regalo mejor que ese. A mi sobrino, tienes nombre de conquistador, y a mí me conquistaste desde el primer segundo, espero que algún día le eches un vistazo a esta tesis. A mi hermano, gracias por tu apoyo incondicional e independiente de lo locas que puedan ser mis ideas. Gracias por estar siempre pendiente de tu hermanita. Te quiero.

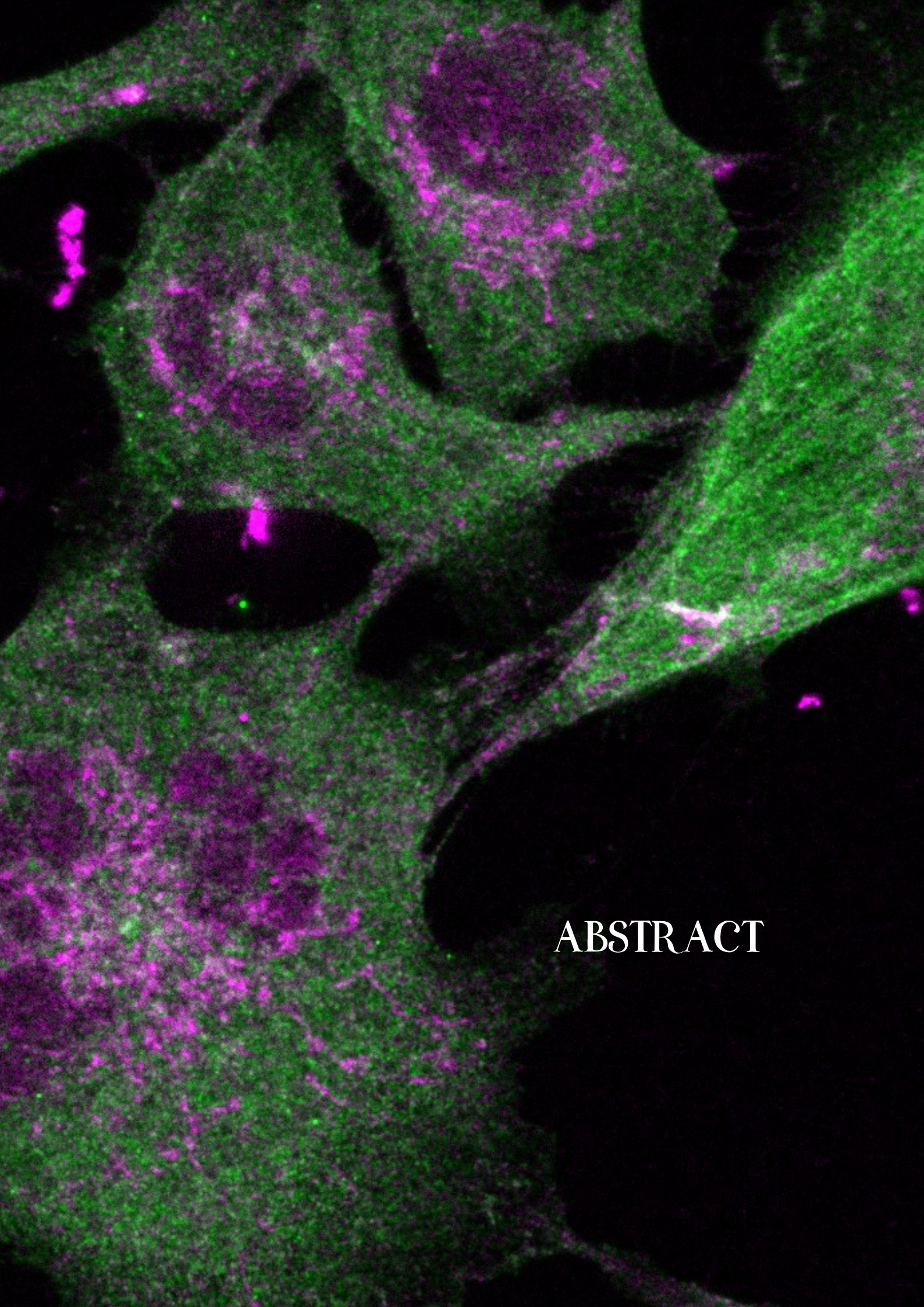
Por último, por supuestísimo que lo mejor para el final, para ti mamá. Gracias por ser madre, padre y todo a la vez, esta tesis no sería lo que es sino fuera por ti, porque yo soy lo que soy gracias a ti. Te adoro.

ABBREVIATIONS

BBB	Blood brain barrier
CNS	Central nervous system
Csk	C-terminal Src kinase
Cx43	Connexin43
Cx43CT	C-terminal domain of Cx43
FAK	Focal adhesion kinase
GSC	Glioma stem cells
IDR	Intrinsically disordered region
IDP	Intrinsically disordered protein
MAPK	Mitogen-activated protein kinases
NGVU	Neuro-glio-vascular Unit
PI3K	Phosphoinositide 3-kinase
PTEN	phosphatase and tensin homolog
SH2	Src homology 2
SH3	Src homology 3
Y	Tyrosine
Y397FAK	autophosphorylatable active form of FAK
Y576 FAK	c-Src-phosphorylatable tyrosine in the active loop of FAK
Y577 FAK	c-Src-phosphorylatable tyrosine in the active loop of FAK
Y416 Src	active form of c-Src
Y527 Src	inactive form of c-Src

INDEX





ABSTRACT

Anti-tumour effects of the c-Src inhibitor peptide, TAT-Cx43$_{266-283}$, in human glioma stem cells

Glioma stem cells (GSCs) constitute one of the challenges in treating glioblastoma, the most severe and frequent brain tumour. They have self-renewal capacity, multilineage differentiation properties, high oncogenic potential, resistance to conventional therapies and aggressive infiltration into the brain parenchyma. All these features make difficult the whole tumour resection and facilitate tumour recurrence. GSCs express very low levels of connexin43 (Cx43), the main gap-junction channel-forming protein in astrocytes. One important channel-independent function of Cx43 is to inhibit the oncogene c-Src, involved in cell proliferation, migration and survival. In fact, restoring Cx43 inhibits the strong activity of this oncogene in glioma cells, including GSCs. Interestingly, a cell-penetrating peptide based on the region of Cx43 that inhibits c-Src, TAT-Cx43$_{266-283}$, reverses GSC phenotype and reduces neurosphere formation. In this thesis, we analysed the anti-tumour effects of TAT-Cx43$_{266-283}$ in human G166 GSCs and more importantly, we set up a protocol for the culture of primary patient-derived GSCs that allowed us to broaden our study to these cells and to gain insight into the anti-tumour mechanism of TAT-Cx43$_{266-283}$.

First, we showed that TAT-Cx43$_{266-283}$ inhibits G166 and patient-derived GSCs proliferation. To corroborate the proposed mechanism for Cx43-c-Src interaction previously described in rat C6 glioma cells and astrocytes, we used a biotinylated TAT-Cx43$_{266-283}$ cell-penetrating peptide as a bait to identify its interacting partners. This confirmed that TAT-Cx43$_{266-283}$ serves as a docking platform that favours the proximity of active c-Src with its endogenous inhibitors, PTEN and Csk, in GSCs. In addition to c-Src inhibition, TAT-Cx43$_{266-283}$ upregulated PTEN contributing to the reduction of GSC proliferation by the downregulation of AKT activity in human GSCs. Since the inhibition of GSC proliferation by TAT-Cx43$_{266-283}$ is lost when PTEN is silenced, it could be proposed that this phosphatase is required for the anti-proliferative effect of TAT-Cx43$_{266-283}$ in GSCs.

Because TAT-Cx43$_{266-283}$ targets c-Src and PTEN, two regulators of the focal adhesion kinase (FAK), which in turn controls migration and invasion, we addressed the effects of TAT-Cx43$_{266-283}$ on FAK activity. FAK active levels decreased after 15 h (Y576 and Y577 FAK) and 24 h (Y397 FAK) of treatment with TAT-Cx43$_{266-283}$ in patient-derived GSCs. The inhibition of this c-Src downstream motility cascade was patent when individual G166 and patient-derived GSC trajectories were tracked because they showed a significant decrease in their lengths. Invasive properties through Matrigel-inserts were

also reduced in G166 and patient-derived GSCs when these cells were exposed to TAT-$Cx43_{266-283}$.Furthermore, we performed a time-lapse microscopy study in fresh tumour explants from the same surgical specimens used to obtain GSCs. Importantly, these movies showed a strong decrease in cell proliferation, migration and survival of those tumour explants treated with TAT-$Cx43_{266-283}$.

Finally, another region of Cx43 (amino acids 274-291) was used to confirm the specificity of TAT-$Cx43_{266-283}$. We showed that TAT-$Cx43_{274-291}$ did not affect significantly migration or proliferation in G166 and patient-derived GSCs, thus verifying TAT-$Cx43_{266-283}$ specificity.

This PhD thesis advances the understanding of the molecular mechanisms triggered by Cx43-Src interaction that includes the PTEN-FAK axis. As a consequence of this signalling pathway, this study shows the anti-tumour effects of TAT-$Cx43_{266-283}$ on the reduction of cell proliferation, migration and survival in two highly relevant glioma models, such as patient-derived GSCs and freshly removed surgical specimens of malignant gliomas.

Efectos antitumorales del péptido inhibidor de c-Src, TAT-Cx43$_{266-283}$, en células madre de glioma humano

Las células madre de glioma constituyen uno de los principales desafíos en el tratamiento del glioblastoma, el tumor cerebral más frecuente y severo. Estas células son capaces de autorrenovarse y de diferenciarse en distintos linajes, poseen un elevado potencial oncogénico, son resistentes a terapias convencionales y se infiltran de forma muy agresiva en el parénquima cerebral sano. Todas estas características dificultan en gran medida la resección completa del tumor y favorecen la recidiva tumoral. Las células madre de glioma expresan niveles muy bajos de conexina 43 (Cx43), la principal proteína formadora de canales de las uniones comunicantes en astrocitos. Una de las funciones de la Cx43, que es independiente de la actividad formadora de uniones comunicantes, es inhibir al oncogén c-Src, involucrado en la proliferación, la migración y la supervivencia celulares. De hecho, la restauración de la Cx43 inhibe la elevada actividad de este oncogén en células de glioma, células madre de glioma inclusive. De forma destacable, un péptido penetrante basado en la región de la Cx43 que inhibe c-Src, TAT-Cx43$_{266-283}$, revierte el fenotipo de células madre de glioma y disminuye la formación de neuroesferas. En esta tesis analizamos los efectos antitumorales de TAT-Cx43$_{266-283}$ en GSCs G166 y, aún más relevante, pusimos a punto un protocolo para la obtención y cultivo de células madre de glioma a partir de muestras de pacientes, lo que nos permitió ampliar el estudio en estas células, así como profundizar en el mecanismo antitumoral de TAT-Cx43$_{266-283}$.

En primer lugar, mostramos que TAT-Cx43$_{266-283}$ inhibe la proliferación de las GSCs, tanto de las G166 como de las derivadas de pacientes. Para corroborar el mecanismo de interacción entre la Cx43 y c-Src descrito en las células de glioma de rata C6 y astrocitos, usamos un péptido penetrante, TAT-Cx43$_{266-283}$ biotinilado, como anzuelo para identificar a las proteínas intracelulares con las que interacciona esta región. Estos experimentos confirmaron que TAT-Cx43$_{266-283}$ actúa como una plataforma estructural que favorece la proximidad de c-Src activo a sus inhibidores endógenos, PTEN y Csk, en las células madre de glioma. Además de inhibir a c-Src, TAT-Cx43$_{266-283}$ aumentó los niveles de expresión de PTEN, contribuyendo a la reducción de la proliferación de células madre de glioma a través de la disminución de la actividad de AKT. Debido a que la inhibición de la proliferación causada por TAT-Cx43$_{266-283}$ desaparece cuando PTEN se silencia, nuestros resultados sugieren que esta fosfatasa es necesaria para el efecto anti-proliferativo de TAT-Cx43$_{266-283}$ en células madre de glioma humano.

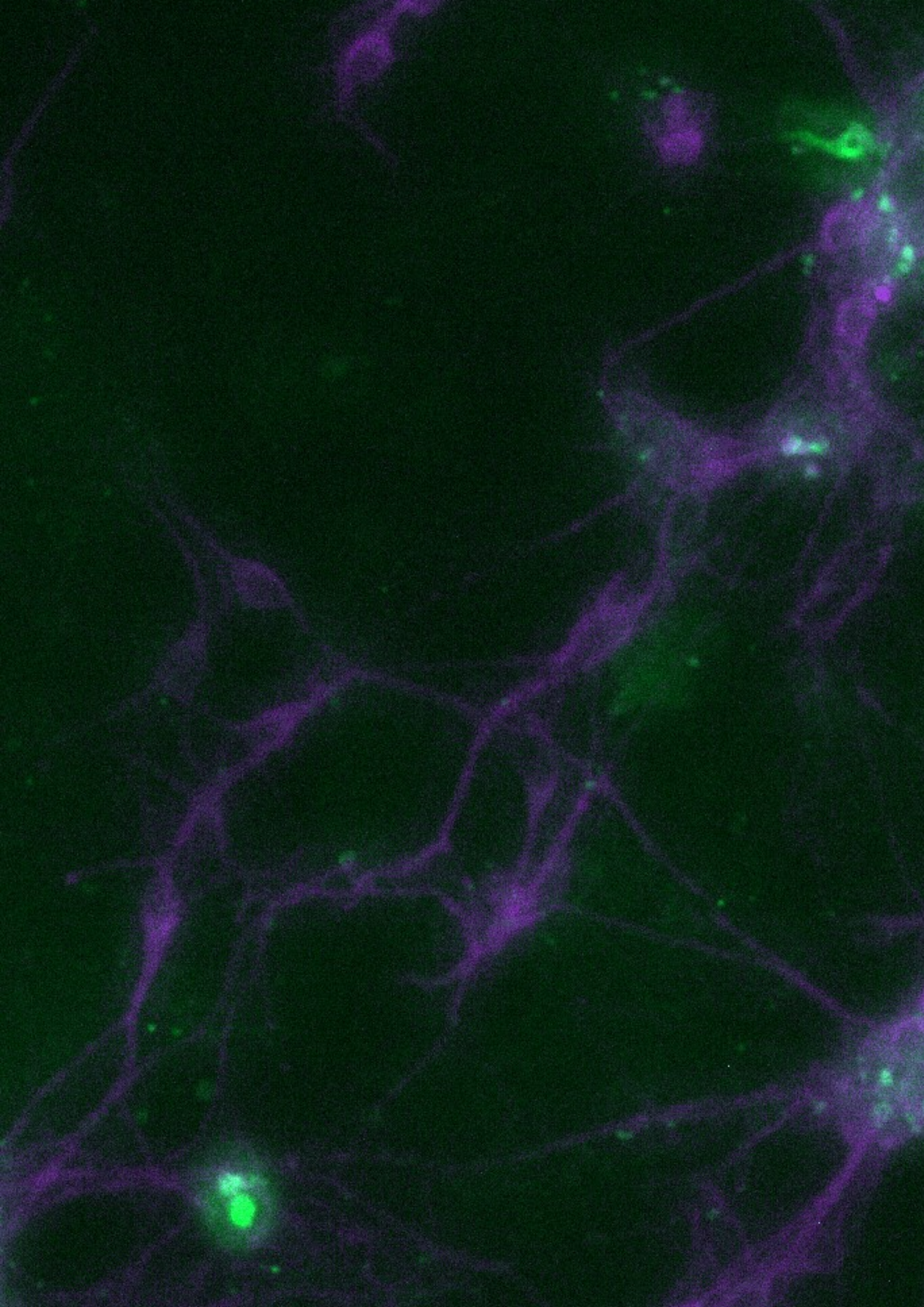
Las actividades de c-Src y PTEN son importantes para regular la actividad de la

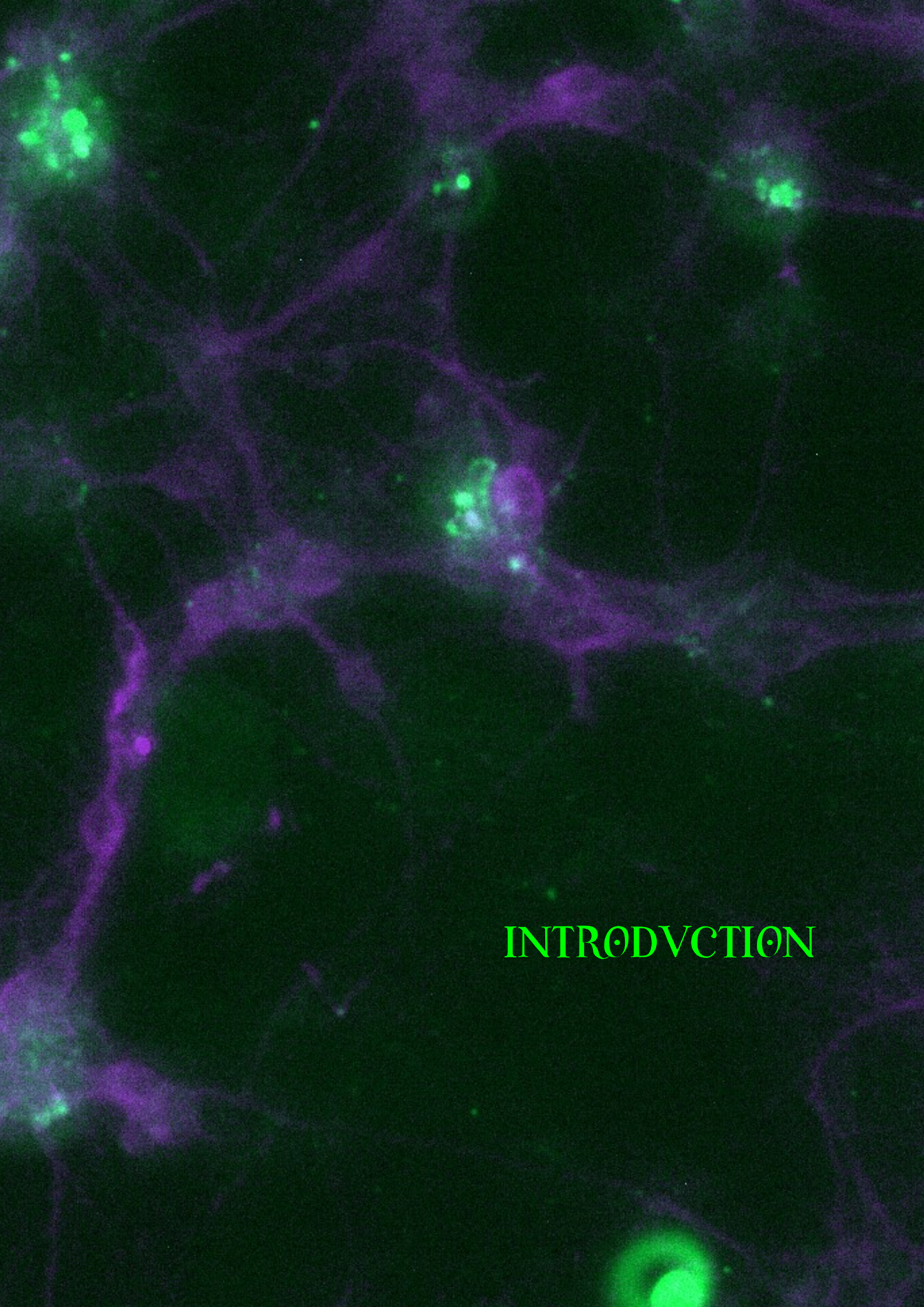
quinasa de adhesión focal (FAK), un importante mediador en la cascada de señalización que dirige la migración y la invasión celulares. Por ello, abordamos el estudio de la quinasa de adhesión focal (FAK), un importante mediador en la cascada de señalización que dirige la migración y la invasión celulares. Por ello, abordamos el estudio de los efectos de la inhibición de c-Src mediada por TAT-$Cx43_{266-283}$ en la actividad de FAK. Los niveles de FAK activo disminuyeron después de 15 h (Y576 y Y577 FAK) y 24 h (Y397 FAK) de tratamiento con TAT-$Cx43_{266-283}$, en las células madre de glioma derivadas de pacientes. La inhibición de esta cascada de señalización involucrada en la motilidad celular se hizo patente cuando se trazaron las trayectorias individuales de las células madre de glioma, que mostraron una disminución significativa en las distancias recorridas. También encontramos una disminución en la capacidad de invasión de las células madre de glioma a través de insertos preincubados con Matrigel, cuando fueron expuestas a TAT-$Cx43_{266-283}$.

A continuación llevamos a cabo un estudio de microscopía de célula viva en explantes de tumores obtenidos a partir de las mismas biopsias utilizadas para la obtención de células madre de glioma. De forma relevante, estas películas mostraron una acusada reducción en la proliferación, la migración y la supervivencia de las células que crecen y migran a partir de los explantes tumorales tratados con TAT-$Cx43_{266-283}$.

Por último, cabe destacar la especificidad de TAT-$Cx43_{266-283}$, que se confirmó mediante la comparación de esta región, comprendida entre los aminoácidos 266 y 283, con otra región de la Cx43, que abarca los aminoácidos 274-291 (TAT-$Cx43_{274-291}$). Efectivamente, TAT-$Cx43_{274-291}$ no mostró efectos significativos sobre la migración o la proliferación de las células madre de glioma G166 ni de las derivadas de pacientes.

Esta Tesis doctoral profundiza en el conocimiento de los mecanismos moleculares que se desencadenan tras la interacción Cx43-Src, que incluyen el eje PTEN-FAK. Como consecuencia de esta vía de señalización, este trabajo muestra los efectos anti-tumorales de TAT-$Cx43_{266-283}$ en la disminución de la proliferación, la migración y la supervivencia celulares, en dos modelos de glioma de gran relevancia por su proximidad a la clínica, como son las células madre de glioma derivadas de pacientes y los explantes de gliomas malignos obtenidos y tratados inmediatamente después de la cirugía.





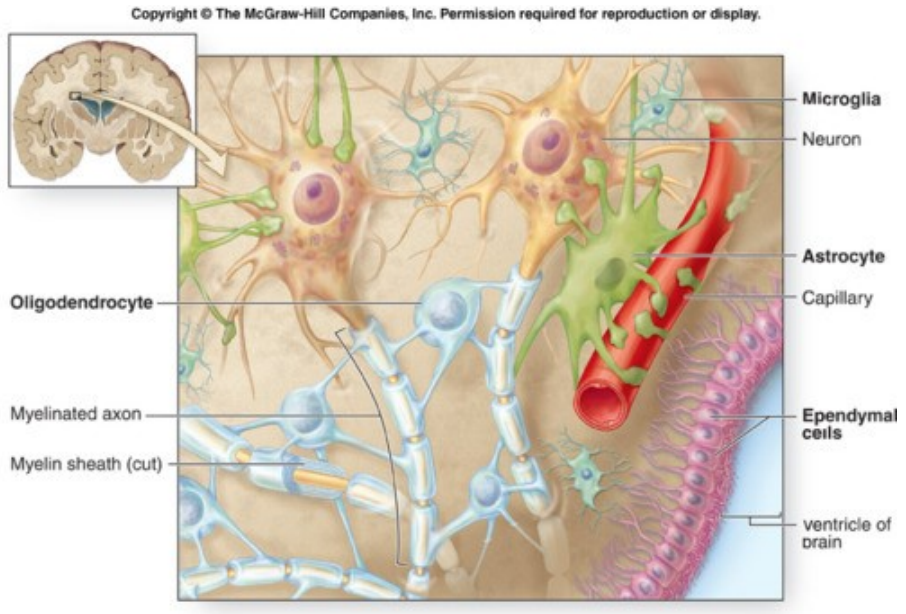
INTRODVCTION

1.Astrocytes

The central nervous system (CNS) is composed by different types of cells: neurons and glial cells. Glial cells can be classified in astrocytes, oligodendrocytes, microglia and ependymal cells (Scheme 1a). Traditionally, the neuron doctrine placed the nerve cell and neuronal synaptic contacts as the very center of the nervous system. However, in the last few decades, the participation in the control of naissance, development, functional activity and death of neuronal circuits have been attributed to astroglial cells (Verkhratsky and Butt, 2017).

Coordinated interaction among cells is critical to perform the extremely complex and dynamic tasks performed by the brain. Astrocytes, indeed, are centrally positioned within the 'neuro-glio-vascular' unit (NGVU), term coined in De Bock et al 2014 to join the importance of glial cells together with the neurovascular unit. Astrocytes are key players linking neurons to the cerebral vasculature, where they form extensive networks that physically and functionally connect neuronal synapses with cerebral vessels (Giaume et al., 2010). In addition, they participate in an active way to control synaptic communication forming a "tripartite synapse" (Araque et al., 1999); regulate metabolic transfer to neurons (Belanger et al., 2011) and cooperate with the rest of the NGVU to control blood-brain barrier (BBB) function (Abbott et al., 2006; Alvarez et al., 2013), local blood supply (Petzold and Murthy, 2011), neuronal development and surveillance/immune function (Hawkins and Davis, 2005).

To carry out some of these functions in a reliable and an efficient



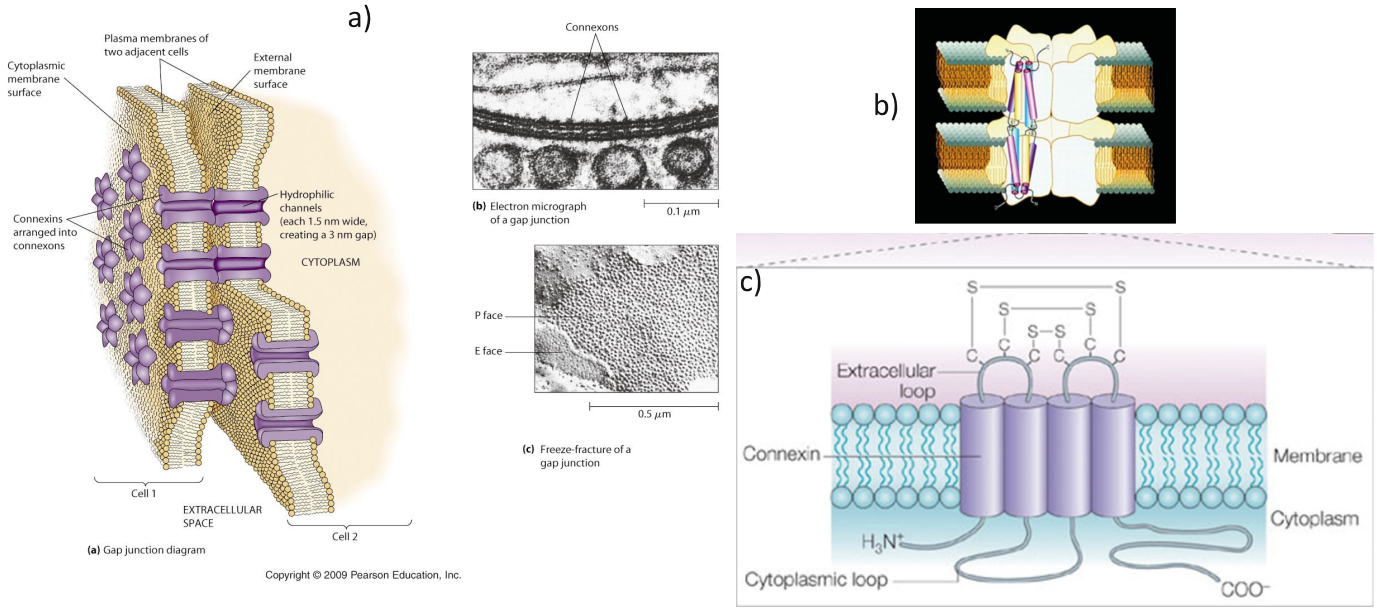
Scheme 1: Cells in the CNS. a) Neurons and glial cells.

manner astrocytes need a strong modality of intercellular communication: gap junctions or connexin-mediated pathways can certainly fulfil this function (Giaume et al., 2010).

2.Gap junctions & hemichannels: the protein connexin43

Gap junction channels facilitate the behaviour of astrocytes as cellular networks (Giaume et al., 2010). They are built of two hemichannels or connexons, one of which is provided by each of the adjacent cells (Scheme 2). Hemichannels are hexamers of connexin molecules that may also form functional channels per se, connecting cytoplasm and extracellular milieu (Giaume et al., 1991). Connexins are tetraspan membrane proteins with two extracellular loops and three intracellular regions represented by the N- and C-terminal domains and the loop linking transmembrane domains 2 and 3 (Scheme 3) (Kumar and Gilula, 1996).

Gap junctions allow the intercellular exchange of small metabolites, second messengers and electrical signals (White, 2003) excluding molecules that exceed 1 kDa in size (Bennett and Verselis, 1992; Alexander and Goldberg, 2003; Bukauskas and Verselis, 2004; Moreno, 2004). Important transjunctional molecules include glucose (Tabernero et al., 1996), cAMP, inositol triphosphate, adenosine, ADP and ATP, to name only a few (Goldberg et al., 2004). Not surprisingly, gap junction intercellular communication is required for processes such as synchronization of myocardial contractions (van der Velden and Jongsma, 2002), neuronal signal transmission via electrical synapses (Sohl et al., 2005), uphold functionality of the



Scheme 2. a)(A) Disposition of connexins within a gap junction. b) Model of the transmembrane domains of six connexin subunits in an oligomeric arrangement to form the hydrophobic spacing (pore) for the gap junction channel. Modified from (Kumar and Gilula, 1996). c) Connexins structure. Modified from (Sohl et al., 2005)

eye's lens (Gong et al., 2007) and modulation of the onset labor (Doring et al., 2006). The plasma membrane normally also contains hemichannels not incorporated into gap junctions. Hemichannels are normally closed but may be activated/open under certain stimuli mediating the release of messenger molecules like ATP and glutamate and thus contributing to paracrine signaling (Wang et al., 2013).

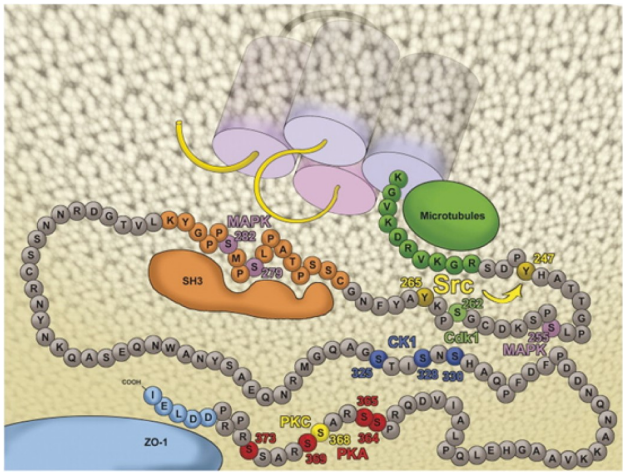
The connexin family consists of 21 isoforms (in humans) that may constitute gap junctions and hemichannels. Connexin43 (Cx43), encoded by GJA1 gene, is the most abundant connexin in mammals. It is widely expressed in different tissues, including the central nervous system where Cx43 is strongly expressed in astrocytes (Giaume et al., 1991).

Interestingly, beyond the multiple channel-dependent functions of Cx43, this protein posseses a large interactome mainly due to its C-terminal domain (Cx43CT).

2.1. Cx43CT

The Cx43CT plays a role in the trafficking, size, localization, and turnover of gap junctions, as well as the level of intercellular coupling via numerous posttranslational modifications and protein-protein interactions (Giepmans, 2004; Herve et al., 2007; Laird, 2010; Thevenin et al., 2013). NMR data indicate that the Cx43CT (amino acids 232 to 382) exists primarily as an elongated random coil, with two regions of alpha-helical structure (Sorgen et al., 2004).

All the structural information available confirms that Cx43CT is an intrinsically disordered region (IDR), with a small amount of alpha-helical content, and consequently, Cx43 is an intrinsically disordered protein (IDP) (Grosely et al., 2013). Importantly, the lack of structure provides a large interaction surface area and a high conformational flexibility allowing to scaffold and interact with numerous other proteins. Thus, the interactome of Cx43 is mainly localized within the CX43CT that contains several known protein-binding regions, such as Src homology (SH)3-, SH2-, microtubule- and postsynaptic density 95/disc-large/zona occludens (PDZ)-binding motifs (Giepmans, 2004; Herve et al., 2007; Laird, 2010) and several residues that can be phosphorylated by important cellular kinases (Solan and Lampe, 2009) (Scheme 3).



Scheme 3. C43CT interactions. Hervé et.

In fact, the Cx43CT contains 27 Serine/Tyrosine(Y) residues and all but 8 can be phosphorylated by several kinases, such as protein kinase A, protein kinase C, mitogen-activated protein kinases (MAPK), Src, cyclin-dependent kinase 1 and casein kinase1 (Tamura et al., 1999b; Huang et al., 2011; Zong et al., 2012).

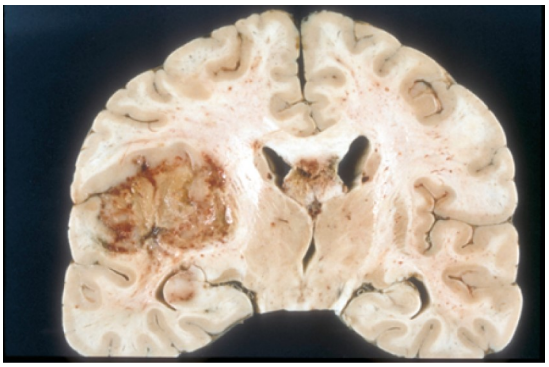
It is noteworthy that some of these protein-interaction motifs and phosphorylation sites overlap within the Cx43CT sequence (residues 266-283)(Tabernero et al., 2016). A disorder Cx43CT may thus offer an ideal substrate for the control of intercellular signalling by permitting a rapid switch between molecular partners (Dunker et al., 2001). In general, the location and cellular conditions, along with post-translational modifications, will dictate which protein associates with the Cx43CT domain. Upon comparing the primary sequences of connexins, Kopanic et al (Kopanic et al., 2014) have recently proposed that several residues within the CT (residues 272-286 in Cx43) constitute a master regulatory domain.

The importance of the Cx43CT as a master regulatory domain is patent when observing that it may modulate some tumorigenic pathways involved in cell proliferation and migration. To name one of our interest, this master regulatory domain contains the sequence required for c-Src binding to Cx43. Cx43 alterations may thus have critical consequences in its downstream pathways. Not surprisingly, certain pathologies show an imbalance in the expression, translation, trafficking or turnover of Cx43 levels. Indeed, Cx43 is downregulated in gliomas, the most common brain tumours (Shinoura et al., 1996; Huang et al., 1999; Soroceanu et al., 2001; Pu et al., 2004; Caltabiano et al., 2010; Sin et al., 2012; Gielen et al., 2013).

3.Glioblastomas

The term “glioma” comprises the majority of malignancies of the central nervous system and encompasses all tumours that are thought to be of glial cell origin. These include astrocytomas, oligodendrogliomas, ependymomas and mixed gliomas. Among them, astrocytomas are the most frequent in adults and have been traditionally classified by the World Health Organization (WHO) into four histological grades (Louis et al., 2007). Grade I (pilocytic astrocytoma) and grade II (diffuse astrocytoma) are low-grade gliomas that usually grow slowly. Grade III (anaplastic astrocytoma) is a highly malignant glioma with increased cellularity, pleomorphism and atypical nuclei. Grade IV, gliobastoma multiforme, consists of poorly differentiated cells with microvascular proliferation and pseudopalisading necrosis (Scheme 4). Glioblastomas are rapidly progressive, very aggressive, diffusely infiltrate the adjacent brain tissue and are one of the most incurable forms of cancer in

humans.



Scheme 4. Human glioblastoma.

Glioblastomas have been classified into 4 distinct subtypes according to their gene expression-based molecular profile: Proneural, Neural, Classical and Mesenchymal subtypes (Verhaak et al., 2010). Despite this heterogeneity, current treatment protocols fall in the "one size fits all" category (Stupp et al., 2005), thus failing to provide effective treatments. In fact, prognosis for patients with glioblastoma remains dismal, with a median survival of 16-19 months (Stupp et al., 2009). To this respect, concerns regarding the low translation rates of basic research findings have increased, together with the realization that cancer is a much more complex disease than previously thought. As a consequence, the concept of personalized treatment has gained popularity in recent years. Interestingly, short-term culture of human glioblastoma explants provides a flexible and rapid platform for screening context-dependent efficacy of therapies in patient-specific fashion. Indeed, this time- and cost-effective approach could be an advance in personalizing glioblastoma treatments (Bayin et al., 2016), reviewed in (Papapetrou, 2016).

In addition to interindividual differences, it is important to consider glioma cell biology, specifically, the high cell heterogeneity found in glioblastomas. P, and patient-derived models can help to approach the challenge of drug resistance that is mainly attributed to glioma stem cells (GSCs).

3.1. Origins and heterogeneity: glioma stem cells

Traditionally it was considered that the pool of precursor cells is fully depleted around birth, and neurogenesis is totally absent in the mature brain. However, the mature brain still has proliferative niches in the human CNS, namely the subventricular zone and the subgranular zone. These provide neural stem cells for neuronal and astroglia replacement, as it appears that neuronal and glial lineages are closely related.

The discovery of neural stem cells in mature brain originated a controversy about glioma origins (Sanai et al., 2005; Dirks, 2010). Recent reviews discuss that gliomas may arise from adult neural stem cells or multipotent neural progenitor cells (Stiles and Rowitch, 2008; Zong et al., 2012) and current evidence indicates that the subventricular zone may be non-contributory in adults (Sanai et al., 2011). Gliomas may also arise from more

differentiated lineages within the brain, including NG2 (neuron-glial antigen 2)-positive oligoden¬drocyte precursor cells (Liu et al., 2011; Sugiarto et al., 2011), astrocytes and even mature neurons (Friedmann-Morvinski et al., 2012; Cuddapah et al., 2014).

In accordance with the hypothesis that attributes the origins of these tumours to proliferative niches in the brain; human gliomas are organized as a cellular and functional hierarchy based on a subpopulation of glioma cells that have stem cell properties. GSCs have potent tumour-initiating ability, self-renewal capacity, multilineage differentiation properties and resistance to standard therapies (Dirks, 2010). GSCs not only generate neural cell types but they are also able to transdifferentiate to tumour endothelial cells (Ricci-Vitiani et al., 2010; Wang et al., 2010). This correlates with enhanced endothelial-GSC associations in peritumoral satellite tumour foci (Hu et al., 2016). In fact, in a similar way to neural stem cell-rich subventricular zone of normal brain tissue (Shen et al., 2004; Ramirez-Castillejo et al., 2006; Tavazoie et al., 2008); the perivascular niche in brain tumours serves as a GSC reservoir, where endothelial cells secrete factors that maintain the tumour-initiating pool (Calabrese et al., 2007; Gilbertson and Rich, 2007; Hambardzumyan et al., 2008; Charles and Holland, 2010; Lathia et al., 2010; Pietras et al., 2014; Lathia et al., 2015), reviewed in (Cuddapah et al., 2014).

Consequently, GSCs constitute an important target and one of the challenges in the treatment of glioblastoma.

3.2. Migration and invasionn

Despite their molecular and genetic differences, and possibly diver¬gent cells of origin, all malignant gliomas share one conserved feature: aggressive invasiveness (Cuddapah et al., 2014). The widespread migration of glioma cells into surrounding brain tissue limits the efficacy of surgical resection and targeted radiotherapy (Giese and Westphal, 1996). In addition, intrinsic or acquired resistance to conventional and targeted therapy further complicates glioblastoma treatment (Galavotti et al., 2013). This resistance has been attributed to GSC niches (Zhou et al., 2009) and altogether leads to 100% recurrence in the most severe grade of gliomas, glioblastomas (Davis et al., 1999; Stupp et al., 2005). (Scheme 5).

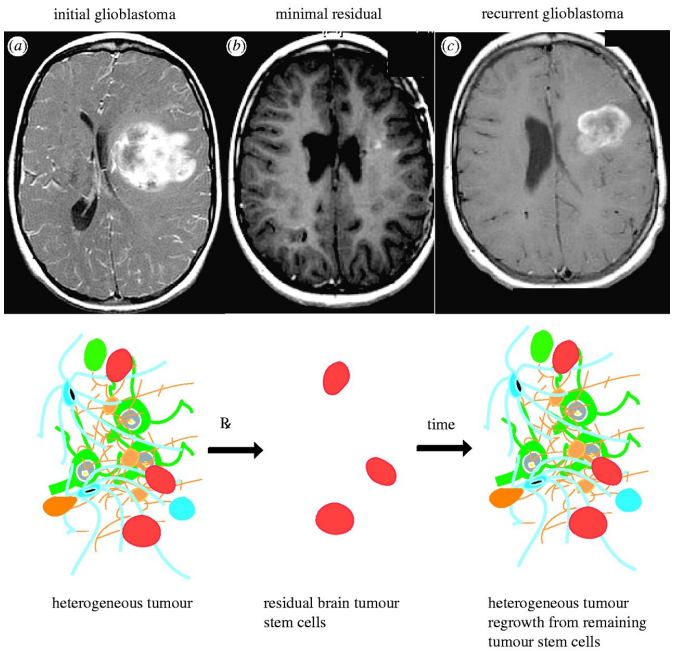
Gliomas are defined as “intraparenchymally metastatic” due to their non-destructive nature infiltration. In fact, glioma cells behave much more like non-malignant brain cells during embryonic development, or adult stem cells in the mature brain because they actively migrate without relying on intravascular or lymphatic metastasis, unlike other high-grade solid cancers

(Cuddapah et al., 2014).

Interestingly, patients belonging to the previously mentioned mesenchymal subtype have the worst prognosis and suppression of the mesenchymal signature severely impairs tumour invasion and progression in preclinical models (Carro et al., 2010). This suggests that mesenchymal genes may enhance the invasive capacity of glioblastoma cells (Galavotti et al., 2013). Indeed, many of patient-derived GSCs have been classified as mesenchymal (Pollard et al., 2009).

In addition, glioma cells recruit microglia, astrocytes and endothelial cells to favour their invasion while also regulating the activity of growth factors and chemokines that increase glioma proliferation and migration (Mentlein et al., 2012; Kwiatkowska and Symons, 2013). For example, astrocytes may provide tumor-stroma interactions that are unique to the brain microenvironment by establishing intercellular gap junctions. Therefore, they may contribute to glioma cell invasion (reviewed in (Sin et al., 2012, Quail and Joyce, 2017, Chen et al., 2016).

To migrate, a cell adheres the leading edge, anchors to the extracellular matrix, and last, detaches of the trailing end (Ridley et al., 2003). Cell attachment is mediated by cell–cell recognition molecules, cadherins, and cell–matrix receptors, mainly integ¬rins, but also neural cell adhesion molecules. In turn, cell detachment requires the activity of proteases that also degrade extracellular matrix components, such as metalloproteinases (Demuth and Berens, 2004; Kwiatkowska and Symons, 2013; Wolfenson et al., 2013). When the extracellular domains of integrins associate with particular extracellular matrix proteins (fibronectin, vitronectin,



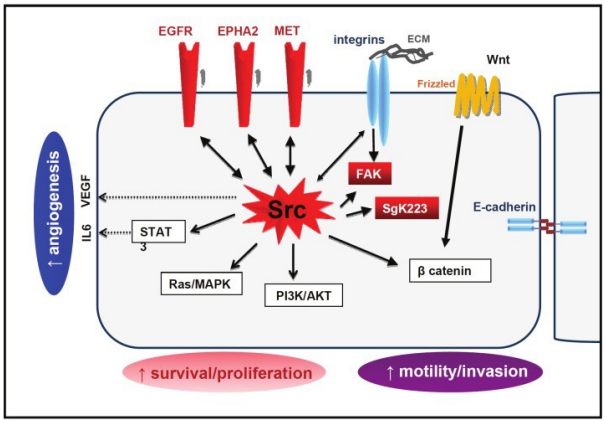
Scheme 5. Tumor recurrence. After tumour resection if GSCs remain in the brain, these cells may cause a relapse.

collagen, laminin), they get activated and recruit many proteins to its cytoplasmic domain to form a complex under the plasma membrane. This protein complex, called focal adhesion complex, links the cell actin cytoskeleton to the extracellular matrix and allows a transmembrane signal transmission from outside to inside the cell and vice versa (Barczyk et al., 2010). The focal adhesion kinase (FAK) interacts with multiple cellular proteins to translate integrin signalling into cell spreading, motility, and invasion. FAK is highly expressed in human tumours and its elevated activity correlates with increased cancer cell motility, invasiveness, and proliferation (Owens et al., 1995; Parsons, 2003).

