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The involvement of FAK and Src in the invasion of cardiomyocytes by *Trypanosoma cruzi*

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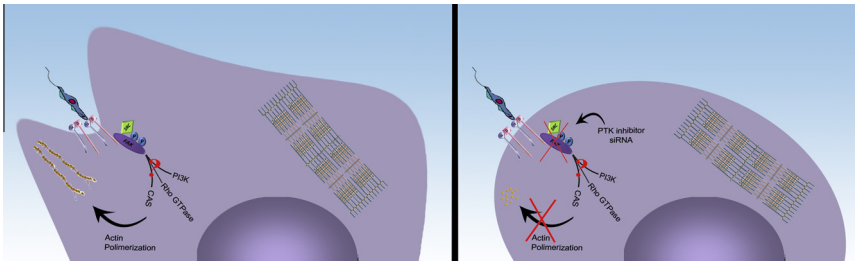
HIGHLIGHTS

- We examined the role of FAK/Src during *Trypanosoma cruzi* invasion process.
- Tyrosine kinase treatments impair *T. cruzi* entry.
- FAK and Src expression and phosphorylation increase during *T. cruzi* invasion.
- knockdown of FAK expression (siRNA or Tet-Fak cells) reduce parasite invasion.

GRAPHICAL ABSTRACT



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ABSTRACT

The activation of signaling pathways involving protein tyrosine kinases (PTKs) has been demonstrated during *Trypanosoma cruzi* invasion. Herein, we describe the participation of FAK/Src in the invasion of cardiomyocytes by *T. cruzi*. The treatment of cardiomyocytes with genistein, a PTK inhibitor, significantly reduced *T. cruzi* invasion. Also, PP1, a potent Src-family protein inhibitor, and PF573228, a specific FAK inhibitor, also inhibited *T. cruzi* entry; maximal inhibition was achieved at concentrations of 25 μM PP1 (53% inhibition) and 40 μM PF573228 (50% inhibition). The suppression of FAK expression in siRNA-treated cells and tetracycline-uninduced Tet-FAK(WT)-46 cells significantly reduced *T. cruzi* invasion. The entry of *T. cruzi* is accompanied by changes in FAK and c-Src expression and phosphorylation. An enhancement of FAK activation occurs during the initial stages of *T. cruzi*-cardiomyocyte interaction (30 and 60 min), with a concomitant increase in the level of c-Src expression and phosphorylation, suggesting that FAK/Src act as an integrated signaling pathway that coordinates parasite entry. These data provide novel insights into the signaling pathways that are involved in cardiomyocyte invasion by *T. cruzi*. A better understanding of the signal transduction networks involved in *T. cruzi* invasion may contribute to the development of more effective therapies for the treatment of Chagas' disease.

1. Introduction

The invasion of mammalian cells is an essential step for the dissemination and persistence of many intracellular pathogens (Hauck et al., 2012; Sibley, 2011). The processes involved in *Trypanosoma cruzi* invasion, the etiologic agent of Chagas' disease, have been intensely discussed in the last decade, and advances in this field have provided new insights into the mechanisms utilized by *T. cruzi* to invade target cells (Butler et al., 2013; Calvet et al., 2012; Caradonna and Burleigh, 2011; Nagajyothi et al., 2011).

The recognition of molecules on the host cell surface triggers the activation of distinct signaling pathways, leading to a mechanism of invasion that can be either dependent or independent of

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the host cell cytoskeleton (Ferreira et al., 2006). The activation of signaling cascades involving both tyrosine kinases and serine/threonine phosphatases have also been described in the *T. cruzi* invasion process (Maeda et al., 2012; Yoshida, 2006; Yoshida and Cortez, 2008). A repertoire of molecules on the surface of the *T. cruzi* is able to bind host cell receptors and to induce parasite invasion. Receptor-ligand binding triggers the activation of protein tyrosine kinase (PTK), phospholipase C (PLC), mTOR or kinin system in mammalian cells, leading to intracellular Ca^{2+} mobilization and parasite entry (Maeda et al., 2012; Scharfstein et al., 2000; Yoshida, 2006). The rise in transient cytoplasmic Ca^{2+} in host cells has been shown to promote actin depolymerization at the parasite adhesion site and activate a lysosome-dependent mechanism of invasion (Tardieux et al., 1994; Yoshida, 2006). However, the participation of the host cell cytoskeleton in this process has also been clearly demonstrated; rapid actin remodeling (Butler et al., 2013; Rosestolato et al., 2002) and the formation of host cell plasma membrane extensions (Barbosa and Meirelles, 1995) enable the engulfment of attached parasites. The phosphatidylinositol 3-kinase (PI3K) pathway has also been shown to regulate *T. cruzi* entry (Barrias et al., 2010; Vieira et al., 2002; Woolsey et al., 2003) and may facilitate cross-talk with a number of other potential signaling pathways, resulting in an intricate signaling network that may be crucial for *T. cruzi* invasion.

Focal adhesion kinase (FAK), a non-receptor tyrosine kinase (125 kDa), is activated by the clustering of integrins or heparan sulfate proteoglycans (HSPGs) (Bass and Humphries, 2002; Hall et al., 2011; Schaller, 2010; Schaller et al., 1995). Autophosphorylation of the Tyr^{397} residue of FAK potentially recruits SH2-containing proteins, including PI3K and members of the Src kinase family (primarily the c-Src protein), that promote the phosphorylation of several FAK tyrosine residues (Guan, 2010; Parsons, 2003). c-Src is a 60 kDa proto-oncogene, non-receptor tyrosine kinase that has multiple substrates and promotes a diverse array of signaling events (Johnson and Gallick, 2007). FAK-activation-mediated actin remodeling is important in many biological functions, such as cell cycle progression and migration (Hall et al., 2011).

