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Research Article

Src-Induced Cisplatin Resistance Mediated by Cell-to-Cell Communication

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Abstract

Cisplatin-induced cell death can be triggered by cell-to-cell communication through gap junctions. Here, we show that activated src produces tyrosine phosphorylation of the gap junction protein connexin 43, decreases gap junction communication, and increases cell survival in response to cisplatin. Experiments with mixed cell populations show that src activity in one cell can confer increased cisplatin survival on neighboring cells, even when the neighboring cells lack such src activity. This work is the first demonstration that expression of an oncogene in one cell can affect the survival of a neighboring cell not expressing the oncogene in response to a chemotherapeutic drug. The *trans*-acting effect of activated src on neighboring cells can be blocked by inhibitors of src kinase or by siRNA-mediated knockdown of src expression, and it can be counteracted by forced up-regulation of connexin 43, via either gene transfer or proteasome inhibition. These results identify a novel pathway of cisplatin resistance that may be amenable to therapeutic intervention. [Cancer Res 2009;69(8):3619–24]

Introduction

Once activated in a cell, oncogenes generally promote increased growth and survival. In addition, oncogene expression in cancer cells commonly confers resistance to chemotherapy and radiation. The increased resistance is thought to occur through cell autonomous mechanisms in which an activated oncogene in a cell confers resistance to that particular cell (1, 2). In this work, we report a novel pathway by which expression of an oncogene, src, in one cell can increase survival of neighboring cells that are not, themselves, expressing the oncogene, to the chemotherapeutic drug, cisplatin.

Prior work by our group identified a pathway of cisplatin toxicity involving cell-to-cell signaling that is dependent on the DNA-dependent protein kinase complex and on gap junction intercellular communication (GJIC; ref. 3). In this pathway, cisplatin damage in one cell triggers a signal that is dependent on DNA-dependent protein kinase and is transmitted via GJIC to a neighboring cell to cause cell death. We therefore hypothesized that factors that modulate GJIC might influence the response of cell populations to cisplatin treatment specifically by affecting the cell-to-cell response pathway. Based on a report that connexin 43 (cx43) is a phosphorylation target of src (4), we asked whether activated src can modulate the intercellular transmission of the death signal in response to cisplatin.

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To test this, we developed a series of genetically defined cell lines derived from mouse embryonic fibroblasts (MEF), including wild-type (wt) MEFs, MEFs expressing activated v-Src, and MEFs deficient in Ku80, a key component of the DNA-dependent protein kinase complex. Because prior work established that Ku80−/− MEFs cannot initiate the cisplatin cell-to-cell death signal but can still receive it, they served as receiver cells to examine the *trans*-acting effects of activated src expression. We report here that expression of activated src in one group of cells attenuates their ability to send a death signal to neighboring cells, thereby conferring resistance to cells in the overall population even if they do not express activated src. In addition, forced overexpression of cx43 by gene transfer or by proteasome inhibition reversed the effect of v-Src, whereas the use of a src kinase inhibitor or the use of siRNAs to knock down src expression blocked it, leading in all four cases to increased sensitivity of neighboring cells to cisplatin. Hence, the work identifies novel pharmacologic strategies to sensitize tumor cells to cisplatin.

Materials and Methods

Cell lines. Wt and Ku80−/− immortalized MEFs were grown in DMEM with 10% fetal bovine serum (FBS). A pcDNA3 vector containing cDNA for v-Src (generous gift from G. Steven Martin, University of California, Berkeley) was stably transfected into wt MEFs. Positive clones (Src1 and Src2) were selected with 800 μg/mL of geneticin. To create the Src1+cx43 cell line, overexpressing human cx43, Src1 cells were stably transfected with human cx43 cDNA cloned into pcDNA 3.1 and a positive clone was selected using 0.1 mg/mL zeocin.

Drug treatment and survival assays. Cells were treated with indicated concentrations of cisplatin (Sigma) for 1 h. In addition, as indicated, cells were treated with 1μmol/L PP2 (Calbiochem) for 2 h before and concurrent with cisplatin treatment or treated with 50 mg/mL of lindane simultaneously with cisplatin treatment.

High- and low-density clonogenic survival experiments and monolayer growth assay with mixed populations of cells were performed as previously described (3) with the following alterations. To measure survival exclusively in Ku80−/− cells, we created a puromycin (puro)-resistant cell line by stably transfecting them with the pCMV-puro-bam vector, and resistant cells were selected and maintained using 2μg/mL of puro (Sigma). For experiments with the proteasome inhibitor, MG132 (A.G. Scientific), Src1 cells were pretreated with MG132 (100 nmol/L) for 24 h, MG132 was removed, and cells were trypsinized and seeded 1:1 with Ku80−/−puro+ cells. Cells were allowed to attach for 3 h, treated with cisplatin, and followed by the monolayer growth assay.

siRNA treatment. Src1 cells were transfected simultaneously with two siRNA oligonucleotides targeting v-Src (UCAACAACACGGAAGGUAAUU, CGGGAGAGCGAGACGACAAUU) or control siRNA molecule designed to target glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA using DharmaFECT 1 transfection reagent (Thermo Scientific). Twenty-four hours posttransfection, the Src1 cells were reseeded 1:1 with Ku80−/−puro+ cells at high density. The next day, mixed cell populations were treated with 5 μg/mL cisplatin for 1 h, washed twice with PBS, trypsinized, and seeded at low density in media containing puro to assay clonogenic survival of the Ku80−/−puro+ cells.