3.3.The Src-PTEN-FAK axis

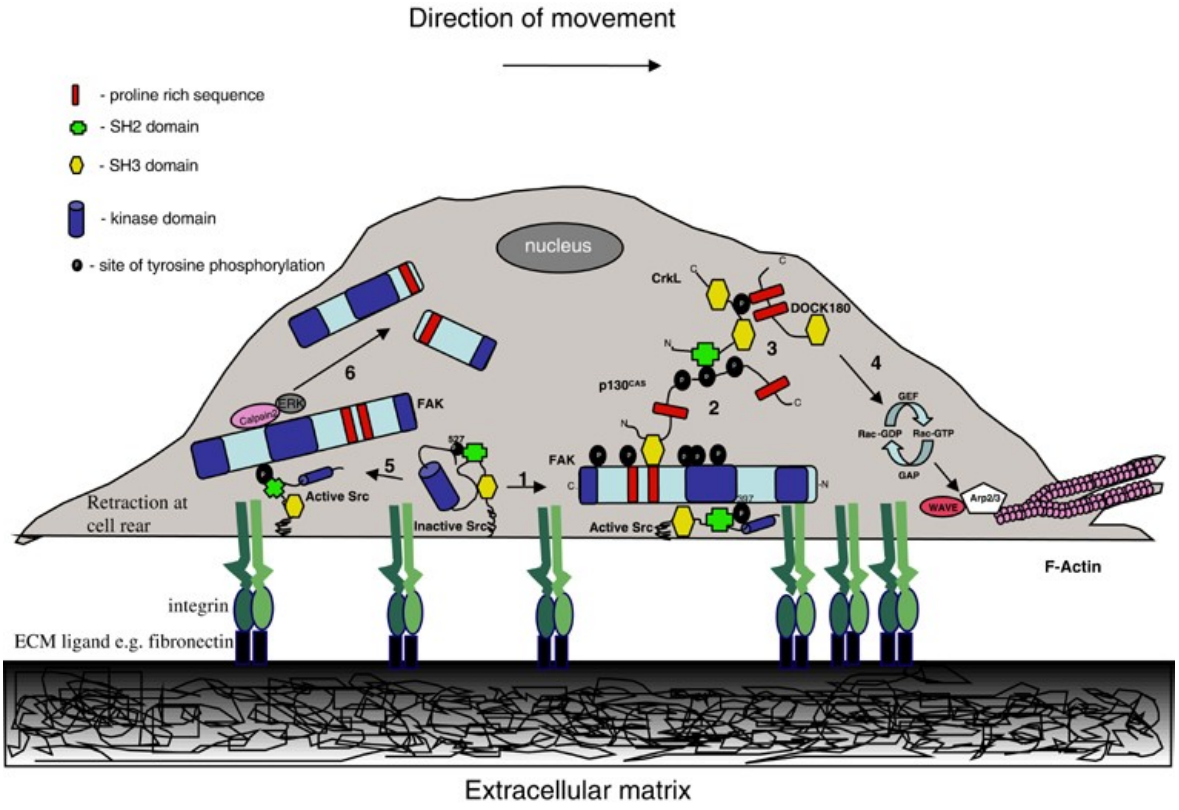
Interestingly, FAK activity is regulated by an axis constituted by the tumour suppressor phosphatase and tensin homolog (PTEN) and the previously mentioned oncogene, c-Src.

c-Src is a non-receptor tyrosine kinase that participates in proliferation, differentiation, survival, vasculogenesis and migration (Thomas and Brugge, 1997) (Scheme 6). Not surprisingly, its imbalance highly contributes to numerous oncogenic pathways (Irby and Yeatman, 2000; Courtneidge, 2002; Frame, 2002; Summy and Gallick, 2003) by modulating many critical proteins; including FAK, which colocalizes with p130CAS, integrin αvβ3, and paxillin to form focal adhesions, and AKT, which is a downstream effector of the PI3K pathway (Schaller et al., 1994; Vuori et al., 1996; Testa and Bellacosa, 2001; Kassenbrock et al., 2002; Westhoff et al., 2004). In addition, c-Src appears to activate the mitogen-activated protein kinase (p44/ 42 MAPK) in cooperation with Grb2/PI3K regulation via activation by platelet-derived growth factor receptor, ultimately instigating tumorigenic transcription and gene expression (Su and Karin, 1996; Conway et al., 1999).



Scheme 6. c-Src involvement in oncogenic pathways.

Likely, a major mechanism by which c-Src regulates motility is the activation of Rac and Cdc42 that work in concert at the leading edge of cells in the formation of lamellipodia and filopodia, respectively (Frame and Brunton, 2002; Pollard and Borisy, 2003). Integrin signaling via Src and FAK activates Rac and Cdc42; this leads to the activation of the WAVE and WASP family of proteins; they



Scheme 7. Architecture of a cell in the process of movement. From (Playford and Schaller, 2004)

activate Arp2/3 complex that serves to initiate new actin filament formation in a branched column, which pushes the leading edge forward (Higgs and Pollard, 2000; Eden et al., 2002; Innocenti et al., 2004)(Scheme 7).

FAK and c-Src form a transient, active complex following the activation of integrins by engagement with extracellular matrix proteins or ligand stimulation of the EFG or PDGF receptors. These cell surface molecules interact with the N-terminal portion of FAK resulting in the autophosphorylation of FAK at Tyr 397 (Ishizawar and Parsons, 2004). This creates a high-affinity docking site for a number of proteins containing SH2 domains, including c-Src and phosphatidylinositol 3-kinase (PI3K) (Schaller, 2001) (Scheme 9 and 10). In addition, a proline-rich sequence upstream of the Y397 FAK conforms to a high-affinity binding site for the SH3 domain of Src (Thomas et al., 1998). Thus, the interaction between FAK and Src occupies both the SH2 and SH3 domains resulting in stabilization of c-Src in its active conformation (Thomas et al., 1999). The binding of c-Src with FAK leads to c-Src phosphorylation of two tyrosine residues (Y576, Y577) in the activation loop. Y576 and Y577 FAK phosphorylation is required for maximum FAK catalytic activity (Calalb et al., 1995; Owen et al., 1999).

In turn, PTEN negatively regulates FAK activity. PTEN is a dual specificity protein and lipid phosphatase with ability to dephosphorylate both tyrosine and serine/threonine sites of acidic peptides

(Myers et al., 1997; Myers et al., 1998). Most of the tumour suppressor function of PTEN is attributed to its ability to control the PI3K/AKT pathway by dephosphorylating the second messenger, phosphatidylinositol 3,4,5-triphosphate (PIP3) (Lee et al., 1999; Vazquez and Sellers, 2000; Simpson and Parsons, 2001).

However, several studies point to its protein phosphatase activity in cell motility, invasion and migration (Maier et al., 1999; Tamura et al., 1999a; Tamura et al., 1999b; Gildea et al., 2004). Indeed, PTEN dephosphorylates tyrosine-phosphorylated FAK (Tamura et al., 1998). The major autophosphorylation site of FAK (Y397) is responsible for the initial in vivo association of PTEN with FAK, required for PTEN-mediated FAK dephosphorylation (Tamura et al., 1999c). Through the dephosphorylation of FAK, PTEN functions as a metastasis suppressor by negatively regulating cell interactions with the extracellular matrix.

4. Molecular alterations in glioblastoma: the connexin43-Src interaction

A wide range of molecular alterations has been described in astrocytomas and includes genetic, epigenetic, transcriptomic, and microRNA (miRNA) changes (Purow and Schiff, 2009; Riemenschneider et al., 2010). It is well described that the levels of Cx43 protein inversely correlate with the degree of malignancy in gliomas (Shinoura et al., 1996; Huang et al., 1999; Soroceanu et al., 2001; Pu et al., 2004; Caltabiano et al., 2010; Sin et al., 2012; Gielen et al., 2013). Simultaneously, gliomas and specifically, GSCs, present a high activity of the oncogene c-Src (Han et al., 2014), which interacts with the Cx43CT (reviewed in: Tabernero et al., 2016).

c-Src interacts with Cx43 by binding to the SH3-binding domain of Cx43, a proline-rich region (amino acids 274–284) and then phosphorylates tyrosine 265 providing an SH2-binding domain with the subsequent phosphorylation at tyrosine 247 (Kanemitsu et al., 1997). As a consequence of these phosphorylations, gap junctional intercellular communication is reduced (Swenson et al., 1990; Giepmans et al., 2001; Lin et al., 2001) and Cx43 turnover is initiated (Solan and Lampe, 2014).

Interestingly, NMR studies show that although binding of c-Src is confined to the SH3-binding domain of Cx43CT, conformational changes resulting from this binding extend for large distances along the Cx43CT (Sorgen et al., 2004) affecting the interaction with other molecular partners.

So far, the phosphorylation of Cx43 promoted by c-Src has been shown to modify the interaction of Cx43 with Zonula occludens-1 (ZO-1), tubulin, autophagy-related proteins (Bejarano et

al., 2014) and the T-cell protein tyrosine phosphatase (TC-PTP), also known as PTPN2 (protein tyrosine phosphatase N2) (Li et al., 2014), which reverts the phosphorylation of Cx43 promoted by Src.

Although the ability of c-Src to phosphorylate Cx43 and inhibit gap junctional intercellular communication is well known (Swenson et al., 1990; Giepmans et al., 2001; Lin et al., 2001), it has been recently shown that the interaction of Cx43 with c-Src can also inhibit the oncogenic activity of Src (Herrero-Gonzalez et al., 2010; Gangoso et al., 2014).

As previously described, Cx43 possesses a c-terminal domain that interacts with numerous proteins that participate in cellular motility; tubulin, debrin or regulate cellular migration via an interaction with calcium/calmodulin-dependent serine protein kinase (CASK; also known as LIN2) (Marquez-Rosado et al., 2012). Not surprisingly, the effects of Cx43 are rather controversial in terms of promoting or impeding glioma cell invasion. Even more intriguing is the differential regulation of invasion depending on the connexin type, which is expressed in tumor cells (reviewed in (Defamie et al., 2014). Indeed, the different outcomes seem to be dependent on models and conditions of experiments. Also, it is important to consider that following the progression of a glioblastoma, changes in Cx43 expression throughout the tumoral mass may provide a clue that Cx43 functions are variable in the same tumour along the different stages.

4.1.The c-Src inhibitor peptide, TAT-$Cx43_{266-283}$

The intracytoplasmic location of c-Src creates the necessity of internalizing Cx43 or the Cx43 region of interest to study the Cx43-Src interaction. However, most peptides and proteins are poorly internalized because they do not efficiently cross the lipid bilayer of the plasma membrane or of endocytic vesicles (Lebleu, 1996). To overcome this limitation, a cell-penetrating peptide that carries the Cx43 region of interest fused to the TAT peptide has been designed (Gangoso et al., 2014). , the TAT peptide is the sequence YGRKKRRQRRR, a domain responsible for the translocation of the human immunodeficiency virus type 1 (HIV) Tat trans-activator protein (Fawell et al 1994 PNAS). The basic nature of the TAT peptide favors the entrance of whatever cargo is attached to it and, as previous works show (Gangoso et al., 2014), TAT-$Cx43_{266-283}$ is efficiently internalized into glioma cells, including GSCs.

Using the TAT peptide fused to different combinations of the Cx43-Src interacting sequence, described by (Kanemitsu et al., 1997) to span from amino acid 245 to amino acid 283 in the Cx43CT, Tabernero and colleagues

(Gangoso et al., 2014; Gonzalez-Sanchez et al., 2016) deepened in the mechanisms of this interaction.

Indeed, the whole sequence inhibits c-Src in GSCs (Gangoso et al., 2014) and recruits c-Src endogenous inhibitors, PTEN and CSK in rat C6 glioma cells (Gonzalez-Sanchez et al., 2016). Intriguingly, the cell-penetrating peptide that lacks the tyrosines phosphorylable by c-Src, Y247 and Y265, TAT-$Cx43_{266-283}$, is also able to carry out these effects. By contrast, the SH3 region, TAT-$Cx43_{274-283}$, is not capable of recruiting PTEN and CSK and consequently it does not inhibit c-Src (Gonzalez-Sanchez et al., 2016).

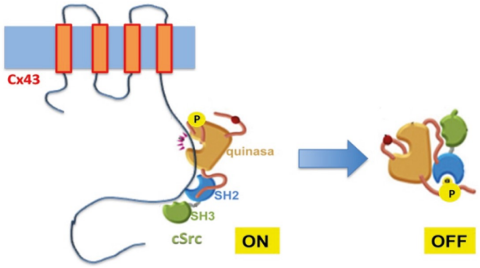
Curiously, while these Cx43-mimetic peptides have the goal of carrying out a Cx43 function that is lost due to their low levels of expression in gliomas, a similar strategy has been used to inhibit Cx43 activity, i.e., peptides Gap19, Gap26 and Gap27. The variety of effects triggered by these peptides have also shown to be effective in different brain pathologies (reviewed in: (De Bock et al., 2014); De bock et al 2014). Consequently, the use of cell-penetrating peptides as targeted molecular therapies might serve to efficiently mitigate CNS pathologies.

4.2. Effects of TAT-$Cx43_{266-283}$ in rat C6 glioma cells

In previous works, Cx43 has shown a crucial effect in the metabolism of astrocytes (Valle-Casuso et al., 2012) and in the reduction of the oncogenic activity of glioma cells through the interaction with c-Src (Herrero-Gonzalez et al., 2010; Yu et al., 2012; Gangoso et al., 2014). However, the mechanism by which the interaction of Cx43 with c-Src inhibits the oncogenic activity of Src has only been recently explored (Gonzalez-Sanchez et al., 2016)(Scheme 14).

c-Src possesses an amino-terminal domain, which may undergo myristoylation, an SH3 and SH2 domain, a tyrosine kinase domain, and a c-terminal negative regulatory element (Thomas and Brugge, 1997). The autophosphorylation at Y416 (active form of c-Src) mediates the strong c-Src activity in glioblastoma cells (Du et al., 2009), contributing to the malignant phenotype (Kmiecik and Shalloway, 1987). In turn, phosphorylation of Y527 (inactive form of c-Src), within the negative regulatory element, inhibits its activity. This inhibition occurs through an intramolecular interaction with the SH2 domain that promotes an intramolecular SH3 domain-mediated interaction (Roussel et al., 1991; Bibbins et al., 1993; Liu et al., 1993; Xu et al., 1997). This modification promotes an intramolecular interaction between the c-terminal of Src and its SH2 domain and an SH3 domain-mediated intramolecular interaction, leading to a closed conformation and inactivation of the protein (Thomas and Brugge, 1997; Xu et al., 1997) (Scheme 8).

Scheme 8. Cx43 inhibits c-Src.

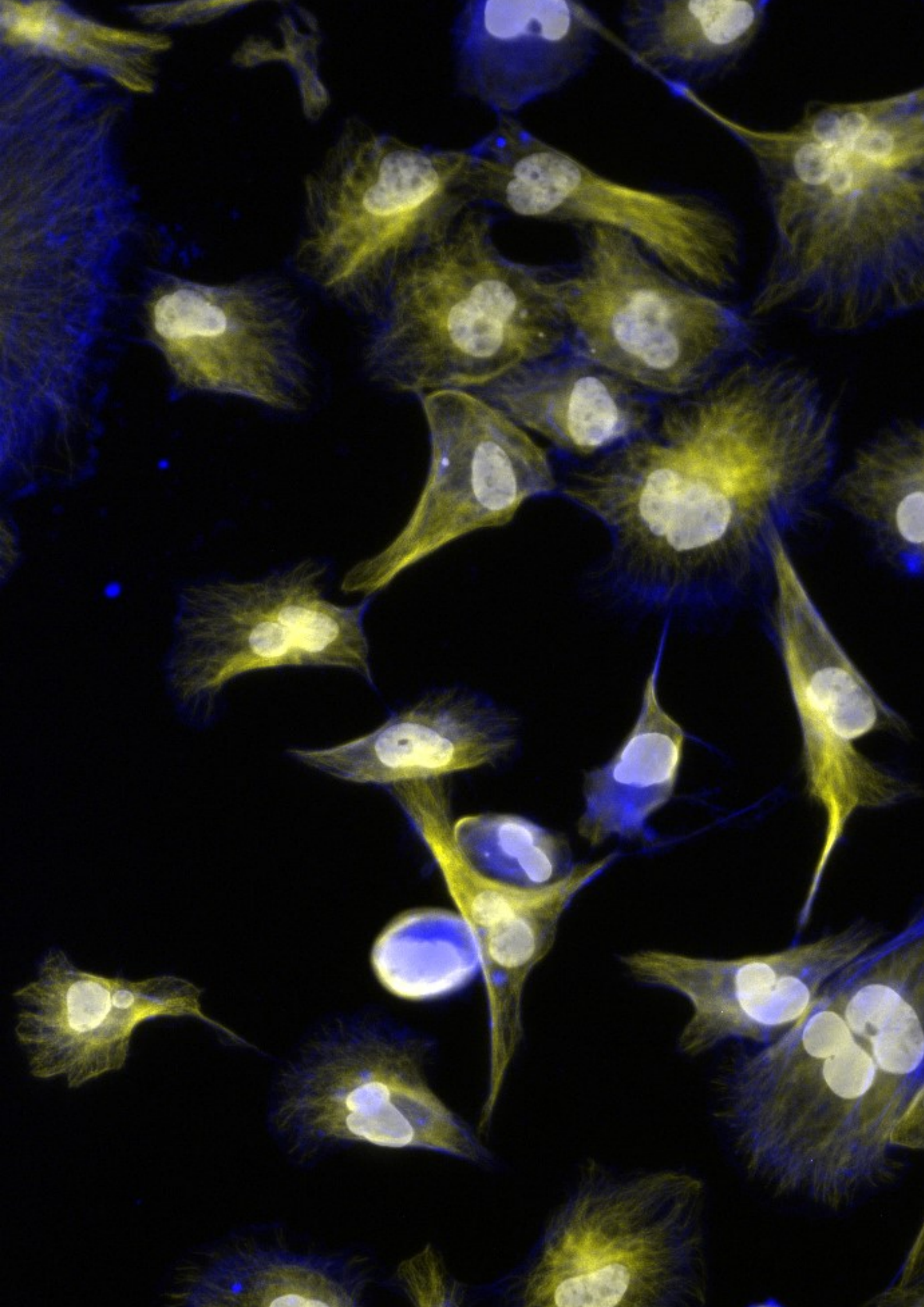


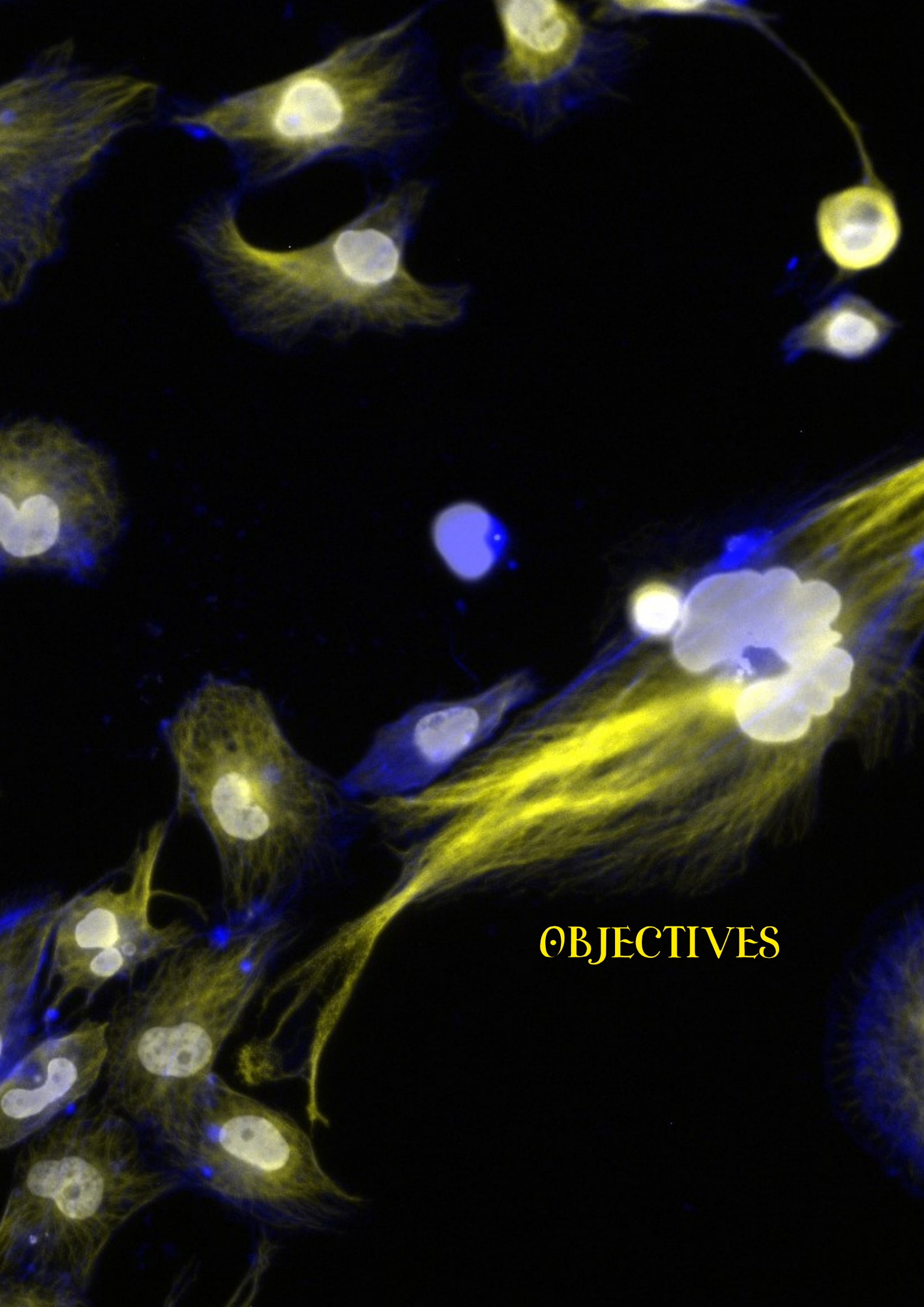
The inactivation of c-Src requires the activity of the C-terminal c-Src kinase (Csk), which phosphorylates Y527 Src (Okada et al., 1991). In addition, several phosphatases, such as PTEN, have been shown to dephosphorylate c-Src at Y416 (Zhang et al., 2011) to complete its inactivation. Cx43 reduces c-Src activity by decreasing Y416 Src and increasing the inactive form of c-Src (Y527 Src) when Cx43 is restored in rat C6 glioma cells (Herrero-Gonzalez et al., 2010). Gonzalez-Sanchez et al showed that Cx43 acts as a scaffolding protein that favours the proximity of c-Src with Csk, that phosphorylates Y527, and with PTEN, that dephosphorylates Y416 (Scheme 15). Mutant Cx43 lacks the ability to bind to c-Src and thus, it does not inhibit this oncoprotein (Herrero-Gonzalez et al., 2010). However, the region 245-283 is able to bind to c-Src and to inhibit it, via the cell-penetrating peptide TAT-$Cx43_{245-283}$ in rat C6 glioma cells. Even TAT-$Cx43_{266-283}$ that contains a shorter region and lacks the tyrosines phosphorylatable by c-Src, Y247 and Y265, is able to act as a docking platform to recruit c-Src with its endogenous inhibitors, Csk and PTEN. Interestingly, the region described to be responsible for Cx43-c-Src binding, the SH3 domain (Sorgen et al., 2004), is not capable of inhibiting c-Src via TAT-$Cx43_{274-283}$ (Gangoso et al., 2014), because this region is not sufficient to act as a docking platform for c-Src negative regulators Csk and PTEN (Gonzalez-Sanchez et al., 2016).

4.3. Effects of TAT-$Cx43_{266-283}$ in human GSC phenotype

Another important consequence of Cx43-Src interaction is the reversion of the GSC phenotype. As mentioned before, this subpopulation is considered a promising therapeutic target against gliomas. Interestingly, the expression of Cx43 in GSCs is very low and when it is restored, GSC phenotype (Yu et al., 2012; Gangoso et al., 2014) and tumorigenicity (Yu et al., 2012) is reduced. Indeed, Cx43 reduces Src activity in GSCs, which is an important self-renewal regulator (Singh et al., 2012), with the subsequent downregulation of Sox-2 (Yu, gangoso), a transcription factor responsible for GSC self-renewal (Gangemi et al., 2009). Sox2 expression is regulated by the inhibitor of differentiation Id1 (Soroceanu et al., 2013) that is also reduced upon restoring Cx43 (Gangoso et al., 2014). Importantly, the effect of Cx43 on GSCs is initiated by inhibitig Src activity promoted by the interaction of Cx43 with Src. In fact, TAT-$Cx43_{266-283}$ can mimic this effect (Gangoso et al., 2014). Intriguingly, Sorgen et al. (Sorgen et al., 2004) described that the interaction of the SH3 domain of Src

affects not just the SH3-binding domain (residues 274–283) but a larger region between amino acids 264–287. In fact, cell-penetrating peptides containing only the consensus SH3-binding site (residues 274–283 in Cx43) were unable to modify GSC phenotype. However, cell-penetrating peptides containing Cx43 residues affected by the binding of the Src SH3 domain (residues 266–283) mimicked the effect of Cx43 in GSCs. Thus, these cell-penetrating peptides reduce Src activity, downregulate Id1 and Sox-2 expression and promote cadherin switching. Consequently, the ability of GSCs to generate neurospheres is decreased and the percentage of cells expressing differentiation markers increased (Gangoso et al., 2014).





OBJECTIVES

As described in the introduction, a cell-penetrating peptide based on the region of the connexin43 (Cx43) that interacts with c-Src and that spans from amino acid 266 to amino acid 283, TAT-$Cx43_{266-283}$, inhibits the oncogenic activity of c-Src in rat C6 glioma cells and GliNS2 human GSCs. Consequently, the main goal of this study was to explore the antitumor properties of TAT-$Cx43_{266-283}$ and to unveil its mechanism in human GSCs, including primary patient-derived GSCs.

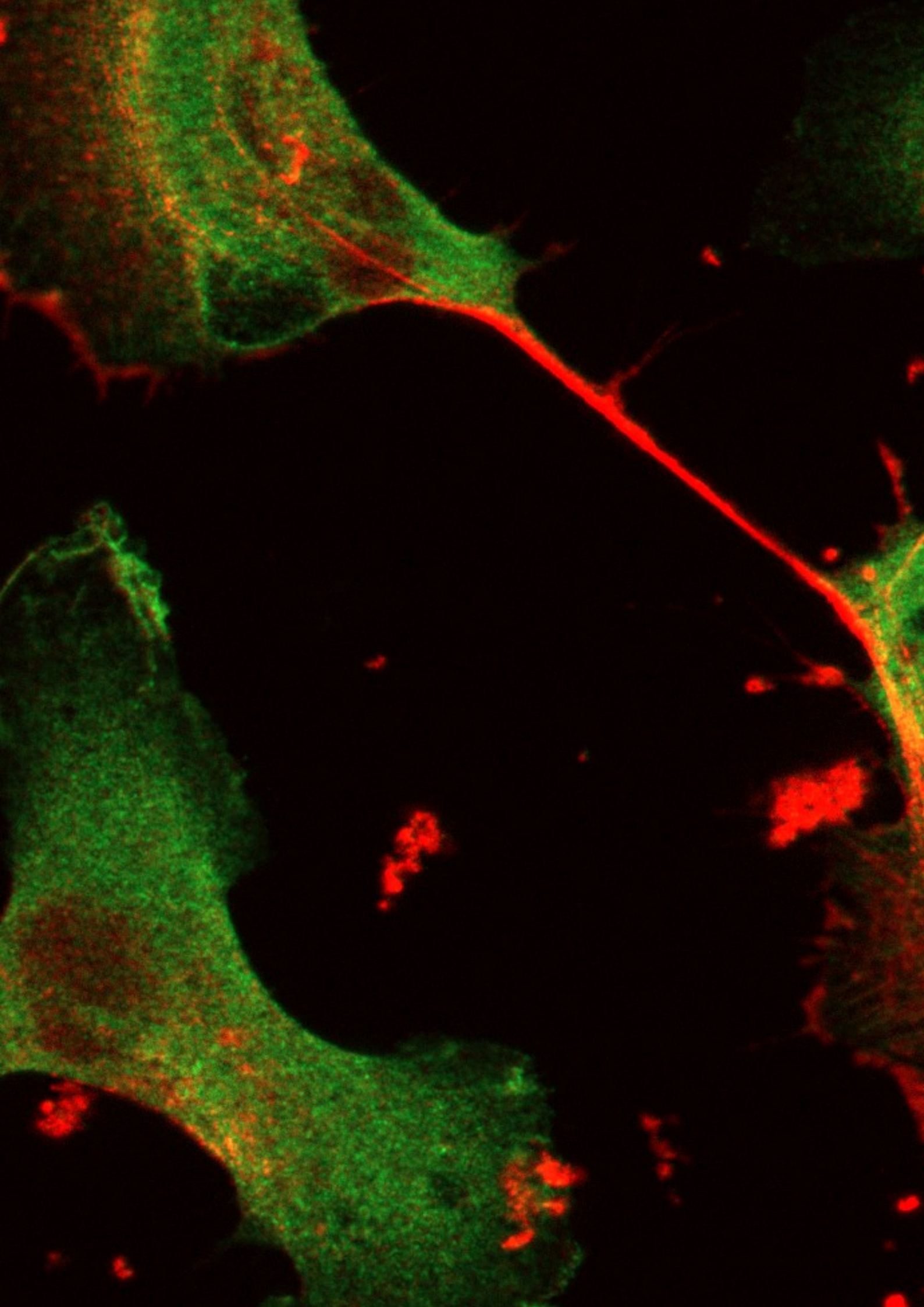
The specific objectives were:

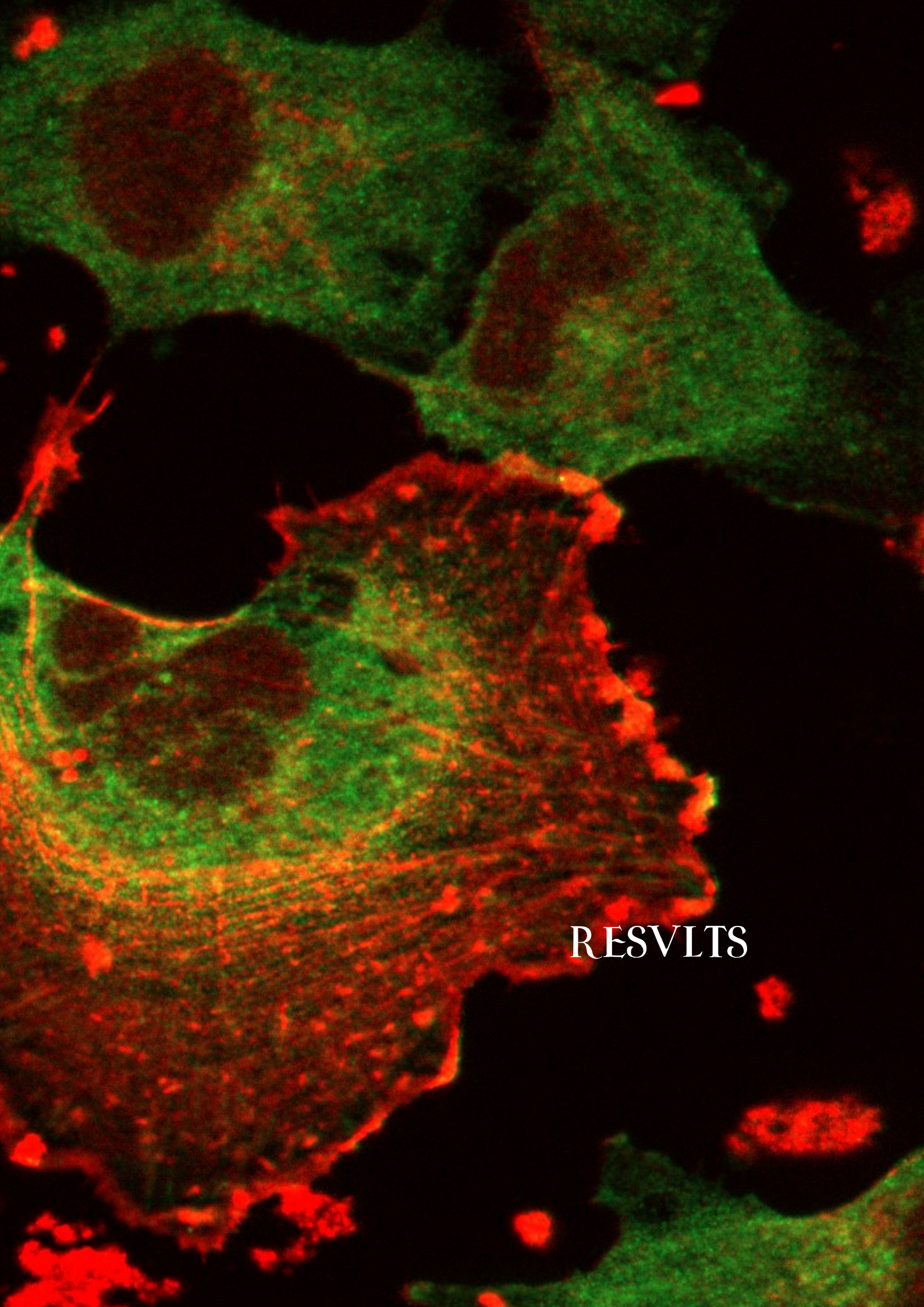
1. To study the effect and mechanism of TAT-$Cx43_{266-283}$ on human GSC proliferation.
2. To study the effect and mechanism of TAT-$Cx43_{266-283}$ on human GSCs migration and invasion.
3. To study the antitumor effects of TAT-$Cx43_{266-283}$ in patient-derived glioblastoma explants.

Como se ha descrito en la introducción, un péptido penetrante basado en la región de la conexina 43 (Cx43) que interacciona con c-Src y que abarca desde el aminoácido 266 al aminoácido 283, TAT-$Cx43_{266-283}$, inhibe la actividad oncogénica de c-Src en células de glioma de rata C6 y células madre de glioma humano GliNS2. Por consiguiente, el principal objetivo de esta tesis fue el estudio de las propiedades anti-tumorales de TAT-$Cx43_{266-283}$ y desvelar su mecanismo en células madre de glioma humano, incluyendo células madre de glioma humano derivadas de pacientes.

Los objetivos específicos fueron:

1. Estudiar el efecto, y el mecanismo de TAT-$Cx43_{266-283}$ en la proliferación de células madre de glioma humano.
2. Estudiar el efecto, y el mecanismo de TAT-$Cx43_{266-283}$ en la migración y la invasión de células madre de glioma humano.
3. Estudiar los efectos anti-tumorales de TAT-$Cx43_{266-283}$ en explantes de glioblastoma derivados de pacientes.





RESVLTS

Efecto y mecanismo de TAT-Cx43$_{266-283}$ en la proliferación de células madre de glioma humano

Connexin43 recruits PTEN and Csk to inhibit c-Src activity in glioma cells and astrocytes. González-Sánchez A, Jaraíz-Rodríguez M, Domínguez-Prieto M, Herrero-González S, Medina JM, Tabernero A. *Oncotarget* 2016; 7:49819-49833.

En este trabajo, establecimos como objetivos: estudiar el efecto de TAT-Cx43$_{266-283}$ en la proliferación de las células madre de glioma humanas G166, y el mecanismo a través del cual se produce este efecto. Para ello, analizamos la proliferación mediante un ensayo de viabilidad por MTT y además, realizamos un estudio por inmunocitoquímica del marcador de proliferación celular, Ki-67. A continuación, comprobamos por Western blot que la actividad de c-Src es menor cuando se tratan estas células con TAT-Cx43$_{266-283}$. Por último, examinamos los niveles de expresión de PTEN y la actividad de AKT, posibles responsables del efecto antiproliferativo de TAT-Cx43$_{266-283}$. Este estudio mostró que debido a la inhibición de c-Src causada por TAT-Cx43$_{266-283}$, se produce una disminución en la degradación de PTEN, causada por esta oncoproteína y, por lo tanto, los niveles de PTEN aumentan, inhibiendo la actividad de AKT y, por consiguiente, la proliferación de estas células. Por último, silenciamos la expresión de PTEN en las células madre de glioma G166 y analizamos la proliferación tras incubarlas con TAT-Cx43$_{266-283}$. En estas condiciones, TAT-Cx43$_{266-283}$ no disminuye la proliferación de las células madre de glioma de forma significativa con respecto a la condición control tratada con TAT. Estos resultados sugieren que PTEN es necesario para que se produzca la cascada de eventos originada por TAT-Cx43$_{266-283}$, que al inhibicr c-Src reduce la proliferación celular.

Por lo tanto, TAT-Cx43$_{266-283}$ reduce la proliferación de células madre de glioma humano G166 como consecuencia de la inhibición de c-Src, que a su vez, produce un aumento en los niveles de PTEN. Esto ocasiona una disminución de la actividad de AKT, un importante efector de la vía PI3K, involucrada en la proliferación y la supervivencia celulares, y que es modulada por PTEN.

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Research Paper

Connexin43 recruits PTEN and Csk to inhibit c-Src activity in glioma cells and astrocytes

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ABSTRACT

Connexin43 (Cx43), the major protein forming gap junctions in astrocytes, is reduced in high-grade gliomas, where its ectopic expression exerts important effects, including the inhibition of the proto-oncogene tyrosine-protein kinase Src (c-Src). In this work we aimed to investigate the mechanism responsible for this effect. The inhibition of c-Src requires phosphorylation at tyrosine 527 mediated by C-terminal Src kinase (Csk) and dephosphorylation at tyrosine 416 mediated by phosphatases, such as phosphatase and tensin homolog (PTEN). Our results showed that the antiproliferative effect of Cx43 is reduced when Csk and PTEN are silenced in glioma cells, suggesting the involvement of both enzymes. Confocal microscopy and immunoprecipitation assays confirmed that Cx43, in addition to c-Src, binds to PTEN and Csk in glioma cells transfected with Cx43 and in astrocytes. Pull-down assays showed that region 266–283 in Cx43 is sufficient to recruit c-Src, PTEN and Csk and to inhibit the oncogenic activity of c-Src. As a result of c-Src inhibition, PTEN was increased with subsequent inactivation of Akt and reduction of proliferation of human glioblastoma stem cells. We conclude that the recruitment of Csk and PTEN to the region between residues 266 and 283 within the C-terminus of Cx43 leads to c-Src inhibition.

INTRODUCTION

Connexin43 (Cx43) is an integral membrane protein that assembles to form gap junction channels and hemichannels in different cell types, including astrocytes, where Cx43 is strongly expressed [1, 2]. However, the levels of Cx43 protein are decreased when these cells acquire a malignant phenotype. In fact, the levels of Cx43 protein are inversely correlated with the degree of malignancy in astrocytomas, being negligible in the majority of glioblastomas, the most common glioma that unfortunately carries the worst prognosis [3–9]. Even in glioma-initiating cells or glioblastoma stem cells (GSCs)-a subpopulation of cells within malignant gliomas that are characterized by their self-renewal capacity, multilineage differentiation properties, high oncogenic potential, and resistance to standard therapies [10]- the levels of Cx43 are negligible [11, 12].

The proto-oncogene tyrosine-protein kinase Src (c-Src) participates in signaling pathways that control a diverse spectrum of biological events, including proliferation, differentiation, survival and migration [13]. c-Src binds to Cx43 through the Src homology 3 (SH3) domain binding motif of Cx43, a proline-rich region (amino acids 274–283), and then phosphorylates tyrosine 265, providing an SH2 domain binding site with subsequent phosphorylation at tyrosine 247 [14]. As a consequence of these phosphorylations, gap junctional intercellular communication is reduced [15–17], and Cx43 turnover is initiated [18]. More recently, it has been found that in addition to these effects, the interaction of Cx43 with c-Src can reciprocally inhibit c-Src activity [11, 19]. Glioblastoma cells exhibit strong c-Src activity [20], which plays an important role in the transforming phenotype of astrocytomas [21]. Autophosphorylation at tyrosine 416 activates c-Src, contributing to the malignant

phenotype [22]. Interestingly, restoring Cx43 to glioma cells reduces c-Src activity by decreasing the active form of c-Src (c-Src phosphorylated at tyrosine 416; Y416 c-Src) and increasing the inactive form of c-Src (c-Src phosphorylated at tyrosine 527; Y527 c-Src) [19]. The inhibition of c-Src is not promoted by mutant Cx43, which lacks the ability to bind to c-Src [19]. Rather, it is promoted by cell-penetrating peptides containing the region of Cx43 involved in c-Src interaction [11]. Collectively, these data indicate that the interaction of Cx43 with c-Src reduces c-Src activity; therefore, these proteins are mutually regulated by a phosphorylation/dephosphorylation loop. c-Src activity is linked to crucial signaling pathways [23]. Consequently, it is not surprising that the inhibition of c-Src promoted by Cx43 reduces the cell cycle [11, 19], glucose uptake [24, 25] or glioma stem cell phenotype [11, 19] (for a review, see [26]).

Despite the relevance of c-Src inhibition for cell biology, the mechanism by which the interaction of Cx43 with c-Src reduces its activity is unknown. The inhibition of c-Src requires the activity of the C-terminal c-Src kinase (Csk), which phosphorylates c-Src at tyrosine 527 [27]. In addition, several phosphatases, such as phosphatase and tensin homolog (PTEN), have been shown to dephosphorylate c-Src at tyrosine 416 [28], an activity that is required to complete the inactivation of c-Src. In this study, we found that a small region located in the C-terminal domain of Cx43 serves as a docking platform for c-Src, PTEN and Csk, favoring the inhibition of the oncogenic activity of c-Src.

RESULTS

Csk and PTEN are involved in the inhibition of glioma cell growth promoted by Cx43

In previous studies, we showed that restoring Cx43 to glioma cells inhibits c-Src activity and consequently their rate of proliferation [11, 19]. Because Csk, through phosphorylation of c-Src at tyrosine 527, is the main enzyme responsible for c-Src inhibition [27], in this study, we addressed the participation of Csk in the antiproliferative effect of Cx43. To do so, the expression of Csk was knocked-down by a specific siRNA (Csk-siRNA) [29] in C6 glioma cells stably transfected with Cx43 (C6-Cx43) or the empty vector (C6-Ires). Figure 1A shows that Csk-siRNA strongly reduced the expression of Csk in both C6-Ires and C6-Cx43 cells at concentrations ranging from 25 to 75 nM. Next, the growth of C6-Ires and C6-Cx43 cells, transfected with 50 nM non-targeting siRNA (NT-siRNA), or Csk-siRNA, was followed using the MTT assay. Figure 1B shows that, as expected, restoring Cx43 reduced the rate of glioma cell growth in the control situation (cells transfected with NT-siRNA). However, when Csk was silenced the effect of Cx43 on glioma cell proliferation was reduced, suggesting the contribution of Csk to the antiproliferative effect of Cx43. It should be mentioned that the growth rate increased when Csk was silenced in both C6-Ires ($p < 0.01$ at days 3 and 4, and $p < 0.001$ for the other days) and C6-Cx43 ($p < 0.05$ at day 3 and $p < 0.001$, for the other days) cells compared with cells transfected with NT-siRNA.

In addition to the phosphorylation at tyrosine 527 by Csk, to be completely inactivated, c-Src needs to be dephosphorylated at tyrosine 416. Several phosphatases, including PTEN, have been shown to dephosphorylate c-Src at tyrosine 416 [28]. To investigate the participation of PTEN in the inhibition of c-Src promoted by Cx43 [19], the levels of Y416 c-Src were analyzed in glioma cells in which PTEN was knocked-down by siRNA [30] (Figure 1C–1E). As expected, Cx43 decreased the ratio of Y416 c-Src / total c-Src in glioma cells transfected with NT-siRNA (Figure 1E). By contrast, when PTEN was silenced by a specific siRNA (PTEN-siRNA), Cx43 could not reduce this ratio (Figure 1E), suggesting that PTEN is involved in the dephosphorylation of c-Src at tyrosine 416. Next, we investigated the participation of PTEN in the antiproliferative effect of Cx43. Our results showed that silencing PTEN reduced the antiproliferative effect of Cx43 on glioma cells compared with glioma cells transfected with NT-siRNA (Figure 1B). The growth rate increased when PTEN was silenced in both C6-Ires ($p < 0.01$ at days 3 and 7, and $p < 0.001$ for the other days) and C6-Cx43 ($p < 0.01$ at days 3 and 7, and $p < 0.001$ for the other days) cells compared with cells transfected with NT-siRNA.

Restoring Cx43 expression in glioma cells increases PTEN by a c-Src-dependent mechanism

Unexpectedly, we observed that the presence of Cx43 increased PTEN protein levels in glioma cells (Figure 1C). Because PTEN is one of the most relevant tumor suppressor proteins in gliomas [31], we decided to explore this effect. To this end, the levels of PTEN were analyzed in C6-Ires and C6-Cx43 cells. Our results showed that restoring Cx43 expression doubled the levels of PTEN protein in glioma cells (Figure 2A and 2C).

To confirm the functionality of PTEN, we analyzed its downstream pathway. PTEN exhibits both lipid and protein phosphatase activities. The main substrate for the lipid phosphatase activity is phosphatidylinositol-trisphosphate (PIP3), which is dephosphorylated by PTEN to generate PIP2. PIP3 is the main activator of Akt [32]. Thus, the higher the activity of PTEN, the lower the activity of Akt. In agreement with this concept, restoring Cx43 expression reduced Akt activity as shown by the concomitant reduction in the levels of Akt phosphorylated at serine 473 and threonine 308 (Figure 2A, 2G and 2H) and the increase in PTEN levels (Figure 2A and 2C).