Several pathogens, including viruses, fungi and bacteria, activate FAK and c-Src after association with integrins and/or HSPGs on the surface of host cells (Boehm et al., 2011; Chen et al., 2008; Kerur et al., 2010; Kim et al., 2012; Slanina et al., 2012; Tegtmeyer et al., 2001). Because integrins and HSPGs have been reported to mediate *T. cruzi* invasion (Bambino-Medeiros et al., 2011; Calvet et al., 2003; Fernandez et al., 1993; Oliveira et al., 2008), we investigated whether parasite entry triggers FAK/c-Src signaling. Our observations that the interaction of *T. cruzi* with cardiomyocytes induces an increase in FAK and c-Src expression and phosphorylation and that parasite entry is impaired by specific protein tyrosine kinase inhibitors (e.g., genistein, PP1 and PF573228) and FAK repression systems (e.g., the silencing of FAK with interference RNA or the use of Tet-FAK(WT)-46 cells in the uninducible state) suggest that FAK and c-Src signaling are involved in the *T. cruzi* invasion process. These data provide novel insights into the signaling pathways that are involved in host cell invasion by *T. cruzi*.

2. Materials and methods

2.1. Cardiomyocyte culture

Cardiac muscle cells were isolated from 18 to 20-day-old mouse embryos as previously described (Meirelles et al., 1986). Briefly, pregnant mice were obtained from Laboratory Animals Breeding Center (CECAL, Fiocruz) and immediately submitted to euthanasia by CO_2 inhalation followed by cervical dislocation. Embryos were removed from the uterus, decapitated and fragments of cardiac tissue were dissociated in phosphate-buffered saline (PBS) containing 0.025% trypsin (Sigma Aldrich, Saint Louis, MO) and 0.01% collagenase (Worthington Biochemical Corporation, Lakewood, NJ) at a pH of 7.2. Subsequently, the isolated cells were either plated (at a density of 1.0×10^5 cells/ml) on 0.01% gelatin-coated glass cover slips (Sigma Aldrich, Saint Louis, MO) in 24-well culture dishes or seeded (at a density of 2.0×10^6 cells/ml) on gelatin-coated 60-mm culture dishes for immunoblotting assays. The cultures were maintained in Dulbecco's Modified Eagle Medium (DMEM; Sigma Aldrich, Saint Louis, MO) that was supplemented with 5% fetal bovine serum (FBS; Sigma Aldrich, Saint Louis, MO), 2.5 mM $CaCl_2$, 1 mM L-glutamine (Sigma Aldrich, Saint Louis, MO) and 2% chicken embryo extract, applied as a source of growth factors (Pajtler et al., 2010), and maintained at 37 °C in a 5% CO_2 atmosphere. All procedures with animals were approved by the animal care and use committee (license LW-37/13, Oswaldo Cruz Foundation).

2.2. Tet-FAK cells inducibly expressing FAK

Tet-FAK(WT)-46 cells are mouse embryo fibroblasts that were initially derived from FAK-null embryos and have been engineered to inducibly express FAK under the control of the tetracycline repression system (Owen et al., 1999). Tet-FAK(WT)-46 cells were kindly provided by Dr. Steven Hanks (Department of Cell Biology, Vanderbilt University School of Medicine, Nashville, Tennessee). The cells were maintained in DMEM that was supplemented with 4500 mg/l D-glucose, 584 mg/l glutamine, 1 mM sodium pyruvate, 1 mM nonessential amino acids (all from GIBCO/BRL, Grand Island, NY), 100 units/ml penicillin, 100 μg/ml streptomycin, 0.25 μg/ml amphotericin B (Sigma Aldrich, Saint Louis, MO) and 10% fetal bovine serum (FBS; Sigma Aldrich, Saint Louis, MO). Tetracycline (Calbiochem) was included at a concentration of 1 mg/ml to maintain the Tet-FAK(WT)-46 cells in the uninduced state, and FAK expression was induced by tetracycline withdrawal for 2 days.

2.3. Cell culture-derived trypomastigotes and T. cruzi–host cell interaction

Cultures of African green monkey kidney epithelial cells (Vero cells) were maintained in RPMI 1640 medium (Cultilab, Campinas, SP) that was supplemented with 5% FBS and 2% L-glutamine and maintained at 37 °C in a 5% CO_2 atmosphere. After reaching semi-confluence, the cells were infected with culture-derived trypomastigote forms of *T. cruzi* (clone Dm28c). Four days post-infection, the trypomastigotes that were released from ruptured *T. cruzi*-infected Vero cells were harvested from the culture supernatant and used to infect cardiomyocyte cultures and Tet-FAK(WT)-46 cells at a ratio of 20 parasites per host cell. The infections were interrupted after 30, 60 and/or 120 min.

2.4. Tyrosine kinase inhibition assay

To test the effect of specific tyrosine kinase inhibitors on *T. cruzi* invasion, cardiomyocyte cultures were incubated for 45 min at 37 °C with non-toxic doses of genistein (25, 50 and 100 μM), a tyrosine inhibitor; PP1 (4-amino-5-(4-methylphenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine; 1, 5, 10, 15, and 25 μM), a potent inhibitor of Src-family tyrosine kinases; or PF573228 (10, 20 and 40 μM), a specific FAK inhibitor (Slack-Davis et al., 2007), in serum-free DMEM prior to the addition of *T. cruzi* parasites. After treatment, the cells were infected for 2 h with clone Dm28c trypomastigotes at a ratio of 20 parasites per host cell. After the interaction of parasites and host cells, the cultures were fixed for 5 min using Bouin's solution (75 ml of saturated aqueous picric acid solution, 25 ml of 40% formaldehyde and 5 ml of glacial acetic acid),

stained for 45 min using Giemsa stain, dehydrated in an acetone-xylol series and mounted in Permount. The tyrosine kinase inhibitors were dissolved in dimethyl sulfoxide (DMSO; Sigma Aldrich, Saint Louis, MO). Neither the inhibitors nor the DMSO itself interfered with the motility of the trypomastigotes or the spontaneous beating of host cells during the course of the experiment. The percent of infection was determined by quantification of a minimum of 200 cells in random selected microscopic fields using a Zeiss Axioplan microscope. The tyrosine kinase inhibition assay was performed three times in duplicate.

