ENGLISH SUMMARY

Effect of connexin43 on c-Src activity in glioma stem cells

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INTRODUCTION

The juctional channel is composed of two hemichannels (fig 1a), each of which is a hexamer of transmembrane connexins (Cx). These transmembrane proteins have a common topology, with four transmembrane domains, two extracellar loops, a cytoplasmic loop, and N- and C- termini located o the cytoplasmatic membrane face (fig 1b). Among the 21 Cxs of mammalian species, connexin43 (Cx43), the most abundant connexin, is an integral membrane protein widely expressed in different tissues. In the CNS, Cx43 is strongly expressed in astrocytes where it exerts a variety of important biological functions. Cx43 assembles to form gap junction channels that facilitate the behavior of astrocytes as metabolic networks (Giaume et al. 2010; Rouach et al. 2008). Cx43 also forms hemichannels responsible for the interchange of molecules and signals between a cell and its extracellular medium (for a review: (Bennett et al. 2003).

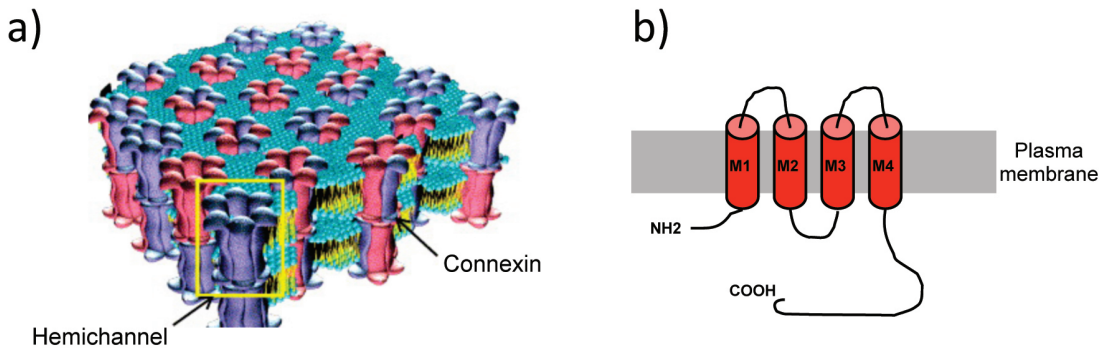

Figure 1. Gap juction channel and Cx43 structure

Furthermore, the intracellular carboxy tail of Cx43 (Cx43CT) interacts with a large number of signaling and scaffolding proteins (Giepmans 2006; Herve et al. 2007), thereby regulating cell functions such as cell adhesion, migration and proliferation (Bates et al., 2007; Cina et al., 2009; for a review: (Kardami et al. 2007; Prochnow and Dermietzel 2008). Since it was first identified (Loewenstein and Kanno 1966), the role of gap junctions in regulating cell proliferation has been widely studied, although there are still several aspects of this process that remain to be elucidated. In addition, the rate of astrocyte cell proliferation is reduced in Cx43 KO mice (Naus et al. 1997). On the other hand, a decrease in Cx43 expression is usually observed in gliomas, the

levels of which are inversely correlated with the degree of malignancy (Huang et al. 1999; Pu et al. 2004; Shinoura et al. 1996; Soroceanu et al. 2001). Interestingly, restoring Cx43 in glioma cells reduces their rate of proliferation (Huang et al. 1998; Zhu et al. 1991).

The Cx43CT is known to interact with c-Src, a non-receptor tyrosine kinase that can regulate cell proliferation. Activated c-Src phosphorylates Cx43 on critical tyrosine residues, Tyr247 and Tyr265, and this event reduces intercellular communication and Cx43 internalization (Giepmans et al. 2001; Gilleron et al. 2008; Lampe and Lau 2004; Lin et al. 2001; Pahujaa et al. 2007; Swenson et al. 1990). Changes in Cx43 expression affect gap junctional communication and astroglial networking but also have an impact on intracellular signalling (Giaume et al. 2010). For instance, the reduction in Cx43 expression in cultured astrocytes by using Cx43-specific small interfering RNA (siRNA) or endothelin-1 triggers a signal transduction pathway that involves c-Src activation (Valle-Casuso et al. 2012). Conversely, the up-regulation of Cx43 in glioma cells reduces the high oncogenic c-Src activity found in these cells (Herrero-Gonzalez et al. 2010). Since one of the characteristics of gliomas is the high oncogenic activity of c-Src (Du et al. 2009) in this study we have the following objetives:

OBJETIVES

1- Effect of connexin43 on c-Src activity in reactive astrocyes after an excitotoxic insult.
2- Effect of connexin43 on c-Src activity in C6 glioma cells.
3- Effect of connexin43 on c-Src activity in glioma stem cells.

1- Effect of connexin43 on c-Src activity in reactive astrocyes after an excitotoxic insult.

MATERIAL AND METHODS

Kainic acid lesions

Adult mice were submitted to intracerebral kainic acid injection as described by Koulakoff et al (Koulakoff et al. 2008). Briefly, mice were deeply anesthetized by intraperitoneal injection of 0.3 mL of 2% avertin and were subjected to a low-pressure injection of 1 nmol kainic acid in 1 µL of phosphate buffered saline (PBS) or 1 µL of PBS alone under stereotaxic guidance (coordinates: 21 mm anteroposterior, 1.5 mm mediolateral, and 0.6 mm dorsoventral from bregma) aiming the injection into the right cerebral cortex. Animals were sacrificed by cervical dislocation at 3, 5 or 7 days post-injection.

After removing the meninges, cortex tissue from the side of kainic acid or PBS injection and that from contralateral control side of the same brain were dissected. Thus, approximately 10 µL of the tissue surrounding the centre of the injection site and the contralateral side were obtained with a pipette tip and immediately frozen on dry ice. The tissue was then resuspended in boiling 2% SDS containing a cocktail of protease inhibitors (Roche, Mannheim, Germany), β-glycerophosphate (10 mM) and orthovanadate (1 mM) to which 5x Laemmli buffer was added. Samples were sonicated on ice, boiled 5 min and stored at −80 °C. Protein concentrations were quantified using the BCA protein assay kit (Pierce, Thermo Fisher).

Western blot analysis

Equivalent amounts of proteins (20 µg per lane) were separated on NuPAGE Novex Bis-Tris (4-12%) minigels (Invitrogen). Proteins were transblotted using Iblot Dry blotting system (Invitrogen). The membranes were cut into several strips for incubation with distinct primary antibodies to compare the amount of each protein in a given sample. Membranes were then blocked for 1 h at room temperature in Tris-buffered saline containing 0.05% Tween (TBST) and 5% non-fat milk powder and incubated overnight at 4 °C with the primary antibodies against Cx43 (Transduction Laboratories

610062 1:250), Y416-Src (Cell Signaling Technology 2101 1:250), total-Src (Cell Signaling Technology 2108 1:500), GFAP (1:500). After extensive washing, membranes were incubated with HRP-conjugated anti-rabbit or anti-mouse antibodies in TBST containing 5% milk. Proteins were developed with a chemiluminescent substrate. Membranes were exposed in a *FujiFilm LAS-4000* mini luminescent image analyser. Membranes were washed with TBST and incubated with mouse antibody against GAPDH (Applied Biosystems AM4300 1:2500) used as a loading control and the values for each protein were normalized to their corresponding GAPDH level. Densitometry analysis was performed using Image J program. The results are expressed as percentages of control values.

Data analysis

Each set of experiments was performed several times (n refers to the number of independent experiments performed on different animals). Statistical significance was carried out with Student's t-test and statistical significance was established as significant when $p < 0.05$.

RESULTS AND DISCUSSION

Cx43 is down-regulated in reactive astrocytes after an excitotoxic insult

Previous studies in our laboratory have shown in cultured astrocytes that an acute reduction in Cx43 expression triggers important changes in astrocyte biology (Herrero-Gonzalez et al. 2009; Valle-Casuso et al. 2012). *In vivo*, Cx43 is down-regulated in astrocytes in response to certain pathological situations (reviewed in: (Giaume et al. 2010). Since the molecular pathways activated in reactive astrocytes after an injury are not fully elucidated, we have investigated whether the changes found in cultured astrocytes after an acute reduction in Cx43 are taking place *in situ* in reactive astrocytes that down-regulate Cx43 expression after injury.

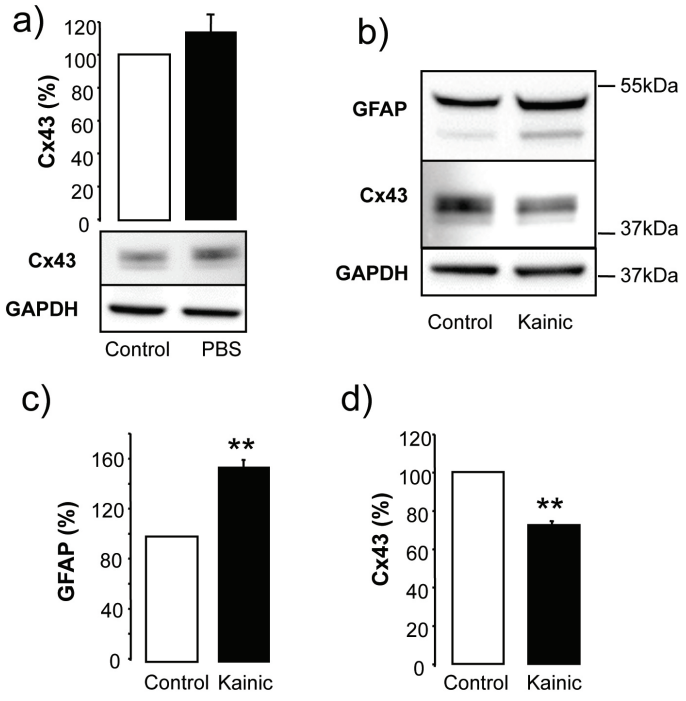

Figure 2. Down-regulation of Cx43 in reactive astrocytes 7 days after kainic acid lesion in adult mouse cortex.