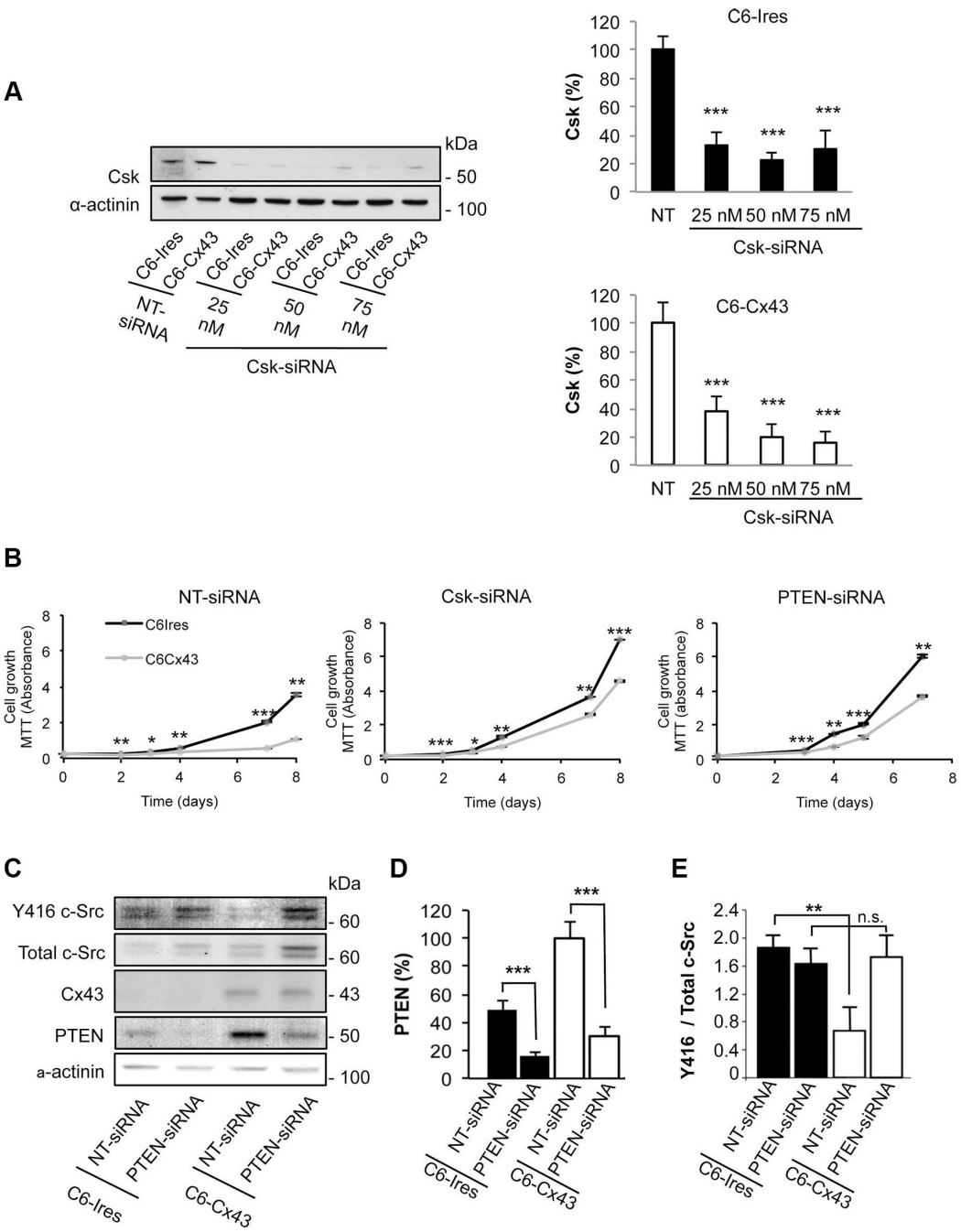

Figure 1: Effect of silencing Csk and PTEN on the reduction of proliferation promoted by Cx43. C6 cells stably transfected with the empty vector (C6-Ires) or the vector containing the Cx43 cDNA (C6-Cx43) were transfected with Csk-siRNA, PTEN-siRNA or non-targeting siRNA (NT-siRNA). (**A**) The levels of Csk were analyzed by Western blotting 72 h after transfection with increasing concentrations of Csk-siRNA or 50 nM NT-siRNA. The results are the means ± s.e.m. ($n = 3$) and they are expressed as the percentage of the corresponding NT-siRNA. ***$p < 0.001$ versus the corresponding *NT-siRNA*. (**B**) The cells transfected with the indicated siRNAs were plated at 2000 cells/cm^2, and the number of living cells was followed by the MTT assay. The results are the means ± s.e.m. ($n = 4$) and they are expressed as the absorbance values of the MTT assay. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$; C6-Ires versus C6-Cx43. (**C**) Cells were transfected with 50 nM NT-siRNA or PTEN-siRNA. After 48 h, PTEN, Cx43, total c-Src and Y416 c-Src levels were analyzed by Western blotting. The results are the means ± s.e.m. ($n = 6$) and they are expressed as the percentage of the C6-Cx43 NT-siRNA (**D**) or as the ratio of Y416 c-Src/total c-Src (**E**). n.s: not significant. **$p < 0.01$ and ***$p < 0.001$.

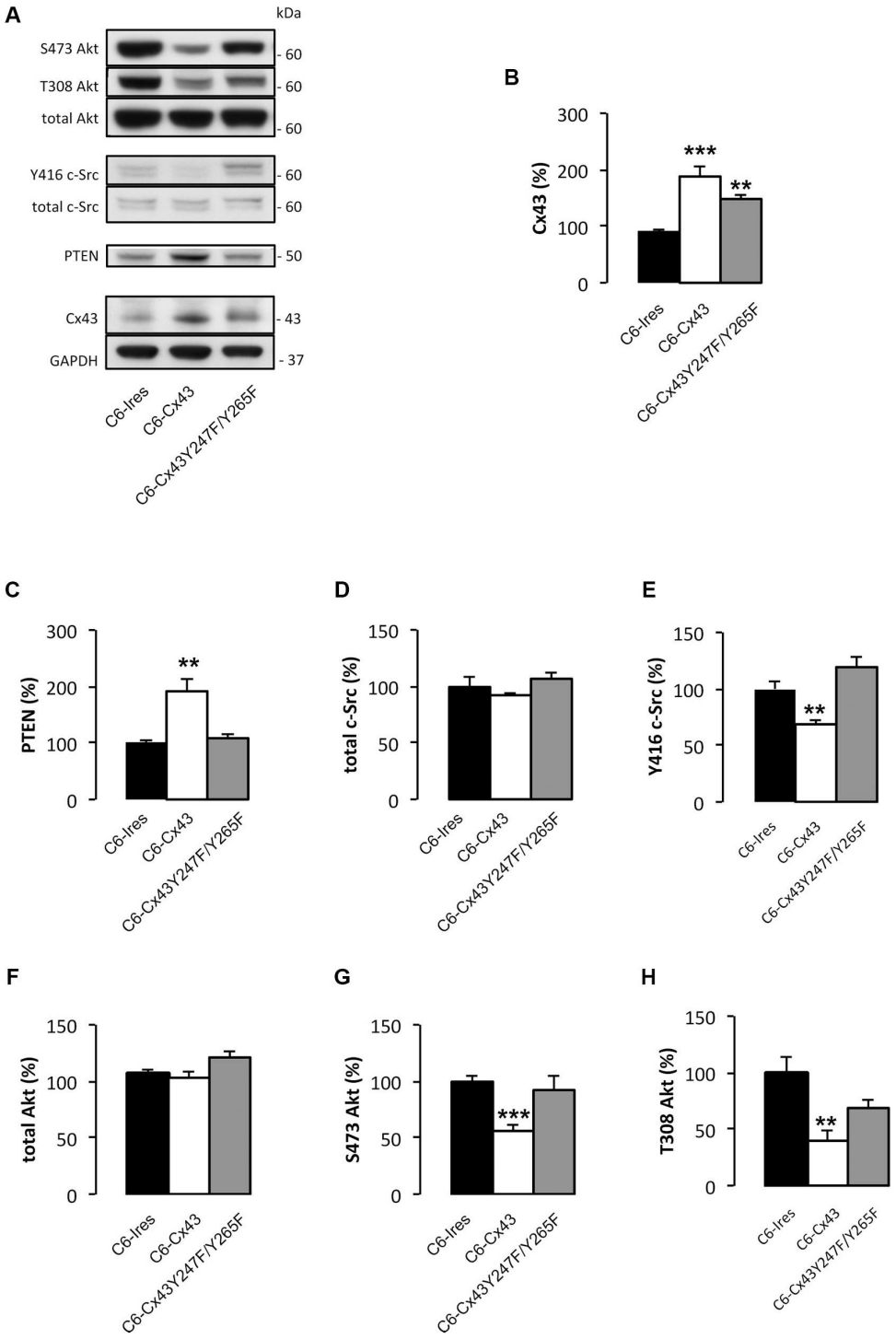

Figure 2: Effect of Cx43 and mutant Cx43 expression on PTEN expression and Akt activity. C6 glioma cells were stably transfected with the empty vector (C6-Ires), the vector containing the Cx43 cDNA (C6-Cx43), or the vector containing the cDNA encoding the double mutant Cx43 in which tyrosine 247 and tyrosine 265 of Cx43 were replaced by non-phosphorylatable phenylalanines (C6-Cx43 Y247F/Y265F). Western blots (**A**) and quantification of Cx43 (**B**), PTEN (**C**), total c-Src (**D**), Y416 c-Src (**E**), total Akt (**F**), S473 Akt (**G**) and T308 Akt (**H**). The results are the means ± s.e.m. ($n = 6$) and they are expressed as percentage of the value generated by C6-Ires cells. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ versus C6-Ires.

Because the activity of c-Src promotes PTEN degradation and reduces its ability to inhibit the PI3K/Akt pathway [33], we analyzed the involvement of c-Src in the effect of Cx43 on PTEN. C6 cells were stably transfected with a double Cx43 mutant in which tyrosines 247 and 265 were replaced with non-phosphorylatable phenylalanines (C6-Cx43Y247F/Y265F). This mutant lacks the ability to bind to c-Src [15, 17] and, consequently, does not inhibit c-Src activity like wild-type Cx43 [19] (Figure 2A, 2D and 2E). Our results showed that the mutant Cx43 did not significantly modify the levels of PTEN (Figure 2A and 2C), suggesting that the Cx43-mediated inactivation of c-Src is required to increase the levels of PTEN. In agreement with the lack of effect on PTEN levels, mutant Cx43 did not significantly modify the levels of active Akt phosphorylated at serine 473 and threonine 308 (Figure 2A, 2G and 2H).

Cx43 binds to PTEN and Csk

The ability of Cx43 to bind to c-Src has been well described [15, 17, 34]. Because our results suggested that Csk and PTEN participate in the inhibition of c-Src activity promoted by Cx43, we postulated that Cx43 could recruit these enzymes to achieve the inactivation of c-Src. To test this hypothesis, glioma cells were transfected with HA-PTEN or the empty vector (pSG5L). After 24 h, HA was immunoprecipitated (Figure 3A). Our results showed that the antibody against HA precipitated HA-PTEN, Cx43, c-Src and Csk in glioma cells transfected with Cx43 but not in glioma cells that lacked Cx43 expression (C6-Ires) or in C6-Cx43 cells transfected with pSG5L, suggesting that restoring Cx43 to glioma cells promotes its binding to PTEN, c-Src and Csk in glioma cells.

To address whether this interaction occurs between endogenously expressed proteins, astrocytes, which naturally express high levels of Cx43 and PTEN, were analyzed. Thus, Cx43 was immunoprecipitated, and the presence of PTEN was identified in the immunocomplex of Cx43 (Figure 3B). Reciprocally, Cx43 was also found after immunoprecipitation with antibodies against Csk in astrocytes. No signals were observed after the immunoprecipitation without IgG (data not shown) or with non-relevant IgGs, such as a mouse monoclonal antibody against hexokinase-1 (Hx-1) or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) used as negative controls.

To confirm the results of immunoprecipitation, immunofluorescence analyses were carried out for Cx43, PTEN and Csk in astrocytes. The distribution of these proteins (Figure 3C; Cx43 in red and PTEN or Csk in green) was analyzed using a confocal microscope. The yellow color in the overlay images confirmed certain areas of colocalization between Cx43 and PTEN or Csk in astrocytes (Figure 3C). This colocalization can be visualized in the Z-projection of the stack images (Figure 3C). In addition, C6 glioma cells were transiently transfected with Cx43, and PTEN (green) and Cx43 (red) were analyzed by immunofluorescence 48h later. The confocal images showed some points of colocalization (yellow) between the two proteins in the overlay image (Figure 3D).

Region of Cx43 involved in the PTEN and Csk binding

Regarding the molecular bases underlying the Cx43-PTEN binding, both Cx43 and PTEN harbor a functional PDZ domain-binding motif (residues 380–382 in Cx43 [35] and residues 401–403 in PTEN [36]) at their C-terminal tail. Therefore, we hypothesized that Cx43 and PTEN could interact through a scaffolding protein containing several PDZ domains. For instance, Zonula occludens-1 (ZO-1) binds to Cx43 through one of its three PDZ domains [35]; therefore, although a direct interaction between ZO-1 and PTEN has not been reported, it might occur in one of the remaining PDZ domains in ZO-1. To test this concept, C6-Cx43 glioma cells or astrocytes were transfected with a mutant PTEN that lacks the PDZ domain-binding motif (residues 1–400; Flag ΔPDZ-PTEN), wild-type PTEN (Flag WT-PTEN) or the empty vector (Flag) (Figure 4). Flag immunoprecipitation revealed that the mutant PTEN retains the ability to bind to Cx43 and c-Src in glioma cells (Figure 4A) and to Cx43, c-Src and Csk in astrocytes (Figure 4B), indicating that the PDZ domain-binding motif is not required for these interactions.

Our previous results suggested that cell-penetrating peptides (CPPs) containing Cx43 residues involved in the c-Src interaction (residues 245–283 and 266–283 but not 274–283 in the C-terminal domain of Cx43 (Cx43CT)) inhibit the activity of c-Src [11]. Therefore, we investigated whether these peptides could recruit the machinery required to inhibit c-Src activity. To address this point, C6 glioma cells were incubated with biotinylated CPPs containing the SH3 domain binding motif and tyrosines phosphorylated by c-Src (from amino acid 245 to 283), the same sequence excluding the tyrosines (from amino acid 266 to 283) or a consensus SH3 domain binding motif (from amino acid 274 to 283) (Figure 5A). These peptides were all fused to the TAT penetrating sequence (YGRKKRRQRRR). After 30 minutes, pull-down assays were performed, the biotinylated peptides were recovered and proteins bound to them were analyzed (Figure 5B). Our results showed that as expected [37], c-Src was found in complex with TAT-Cx43-266-283-B, TAT-Cx43-245-283-B and, to a lesser extent, TAT-Cx43-274-283-B. Interestingly, PTEN and Csk were mainly bound to TAT-Cx43-266-283-B and, to a lesser extent, TAT-Cx43-245-283-B, suggesting that this region of Cx43 (266–283) recruits not only c-Src but also PTEN and Csk. These proteins were not found when the cells were incubated with TAT-B, confirming that the sequence of Cx43 containing the residues 266–283 is sufficient to recruit c-Src, PTEN and Csk. The cellular internalization of these peptides was confirmed by fluorescence microscopy (Figure 5C).

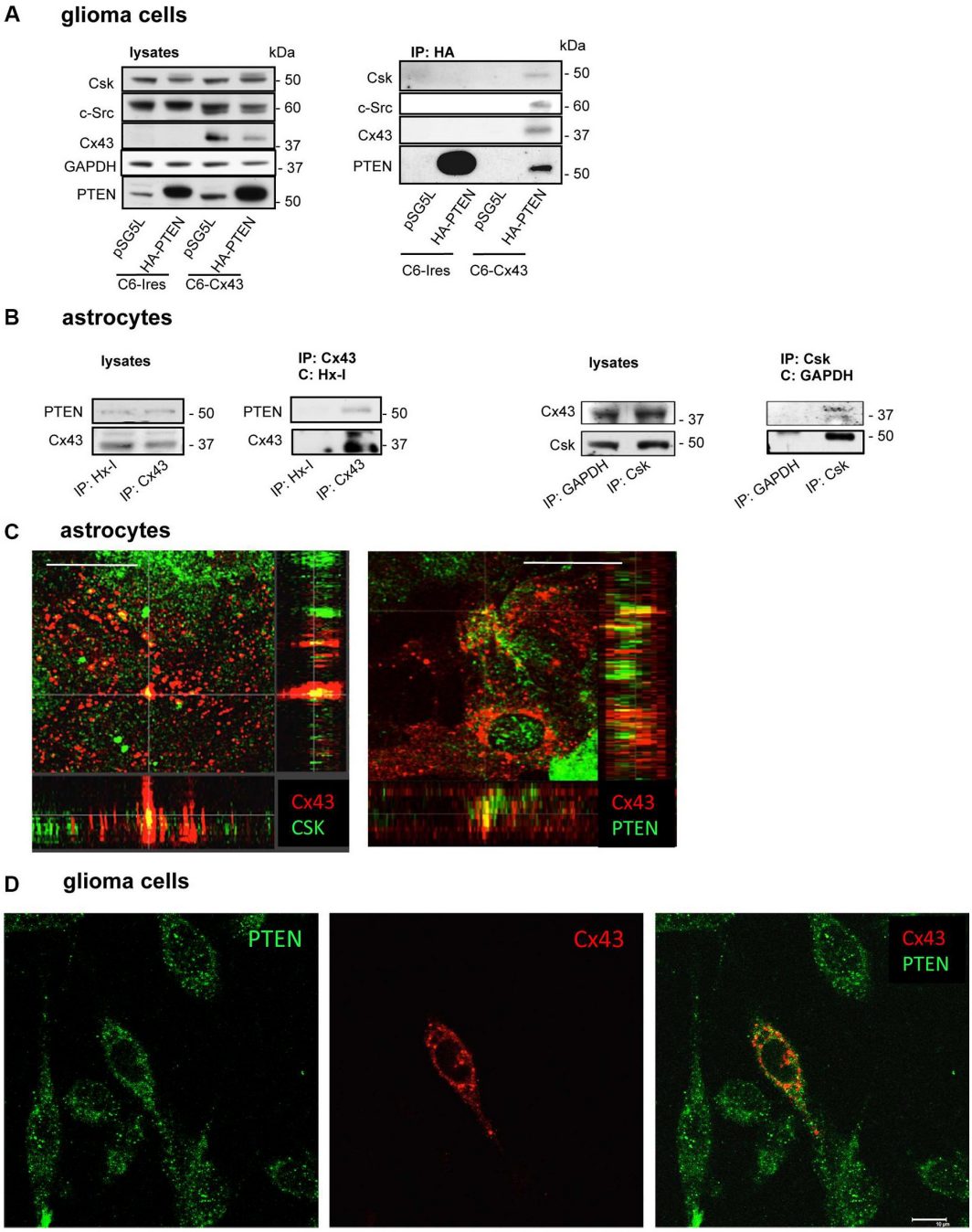

Figure 3: Interaction between Cx43, c-Src, Csk and PTEN in C6 glioma cells and astrocytes. (**A**) C6 glioma cells stably transfected with the empty vector (C6-Ires) or the vector containing the Cx43 cDNA (C6-Cx43) were transfected with HA-PTEN or pSG5L (empty vector). After 24 h, the cells were lysed and immunoprecipitated with anti-HA antibodies. Western blotting before (lysates) and after HA immunoprecipitation for PTEN, Cx43, c-Src and Csk showing the presence of Csk, c-Src and Cx43 in the immunocomplex obtained with HA-PTEN in glioma cells expressing Cx43. (**B**) Astrocytes from primary culture were lysed and immunoprecipitated with antibodies against Cx43 or Csk. Antibodies against hexokinase-1 (Hx-1) and GAPDH were used as controls. (**C**) Colocalization of Cx43 with Csk and PTEN in astrocytes. Confocal images show Cx43 (red) and Csk or PTEN (green) and their colocalization (yellow). Scale bars: 15 μm. Orthogonal projections along the z-axis of the images are shown at the bottom and right. (**D**) Colocalization of PTEN and Cx43 in C6 glioma cells transiently transfected with the construct containing Cx43 cDNA. The confocal images show PTEN (green), Cx43 (red) and overlay image with some points of colocalization (yellow). Note that only one Cx43-transfected cell is shown. Scale bar: 10 μm.

TAT-Cx43-266-283 inhibits human glioblastoma stem cell growth in a PTEN-dependent fashion

To confirm that the recruitment of PTEN and Csk to the sequence of Cx43 containing residues 266–283 is sufficient to inhibit c-Src and its downstream pathway, we tested the effect of the peptide TAT-Cx43-266-283 on G166 human glioblastoma stem cells (GSCs) [38].

Our results showed that TAT-Cx43-266-283 reduced c-Src activity as evidenced by decreased levels of Y416 c-Src, increased PTEN protein levels and subsequently reduced Akt activity (T308 Akt) (Figure 6A).

As a consequence of the inhibition of these proliferative pathways, TAT-Cx43-266-283 reduced GSC proliferation compared with the control or TAT peptides (Figure 6B–6D and Supplementary Figure S1). Interestingly, when PTEN was knocked-down by siRNA (Figure 6E), TAT-Cx43-266-283 was not able to significantly affect GSC growth. This result was in contrast to the observations made in GSCs transfected with a NT-siRNA (Figure 6F) and confirms the participation of PTEN in the inhibition of c-Src that is promoted by the sequence of Cx43 containing the residues 266–283.

DISCUSSION

High-grade glioma cells exhibit reduced levels of Cx43 protein [3–9] but increased c-Src activity [20]. Importantly, restoring Cx43 expression inhibits the activity of c-Src [11, 19], reduces glioma cell proliferation [39, 40] and reverses glioma stem cell phenotype [11, 12]. In this study, we revealed the mechanism by which Cx43 inhibits the oncogenic activity of c-Src. Our results showed that Cx43, in addition to c-Src, recruits Csk and PTEN, which are the enzymes required to inhibit c-Src activity.

The Csk-mediated phosphorylation of c-Src at tyrosine 527 is the best-described mechanism for c-Src inactivation [41]. The completion of this inhibition requires the dephosphorylation of c-Src at tyrosine 416 [42]. Our results suggest that in C6 glioma cells, as in other cell types [28, 43], PTEN is the phosphatase that catalyzes this process. Indeed, silencing PTEN prevented the reduction of Y416 c-Src that was promoted by Cx43 in glioma cells. In addition, when either Csk or PTEN was silenced and c-Src activity could not be inhibited, the antiproliferative effect of Cx43 on glioma cells was reduced. It can be concluded that Csk and PTEN participate in the antiproliferative effect of Cx43 by inhibiting c-Src.

Intriguingly, our results showed that restoring Cx43 expression in glioma cells up-regulates the levels of functionally active PTEN protein. Indeed, PI3K/Akt, the main PTEN downstream pathway, is subsequently inhibited. Because the mutant Cx43 that lacks the ability to inhibit c-Src activity [19] did not modify PTEN levels or activity, it could be proposed that the effect of Cx43 on PTEN levels is a consequence of c-Src inhibition. In agreement with this proposal, the activity of c-Src promotes PTEN degradation and the subsequent activation of the PI3K/Akt pathway [33]. By showing that Cx43 up-regulates PTEN, one of the

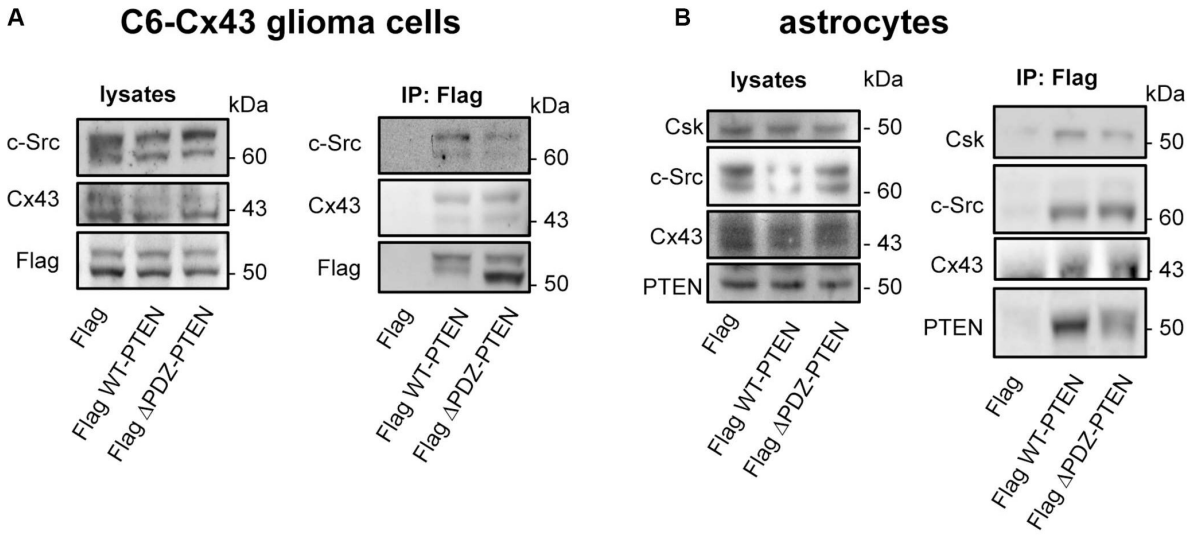

Figure 4: PTEN PDZ domain-binding motif is not required for PTEN interaction with Cx43, c-Src, and Csk. C6-Cx43 cells (**A**) or astrocytes (**B**) were transfected with Flag WT-PTEN, Flag ΔPDZ-PTEN (1-400; PTEN lacking the PDZ domain-binding motif) or Flag (control). After 48 h, the cells were lysed, and immunoprecipitations were carried out with antibodies against Flag. Western blotting before (lysates) and after Flag immunoprecipitation for Flag, Cx43, c-Src, PTEN and Csk showing the presence of c-Src and Cx43 in the immunocomplex obtained with both WT-PTEN and ΔPDZ-PTEN in glioma cells expressing Cx43 and the presence of c-Src, Csk and Cx43 in the immunocomplex obtained with both WT-PTEN and ΔPDZ-PTEN in astrocytes that endogenously express Cx43.

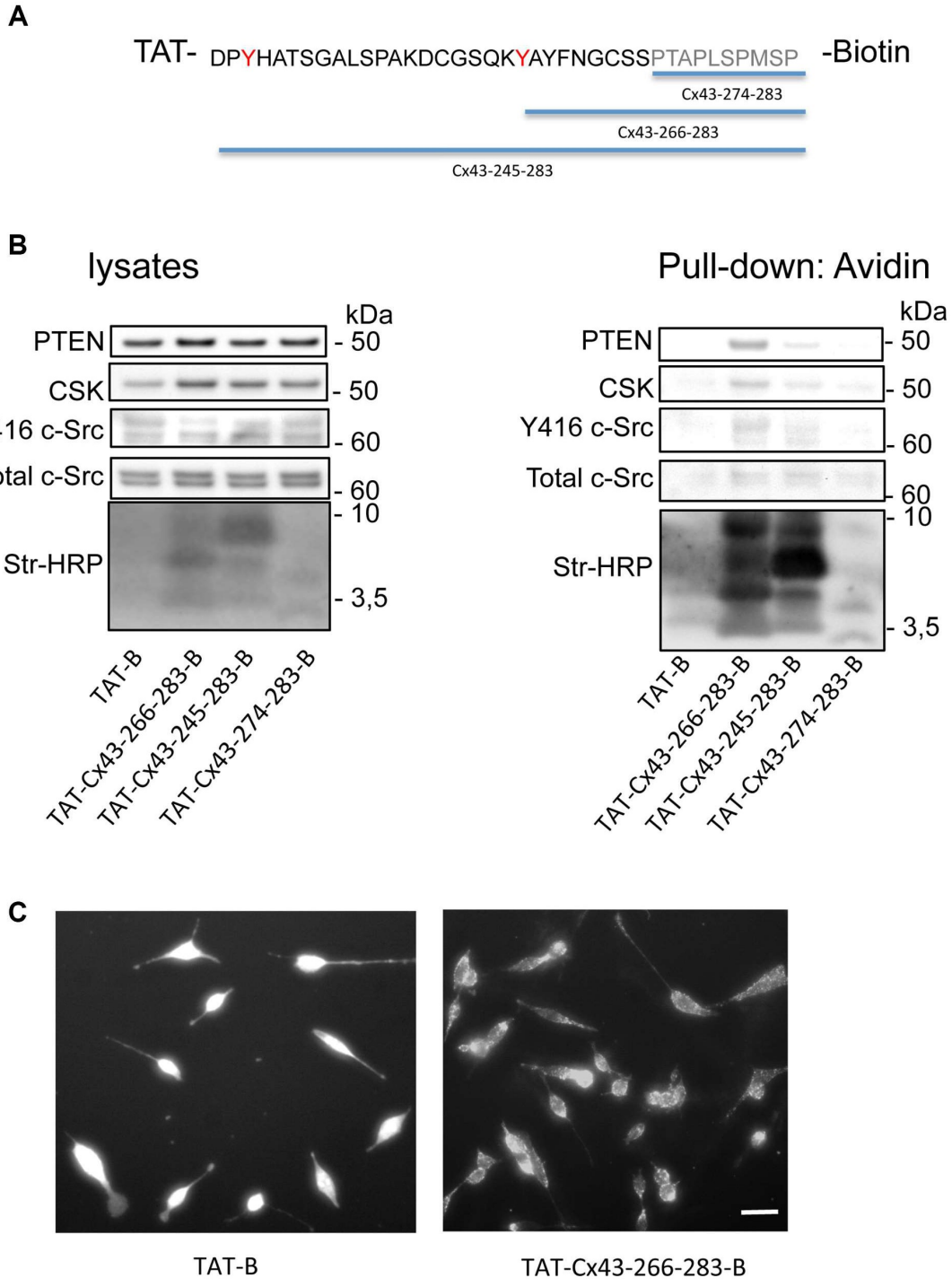

Figure 5: The Cx43 region involved in the recruitment of PTEN and Csk. C6 glioma cells were incubated with several cell-penetrating peptides containing the indicated sequences of Cx43 fused to biotin for 30 min. (**A**) The SH3 domain binding motif is shown in grey and the tyrosines phosphorylated by c-Src in red. (**B**) After 30 min, the cells were lysed, and pull-down assays were carried out with avidin-conjugated agarose beads. Western blots before (lysates) and after avidin pull-down for c-Src, Csk and PTEN showing the enrichment of PTEN and Csk in the complex obtained with TAT-Cx43-266-283-Biotin and, to a lesser extent, with TAT-Cx43-245-283-Biotin. Str-HRP, HRP-conjugated streptavidin. (**C**) After 30 min, the cells were fixed, and the uptake of peptides bound to biotin was analyzed by fluorescence microscopy. Scale bars: 15 µm.

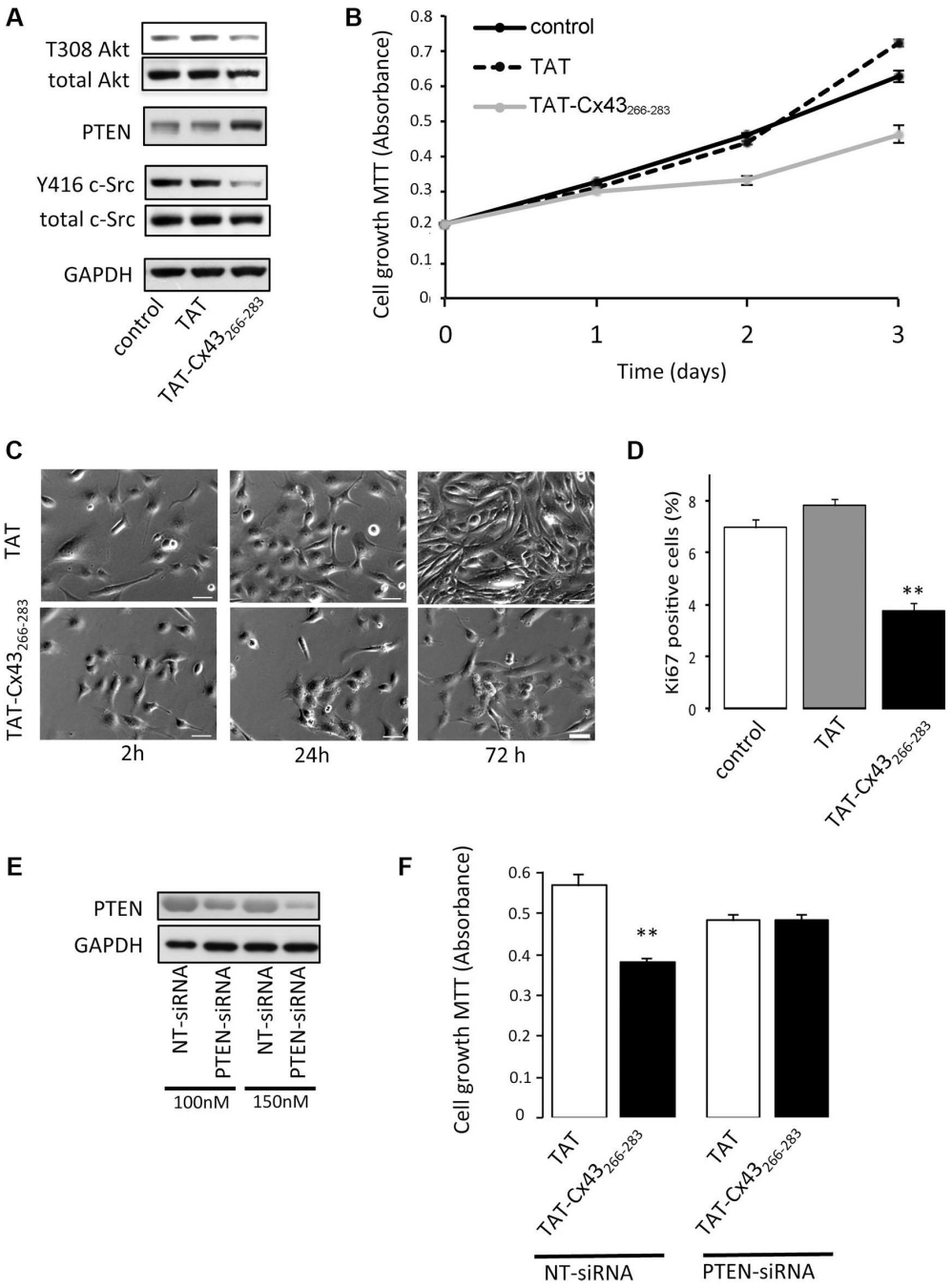

Figure 6: Involvement of PTEN in the antiproliferative effect of TAT-Cx43-266-283 on G166 human glioblastoma stem cells. G166 cells were incubated with 50 µM TAT or TAT-Cx43-266-283 for the indicated times. (**A**) After 48 h, total c-Src, Y416 c-Src, PTEN, total Akt and T308 Akt levels were analyzed by Western blotting. (**B**) The cells were plated at a density of 5000 cells/cm^2 and the number of living cells was monitored over a 3 day period by the MTT assay. Means ± s.e.m. (n = 3). (**C**) Representative phase-contrast images showing the inhibition of proliferation promoted by TAT-Cx43-266-283. Scale bars: 50 µm. (**D**) Percentage of Ki-67-positive cells found after 48h. Means ± s.e.m (n = 3). ** $p < 0.01$ versus control. (**E**) G166 cells were plated at 7500 cells/cm^2 and transfected with PTEN-siRNA or non-targeting siRNA (NT-siRNA). After 48 h, PTEN levels were analyzed by Western blotting. (**F**) G166 cells were plated at 7500 cells/cm^2 and transfected with 150 nM NT-siRNA or PTEN-siRNA. After 24 h, the cells were incubated with 50 µM TAT or TAT-Cx43-266-283 for 48 h and the number of living cells was analyzed by the MTT assay. Means ± s.e.m. (n = 3). **$p < 0.01$ versus TAT NT-siRNA.

most relevant tumor suppressor genes in gliomas [44, 45], and inhibits PI3K/Akt, an important proliferative pathway that is frequently up-regulated in glioma [46], these results expand our knowledge of the mechanism by which Cx43 suppresses tumor growth (Figure 7).

This study shows that in astrocytes, that express high levels of endogenous Cx43, there is also an interaction between Cx43, c-Src, Csk and PTEN, indicating that the regulation of c-Src activity is a physiological function of Cx43. c-Src phosphorylates tyrosines 265 and 247 [14], which strongly affects Cx43 structure and function [26, 37] and the interaction of Cx43 with several partners [47–49]. To terminate these effects, the phosphatase TC-PTP is recruited to remove the phosphates at tyrosines 265 and 247 of Cx43 [50]. However, if c-Src remained active, it would continue to phosphorylate Cx43. Our results suggest that Cx43 phosphorylation by c-Src could also be a signal to recruit PTEN and Csk to this complex. The inhibition of c-Src activity promoted by PTEN and Csk would collaborate with TC-PTP to terminate this pathway, which is required to recycle Cx43 [51]. In this way, the transient and dynamic interactions of these proteins would contribute to the maintenance of the homeostasis of the gap junction channel and hemichannel activities.

Although both PTEN and Cx43 contain PDZ-binding motifs, our results indicated that these regions

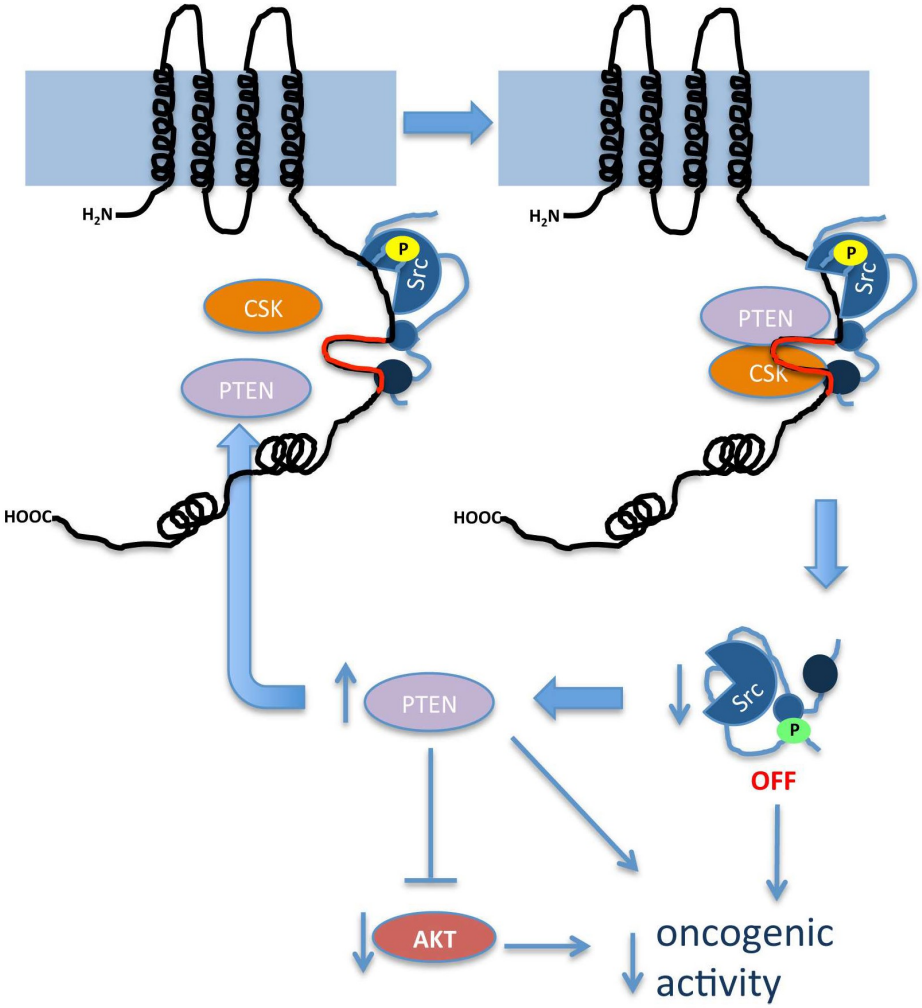

Figure 7: Proposed mechanism by which Cx43 inhibits the activity of c-Src. Our results showed that the region of Cx43 that comprises amino acids 266-283 (in red), located in one of the intrinsically disordered regions of Cx43CT, serves as a docking platform for the active form of c-Src (c-Src phosphorylated at tyrosine 416, shown in yellow), Csk and PTEN. The proximity of these proteins facilitates the phosphorylation of c-Src at tyrosine 527 (green) catalyzed by Csk and the removal of phosphate at tyrosine 416 (yellow) catalyzed by PTEN with the subsequent inhibition of c-Src activity. c-Src inhibition prevents PTEN degradation creating a positive feed-forward loop to guarantee the long-term regulation of this process. As a consequence of increased PTEN, Akt activity decreases. This signaling pathway contributes to a reduction in the oncogenic activity of these proteins in glioma cells.

are not required for the interaction between these proteins. Instead, the region of Cx43 that is involved in its interaction with c-Src (residues 266–283) is sufficient to recruit PTEN and Csk. In agreement with this finding, CPPs containing this sequence (TAT-Cx43-266-283) inhibited the oncogenic activity of c-Src [11], increased PTEN, reduced Akt activity and subsequently decreased human glioblastoma stem cell proliferation.

Since it was first described, the relationship between Cx43 and cancer has been deeply investigated [52]. However, it should be noted that although restoring Cx43 expression in glioma cells reduces proliferation, it can also have detrimental effects due to gap junctional communication or cytoskeletal interactions [53–56]. There is interest in the short sequence (residues 266–283) of Cx43 because it inhibits the oncogenic activity of c-Src and lacks the ability to interact with the cytoskeleton or to form gap junction channels. Nevertheless, unexpected effects cannot be ruled out. Therefore, further studies should be carried out to explore the therapeutic potential of this sequence in depth.

In conclusion, we propose that the Cx43-mediated inhibition of c-Src involves the recruitment of the c-Src inhibitors Csk and PTEN to residues 266–283 within Cx43CT. Furthermore, upon c-Src inhibition, Cx43 up-regulates PTEN and subsequently inactivates Akt to sustain its tumor suppressor properties (Figure 7). These results stress the relevance of Cx43 residues 266–283 for the development of new therapies to reduce the oncogenic activity of c-Src. In contrast to the available c-Src inhibitors, this approach uses an endogenous inhibitory mechanism, making off-target effects less likely.

MATERIALS AND METHODS

Cell cultures

Astrocytes in primary culture were prepared from the forebrains of 1- to 2-day-old Wistar rats and cultured in DMEM (Sigma-Aldrich Química, Madrid, Spain) supplemented with 10% FCS (Gibco, Life Technologies, Madrid, Spain), as previously described [57]. C6 glioma cells (ATCC, Manassas, USA) were cultured in DMEM supplemented with 10% FCS as previously described [19]. The G166 human GSC line was obtained from BioRep (Milan, Italy). The cells were grown in petri dishes coated with 10 mg/ml laminin (Life Technologies). The growth medium was RHB-A (StemCells, Cambridge, UK) supplemented with 1% N2, 2% B27 (Life Technologies), 20 ng/ml EGF and 20 ng/ml b-FGF (PeproTech, London, UK) as described previously [11].

Plasmid constructs and cell transfection

The pIRES-Cx43 construct was generated as previously reported [19] by ligating a PCR-amplified fragment encoding the rat Cx43 sequence (NM_012567) into the *Xho*I-*Bam*HI sites of the bicistronic pIRES2-DsRed2 vector (Clontech, Palo Alto, CA, USA). The Cx43Y247F/Y265F mutation was introduced into pIRES-Cx43 by site-directed mutagenesis [19]. C6-Ires, C6-Cx43 and C6-Cx43 Y247F/Y265F clones were generated and characterized as previously described [19]. Unless otherwise specified, the C6 glioma cells were stably transfected with the empty vector (C6-Ires), the construct containing Cx43 (C6-Cx43) or the construct containing the mutant Cx43 (C6-Cx43Y247F/Y265F) using Lipofectamine 2000 (Life Technologies). For stable transfection cells were selected with 0.5 mg/ml G418 (Promega, Madison, WI, USA) in DMEM supplemented with 10% (v/v) FCS.

The pSG5L, pSG5L HA-PTEN, pCMV FLAG WT-PTEN and pCMV FLAG ΔPDZ-PTEN (1–400) plasmids were a gift from William Sellers and Hong Wu and were obtained from Addgene (Addgene plasmid 10737, 10750, 22231 and 22232, respectively) [58, 59]. Cells were transiently transfected using Lipofectamine 2000, as described above.

Transfection of siRNA

C6-Cx43 clone 7 cells were transfected with a validated non-targeting siRNA (NT-siRNA), a siRNA specific for Csk (Csk-siRNA) or a siRNA specific for PTEN (PTEN-siRNA) (BioNova Científica S.L., Madrid, Spain). Cells were transfected with the double-strand siRNA complexed with 3 µl/ml Lipofectamine 2000 (Life Technologies) in culture medium without antibiotics. The cells were maintained in the presence of the oligonucleotides in culture medium without antibiotics for 6 h. The extent of siRNA-mediated down-regulation of Csk or PTEN expression was evaluated by Western blot analysis. The Csk-siRNA sequences were as follows: sense 5′-AGUACCCAGCAAAUGGGCATT-3′ and antisense 5′-UGCCCAUUUGCUGGGUACUTT-3′ [29]. The PTEN-siRNA sequences were as follows: sense 5′-GUUAGCAGAAACAAAAGGAGATT-3′ and antisense 5′-UCUCCUUUUGUUUCUGCUAACTT-3′ [30].

Peptide treatments

The synthetic peptides (> 90% pure) were obtained from GenScript (Piscataway, NJ, USA). All peptides used were biotinylated via a C-terminal lysine. YGRKKRRQRRR-Lys(biotin) was used as the TAT sequence (TAT-B), which is responsible for cell penetration of the peptides [60]. The sequence for TAT-Cx43-245-283-B was YGRKKRRQRRRDPYHATSGAL SPAKDCGSQKYAYFNGCSSPTAPLSPMSP-Lys(biotin), the sequence for TAT-Cx43-266-283-B was YGRKKRRQ RRRAYFNGCSSPTAPLSPMSP-Lys(biotin) and the sequence for TAT-274-283-B was YGRKKRRQRRR PTAPLSPMSP-Lys(biotin). Peptides were used at 50 µM in culture medium at 37 °C for the indicated times.

MTT assay

Cells cultured at 37°C in 24-well-plates were incubated in the dark for 75 min with 300 µl of DMEM containing 0.5 mg/ml MTT. The medium was then removed, and the cells were incubated for 10 min in the dark with dimethyl sulfoxide (500 µl/well). Finally, the absorbance was measured at a wavelength of 570 nm using a microplate reader (Appliskan 2001; Thermo Electron Corporation, Waltham, MA, USA).