2.5. Transfection of FAK siRNA

A modified version of the manufacturer's protocol was used for the silencing of FAK with small-interference RNAs (siRNA, Santa Cruz Biotechnology, CA). Briefly, 1.5×10^5 or 2×10^6 cells seeded into 24-well plates or 60-mm tissue culture dishes, respectively, were transfected with lipofectamine (Invitrogen, Carlsbad, CA) and 5 μg/ml of FAK siRNA (sc-35353; Santa Cruz) or control nonsilencing siRNA (sc-37007; control siRNA-A from Santa Cruz). Solution A, which consisted of 1 μl of lipofectamine for every 25 μl of OPTI-MEM (Invitrogen, Grand Island, NY), was incubated at room temperature for 45 min. Solution B, consisting of 1 μl of FAK siRNA for every 40 μl of OPTI-MEM, was then added to solution A. After 20 min of incubation, the mixture was applied to the cells in an appropriate volume of OPTI-MEM. Twenty-four hours after transfection, the cells were infected for 2 h with culture-derived trypomastigotes (clone Dm28c) at a ratio of 20 parasites per host cell. Protein knockdown effectiveness was analyzed by immunoblotting after 24 h of siRNA transfection.

2.6. Protein extraction and immunoblotting assays

After parasite-host cell interaction, the cultures were washed with cold PBS (pH 7.2) and lysed in lysis buffer (1% Triton X-100; 10 mM Tris–HCl, pH 7.4; 10 mM sodium pyrophosphate; 100 mM sodium fluoride; 10 μg/ml aprotinin; 1 mM PMSF; and 0.25 mM sodium orthovanadate). Loading buffer (62.5 mM Tris–HCl, pH 6.8; 2% SDS; 10% glycerol; 0.01% bromophenol blue; and 5% β-mercaptoethanol) was added to cell lysates and heated at 100 °C. The amount of protein was quantified using the Follin–Lowry method, and a total of 20 μg of protein was resolved by SDS–PAGE. Subsequently, the proteins were transferred to nitrocellulose membranes (BioRad, Hercules, CA) in transfer buffer (25 mM Tris–HCl; 192 mM glycine; and 10% methanol, pH 8.3). The membrane was then blocked with 5% skim milk in TBS containing 0.05% Tween 20 (TBST; Sigma Aldrich, Saint Louis, MO) for 40 min prior to an overnight incubation at 4 °C with anti-FAK antibody (1:2000; Santa Cruz Biotechnology, CA) or blocked with 5% BSA in TBST before a similar incubation with either anti-pY^{397}FAK (1:1000; Invitrogen, Frederick, MD), anti-c-Src (1:500; Santa Cruz Biotechnology, CA) or anti-pY^{418} Src (1:2000; Millipore) antibodies. A loading control was performed using mouse anti-GAPDH (36 kDa; Ambion, Inc.). After being washed, the membranes were incubated with anti-rabbit IgG or anti-mouse IgG HRP-labeled antibody (1:3000; Thermo Scientific, Rockford, IL) for 1 h, washed with TBST and incubated with chemoluminescent kit reagents (PIERCE; Thermo Scientific, Rockford, IL); subsequently, the membranes were exposed to X-ray film. Bands corresponding to proteins of interest were quantified by optical densitometry. The data shown represent the means of at least 3 independent experiments.

2.7. Statistical analysis

The films were scanned, and densitometric analysis was performed using ImageJ version 1.38 (Windows version of NIH Image, http://rsb.info.nih.gov/nih-image/). The results were normalized by the GAPDH values for each sample in the same experiment. Student's *t*-test was used to determine the significance of differences between the means in the Western blot assays; a p value $\leqslant 0.05$ was considered significant.

3. Results

3.1. FAK and Src are involved in T. cruzi invasion

Actin dynamics are regulated by numerous signaling pathways, including protein tyrosine kinases (Gunst, 2004; Rohatgi et al., 2001). Thus, because actin rearrangement plays a key role in *T. cruzi* invasion (Barbosa and Meirelles, 1995; Rosestolato et al., 2002), we evaluated the involvement of tyrosine kinase phosphorylation in regulating the entry of *T. cruzi* into cardiomyocytes. Tyrosin kinases inhibitors were used as strategy to blockage non-receptor tyrosin kinases (NRTK) (Fig. 1). Whole cell lysates were used to assess the phosphorylation status of pY^{397}FAK (Fig. 1A) and pY^{418}Src (Fig. 1B) after treatment of cardiomyocytes with tyrosine kinases inhibitors. Western blot analysis revealed a significant decrease in the phosphorylation level of FAK and c-Src with genistein (a potent general tyrosine kinase inhibitor), PP1 (a potent inhibitor of Src-family tyrosine kinase) and PF573228 (a specific inhibitor of FAK Tyr^{397} residue phosphorylation), but markedly lower level was achieved in PF573228 (90%) and PP1 (59%) treatment. Then, prior to infection for 2 h with clone Dm28c trypomastigotes, primary cultures of cardiomyocytes were pretreated with nontoxic concentrations of genistein (25, 50 and 100 μM) for 45 min at 37 °C. The inhibition of tyrosine kinases impaired the invasion of cardiomyocytes by *T. cruzi*, leading to a significant, dose-dependent reduction of the observed infection levels (Fig. 1). A maximal inhibition was achieved after treatment with 100 μM genistein (Fig. 1C), resulting in a decrease in the infection level from 34% (control value; $p \leqslant 0.0006$) to 20%, representing an inhibition of approximately 41%.

To investigate whether FAK signaling orchestrates *T. cruzi* entry, we evaluated the effect of PF573228 on parasite invasion. A dose-dependent reduction of parasite invasion was achieved when cardiomyocyte cultures were treated with PF573228 prior to infection (Fig. 1D). The lowest infection level (17%), compared to untreated cells (35%), was achieved with the highest concentration of PF573228 (40 μM), representing a 50% reduction in the invasion of *T. cruzi*. Because the autophosphorylation of FAK (Tyr^{397} residue) leads to the binding of Src-SH2 domain that promotes the upstream phosphorylation of FAK, we assessed the participation of FAK/Src in *T. cruzi* invasion using PP1 a Src family tyrosine kinase inhibitor. The treatment of cardiomyocytes with different concentrations of PP1 (1, 5, 10, 15 and 25 μM) led to a substantial, dose-dependent decrease in *T. cruzi* invasion. The observed infection levels declined from approximately 34% to 16% ($p \leqslant 0.0006$) after treatment with the highest dose of PP1 (25 μM), corresponding to a 53% inhibition (Fig. 1E).