A Western blot and quantification for Cx43 and GAPDH expression 7 days after the injection of PBS showing no significant differences in Cx43 expression in the injected area compared to control. **B** Representative Western blot of GFAP, Cx43 and GAPDH expression in the same samples showing the increase in GFAP and the decrease in Cx43 in the lesioned area compared to control. GAPDH was used as a loading control. Western blots for GFAP **(C)** and Cx43 **(D)** were quantified and normalized against GAPDH. The results are expressed as percentages of control values and they are the means ± SEM (n=5). **p < 0.01 versus control (Student's t-test).

To do so, we have analysed reactive astrocytes present in a lesioned area resulting of an excitotoxic insult induced by an injection of kainic acid in the cortex of adult mice.

In this model of brain injury, down-regulation of Cx43 has been previously characterized (Koulakoff et al. 2008). Tissue samples were collected from the area surrounding the injection site (Kainic) and from the contralateral healthy hemisphere (control) of the same brain. When the vehicle solution (PBS) was injected, no significant changes in Cx43 expression were detected as compared with the contralateral hemisphere after 7 days (Figure 2A). We analysed the expression of GFAP and Cx43 in each sample was analysed in Western blots after kainic acid inyection (Figure 2B). The quantification of these Western blots corroborated immunohistochemical analyses, since GFAP increased by about 50% (Figure 2C) and Cx43 decreased by about 30% (Figure 2D) in kainic acid-lesioned cortical area as compared with the contralateral hemisphere.

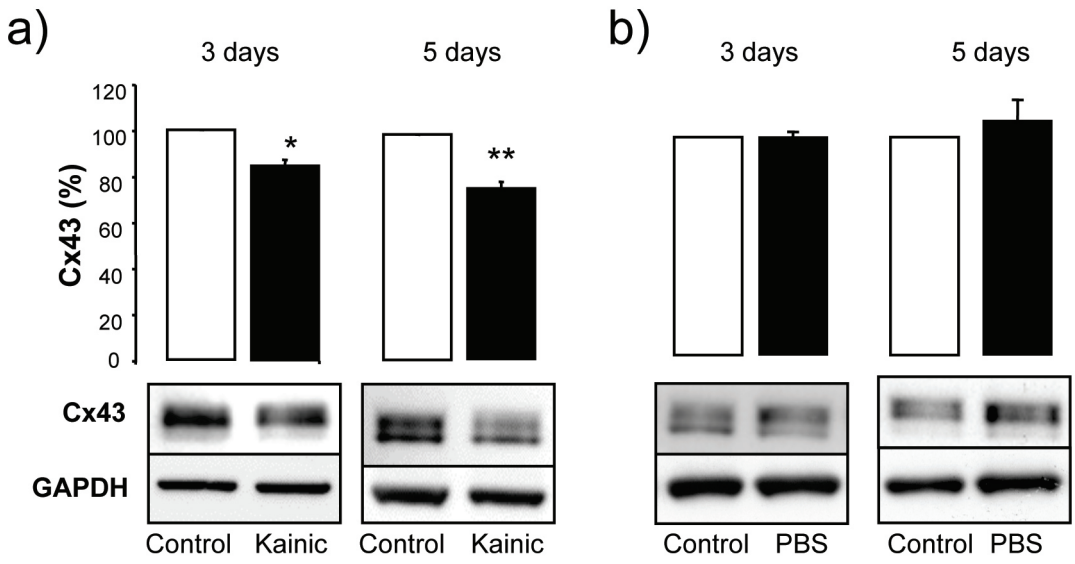

Figure 3. Time course of CX43 in reactive astrocytes after kainic acid lesion.

A, Western blot of Cx43 and GAPDH showing the decrease in Cx43 expression at 3 and 5 days after kainic acid injection. **B,** Western blot of Cx43 and GAPDH showing no significant changes in Cx43 expression at 3 or 5 days after PBS injection.

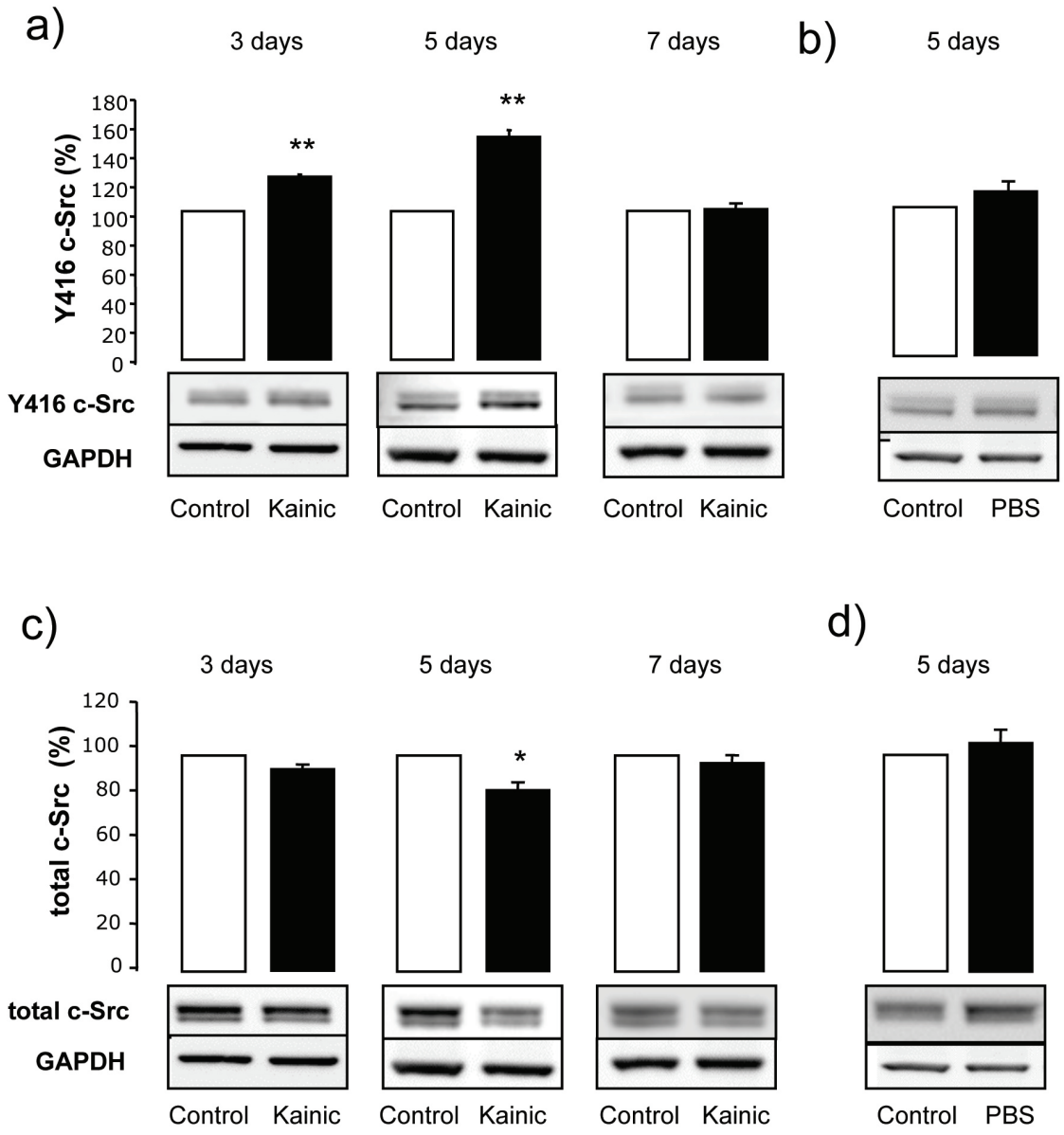

Figure 4. Time course of c-Src activity, total c-Src in reactive astrocytes after kainic acid lesion.

A, Western blot of Y416 c-Src and GAPDH collected from the lesioned and control areas, at 3, 5 or 7 days after unilateral kainic acid injection. Note the increase in the active form of c-Src (Y416 c-Src) in the lesioned area compared to the contralateral hemisphere (control) at 3 and 5 days. **B**, Western blot for Y416 c-Src and GAPDH at 5 days after the injection of PBS showing no significant differences in the injected area compared to control. **C**, Western blot of total c-Src and GAPDH collected from the lesioned and control areas, at 3, 5 or 7 days after unilateral kainic acid injection. **D**, Western blot for total c-Src and GAPDH at 5 days after the injection of PBS showing no significant differences in the injected area compared to control.

We also analysed the expression of Cx43 at 3 and 5 days post-injection. Our results show that the expression of Cx43 decreased by about 18% and 22% in the lesioned area, 3 and 5 days after kainic acid injection, respectively (Figure 3A). It should be mentioned that PBS injection did not affect the expression of Cx43 (Figure 3B).

c-Src activity in reactive astrocytes with low Cx43

Since, c-Src activation is one of the initiating events found after an acute reduction of Cx43 in cultured astrocytes, we have investigated the activity of c-Src after cortical injury by analysing the levels of c-Src phosphorylated at Tyr 416, which is the active form of the enzyme (Kmiecik and Shalloway 1987). Our results show that the levels of Y416 c-Src did not change in the lesioned area when compared to the contralateral hemisphere 7 days after the kainic acid injection (Figure 4A). We have analysed the levels of Y416 c-Src at earlier stages. Interestingly, our results show that the levels of Y416 c-Src increased by about 20% and 50% in the lesioned area 3 and 5 days after the kainic acid injection, respectively (Figure 4A). PBS injection did not affect the expression of Y416 c-Src (Figure 4B). The total level of c-Src was not modified or slightly reduced after 5 days (Figure 4C), indicating that c-Src is transiently activated by phosphorylation on tyrosine 416 in the lesioned area.

In their reaction to injury, astrocytes leave their quiescent state and become activated. During this process, they up-regulate intermediate filament forming proteins, GFAP and vimentin and, in certain situations, undergo cell proliferation (Pekny and Nilsson 2005). Our results show that in a cortical lesion induced by a kainic acid injection, astrocytes up-regulate GFAP expression after 7 days. As previously reported, reactive astrocytes located within the lesioned area showed a reduction in the expression of Cx43 (Koulakoff et al. 2008).