Western blot analysis

Western blotting was performed as previously described [11]. Briefly, equivalent amounts of proteins (20 μg per lane) were separated on NuPAGE Novex Bis-Tris (4–12% or 10%) midigels (Life Technologies). Proteins were transblotted using an iBlot dry blotting system (Life Technologies). The membranes were cut into several strips to be immunoblotted with distinct antibodies, thus allowing for comparative analysis of the amount of each protein in the same sample. Membranes were then blocked for 1 h at room temperature in Tris-buffered saline containing 0.05% Tween 20 (TTBS) and 7% non-fat milk powder before being incubated overnight at 4 °C with the primary antibodies. The primary antibodies used were: Cx43 (1:250; BD Biosciences, Madrid, Spain; Ref. 610062); total c-Src (1:500; Ref. 2108); Y416 c-Src (1:250; Ref. 2101), Y527 c-Src (1:500; Ref. 2105), Csk (1:500; Ref. 4980), PTEN (1:500; Ref. 9552), total Akt (1:1000; Ref. 9272), S473 Akt (1:2000; Ref. 4060), T308 Akt (1:1000; Ref. 4056) (all from Cell Signaling, Danvers, MA, USA), GAPDH (1:6000; Ambion; Ref. AM4300) or α -actinin (1:1000; Merck Millipore, Darmstadt, Germany; Ref. MAB1682). After extensive washing, the membranes were incubated with HRP-conjugated antibodies (Santa Cruz Biotechnology, Inc., Dallas, TX, USA; Refs. sc-2030 and sc-2005) in TTBS. The proteins were developed with a chemiluminescent substrate. Densitometry analysis of the bands was performed using the Image J program (Wayne Rasband; NIH, Bethesda, MD, USA). The amounts of GAPDH or α-actinin recovered in each sample served as the loading control, and the values for each protein were normalized to their corresponding GAPDH or α-actinin level.

Immunofluorescence

Immunofluorescence was performed as previously described [11]. The cells were incubated overnight at 4ºC with primary antibodies against Cx43 (1:100; BD Biosciences; Ref. 610062), Csk (1:100), and PTEN (1:100) (both from Cell Signaling; Refs. 4980 and 9552). The secondary antibodies used were as follows: Cy5 goat anti-mouse (1:500; Jackson ImmunoResearch, Baltimore, PA, USA; Ref. 115-175-003) and Alexa Fluor 488 goat anti-rabbit (1:1000; Life Technologies, Ref. A-11029). The cells were mounted using the SlowFade Gold Antifade kit (Life Technologies) and were analyzed using a Leica DM-IRE2 confocal microscope and LCS Lite software (Leica Microsystems, Wetzlar, Germany).

Co-immunoprecipitation

Twenty-one DIV astrocytes or C6 cells grown to confluence in 10-cm dishes were washed with ice-cold PBS and lysed at 4°C in 1 ml of SDS-free RIPA buffer (10 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.2% Triton X-100, 2 mM EDTA, 2 mM EGTA, 1 mM PMSF, 1:100 (v/v) protease cocktail (Cocktail III; Calbiochem Merck-Millipore, Billerica, MA, EE.UU), 1 mM NaF and 0.1 mM Na_3VO_4). Lysates were centrifuged at 11000 × g for 10 min at 4°C, and the supernatants were recovered. A 25-µl aliquot was used to analyze the protein content (lysates), and the remaining lysate (immunoprecipitation) was incubated with 2 µg of mouse monoclonal antibody against hexokinase I (Merck Millipore; Ref. MAB1532), mouse monoclonal antibody against HA (Roche, Basel, Switzerland Ref.11 583 816 001), mouse monoclonal antibody against Cx43 (BD Biosciences; Ref. 610062), rabbit monoclonal antibody against Csk (Cell Signaling; Ref. 4980) or rabbit monoclonal antibody against GAPDH (Ambion; Ref. AM4300) for 12 h at 4°C with gentle shaking. The immunocomplexes were sequestered by adding 50 µl of Protein-A Sepharose CL-4B (GE Healthcare, Madrid, Spain), which was previously saturated with 5% (w/v) albumin and gently shaken at 4°C for 4 h. The Protein-A beads containing the immunocomplexes were collected by centrifugation (11000 × g for 1 min at 4°C). The beads were then washed four times with buffer A (10 mM Tris-HCl (pH 8.0), 150 mM NaCl, Triton X-100 and 2 mM EDTA), once with buffer B (10 mM Tris-HCl (pH 8.0), 500 mM NaCl, 0.2% Triton X-100 and 2 mM EDTA) and once with buffer C (10 mM Tris-HCl (pH 8.0)). The bound proteins were eluted with SDS sample buffer at 95°C for 5 min. After centrifugation, the supernatants were analyzed by Western blotting.

For Flag fusion proteins, the lysis buffer contained 20 mM Tris-HCl (pH 8.0), 137 mM NaCl, 1% IGEPAL (p/v), 1 mM PMSF, 1:100 (v/v) protease cocktail (Cocktail III, Calbiochem), 1 mM NaF and 0.1 mM Na_3VO_4. The lysates were incubated with anti-Flag M2 Affinity Gel (40 µl per ml; Sigma, Saint Louis, MO, USA; Ref.F7425) for 12 h at 4°C with gentle shaking. The anti-Flag beads containing the immunocomplexes were collected by centrifugation (11000 × g for 1 min at 4°C) and washed five times with lysis buffer, and the bound proteins were eluted and analyzed by Western blotting.

Avidin pull-down assay

Cells grown in 3.5-cm dishes were incubated with 50 µM biotinylated peptides for 30 min. Proteins were then collected in 1 ml of lysis buffer (20 mM Tris-HCl (pH 8.0), 137 mM NaCl, 1% IGEPAL, 1 mM PMSF, protease

cocktail (1:100; Cocktail III, Calbiochem), 1 mM NaF and 0.1 mM Na_3VO_4). Lysates were centrifuged at 11000 × *g* for 10 min at 4°C, and the supernatants were recovered. A 25-µl aliquot of each lysate was used to analyze the protein content, and the remaining lysate was incubated with NeutrAvidin-Agarose (Thermo Scientific, Rockford, IL, USA; Ref. 29200) for 12 h at 4°C with gentle shaking. The avidin beads bound with the peptides were collected by centrifugation (3000 × *g* for 1 min at 4°C). The beads were then washed five times with lysis buffer, and the bound proteins were eluted and analyzed by Western blotting. To detect biotinylated peptides, the membranes were incubated with HRP-conjugated streptavidin in TTBS (1:40000, Ref. 434323, Life Technologies) and then developed with a chemiluminescent substrate.

In parallel, the cells were incubated with 50 µM biotinylated peptides for 30 min. The cells were then washed with PBS at 4ºC and fixed with 4% paraformaldehyde for 20 min. After washing, the cells were incubated with Cy2-conjugated streptavidin (1:500; Jackson ImmunoResearch, Baltimore, USA; Ref. 016-220-084) for 1 h, mounted and visualized as described previously.

Statistical analyses

The results were expressed as the means ± s.e.m. of at least three independent experiments. Statistical analyses were carried out using Student's *t*-test when two groups were compared. For the comparison of more than one group, analysis of variance (one-way ANOVA) was used, followed by an appropriate post-test (Dunnet or Tukey). Values were considered significant with a p value less than 0.05.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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CAPíTVLO 2

Estudio del mecanismo de la inhibición de c-Src mediante TAT-Cx43$_{266-283}$ biotinilado en células madre de glioma humano

Biotinylated cell-penetrating peptides to study intracellular protein-protein interactions. Jaraíz-Rodríguez M, González-Sánchez A, García-Vicente L, Medina JM, Tabernero A. *JoVE*; en prensa.

Para llevar a cabo el estudio del mecanismo a través del cual el péptido penetrante, TAT-Cx43$_{266-283}$, inhibe c-Src en células madre de glioma humano, en este trabajo diseñamos un método para estudiar esta interacción en un contexto intracelular. A diferencia de los ensayos de inmunoprecipitación con lisados celulares, nos basamos en las propiedades de la afinidad avidina-biotina para utilizar el péptido penetrante como anzuelo de las proteínas con las que interacciona esta región, y realizar un *pull-down* del complejo formado. Para ello, el péptido control TAT y el péptido con la región de interés, TAT-Cx43$_{266-283}$, se unieron a biotina en su extremo terminal. La internalización de los péptidos gracias al péptido TAT, facilita que la interacción se produzca en un contexto intracelular. En primer lugar, nos aseguramos de que los péptidos unidos a biotina ejercían los mismos efectos que TAT o TAT-Cx43$_{266-283}$ sobre la morfología y la proliferación de las células madre de glioma humano G166. Una vez confirmado, los péptidos penetrantes biotinilados se incubaron durante 30 minutos en células madre de glioma G166, se lisaron las células y se llevó a cabo el *pull-down* con Neutravidina-agarosa. De esta forma, al eluir estos complejos Neutravidina-biotina, el ensayo de Western-blot mostró que TAT-Cx43$_{266-283}$ interacciona con las proteínas propuestas para su mecanismo de acción, es decir, c-Src y sus inhibidores endógenos Csk y PTEN. Por el contrario, la quinasa de adhesión focal (FAK) que interacciona con c-Src pero no con la Cx43, no interacciona con TAT-Cx43$_{266-283}$, a diferencia de la caveolina-1 (Cav-1), que se une tanto a TAT-Cx43$_{266-283}$-B como a TAT-B, sugiriendo que esta proteína puede estar implicada en el mecanismo de internalización de los péptidos.

TITLE:
Biotinylated cell-penetrating peptides to study intracellular protein-protein interactions

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TAT, Biotin-avidin, Cell-penetrating peptides, Pull-down, Intracellular interactions, Protein-protein interactions, Western blot

SHORT ABSTRACT:
This is a protocol to study intracellular protein-protein interactions based on the biotin-avidin pull-down system with the novelty of combining cell-penetrating sequences. The main advantage is that the target sequence is incubated with living cells instead of cell lysates and therefore the interactions will occur within the cellular context.

LONG ABSTRACT:
Here we present a protocol to study intracellular protein-protein interactions that is based on the widely used biotin-avidin pull-down system. The modification presented includes the combination of this technique with cell-penetrating sequences. We propose to design cell-penetrating baits that can be incubated with living cells instead of cell lysates and therefore the interactions found will reflect those that occur within the intracellular context. Connexin43 (Cx43), a protein that forms gap junction channels and hemichannels is down-regulated in high-grade gliomas. The Cx43 region comprising amino acids 266-283 is responsible for the inhibition of the

oncogenic activity of c-Src in glioma cells. Here we use TAT as the cell-penetrating sequence, biotin as the pull-down tag and the region of Cx43 comprised between amino acids 266-283 as the target to find intracellular interactions in the hard-to-transfect human glioma stem cells. One of the limitations of the proposed method is that the molecule used as bait could fail to fold properly and, consequently, the interactions found could not be associated with the effect. However, this method can be especially interesting for the interactions involved in signal transduction pathways because they are usually carried out by intrinsically disordered regions and, therefore, they do not require an ordered folding. In addition, one of the advantages of the proposed method is that the relevance of each residue on the interaction can be easily studied. This is a modular system; therefore, other cell-penetrating sequences, other tags, and other intracellular targets can be employed. Finally, the scope of this protocol is far beyond protein-protein interaction because this system can be applied to other bioactive cargoes such as RNA sequences, nanoparticles, viruses or any molecule that can be transduced with cell-penetrating sequences and fused to pull-down tags to study their intracellular mechanism of action.

INTRODUCTION:

Protein-protein interactions are essential for a great variety of cellular processes. To fully understand these processes, methods for identifying protein interactions within the complex intracellular environment are required. One of the most used methods to identify interaction partners of a protein is to use that protein or a mimetic peptide of a part of that protein as bait in affinity pull-down experiments followed by detection of binding proteins. The avidin-biotin system is frequently used because of the high affinity, specificity and stable interaction between avidin and biotin[1,2]. Usually, biotin is covalently bound to the bait (protein or peptide) and after a period of incubation with the cell lysates to allow the establishment of interactions, the biotinylated bait bound to its intracellular partners is pulled down with avidin or avidin derivatives conjugated with support beads. Then, bait-protein interactions are detected after washing, elution, and analysis by denaturing electrophoresis followed by Western blot. One of the problems of this technique is that the interactions between the protein of interest and its intracellular partners are taking place outside of the cellular context. This is especially important for the interactions involved in signal transduction pathways because they take place in specific intracellular locations, they are transient and they are typically carried out by not abundant proteins. Therefore, within the cell lysates these interactions can be masked by other more abundant proteins or by proteins that usually are not in close proximity.

Cell-penetrating peptides (CPPs) are short peptides (≤40 amino acids), composed mostly of cationic amino acids that are capable of transporting a wide range of molecules into almost any cell[3]. Cargoes such as proteins, plasmid DNA, siRNA, viruses, imaging agents, and various nanoparticles have been conjugated to CPPs and efficiently internalized[4,5]. Because of this transporting ability they are also known as protein transduction domains (PTDs), membrane translocating sequences (MTSs), and Trojan peptides. Among the CPPs, the TAT peptide from the HIV transactivator protein TAT[6] has been one of the most widely studied [7-9]. TAT is a

nonapeptide that contains 6 arginine and 2 lysine residues and consequently is highly cationic. Substitution studies have demonstrated that the net positive charge of TAT is necessary for electrostatic interactions with the plasma membranes of eukaryotic cells and its subsequent internalization[10]. Similarly to other CPPs, positively-charged TAT strongly binds electrostatically to the various negatively-charged species present at the extracellular surface of cell membranes, including lipid head groups, glycoproteins and proteoglycans[3,10]. The bioactive cargoes transported by TAT become immediately free in the cytosol to reach their intracellular partners.

Here we present a method that combines the TAT CPP with biotin to study intracellular interactions. The aim is to design cell-penetrating baits by fusing the target biomolecule to TAT and to biotin. The main advantage of this proposal is that the interactions between the bait and its partners will take place within its cellular context. To show the efficacy of this method we used as bait a small sequence of the protein Cx43 that has been reported to interact intracellularly with the proto-oncogene c-Src[11-13]. Cx43 is an integral membrane protein that is widely expressed in astrocytes[14] and is down-regulated in high-grade gliomas, the most common malignant tumor of the central nervous system[15-18]. It has been previously shown that the Cx43 region that interacts with c-Src (amino acids 266-283 in human Cx43; Pubmed: P17302) fused to TAT (TAT-$Cx43_{266-283}$) inhibits the oncogenic activity of c-Src in glioma cells and glioma stem cells (GSCs)[19,20,21]. To design the intracellular bait, $Cx43_{266-283}$ has been fused to TAT at the N-terminus (TAT-$Cx43_{266-283}$) and to biotin at the C-terminus (TAT-$Cx43_{266-283}$-B). This strategy has been successfully used in the rat glioma C6 cell line to identify c-Src, c-terminal Src kinase (CSK) and phosphatase and tensin homolog (PTEN) as intracellular partners of this region of Cx43[20]. Here, we describe this method testing its efficacy in human GSCs, which are very relevant for glioma therapy but much harder to transfect than non-stem glioma cells.

PROTOCOL:

All experimental procedures were carried out at the University of Salamanca.

1. Cells

1.1. Two days before starting the procedure, plate the cells at the required density to be confluent the day of the experiment. Plate the cells in flasks or plates. However, the protein extraction will be easier from plates. It is convenient to prepare at least 4 plates of 78 cm^2 or 2 flasks of 150 cm^2 per condition per experiment, to be sure that the results are consistent.

1.2. In this study, plate human G166 GSCs in 4 flasks of 150 cm^2, cultured in RHB-A stem cell medium supplemented with 2% B27, 1% N2, 20 ng/mL EGF and b-FGF as described by Pollard et al.[22]. Process when confluence is reached.
NOTE: For instance, when 5 x 10^6 G166 cells were plated in a 150 cm^2 flask, they were processed 2 days after plating.

2. Biotinylated CPPs

2.1. Spin the vials containing the lyophilized biotinylated CPPs (BCPPs) at 8200 x g for 30 s, to avoid some of the powder remaining on the lid. Include a control BCPP to be sure that the interactions found are specific of the target sequence.
NOTE: In this study, the control BCPP was TAT-biotin (TAT-B) and the treatment BCPP was TAT-$Cx43_{266-283}$-B. Other controls could be used, such as TAT fused to scrambled or mutated fragments bond to biotin.

2.2. Dissolve the BCPPs in the corresponding culture medium to the stock solution indicated by the manufacturer; for instance, to obtain a stock solution of 2 mg/mL BCPP to treat GSCs add 0.5 mL of GSC culture medium to one vial containing 1 mg of the BCPP. Vortex, and make sure the peptide is well dissolved.

3. Tubesn

Note: Prepare at least twelve 1.5 mL tubes per condition required in the Section 7.

3.1. Mark the first 3 tubes per condition. They will have the total volume of cellular lysates obtained in step 6.4.

3.2. Mark an A on 3 tubes per condition. These tubes will have the first supernatants obtained after lysing and spinning the cellular lysates, step 7.2.

3.3. Mark a B on 3 tubes per condition. These tubes will have a small aliquot of the first supernatants. These lysates will serve as the Western blot samples in step 7.3.

3.4. Mark a C on 3 tubes per condition. These tubes will have the supernatants obtained after the pull-down with NeutrAvidin, step 7.7.

Note: This is important in case the Western blot showed no signal for proteins, meaning the proteins were not pulled down or were lost at some step of the procedure. If this were the situation, repeat the process to pull down the proteins.

4. Cellular treatment with the BCPPs

4.1. Aspirate the culture medium.

4.2. Replace the corresponding volume of fresh medium required to incubate the BCPPs in the smallest possible volume of medium according to the incubation times. It is very important that in any case, the medium covers completely the whole surface of the plate/flask so that the cells do not dry out; for instance, 6 mL per 150 cm^2 for a 30-min incubation.

4.3. Add the volume of the stock solution of BCPP to the cell cultures to reach the concentration that has been proved to be effective. In this study 50 μM TAT-$Cx43_{266-283}$-B has been proved to reduce GSC proliferation.

4.3.1. Therefore, add 92.8 µL of 2 mg/mL TAT-Cx43$_{266-283}$-B (MW=3723.34 g/mol) per mL of culture medium to obtain a final concentration of 50 µM TAT-Cx43$_{266-283}$-B. If the volume to be added is different for control peptides, complete with culture medium up to the same final volume. For instance, 49.1 µL TAT-B (MW=1914.31 g/mol) plus 43.7 µL GSC medium were added per mL of culture medium to obtain a final concentration of 50 µM TAT-B.

4.4. Place the cells in the incubator at 37 °C and 5% CO_2 for 30 min to make sure that the interactions between the BCPP and its intracellular partners take place. If the interaction takes longer, incubate for longer times or adjust times in case the experiment consisted of a time-course. In addition, the interaction of interest can be promoted or prevented by stimulating different intracellular signaling pathways.

5. Buffers and solutions.

5.1. Prepare PBS pH 7.4: 136 mM NaCl; 2.7 mM KCl; 7.8 mM Na2HPO4·2H2O; 1.7 mM KH2PO4.

5.2. Prepare protein lysis buffer: 20 mM Tris-HCl (pH 8.0), 137 mM NaCl, 1% IGEPAL, Prior to use, add the following: 1/100 (v/v) Protease Inhibitor Cocktail, 1 mM Sodium Fluoride, 1 mM Phenylmethanesulfonyl fluoride (PMSF) and 0.1 mM Sodium orthovanadate.

5.3. Prepare Laemmli buffer: (4x: 0.18 M Tris-HCl pH 6.8; 5 M glycerol; 3.7 % SDS (p/v); 0.6 M β-mercaptoethanol or 9 mM DTT; 0.04 % (v/v) bromophenol blue (BB)).

6. Protein extraction

Note: Protein extraction was performed as previously described[20,23]. **Carry out this whole section of the procedure at 4 °C.**

6.1. Aspirate the culture medium completely.

6.2. Wash 3 x 10 mL of with ice-cold phosphate buffered saline (PBS) per 150 cm^2 very carefully to avoid cell detachment.

6.3. To obtain the cell lysate, add 3 mL of lysis buffer per 150 cm^2 and thoroughly scrape the surface by using a cell scraper. Tilting the plate/flask to about 45 degrees will make easier to gather the cell lysates into their corresponding tubes.

6.4. Pour 1 mL of the cellular lysate per tube in three 1.5 mL tubes. These tubes will be marked with the condition and the replicate as indicated in step 3.1.

7. Pull-down

7.1. Centrifuge the 1.5 mL tubes at 11000 x g for 10 min at 4 °C.

7.2. Transfer the supernatants to new tubes (A).

7.3. Take an aliquot of this supernatant per condition to different tubes (B), i.e. 50 µL per tube. Add 4x Laemmli buffer (16.6 µL for 50 µL of lysate) and freeze at -20 °C. These lysates will serve as usual Western blot samples.

7.4. Homogenize the NeutrAvidin Agarose very thoroughly by gentle shaking. Cut the tips of the pipette tips to increase their diameter and improve the pipetting of the beads. Add 50 µL of agarose per mL of cell lysate in the (A) tubes.

7.5. Incubate at 4 °C overnight with very gentle shaking to allow the agarose to interact with BCPPs bound to their intracellular partners.

7.6. Centrifuge at 3000 x g for 1 min at 4 °C to collect the complex of NeutrAvidin with proteins.

7.7. Carefully remove the supernatants and transfer them to clean tubes (C). Keep them to use them in case the pull-down is not successful.

7.8. Washing steps: add fresh lysis buffer to the pellets (tubes (A)), resuspend by inversion and repeat the steps 7.6) and 7.7) for 5 more times. From here on the supernatants can be discarded.

7.9. Remove the supernatants and add 2x Laemmli buffer to the desired final volume (40 µL per 1.5 mL tube for pellets obtained from 150 cm^2 flasks of confluent cells).

7.10. Elute at 100 °C for 5 min to dissociate the interactions between proteins and centrifuge for 30 s at 8200 x g to pellet NeutrAvidin beads.

7.11. Take the supernatants, containing the dissociated proteins, with capillary tips to new tubes (D). The beads can be kept in case repeating elution steps is required.

Note: The supernatants (tubes D) are now ready to be loaded in Western blot or to freeze at -20 °C.

8. Western blot

Note: Western blotting was performed as previously described24.

8.1. Load equivalent volume of each sample per lane on Bis-Tris (4-12%) midigels in a Midi-Cell Electrophoresis System.

8.2. Transblot proteins using a dry blotting system into a nitrocellulose regular stack.

8.3. Stain the membrane with 10% Ponceau for 10 min.

8.4. Wash the membranes 3 x 5 min with 5 mL of TTBS.

8.5. Block the membranes with 7% milk in TTBS for 1 h with gentle shaking in tubes or small boxes. Make sure the volume used is enough to cover the membranes and that they do not dry, i.e. 40 mL per whole midi membrane.
8.6. Wash 3 x 5 min with 5 mL of TTBS.

8.7. Incubate overnight at 4 °C with the primary antibody against the protein of interest with gentle shaking.

8.8. Wash 3 x 5 min with 5 mL of TTBS.

8.9. Incubate the membrane at room temperature with the correspondent peroxidase-conjugated secondary antibody in TTBS for 1 h.

8.10. Wash 3 x 5 min with 5 mL of TTBS.

8.11. Develop with a chemiluminescent substrate in a chemiluminescence system.

9. Problem solving
9.1. If after developing the Western blot any of the samples showed no signal at all for proteins, check the Ponceau.

Note: The membrane in Ponceau should show undefined and numerous bands along the lanes loaded. If the bands are not very noticeable, the quantity of proteins loaded may not be enough or the transference may have gone incorrectly. Consider repeating the Western blot.

9.2. If there is no stain at all in the Ponceau membrane in any lane, repeat the whole procedure from the step 7.4 in the (C) tubes.

9.3. If after developing the Western blot a protein signal is very weak but the Ponceau showed proteins, incubate again the membrane with the primary antibody for longer time. If the signal is still rather weak, incubate again at room temperature.

10. Other techniques used in this article
10.1. Immunocytochemistry of BCPPs
10.1.1. Fix cells in 4% paraformaldehyde (0.2 mL per cm2) for 20 min.

10.1.2. Wash 3x 5 min with PBS (0.2 mL per cm2).

10.1.3. Apply the corresponding antibody (at least 0.15 mL per cm2) diluted in antibody solution at the manufacturer's indicated concentration against your protein of interest overnight at 4 °C.

10.1.4. Incubate with a fluorophore-conjugated secondary antibody (0.15 mL per cm^2) for 2 h at room temperature.

10.1.5. Repeat steps 10.1.2-10.1.4 with other antibodies against your proteins of interest. Be careful not to use the same species for different primary antibodies or same wavelength fluorophores for secondary antibodies.

10.1.6. Mount the cells using the antifade reagent (0.005 mL per cm^2).

10.1.7. Analyze on a fluorescence microscope connected to a digital camera.

10.2. MTT assay

10.2.1. Culture the cells at 37 °C in 24-well plates.

10.2.2. Incubate the cells in the dark for 75 min with 0.15 mL of culture medium per cm2 containing 0.5 mg/mL MTT.

10.2.3. Aspirate the medium and incubate the cells for 10 min in the dark with dimethyl sulfoxide (0.25 mL per cm2) with mild shaking.

10.2.4. Measure the absorbance at a wavelength of 570 nm using a microplate reader.

REPRESENTATIVE RESULTS:

Before using BCPPs to study intracellular interaction, it is critical to compare the effects of BCPP vs CPP to validate the results obtained with BCPP. Consequently, to study whether the inclusion of biotin modifies the activity of the target sequence, we first analyzed the effect of TAT-$Cx43_{266-283}$-B compared with TAT-$Cx43_{266-283}$ on G166 GSCs morphology. To do so, we performed some immunofluorescence analyses of two cytoskeletal proteins, F-actin and α-tubulin after 24 h of treatment. Figure 1 shows that G166 GSCs in the presence of 50 μM TAT-$Cx43_{266-283}$ or TAT-$Cx43_{266-283}$-B acquire a more rounded shape compared to the elongated and expanded cellular prolongations shown in the controls (TAT or TAT-B). In fact, Figure 1b shows that actin filaments are mostly assembled as actin networks when the cells were treated with TAT-$Cx43_{266-283}$ or TAT-$Cx43_{266-283}$-B while they form more actin bundles in the control cells (treated with TAT or TAT-B)[25]. In contrast, α-tubulin distribution does not vary between the different conditions. These results showed that the presence of biotin did not modify the effect of the target sequence on the morphology of G166 GSCs. In previous studies[20,21], we showed that TAT-$Cx43_{266-283}$ reduced G166 GSCs proliferation. In this study, we investigated whether TAT-$Cx43_{266-283}$-B exerts the same effects in the growth as TAT-$Cx43_{266-283}$. To do so, we analyzed the G166 GSCs proliferation by MTT assay after 72 h of treatment. The MTT assay is a colorimetric assay for assessing cell metabolic activity. MTT is metabolized by NAD(P)H oxidoreductase enzymes in mitochondria reflecting the number of viable cells present. Figure 2 shows that the reduction in the G166 GSCs cell viability is not significantly different when cells were treated with 50 μM TAT-$Cx43_{266-283}$ or 50 μM TAT-$Cx43_{266-283}$-B. Indeed, both significantly diminished G166 GSCs proliferation as compared to the control, TAT or TAT-B.

Once we confirmed that the effect of our target sequence in G166 GSCs (TAT-$Cx43_{266-283}$) was not modified by the inclusion of biotin at the C-terminus (TAT-$Cx43_{266-283}$-B), we investigated the intracellular partners of this sequence following the protocol described in this study (Figure 3). Because caveolae have been involved in the mechanism of TAT internalization[26], we analyzed the presence of caveolin-1 (Cav-1) in the pull-downs. Western blot analysis (Figure 4) showed that TAT-B and TAT-$Cx43_{266-283}$-B interact with Cav-1. However, the ability of TAT-$Cx43_{266-283}$-B to recruit c-Src, PTEN and CSK is stronger than that found with TAT-B. Focal adhesion kinase (FAK) is a substrate of c-Src that has not been shown to interact with Cx43. Indeed, FAK did not show any significant interaction with either TAT-B or TAT-$Cx43_{266-283}$-B.

To confirm the interaction between TAT-$Cx43_{266-283}$-B and c-Src, G166 GSCs were incubated with 50 μM TAT-$Cx43_{266-283}$-B for 30 min and their localization was followed with fluorescent streptavidin by confocal microscopy (Figure 5). The results showed that the intracellular distribution of TAT-$Cx43_{266-283}$-B is close to the plasma membrane (shown by phosphatidylserine staining) and matches with that of c-Src. In fact, co-localization analyses revealed some points of co-localization (white) between TAT-$Cx43_{266-283}$-B and c-Src in the merge image. Consequently, confocal microscopy studies confirm the results obtained with the BCPP pull-down protocol described in this study.

Figure 1: Effect of BCPP and CPP on GSC morphology.
G166 GSCs were plated at a low density (2 x 104 cells / cm^2) and after 24 h they were incubated with 50 μM control CPP (TAT), control BCPP (TAT-B), treatment CPP (TAT-$Cx43_{266-283}$) or treatment BCPP (TAT-$Cx43_{266-283}$-B). a) F-actin (red), α-tubulin (green) and merged + DAPI immunostaining of the same field showing G166 GSCs morphology. Bars = 50 μm. b) F-actin immunostaining showing the different distribution of F-actin in G166 GSCs after incubation for 24 h with 50 μM control CPP (TAT) or control BCPP (TAT-B) as compared with 50 μM treatment CPP (TAT-$Cx43_{266-283}$) or BCPP (TAT-$Cx43_{266-283}$-B). Bars = 10 μm.

Figure 2: Effect of BCPP and CPP on GSC viability.
G166 GSCs were plated at 5500 cells/cm^2 in 24-multiwell plates and incubated with 50 μM control peptides, CPP (TAT) or BCPP (TAT-B), or 50 μM treatment peptides, CPP (TAT-$Cx43_{266-283}$) or BCPP (TAT-$Cx43_{266-283}$-B). The cell viability was analyzed using a MTT assay after 72 h. The results are expressed as MTT absorbances and are the mean ± s.e.m. of at least 3 experiments (++ $p<0.01$ vs control. ** $p<0.01$, *** $p<0.001$ vs TAT or TAT-B; one-way ANOVA with Tukey post-test). Note that there are not significant differences between the effects of CPPs vs BCPPs.

Figure 3: Protocol diagram.
Step by step graphical depiction of the procedure as described in the section “Protocol”, from the incubation of the BCPPs until the eluted BCPPs and their interacting proteins were obtained. 1) Incubate culture cells with BCPPs at the

desired concentration for the required time. 2) During the incubation, the BCPPs are internalized and they interact with their intracellular partners. 3) Wash the cells three times on ice with ice-cold PBS. 4) Lyse the cells to extract proteins. 5) Transfer cell lysates to tubes. 6) Spin at 11000 x g for 10 min at 4 °C. 7) Transfer the supernatants to new tubes (A) and keep a small aliquot of the lysates to process as regular Western blot samples in tube (B). 8) Resuspend the NeutrAvidin Agarose beads and add 50 µL to each tube A using a cut pipette tip. 9) Incubate with gently shaking for 12 h at 4 °C to allow the NeutrAvidin agarose beads to interact with BCPPs and their partners. 10) Spin for 1 min at 3000 x g to pellet the beads with the biotinylated baits and their interacting proteins bound to them. 11) Transfer supernatants to new tubes (C) and keep them to use in case the pull-down need to be repeated. 12) Wash the pellet five times with fresh lysis buffer, resuspend by inversion, spin for 1 min at 3000 x g and discard the supernatant. 13) Remove all the supernatant carefully. 14) Add the desired volume of 4x Laemmli buffer and elute the proteins at 100 °C for 5 min. 15) Spin at 8200 x g for 30 s to pellet the beads. 16) Transfer the eluted proteins found in the supernatant with capillary tips to new tubes (D). 17) Load onto gels for Western blot analysis.

Figure 4: Study of the intracellular interactions of TAT-Cx43$_{266-283}$-B in G166 GSCs by pull-down followed by Western blot.
G166 GSCs were incubated with 50 µM TAT-B or TAT-Cx43$_{266-283}$-B. After 30 min the cells were lysed and TAT-B or TAT-Cx43$_{266-283}$-B attached to their intracellular partners were pulled down with NeutrAvidin beads. The eluted proteins were loaded and analyzed by Western blot to study the levels of FAK, c-Src, CSK, PTEN and Cav-1. Note that Cav-1 interacts with both TAT-B and TAT-Cx43$_{266-283}$-B, c-Src, PTEN and CSK interact preferentially with TAT-Cx43$_{266-283}$-B and FAK did not show any interaction with either TAT-B or TAT-Cx43$_{266-283}$-B.

Figure 5: Confirmation of TAT-Cx43$_{266-283}$-B intracellular interactions in G166 GSCs by confocal microscopy.
G166 GSCs were incubated with 50 µM TAT-Cx43$_{266-283}$-B. After 30 min, cells were fixed and processed to localize TAT-Cx43$_{266-283}$-B with Cy2-Streptavidin (green), c-Src by immunofluorescence (red) and phosphatidylserine with annexin V (blue). Note some points of co-localization (white) between TAT-Cx43$_{266-283}$-B and c-Src close to the plasma membrane in the merge images.

DISCUSSION:
There are many methods to study protein-protein interactions. The method presented in this study is based on the widely used biotin-avidin pull-down system in which a biotinylated bait is incubated with cell lysates to allow the establishment of interactions. The modification presented in this study includes the combination of this technique with cell-penetrating sequences. We propose to design cell-penetrating baits that can be incubated with living cells instead of cell lysates and therefore the interactions found will reflect those that occurred within the cellular context.

Here we use TAT as the cell-penetrating sequence, biotin as the pull-down tag and

the region of Cx43 comprised between amino acids 266-283 as the target to find intracellular interactions in human GSCs. The structural basis for the interaction between Cx43 and c-Src is well known[11,12]. This is an important interaction because it inhibits the oncogenic activity of c-Src in glioma cells[24,13]. In fact, CPPs containing this region (TAT-$Cx43_{266-283}$) mimic the antioncogenic properties of Cx43 on glioma cells[19,20,21]. In rat glioma C6 cells, the mechanism by which TAT-$Cx43_{266-283}$ inhibits c-Src includes the recruitment of c-Src together with its endogenous inhibitors CSK and PTEN[20]. It should be mentioned that GSCs are very interesting as a target in glioma therapy, because they constitute a subpopulation that is resistant to conventional treatments and therefore responsible for the recurrence of this malignant brain tumors[27]. Furthermore, they are hard-to-transfect cells and therefore the study of intracellular interactions becomes more difficult. CPPs are rapidly and efficiently internalized in GSCs[19] favoring their use for the study of intracellular interactions. In this study, using CPPs fused to biotin we confirm the interaction of the $Cx43_{266-283}$ sequence with c-Src together with its endogenous inhibitors CSK and PTEN in human GSCs.

This method is very powerful to study the intracellular mechanism of bioactive compounds. However, it is very important to confirm that the biological effect of the biotinylated cell penetrating bait is not different from that obtained with the non-biotinylated one. This step is required to associate the interactions found with the effect of the bioactive compound. In addition, the stability of the compound, its possible degradation by proteases as well as its possible toxicity, should be carefully tested and taken into account before planning the experiment. In the example presented, the anti-proliferative effect of TAT-$Cx43_{266-283}$ on G166 human GSCs has been previously documented[20]. In this study, we confirm that the anti-proliferative effect of TAT-$Cx43_{266-283}$-B and of TAT-$Cx43_{266-283}$ is very similar. In addition, the analysis of cellular morphology revealed that α-tubulin and F-actin distribution is very similar in G166 GSCs treated with TAT-$Cx43_{266-283}$-B or with TAT-$Cx43_{266-283}$. Altogether, these results indicate that the inclusion of biotin at the c-terminus of TAT-$Cx43_{266-283}$ did not modify the effects of this compound on human GSCs. However, if biotin would modify the effects of the bioactive molecule, other tags for protein purification can be tested, such as the FLAG octapeptide (DYKDDDDK)[28], the human influenza hemagglutinin-derived tag HA (YPYDVPDYA) or glutathione S-transferase (GST)[29]. Similarly, if TAT does not target the cell population of interest, other cell penetrating sequences, such as penetratin, MPG (for a review, see[30]) or cell specific sequences can be used[31].

In addition to study proteins that specifically interact with the target sequence, ideally, the presence of proteins that interact with both the control and the target sequence and proteins that do not interact with them, as positive and negative controls, should be addressed. In this sense, we found Cav-1 in the control and treated situation, suggesting that the caveolae have been involved in the mechanism of internalization, as it has been previously shown[26]. Furthermore, FAK, which interacts with c-Src but is supposed not to interact with the Cx43 c-terminal, was absent in both the control and treated situation. These results reinforce the specificity of the interaction

between TAT-$Cx43_{266-283}$-B, c-Src, CSK and PTEN. To confirm the results obtained with this protocol, confocal microscopy can be used to visualize the distribution of the interacting proteins and to study their co-localization. Thus, we found that TAT-$Cx43_{266-283}$-B and c-Src exhibit a similar intracellular distribution with some points of co-localization confirming the results obtained with the pull-down experiments. In fact, TAT-$Cx43_{266-283}$-B is distributed close to the plasma membrane suggesting that the cargo, in this study $Cx43_{266-283}$, directs the molecule to its intracellular partners.

One of the limitations of the proposed method is that the molecule used as bait could fail to fold properly and the expected effects would not be found. In this situation, the interactions found could not be associated to the effect. However, this method can be especially interesting for the interactions involved in signal transduction pathways because they are usually carried out by intrinsically disordered regions[32] and therefore they do not require an ordered folding. In addition, one of the advantages of the proposed method is that the time course of the interaction can be followed, which is especially relevant for transient interactions. Furthermore, the relevance of each residue on the interaction can be easily studied. Indeed, it is possible to study the relevance of posttranslational modifications on protein-protein interaction, for instance, by phosphomimetic substitution of glutamate for serine or threonine. Similarly, substitution of serine or threonine for alanine or tyrosine for phenylalanine allows testing the effect of non-phosphorylatable serine, threonine or tyrosine. To mimic phospho-tyrosine, the most accurate way is the substitution of Tyr for p-Tyr[33].

Finally, the scope of this protocol is far beyond protein-protein interaction because this system can be applied to other bioactive cargoes such as RNA sequences, nanoparticles, viruses or other molecules that can be fused to biotin and transduced with CPP to study their intracellular mechanism of action.

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DISCLOSURES:
The authors have nothing to disclose.

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Figure 1.

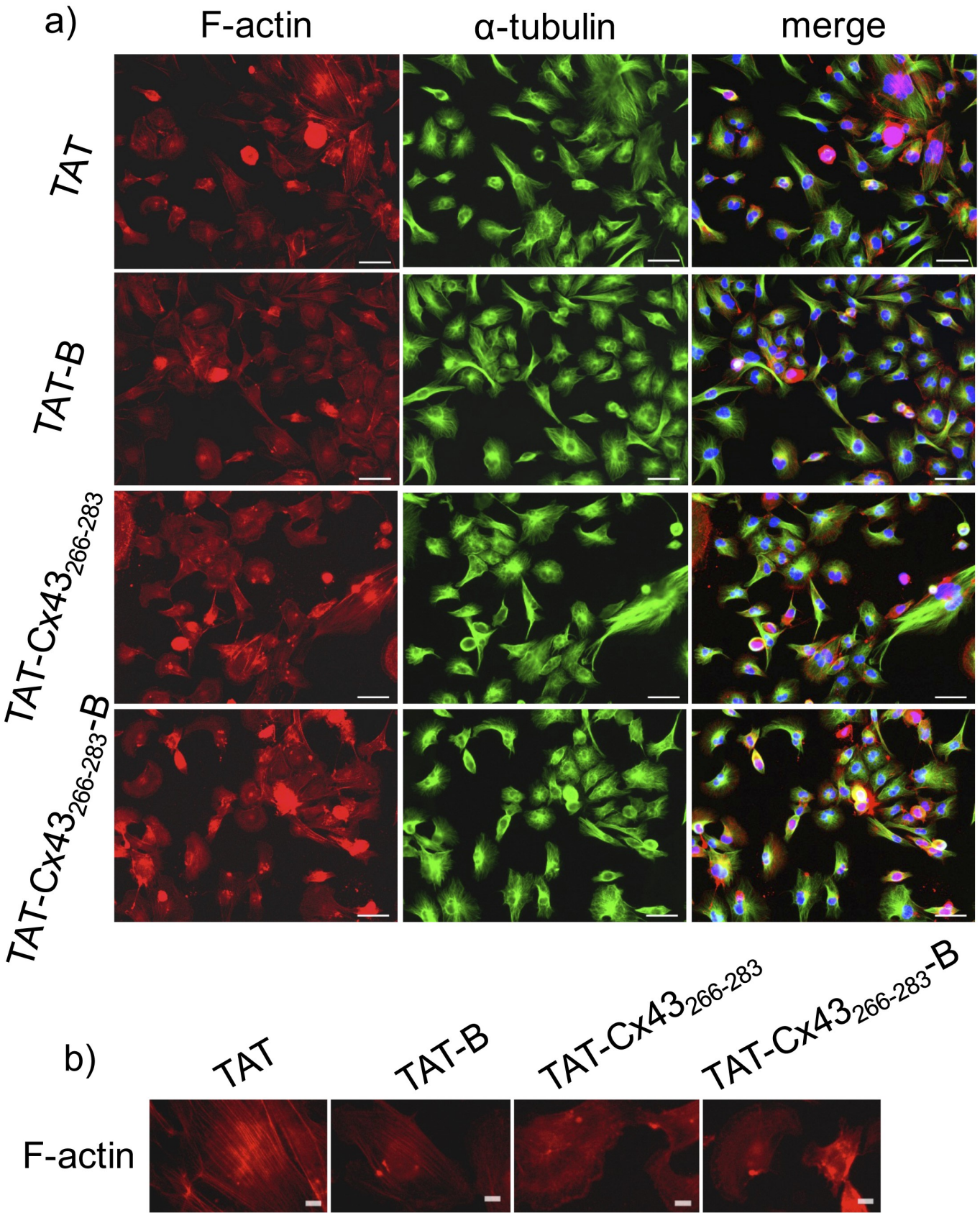

a)
F-actin
α-tubulin
merge
TAT
TAT-B
TAT-Cx43266-283
TAT-Cx43266-283-B
b)
TAT
TAT-B
TAT-Cx43266-283
TAT-Cx43266-283-B
F-actin

Figure 2.

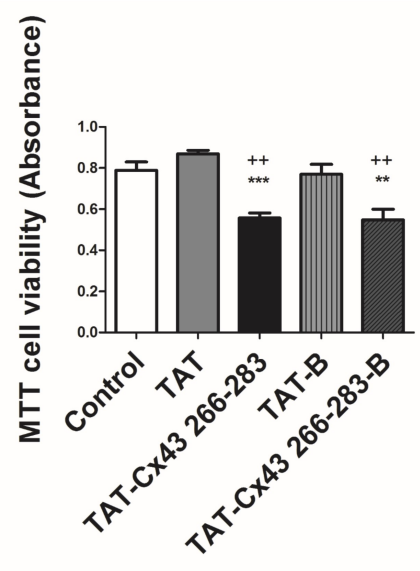


Figure 3

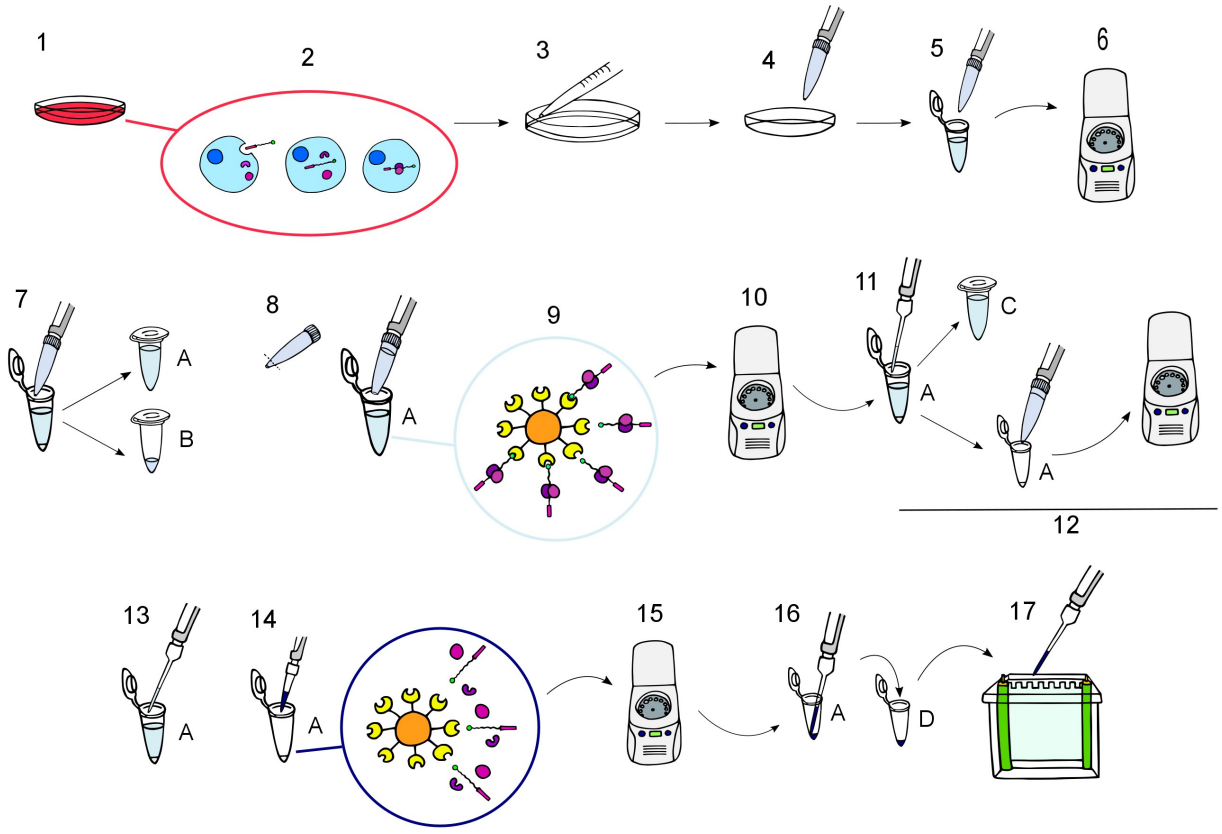


Figure 4.