3.2. T. cruzi invasion induces FAK phosphorylation

To further determine whether the phosphorylated state of FAK changes during *T. cruzi* invasion, we evaluated the ability of *T. cruzi* to induce FAK phosphorylation at early stages of infection. Cardiomyocyte cultures were infected with clone Dm28c trypomastigotes for 30, 60 and 120 min, and total FAK and c-Src expression and phosphorylation levels were evaluated by immunoblotting assays (Fig. 2). Interesting, the initial stage of *T. cruzi*–host cell interaction, which is associated with receptor-ligand recognition, corresponds to an increase in pY^{397}FAK, with significant increases of 33%

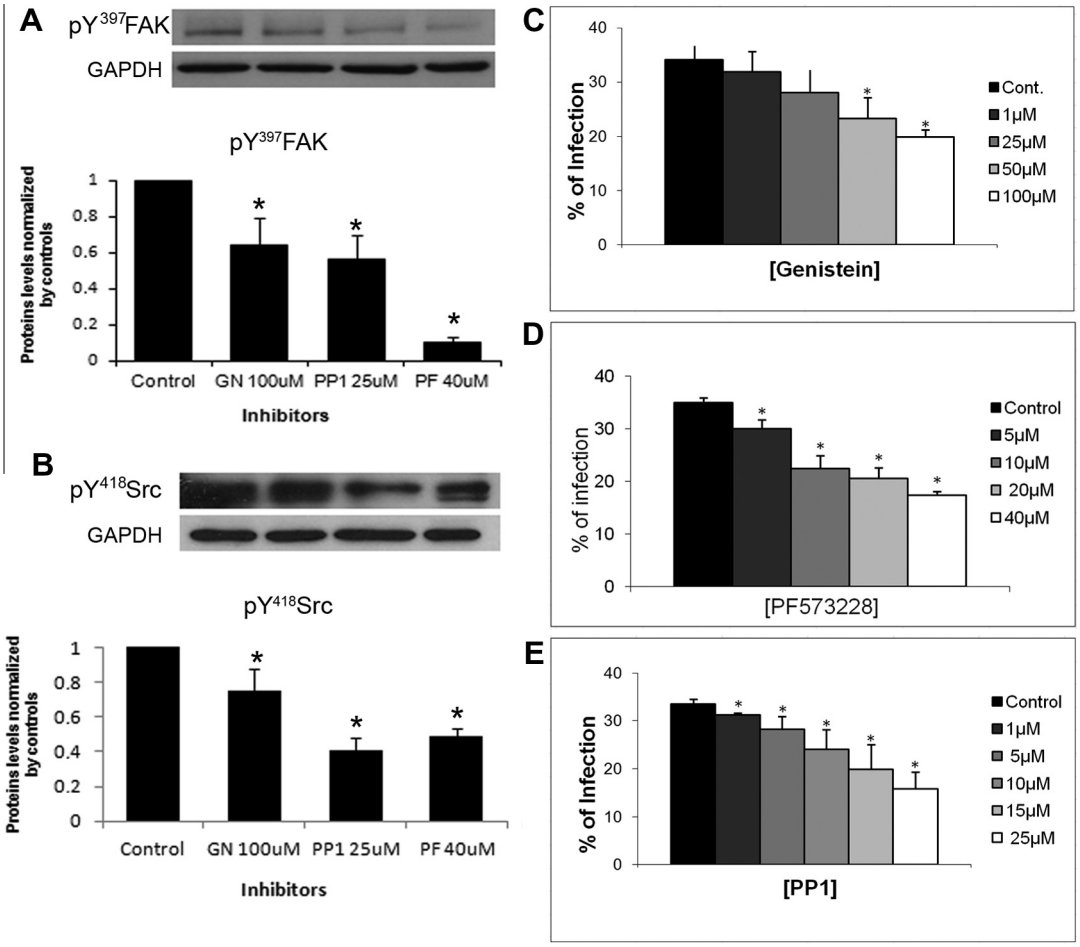

Fig. 1. The effect of protein tyrosine kinases (PTKs) inhibitors on *T. cruzi* invasion. The treatment of cardiomyocytes with 100 μM genistein, 25 μM PP1 and 40 μM PF573228 led to a reduction of FAK (A) and Src (B) phosphorylation. Immunoblotting was performed with an anti-pY^{397}FAK (A) and anti-Y^{418}Src (B) antibodies. GAPDH was used as internal control. The PTKs inhibitors block *T. cruzi* entry in a dose-dependent manner. (C) A maximal inhibition of *T. cruzi* invasion was achieved after treatment with 100 μM genistein, which resulted in an inhibition of approximately 41%. (D) PF573228 treatment led to a 50% reduction in parasite invasion levels when cardiomyocyte cultures were treated with a 40-μM dose of the inhibitor. (E) The infection levels also declined by 53% after treatment with a high dose of PP1 (25 μM). *Represents $p \leqslant 0.05$, as determined using Student's *t*-test.

($p \leqslant 0.0339$) and 36% ($p \leqslant 0.003$) in FAK activation after 30 and 60 min of infection, respectively. Subsequently, pY^{397}FAK levels reduced to steady-state levels compared to uninfected cardiomyocyte cultures (Fig. 2B). Total FAK expression levels were not altered by *T. cruzi* invasion (Fig. 2A), suggesting that *T. cruzi* infection did not affect FAK synthesis but instead induced the activation of endogenous FAK. In contrast, the total expression level of c-Src (60 kDa), a tyrosine kinase protein that is associated with FAK activation, was increased by approximately 2.0-fold at the early stages of infection (30 and 60 min); this was followed by a significant 22% reduction ($p \leqslant 0.05$) in c-Src levels after 120 min of infection in comparison to the control cultures (Fig. 2C). Remarkably, the level of pY^{418}Src also increased after 30 min (86%, $p \leqslant 0.008$) and 60 min (93%, $p \leqslant 0.002$) of *T. cruzi* infection (Fig. 2D). Like c-Src expression, Src phosphorylation was also decreased at later time (120 min) of infection (22%).