The interaction between Cx43 and c-Src mutually regulates their activities Giepmans et al. 2001; Lin et al. 2001; Swenson et al. 1990). Previous works in our laboratory has shown that modifications of Y416 c-Src precede changes in glucosa transporters and hexokinases (Valle-Casuso et al. 2012). Since c-Src is a well-known activator of cell proliferation (Kmiecik and Shalloway 1987), it has been proposed as the link between Cx43 and glucose uptake and proliferation (Valle-Casuso et al. 2012). Interestingly, in this study we show that c-Src activation coincides with the reduction in Cx43 expression, indicating that c-Src could mediate the regulatory effect of Cx43 on

glucose uptake and proliferation in astrocytes after an excitotoxic insult. These data suggest that c-Src could be a promising target to modulate the response of astrocytes under certain pathological situations.

This work has been published as a part of the paper *"Reduced connexin43 expression correlates with c-Src activation, proliferation, and glucose uptake in reactive astrocytes after an excitotoxic insult"* in Glia 2012 60: 2040-9. (Figs 1 and 5 and fig2 supplementary).

2- Effect of connexin43 on c-Src activity in C6 glioma cells

MATERIAL AND METHODS

Cell cultures

C6-Cx43 glioma cells were cultured in DMEM supplemented with 10% (v/v) FCS, penicillin G (50 U/ml), streptomycin (37.5 U/ml) and amphotericin B (10^{-4} mg/ml). Cells were plated on Petri dishes coated with poly-L-lysine. The cells were maintained at 37ºC in an atmosphere of 95% air/5% CO_2 and with 90-95% humidity.

Transfection of siRNA

C6-Cx43 cells were transfected with a validated non-targeting siRNA (NT-siRNA) or with a siRNA specific for Cx43 (Cx43-siRNA) as previously reported (Herrero-Gonzalez et al., 2009)

Western blot analysis

Equivalent amounts of proteins (20 µg per lane) were separated on NuPAGE Novex Bis-Tris (4-12%) minigels (Invitrogen). Proteins were transblotted using either XCell II blot module (Invitrogen). The membranes were cut into several strips for incubation with distinct primary antibodies to compare the amount of each protein in a given sample. Membranes were then blocked for 1 h at room temperature in Tris-buffered saline containing 0.05% Tween (TBST) and 5% non-fat milk powder and incubated overnight at 4 °C with the primary antibodies against Cx43 (Transduction Laboratories 610062 1:250), Y416-Src (Cell Signaling Technology 2101 1:250), total-Src (Cell Signaling Technology 2108 1:500). After extensive washing, membranes were incubated with HRP-conjugated anti-rabbit or anti-mouse antibodies in TBST containing 5% milk. Proteins were developed with a chemiluminescent substrate. Membranes were exposed in a *FujiFilm LAS-4000* mini luminescent image analyser. Membranes were washed with TBST and incubated with mouse antibody against GAPDH (Applied Biosystems AM4300 1:2500) used as a loading control and the values for each protein were normalized to their corresponding GAPDH level. Densitometry analysis was performed using Image J program. The results are expressed as percentages of control values.

Data analysis

Each set of experiments was performed several times (n refers to the number of independent experiments performed on different animals). Statistical significance was carried out with Student's t-test and statistical significance was established as significant when $p < 0.05$.

RESULTS AND DISCUSSION

Cx43 interacts with several proteins allowing to bind Cx43 to a variety of scaffolding and regulatory proteins (Giepmans 2006; Herve et al. 2007). One such protein is c-Src, a non-receptor tyrosine kinase that is very active in gliomas (Du et al. 2009) and that is known to be oncogenic (Thomas and Brugge 1997). Autophosphorylation at Tyr-416 activates c-Src (Kmiecik and Shalloway 1987; Xu et al. 1997) and therefore, we followed c-Src phosphorylated at Tyr-416 (Y416 c-Src) to monitor the activity of c-Src. Herrero-Gonzalez and col shows that the presence of Cx43 diminished the activity of c-Src by more than 60% in glioma C6 cells (Herrero-Gonzalez et al. 2010). To confirm the down-regulation of c-Src activity promoted by Cx43, the activity of c-Src was analyzed in C6-Cx43 cells after silencing Cx43 expression by siRNA (Fig 5). Our results show that the knock-down of Cx43 in C6-Cx43 reverted the effect on c-Src activity. Thus, while the levels of total c-Src were not modified, the levels of Y416 c-Src were increased by about 45% in C6-Cx43 transfected with Cx43-siRNA .

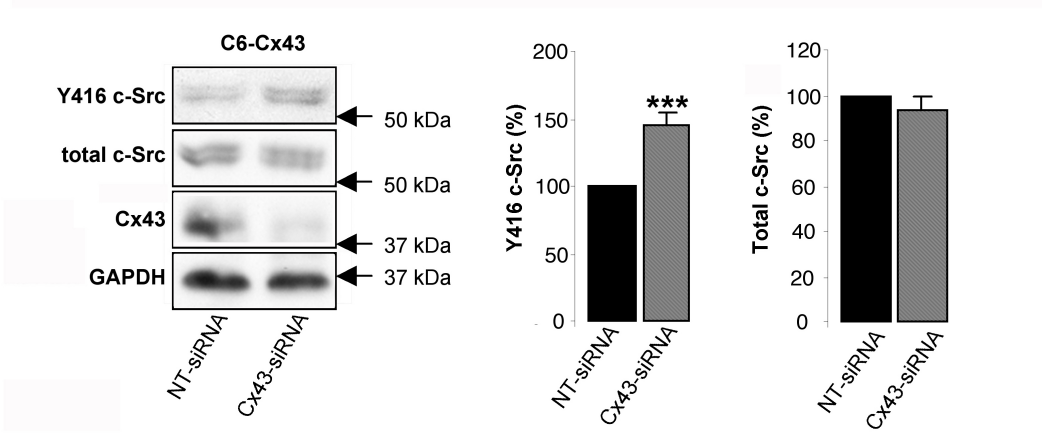

Fig 5. Effect of connexin43 on c-Src activity in glioma C6 cells

Quantification of Y416 c-Src and total c-Src in Western blots of C6-Cx43 cells transfected with NT-siRNA or with Cx43-siRNA for 72 hours. Values were normalized to GAPDH. The results are the means ± SEM (n=6) and they are expressed as percentages relative to the level found in cells transfected with NT-siRNA. Statistical differences when compared with cells transfected with NT-siRNA are given as ***p<0.001 (Student's t-test).

Previous studies presented in our laboratory show that restoring Cx43 to glioma cells diminishes the oncogenic activity of c-Src as judged by the decrease in the active form (Y416 c-Src) (Herrero-Gonzalez et al. 2010). Our results here confirm the down-regulation of c-Src activity promoted by Cx43. It has been shown that the activity of c-Src is specifically elevated in malignant gliomas (Du et al. 2009). Therefore, the results presented in this study togheter with previous work in our laboratory, showing that Cx43 inhibits the strong onocgenic activity of c-Src in glioma C6 cells, this could help to design new therapies against gliomas.

This work has been published as a part of the paper *"Connexin43 inhibits the oncogenic activity of c-Src in C6 glioma cells"* in the journal Oncogene 29:5712-23. (Fig4).

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3- Effect of connexin43 on c-Src activity in glioma stem cells

This work has been submitted for publication in “Stem Cell” with the titled *“The inhibition of c-Src mediates the reversion of glioma stem-like phenotype promoted by connexin43”*

The inhibition of c-Src mediates the reversion of glioma stem-like phenotype promoted by connexin43

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Jose M Medina: financial support, data interpretation and manuscript revision.

Arantxa Tabernero: conception and design, performance of experiments, financial support, data analysis and interpretation, manuscript writing.

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Running Title: c-Src mediates the effect of Cx43 on GSC

Abstract

Connexin43 (Cx43), the main gap junction channel-forming protein in astrocytes, is down-regulated in malignant gliomas. These tumors are composed of a heterogeneous population of cells that include many with stem-cell-like properties, called glioma stem cells (GSCs), which are highly tumorigenic and lack Cx43 expression. Interestingly, restoring Cx43 reverses GSC phenotype and consequently reduces their tumorigenicity. In this study we investigated the mechanism by which Cx43 exerts its antitumorigenic effect on GSCs. We have focused on the tyrosine kinase c-Src, which interacts with the intracellular carboxy tail of Cx43. We found that Cx43 regulates c-Src activity and proliferation in human GSCs expanded in adherent culture. Thus, restoring Cx43 to GSCs inhibited c-Src activity, which in turn promoted the down-regulation of the inhibitor of differentiation Id1. Id-1 sustains stem-cell phenotype since it controls the expression of Sox2, responsible for stem-self renewal, and promotes cadherin switching, involved in epithelial-mesenchymal transition. Our results show that both the ectopic expression of Cx43 and the inhibition of c-Src reduced Sox2 expression and promoted the switch from N- to E-cadherin, suggesting that Cx43 by inhibiting c-Src down-regulates Id1 with the subsequent changes in Sox2 and cadherin expression. These results confirm the relevance of the interaction between Cx43 and c-Src in the regulation of the malignant phenotype and pinpoint this interaction as a promising therapeutic target.

Key words: CNS / gap junctions / glia / Sox-2/ Id1/ cadherin

Introduction

Connexin43 (Cx43), the most abundant connexin in mammals, is an integral membrane protein widely expressed in different tissues. In the CNS, Cx43 is strongly expressed in astrocytes where it exerts a variety of important biological functions. Cx43 assembles to form gap junction channels and hemichannels that facilitate the behavior of astrocytes as cellular networks [1] [2] and the interchange of molecules between the cells and their extracellular medium [3]. In addition, since it was first identified [4], the role of Cx43 and gap junctions in regulating malignant phenotype has been widely studied, although there are still several aspects of this process that remain elusive [5] [6]. In gliomas, the most common neoplasia of the central nervous system, the expression of Cx43 is often reduced [7-10] and restoring Cx43 to these cells reduces their rate of proliferation [11, 12]. Patients diagnosed with a glioblastoma multiforme, the most aggressive form of glioma, have a median survival of 1–2 years [13]. These tumors are composed of a heterogeneous population of cells that include many with stem-cell-like properties, called glioma-initiating cells or glioma stem cells (GSCs). GSCs are characterized by their self-renewal capacity, their multilineage differentiation properties, their high oncogenic potential, and their resistance to standard therapy [14]. There is an intense scientific debate on GSCs in gliomagenesis [15, 16] that suggests that glioma stem cell phenotype is dynamic and therefore the reversion of glioma stem cell phenotype appears as a promising therapeutic strategy. Interestingly, it has been recently shown that restoring Cx43 reverses glioma stem cell phenotype and consequently reduces their tumorigenicity [17]. Despite of the relevance of this function, the mechanism by which Cx43 exerts this effect is unknown.