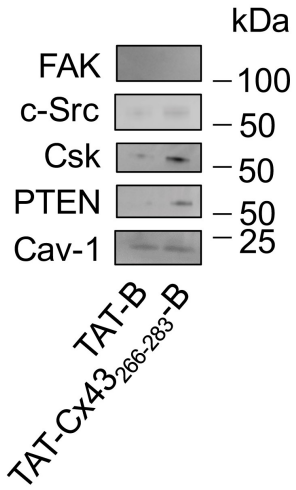
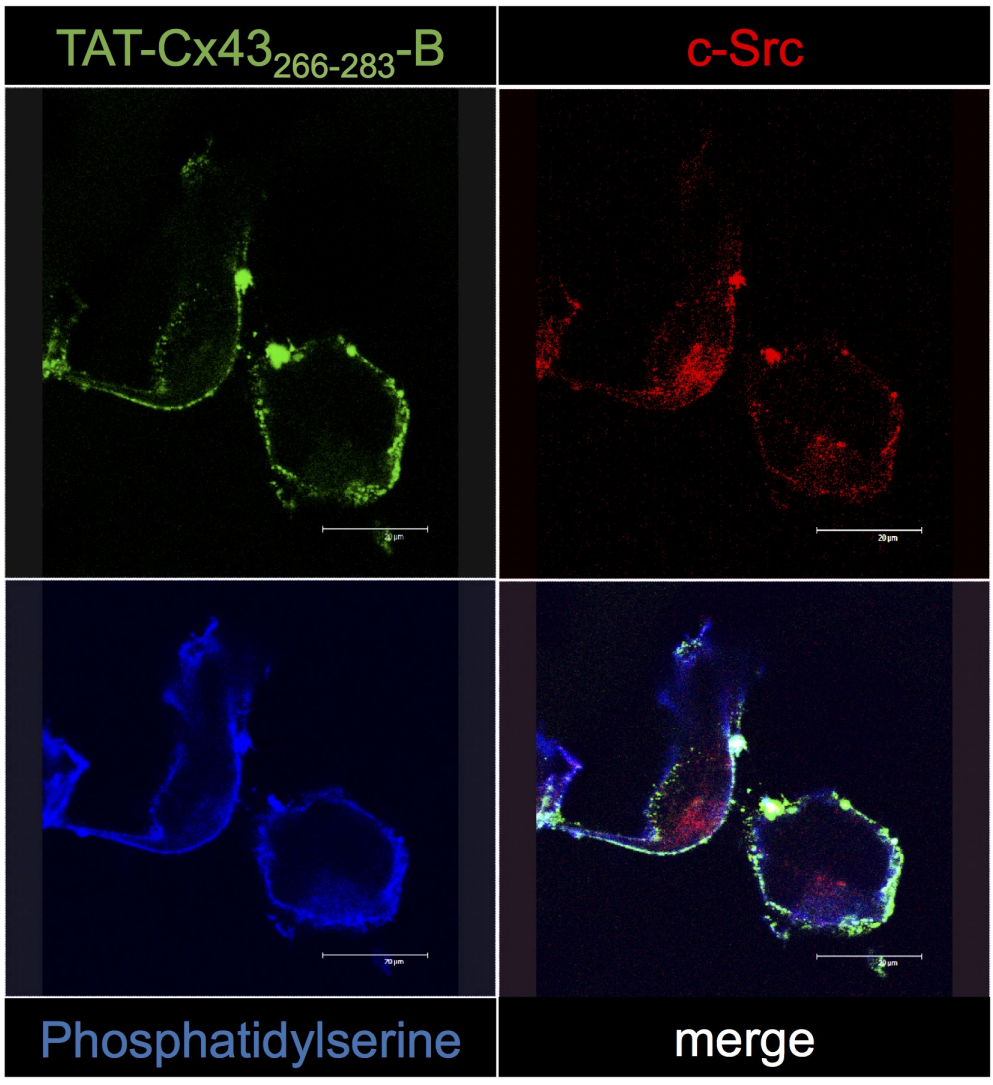


Figure 5.



CAPíTVLO 3

Efecto y mecanismo de TAT-Cx43$_{266-283}$ en la migración y la invasión de células madre de glioma derivadas de pacientes

A short region of connexin43 reduces human glioma stem cell migration, invasion ad survival through Src, PTEN and FAK. Jaraíz-Rodríguez M, Tabernero MD, González-Tablas M, Otero A, Orfao A, Medina JM, Tabernero A. *Stem Cell Reports* (2017), http://dx.-doi.org/10.1016/j.stemcr.2017.06.007, en prensa.

Teniendo en cuenta que una de las proteínas con las que interacciona c-Src, es la quinasa de adhesión focal (FAK), necesaria para el establecimiento de las adhesiones focales que permiten la migración e invasión, en este trabajo estudiamos si la inhibición de c-Src mediante TAT-Cx43$_{266-283}$, afecta a la migración e invasión de células madre de glioma derivadas de pacientes. Para llevar a cabo este objetivo, pusimos a punto la obtención y cultivo de células madre de glioma a partir de biopsias de glioblastoma de pacientes, en colaboración con el Servicio de Neurocirugía del Hospital Universitario de Salamanca. Además, también estudiamos los efectos antitumorales de TAT-Cx43$_{266-283}$ en explante de gliomas de los mismos pacientes a partir de los cuales obtuvimos las células madre de glioma. Para ello, analizamos los niveles de c-Src, PTEN y FAK mediante Western blot, y realizamos un estudio de la movilidad y la invasión de estas células en presencia de TAT-Cx43$_{266-283}$. Los resultados mostraron que TAT-Cx43$_{266-283}$ disminuye la actividad de c-Src, y como consecuencia, se produce un aumento de los niveles de PTEN y se reduce la actividad de FAK. Mediante microscopía de célula viva determinamos que TAT-Cx43$_{266-283}$ disminuye la movilidad de estas células y, además, la capacidad invasiva para atravesar membranas recubiertas con una mezcla de componentes de la matriz extracelular. TAT-Cx43$_{266-283}$ también produjo un acusado efecto antitumoral en los explantes derivados de pacientes, disminuyendo la proliferación, migración y supervivencia de estas células. Por último, es importante mencionar que se trata de un efecto específico de esta secuencia, ya que otro péptido penetrante, TAT-Cx43$_{274-291}$, no disminuye ni la migración ni la proliferación de estas células.

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A Short Region of Connexin43 Reduces Human Glioma Stem Cell Migration, Invasion, and Survival through Src, PTEN, and FAK

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SUMMARY

Connexin43 (CX43), a protein that forms gap junction channels and hemichannels in astrocytes, is downregulated in high-grade gliomas. Its relevance for glioma therapy has been thoroughly explored; however, its positive effects on proliferation are counterbalanced by its effects on migration and invasion. Here, we show that a cell-penetrating peptide based on CX43 (TAT-Cx43$_{266-283}$) inhibited c-Src and focal adhesion kinase (FAK) and upregulated phosphatase and tensin homolog in glioma stem cells (GSCs) derived from patients. Consequently, TAT-Cx43$_{266-283}$ reduced GSC motility, as analyzed by time-lapse microscopy, and strongly reduced their invasive ability. Interestingly, we investigated the effects of TAT-Cx43$_{266-283}$ on freshly removed surgical specimens as undissociated glioblastoma blocks, which revealed a dramatic reduction in the growth, migration, and survival of these cells. In conclusion, a region of CX43 (amino acids 266–283) exerts an important anti-tumor effect in patient-derived glioblastoma models that includes impairment of GSC migration and invasion.

RESULTS

INTRODUCTION

Connexin43 (CX43) is an integral membrane protein that is widely expressed in astrocytes (Giaume et al., 2010) and is downregulated in high-grade gliomas, the most common malignant tumor of the CNS (Crespin et al., 2016; Huang et al., 1999; Pu et al., 2004; Shinoura et al., 1996; Soroceanu et al., 2001). Patients diagnosed with glioblastoma multiforme, the most aggressive form of glioma, have a median survival rate of 1–2 years (Gilbert et al., 2014) because of the infiltrative nature of these tumors, which facilitates recurrence after surgery and standard therapy. These tumors are composed of a heterogeneous population of cells, including many with stem cell-like properties, called glioma-initiating cells or glioma stem cells (GSCs). GSCs are characterized by their self-renewal capacity, high oncogenic potential, resistance to standard therapies (Chen et al., 2012; Dirks, 2010), and high invasive capacity (Cheng et al., 2011; Garcia et al., 2010).

GSCs express very low levels of CX43. When this protein is restored, the stem cell phenotype of GSC lines is reversed and their tumorigenicity is reduced (Gangoso et al., 2014; Yu et al., 2012). Our previous studies revealed that CX43 exerts this effect through the inhibition of c-Src activity. Thus, restoring CX43 inhibited the oncogenic activity of c-Src in different glioma cell lines (Gangoso et al., 2014; Gonzalez-Sanchez et al., 2016; Herrero-Gonzalez et al., 2010). Inhibition of c-Src is caused by a short region, residues 266–283, within the C-terminal domain of CX43 that recruits c-Src together with its inhibitors, C-terminal Src kinase (CSK) and phosphatase and tensin homolog (PTEN) (Gonzalez-Sanchez et al., 2016). In fact, cell-penetrating peptides containing the CX43 residues 266–283 (TAT-Cx43$_{266-283}$) are able to mimic these effects. Indeed, as a result of c-Src inhibition, TAT-Cx43$_{266-283}$ increased PTEN, with subsequent inactivation of AKT (Gonzalez-Sanchez et al., 2016), downregulated the expression of the inhibitor of differentiation (ID1), and the transcription factor, SOX-2, and promoted cadherin switching (Gangoso et al., 2014) in the GSC lines GliNS2 and G166. Consequently, the ability of GlinNS2 and G166 GSCs to proliferate and generate neurospheres decreased and the percentage of cells expressing differentiation markers increased in the presence of TAT-Cx43$_{266-283}$ (Gangoso et al., 2014; Gonzalez-Sanchez et al., 2016).

One important substrate of c-Src is focal adhesion kinase (FAK). c-Src binds to FAK and phosphorylates the tyrosine residues 576 and 577 in the activation loop of FAK (Calalb et al., 1995), thereby maximizing its kinase activity and creating additional protein binding sites (Mitra and Schlaepfer, 2006). This active FAK-Src complex stimulates the activity of RAC1 and CDC42 to increase membrane protrusions and mediates the transient suppression of RHOA-GTP levels, thereby facilitating cell spreading. Not surprisingly, both *Src*–/– and FAK (*Ptk2*)–/– fibroblasts display impaired migration (Ilić et al., 1995; Klinghoffer et al., 1999). c-Src and FAK are required for the processes of cell invasion and migration in tumor cells (Carragher

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et al., 2006; van Nimwegen et al., 2005), including glioblastoma (Du et al., 2009; Lindemann et al., 2011) and GSCs (Frolov et al., 2016; Liu et al., 2016). In fact, the activity of both c-Src and FAK are augmented in glioblastomas (Riemenschneider et al., 2005; Zhang et al., 2011). In addition, PTEN, as a tyrosine phosphatase, interacts directly with and dephosphorylates FAK (Cai et al., 2005; Tamura et al., 1998), leading to suppression of glioma cell migration and invasion (Park et al., 2002).

As previously mentioned, the major obstacle to developing a cure for glioblastoma is its diffuse invasion property, which enables GSCs to escape complete surgical resection, chemotherapy, and radiation therapy. Therefore, an important therapeutic aim is to reduce GSC motility and invasion. Because TAT-Cx43$_{266-283}$ inhibits c-Src activity and upregulates PTEN, in this study, we investigated whether TAT-Cx43$_{266-283}$ reduces FAK activity with subsequent impairment in GSC motility and invasive capacity.

RESULTS

TAT-Cx43$_{266-283}$ Inhibits c-Src Activity and Upregulates PTEN in Primary Glioblastoma Stem Cells

Our previous studies showed that cell-penetrating peptides containing the residues of CX43 involved in the interaction with c-Src (residues 266–283) fused to the TAT-penetrating sequence (TAT-Cx43$_{266-283}$) inhibit c-Src activity in the human GSC lines GliNS2 and G166 (Gangoso et al., 2014; Gonzalez-Sanchez et al., 2016). In this study, we investigated whether this effect also occurs in GSCs derived directly from patients (primary GSCs). To do so, primary GSCs were obtained and cultured from human glioblastoma biopsies immediately after surgery, as described previously (Pollard et al., 2009; Thirant et al., 2011). As expected, most of the cells in these cultures expressed the stem cell markers SOX-2 and Nestin (Figures 1A and S1). The c-Src activity was analyzed by measuring the levels of c-Src phosphorylated at Tyr416 (Y416 c-Src), the active form of this tyrosine kinase (Kmiecik and Shalloway, 1987). Our results showed that the level of activated c-Src decreased after 24 hr of incubation with 50 μM TAT-Cx43$_{266-283}$ compared with cells incubated with 50 μM TAT-penetrating peptide (TAT) or with control (Figures 1B and 1C) in primary GSCs derived from five glioblastoma patients (G9, G12, G13, G15, and G16). These results indicate that TAT-Cx43$_{266-283}$ inhibits c-Src activity also in primary GSCs.

As a result of c-Src inhibition, TAT-Cx43$_{266-283}$ increases PTEN, with subsequent AKT inactivation and a reduction in G166 GSC proliferation and survival (Gonzalez-Sanchez et al., 2016). Because PTEN is one of the most relevant tumor suppressor proteins in gliomas (Cancer Genome Atlas Research Network, 2008), in this study, we analyzed the effect of TAT-Cx43$_{266-283}$ on PTEN in primary GSCs. Our data revealed that TAT-Cx43$_{266-283}$ upregulated the PTEN levels in these primary GSCs (Figures 1B and 1D).

TAT-Cx43$_{266-283}$ Targets FAK

FAK autophosphorylation at Tyr397 (Y397 FAK) creates a binding site for c-Src that phosphorylates FAK at Tyr576 (Y576 FAK) and Tyr577 (Y577 FAK), promoting maximal FAK catalytic activity. In fact, FAK phosphorylation at Y576 and Y577 is required for maximal Y397 phosphorylation (Ruest et al., 2000). In addition, PTEN dephosphorylates Y397 FAK, which decreases FAK activity (Tamura et al., 1998). Therefore, in this study, we investigated whether changes in the c-Src activity and PTEN levels promoted by TAT-Cx43$_{266-283}$ impact FAK activity in GSCs.

Our results showed that, except for G12 GSCs, in which changes were not statistically significant, incubation with 50 μM TAT-Cx43$_{266-283}$ for 24 hr decreased the Y397 FAK phosphorylation levels compared with incubation with TAT or control in primary GSCs (Figures 1B and 1E).

We also analyzed the effect of TAT-Cx43$_{266-283}$ on FAK residues that were directly phosphorylated by c-Src (Y576 and Y577). Figure 2A shows that primary G9 GSCs incubated with 25 or 50 μM TAT-Cx43$_{266-283}$ for 15 hr had lower levels of Y576 and Y577 FAK compared with those incubated with the same TAT concentrations. In fact, as soon as 4 hr after 50 μM TAT-Cx43$_{266-283}$ incubation, the c-Src activity decreased in primary GSCs, although no changes in FAK phosphorylation were found (Figure S2). However, 2 hr later, i.e., 6 hr after incubation with 50 μM TAT-Cx43$_{266-283}$, both the c-Src activity and FAK phosphorylation levels decreased compared with the TAT treatment (Figure S2), suggesting that inhibition of c-Src activity leads to a reduction in FAK phosphorylation. Similarly, FAK phosphorylation at Y576 and Y577 was reduced by TAT-Cx43$_{266-283}$ in G166 GSCs (Figure 2B), a cell line in which the anti-tumor effect of TAT-Cx43$_{266-283}$ has been shown to be caused by c-Src inhibition (Gonzalez-Sanchez et al., 2016).

TAT-Cx43$_{266-283}$ Reduces GSC Motility

FAK plays a key role in both normal and tumor cell migration downstream of growth factor and integrin receptors (McLean et al., 2005). Because TAT-Cx43$_{266-283}$ inhibits FAK activity (Figures 1B, 1E, and 2), we investigated the effect of TAT-Cx43$_{266-283}$ on GSC migration. Primary G9, G13, G16, or G166 GSCs were exposed to TAT or TAT-Cx43$_{266-283}$ for 15 hr, and their movements were followed by time-lapse microscopy (representative movies are shown as Movies S1, S2, and S3). These movies show that 50 μM TAT-Cx43$_{266-283}$ greatly reduced cell motility in primary G9 GSCs (Movie S3) compared with the

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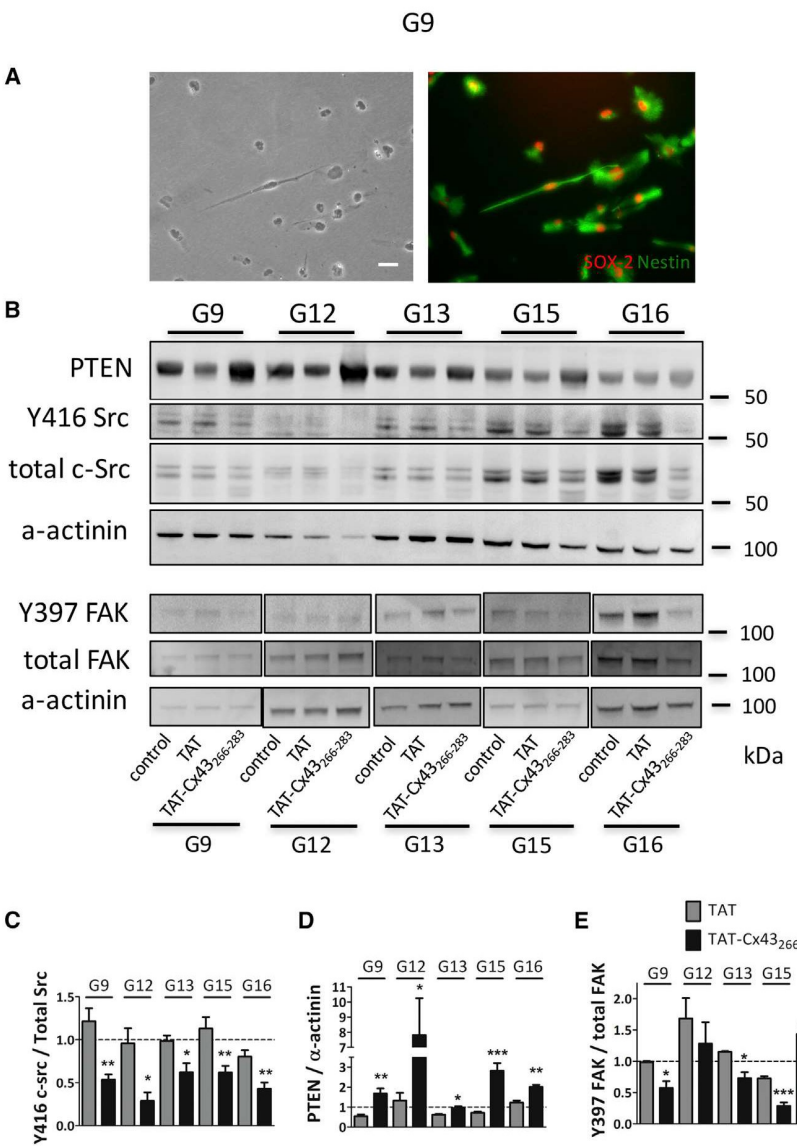

Figure 1. TAT-Cx43$_{266-283}$ Targets c-Src, PTEN, and FAK in Primary GSCs

(A) Phase-contrast images (left) and SOX-2 (red) and Nestin (green) immunostaining (right) of the same field showing that primary G9 GSCs express these GSC markers. Scale bar, 50 μm.

(B) Primary G9, G12, G13, G15, and G16 GSCs were incubated with 50 μM TAT or 50 μM TAT-Cx43$_{266-283}$. After 24 hr, the PTEN, total c-Src, Y416 c-Src, total FAK, and Y397 FAK levels were analyzed by western blotting.

(C–E) Quantification of the Y416 c-Src/total c-Src (C), PTEN/α-actinin (D), and Y379 FAK/total FAK (E) ratios. The results were normalized with their corresponding controls (assigned a value of 1; dotted line) and are the means ± SEM of at least three independent experiments (ANOVA; ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$; TAT-Cx43$_{266-283}$ versus TAT). See also Figure S1.

corresponding concentrations of TAT (Movie S2) or with the control (Movie S1). To quantify these effects, individual cell trajectories were tracked. Our results clearly show that the trajectories described for 12–14 hr by G166 GSCs (Figure 3A) and primary G9, G13, and G16 GSCs (Figures 3C, 4A, and 4C), were shorter when they were exposed to 25 or 50 μM TAT-Cx43$_{266-283}$ compared with TAT or control. Indeed, TAT-Cx43$_{266-283}$, at concentrations ranging from 25 to 50 μM, caused a significant reduction in the path length described by both G166 and primary GSCs (Figures 3B, 3D, 4B, and 4D).

To test whether these effects were carried out specifically by TAT-Cx43$_{266-283}$, we checked the effect of the 274–291 CX43 sequence fused to TAT (TAT-Cx43$_{274-291}$). It should be mentioned that the 274–291 CX43 sequence does not recruit c-Src together with its inhibitors, PTEN and CSK (Gonzalez-Sanchez et al., 2016), and consequently does not reduce Src activity with the subsequent reversion of the GSC phenotype in GliNS2 GSCs (Gangoso et al., 2014). Our results showed that TAT-Cx43$_{274-291}$ did not significantly modify the length of the primary G13 and G16 GSC trajectories (Figures 4B and 4D). To confirm the participation of FAK in the mechanism by which TAT-Cx43$_{266-283}$ reduced GSC motility, FAK activity was inhibited with 5 μM FAK inhibitor 14. Figures 4B and 4D reveal that G13 and G16 GSC motility was strongly reduced by the FAK inhibitor compared with the control, indicating that FAK activity is required for GSC motility.

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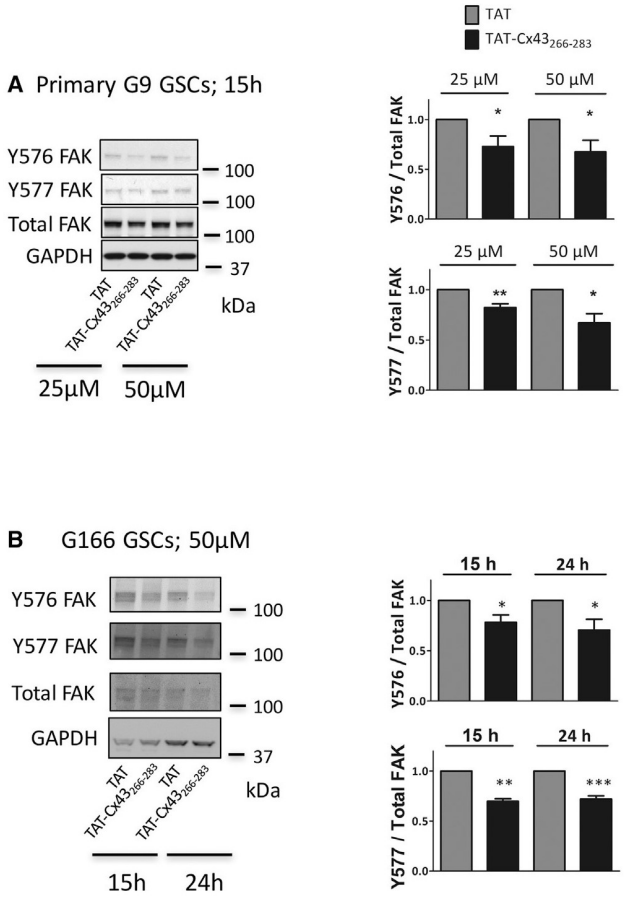

Figure 2. TAT-Cx43$_{266-283}$ Reduces Y576 and Y577 FAK Phosphorylation in GSCs

Primary G9 (A) or G166 (B) GSCs were incubated with TAT or TAT-Cx43$_{266-283}$. Western blot analysis for FAK, Y576 FAK, and Y577 FAK. The results were normalized with their corresponding TAT (assigned a value of 1) and are the means ± SEM of at least four independent experiments (***p < 0.001, **p < 0.01, *p < 0.05; TAT-Cx43$_{266-283}$ versus TAT, t test). See also Figure S2.

In fact, the effect of FAK inhibitor 14 plus TAT-Cx43$_{266-283}$ did not affect or slightly increased this effect compared with the FAK inhibitor alone (Figures 4B and 4D), suggesting that Src-dependent pathways other than FAK have a minor contribution to the effect of TAT-Cx43$_{266-283}$ on migration.

TAT-Cx43$_{266-283}$ Reduces GSC Invasion

Because TAT-Cx43$_{266-283}$ affects c-Src, PTEN, and FAK, three proteins that share common pathways to promote tumor cell invasion, we further hypothesized that TAT-Cx43$_{266-283}$ could decrease the invasive capacity of GSCs. To address this point, cultures of primary G9 or G166 GSCs were established in a Matrigel transwell system. Matrigel is an assortment of extracellular matrix proteins, mainly laminin, collagen IV, and enactin, and it is considered to be a reconstituted basement membrane preparation (Hughes et al., 2010). Matrigel occludes transwell membrane pores, blocking non-invasive cells from migrating through. In contrast, invasive cells can degrade the matrix and move through the extracellular layer and adhere to the bottom of the transwell.

Typical fields obtained from the bottom of transwells after 15 hr of Matrigel invasion, as shown in Figure 5, show that the ability of the cells to invade the basement membrane was significantly compromised in TAT-Cx43$_{266-283}$-treated primary G9 and G166 GSCs (Figures 5A and 5B) relative to TAT-treated cells. When quantifying these results, we observed that TAT-Cx43$_{266-283}$ decreased the invasion capacity of the primary G9 and G166 GSCs by approximately 63%–66% and 34%–37%, respectively, relative to TAT-treated cells. It should be mentioned that TAT, even at 100 μM, only slightly (11% in primary G9 GSCs) modified or did not modify the invasive ability of these cells compared with the control (Figure S3). In addition, the observed reduction in the number of invading cells was not due to a decrease in cell proliferation or cytotoxicity, because TAT-Cx43$_{266-283}$ applied at such concentrations (up to 50 μM) for 15 hr did not affect or only slightly affected (9% and 13% in G9 at 25 and 50 μM, respectively; TAT-Cx43$_{266-283}$ versus TAT) the viability of primary G9 and G166 GSCs (Figures S4A and S4B).

TAT-Cx43$_{266-283}$ Dramatically Reduced Growth, Migration, and Survival of Glioblastoma Cells from Freshly Removed Surgical Malignant Glioma Specimens

To test the effect of TAT-Cx43$_{266-283}$ on GSC migration and invasion, concentrations ranging from 25 to 50 μM for 15 hr were chosen to avoid the effects related to GSC viability (Figures S4A–S4D). TAT-Cx43$_{266-283}$ inhibits the oncogenic activity of c-Src, which in addition to its effect on migration is involved in a diverse spectrum of cancer tumor events, including the stem cell phenotype, survival, differentiation, or proliferation (Zhang et al., 2011). In fact, Figures S4E and S4F and our previous studies show that TAT-Cx43$_{266-283}$ at concentrations ranging from 50 to 100 μM for 72 hr inhibited GSC proliferation (Gonzalez-Sanchez et al., 2016) and their stem cell phenotype (Gangoso et al., 2014).

It has been proposed that the culture of human glioblastoma explants provides a flexible and rapid platform for drug screening in a patient-specific fashion (Bayin et al., 2016). Therefore, in addition to studying the effect of TAT-Cx43$_{266-283}$ on GSC migration and invasion, we investigated its anti-tumor effect on freshly removed surgical specimens as undissociated tumor blocks. Thus, explants from

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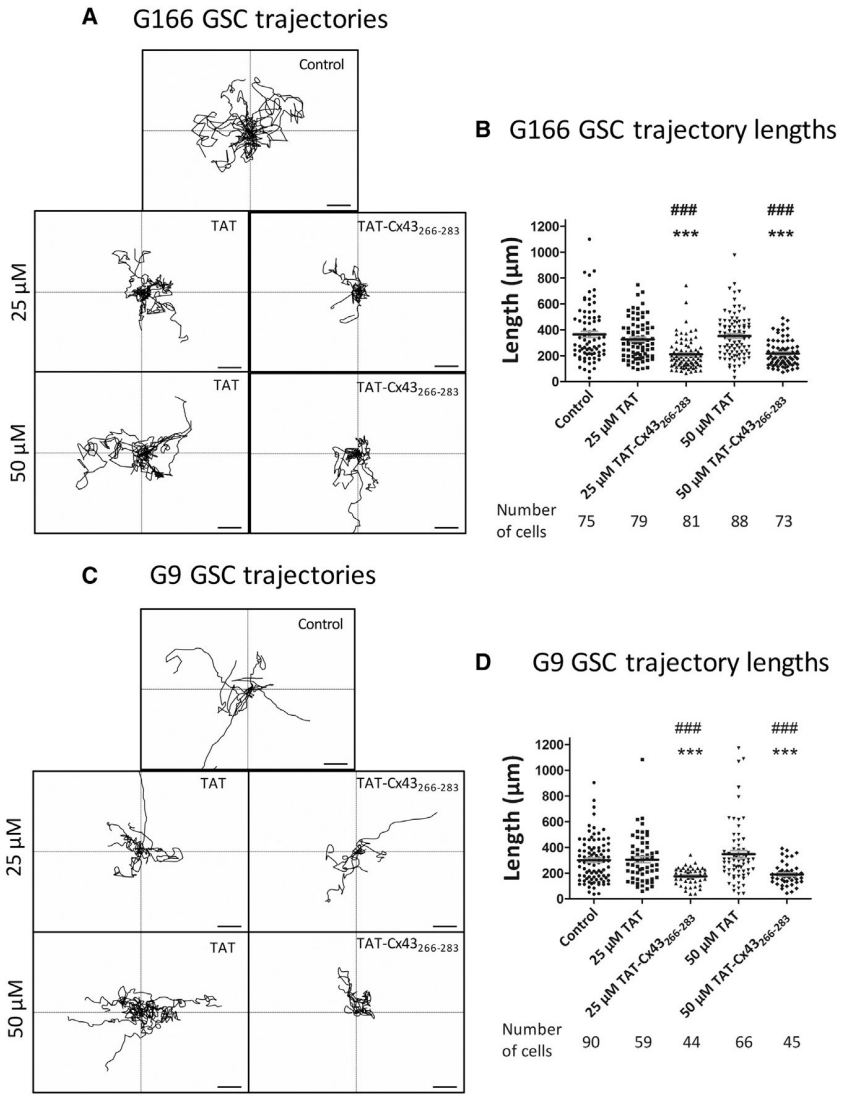

Figure 3. TAT-Cx43$_{266-283}$ Reduces GSC Motility

G166 (A and B) or primary G9 GSCs (C and D) were incubated with 25 or 50 µM TAT or TAT-Cx43$_{266-283}$, and their random movements were recorded by time-lapse microscopy for 12 hr (representative movies from primary G9 GSCs are available as Supplemental Information Movies S1, S2, and S3). Track-plots showing the trajectories described by nine representative G166 GSCs (A) or seven primary G9 GSCs (C). The origin of each cell trajectory is at the intersection of the x and y axes. Scale bars, 50 µm. (B and D) The migration length was extracted from the track-plots using the total path length described by each individual cell in at least six movies from three independent experiments. The line bisecting the plot corresponds to the mean (ANOVA; ***$p < 0.001$; TAT-Cx43$_{266-283}$ versus control, ###$p < 0.001$; TAT-Cx43$_{266-283}$ versus TAT). Non-significant differences were found when control, 25 µM TAT and 50 µM TAT levels were compared. The trajectories of cells that died during the time lapse were not considered. See also Movies S1, S2, and S3.

the same tumors used to obtain GSCs were cultured immediately after surgery, exposed to 100 µM TAT or TAT-Cx43$_{266-283}$, and cell behavior was monitored by time-lapse microscopy. Despite the different cell morphologies and features found in each tumor, in the control the cells grew rapidly and exhibited high motility and proliferation consistent with the strong aggressiveness of these tumors (Figures 6A and 6B; Movies S4, S6, and S8 for primary G9, G13, and G16 GSCs, respectively; all were treated with 100 µM TAT). Because the explants were cultured in GSC medium, which favors the growth and survival of GSCs, most of the cells expressed SOX-2 and Nestin (Figure 6C). Interestingly, the movies clearly show that 100 µM TAT-Cx43$_{266-283}$ reduced cell motility and proliferation and promoted cell death (Movies S5, S7, and S9 for G9, G13, and G16, respectively). Consequently, the change in cell number from the beginning to the end of the experiment was strongly reduced in G9, G13, or G16 explants by 100 µM TAT-Cx43$_{266-283}$ compared with treatments with 100 µM TAT or with their corresponding controls (Figures 6A and 6B; Movies S5, S7, and S9 for G9, G13, and G16, respectively).

It should be mentioned that, over the course of these experiments, we received two surgical specimens that were finally diagnosed as neuroblastoma (N6) and oligodendroglioma (O17) instead of glioblastoma. Intriguingly, 100 µM TAT-Cx43$_{266-283}$ rapidly promoted tumor cell death in the neuroblastoma and oligodendroglioma explants (data not shown), as it did in the glioblastoma explants, suggesting that the anti-tumor effect of TAT-Cx43$_{266-283}$ is not restricted to glioblastoma.

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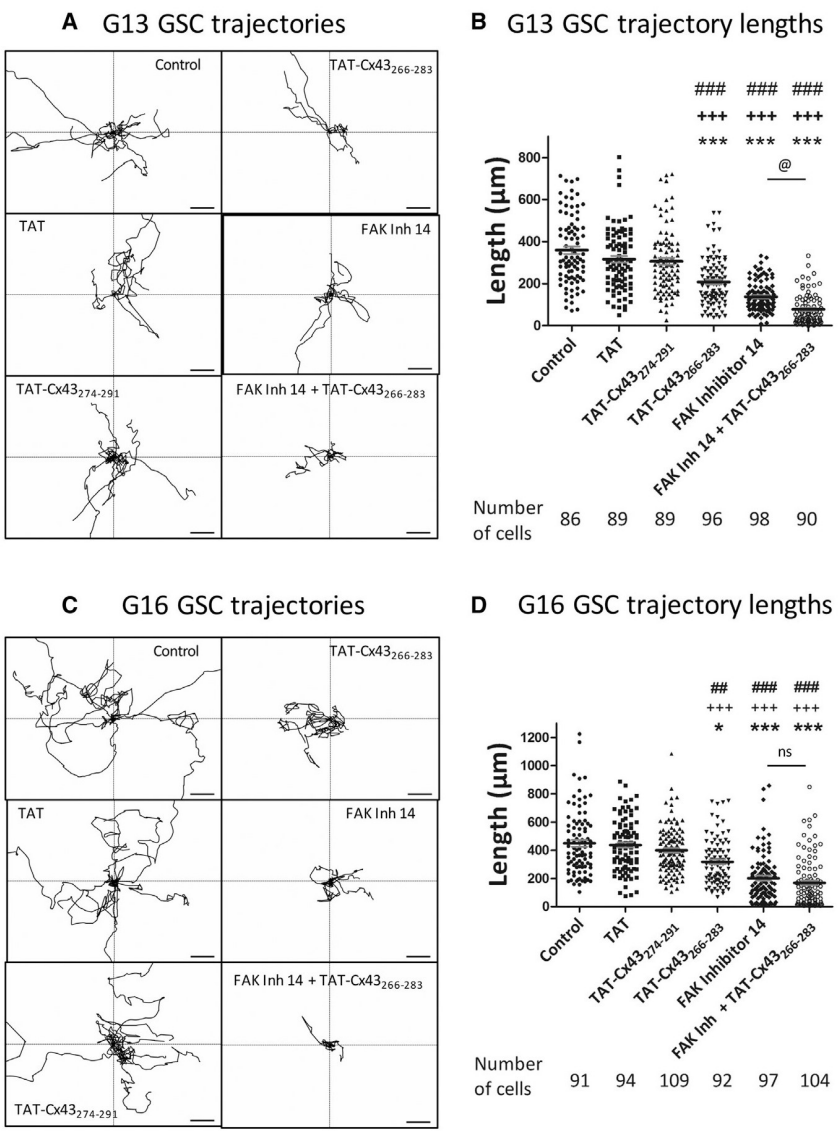

Figure 4. Specificity of the Effect of TAT-Cx43$_{266-283}$ on FAK-Dependent GSC Motility

Primary G13 (A and B) or G16 (C and D) GSCs were incubated with 50 µM TAT, TAT-Cx43$_{274-291}$, TAT-Cx43$_{266-283}$, 5 µM FAK inhibitor 14 (FAK inh 14), or 5 µM FAK inh 14 + TAT-Cx43$_{266-283}$, and their random movements were recorded by time-lapse microscopy for 14 hr. Track-plots showing the trajectories described by at least seven representative G13 (A) or G16 (C) GSCs for 14 hr. The origin of each cell's trajectory is at the intersection of the x and y axes. Scale bars, 50 µm. The migration length was extracted from the track-plots using the total path length described by each individual cell in at least ten movies from three independent experiments. The line bisecting the plot corresponds to the mean (ANOVA; ***$p < 0.001$, *$p < 0.05$; versus control; $^{+++}p < 0.001$; versus TAT; $^{###}p < 0.001$, $^{##}p < 0.01$; versus TAT-Cx43$_{274-291}$; $^{@}p < 0.05$; FAK inh 14 versus FAK inh 14 + TAT-Cx43$_{266-283}$; ns, not significant). Non-significant differences were found when the control, 50 µM TAT and 50 µM TAT-Cx43$_{274-291}$ were compared. The trajectories of cells that died during the time lapse were not considered.

DISCUSSION

Although the relationship between connexin and cancer was first described 50 years ago, the interest in this protein for use in the development of cancer therapies is increasing (Aasen et al., 2016). Our previous studies showed that restoring CX43 in glioma cells that express negligible levels of CX43 inhibits the activity of c-Src (Gangoso et al., 2014; Herrero-Gonzalez et al., 2010; Tabernero et al., 2016). This effect is caused by the recruitment of c-Src, together with its inhibitors CSK and PTEN, to a short region of CX43 from amino acids 266–283 (Gonzalez-Sanchez et al., 2016). Indeed, a cell-penetrating peptide containing this region, TAT-Cx43$_{266-283}$, is able to recruit these proteins and consequently inhibits c-Src and upregulates PTEN, mimicking the anti-proliferative effect and reversion of the stem cell phenotype promoted by CX43 in the GSC lines GliNS2 and G166 (Gangoso et al., 2014; Gonzalez-Sanchez et al., 2016). In the present study, we found that TAT-Cx43$_{266-283}$ inhibited c-Src activity and upregulated PTEN also in GSCs derived directly from patients. However, it should be considered that, although restoring CX43 in glioma cells reduces c-Src oncogenic activity, it can also have detrimental effects because of the increase in cell invasion (Naus et al., 2016). Interestingly, this study revealed that, by inhibiting c-Src and increasing PTEN, TAT-Cx43$_{266-283}$ reduced FAK activity, with subsequent inhibition in GSC migration and invasion.

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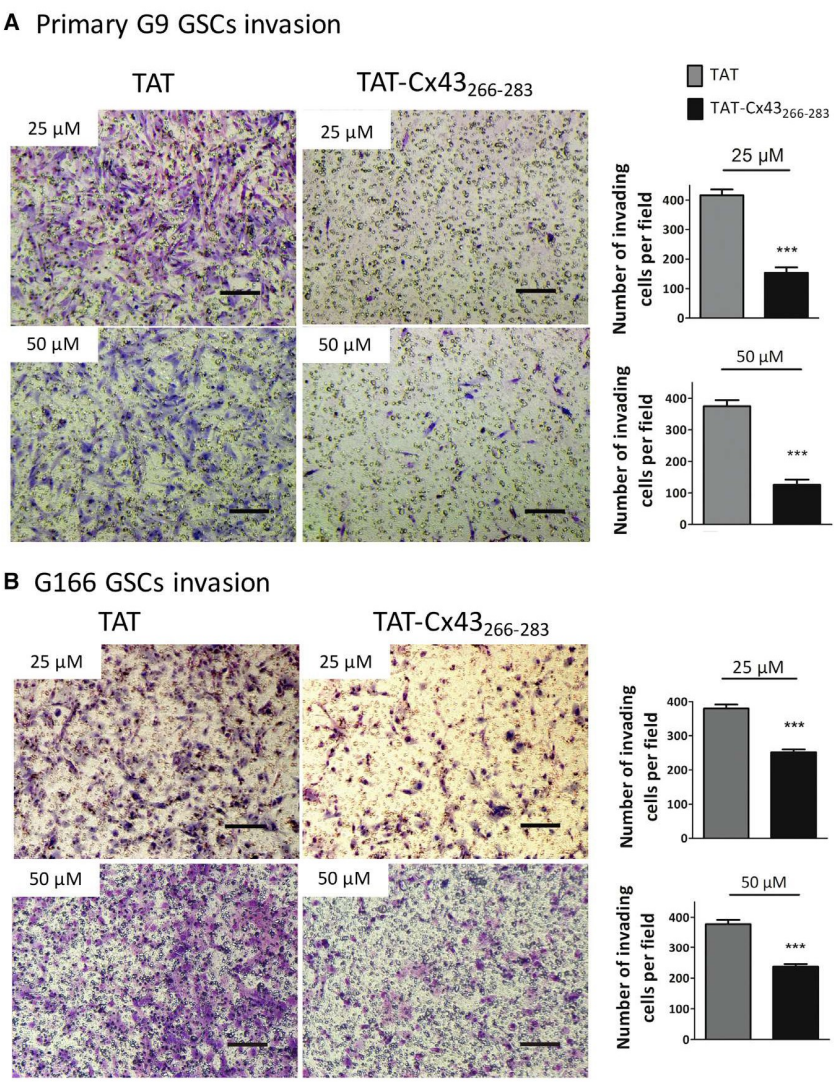

Figure 5. TAT-Cx43$_{266-283}$ Reduces GSC Invasion

Primary (A) or G166 (B) GSC invasion was analyzed using a transwell Matrigel invasion assay. Cells were incubated with TAT or TAT-Cx43$_{266-283}$ and were allowed to invade for 15 hr. The results are expressed as the number of invading cells per field ± SEM. At least five fields per insert in nine inserts from three independent experiments were counted. Scale bars, 100 μm (***$p < 0.001$; t test). See also Figures S3 and S4.

It is well known that CX43 plays a significant role in cell migration, impacting adhesion (Elias et al., 2007), cytoskeletal rearrangements, and invasiveness (reviewed in Naus et al., 2016). Thus, the C-terminal domain of CX43 interacts with crucial proteins for cell motility, such as ZO-1 (Toyofuku et al., 2001), tubulin (Saidi Brikci-Nigassa et al., 2012), debrin (Ambrosi et al., 2016), and binding partners of these proteins, such as F-actin. In addition, CX43 can increase gap junctional communication between tumor and stromal cells, causing a diffusion of molecules that contribute to tumor cell invasion or metastasis, such as microRNAs (Hong et al., 2015) or cGAMP (Chen et al., 2016). Furthermore, the C-terminal domain of CX43 can regulate signaling molecules that are involved in migration, such as p38 MAPK (Behrens et al., 2010) or c-Src (Herrero-Gonzalez et al., 2010). Therefore, it is not surprising that the resulting effect of CX43 on migration and invasion varies depending on the cellular context (Naus et al., 2016), such as the tumor microenvironment or level of activity of the partners of CX43. For instance, in glioma cells, some studies show that CX43 increases invasiveness (Lin et al., 2002; Oliveira et al., 2005; Osswald et al., 2015; Strale et al., 2012). However, others show the opposite effect: that downregulation of CX43 promotes glioma migration (Aftab et al., 2015).

To avoid this multifactorial effect, we used a short region of CX43 (from amino acids 266–283). This region lacks cytoskeleton-binding motifs and is not able to form gap junction channels; however, it retains its ability to inhibit c-Src activity (Gangoso et al., 2014; Gonzalez-Sanchez et al., 2016). As mentioned in the Introduction, c-Src is a key regulator of glioma cell migration and invasion because it phosphorylates and activates FAK (Calalb et al., 1995) and

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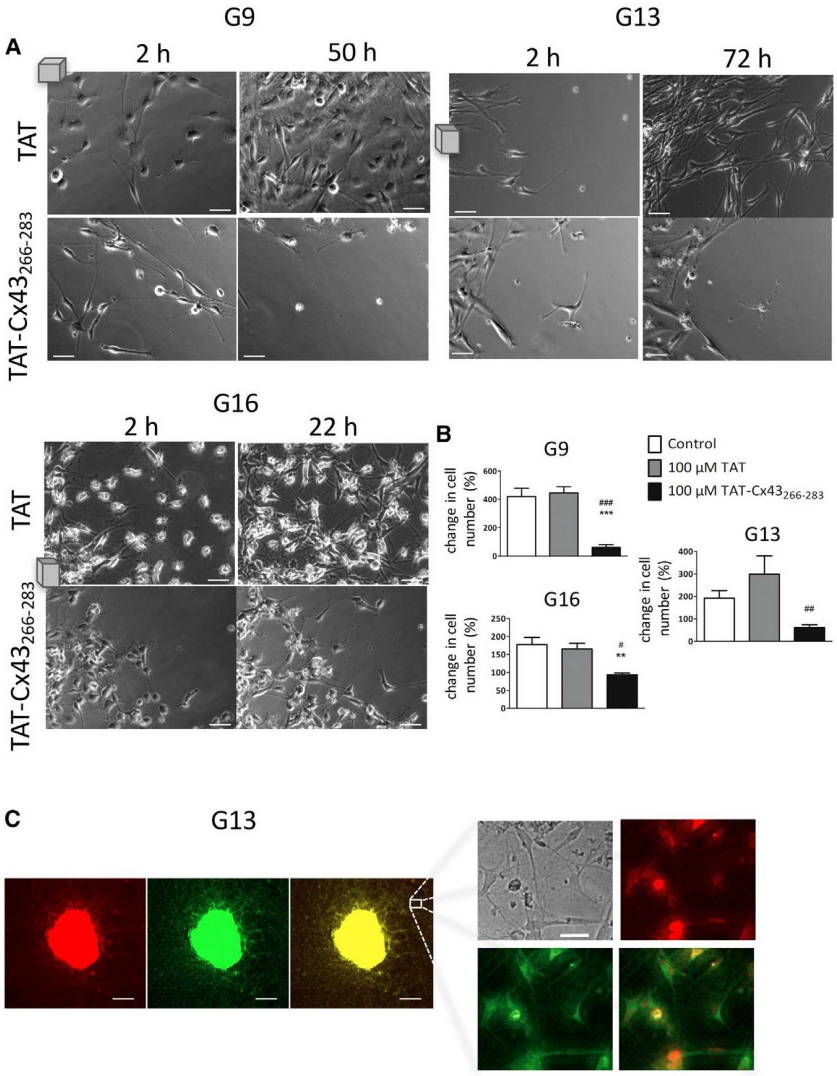

Figure 6. TAT-Cx43$_{266-283}$ Reduces Growth, Migration, and Survival in Patient-Derived Glioblastoma Explants

G9, G13, and G16 glioma explants were cultured in GSC medium and incubated in the absence (control) or presence of 100 μM TAT or TAT-Cx43$_{266-283}$ for the indicated times.