3.3. Knockdown of FAK impairs T. cruzi invasion

The role of FAK in *T. cruzi* invasion was also investigated using small-interfering RNAs (siRNA) technology in transient assays. Immunoblot analysis revealed that the transfection of cardiomyocytes with 5 μg/ml of FAK siRNA efficiently reduced the expression of FAK and the activation of pY^{397}FAK by 80% and 84%, respectively, in comparison with control siRNA or lipofectamine-untreated cells (Fig. 3A). The knockdown of FAK significantly suppressed the ability of cardiomyocytes to internalize the parasites, decreasing the infection level to 42% of the control (Fig. 3B). The general aspect of control (Fig. 3C) and siRNA FAK treated cardiomyocyte cultures (Fig. 3D) clearly demonstrates that down-regulation of FAK inhibits parasite entry. This issue was also investigated using a tetracycline repression system, Tet-FAK(WT)-46 cells (WT-FAK cells), as a tool to evaluate the role of FAK signaling in *T. cruzi* entry. WT-FAK cells that are treated with tetracycline (WT-FAK-uninduced cells) are a suitable model system because an identical inducible cell population serves as a control. Therefore, WT-FAK cells were cultured in the presence or absence of tetracycline for 48 h prior to *T. cruzi* infection (2 h). To further confirm the effect of tetracycline in FAK regulation, untreated and tetracycline-treated cells were examined by Western blot. As shown in Fig. 4A, tetracycline treatment reduced both expression and activation of FAK in Tet-FAK cells. In these cells, the down-regulation of FAK after 48 h of tetracycline treatment led to an 82% reduction of FAK and pY^{397}FAK. The infection of WT-FAK cells after 48 h of tetracycline treatment resulted in a 40% decrease in parasite invasion in comparison to the control

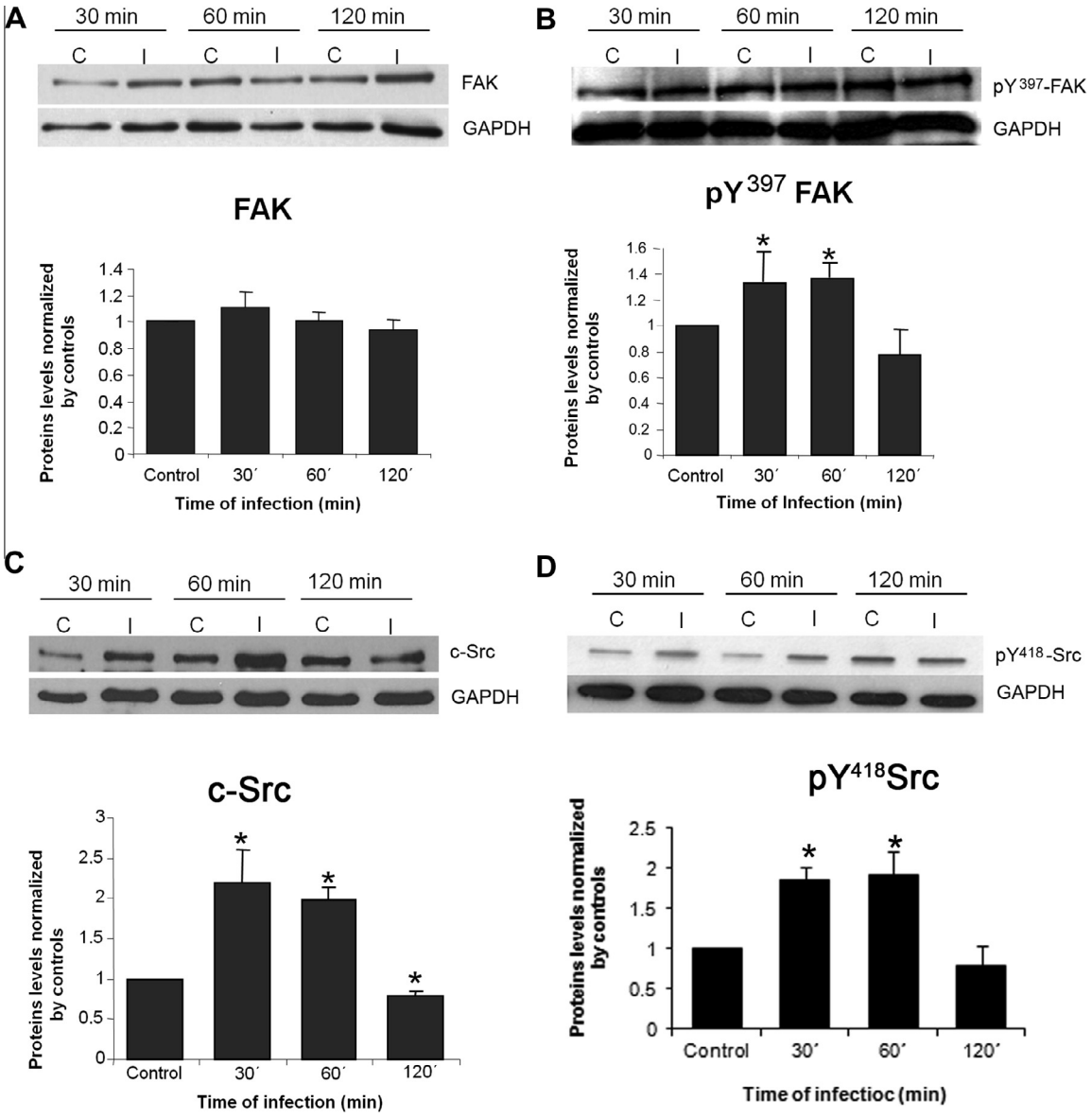

Fig. 2. FAK/SRC activity is involved in cardiomyocyte invasion by *T. cruzi*. The expression of FAK, c-SRC and phosphorylated FAK (pY^{397}FAK) and Src (pY^{418}Src) was evaluated in non-infected (C) and *Trypanosoma cruzi*–infected cardiomyocytes (I) after 30, 60 and 120 min of interaction. Immunoblot assays revealed that *T. cruzi* induces FAK and Src phosphorylation during the invasion process. Although FAK expression was not altered after infection (A), the level of pY^{397}FAK increased by 33% and 36% after 30 and 60 min of infection, respectively, and subsequently reduced to a steady-state level after 120 min (B). A 2-fold increase in c-Src expression (C) was associated with pY^{397}FAK (B) and pY^{418}Src (D) enhancement, which also exhibited a decline after 120 min. The level of Src phosphorylation increased by 86% and 93% after 30 min and 60 min of infection, respectively. The GAPDH signal was used to normalize loading differences between the lanes. Densitometric analyses were performed by normalizing FAK and Src expression and phosphorylation to the simultaneously measured GAPDH levels. Protein/GAPDH = D.O. *Represents $p \leqslant 0.05$, as determined using Student's *t*-test.

cells (i.e., WT-FAK induced cells) (Fig. 4B). Images of inducible (Fig. 4C) and uninducible *T. cruzi*-infected Tet-FAK cultures (Fig. 4D) noticeably demonstrate that FAK down-regulation impairs *T. cruzi* invasion.