The intracellular carboxy tail of Cx43 (Cx43CT) interacts with a large number of signaling and scaffolding proteins [18, 19], thereby regulating cell functions such as cell adhesion, migration and proliferation [20, 21, 22, 23]. One of these interacting proteins is the non-receptor tyrosine kinase c-Src, which participates in the stem cell phenotype [24, 25]. We have recently shown that the interaction between Cx43 and c-Src promotes a reduction in the oncogenic activity of c-Src in rat C6 glioma cells [26]. In this study we investigated whether the reversion of GSC phenotype promoted by Cx43 is triggered by the interaction between Cx43 and c-Src.

Material and Methods

Animals

Albino Wistar rats, fed ad libitum on a stock laboratory diet (49.8% carbohydrates, 23.5% protein, 3.7% fat, 5.5% wt/vol minerals and added vitamins and amino acids), were used for the experiments. Rats were maintained on a 12-h light–dark cycle. Postnatal day 1 newborn rats were used to prepare astrocyte cultures. The animals were obtained from the animal facility of the University of Salamanca and their use for this study was approved by the bioethics committee of this institution.

Cell Cultures

GSC lines (G166, GliNS2, G144, G179) were obtained from BioRep [27]. The cells were grown in RHB-A medium (StemCells, Inc) supplemented with 1% N2, 2 % B27 (Life Techonologies), 20 ng/ml EGF and 20 ng/ml b-FGF (Peprotech), as described previously [27]. Culture plates were coated with 10 μg/ml laminin (Life Technologies) for 2 hours prior to use. The cells were maintained at 37ºC in an atmosphere of 95% air / 5% CO_2 and with 90-95% humidity.

OB1 and TG10 cells were cultured from tumor samples obtained from surgical resections carried out on patients at Sainte Anne Hospital (Paris, France). This study was approved by the Institutional Review Board and informed consent was obtained from all patients. The tumors were high-grade gliomas (glioblastoma), according to the WHO classification. Glioma stem-like cells were obtained as previously described [28]. Briefly, tumor samples were dissociated to form a single-cell suspension, which was plated on serum-free DMEM/F12 supplemented with B27, heparin (Stem Cell Technologies) and human recombinant EGF and FGF-2 (Sigma), both at a final concentration of 20 ng/ml. Neurosphere cultures were then passaged every 11 days, by mechanical dissociation, to give a concentration of 50,000 cells/ml in fresh medium, in non coated T25 or T75 flasks.

Astrocytes in primary culture were prepared from the forebrains of 1-to 2 days-old Wistar rats and cultured in DMEM supplemented with 10 % FCS as previously described [29]. C6 glioma cells were cultured in DMEM supplemented with 10 % FCS as previously described [26].

Plasmid constructs and cell transfection

The pIres-Cx43 construct was generated by ligating a PCR-amplified fragment encoding the human Cx43 sequence (accession number: NM_000165) into the AgeI-

BamHI sites of the bicistronic pIRESpuro2 vector that encodes the puromycin resistance gene. The fragment encoding the human Cx43 sequence was obtained by RT-PCR performed on a template of total RNA isolated from GliNS2 cells using the following primers: 5'- tatataccggtatgggtgactggagcgcctt -3' and 5'- cgggatcccgctagatctccaggtcatcag -3'. GliNS2 glioma cells were transfected with the construct containing Cx43 (Ires-Cx43) or with the empty vector (Ires) by electroporation using Neon Transfection System (Life technologies) according to the manufacturer's instructions. Transfected cells were selected with puromycin (0.1 mg/ml).

Transfection of siRNA

G166 cells were transfected with a validated non-targeting siRNA (NT-siRNA) or with a siRNA specific for Cx43 (Cx43-siRNA). Cells were transfected with the double-strand siRNA (25 nM or 50 nM) complexed with 2.5 µl/ml Lipofectamine 2000 (Life technologies) in culture medium without antibiotics. The sequences of the Cx43 siRNAs were: sense 5'-gcugguuacuggugacagatt-3'; and antisense 5'-ucugucaccaguaaccagctt-3'. 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 Cx43 expression was evaluated in Western blots.

Western blot analysis

Equivalent amounts of proteins (20 µg per lane) were separated on NuPAGE Novex Bis-Tris (4-12%) minigels (Life technologies). According to their molecular size, proteins were transblotted using either XCell II blot module or 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 (TTBS) and 7% non-fat milk powder before being incubated overnight at 4 °C with the primary antibodies against Cx43 (Transduction Laboratories 610062, 1:250), Y416-Src (Cell Signaling Technology 2101, 1:250), total-Src (Cell Signaling Technology 2108 and 2110, 1:500), Id1 (Santa Cruz Biotechnolgy Inc. sc-488, 1:500), Sox2 (Abcam ab97959, 1:1000), E-cadherin (S Santa Cruz Biotechnolgy Inc sc-7870, 1:100), N-cadherin (S Santa Cruz Biotechnolgy Inc sc-7939, 1:500). Antibody against GAPDH (Applied Biosystems AM4300, 1:5000) or α-actinin (Merk Millipore MAB1682, 1:1000) were used as a loading control. After extensive washing, membranes were incubated with HRP-conjugated anti-rabbit or anti-mouse antibodies in TTBS. Proteins were developed with a chemiluminiscent substrate. Densitometry analysis of the bands was performed using

Image J program (Wayne Rasband, NIH). The amounts of GAPDH or α-actinin recovered in each sample served as loading control and the values for each protein were normalized to their corresponding GAPDH or α-actinin level.

Inmunocytochemistry

Cells fixed with 4% (w/v) formaldehyde in PBS for 20 min and then were blocked for 1 h at 4ºC. The cells were incubated overnight at 4ºC with primary antibodies. Specific dilutions of primary antibodies used are as follows: Cx43 (Transduction Laboratories 610062, 1:200), Id1 (Santa Cruz Biotechnolgy Inc. sc-488, 1:500), Sox2 (Abcam ab97959 1:500), Ki-67 (Sigma P6834, 1:200), N-cadherin (Santa Cruz Biotechnolgy Inc sc-7939, 1:200). The cells were mounted using the Slowfade Gold Antifade Kit and they were analyzed on a Leica inverted fluorescence microscope connected to a digital video camera (Leica DC100). For Ki-67 quantification, the nuclei were stained with DAPI (1.25 µg/ml) for 5 min and at least 6 photomicrographs were taken from each plate and the number of nuclei (DAPI staining) and Ki-67 positive cells were counted. The percentage of Ki-67 positive cells was calculated from the total number of cells (DAPI staining).

MTT assay

Cells cultured at 37ºC in 4-well-plates were incubated in the dark for 75 min with 300 µl/ml 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 dimethyl sulfoxide (250 µl/well) with mild shaking. Finally, the absorbance was measured at a wavelength of 570 nm using a microplate reader (Multiskan Ascent, Thermo Electron Corporation).

Statistical analyses

The results are the means ± SEM of at least three independent experiments. Statistical analyses were carried out with the Student's *t*-test. Values were considered significant when $p<0.05$.

Results

Expression of Cx43 and activity of c-Src in glioma stem cells

It has been previously shown that GSCs obtained from patient glioma specimens or from the glioma cell line U87 express low levels of connexin43 [17]. In this study we confirmed the low expression of Cx43 in GSCs obtained from glioblastoma patients, termed OB1 and TG10 [30], as compared to rat astrocytes or even to rat C6 glioma cells (Figure 1A). Gliomas, like many other tumors, exhibit a high oncogenic activity of c-Src [31]. We analyzed c-Src activity in OB1 and TG10 by measuring the levels of c-Src phosphorylated at Tyr-416 (Y416 c-Src), the active form of this tyrosine kinase [32, 33]. Figure 1A shows that the activity of c-Src was higher in OB1 and TG10 GSCs than that found in rat astrocytes and similar to that found in rat C6 glioma cells. Recently, GSCs obtained from glioblastoma patients (GliNS2, G179, G166, G144) have been expanded in adherent culture, characterized and deposited in a cell repository [27]. We took advantage of the purity and stability of these cells in culture, which can be genetically manipulated to study the role of Cx43 on glioma stem cell biology. Figure 1B shows that the levels of Cx43 were lower in the four GSC lines tested (GliNS2, G179, G166, G144) than those found in the rat C6 glioma cell line or in rat astrocytes obtained from primary culture. Although with different profiles, all of them expressed c-Src, which in GliNS2 and G179 cells is mostly phosphorylated on Tyr 416, i.e., in the active conformation (Figure 1C). It should be noted that GliNS2 and G179 cells expressed lower levels of Cx43 than G166 or G179 cells. Consequently, in order to investigate the role of Cx43 on GSCs, GliNS2 cells were used for the gain-of-function experiments whereas G166 were used for the loss-of function experiments.

Effect of connexin43 on c-Src activity in glioma stem cells

We have previously shown that restoring Cx43 expression in rat C6 glioma cells reduces the activity of c-Src [26] while silencing Cx43 in cultured astrocytes increases c-Src activity [34]. In this study we investigated whether Cx43 also regulates c-Src activity in GSCs. To this end, gain- and loss-of-function experiments for Cx43 were carried out and c-Src activity was analyzed.

Since G166 cells express a certain level of Cx43, they were used in loss-of function experiments. To knockdown Cx43, these cells were transfected with a specific siRNA for Cx43 (Cx43-siRNA) or a non-target siRNA (NT-siRNA) used as control. Figure 2A shows that as soon as 2-days after transfection with 50nM Cx43-siRNA, the levels of Cx43 were strongly reduced in G166 cells. This effect was maintained to a lesser extent 4-days post transfection. Concomitantly, the levels of Y416 c-Src were

increased by about 100% and 40% after 2- and 4-days, respectively (Figure 2B). The total amount of c-Src was slightly increased (by about 30%) 4 days post transfection (Figure 2C).