(A) Phase-contrast images of the same field showing cells growing and spreading from G9, G13, or G16 glioblastoma explants at the beginning and at the end of the experiment. Scale bars, 50 μm. Cells were recorded by time-lapse microscopy. The blocks indicate the location of the glioblastoma explants. The full movies are available as Supplemental Information Movies S4, S5, S6, S7, S8, and S9, showing the reduction in the cell growth, migration, and survival of the explants treated with TAT-Cx43$_{266-283}$.

(B) Change in cell number per field from the beginning to the end of the experiment expressed as percentage of the cells found at the beginning of the experiment. The results are the means ± SEM of three to six fields in explants from G9, G13, or G16 tumors (ANOVA; ***$p < 0.001$, **$p < 0.01$; TAT-Cx43$_{266-283}$ versus control; $^{###}p < 0.001$, $^{##}p < 0.01$, $^{#}p < 0.05$; TAT-Cx43$_{266-283}$ versus TAT).

(C) SOX-2 (red), Nestin (green) immunostaining, and merged images of the same field showing a representative explant from the G13 tumor. Scale bars, 500 μm. A magnified inset showing that most cells express these GSC markers. Scale bar, 50 μm. See also Movies S4, S5, S6, S7, S8, and S9.

promotes degradation of the tumor suppressor protein PTEN (Lu et al., 2003), whose phosphatase activity inhibits FAK (Cai et al., 2005; Tamura et al., 1998) (Figure 7A). Indeed, our results show that TAT-Cx43$_{266-283}$ inhibits c-Src activity and upregulates PTEN, with a subsequent decrease in FAK activity (Figure 7B). Src, PTEN, and FAK are crucial to trigger a cascade of events that lead to cell migration and invasion (Mitra and Schlaepfer, 2006; Yamada and Araki, 2001). Consistent with these studies, our time-lapse movies revealed that random GSC movement is reduced by the presence of TAT-Cx43$_{266-283}$, as judged by the length of the GSC trajectories. Furthermore, TAT-Cx43$_{266-283}$ strongly impaired the ability of GSCs to degrade the extracellular matrix (Matrigel) and invade transwells, consistent with a reduction in their invasive capacity.

Despite the intertumoral heterogeneity, all malignant gliomas share one conserved feature, aggressive invasiveness (Cuddapah et al., 2014), which is one of the most important consequences of the oncogenic activity of c-Src (Lund et al., 2006), specifically in GSCs (Liu et al., 2016). These cells exhibit a high level of c-Src activity (Han et al., 2014) and PTEN deficiency (Duan et al., 2015), which are required to invade the brain parenchyma, escaping surgery (Cheng et al., 2011; Garcia et al., 2010). As GSCs are resistant to conventional therapy and are highly tumorigenic, they are thought to be the main cause of recurrence after surgery and are consequently responsible for the poor survival of these patients (Chen et al., 2012; Dirks, 2010). Therefore, the results presented in this study showing inhibition of primary GSC migration

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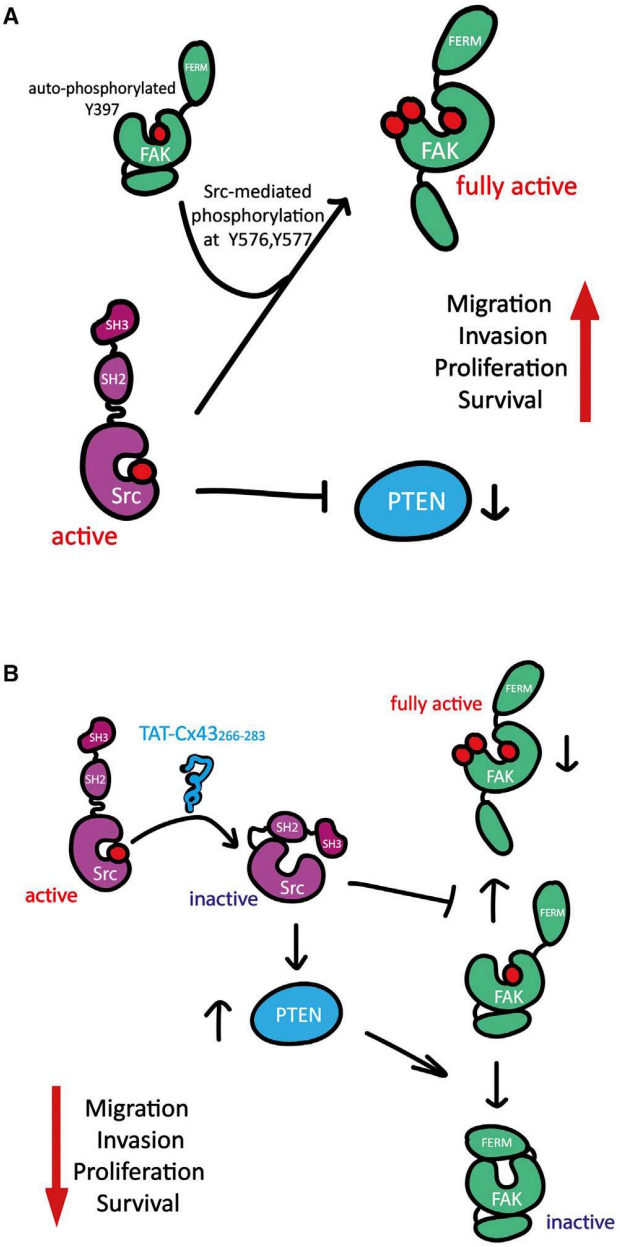

Figure 7. TAT-Cx43$_{266-283}$ Targets Src, PTEN, and FAK in GSCs

(A) In GSCs, active c-Src phosphorylates FAK at Y576 and Y577, thus fully activating FAK. Active c-Src also promotes the degradation of PTEN, an important inhibitor of FAK. The activated Src-FAK axis, together with low levels of PTEN are crucial to trigger the cascade of events leading to cell migration, invasion, proliferation, and survival. (B) TAT-Cx43$_{266-283}$ inhibits c-Src activity together with a strong upregulation of PTEN expression. As a consequence, there is a decrease in the phosphorylation of FAK at Y576 and Y577 by the low levels of active c-Src. In addition, the PTEN upregulation contributes to FAK inactivation by dephosphorylation. Altogether, these molecular changes reduce the capacity of the GSCs to migrate, invade, proliferate, and survive.

and invasion promoted by TAT-Cx43$_{266-283}$ are very promising for therapeutic development against this incurable disease.

Notably, TAT-Cx43$_{266-283}$ targets Src and PTEN, which are critical nodes in signaling pathways that impact proliferation, migration, invasion, and survival (Knobbe et al., 2002; Zhang et al., 2011). Furthermore, *PTEN* is one of the most frequent genetic alterations found in glioblastomas (Cancer Genome Atlas Research Network, 2008) and, although genetic analyses did not show any *SRC* alterations in glioblastoma, tyrosine kinase phosphorylation assays have shown that Src activation is a common event in glioblastoma (Du et al., 2009). Therefore, it is not unexpected that TAT-Cx43$_{266-283}$ exerted a potent anti-tumor effect on most of the glioblastoma patient explants analyzed in this study (Movies S4, S5, S6, S7, S8, and S9). In fact, these results should be highlighted because of the accuracy of patient-derived models in the identification of effective cancer treatments (Bayin et al., 2016; Crystal et al., 2014).

In conclusion, a region of CX43 (amino acids 266–283), by targeting c-Src, PTEN, and FAK, exerts a potent anti-tumor effect in patient-derived glioblastoma cells, which includes an impairment of GSC migration and invasion. This study reinforces the relevance of this sequence and encourages further research for the development of new therapies against glioblastoma.

EXPERIMENTAL PROCEDURES

Ethics Statement

Patients provided written informed consent to participate in the study, and tumor samples and cell culture brain tumor samples were obtained following local ethical board approval at the Service of Neurosurgery in the Hospital Universitario de Salamanca (Spain). The study was approved by the bioethics committee of the University of Salamanca and Junta de Castilla y León (Spain).

GSC Cultures

G166 GSCs were obtained from BioRep (Milan, Italy) (Pollard et al., 2009). Primary GSCs were obtained and cultured as described previously (Thirant et al., 2011). In brief, immediately after surgery, the tumor samples (G9, G12, G13, G15, and G16, diagnosed as classic glioblastomas; N6, a neuroblastoma; and O17, an oligodendroglioma) were washed and deprived of vessels in PBS. After mechanical dissociation, the samples were subjected to enzymatic dissociation with Accutase (Sigma-Aldrich Química, Madrid, Spain) for 15–20 min at 37°C. These solutions were then filtered and centrifuged at 1,000 × *g* for 5 min. The G166 and primary GSCs were cultured in RHB-A medium (Takara Bio, Condalab, Madrid, Spain) supplemented with 2% B27 (Life Technologies, Thermo Fisher Scientific, Waltham, USA), 1% N2 supplement (Life Technologies), 20 ng/mL epidermal growth factor (EGF), and 20 ng/mL basic fibroblast growth factor (b-FGF) (PeproTech,

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London, UK) under adherent conditions as described by Pollard et al. (2009). Culture plates were coated with 10 μg/mL laminin (Life Technologies) for 2 hr before use. The cells were maintained at 37°C in an atmosphere of 95% air/5% CO_2 and with 90%–95% humidity. The G166 and primary GSCs were grown to confluency, dissociated using Accutase, and then split. We routinely used cultures expanded for no more than 15 passages.

Cell Treatments

The synthetic peptides (>85% pure) were obtained from GenScript (Piscataway, NJ, USA). YGRKKRRQRRR was used as the TAT sequence, which is responsible for the cell penetration of the peptides (Gangoso et al., 2014). The TAT-$Cx43_{266-283}$ sequence was TAT-AYFNGCSSPTAPLSPMSP and the TAT-$Cx43_{274-291}$ sequence was TAT-PTAPLSPMSPGYKLVTG. The peptides were used at different concentrations (25, 50, or 100 μM) in culture medium at 37°C for the indicated time. FAK inhibitor 14 (SML0837) was obtained from Sigma and used at 5 μM.

Immunocytochemistry

Cells were fixed in 4% paraformaldehyde for 20 min. A mouse monoclonal antibody against human Nestin (1:200; Abcam, Cambridge, UK; Ref. ab18102) was applied overnight at 4°C, followed by incubation with an Alexa Fluor 488-conjugated anti-mouse immunoglobulin G (IgG) antibody (1:1,000; Life Technologies; Ref. A-11029) for 2 hr. A rabbit polyclonal antibody against SOX-2 (1:200; Abcam; Ref. ab97959) was applied overnight at 4°C, followed by incubation with an Alexa Fluor 594-conjugated anti-rabbit IgG antibody (1:1,000; Life Technologies; Ref. A-11012) for 2 hr. The cells were then mounted using a SlowFade Light Antifade Kit (Life Technologies), and they were analyzed on a Leica inverted fluorescence microscope connected to a digital video camera (Leica DC 100; Leica Microsystems, Wetzlar, Germany).

Migration Assays

G166 or primary GSCs were plated at a low density (5,000 cells/cm^2) in 24-well plates. Once the cells were attached, TAT or TAT-$Cx43_{266-283}$ was added at 25 or 50 μM, and the cells were allowed to equilibrate for 1–3 hr in the microscope incubator before imaging. Random cell movement was recorded by time-lapse live-cell imaging for 12–14 hr. The total duration of the treatment was always 15 hr. Every 10 min, phase-contrast photographs of each experimental condition were taken with an inverted Zeiss Axio Observer Z1 microscope for live-cell imaging (Carl Zeiss Microscopy, LLC, USA) coupled to an AxioCam MRm camera. The system included an automated XY stage controller and a humidified incubator set at 37°C and 5% CO_2. Image stacks were processed, and cell movement was manually tracked and further analyzed using Zen imaging software (Carl Zeiss Microscopy). Average cell length was calculated in at least six independent movies from three independent experiments.

Glioblastoma Explant Cultures

The tumor samples, immediately after surgery, were washed, deprived of vessels in PBS and finely minced into approximately 1-mm^3 pieces. The explants were plated individually in 24-well plates and cultured in RHB-A medium (Takara Bio, Condalab, Madrid, Spain) supplemented with 2% B27 (Life Technologies, Thermo Fisher Scientific, Waltham, USA), 1% N2 supplement (Life Technologies), 20 ng/mL EGF, and 20 ng/mL b-FGF (PeproTech, London, UK), as described previously (Bayin et al., 2016). Culture plates were coated with 10 μg/mL laminin (Life Technologies) for 2 hr before use. The tumor blocks were maintained at 37°C in an atmosphere of 95% air/5% CO_2 and with 90%–95% humidity. Once the explants were attached (after 24–72 hr), TAT or TAT-$Cx43_{266-283}$ was added at 100 μM, and the cells were allowed to equilibrate for 1–3 hr in the microscope incubator before imaging. The cells were recorded by time-lapse live-cell imaging for the indicated times. Every 10 min, phase-contrast photographs of each experimental condition were taken with an inverted Zeiss Axio Observer Z1 microscope for live-cell imaging (Carl Zeiss Microscopy, LLC, USA) coupled to an AxioCam MRm camera. The system included an automated XY stage controller and a humidified incubator set at 37°C and 5% CO_2. Image stacks were processed using Zen imaging software (Carl Zeiss Microscopy).

Invasion Assays

Cell invasion was measured in Matrigel (Corning, Amsterdam, The Netherlands)-coated transwell inserts (Merck Millipore, Madrid, Spain) containing polyethylene terephthalate filters with 8-μm pores. The inserts were coated with 100 μL of 1 mg/mL Matrigel matrix according to the manufacturer's recommendations. Next, 7.5×10^4 cells in 200 μL of serum-free medium were plated in the upper chamber, whereas 500 μL of medium supplemented with 10% fetal bovine serum (Gibco, Life Technologies) was added to the lower well. The indicated treatments were added, and the cells were allowed to invade for 15 hr. Non-invading cells were carefully removed with wet cotton swabs from the top of the membranes. The invading cells of the lower surface were fixed with 4% paraformaldehyde for 10 min, washed with PBS, and stained with Giemsa for 10 min. The inserts were washed with PBS and allowed to dry. The invading cells were counted in at least five random fields per insert from three independent experiments. Images were taken using a Leica microscope connected to a digital camera (Leica DFC500).

Western Blot Analysis

Western blotting was performed as described previously (Herrero-Gonzalez et al., 2010). In brief, equivalent amounts of proteins (20 μg per lane) were separated on NuPAGE Novex Bis-Tris (4%–12%) midi gels (Life Technologies). The proteins were transblotted using an iBlot dry blotting system (Life Technologies). After blocking, the membranes were incubated overnight at 4°C with the primary antibodies against Y416 Src (1:200; Cell Signaling, Danvers, MA, USA; Ref. 2101), total Src (1:500; Cell Signaling; Ref. 2108), PTEN (1:500; Cell Signaling; Ref. 9556S), Y397 FAK (1:1,000; Life Technologies; Ref. 44-624G), Y576 FAK (1:500; Life Technologies; Ref. 44652G), Y577 FAK (1:500; Life Technologies; Ref. 44-614G), and total FAK (1:500; Life Technologies; Ref. AHO0502). The antibodies against glyceraldehyde phosphate dehydrogenase (GAPDH, 1:15,000; Ambion, Thermo Fisher Scientific; Ref. AM4300) or alpha-actinin (1:1,000; Chemicon International, Merck Millipore; Ref. MAB1682) were used as a loading control. After extensive washing, the membranes were incubated

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with peroxidase-conjugated anti-rabbit IgG or the anti-mouse IgG antibody (Santa Cruz Biotechnology, Dallas, TX, USA; Refs. sc-2030 and sc-2005) in TTBS and developed with a chemiluminescent substrate (Western Blotting Luminol Reagent; Santa Cruz Biotechnology). X-ray films were obtained from Fujifilm (Madrid, Spain).

MTT Assay

Cells cultured at 37°C in 24-well plates were incubated in the dark for 75 min with 300 μL of RHB-A medium containing 0.5 mg/mL MTT (Sigma). The medium was then removed, and the cells were incubated for 10 min in the dark with DMSO (500 μL per well) with mild shaking. Finally, the absorbance was measured at a wavelength of 570 nm using a microplate reader (Appliskan 2001; Thermo Electron Corporation, Thermo Scientific, Madrid, Spain).

Statistical Analysis

The results are expressed as the mean ± SEM of at least three independent experiments. Statistical analyses were carried out using Student's t test when two groups were compared. For the comparison of more than two groups, an ANOVA (one-way ANOVA) was used, followed by the appropriate post-test (Tukey). Values were considered significant when $p < 0.05$.

SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures and nine movies and can be found with this article online at http://dx.doi.org/10.1016/j.stemcr.2017.06.007.

AUTHOR CONTRIBUTIONS

M.J.-R., contributed to the experimental design and development, data acquisition, analysis, and interpretation, drafting and revision of the article for important intellectual content, and approved the final version for publication. M.D.T., design and custody of patients' written informed consent, design and processing of ethical board approval at the Service of Neurosurgery in the Hospital Universitario de Salamanca (Spain), transfer of patient-derived samples, revision of the article for important intellectual content, and approved the final version for publication. M.G.-T., design and processing for ethical board approval at the Service of Neurosurgery in the Hospital Universitario de Salamanca (Spain), transfer of patient-derived samples, revision of the article for important intellectual content, and approved the final version for publication. A. Otero, glioblastoma surgery, diagnosis, obtained written informed consent from patients, revised the article for important intellectual content, and approved the final version for publication. A. Orfao, design of patients' written informed consent, design and processing for approval from the ethical board at the Service of Neurosurgery in the Hospital Universitario de Salamanca (Spain), revision of the article for important intellectual content, and approved the final version for publication. J.M.M., contributed to the experimental design and data interpretation, revised the article for important intellectual content, and approved the final version for publication. A.T., conceived and designed the experiments, designed and processed documentation for bioethics committee approval from the University of Salamanca and Junta de Castilla y León (Spain), supervised the experimental development and analysis, interpreted the data, drafted the article, and approved the final version for publication.

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Stem Cell Reports, Volume *9*

Supplemental Information

A Short Region of Connexin43 Reduces Human Glioma Stem Cell Migration, Invasion, and Survival through Src, PTEN, and FAK

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Supplemental Figures and legends

Figure S1

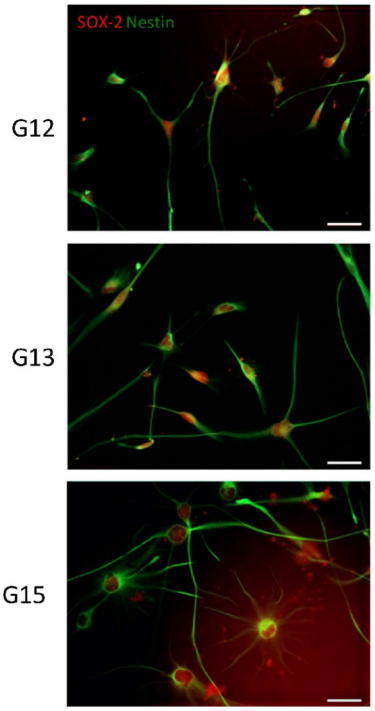


Figure S1. SOX-2 and Nestin immunostaining in primary G12, G13, and G15 GSCs. Related to Figure 1.

SOX-2 (red) and Nestin (green) merged immunostaining showing that primary G12, G13, and G15 GSCs, obtained as described in the Experimental procedures section, express these GSC markers. Bar = 50 μm.

Figure S2

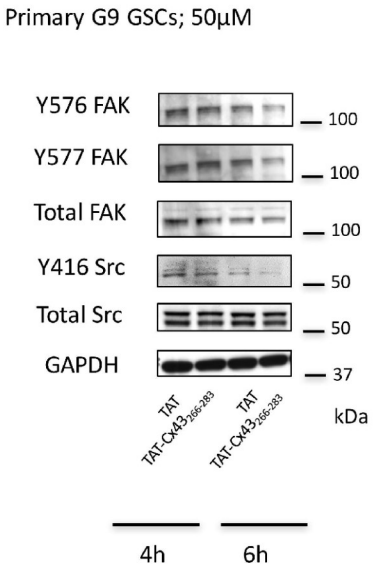


Figure S2. **Effect of TAT-Cx43$_{266-283}$ on c-Src and FAK activity in primary GSC and G166 GSC after 4 and 6h. Related to Figure 2.**

Primary G9 GSCs were incubated with 50 µM TAT or 50 µM TAT-Cx43$_{266-283}$. After 4 and 6 h, the total c-Src, Y416 c-Src, total FAK, Y576 FAK and Y577 FAK levels were analyzed by Western blotting. GAPDH was used as a loading control.

Figure S3

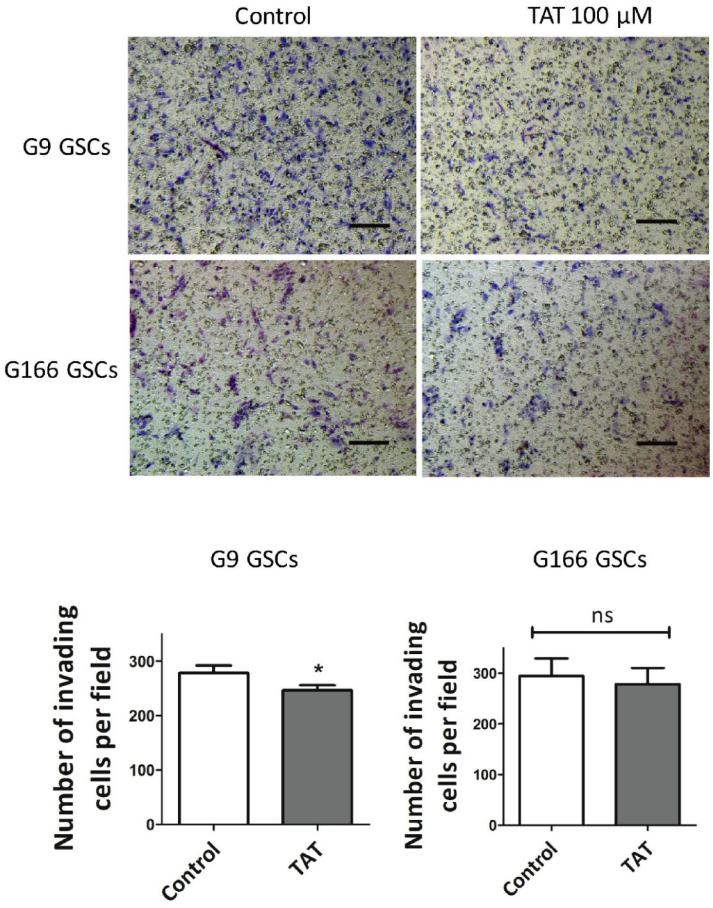


Figure S3. Effect of TAT on primary G9 GSC and G166 GSC invasion. Related to Figure 5.

Representative photomicrographs and the quantification of invading cells from the analysis of the transwell Matrigel invasion assay as described in the Experimental procedures section. Control or 100 µM TAT-treated G9 GSCs or G166 GSCs were allowed to invade for 15 h. The results are expressed as the number of invading cells per field ± s.e.m. At least five fields per insert in nine inserts from three independent experiments were counted. Bar = 100 µm. (*p<0.05; ns, not significant; TAT vs control, t-test).

Figure S4

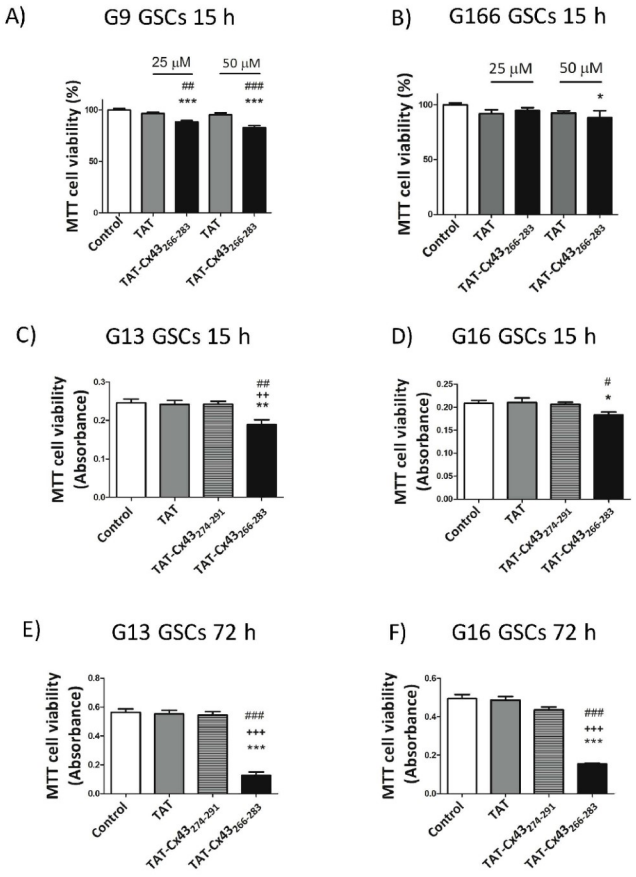


Figure S4. **Effect of TAT-Cx43$_{266-283}$ on primary GSC and G166 GSC viability after 15 and 72h. Related to Figure 5.**

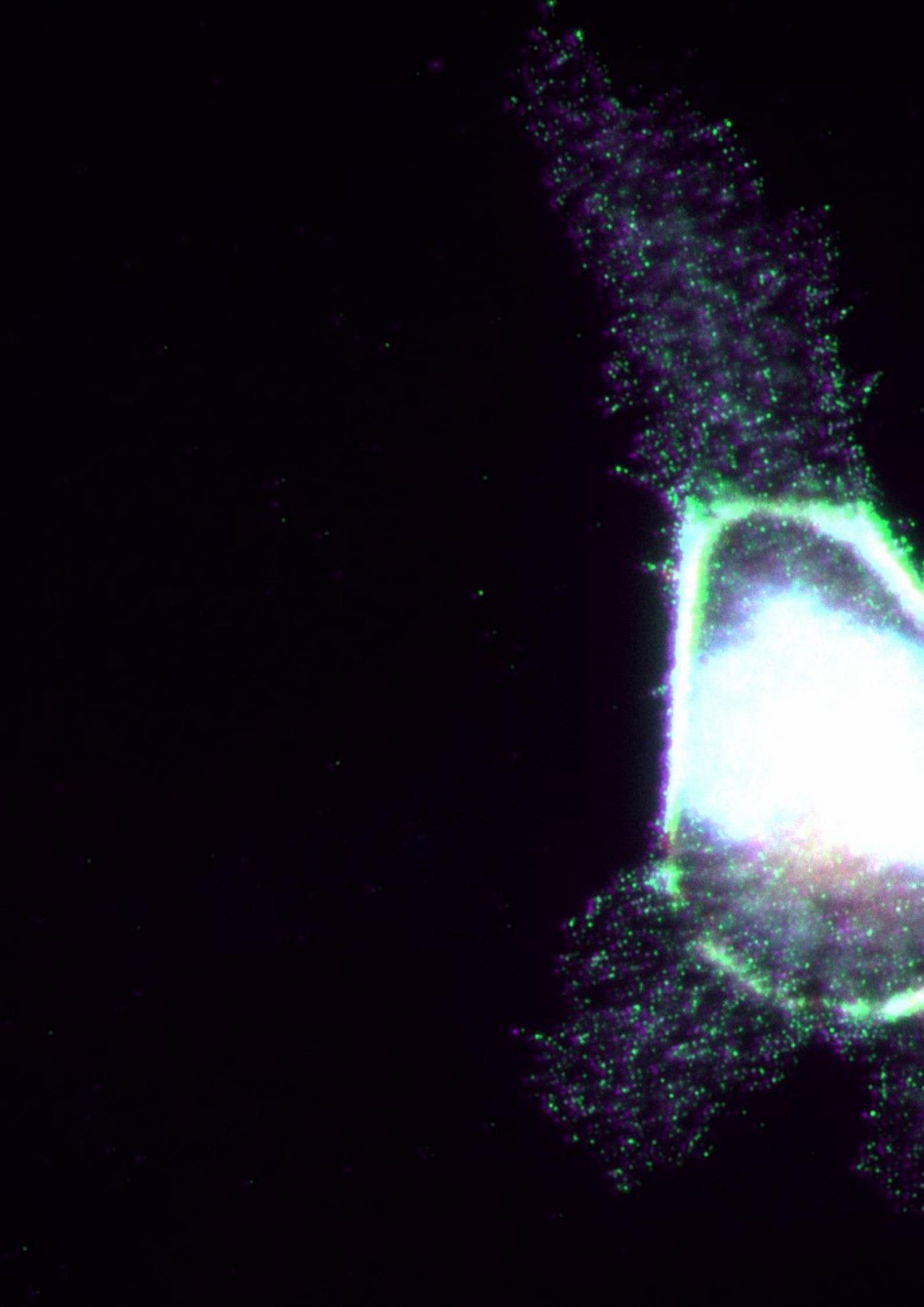
Primary G9 GSCs or G166 GSCs were plated at 5500 cells/cm^2 and incubated with 25 or 50 µM TAT or TAT-Cx43$_{266-283}$ for 15 h **(A, B)**. Primary G13 or G16 GSCs were plated at 5500 cells/cm^2 and incubated with 50 µM TAT, 50 µM TAT-Cx43$_{266-283}$ or TAT-Cx43$_{274-291}$ for 15 h **(C, D)**. Primary G13 or G16 GSCs were plated at 5500 cells/cm^2 and incubated with 50 µM TAT-Cx43$_{266-283}$ or TAT-Cx43$_{274-291}$ for 72 h **(E, F)**. The cell viability was analyzed using a MTT assay as described in the Experimental procedures section. The results are expressed as the percentages of the absorbance found in the control and are the mean ± s.e.m. of 3 experiments (ANOVA ***p<0.001; **p<0.01; *p<0.05; TAT-Cx43$_{266-283}$ vs Control; ###p<0.001; ##p<0.01; #p<0.05, TAT-Cx43$_{266-283}$ vs TAT; +++p<0.001; ++p<0.01; TAT-Cx43$_{266-283}$ vs TAT-Cx43$_{274-291}$).

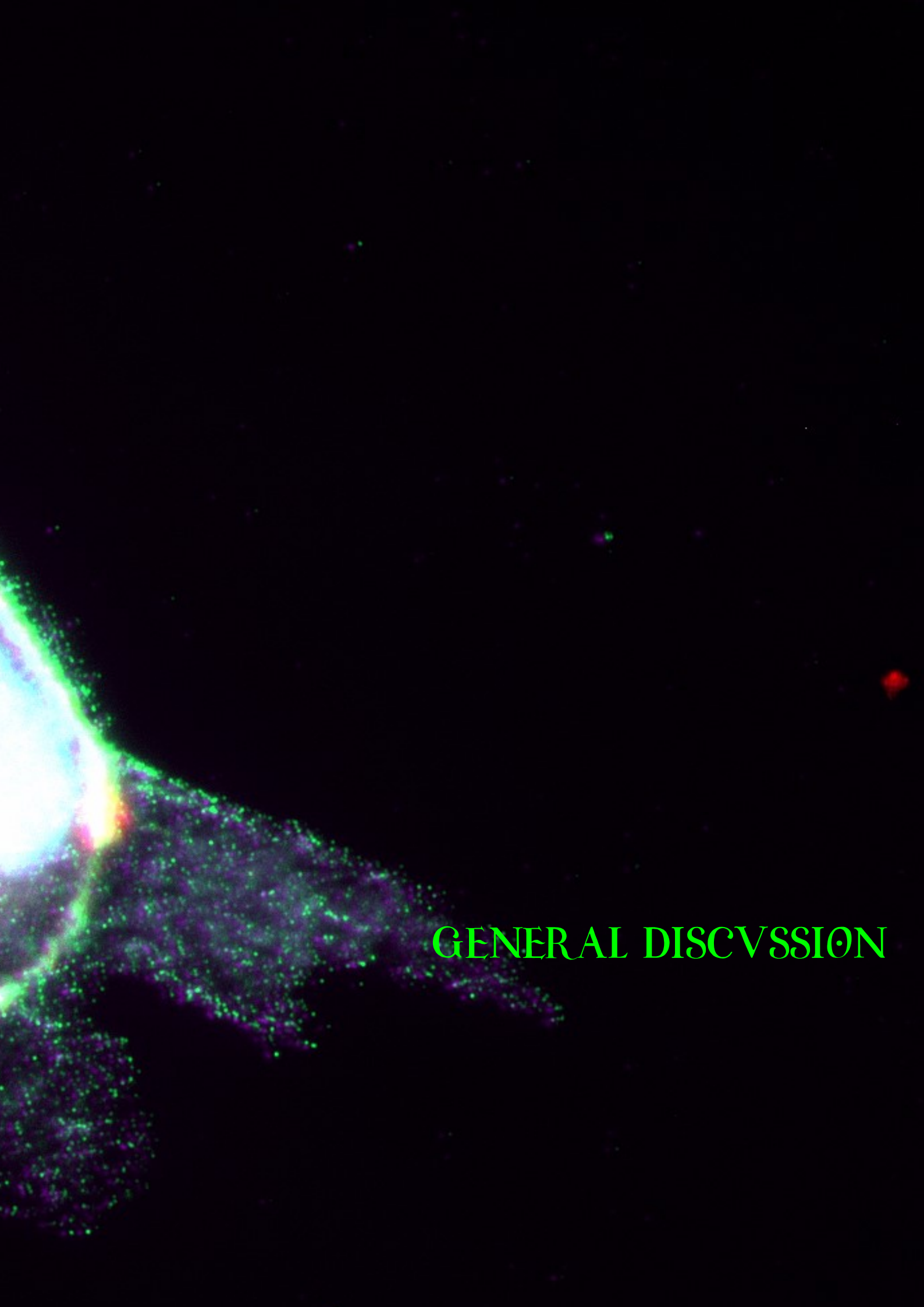
Movies S1-S3. Effect of TAT-Cx43$_{266-283}$ on primary G9 GSC motility. Related to Figure 3.

Primary G9 GSCs plated at a low density were incubated in the absence (Control; **S1**) or presence of 50 μM TAT **(S2)** or 50 μM TAT-Cx43$_{266-283}$ **(S3)**. Phase-contrast time-lapse movies show the random movement of primary G9 GSCs. Images were acquired for 12 h at 10 min intervals as described in the Experimental procedures section. Note the reduction in the motility of primary G9 GSCs promoted by TAT-Cx43$_{266-283}$. Frame size= 480 x 360 pixels. Frame rate= 10 frames per second.

Movies S4-S9. Effect of TAT-Cx43$_{266-283}$ on G9, G13 and G16 explants. Related to Figure 6.

Tumor explants plated immediately after surgery were incubated in the presence of 100 μM TAT **(S4, S6, S8)** or 100 μM TAT-Cx43$_{266-283}$ **(S5, S7, S9)**. Phase-contrast time-lapse movies showing the spread and growth of cells from the tumor blocks (**S4** and **S5** for G9; **S6** and **S7** for G13; **S8** and **S9** for G16, respectively). Images were acquired for the indicated times at 10 min intervals as described in the Experimental procedures section. Note the reduction in the survival, migration and growth of glioblastoma cells promoted by TAT-Cx43$_{266-283}$. Frame size= 480 x 360 pixels. Frame rate= 15 frames per second.




GENERAL DISCVSSION

The expression of Cx43, the main protein forming gap junction channels and hemichannels in astrocytes, is reduced in malignant gliomas (Shinoura et al., 1996; Huang et al., 1999; Soroceanu et al., 2001; Pu et al., 2004; Caltabiano et al., 2010; Gielen et al., 2013; Lathia et al., 2015). Although restoring Cx43 has shown strong anti-tumor effects in different models of glioma (Naus et al., 1992; Herrero-Gonzalez et al., 2010; Yu et al., 2012; Gangoso et al., 2014), recent data suggest a controversial effect of this protein on glioma cell invasion that depends on the cell context (reviewed in: (Lathia et al., 2015). It should be mentioned that migration and invasion constitute both key processes in glioblastoma recurrence (Cuddapah et al., 2014). Consequently, the anti-proliferative effects of Cx43 may be counterbalanced by its migrating detrimental properties.

The C-terminal tail of Cx43 possesses a broad interactome (Aasen et al., 2016), which is partly responsible for this variety of effects. Among these effects, the role of Cx43-Src interaction has been described to be relevant for some of the anti-tumour effects of Cx43 because it has the ability to inhibit c-Src activity. Thus, the design of a cell-penetrating peptide based on the region of the Cx43CT responsible for c-Src inhibition, TAT-$Cx43_{266-283}$, provides an excellent tool to restrict the effects to c-Src activity and tackle with problems derived of using the whole Cx43 protein (Tabernero et al., 2016). TAT-$Cx43_{266-283}$ reproduces the effects of Cx43 on inhibiting c-Src with the subsequent effects on C6 rat glioma cell proliferation and the reversion of the GSC phenotype.

As it has been described, GSCs constitute one important challenge in treating glioblastoma because they have high oncogenic potential, resistance to conventional therapies and aggressive infiltration into the brain parenchyma. All these features make difficult the whole tumour resection and facilitate tumour recurrence (Davis et al., 1999; Stupp et al., 2005). In this regard, in collaboration with the Neurosurgery Hospital Service in this Thesis we set up a protocol to obtain and culture patient-derived GSCs. It should be mentioned that a rising number of publications based on patient-derived GSCs has revealed the relevance of this approach to implement precision oncology (Crystal et al., 2014).

Therefore, the overall goal of this PhD thesis was to study the anti-tumor effect and mechanism of TAT-$Cx43_{266-283}$ in patient-derived GSCs.

Effect and mechanism of TAT-$Cx43_{266-283}$ on human GSC proliferation

It has been previously shown that Cx43 exerts its antiproliferative effect in rat C6 glioma cells by inhibiting c-Src activity (Herrero-Gonzalez et al., 2010).

Indeed, while all these effects are also triggered by the region of Cx43 that comprises amino acids 266-283 via a cell-penetrating peptide, TAT-$Cx43_{266-283}$ (Gangoso et al., 2014; Gonzalez-Sanchez et al., 2016), the mutant of Cx43 C6-Cx43 Y247F/Y265F that is unable to inhibit c-Src, does not retain this capacity (Herrero-Gonzalez et al., 2010).

In addition to inhibit glioma cell proliferation, Cx43 has been described to exhibit GSC differentiation properties (Yu et al., 2012; Gangoso et al., 2014). Interestingly, the effects of Cx43 in reversing GSC phenotype are also mediated by c-Src inhibition with the subsequent downregulation of the inhibitor of differentiation Id1 (Yu et al., 2012; Gangoso et al., 2014) . Id1, whose expression is regulated by c-Src activity (Gautschi et al., 2008), regulates the expression of Sox2 (Soroceanu et al., 2013), a transcription factor responsible for stem cell self-renewal (Gangemi et al., 2009; Singh et al., 2012). Importantly, TAT-$Cx43_{266-283}$ mimicked the effects of Cx43 on GSC phenotype by reducing Id1 and Sox-2 expression (Gangoso et al., 2014).

Once established the effect of TAT-$Cx43_{266-283}$ on the reversion of the stem cell phenotype, in this study we aimed to investigate the effect of TAT-$Cx43_{266-283}$ on GSC proliferation. First, we revealed that TAT-$Cx43_{266-283}$ reduced the rate of proliferation of G166 human GSCs, as judged by growth curves and Ki-67 expression, a marker of cell proliferation. In fact, this effect was stronger than that found in C6 rat glioma cells. More importantly, TAT-$Cx43_{266-283}$ inhibited the proliferation of the G9, G13 and G16 patient-derived GSCs obtained along the period of this Thesis. Furthermore, c-Src activity was reduced by TAT-$Cx43_{266-283}$ in these GSCs, suggesting that by inhibiting c-Src, TAT-$Cx43_{266-283}$ inhibits the proliferation also in human GSCs, including primary patient-derived GSCs.

Intriguingly, the mechanism underlying c-Src inhibition by Cx43 has only been recently explored (Gonzalez-Sanchez et al., 2016). In astrocytes and C6 rat glioma cells, Cx43 recruits Csk, responsible for Y527 c-Src phosphorylation (the inactive form) and PTEN, that dephosphorylates Y416 c-Src (the active form). The recruitment ability of Cx43 to bring active c-Src, Csk and PTEN to proximity and therefore, to prompt the c-Src inhibition cascade of events, resides in the amino acids 266-283. Indeed, TAT-$Cx43_{266-283}$ but not TAT-$Cx43_{274-283}$ recruit c-Src, CSK and PTEN in C6 glioma cells (Gonzalez-Sanchez et al., 2016).

By biotinylating TAT-$Cx43_{266-283}$, we could study this mechanism in an intracellular context. The pull-down of G166 GSCs cellular lysates incubated with TAT-$Cx43_{266-283}$-B for 30 minutes corroborated the hypothesized mechanism for the Cx43-mediated c-Src inhibition in GSCs: the cell-penetrating

peptide, TAT-$Cx43_{266-283}$, exerts its anti-tumour effect by favouring the proximity of c-Src with its endogenous inhibitors PTEN and Csk. This leads to c-Src inhibition, which initiates a cascade of anti-tumour effects. Indeed, the use of TAT-$Cx43_{266-283}$-B as a bait to identify the proteins attached to this region in G166 GSCs confirmed the results obtained in rat C6 glioma cells, conferring to this mechanism a broader spectrum not restricted to a single type of cells.

The use of cell-penetrating peptides attached to biotin or to other tags, as well as countless modifications, provides a much more realistic insight into the intracellular mechanisms of protein-protein interaction as compared to the traditional approach of immunoprecipitating cellular lysates with the protein of interest. In this case, proteins with high affinity may interact under these conditions, whereas in an intracellular context they could never even meet each other. On the contrary, low abundant or low affinity proteins are usually difficult to reveal by immunoprecipitation assays because the context does not favour their approach.

Cx43CT is an IDR (Grosely et al., 2013), which means that it does not acquire a folded structure and facilitates the previously mentioned large and dynamic interactome within the cytoplasm. The flexible conformation of the Cx43CT IDR is important for c-Src, PTEN and Csk to acquire proximity and it is reproduced by TAT-$Cx43_{266-283}$. Because folding in the required conformation is an important factor to consider when designing a bait to study intracellular protein-protein interactions, the fact that this region does not require a folded conformation favours its use as a bait to study their intracellular partners.

Altogether, our data suggest that the anti-proliferative effect of Cx43 and TAT-$Cx43_{266-283}$ seems to rely on their capacity to serve as a docking platform for c-Src and its endogenous inhibitors, CSK and PTEN.

The inhibition of c-Src prevents PTEN degradation (Lu et al., 2003) creating a positive feed-forward loop that guarantees the long-term regulation of c-Src inhibition. PTEN is a phosphatase whose importance as an inhibitor in critical molecular pathways involved in tumorigenic processes such as PI3K/AKT/mTOR (Lee et al., 1999; Vazquez and Sellers, 2000; Simpson and Parsons, 2001) is widely described. Not surprisingly, epigenetic events such as promoter methylation, COOH-terminal phosphorylation, oxidation events, and other protein modifications may result in loss of PTEN function as found in glioblastoma and other cancers (Whang et al., 1998; Vazquez et al., 2001). As mentioned before, AKT, a pathway downregulated by PTEN, promotes proliferation and survival. In fact, restoring Cx43 to C6 glioma cells, by inhibiting c-Src, up-regulated PTEN and

inhibited AKT (Gonzalez-Sanchez et al., 2016), suggesting the participation of PTEN-AKT pathway in the antiproliferative effect of Cx43 in these cells. In agreement with these results, G166 and patient-derived GSCs treated with TAT-$Cx43_{266-283}$ showed an upregulation in PTEN levels caused by c-Src inhibition. As a negative regulator of the Pi3K/AKT pathway, PTEN upregulation by TAT-$Cx43_{266-283}$ reduced the phosphorylation of AKT at threonine 308 in G166 GSCs, suggesting that PTEN is functional. PTEN also showed to be necessary for the TAT-$Cx43_{266-283}$-mediated c-Src inhibition and subsequent anti-proliferative effect on GSCs. In fact, the reduction in proliferation promoted by this cell-penetrating peptide is decreased when PTEN is silenced. These data indicate that the effects of TAT-$Cx43_{266-283}$ on GSC proliferation are dependent on PTEN functionality.

As mentioned above, we analyzed TAT-$Cx43_{266-283}$-mediated c-Src inhibition in patient-derived GSCs obtained from 5 different patients: G9, G12, G13, G15 and G16. Despite the intrinsic interindividual differences, all of them showed a reduction in c-Src activity. In addition and corroborating our previous results in G166 GSCs, probably as a consequence of c-Src inhibition by TAT-$Cx43_{266-283}$ that prevents PTEN degradation, G9, G12, G15 and G16 showed an upregulation in PTEN levels. Only G13 did not show this effect despite its c-Src inhibition was also significant.