4. Discussion

Emerging evidence has revealed that protein tyrosine kinases (PTKs) regulate critical early events during microbe infection. Additionally, interactions between signal pathways have been shown to enhance parasite invasion and, consequently, the success of infections (Tegtmeyer et al., 2001). The involvement of Ca^{2+} in signaling cascades has been shown to trigger the invasion of many pathogens (Hu et al., 2005; TranVan Nhieu et al., 2004), including *T. cruzi* (Burleigh and Andrews, 1998). During *T. cruzi*–host cell interactions, Ca^{2+} mobilization has been observed in both parasites and target cells at early stages of infection (Maeda et al., 2012; Yoshida, 2006). In mammalian cells, the rise in transient cytosolic Ca^{2+} has been shown to result from the release of Ca^{2+} from cellular compartments, such as the endoplasmic reticulum; this release is the result of Ca^{2+} ATPase activation in response to protein G, phospholipase C (PLC) and IP_3 (Burleigh and Woolsey, 2002). In turn, the involvement of FAK and Src in transmembrane receptor-activated signaling pathways, primarily those of integrin and heparan sulfate proteoglycan (Bass and Humphries, 2002; Schaller et al., 1995), has also been shown to regulate Ca^{2+} channels. Because both integrin and heparan sulfate proteoglycan (HSPG) mediate *T. cruzi* invasion (Fernandez et al., 1993; Oliveira et al., 2008), protein tyrosine kinases, including focal adhesion kinase (FAK) and Src, may play a

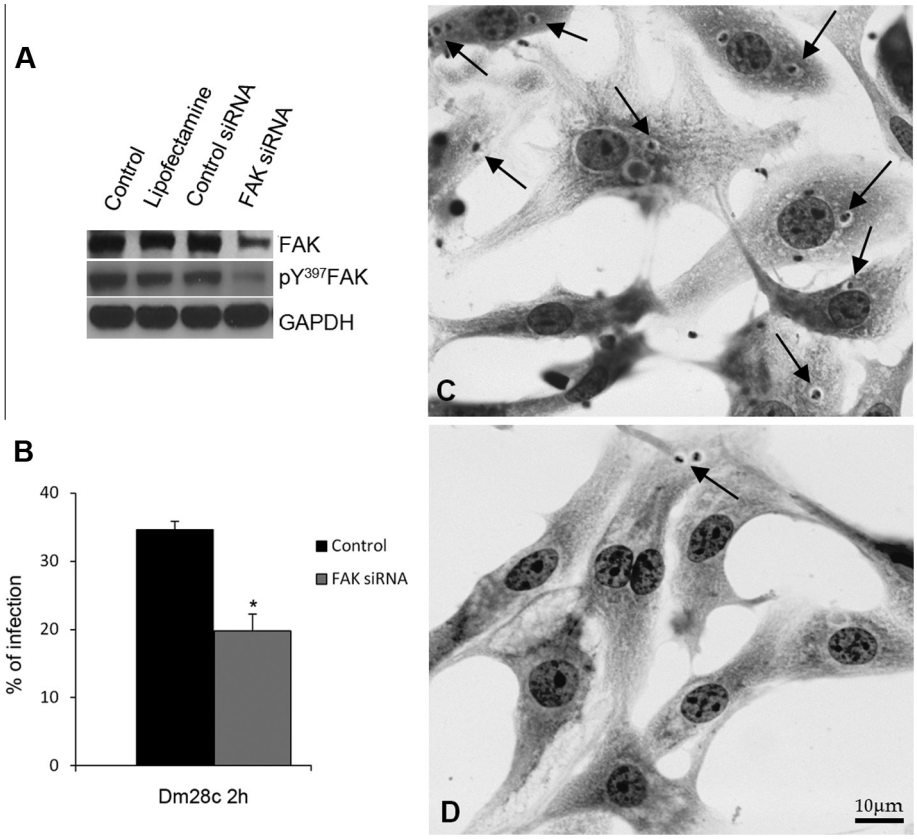

Fig. 3. RNA interference-mediated silencing of focal adhesion kinase inhibits *T. cruzi* invasion. Cardiomyocyte cultures were transfected for 24 h with siRNA targeting FAK. (A) Immunoblotting assays confirmed the knockdown of pY^{397}FAK after siRNA transfection when compared to the control siRNA. GAPDH expression levels were determined as controls. (B) The down-regulation of pY^{397}FAK reduced *T. cruzi* invasion by 42% in comparison to untreated controls. (C, D) Image of *T. cruzi*-infected cardiomyocyte cultures after transfection with control siRNA (C) or FAK siRNA (D). Note that FAK suppression reduces *T. cruzi* invasion (D), showing few intracellular parasites (arrow). The bars represent the average of infection level of triplicate experiments ± SD. *Represents $p \leqslant 0.05$, as determined using Student's *t*-test. Bar = 10 μm.

key role in the *T. cruzi* invasion process. Our findings provide insights into a potential role of FAK and Src activity in *T. cruzi* invasion.

The treatment of cardiac muscle cells with genistein, an isoflavone that has been shown to be a highly-specific inhibitor of protein tyrosine kinases (Akiyama and Ogawara, 1991), reduced the invasion by *T. cruzi* in a dose-dependent manner. Our data are in accordance with a previous report that also demonstrated the involvement of host-cell PTK in *T. cruzi* entry (Procópio et al., 1998). In addition, several studies have demonstrated the participation of PI3K in *T. cruzi* entry into both professional and non-professional phagocytic cells (Vieira et al., 2002; Woolsey et al., 2003), revealing that this parasite exploits cellular protein kinase signaling pathways to enter target cells.