GliNS2 cells were used in gain-of-function experiments due to the lack of Cx43 expression. Thus, these cells were transfected with increasing concentrations of the construct containing Cx43 (Ires-Cx43) or with the empty vector (Ires). Figure 2D shows that the increase in Cx43 expression promoted a decrease in the levels of Y416 c-Src, even at the lowest concentration of Ires-Cx43, 2 days post transfection. Indeed, the levels of Y416 c-Src decreased by about 35% and 25%, 2 and 5 days after the transfection of 1 µg/µl Ires-Cx43, respectively (Figure 2E and F). The total amount of c-Src was not significantly modified by the expression of Cx43 (Figures 2E and 2G).

Effect of connexin43 on glioma stem cell proliferation

Next, we analyzed the effect of modulating Cx43 expression on the rate of GSC proliferation. As shown in figure 3A, the density of G166 cells transfected with Cx43-siRNA was higher than that found in G166 cells transfected with NT-siRNA (Figure 3A). This observation was confirmed by analyzing the rate of growth by MTT (Figure 3B). In order to find out whether the increase in the number of cells was due to an increased proliferation, the expression of the proliferation marker Ki-67 was analyzed (Figure 3C). Our results show that the percentage of Ki-67 positive cells strongly increased 2 days after transfection with Cx43-siRNA and, to a lesser extent, 5 days after transfection (Figure 3D). Moreover, restoring Cx43 in GliNS2 cells produced the opposite effects (Figure 4). Thus, transfecting GliNS2 with Cx43 reduced the rate of cell growth as judged by phase contrast images (Figure 4A) and MTT assay (Figure 4B). This reduction was due to a decrease in the rate of proliferation, since the percentage of Ki-67-positive cells strongly decreased 2 days after the transfection with Cx43 (Figures 4C and D).

Effect of connexin43 on the expression of Sox2, E-cadherin, N-cadherin and Id1

Recent evidence suggests that glioma stem cell phenotype can be reverted. Thus, Yu et al have shown that transfecting Cx43 into GSCs, obtained from the U87 cell line and cultured in serum containing medium, reduced the expression of stemness markers, such as Sox2 and up-regulated the expression of E-cadherin [17]. Our results confirm that restoring Cx43 reduces the expression of Sox2 and up-regulates E-cadherin in GliNS2 cells, even in the absence of serum that could help stem cell differentiation. Thus, the immunocytochemistry revealed that the levels of Sox2 decreased in most cells (Figure 5A); more specifically, Sox2 was down-regulated in

cells containing high levels of Cx43 (Figure 5B). Western blot analysis confirmed the reduction of Sox2 and the up-regulation of E-cadherin expression by restoring Cx43 in GSCs (Figure 5C). Interestingly, we found a down regulation of N-cadherin in cells expressing Cx43 (Figure 5D), suggesting that Cx43 promoted a cadherin switching in GSCs [35].

Sox2 expression [36] and the switching from N-cadherin to E-cadherin [37] have been reported to be regulated by the inhibitor of differentiation Id1. Therefore, we analyzed the levels of Id1 under our experimental conditions. Interestingly, our results revealed that restoring Cx43 reduced the levels of Id1 in GliNS2 cells when analyzed by either immunocytochemistry or Western blot (Figures 5E-G).

Participation of c-Src in the effect of Cx43 on glioma stem cell phenotype

Since restoring Cx43 decreased c-Src activity, Id1 and Sox2 expression and switched cadherin expression, we analyzed the effect of c-Src inhibition on these proteins to address the possible participation of c-Src on the effect of Cx43 on GSC phenotype. Our results show that the inhibition of c-Src activity promoted by dasatinib strongly decreased Id1 expression in a time- and a dose-dependent way (Figure 6A). Furthermore, the inhibition of c-Src decreased Sox2 (Figure 6B) and N-cadherin expression while increasing E-cadherin expression (Figure 6C).

Discussion

As it has been previously reported [17] our study confirms that glioma stem cells express very low levels of the gap junctional protein Cx43. Interestingly, the ectopic expression of Cx43 in GSCs strongly reduces the tumorigenic properties of these cells [17]. Thus, the size of the tumors generated by Cx43-transfected GSCs was smaller and consequently these animals showed prolonged survival [17]. However, little is known about the mechanism underlying this important effect of Cx43 on GSCs. We previously found that Cx43 decreased the oncogenic activity of c-Src in rat glioma C6 cell line, therefore in this study we investigated whether c-Src was involved in the antitumorigenic effect exerted by Cx43 on GSCs.

Our results show that changes in the levels of Cx43 are accompanied by changes in the oncogenic activity of c-Src in GSCs. Thus, silencing Cx43 increased c-Src activity and augmented the rate of GSC proliferation while the transfection with Cx43 decreased c-Src activity and GSC proliferation. Src activity has been tightly linked to stem cell self-renewal, expansion [24, 25] and resistance to anticancer treatments [38]. Therefore, these data suggest that Cx43 could exert its antitumorigenic activity through the inhibition of c-Src activity.

The relevance of GSCs as therapeutic target has been highlighted because their stemness phenotype can be reverted [15, 16]. Interestingly, the results presented in this work and other studies [17] show that restoring Cx43 in GSCs decreases the expression of Sox2, which is a transcription factor responsible for stem cell self-renewal [25, 39] and is frequently used as a marker of stem cells. Since Sox2 regulates GSC proliferation, it could be suggested that the reduction in GSC proliferation reported in the present study after restoring Cx43 could be mediated by the down-regulation of this transcription factor.

The expression of Sox2 is regulated by the inhibitor of differentiation Id1 [36], which belongs to the family of Id transcriptional regulators. Id1 inhibits the DNA-binding of basic helix-loop-helix transcription factors involved in cellular differentiation [40] and is a promising therapeutic target because it is specifically up-regulated in high-grade gliomas [36] and more specifically in GSCs [41]. Intriguingly, our results show that restoring Cx43 to GSCs reduced the expression of Id1. It should be mentioned that Id1 expression is regulated by c-Src activity [42]. Indeed, our data shows that dasatinib, an inhibitor of c-Src activity, reduced Id1 and Sox2 expression in GSCs. Therefore it could be proposed that by decreasing c-Src activity Cx43 down-regulates the expression of Id1 and consequently the expression of the transcription factor Sox2 (Figure 7).

In addition to its role in stem cell self-renewal, the transcriptional regulator Id1 plays a critical role in modulating the invasiveness of glioma cells. Id1 knockdown dramatically reduces cell invasion that is accompanied by profound morphological changes and robust reduction in expression levels of mesenchymal markers [36]. Thus, Id1 knockdown promotes a switch from N-cadherin to E-cadherin [37] contributing to the reduction of the mesenchymal phenotype [35]. Consistent with this, Yu et al [17] and the present study show that restoring Cx43 to GSCs increased the expression of E-cadherin. Furthermore, our results reveal that Cx43 promotes a switch from N-cadherin to E-cadherin that could be a consequence of the reduction in Id1 expression promoted by the inhibition of c-Src activity (Figure 7). In fact, our results show that in addition to its effect on Id1 expression, the inhibition of c-Src with dasatinib promoted the switch from N-cadherin to E-cadherin in GSCs.

Together, these data suggest that Cx43 inhibits c-Src activity, which in turns reduces the expression of the transcriptional regulator Id1. Since Id1 has been reported to regulate Sox2 expression [36] and to promote cadherin switching [37], it is not unexpected that restoring Cx43 reduces the tumorigenic ability of GSCs (Figure 7). These results stress the relevance of the interaction between Cx43 and c-Src in the regulation of the malignant phenotype and pinpoint this interaction as a promising therapeutic target.

CONFLICT OF INTEREST:

The authors declare no conflict of interests.

ACKNOWLEDGMENTS:

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Figure Legends

Figure 1. **Connexin43 (Cx43) expression and c-Src activity in glioma stem cells (GSCs)**. **(A)** Western blot of Cx43, Y416 c-Src and total c-Src in OB1 TG10 GSCs [28]. (**B**) Western blot of Cx43 expressed by G166, GliNS2, G179 and G144 GSC lines characterized in [27]. (**C**) Western blot of Y416 c-Src and total c-Src G166, GliNS2, G179 and G144 cells. Rat C6 glioma cell line and rat astrocytes from primary culture were used as controls.

Figure 2. Effect of Cx43 on c-Src activity. **(A-C)** G166 cells were transfected with 50 nM NT-siRNA or Cx43-siRNA. **(A)** Western blot of Y416 c-Src, total c-Src, Cx43 and GAPDH, 2 or 4 days after transfection. **(B)** Y416 c-Src and **(C)** total c-Src quantification. ***p < 0.001, **p < 0.01, *p < 0.05 versus the corresponding NT-siRNA. **(D-G)** GliNS2 cells were transfected with the empty vector (Ires) or with the vector containing the Cx43 cDNA (Ires-Cx43). **(D)** Levels of Y416 c-Src, Cx43 and α-actinin detected by Western blotting after transfection with increasing plasmid concentrations. **(E)** Time course of Y416 c-Src, total c-Src, Cx43 and GAPDH after transfecting cells with 1 µg/µL Ires or Ires-Cx43. **(F)** Y416 c-Src and **(G)** total c-Src quantification after transfecting cells with 1 µg/µL Ires or Ires-Cx43 for 2 or 5 days. **p < 0.01, versus Ires. Abbreviations: NT-siRNA, non-targeting siRNA; Cx43-siRNA, siRNA against connexin43; Cx43, connexin43; GAPDH, Glyceraldehyde 3-phosphate dehydrogenase; AU, arbitrary units.

Figure 3. Effect of silencing Cx43 on G166 cell proliferation. G166 cells were transfected with 50 nM NT-siRNA or Cx43-siRNA. **(A)** Phase contrast photomicrographs showing the increase in cell density 4 days after the transfection with Cx43-siRNA. **(B)** The number of living cells was followed for 6 days by MTT assay. The results are expressed as the percentages of the higher value of absorbance found (NT-siRNA, 6^{th} day). **p < 0.01 versus the corresponding NT-siRNA. **(C)** DAPI, Ki-67 and merged photomicrographs of the same field showing Ki-67 positive cells (arrow) after 2 or 4 days. Scale bar = 20 μm **(D)** Percentage of Ki-67 positive cells. ***p < 0.001, **p < 0.01 versus the corresponding NT-siRNA. Abbreviations: NT-siRNA, non-targeting siRNA; Cx43-siRNA, siRNA against connexin43; Cx43, connexin43; DAPI, 4',6-diamidino-2-phenylindole.