Considering all the results, the proposed sequence of events is that TAT-$Cx43_{266-283}$-mediated inhibition of c-Src causes the upregulation of PTEN with subsequent reduction of AKT activity and GSC proliferation, even in patient-derived GSCs.

Effect and mechanism of TAT-$Cx43_{266-283}$ on human GSC migration and invasion

GJA1, the gene that codes for Cx43, has been described as one of the genes that promote epithelial cell migration (Simpson et al., 2008). Indeed, radial glial progenitors require Cx43 for a proper neurogenesis (Kunze et al., 2009) and Cx43KO cells fail to accomplish directional cell migration (Francis et al., 2011). As opposed to the physiological role of Cx43 in migration, the amount of controversial data that involve Cx43 in glioma migration is huge, but it seems to favor invasion (reviewed in(Lathia et al., 2015). To start, we can establish a difference between channel and non-channel implications of Cx43 in terms of glioma migration.

Probably through the establishment of intercellular gap junctions between astrocytes and glioma cells, the last ones exchange tumorigenic miRNAs that play in their favor turning surrounding cells into a more permissive phenotype (Hong et al., 2015). It seems, though, that migration of glioma cells is

optimal when gap junction intercellular communication is low between them but high with the stromal cells (reviewed in: (Lathia et al., 2015). To our knowledge, gap junctions between GSCs and astrocytes have not been described. However, neural progenitor cells and astrocytes and even microglia have shown to communicate through gap junctions (Talaveron et al., 2014). This raises the interesting question about gap junction formation between GSCs and stroma cells and their possible role in GSC infiltration, as obvious similarities in the molecular biology of migration between glioma and neural progenitor cells have been described (Dirks, 2001).

Concerning independent channel functions, while the Cx43 extracellular loops seem essential in the cellular adhesive properties and consequently, in migration . The influence of this protein in migration might also be due to the previously mentioned Cx43CT large interactome. The Cx43CT interacts and associates with cytoskeletal proteins including N-cadherin (Wei et al., 2005), E-cadherin, alpha-catenin (Fujimoto et al., 1997), beta-catenin (Ai et al., 2000), p120 (Wei et al., 2005), p150 (Lin et al., 2001), vinculin (Xu et al., 2006), ZO-1 and various actin-binding proteins such as drebrin (Butkevich et al., 2004). To this respect, the presence of the Cx43CT for human glioma cell migration seems to be necessary (Crespin et al., 2010) and particularly the region 257-382 sufficient to promote migration in Hela cells (Behrens et al., 2010). A good example of a mechanism through which the Cx43CT mediates migration is the activation of p38 MAPK (Behrens et al., 2010). p38 MAPK is known to mediate actin organization and cell migration induced by growth factors (Rousseau et al., 1997; Huang et al., 2004; Lamalice et al., 2006) and Cx43CT region could be a scaffold protein to improve phosphorylation of p38.

Precisely an essential feature of the cell-penetrating peptide TAT-$Cx43_{266-283}$ is the lack of channel forming properties, tubulin-binding domain region or ZO1-binding region, which prevents some of the possibilities of positive effects on migration. Also, the last few amino acids in TAT-$Cx43_{266-283}$ are those corresponding with the SH3 domain, attempting to delimit its interaction to c-Src and exclude other protein interactions. As previously addressed, this region inhibits c-Src (Gangoso et al., 2014; Gonzalez-Sanchez et al., 2016). Because c-Src is a non-receptor kinase linked to crucial signaling pathways (Du et al., 2009), Src Family kinases inhibitors have been explored in glioblastoma and other cancers. In effect, c-Src inhibitors such as dasatinib show a reduction in GSC migration (Nomura et al., 2007; de Groot and Milano, 2009; Sikkema et al., 2009). Intriguingly, these inhibitors have not shown the ability of Cx43 or TAT-$Cx43_{266-283}$ to affect GSC growth (Han et al., 2014),

suggesting that the mechanism by which c-Src is inhibited is important to encompass all the oncogenic effects.

c-Src participates in migratory pathways mainly through the interaction with FAK, an important mediator of migration and invasion in human GSCs. FAK activity builds upon a series of events: Y397 autophosphorylation creates a binding site for c-Src; c-Src phosphorylates Y576 and Y577 in the activation loop of FAK (Calalb et al., 1995), thereby prompting its full kinase activity (Mitra and Schlaepfer, 2006) and facilitating tumoural migration and invasion. Conversely, this pathway is modulated by PTEN that interacts directly with and dephosphorylates FAK (Tamura et al., 1998; Cai et al., 2005), leading to suppression of glioma cell migration and invasion (Park et al., 2002).

The involvement of c-Src and PTEN in FAK activity and migratory pathways bring in the interest and underlie one of the main goals of this thesis: to study the effects of TAT-$Cx43_{266-283}$ on Src-PTEN-FAK axis and the corresponding repercussions on GSC migration and invasion.

Importantly, our data revealed that TAT-$Cx43_{266-283}$ reduces Y397 FAK, which is dephosphorylated by PTEN, and also Y576 and Y577, which are c-Src-dependent sites. While the effects of TAT-$Cx43_{266-283}$ on c-Src inhibition are detectable at 4 h post-treatment, Y576 and Y577 FAK reduction is not evident until 6 h post-treatment in primary G9 GSCs, suggesting that the inhibition of FAK activity is caused by c-Src inhibition.

Once we determined that TAT-$Cx43_{266-283}$ inhibits the activity of FAK, we investigated whether this could be translated into a reduction of GSC migration and invasion. To do so, we tracked individual random movement of G166 and primary G9 GSCs for 15 h of treatment with TAT or TAT-$Cx43_{266-283}$, at concentrations ranging from 25 to 50 µM. Our results showed that even at 25 µM, TAT-$Cx43_{266-283}$ reduces the length of the trajectories described by both types of GSCs as compared with controls and TAT. To confirm the importance of FAK in GSC migration, we extended the analyses to 2 other patient-derived GSCs, G13 and G16, corroborating that TAT-$Cx43_{266-283}$ reduces the motility of GSCs.

FAK is an important mediator in GSC migration. In fact, the inhibition of FAK activity with the FAK inhibitor 14 resulted in a strong inhibition of GSC motility. In addition, this effect was not importantly increased by TAT-$Cx43_{266-283}$ suggesting that both agents share a common pathway to inhibit migration, i.e., the inhibition of FAK.

Considering the aggressive phenotype of GSCs that tortuously infiltrate into the brain parenchyma; we studied the effects of TAT-$Cx43_{266-283}$ on G166 and primary G9 GSCs invasion. To invade, in addition to migrate, cells need to degrade the extracellular matrix that they encounter along their way. Our

results revealed that TAT-Cx43$_{266-283}$ reduces the ability to invade a basement of extracellular matrix proteins in human G166 and primary G9 GSCs.

Even though concentrations up to 50 µM TAT-Cx43$_{266-283}$ inhibit G166 and patient-derived GSCs proliferation, after 15 h of treatment the effects are still modest. Consequently, the strong effects on migration and invasion are not attributable to the reduction of proliferation caused by TAT-Cx43$_{266-283}$. As a whole, our results show that TAT-Cx43$_{266-283}$ by targeting the axis c-Src-PTEN-FAK, inhibits two crucial features of malignant gliomas: migration and invasion.

Specificity of TAT-Cx43$_{266-283}$

To exclude TAT-Cx43$_{266-283}$ toxicity, and to ensure its specificity, we used a different cell-penetrating peptide with the region of Cx43 that comprises amino acids 274-291. This region includes the SH3 domain and next 8 amino acids up to complete the same number of amino acids as in TAT-Cx43$_{266-283}$.

As it has been described TAT-Cx43$_{266-283}$ reduces the random movement of primary G13 and G16 GSCs. More importantly, TAT-Cx43$_{274-291}$ is not capable of recruiting PTEN and CSK or inhibiting c-Src as previously mentioned (Gangoso et al., 2014, Gonzalez-Sanchez et al., 2016) and neither is able to reduce GSC migration or proliferation, as shown in this work. Consequently, these results highlight the specificity of the region 266-283 within Cx43 to inhibit GSC migration, especially when compared to the complex effect exerted by the whole protein.

Interestingly, the effects of TAT-Cx43$_{266-283}$ are not only specific to this region, but also selective to the cell type. Indeed, our results showed that TAT-Cx43$_{266-283}$ selectively affects GSCs without affecting neuron or astrocyte viability (see Additional results and Additional videos S14-S16).

Anti-tumour effects of TAT-Cx43$_{266-283}$ in patient-derived glioblastoma explants

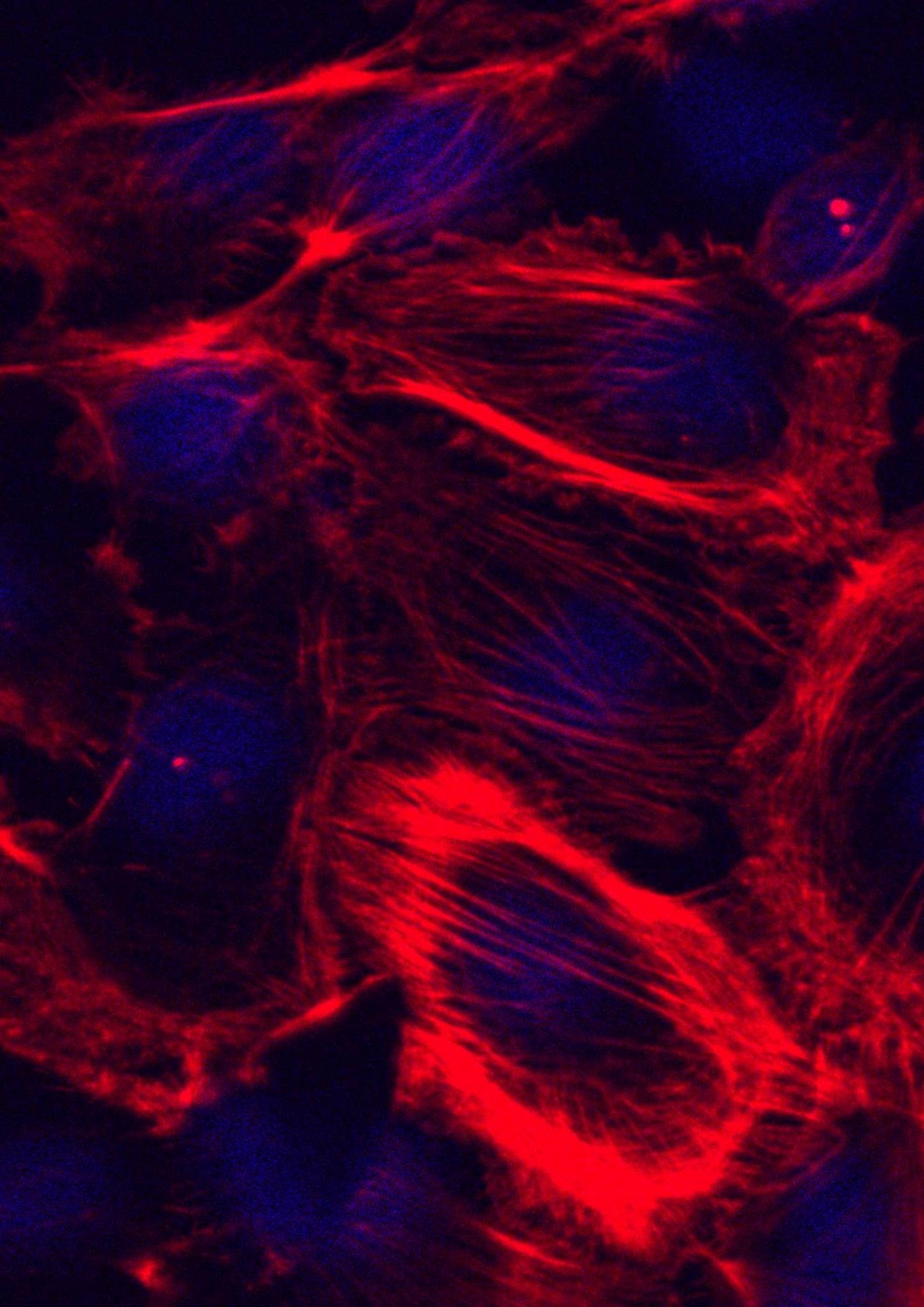
In previous works and throughout this PhD thesis, the cellular models used to study the Cx43-Src interaction have shown an evolution: astrocytes, rat C6 glioma cells, GliNS2 GSCs, G166 GSCs and patient-derived GSCs (G9, G12, G13, G15, G16). In fact, in the latest studies to specifically target c-Src, only the Cx43 region responsible for Cx43-Src interaction has been used via a cell-penetrating peptide, TAT-Cx43$_{266-283}$.

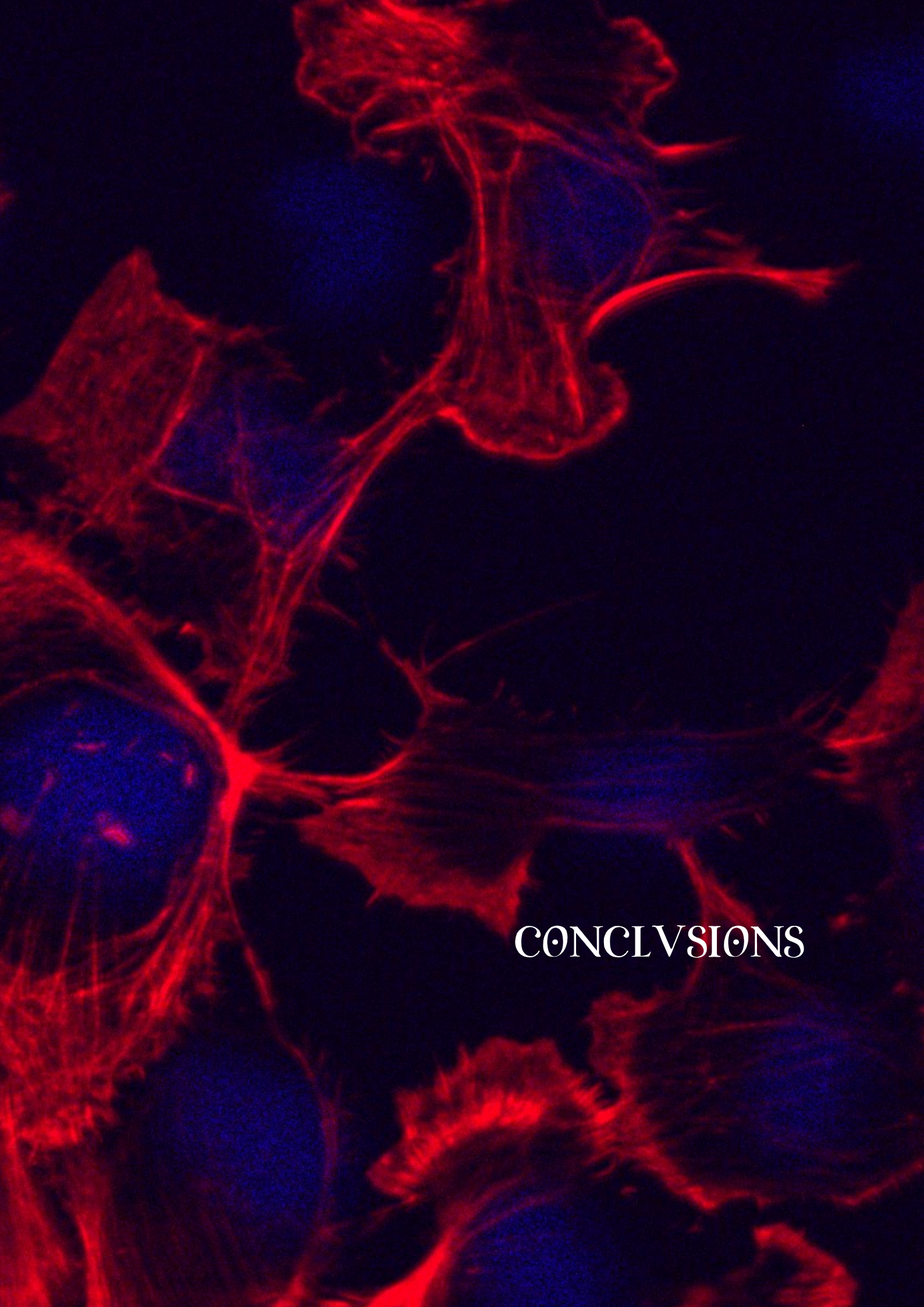
To further explore the effects of Cx43-Src interaction, our approach consisted of patient-derived tumour explants obtained from the same surgical biopsies used to obtain GSCs. These tumour blocks of similar size were allowed to adhere to laminin and cultured

individually in GSC medium. After attachment, glioblastoma explants were treated with 100 µM TAT or TAT-Cx43$_{266-283}$ and recorded by time-lapse microscopy. These movies revealed that TAT-Cx43$_{266-283}$ strongly impairs the growth, migration and survival of GSCs spreading out from at least patient-derived G9, G13 and G16 glioblastoma explants as compared to controls and TAT. Probably, the combination between GSC medium, that favours only stem cell survival, and the TAT-Cx43$_{266-283}$-mediated differentiation contributed to the death of cells coming out of the tumour explants. Interestingly, these effects were not limited to glioblastoma explants. We received one neuroblastoma specimen (N6) and one oligodendroglioma (O17)and processed them equally. Both of them showed similar effects to glioblastoma explants when treated with TAT-Cx43$_{266-283}$ (see supplemental videos S10-S11 for N6 and S12-S13 for O17, in Additional videos), suggesting that the effects of TAT-Cx43$_{266-283}$ may not be exclusive to glioblastoma stem cells.

The results obtained with glioblastoma explants should be highlighted because It has been proposed that this model provides an accurate, flexible and rapid platform for drug screening in a patient-specific fashion (Bayin et al., 2016), suggesting that TAT-Cx43$_{266-283}$ could be, in fact, a good structural base for the design of new therapies against this incurable disease.

Altogether, the results presented in this PhD thesis establish a nexus between molecular biology of the Cx43-Src interaction, targeted molecular therapies as the cell-penetrating peptide TAT-Cx43$_{266-283}$ and personalized medicine through patient-derived GSCs and explants. Advances to this respect are critical to mitigate the poor prognosis of patients diagnosed with glioblastoma.





CONCLVSIONS

1.The cell-penetrating peptide based on the region of the connexin43 (Cx43) that spans from amino acid 266 to amino acid 283, TAT-$Cx43_{266-283}$, inhibits the proliferation of human glioma stem cell (GSC), including primary patient-derived GSCs.

2.By using biotinylated cell-penetrating peptides as baits we have identified c-Src, CSK and PTEN as the intracellular partners of TAT-$Cx43_{266-283}$ in human GSCs. These results confirm that TAT-$Cx43_{266-283}$ favours the proximity of c-Src with its endogenous inhibitors PTEN and Csk. This leads to c-Src inhibition, which initiates a cascade of antitumor effects.

3.In addition to c-Src inhibition, TAT-$Cx43_{266-283}$ upregulated PTEN and inhibited AKT activity in human GSCs. Since the inhibition of GSC proliferation by TAT-$Cx43_{266-283}$ is lost when PTEN is silenced, it could be proposed that the inhibition of c-Src causes the upregulation of PTEN with subsequent reduction of AKT activity and glioma cell proliferation.

4.TAT-$Cx43_{266-283}$ reduced the phosphorylation of the focal adhesion kinase (FAK) at tyrosines 576 and 577 FAK, which are c-Src-dependent sites and also at tyrosine 397, which is dephosphorylated by PTEN. As these tyrosines are required for FAK activity, we concluded that TAT-$Cx43_{266-283}$ inhibits the activity of this important mediator of migration and invasion in human GSCs.

5.TAT-$Cx43_{266-283}$ reduced the random movement and the ability to invade a basement of extracellular matrix proteins in human GSCs, suggesting that TAT-$Cx43_{266-283}$, by targeting the axis c-Src-PTEN-FAK, inhibits two crucial features of malignant gliomas, migration and invasion.

6.The inhibition of proliferation and migration promoted by TAT-$Cx43_{266-283}$ is specific of this Cx43 region because TAT-$Cx43_{274-291}$, did not affect these GSC properties. These results reinforce the mechanism proposed, because TAT-$Cx43_{274-291}$ is a cell penetrating peptide based on Cx43 that does not recruit CSK and PTEN and does not inhibit c-Src.

7.TAT-$Cx43_{266-283}$ strongly impaired the growth, migration and survival of GSCs spreading from patient-derived glioblastoma explants obtained from the same surgical specimens used to obtain GSCs.

Final conclusion

Altogether, our results show the anti-tumour effects and mechanism of TAT-$Cx43_{266-283}$. This peptide strongly reduces cell proliferation, migration and survival in two highly relevant glioma models, such as patient-derived GSCs and freshly removed surgical specimens of malignant gliomas. Therefore, this structure could be the base for the design of new therapies against this devastating disease.

1.El péptido penetrante basado en la región de la conexina 43 (Cx43) que abarca desde el aminoácido 266 al aminoácido 283, TAT-$Cx43_{266-283}$, inhibe la proliferación de células madre de glioma humano, incluyendo células madre de glioma primarias derivadas de pacientes.

2.Mediante el uso de péptidos penetrantes biotinilados como anzuelo, hemos identificado a c-Src, CSK y PTEN como proteínas intracelulares con las que interacciona TAT-$Cx43_{266-283}$. Estos resultados confirman que TAT-$Cx43_{266-283}$ actúa como una plataforma estructural que favorece la proximidad de c-Src con sus inhibidores endógenos, PTEN y CSK en células madre de glioma humano. Ésto causa la inhibición de c-Src, con la consiguiente cascada de efectos antitumorales.

3.Además de inhibir c-Src, TAT-$Cx43_{266-283}$ aumentó los niveles de expresión de PTEN e inhibió la actividad de AKT en células madre de glioma humano.Puesto que la inhibición de la proliferación causada por TAT-$Cx43_{266-283}$ se reduce cuando PTEN se silencia, podemos sugerir que la inhibición de c-Src origina el aumento de PTEN con la consiguiente disminución de la actividad de AKT y la proliferación de células de glioma.

4.TAT-$Cx43_{266-283}$ redujo la fosforilación de la kinasa de adhesión focal (FAK) en las tirosinas 576 y 577, que son residuos dependientes de c-Src, y también la tirosina 397, que es desfosforilada por PTEN. Ya que estas tirosinas son necesarias para la actividad de FAK, podemos concluir que TAT-$Cx43_{266-283}$ inhibe la actividad de este importante mediador de la migración y la invasión de células madre de glioma humano.

5.TAT-$Cx43_{266-283}$ disminuyó el movimiento de las células madre de glioma humano, así como su capacidad de invadir una membrana recubierta de proteínas de la matriz extracelular. Estos resultados sugirien que TAT-$Cx43_{266-283}$ a través del eje Src-PTEN-FAK, inhibiendo dos de las más importantes características de los gliomas malignos, la migración y la invasión.

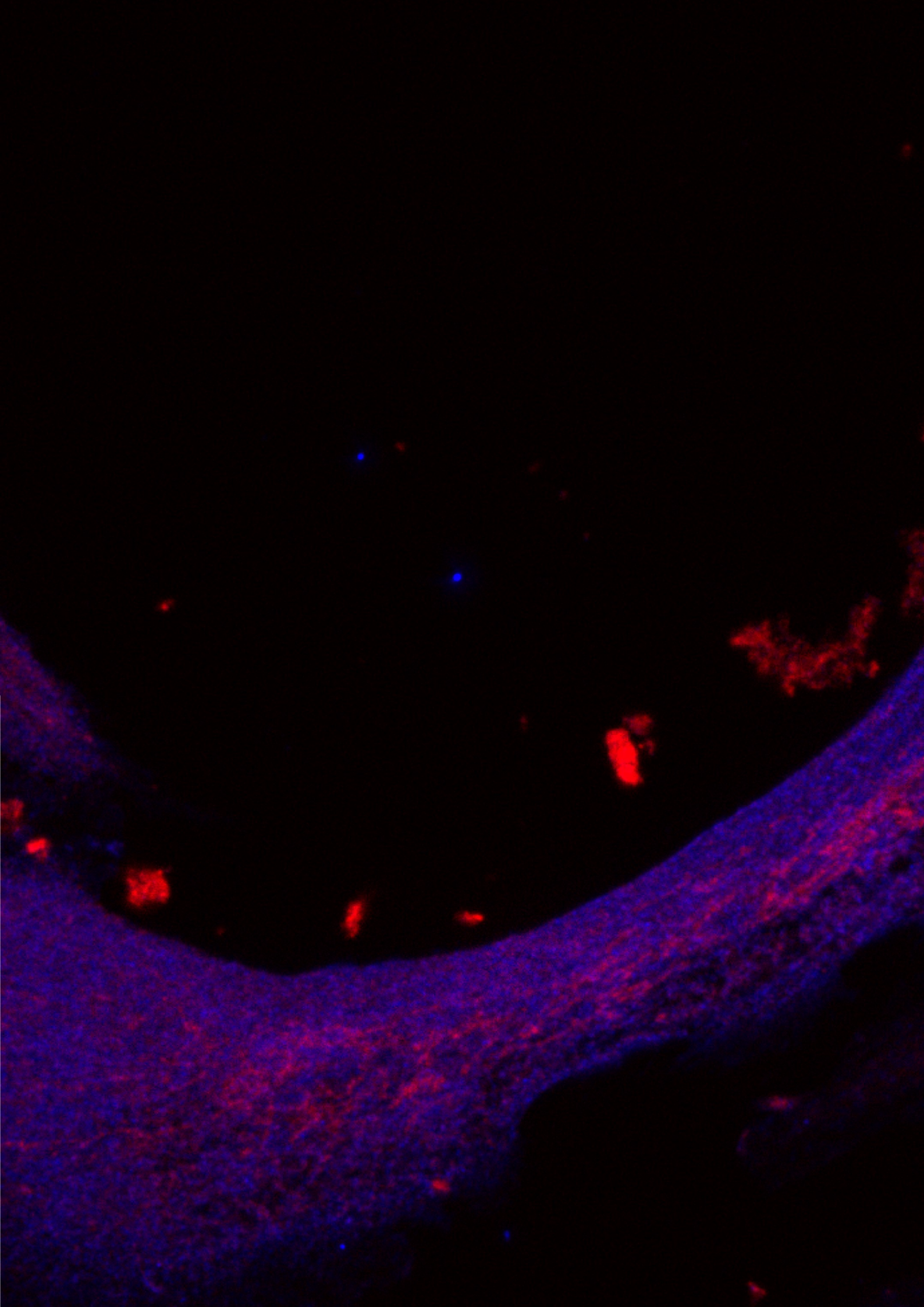
6.La inhibición de la proliferación y la migración promovida por TAT-$Cx43_{266-283}$, es específica de esta región de la Cx43, porque TAT-$Cx43_{274-291}$, no afectó a estas propiedades de las células madre de glioma humano. Estos resultados refuerzan el mecanismo propuesto, ya que TAT-$Cx43_{274-291}$ es un péptido penetrante basad en la Cx43

que no recluta a CSK ni a PTEN, y que no inhibe a c-Src.

7.TAT-Cx43$_{266-283}$ ejerce una acusada inhibición de la proliferación, la migración y la supervivencia de las células madre de glioma que se expanden a partir de explantes de glioblastoma obtenidos de pacientes inmediatamente tras la cirugía.

Conclusión final

Los resultados recogidos en esta memoria muestran los efectos anti-tumorales y el mecanismo de acción de TAT-Cx43$_{266-283}$. Este péptido disminuye la proliferación, migración y supervivencia celular en dos modelos de glioma de gran relevancia por su proximidad a la clínica, como son las células madre de glioma derivadas de pacientes y los explantes de gliomas malignos obtenidos y tratados inmediatamente después de la cirugía. Por tanto, esta estructura podría ser la base para el diseño de fármacos contra esta devastadora enfermedad.



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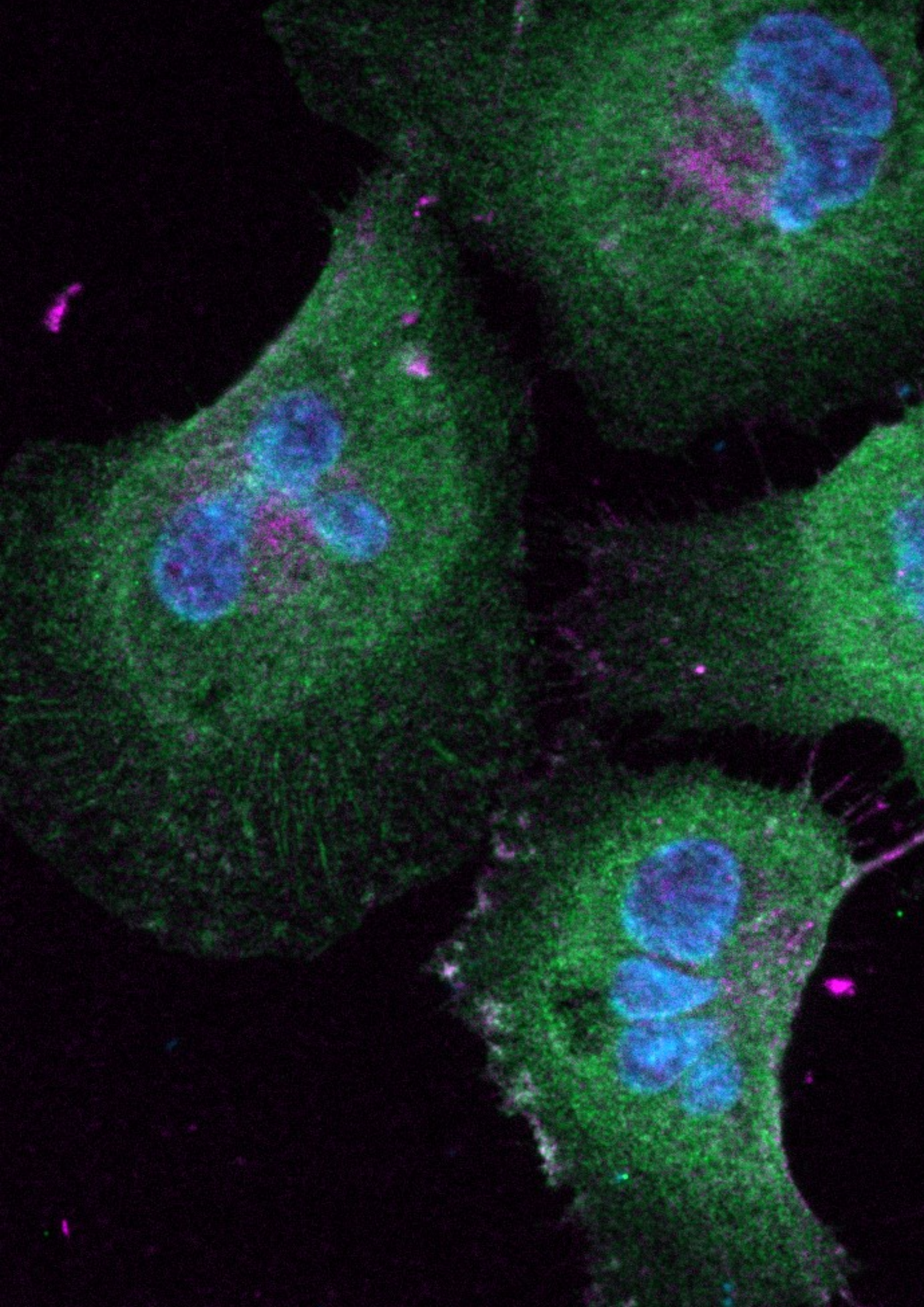

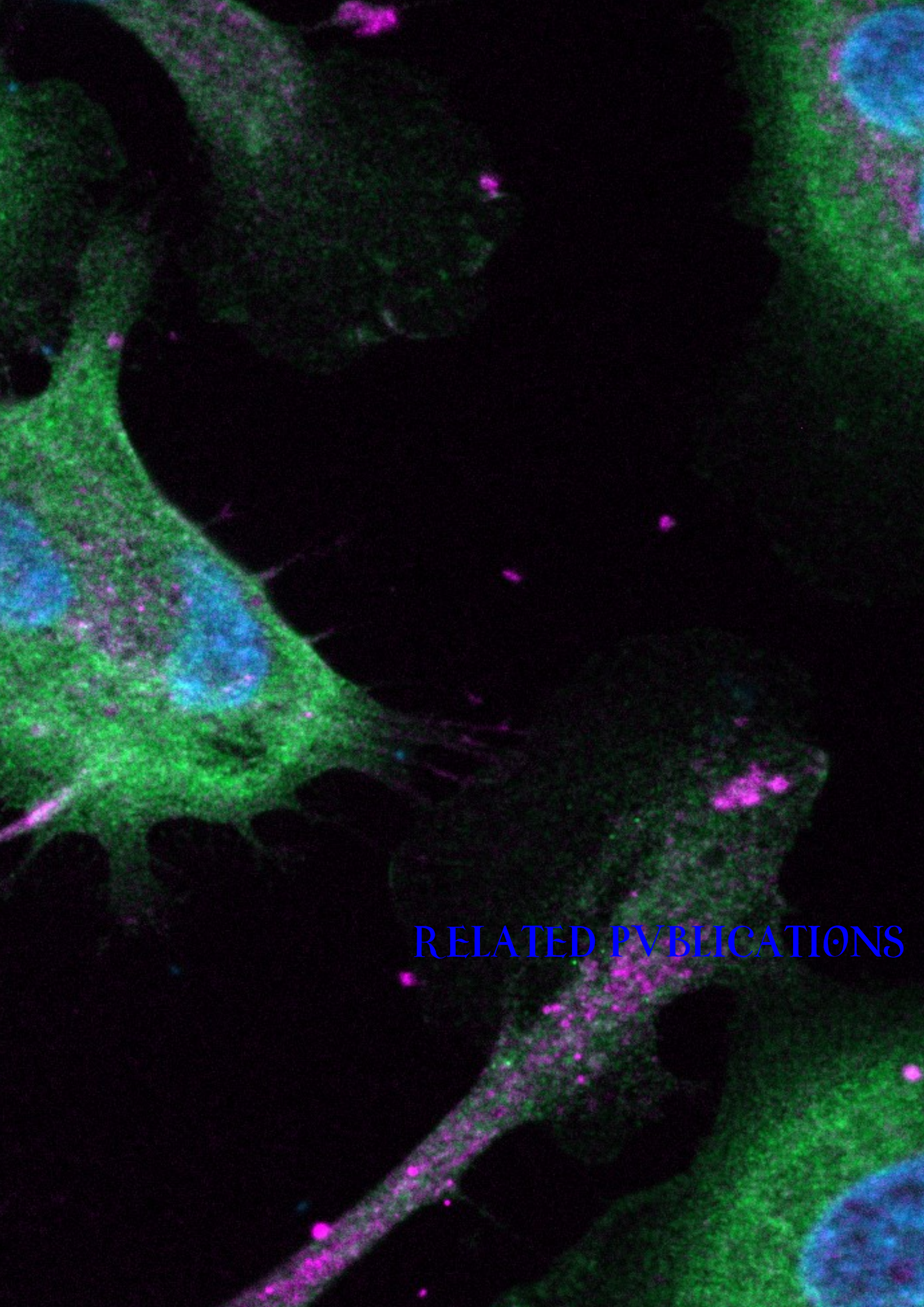

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REVIEW

THE ROLE OF CONNEXIN43–SRC INTERACTION IN ASTROCYTOMAS: A MOLECULAR PUZZLE

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Abstract—Connexin43 (Cx43) as a building block of gap junction channels and hemichannels exerts important functions in astrocytes. When these cells acquire a malignant phenotype Cx43 protein but not mRNA levels are downregulated, being negligible in high-grade astrocytoma or glioblastoma multiforme, the *most common* and deadliest of *malignant* primary *brain tumors in adults*. Some microRNAs associated to glioma target Cx43 and could explain the lack of correlation between mRNA and protein levels of Cx43 found in some high-grade astrocytomas. More importantly, these microRNAs could be a promising therapeutic target. A great number of studies have confirmed the relationship between cancer and connexins that was proposed by Loewenstein more than 40 years ago, but these studies have also revealed that this is a very complex relationship. Indeed, restoring Cx43 to glioma cells reduces their rate of proliferation and their tumorigenicity but this tumor suppressor effect could be counterbalanced by its effects on invasiveness, adhesion and migration. The mechanisms underlying these effects suggest the participation of a great variety of proteins that bind to different regions of Cx43. The present review focuses on an intrinsically disordered region of the C-terminal domain of Cx43 in which converges the interaction of several proteins, including the proto-oncogene Src. We summarize data that indicate that Cx43–Src interaction inhibits the oncogenic activity of Src and promotes a conformational change in the structure of Cx43 that allosterically modifies the binding to other important signaling proteins. As a consequence, crucial cell functions, such as proliferation or migration, could be strongly affected. We propose that the knowledge of the structural basis of the antitumorigenic effect of Cx43 on astrocytomas could help to design new therapies against this incurable disease.

This article is part of a Special Issue entitled: Astrocyte-Neuron Interact.

Key words: connexin, Src, glioma, proliferation, glucose uptake, migration.

Contents

INTRODUCTION

The term 'glioma' comprises the majority of malignancies of the central nervous system and encompasses all tumors that are thought to be of glial cell origin. These include astrocytomas, oligodendrogliomas, ependymomas and mixed gliomas. Among them astrocytomas are the most frequent in adults and have been traditionally classified by the World Health Organization (WHO) into four histological grades (Louis et al., 2007). Grade I (pilocytic astrocytoma) and grade II (diffuse astrocytoma) are low-grade gliomas that usually grow slowly. Grade III (anaplastic astrocytoma) is a highly malignant glioma with increased cellularity, pleomorphism and atypical nuclei. Grade IV

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Abbreviations: Cx43, connexin43; Cx43CT, C-terminal domain of connexin43; GLUT-3, glucose transporter-3; GSC, glioma stem cells; HIF, hypoxia-inducible factor; HK2, type II hexokinase; IDP, intrinsically disordered protein; IDR, intrinsically disordered region; MAPK, mitogen-activated protein kinases; miRNA, microRNA; MTOC, microtubule organizing center; PDZ, postsynaptic density 95/disk-large/zona occludens; pRb, retinoblastoma protein; PTEN, phosphatidylinositol 3,4,5-trisphosphate 3-phosphatase and dual-specificity protein phosphatase; PTPμ, protein tyrosine phosphatase μ; SH3, Src homology 3; TC-PTP, T-cell protein tyrosine phosphatase; WHO, World Health Organization; ZO-1, Zonula occludens-1.

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(glioblastoma multiforme) consists of poorly differentiated cells with microvascular proliferation and pseudopalisading necrosis. More recently, a gene expression-based molecular classification of glioblastoma into Proneural, Neural, Classical, and Mesenchymal subtypes has been proposed (Verhaak et al., 2010). Unfortunately, high-grade gliomas (WHO grade III and IV tumors) are the most common type of glioma in adults (Ostrom et al., 2014). Glioblastomas are rapidly progressive, very aggressive, diffusely infiltrate the adjacent brain tissue and are one of the most incurable forms of cancer in humans. Despite significant advances in diagnostics and therapeutics over the past decades, prognosis for patients with glioblastoma remains dismal, with a median survival of 16–19 months (Stupp et al., 2009), which indicates that a great research effort is required to propose new therapeutic strategies against this devastating disease.

A wide range of molecular alterations has been described in astrocytomas and includes genetic, epigenetic, transcriptomic, and microRNA (miRNA) changes (Purow and Schiff, 2009; Riemenschneider et al., 2010). In this review, we will focus on the alterations found in connexin43 (Cx43), an integral membrane protein encoded by GJA1 gene. Cx43 is the most abundant connexin in mammals, widely expressed in different tissues, including the central nervous system, where Cx43 is strongly expressed in astrocytes (Giaume et al., 1991). Cx43 assembles to form gap junction channels and hemichannels (Fig. 1A) that facilitate the behavior of astrocytes as cellular networks (Giaume et al., 2010) and the interchange of molecules between the cells and their extracellular medium (Bennett et al., 2003). Thus, Cx43 is highly expressed in astrocytes but this expression is downregulated when these cells acquire a malignant phenotype. It is well described that the levels of Cx43 protein inversely correlate with the degree of malignancy in astrocytomas. In fact, the levels of Cx43 protein in the vast majority of glioblastomas are negligible (Shinoura et al., 1996; Huang et al., 1999; Soroceanu et al., 2001; Pu et al., 2004; Caltabiano et al., 2010; Sin et al., 2012; Gielen et al., 2013).

Interestingly, restoring Cx43 to glioma cells reduces their rate of proliferation (Zhu et al., 1991; Huang et al., 1998) and their tumorigenicity (Yu et al., 2012). Although there are still many open questions to understand the mechanism by which Cx43 controls proliferation, most of the studies pinpoint the C-terminal domain of Cx43 (Cx43CT) responsible for the antiproliferative effect (Moorby and Patel, 2001; Zhang et al., 2003b). As recently reviewed (Naus and Laird, 2010; Sin et al., 2012), this tumor suppressor effect could be counterbalanced by its effects on invasiveness (Zhang et al., 2003a), adhesion (Elias et al., 2007) and migration (Matsuuchi and Naus, 2013). Several interesting reviews about the link of connexins with cancer, including astrocytomas have appeared in recent years (Mesnil et al., 2005; Vinken et al., 2006; Naus and Laird, 2010; Sin et al., 2012). Collectively, these studies have confirmed the relationship between cancer and connexins that was proposed by Loewenstein more than 40 years ago (Loewenstein and Kanno, 1966), but they have also revealed that this is a very complex relationship in which a great variety of molecular partners are participating. Therefore, the knowledge of the structural basis of the antitumorigenic effect of Cx43 on astrocytomas is necessary to design new therapies against this devastating disease. The present review focuses on a specific region of the Cx43CT in which converges the interaction of several partners, including the proto-oncogene Src. We summarize data that indicate that Cx43–Src interaction inhibits the oncogenic activity of Src and promotes a conformational change in the structure of Cx43 that allosterically modifies the binding to other important signaling proteins. As a consequence, crucial cell functions, such as proliferation or migration, could be strongly affected.

CX43 PROTEIN BUT NOT MRNA IS DOWNREGULATED IN HIGH-GRADE ASTROCYTOMAS

Importantly, in some of high-grade astrocytomas the levels of mRNA do not mirror with those of the protein (Caltabiano et al., 2010; Sin et al., 2012; Gielen et al., 2013). Thus, Caltabiano et al. analyzed 32 astrocyte tumors, and they found that 90% of the high-grade astrocytomas (7/7 grade III, 10/13 grade IV) demonstrated an intracytoplasmic positivity for Cx43 mRNA, despite the fact that 80% of the high-grade astrocyte tumors (5/7 grade III and 11/13 grade IV) demonstrated a marked reduction or negativity for Cx43 immunostaining labeling. Gielen et al. analyzed the glioma repository of The Cancer Genome Atlas (TCGA) to examine the DNA copy number and mRNA expression profile of Cx43/GJA1 in 372 grade III and IV astrocytomas. They found that Cx43/GJA1 has a tendency to be deleted in glioblastoma (11.3%) and that while 27.3% of glioblastoma exhibits a twofold downregulation of Cx43 mRNA, 20.3% of GBM show a twofold upregulation of Cx43 mRNA, compared to normal tissues. These authors performed a screen of a brain tumor tissue array and confirmed minimal Cx43 protein levels in glioblastomas.

Together, these data suggest that Cx43 mRNA levels in gliomas do not predict the levels of the protein. Consequently, the reduced levels of Cx43 protein found in high-grade glioma are not mainly due to a reduced genetic transcription, but to alterations in post-transcriptional mechanisms (Klotz, 2012). The inhibition of translation and/or the enhanced degradation of the protein could be the primary mechanism for Cx43 protein downregulation, as described for other key regulators of proliferation such as p27 (kip1), which is frequently decreased maintaining mRNA levels unchanged in many human cancers, including glioblastomas (Blain et al., 2003; Gillies and Lorimer, 2007).