The participation of host cell kinases has also been reported as a mechanism of invasion of a variety of pathogens (Hong et al., 2006; Martiny et al., 1996; Monteiro da Silva et al., 2007). In similar studies that used kinase inhibitors, the invasion of the fungus *Fonsecaea pedrosoi*, the principal etiologic agent of chromoblastomycosis, was blocked by 77% and 52% when macrophages and epithelial cells, respectively, were pretreated with genistein (Limongi et al., 2003). In human brain microvascular endothelial cells (HBMEC), the inhibition of PTKs by genistein decreased the uptake of *Neisseria meningitidis* and inhibited the secretion of cytokines, such as interleukin-6 (IL-6) and interleukin-8 (IL-8) (Sokolova et al., 2004), suggesting that PTKs regulate important events in the interactions between parasites and host cells. In addition, it has been shown that PTK mediates the entry of *Leishmania donovani* promastigotes into macrophages, a process that does not depend on parasite virulence, without interfering with intracellular parasite proliferation (Ghosh and Chakraborty, 2002). Inhibition of PTKs with genistein reduces Leishmania promastigote entry but also decreases the macrophages microbicidal activity, leading to enhancement of parasite burden (Martiny et al., 1996). Phosphorylation of proteins has also been evidenced in the invasion of *Toxoplasma gondii* in macrophage (Ferreira et al., 2003). Treatment of macrophages or tachyzoites with genistein reduced the parasite entry, suggesting that tyrosine kinases are involved in *T. gondii*–host cell interaction.

We also attempted to determine whether FAK and c-Src signaling pathways are activated during *T. cruzi* invasion. The treatment of cardiomyocytes with PF573228 blocked FAK autophosphorylation (pY^{397}FAK) and led to a 50% reduction in the entry of *T. cruzi* parasites, suggesting a key role of the FAK signaling pathway in the *T. cruzi* invasion process. Given that FAK activation is tightly coupled with the generation of an active FAK/Src complex, which determines the maximal catalytic activity of FAK (Parsons, 2003), we used PP1, a Src-family tyrosine kinase inhibitor, to evaluate the role of the FAK/Src complex in *T. cruzi* entry. Our results revealed that FAK/Src inhibition impaired the invasion of cardiomyocytes by *T. cruzi*, achieving a maximum inhibition (53%) after treatment with 25 μM PP1, suggesting that cooperation between FAK and Src drives the entry of *T. cruzi* into cardiomyocytes.

Dual-activation FAK-Src signaling orchestrates the remodeling of the actin cytoskeleton and participates in the invasion mechanisms of several pathogens (Reddy et al., 2000). The FAK/Src activation that is required for *Staphylococcus aureus* invasion is controlled by the interaction between cell wall-attached fibronectin-binding

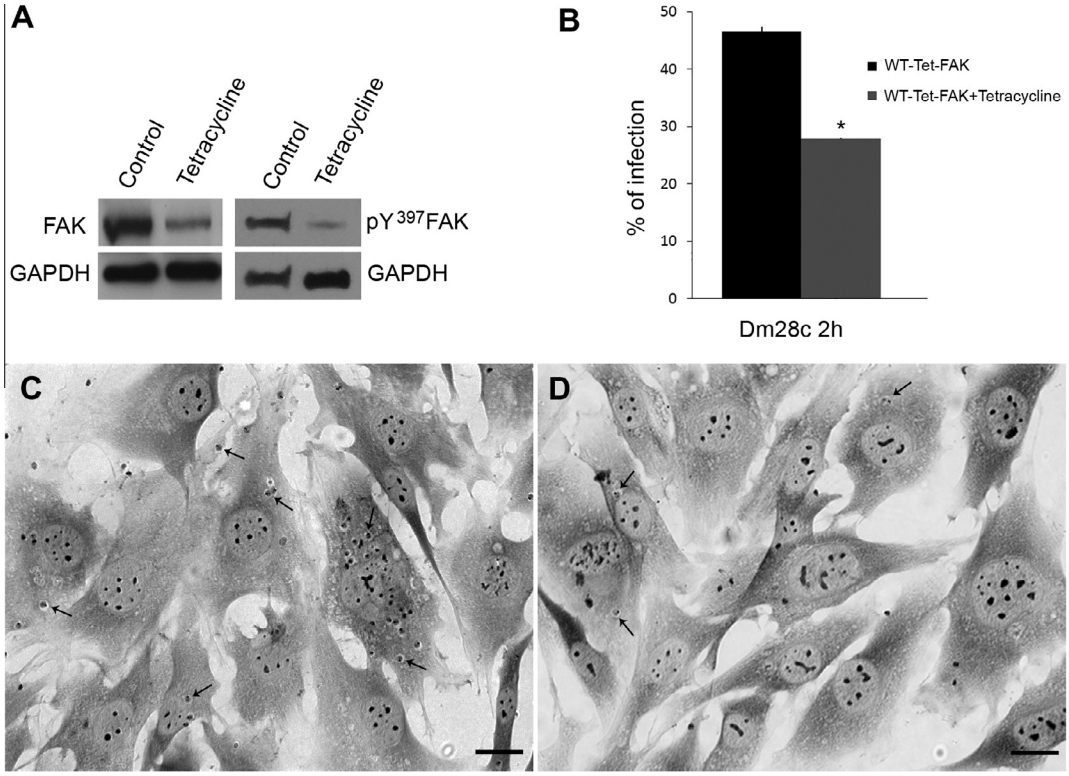

Fig. 4. The suppression of FAK expression in tetracycline-uninduced Tet-FAK(WT)-46 cells significantly reduced *T. cruzi* invasion. TetFAK cells were used to evaluate the requirement of FAK activation in *T. cruzi* invasion. (A) The down-regulation of FAK and pY^{397}FAK after 48 h of treatment of Tet-FAK(WT)-46 cells with tetracycline was demonstrated by immunoblotting assays. (B) 48 h-Tetracycline treated Tet-FAK(WT)-46 cells (uninduced cells) exhibited a decrease in the level of *T. cruzi* infection, showing a 40% inhibition of parasite invasion. (C, D) General aspects of induced (C) and uninduced (D) Tet-FAK(WT)-46 cultures infected by *T. cruzi* (2 h), showing intracellular parasites (arrows). Bars represent the average of infection level of triplicate experiments ± SD. *Represents $p \leqslant 0.05$, as determined using Student's *t*-test. Bar = 20 μm.