Figure 4. Effect of restoring Cx43 on GliNS2 cell proliferation. GliNS2 cells were transfected with 1 μg/μL of the empty vector (Ires) or with the vector containing the

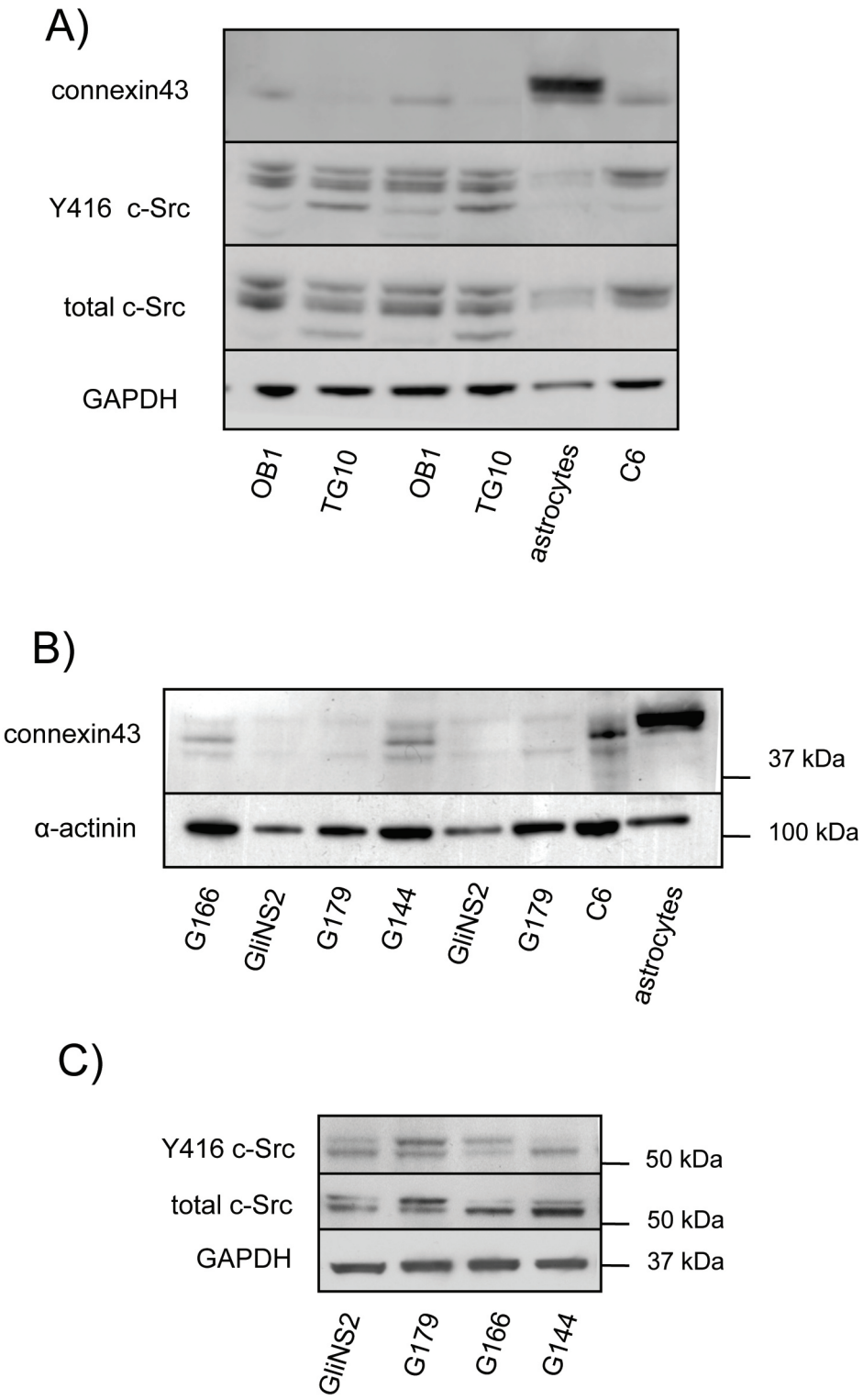
Cx43 cDNA (Ires-Cx43). **(A)** Phase contrast photomicrographs showing the reduction on cell density 5 days after the transfection with Ires-Cx43. **(B)** The number of living cells was followed for 7 days by MTT assay. The results are expressed as the percentages of the higher value of absorbance found (Ires, 7th day). **p < 0.01 versus the corresponding Ires. **(C)** DAPI, Ki-67 and merged photomicrographs of the same field showing Ki-67 positive cells (arrow) after 2 or 5 days. Scale bar = 20 μm **(D)** Percentage of Ki-67 positive cells. *p < 0.05 versus the corresponding Ires. Abbreviations: Cx43, connexin43; DAPI, 4',6-diamidino-2-phenylindole.

Figure 5. Effect of restoring Cx43 on Sox2, E-cadherin, N-cadherin and Id1 expression in GSC. GliNS2 cells were transfected with the empty vector (Ires) or with the vector containing the Cx43 cDNA (Ires-Cx43). **(A)** Immunostaining and phase contrast from the same field showing the decrease in Sox2 expression in Cx43-transfected GSCs. Scale bar= 20 μm. **(B)** Double immunostaining showing the lack of Sox2 in Cx43-expressing cells. Scale bar = 10 μm. **(C)** Western blot for Cx43, Sox2, E-cadherin and alpha-actinin as a loading control. **(D)** Double immunostaining showing the down-regulation of N-cadherin in Cx43-expressing cells. Scale bar = 10 μm. **(E)** Immunostaining and phase contrast from the same field showing the decrease in Id1 expression in Cx43-transfected GSC. Scale bar= 20 μm. **(F)** Double immunostaining showing the lack of Id1 in Cx43-expressing cells. Scale bar = 10 μm. **(G)** Western blot for Cx43 and Id1. Abbreviations: Cx43, connexin43.

Figure 6. Participation of c-Src in the reversion of GSC phenotype. (A-C) GliNS2 cells were incubated with Dasatinib for the indicated times and concentrations. **(A)** Western blot for total c-Src, Y416 c-Src and Id1. **(B)** Western blot for Sox2. **(C)** Western blot for E-cadherin and N-cadherin. Abbreviations: Cx43, connexin43.

Figure 7. Proposed mechanism. Our results show that restoration of Cx43 inhibits c-Src activity in GSC, which in turn down-regulates the expression of the inhibitor of differentiation Id1. Since Id1 regulates Sox2 expression [36] and cadherin switching [37], it could be proposed that this regulator of transcription is mediating the effect of Cx43 on Sox2 expression and cadherin switching. Down-regulation of Sox2 reduces GSC self-renewal [39] and cadherin switching participates in epithelial-mesenchymal transition (EMT) and invasion [35]. Consequently, we propose that Cx43 triggers this pathway to revert GSC phenotype and consequently tumorigenicity.

Fig. 1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37 kDa
100 kDa
G166
GliNS2
G179
G144
GliNS2
G179
C6
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Fig. 2