Some miRNAs involved in gliomagenesis target Cx43

One of the cellular mechanisms responsible for post-transcriptional regulation is the synthesis of miRNAs, which are endogenous small noncoding transcripts of 21–23 nucleotides that downregulate gene expression by conducting mRNA degradation or by inhibiting protein

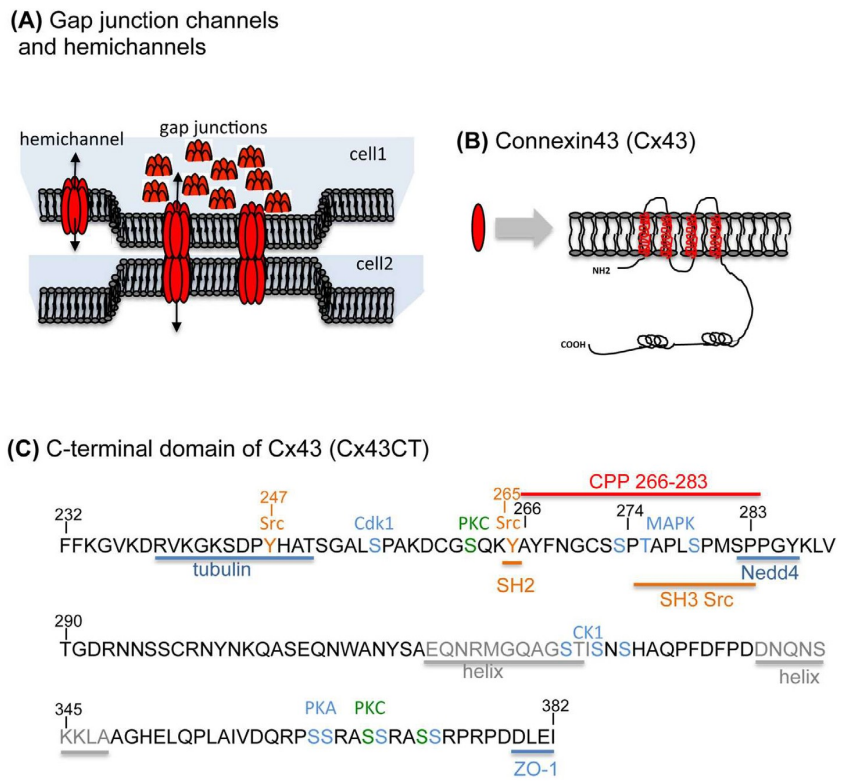

Fig. 1. Structure and interactions of connexin43 (Cx43). (A) Cx43 forms gap junction channels and hemichannels. Gap junctions channels are formed by two hemichannels, called connexons, each composed of six connexin43 protein subunits. (B) Cx43 structure. Cx43 organizes as tetraspan membrane proteins with two extracellular loops and three intracellular regions represented by the N- and C-terminal domains and the loop linking transmembrane domains. (C) C-terminal domain of Cx43 (Cx43CT). Cx43CT comprises amino acids 232–382 and is an elongated random coil, with two regions of alpha-helical structure (amino acids in gray) (Sorgen et al., 2004). Some binding-domains are underlined and their molecular partners are indicated. Phosphorylation sites are shown in different colours together with the corresponding kinases. Cdk1, cyclin-dependent kinase 1; CK1, casein kinase 1; CPP, cell-penetrating peptide; MAPK, mitogen-activated protein kinases; Nedd4, Neural precursor cell expressed developmentally down-regulated protein 4; PKA, protein kinase A; PKC, protein kinase C.

translation. In recent years the role of miRNAs in cancer has been demonstrated in many types of tumors including glioblastomas where they can control processes such as proliferation, invasion, angiogenesis and apoptosis (Chan et al., 2005; Ernst et al., 2010). Interestingly, some miRNAs involved in gliomagenesis downregulate Cx43 expression. This is the case of miRNA-125b that binds to the Cx43 3′-UTR to downregulate Cx43 expression and promotes glioma cell-line growth, clone formation and inhibits apoptosis *in vitro*. The overexpression of Cx43 could counteract the effects of miRNA-125b on glioma cell proliferation and anti-apoptosis, suggesting that Cx43 downregulation is involved in the miR-125b tumorigenic effect (Jin et al., 2013). Other miRNAs can repress the expression of Cx43 in gliomas, for instance, miRNAs 221–222 (Hao et al., 2012). miRNA-221 and miRNA-222, encoded in tandem on chromosome X, have frequently been found to be dysregulated in astrocytomas (Ciafrè et al., 2005; Conti et al., 2009).

One feature of miRNAs that highlights their relevance in cancer biology is the fact that each miRNA can target and downregulate the expression of several mRNAs to affect cell behavior. For instance, miRNAs 221–222 despite repressing Cx43, regulate important tumor-related proteins such as, p27, an important repressor of the progression through the cell cycle (Gillies and Lorimer, 2007), phosphatidylinositol 3,4,5-trisphosphate 3-phosphatase and dual-specificity protein phosphatase (PTEN), a key regulator of cell growth and apoptosis and one of the most commonly altered tumor suppressors in gliomas (Garofalo et al., 2009) and protein-tyrosine phosphatase μ (PTPμ), an important mediator of invasiveness (Quintavalle et al., 2012). Therefore, the synthesis of miRNAs 221–222 in one cell can reduce simultaneously the expression of Cx43, p27, PTEN and PTPμ, which are important tumor suppressor proteins, favoring tumoral transformation.

In addition, Cx43 is a target of miR-19a/b (Danielson et al., 2013), which is part of the cluster miR17-92, a potential oncogene firstly described in lymphomas (He et al., 2005) and thereafter in several other tumors including gliomas (Ernst et al., 2010). This cluster, located on human chromosome 13 (chromosome 14 in mice) encodes a polycistronic miRNA gene, which is matured into six functional miRNAs, miR-17, miR-18a, miR-19a, miR-19b, miR-20 and miR-92. Among them, miR-19a/b have shown to be overexpressed in gliomas and their expression correlates with malignancy (Jia et al., 2013).

All together these data suggest that miRNAs could be involved in the downregulation of Cx43 protein found in high-grade gliomas. Downregulation mediated by miRNA is typically the consequence of imperfect base pairing of miRNAs with sequences in the 3′ untranslated regions of target mRNAs. This causes inhibition of translation without mRNA degradation, and therefore could explain the lack of correlation between mRNA and protein levels of Cx43 found in some high-grade gliomas (Caltabiano et al., 2010; Sin et al., 2012; Gielen et al., 2013). More importantly, miRNAs could be an interesting target for glioma therapy.

THE RELEVANCE OF THE CX43CT FOR ASTROCYTOMAS

The connexin family consists of 21 isoforms in humans, named according to their predicted molecular masses in kilodaltons. At the molecular level, connexins organize as tetraspan membrane proteins with two extracellular loops and three intracellular regions represented by the N- and C-terminal domains and the loop linking transmembrane domains 2 and 3 (Fig. 1B) (Kumar and Gilula, 1996). Connexin isoforms present a high level of homology except for the intracytoplasmic loop and C-terminal domain.

The Cx43CT plays a role in the trafficking, size, localization, and turnover of gap junctions, as well as the level of intercellular coupling via numerous posttranslational modifications and protein–protein interactions (Giepmans, 2004; Herve et al., 2007; Laird, 2010; Thévenin et al., 2013). Early structural studies of Cx43, which used electron crystallography, and the more recent X-ray crystal structure of Cx26 have provided a significant amount of information about channel architecture (Unger et al., 1999; Maeda et al., 2009). NMR data indicate that the Cx43CT (amino acids 232–382) exists primarily as an elongated random coil, with two regions of alpha-helical structure (Fig. 1B) (Sorgen et al., 2004). All the structural information available confirms that Cx43CT is an intrinsically disordered region (IDR), with a small amount of alpha-helical content, and consequently, Cx43 is an intrinsically disordered protein (IDP) (Grosely et al., 2013). The relevance of IDPs has been revealed over the last decade (for a review, see: Babu et al., 2011) because of their role in binding, signaling and regulatory functions. The lack of structure provides a large interaction surface area and a high conformational flexibility allowing IDPs to scaffold and interact with numerous other proteins. Due to this structural plasticity IDPs operate dynamically within numerous functional pathways, behaving as hubs and nodes in signaling networks. In addition, IDRs tend to be phosphorylated/dephosphorylated increasing the conformational versatility of these proteins. As a result, altered expression of IDPs is associated with many diseases; for instance, evidence suggests that approximately 80% of proteins participating in processes driving cancer contain IDRs (Iakoucheva et al., 2002). Importantly, all the features mentioned above for IDPs are present in Cx43.

The interactome of Cx43 is mainly localized within the Cx43CT IDR that contains several known protein-binding regions, such as Src homology (SH)3-, SH2-, microtubule- and postsynaptic density 95/disk-large/zona occludens (PDZ)-binding motifs (Giepmans, 2004; Herve et al., 2007; Laird, 2010) and several residues that can be phosphorylated by important cellular kinases (Solan and Lampe, 2009). In fact, Cx43CT contains 27 Ser/Tyr residues and all but eight can be phosphorylated by several kinases, such as protein kinase A, protein kinase C, mitogen-activated protein kinases (MAPK), Src, cyclin-dependent kinase 1 and casein kinase1 (Lampe and Lau, 2000; Pahujaa et al., 2007; Huang et al., 2011). It is noteworthy that some of these protein-interaction motifs and phosphorylation sites overlap within the Cx43CT sequence (residues 266–283; Fig. 1C). This sequence is within an IDR and therefore allows a rapid switch between molecular partners. In general, the location and cellular conditions, along with post-translational modifications, will dictate which protein associates with the CT domain. Upon comparing the primary sequences of Cxs, Kopanic et al. (Kopanic et al., 2014) have recently proposed that several residues within the CT (residues 272–286 in Cx43) constitute a master regulatory domain, i.e., an IDR with known sites of phosphorylation and overlapping sequence motifs that enable binding with multiple molecular partners.

Interestingly, this master regulatory domain contains the sequence required for Src binding to Cx43 (Fig. 1C). Src is a proto-oncogene that plays key roles in the development of several types of tumors (Frame, 2004) including glioblastomas (Du et al., 2009). In this review we propose that Cx43–Src interaction modifies Cx43 function, the binding to other molecular partners and the oncogenic activity of Src, impacting several important aspects related to astrocytoma biology.

CX43–SRC INTERACTION ALLOSTERICALLY AFFECTS THE BINDING OF SEVERAL PARTNERS

Src is a non-receptor tyrosine kinase that participates in signaling pathways that control a diverse spectrum of biological events, including proliferation, differentiation, survival and migration (Thomas and Brugge, 1997). Src interacts with Cx43 by binding to the Src homology 3 (SH3)-binding domain of Cx43, a proline-rich region (amino acids 274–284) and then phosphorylates tyrosine 265 providing an SH2-binding domain with the subsequent phosphorylation at tyrosine 247 (Fig. 1C) (Kanemitsu et al., 1997). As a consequence of these phosphorylations gap junctional intercellular communication is reduced (Swenson et al., 1990; Lin et al., 2001; Giepmans et al., 2001a) and Cx43 turnover is initiated (Solan and Lampe, 2014).

Interestingly, NMR studies show that although binding of Src is confined to the SH3-binding domain of Cx43CT, conformational changes resulting from this binding extend for large distances along the Cx43CT (Sorgen et al., 2004) affecting the interaction with other molecular partners (Fig. 2A). So far, the phosphorylation of Cx43 promoted by Src has been shown to modify the interaction

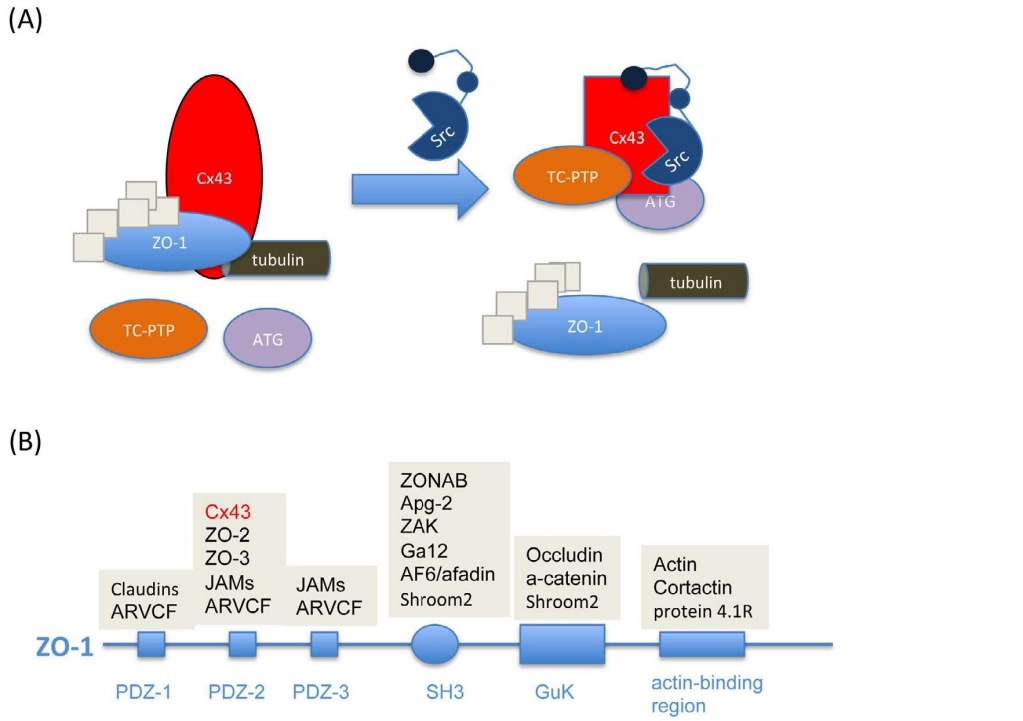

Fig. 2. Cx43–Src interaction allosterically affects the binding of several partners. (A) The phosphorylation of connexin43 (Cx43) promoted by Src modifies Cx43 structure and disrupts, at least, the interaction of Cx43 with Zonula occludens-1 (ZO-1) and its binding partners (small beige squares mentioned in B) (Toyofuku et al., 2001), tubulin (Saidi Brikci-Nigassa et al., 2012) and promotes the interaction of autophagy-related proteins (ATG) (Bejarano et al., 2014) and the phosphatase TC-PTP, which reverts the phosphorylation of Cx43 promoted by Src (Li et al., 2014). (B) ZO-1 structure and binding partners (reviewed in Thévenin et al., 2013). ARVCF, armadillo repeat gene deleted in velo-cardio-facial syndrome; Ga12, G-protein subunit alpha-12; GuK, guanylate kinase; JAM, Junctional Adhesion Molecule; PDZ, postsynaptic density 95/disk-large/zona occludens; SH3, Src homology 3; ZAK, ZO-1 associated kinase; ZONAB, ZO-1 – associated nucleic acid binding protein.

of Cx43 with Zonula occludens-1 (ZO-1), tubulin, autophagy-related proteins (Bejarano et al., 2014) and the T-cell protein tyrosine phosphatase (TC-PTP), also known as *PTPN2* (protein tyrosine phosphatase N2) (Li et al., 2014), which reverts the phosphorylation of Cx43 promoted by Src. In this review we will summarize data from ZO-1 and tubulin interactions since the structural details have been thoroughly documented and are tightly linked to important aspects of glioma biology.

Cx43–ZO-1 interaction

ZO-1 is a tight junction-associated protein that belongs to the membrane-associated guanylate kinase (MAGuK) family and is composed of three PDZ domains and one Src homology (SH3) and guanylate kinase (GuK) domain (Fig. 2B) (González-Mariscal et al., 2000). The modular organization of these proteins allows them to function as scaffolds, which associate with transmembrane tight junction proteins, the cytoskeleton and signal transduction molecules (Hartsock and Nelson, 2008). ZOs and some of their partners, such as beta-catenin, can shuttle between the membrane and the nucleus, where they may regulate the expression of genes involved in cell malignancy (Polette et al., 2007).

Cx43 interacts with the second PDZ domain of ZO-1 protein through its PDZ-binding domain (last four amino acids in Cx43) (Giepmans and Moolenaar, 1998; Toyofuku et al., 1998) (Fig. 1C). Thus, the second PDZ domain (PDZ-2; amino acids 160–290 in ZO-1) shows a strong binding to Cx43CT (K_D = 386 nM) (Duffy et al., 2003) and affects the resonance peaks of the last 19 amino acids of the Cx43CT, a region larger than what was expected from the putative "PDZ-binding domain" of Cx43 (Sorgen et al., 2004). Cx43–ZO-1 interaction regulates the rates of channel assembly, turnover and the localization of Cx43 at the cell surface, which could regulate inversely gap junctional communication and hemichannel activity (Rhett et al., 2011). Localization of ZO-1 in the plasma membrane is important to avoid cellular malignization caused by the cytoplasmic localization of its PDZ domains (Reichert et al., 2000). The interaction of the Cx43CT with these domains could prevent the cytoplasmic localization and therefore malignization. In fact, Cx43 interacts with ZO-1 in a cell-cycle phase-specific manner. Thus, Cx43 preferentially interacts with ZO-1 when cells are in G0 phase, which coincides with an efficient assembly and stabilization of gap junctions (Singh et al., 2005).

Remarkably, Toyofuku et al. (Toyofuku et al., 2001) reported that phosphorylation of Cx43 on Y247 by Src disrupted the interaction of Cx43 with ZO-1 (Fig. 2A). The

structural analyses performed by Sorgen et al. (2004) confirmed the regulation of Cx43–ZO-1 interaction promoted by Src. Src-mediated tyrosine phosphorylation and SH2 domain binding may induce a structural change in the C-terminal region of Cx43, thereby hindering the interaction between the C-terminal tetrapeptide and the ZO-1 PDZ-2 domain (Toyofuku et al., 2001). Since ZO-1 contains several docking domains to act as a scaffolding protein, the disruption of Cx43–ZO-1 interaction promoted by Src could result in changes in the interaction of Cx43 with several other proteins bound to ZO-1 (Fig. 2).

Cx43–microtubule interaction

Cx43 interacts directly with microtubules through a tubulin-binding domain located in the juxtamembrane region of the Cx43CT (Giepmans et al., 2001b). This is a short region of 26 amino acid residues (234–259), which is unstructured and adopts a helix conformation upon binding with tubulin. The structural analyses show that Cx43 interacts with tubulin through the minimal sequence 239–250 (Fig. 1C) (Saidi Brikci-Nigassa et al., 2012). Interestingly, the Cx43–tubulin interaction is prevented by the phosphorylation of Y247 by Src because the aromatic ring of tyrosine-247 is within the tubulin-binding region and is directly involved in Cx43–tubulin binding. Consequently, Cx43 phosphorylation by Src has a negative impact on tubulin recognition (Saidi Brikci-Nigassa et al., 2012).

Cx43–tubulin interaction is required for Cx43 cell surface trafficking (Lauf et al., 2002) and, also modulates cell polarity and directional cell migration (Francis et al., 2011). To accomplish directional cell migration, the cytoskeleton requires to be aligned in a polarized way with the microtubule organizing center (MTOC) and the Golgi needs to be positioned forward facing at the cell's leading edge (Kupfer et al., 1982; Magdalena et al., 2003). Cx43 knockout cells fail to realign their MTOC and Golgi with the direction of cell migration. Forced expression of Cx43 rescues this phenotype but forced expression of Cx43 with deletion of its tubulin-binding domain (Cx43dT) recapitulates the cell migration defects seen in Cx43KO cells (Francis et al., 2011), indicating the participation of the tubulin-binding domain of Cx43 in cell polarity and directional cell migration. In these Cx43KO cells, the establishment of cell polarity is also compromised by the loss of stabilized microtubules. In addition, directional cell migration is disturbed by alterations in the organization of the actin cytoskeleton and focal adhesion contacts caused by the absence of Cx43 (Xu et al., 2006; Rhee et al., 2009). Using a screening approach, Simpson et al. (2008) identified Cx43 as one of the genes that regulate epithelial cell migration, they found that Cx43-knockdown cells showed minimal cell–cell adhesion, poor front-rear polarity and consequently, erratic migration. Altogether, these findings support the role of Cx43 in modulating cell motility through the interaction with tubulin and other proteins associated with the cytoskeleton, such as N-cadherin (Wei et al., 2005), E-cadherin, alpha-catenin (Fujimoto et al., 1997), beta-catenin (Ai et al., 2000), p120 (Wei et al., 2005), p150 (Prochnow and Dermietzel, 2008), vinculin (Xu et al., 2006), ZO-1 (see above) and various actin associated proteins including drebrin (Butkevich et al., 2004), reviewed in (Matsuuchi and Naus, 2013).

The aggressive and widespread migration of glioma cells into surrounding brain tissue is a hallmark of astrocytic tumors (Cuddapah et al., 2014). The efficacy of surgical resection and targeted radiotherapy is limited by the infiltrative non-destructive nature of these tumors (Giese and Westphal, 1996), defined as "intraparenchymally metastatic" (Bernstein, 1996). As mentioned above, Cx43 interaction with tubulin clearly affects directional cell migration. Consequently, the tubulin-binding region of Cx43 (residues 239–250) and the effect of Src activity on this binding could be highly relevant in the complex field of glioma cell migration.

CX43–SRC INTERACTION INHIBITS THE ONCOGENIC ACTIVITY OF SRC

Although the ability of Src to phosphorylate Cx43 and inhibit gap junctional intercellular communication is well known, it has been recently shown that the interaction of Cx43 with Src can also inhibit the oncogenic activity of Src (Fig. 3) (Herrero-Gonzalez et al., 2010; Gangoso et al., 2014). Glioblastoma cells exhibit a strong oncogenic Src activity (Du et al., 2009), which plays an important role in the transforming phenotype of astrocytomas (Weissenberger et al., 1997). Autophosphorylation at Tyr-416 activates Src contributing to the malignant phenotype (Kmiecik and Shalloway, 1987). Interestingly, restoring Cx43 to glioma cells reduces Src activity, by decreasing the active form of Src (phosphor-Src Tyr416) and increasing the inactive form of Src (phosphor-Src Tyr527) (Herrero-Gonzalez et al., 2010). Collectively, these data indicate that Cx43 and Src are mutually regulated by a phosphorylation/dephosphorylation loop. It should be mentioned that the mechanism by which the interaction of Cx43 with Src reduces Tyr-416 phosphorylation and increases Tyr-527 phosphorylation on Src remains to be elucidated.

Since the oncogenic activity of Src is linked to crucial signaling pathways, including the Ras-Raf-MEK-ERK (MAPK/-extracellular signal-regulated kinase (ERK)) and phosphatidylinositol 3 kinase (PI3K)/Akt pathways (Frame, 2004), it is not surprising that the inhibition of Src activity promoted by its interaction with Cx43 affects the regulation of the cell cycle, glucose uptake and even glioma stem cell (GSC) phenotype, as described in Fig. 3.

Effects on the cell cycle

The antiproliferative effect of Cx43 has been widely described. The expression of several proteins, such as p27, p21, Skp2, CCN1/Cyr61 or CCN3/NOV (Zhang et al., 2003c; Sin et al., 2008), changes as a consequence of the levels of Cx43 and can contribute to understand the downstream pathways involved in the antiproliferative effect of Cx43 in glioma cells. However, the initial events leading to these changes are just beginning to be elucidated. One of the proposed initial steps in the antiproliferative effect of Cx43 is the interaction of Cx43 with Src (Herrero-Gonzalez et al., 2010; Gangoso et al., 2014).

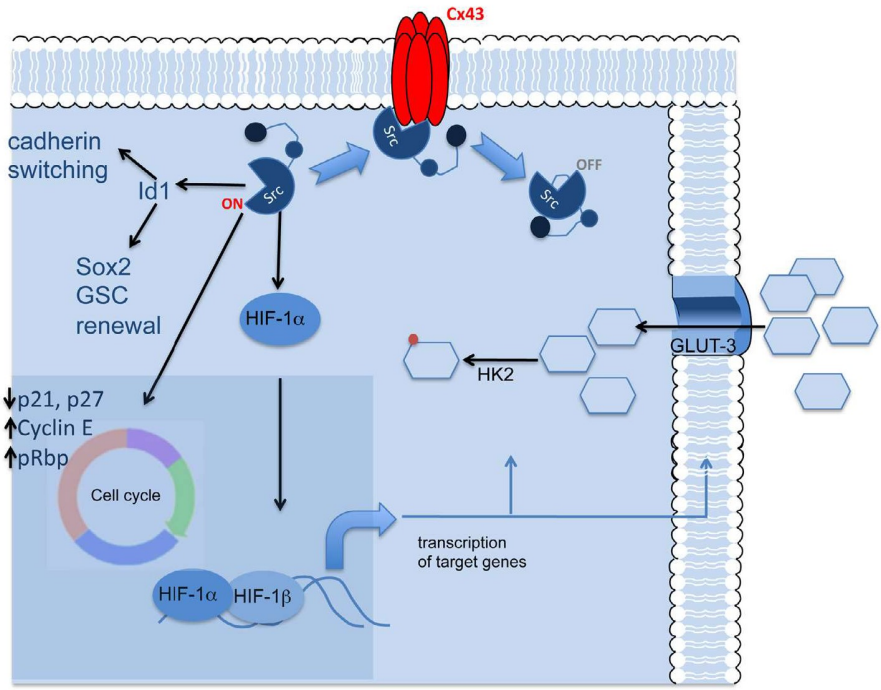

Fig. 3. Cx43–Src interaction inhibits Src activity. Restoring connexin43 (Cx43) to glioma cells inhibits Src activity. Consequently, Cx43 prevents the reduction of p27 and the increase in retinoblastoma protein (pRb) phosphorylation and cyclin E expression leading to G0/G1 cell-cycle arrest. Similarly, downregulation of Cx43 in astrocytes promotes Src activation with the subsequent upregulation of hypoxia-inducible factor-1α (HIF-1α), which in turn induces the transcription of glucose transporter-3 (GLUT-3) and type II hexokinase (HK2) to increase the rate of glucose uptake and proliferation. Finally, Cx43 or cell-penetrating peptides containing Cx43 residues affected by the binding of the Src SH3 domain (CPP 266–283, Fig. 1C) reduce Src activity, downregulate Id1 and Sox-2 expression and promote cadherin switching. Consequently, the ability of glioma stem cells (GSCs) to generate neurospheres is decreased and the percentage of cells expressing differentiation markers is increased, suggesting a reversion of GSC phenotype.

In this sense, this interaction triggers a signaling pathway that reduces their rate of proliferation. Interestingly, Cx43 lacking its Src phosphorylation sites does not inhibit glioma cell proliferation (Herrero-Gonzalez et al., 2010) pinpointing Cx43–Src interaction as an initial event in the antiproliferative effect exerted by Cx43 (Herrero-Gonzalez et al., 2010; Gangoso et al., 2014).

Restoring Cx43 to glioma cells decelerates the passage of cells from G0/G1 to the S phase of the cell cycle. Among the factors responsible for this effect is the reduced expression of cyclin E, a step required for the passage of cells from the G1 to S phase. It should be mentioned that Cx43 prevents the increase in retinoblastoma protein (pRb) phosphorylation that occurs in glioma cells upon entering S-phase (Herrero-Gonzalez et al., 2010). Since the phosphorylation of pRb releases E2F, a transcription factor implicated in cyclin E expression (Geng et al., 1996), the reduction in pRb phosphorylation could provoke a decrease in cyclin E. Moreover, pRb is phosphorylated by cyclin-dependent kinases, which are inhibited by several regulators such as p21 and p27. Since Cx43 upregulates p21 and p27 (Koffler et al., 2000; Zhang et al., 2003c; Sanchez-Alvarez et al., 2006; Herrero-Gonzalez et al., 2010) this effect of Cx43 might reduce pRb phosphorylation and prevent the upregulation of cyclin E, with the ensuing arrest in the progression from G0/G1 to S phase of the cell cycle in glioma cells. In agreement with the role of Cx43–Src interaction in the initial steps of the regulation of cell proliferation, Cx43 lacking its Src phosphorylation sites did not modify p27, pRb phosphorylation or cyclin E expression (Herrero-Gonzalez et al., 2010).

It should be mentioned that the phosphorylation of p27 by Src promotes p27 degradation (Chu et al., 2007; Grimmler et al., 2007). Consequently, the inhibition of Src activity promoted by Cx43 (Herrero-Gonzalez et al., 2010; Gangoso et al., 2014) could also serve to upregulate p27 expression and activity, ultimately inhibiting the upregulation of cyclin E and therefore, the passage from G1 to S phase of the cell cycle (Fig. 3).

Effects on glucose uptake

One interesting feature of Cx43 is the relationship with glucose uptake. Under physiological situation this channel allows the distribution of glucose and other metabolic substrates throughout the network of astrocytes communicated through gap junctions (Tabernero et al., 1996; Giaume et al., 1997, 2010). This astroglial metabolic network provides glucose to sustain neuronal activity (Rouach et al., 2008). Interestingly, the inhibition of gap junctional communication or the reduction of Cx43 expression results in an increase in the rate of glucose uptake (Fig. 3) (Herrero-Gonzalez et al., 2009). This effect includes the induction of the high-affinity glucose transporter-3 (GLUT-3), and the enzyme that

phosphorylates and traps glucose, type II hexokinase (HK2) (Sanchez-Alvarez et al., 2004; Herrero-Gonzalez et al., 2009; Gangoso et al., 2012). It should be stressed that GLUT-3 and HK2 are not normally expressed by astrocytes but are tightly linked to the oncogenic phenotype of glioma cells (Wolf et al., 2011; Flavahan et al., 2013).

Hypoxia-inducible factor (HIF)-1α/β heterodimer is a master transcription factor for several genes involved in glucose uptake, angiogenesis, glycolysis, pH balance and metastasis, including GLUT-3 and HK2 (revised in Denko, 2008). While HIF-1β is stable and constitutively expressed, HIF-1α is highly regulated, as well as susceptible to oxygen-dependent degradation due to the sequential action of oxygen-dependent prolyl hydroxylases and the VHL ubiquitin ligase. It should be mentioned that although HIF-1α is mainly activated under hypoxia, several factors activate HIF-1α under normoxic conditions. Intriguingly, oncogenes, such as Src prevent hydroxylation-dependent ubiquitinylation of HIF-1α, thus stabilizing it under normoxic conditions (Chan et al., 2002; Karni et al., 2002; Lee et al., 2011). In fact, downregulation of Cx43 in astrocytes promotes Src activation with the subsequent upregulation of HIF-1α, which in turn induces the transcription of GLUT-3 and HK2 to increase the rate of glucose uptake (Fig. 3) (Gangoso et al., 2012; Valle-Casuso et al., 2012).

Glioma cells, like many other cancer cells adapt their metabolism to the tumor environment, a process known as "the Warburg Effect" (Warburg et al., 1927) that begins by an increase in the rate of glucose uptake and a metabolic shift to aerobic glycolysis, which is associated with a survival advantage as well as the generation of substrates necessary in rapidly proliferating cells (Wolf et al., 2011). In fact, GLUT-3, the high-affinity transporter for glucose contributes to tumor progression by maintaining the GSC phenotype (Flavahan et al., 2013) and enzymes such as HK2, key mediators of aerobic glycolysis, promote tumor growth in gliomas (Wolf et al., 2011). As mentioned above, downregulating Cx43 in astrocytes activates Src, which in turn upregulates HIF-1α leading to the transcription of GLUT-3 and HK2 (Fig. 3). These metabolic changes are probably designed to sustain the higher rate of cell proliferation observed under these circumstances (Tabernero et al., 2006; Herrero-Gonzalez et al., 2009). Therefore it could be proposed that the regulation of the cell cycle and glucose uptake in these cells could be linked in a common node involving Cx43–Src interaction (Fig. 3).

Effects on GSC phenotype

Another important consequence of Cx43–Src interaction is the reversion of the GSC phenotype. Malignant gliomas are composed of a heterogeneous population of cells that include many with stem-cell-like properties, called glioma-initiating cells or GSCs. GSCs are characterized by their self-renewal capacity, their multilineage differentiation properties, their high oncogenic potential, and their resistance to standard therapies (Dirks, 2010), consequently this subpopulation is considered a promising therapeutic target against gliomas. Interestingly, the expression of Cx43 in GSCs is very low (Yu et al., 2012; Gangoso et al., 2014) and when Cx43 expression is restored, GSC phenotype (Yu et al., 2012; Gangoso et al., 2014) and tumorigenicity (Yu et al., 2012) is reduced. Indeed, Cx43 reduces Src activity in GSCs, which is an important self-renewal regulator (Singh et al., 2012), with the subsequent downregulation of Sox-2 (Yu et al., 2012; Gangoso et al., 2014), a transcription factor responsible for GSC self-renewal (Fig. 3) (Gangemi et al., 2009). Sox-2 expression is regulated by the inhibitor of differentiation Id1 (Soroceanu et al., 2013) that was also reduced upon restoring Cx43 (Fig. 3) (Gangoso et al., 2014). Importantly, the effect of Cx43 on GSCs is initiated by inhibiting Src activity promoted by the interaction of Cx43 with Src. In fact, a cell-penetrating peptide containing the region of Cx43 that interacts with Src can mimic this effect (Fig. 3) (Gangoso et al., 2014). Intriguingly, Sorgen et al. (Sorgen et al., 2004) described that the interaction of the SH3 domain of Src affects not just the SH3-binding domain (residues 274–283, Fig. 1C) but a larger region between amino acids 264–287. In fact, cell-penetrating peptides containing only the consensus SH3-binding site (residues 274–283 in Cx43) were unable to modify GSC phenotype. However, cell-penetrating peptides containing Cx43 residues affected by the binding of the Src SH3 domain (residues 266–283, Fig. 1C) mimicked the effect of Cx43 on GSCs (Fig. 3). Thus, these cell-penetrating peptides reduced Src activity, downregulated Id1 and Sox-2 expression and promoted cadherin switching. Consequently, the ability of GSCs to generate neurospheres decreased and the percentage of cells expressing differentiation markers increased (Gangoso et al., 2014). As mentioned above residues 266–283 in Cx43 constitute an IDR that has been proposed as a master regulatory domain within Cx43 (Fig. 1C) (Kopanic et al., 2014). The results obtained with cell-penetrating peptides (residues 266–283 in Cx43) on GSC phenotype confirm the relevance of Cx43–Src interaction and suggests that this short sequence could mimic the beneficial antitumorigenic effect of Cx43 found in high-grade astrocytomas.

CONCLUDING REMARKS

Cx43 as a building block of gap junction channels and hemichannels exerts important functions in astrocytes (Fig. 1A). When these cells acquire a malignant phenotype the levels of Cx43 protein, but not always those of mRNA, are downregulated, specifically in high-grade astrocytomas. The involvement of some miRNAs on the inhibition of Cx43 translation could explain the lack of correlation between Cx43 mRNA and protein levels and, more importantly, could be an interesting therapeutic target for high-grade astrocytomas that deserves further investigation.

The studies of the structure of Cx43 have revealed that its function relies on the interaction with other proteins, from homophilic interactions to form gap junction channels and hemichannels to heterophilic interactions with a great variety of proteins and enzymes (Fig. 1) (Giepmans, 2004; Herve et al., 2007) that impact important cellular functions such as proliferation, metabolism,

migration or survival. Since all of these functions are crucial for the progression of tumors, including astrocytomas, restoring Cx43 has been widely investigated as a therapeutic strategy (Zhu et al., 1991; Huang et al., 1998; Moorby and Patel, 2001; Zhang et al., 2003b; Yu et al., 2012). Most of the studies suggest that Cx43 exerts a beneficial role in reducing cell proliferation but regarding migration, the majority of studies show that Cx43 increases invasiveness (Lin et al., 2002; Zhang et al., 2003a; Oliveira et al., 2005; Behrens et al., 2010), however, other studies report the opposite, i.e., a reduction of migration promoted by Cx43 (McDonough et al., 1999; Mori et al., 2006; Simpson et al., 2008; Yu et al., 2012). In light of the data summarized in this review, these context-dependent effects could be attributed to different levels of some Cx43-interacting partners. Consequently, the knowledge of the structural basis of Cx43 interaction with its molecular partners and the effects promoted by these interactions will help to understand this complex disease.

Based on the data summarized in this review we propose that the sequence of Cx43 postulated by Kopanic et al. (Kopanic et al., 2014) (residues 272–286 in Cx43) as a master regulatory domain is indeed crucial for the regulation of Cx43 function and Src activity. In fact, Cx43–Src interaction modifies Cx43 structure affecting allosterically the binding of at least tubulin, ZO-1 and its interacting partners, TC-PTP and autophagy related proteins (Fig. 2). The inhibition of the oncogenic activity of Src promoted by Cx43 impacts glioma cell cycle, glucose uptake, and even GSC phenotype (Fig. 3). In fact, the results obtained with cell-penetrating peptides containing the Src-interacting region (residues 266–283 in Cx43) indicate that this short sequence reverts GSC phenotype and could be a source of inspiration for new glioma therapies. Since these cell-penetrating peptides lack cytoskeleton-binding or extracellular adhesion domains they could overcome the deleterious effect of Cx43 on adhesion, migration and invasiveness. We are aware that there are still several pieces left in this puzzle but the structural and functional data available suggest that this master sequence of Cx43 containing the Src-interacting region could be a key piece to figure out most of the final image.

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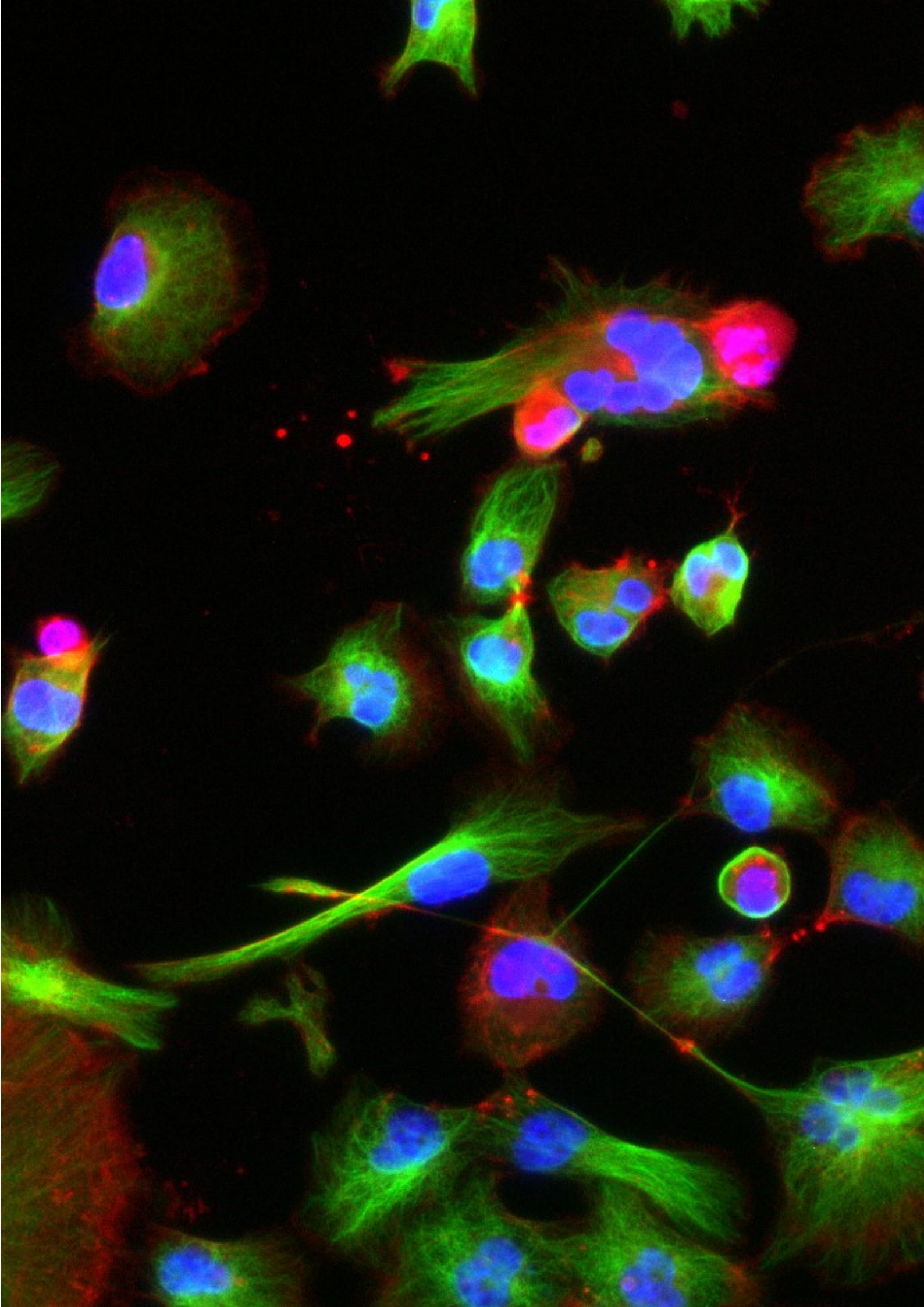
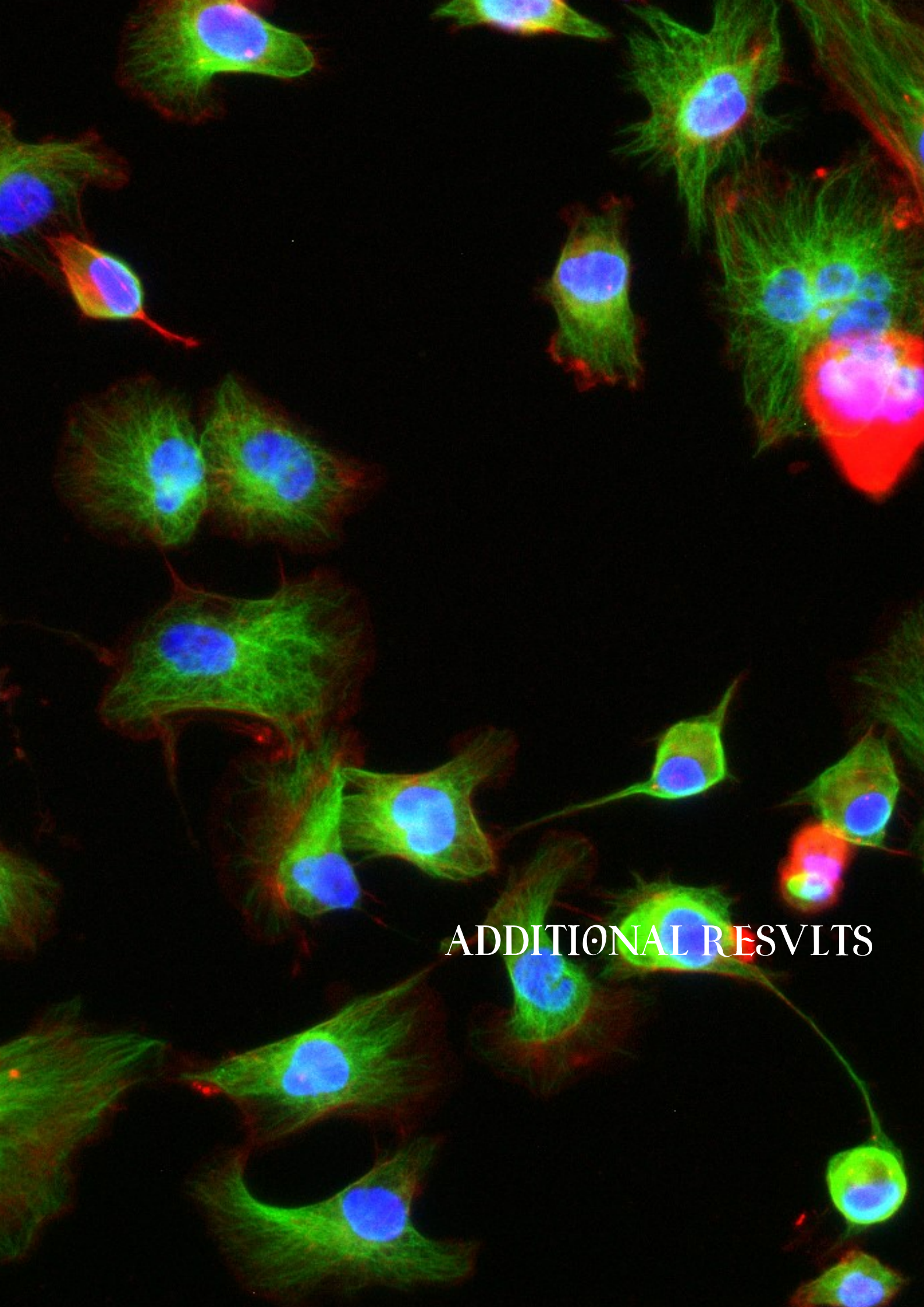
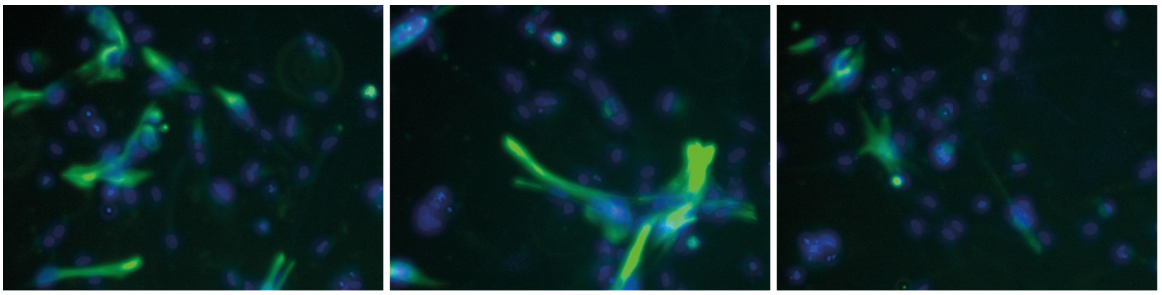




ADDITIONAL RESVLTS

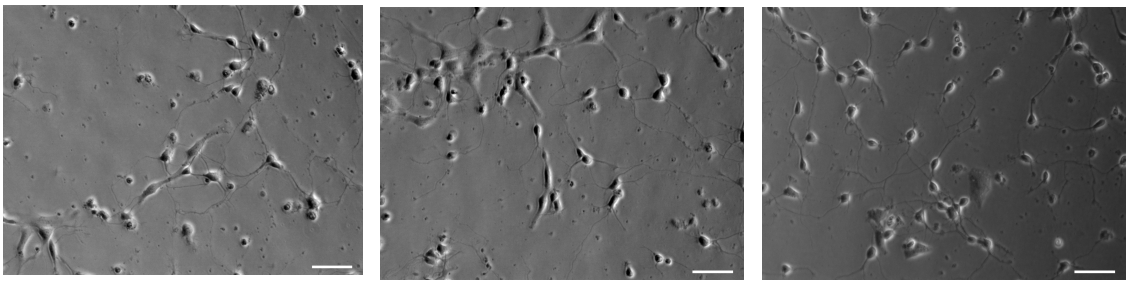
Additional Figure 1



Control TAT 50 µm TAT-Cx43$_{266-283}$ 50 µm

Additional Fig.1. Selective effect of TAT-Cx43$_{266-283}$ on G166 GSCs vs astrocytes. Primary rat astrocytes were plated on 24-well plates and allow to form a monolayer. 10.000 G166 GSCs were added on top of the astrocytes and allowed to integrate among them. The cocultures were incubated with 50 µM TAT or TAT-Cx43$_{266-283}$ for 72 hours, when they were fixed with 4%PFA. a) DAPI (blue) and Nestin (green) immunostaining of Control, TAT and TAT- Cx43$_{266-283}$ respectively showing that G166 GSCs are reduced when exposed to the last condition. Bar = 50 µm.

Additional Figure 2



Control TAT TAT-Cx43$_{266-283}$

Additional Fig.2. Representative phase-contrast photomicrographs of neurons incubated for 48 hours with 50 µM TAT or TAT-Cx43-$_{266-283}$. Bar = 50 µm.

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