proteins A and B and host cell integrin through the extracellular matrix protein fibronectin (Agerer et al., 2003). Bacteria uptake is inhibited in Src-deficient cells, indicating that FAK/Src kinases regulate the cytoskeletal remodeling that enables the entry of bacteria. *Neisseria meningitides* also induces FAK/Src complex and cortactin activation and their cooperative interplay lead to endocytosis of the bacteria by human brain microvascular endothelial cells (Slanina et al., 2012). The binding of the IpA protein of *Shigella flexneri* to $\alpha_5\beta1$ integrin also promotes FAK activation, which stimulates actin rearrangement at the bacterial adhesion site (Watarai et al., 1996). *Campylobacter jejuni* also exploits integrin signal pathway and FAK coordenates Rac1 GTPase activity for its entry in epithelial cells (Boehm et al., 2011). The recruitment of FAK to focal plates at sites of *Escherichia coli* invasion in brain microvascular endothelium cells, in addition to evidence of FAK phosphorylation, have demonstrated the important role of FAK in regulating actin rearrangements during *E. coli* internalization (Reddy et al., 2000). Activation of FAK also contributes to *Cryptococcus neoformans* transmigration across the blood–brain barrier (Kim et al., 2012). In malaria, red blood cells that are infected by protozoan *Plasmodium* sp present proteins on their surfaces that activate Src and FAK for subsequent MAPK activation, leading to the adherence of infected cells to the endothelium, which impairs the removal of parasitized cells by macrophages (Yipp et al., 2003).

To test whether the activation of FAK and Src is required for the entry of *T. cruzi* into cardiomyocytes, we analyzed the expression of Src, FAK and phosphorylated FAK ([pY^{397}]-FAK) during *T. cruzi*–cardiomyocyte interactions. Although the total FAK expression level was unchanged, *T. cruzi* induced a significant increase in FAK phosphorylation in cardiomyocytes. FAK phosphorylation takes place during the initial stage of *T. cruzi*–cardiomyocyte interaction (30 and 60 min), with a concomitant increase in the level of Src expression and phosphorylation. Downstream of FAK phosphorylation, a corresponding reduction in Src expression after 2 h of interaction is observed, suggesting that FAK/Src are involved in an integrated signaling pathway that coordinates parasite entry. Evidence that *T. cruzi* parasites recognize both integrin and HSPG on the surface of the target cells supports the idea that this parasite activates the FAK/Src signaling pathway (Calvet et al., 2003; Fernandez et al., 1993; Oliveira et al., 2008). The binding of parasite surface ligands to host-cell transmembrane receptors may induce FAK autophosphorylation and recruit Src, which amplifies tyrosine phosphorylation and results in actin rearrangement.

Integrin and HSPG have been implicated in the invasion pathways of a variety of pathogens (Chen et al., 2008). The association of type 1 pili, the filamentous adhesive organelles of uropathogenic *E. coli*, with $\alpha_3\beta1$ integrins elicits bacterial entry via FAK/Src activation and cortactin phosphorylation (Eto et al., 2007). Interestingly, HSPG-integrin cooperation activates host cell signaling pathways that are involved in the entry of Kaposi's sarcoma-associated herpesvirus (Kerur et al., 2010; Veettil et al., 2006). Successive binding of the viral envelope glycoprotein gpB to heparan sulfate and integrin induces a signaling cascade that involves FAK, Src, PI3K and RhoA GTPase, resulting in the uptake of the virus via endocytosis (Veettil et al., 2006). The binding of human papillomavirus type 16 to heparan sulfate also triggers FAK activation through integrin and mediates the virus infection in human adult keratinocytes cell line (Abban and Meneses, 2010), reinforcing that HSPG-integrin cooperation may also induce FAK signaling and elicit virus entry. In addition, the attachment of *Entamoeba histolytica* trophozoites to fibronectin on the surface of endothelial cells triggers integrin-dependent signaling through the association of FAK/Src, paxillin and vinculin (Flores-Robles et al., 2003). An obligate intracellular pathogen, *Orientia tsutsugamushi*, the causative agent of scrub

typhus, activates integrin-mediated signal transduction pathways including FAK/Src and induces actin rearrangements at the infection site in non-phagocytic host cells (Cho et al., 2010).

The knockdown of FAK expression by siRNA or the use of a tetracycline inducible system (i.e., Tet-FAK(WT)-46 cells) further demonstrates the role of FAK signaling in the invasion of *T. cruzi* parasites. The down-regulation of FAK through siRNA or tetracycline treatment significantly reduced trypomastigote internalization by cardiomyocytes and uninduced Tet-FAK cells, respectively. The specific transmembrane molecule responsible for the activation of FAK signaling during cardiomyocyte invasion by *T. cruzi* is still unknown; however, it may be coordinated by integrin, HSPG or cross-talk between both receptors, as is observed in Kaposi's sarcoma-associated herpesvirus (Veettil et al., 2006) and Human Papillomavirus Type 16 infections (Abban and Meneses, 2010), because both surface molecules are known to elicit *T. cruzi* invasion (Calvet et al., 2003; Fernandez et al., 1993). This issue will be the focus of further investigations.

In summary, the results shown here demonstrated the role of the FAK signaling pathway in the invasion of cardiomyocytes by *T. cruzi*. The inhibition of total tyrosine kinase, FAK and Src tyrosine kinase family activity by specific inhibitors impairs parasite entry. The entrance of *T. cruzi* is associated with an increase in Src and FAK phosphorylation and may modulate the actin cytoskeleton, resulting in an endocytic invasion process. The previous observation of cardiomyocyte membrane extensions during *T. cruzi* invasion and the inhibition of parasite entry upon treatment of these cells with cytochalasin D (Barbosa and Meirelles, 1995) demonstrate the involvement of the host cell cytoskeleton in parasite internalization. This process may be regulated by FAK/Src activation and/or crosstalk between this signaling cascade and PI3K and Rho GTPases. A better understanding of these pathways may contribute to our knowledge of the molecular and cellular events that occur during the interactions of *T. cruzi* parasites and cardiomyocytes and may reveal new therapeutic targets for the treatment of Chagas' disease.

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