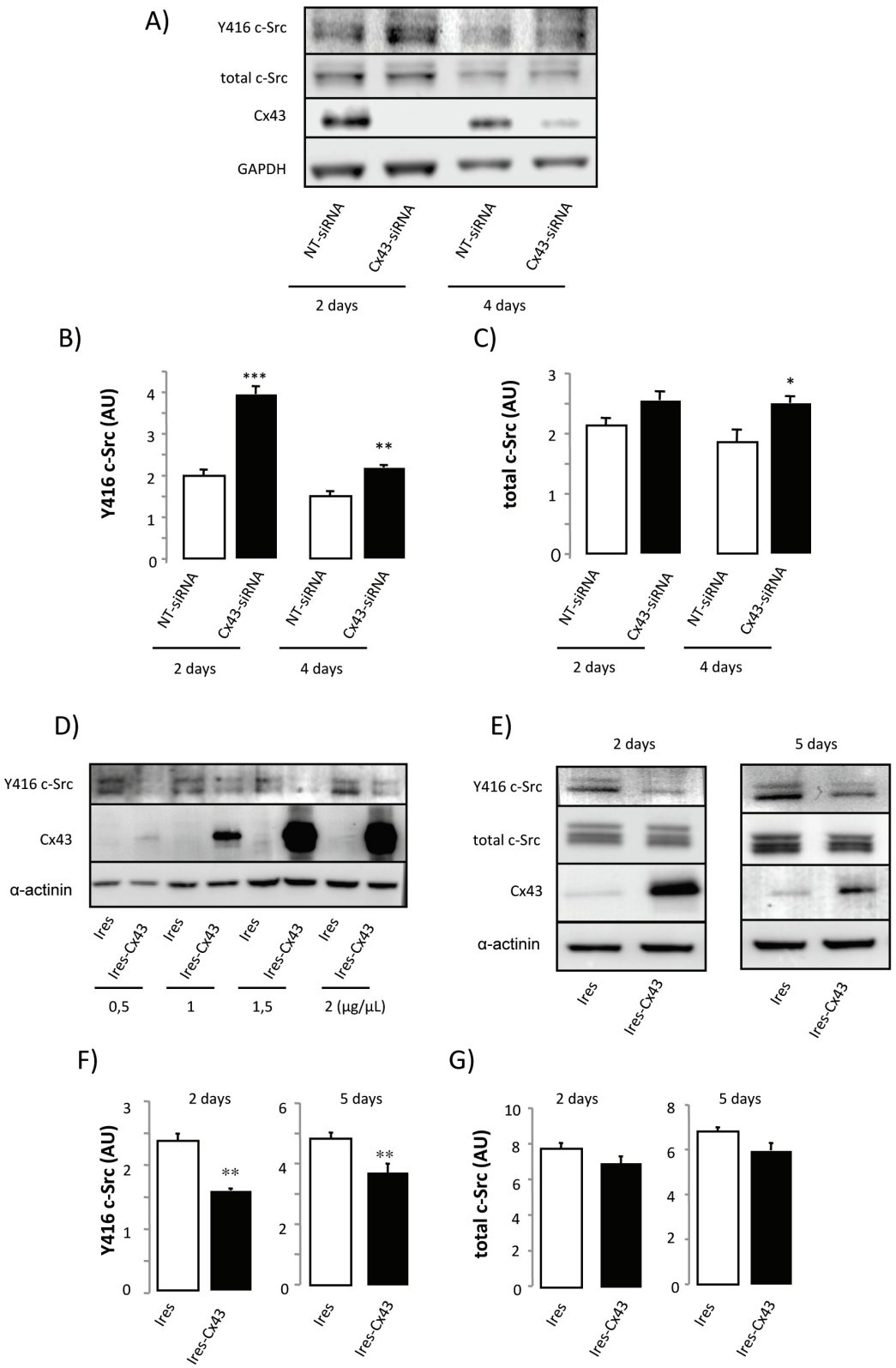

A)
Y416 c-Src
total c-Src
Cx43
GAPDH
NT-siRNA
Cx43-siRNA
NT-siRNA
Cx43-siRNA
2 days
4 days
B)
Y416 c-Src (AU)
***
**
NT-siRNA
Cx43-siRNA
NT-siRNA
Cx43-siRNA
2 days
4 days
C)
total c-Src (AU)
*
NT-siRNA
Cx43-siRNA
NT-siRNA
Cx43-siRNA
2 days
4 days
D)
Y416 c-Src
Cx43
α-actinin
Ires
Ires-Cx43
Ires
Ires-Cx43
Ires
Ires-Cx43
Ires
Ires-Cx43
0,5
1
1,5
2 (µg/µL)
E)
2 days
5 days
Y416 c-Src
total c-Src
Cx43
α-actinin
Ires
Ires-Cx43
Ires
Ires-Cx43
F)
2 days
5 days
Y416 c-Src (AU)
**
**
Ires
Ires-Cx43
Ires
Ires-Cx43
G)
2 days
5 days
total c-Src (AU)
Ires
Ires-Cx43
Ires
Ires-Cx43

Fig. 3

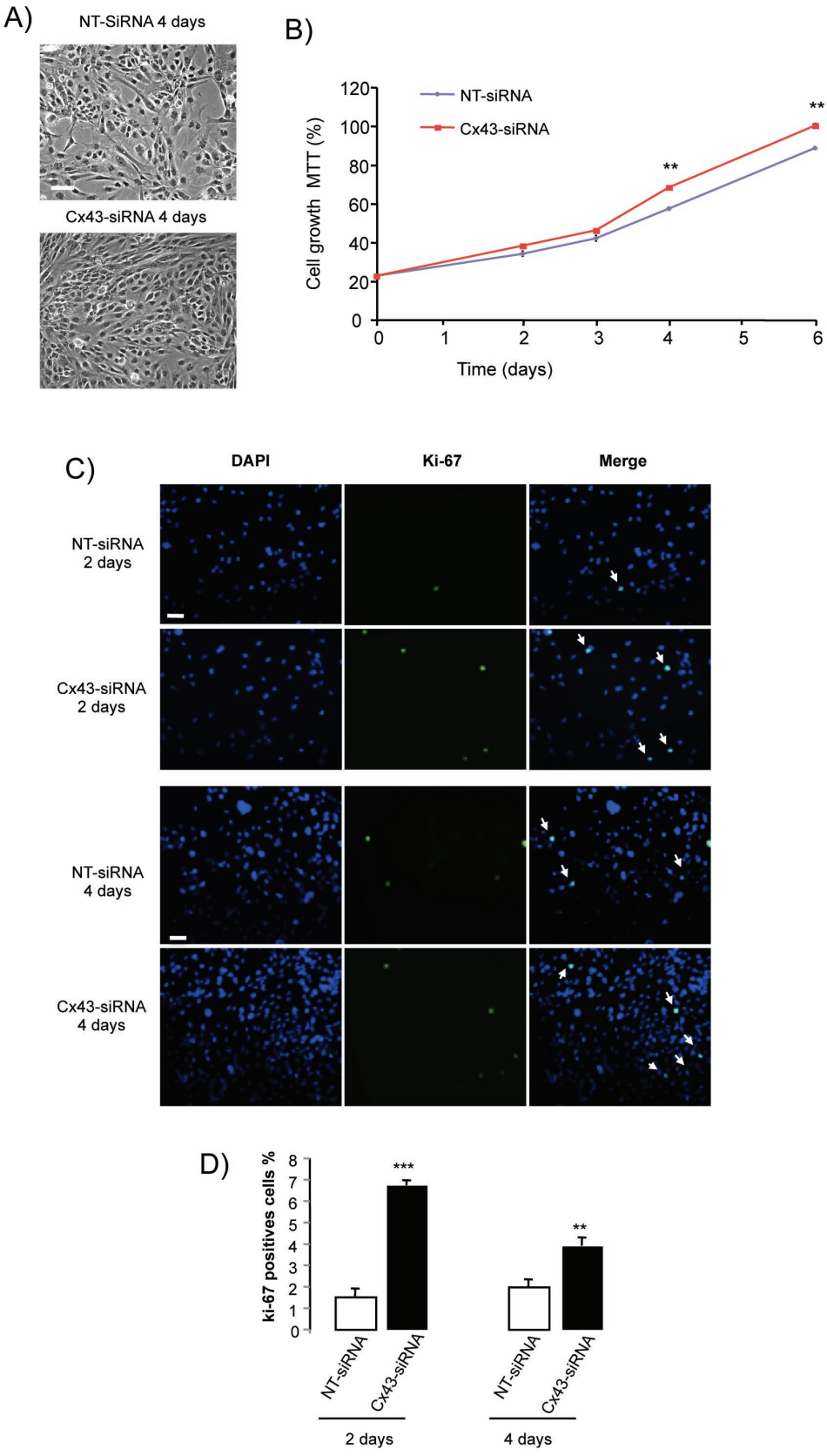

A)
NT-SiRNA 4 days
Cx43-siRNA 4 days
B)
NT-siRNA
Cx43-siRNA
Cell growth MTT (%)
Time (days)
**
**
C)
DAPI
Ki-67
Merge
NT-siRNA 2 days
Cx43-siRNA 2 days
NT-siRNA 4 days
Cx43-siRNA 4 days
D)
ki-67 positives cells %
***
**
NT-siRNA
Cx43-siRNA
2 days
4 days

Fig. 4

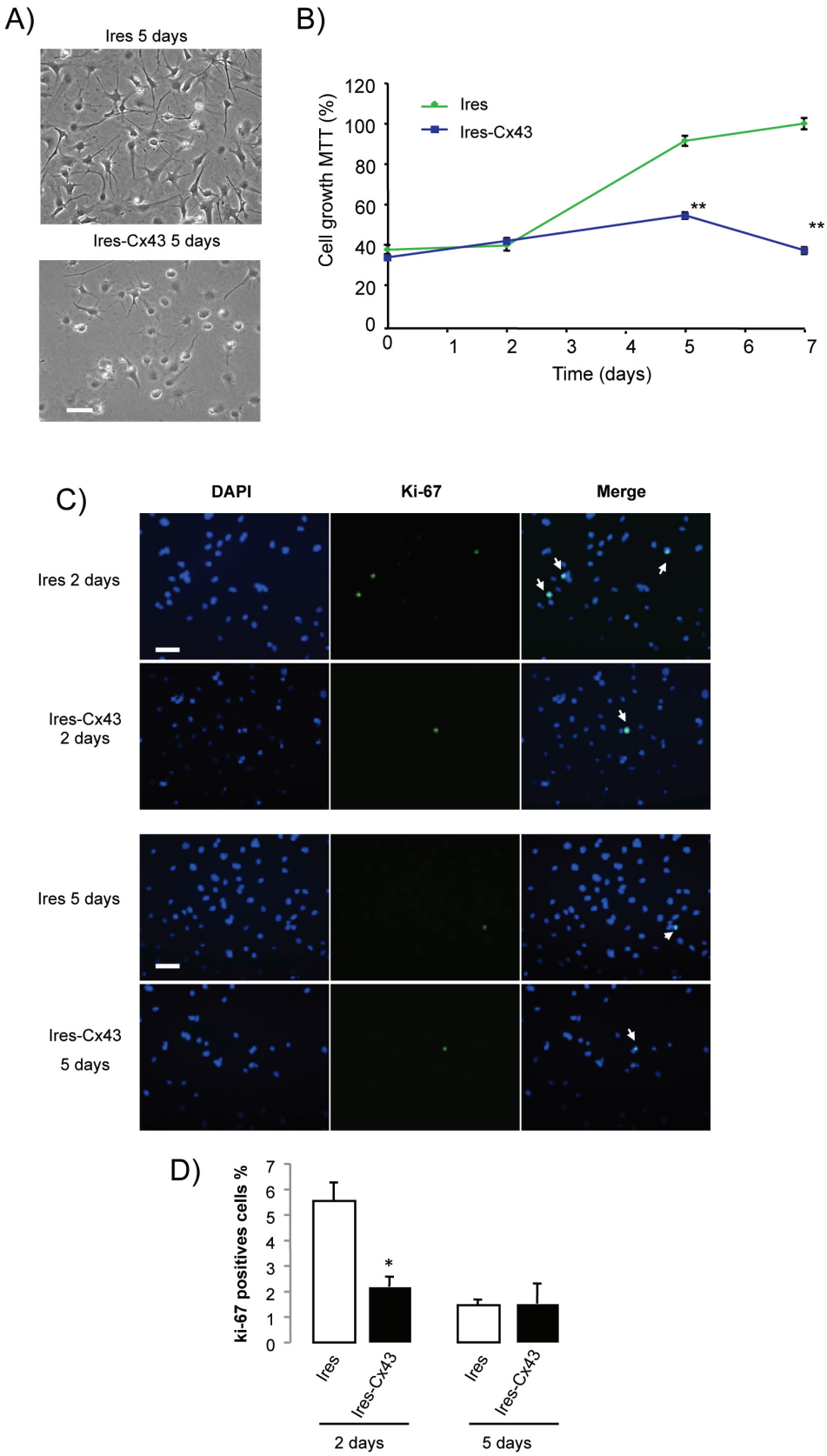

A)
Ires 5 days
Ires-Cx43 5 days
B)
Ires
Ires-Cx43
Cell growth MTT (%)
Time (days)
**
**
C)
DAPI
Ki-67
Merge
Ires 2 days
Ires-Cx43 2 days
Ires 5 days
Ires-Cx43 5 days
D)
ki-67 positives cells %
*
Ires
Ires-Cx43
Ires
Ires-Cx43
2 days
5 days

Fig. 5

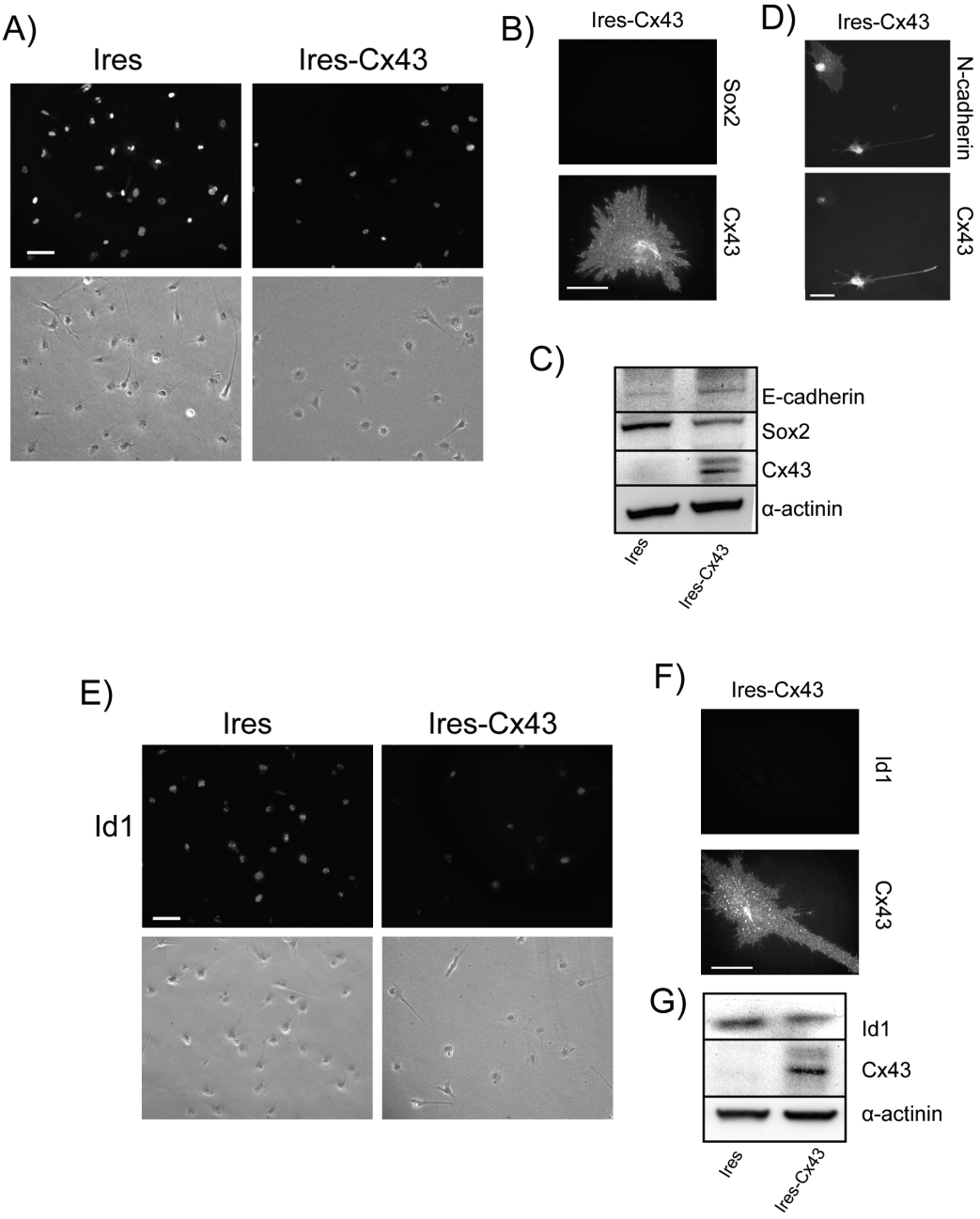

A)
Ires
Ires-Cx43
B)
Ires-Cx43
Sox2
Cx43
D)
Ires-Cx43
N-cadherin
Cx43
C)
E-cadherin
Sox2
Cx43
α-actinin
Ires
Ires-Cx43
E)
Ires
Ires-Cx43
Id1
F)
Ires-Cx43
Id1
Cx43
G)
Id1
Cx43
α-actinin
Ires
Ires-Cx43

Fig. 6

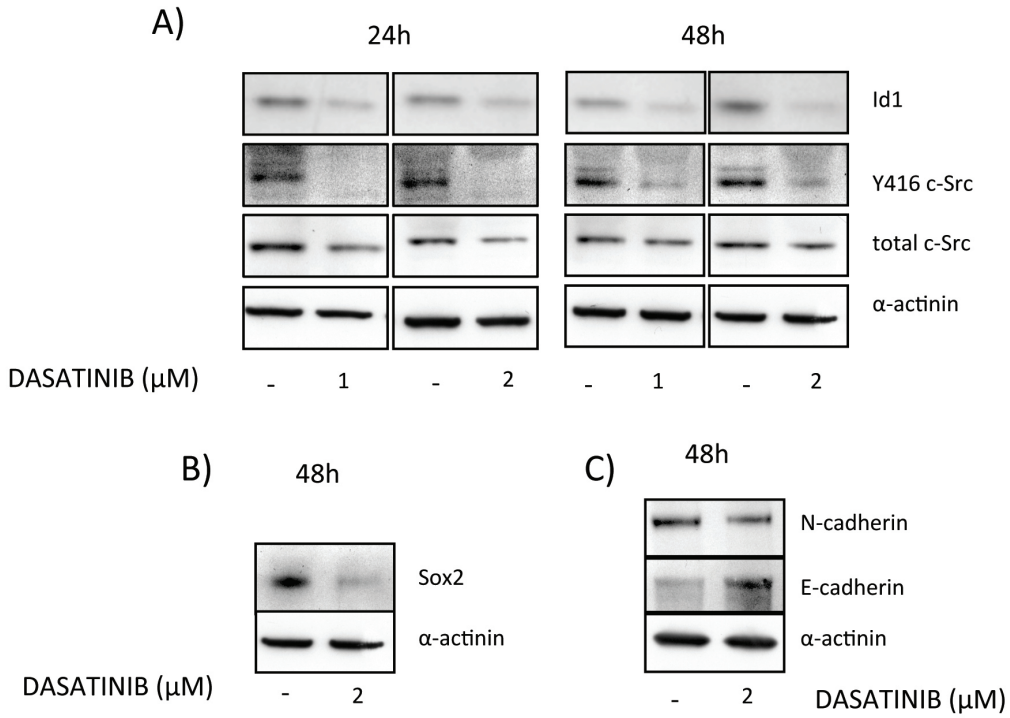
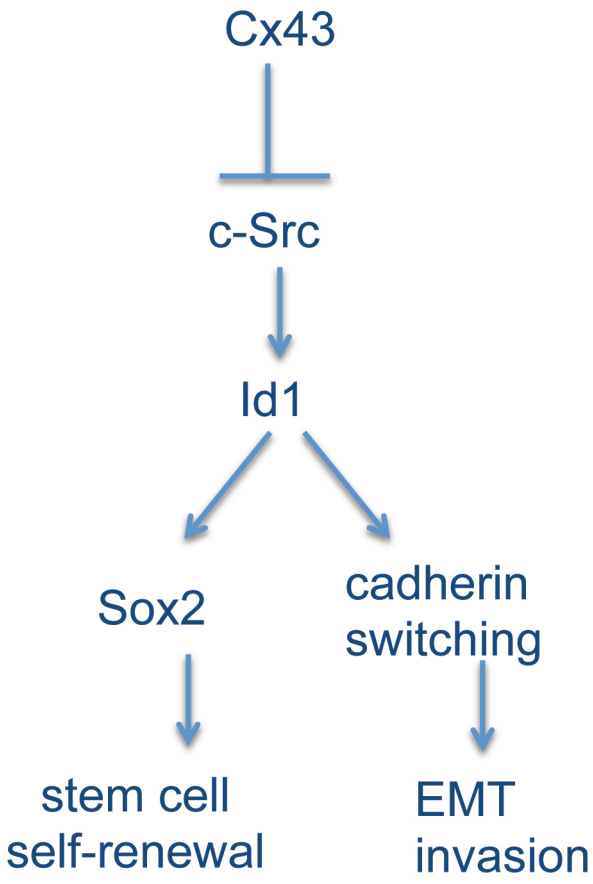

A)
24h
48h
Id1
Y416 c-Src
total c-Src
α-actinin
DASATINIB (μM)
-
1
-
2
-
1
-
2
B)
48h
Sox2
α-actinin
DASATINIB (μM)
-
2
C)
48h
N-cadherin
E-cadherin
α-actinin
-
2
DASATINIB (μM)

Fig. 7


Cx43
c-Src
Id1
Sox2
cadherin
switching
stem cell
self-renewal
EMT
invasion

CONCLUSIONS

1-The reactive gliosis provoked by the neuronal lesion promoted by kainic acid in mice cortex induces a decrease in connexin43 expression and an increase in the activity of c-Src. These data suggest that connexin43 is linked to c-Src in astrocytes in vivo.

2- The human gliomas including glioma stem cells, present a decrease in Cx43 expression and a high activity of c-Src.

3- Modulation of connexin43 in human glioma stem cells regulates the protooncogen c-Src activity and also cell proliferation.

4- Restoring connexin43 induces the decrease of Sox2 expression and promotes cadherin switching. Confirm that connexion-43 reverses the characteristic phenotype of glioma stem cell.

5- Inhibition of c-Src activity or restoration of Cx43 expression in glioma stem cells reduces Id1 expression, an important stem-like phenotype regulator. These results suggest that connexin43 inhibits c-Src activity, which in turn reduces the expression of the transcriptional regulator Id1 and consequently reverses glioma stem-like phenotype.

6- We have designed a cell penetrating peptide that mimics the effects of connexin43 on glioma stem-like phenotype and therefore with potential therapeutic opportunities.