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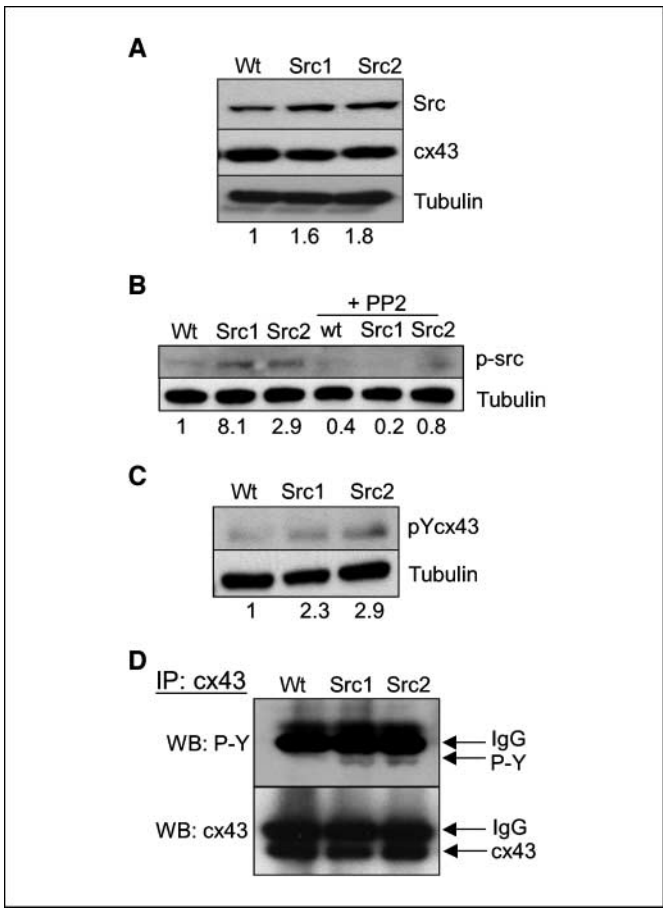

Figure 1. v-Src expression mediates connexin phosphorylation. Wt MEFs were transfected with a v-Src expression vector and analyzed for v-Src levels and cx43 phosphorylation. *A,* Western blot (*WB*) for src expression in wt MEFs in two subclones, Src1 and Src2. Band intensities for src expression were quantified and normalized to tubulin. The values given below the respective lanes indicate fold level of expression of src in relation to wt cells. *B,* Western blot for tyrosine phosphorylated src in the presence (+*PP2*) or absence of the src inhibitor PP2. *C,* Western blot for tyrosine phosphorylated cx43 using a cx43 phospho-specific antibody (*pYcx43*). *B* and *C,* values below each lane indicate levels of phospho-protein in relation to wt cells (normalized to tubulin). *D,* immunoblot analysis with antiphosphotyrosine antibody (*top*) and anti-cx43 antibody (*bottom*) of samples from the indicated cells first immunoprecipitated with anti-cx43 antibody. *IP,* immunoprecipitaion.

Assays for cell communication. The Lucifer yellow dye transfer assay was performed as previously described (5). Briefly, cell monolayers were overlayed with 0.05% Lucifer yellow in 2 mL of DMEM + 10% FBS, then two linear scrapes were made through the monolayer using a scalpel blade. Treated monolayers were incubated at 37°C for 5 min to allow for dye uptake, and then dye containing media was removed and monolayers were washed thoroughly with PBS and returned to normal media. Dye transfer from cells at the edge of the scrape to neighboring cells (as a measure of gap junction communication) was visualized using Zeiss Axiovert 200 fluorescent microscope. The flow cytometry assay was adapted from Czyz and colleagues (6). Briefly, donor cells were trypsinized, resuspended in 0.3 mol/L glucose, and preloaded for 30 min with 50 nmol/L calcein AM and 90 nmol/L DiI (Inivitrogen). Preloaded donor cells were washed thrice with PBS, and added to a monolayer of unstained recipient cells of the same type at a ratio of 1:25 (donor/recipient). Donor and recipient cells were cocultured for 3 h then collected by trypsinization, resuspended in PBS, and analyzed immediately on Becton Dickinson FACSCalibur. Data were analyzed by FlowJo software. In the indicated experiments, cells were pretreated with MG132 for 24 h.

Western blotting and immunoprecipitation. Cell lysates were collected and processed for Western blot as previously described (7). Immunoprecipitation was performed as previously described (8). Primary antibodies were as follows: anti–v-Src (Ab-1; Calbiochem), anti–p-Src (Tyr416; Cell Signaling), anti-phosphotyrosine (Cell Signaling), anti-cx43 (BD Transduction Laboratories), anti–p-cx43 (Tyr-265; Santa Cruz), and anti–γ-tubulin clone GTU-88 (Sigma). Gel images analyzed with NIH image software.

Results

v-Src expression alters connexin phosphorylation and function. To examine the role of activated src in cisplatin response, we transfected wt MEFs with v-Src cDNA. Stable clones were selected and tested for v-Src expression. Western blotting confirmed that two clones (Src1 and Src2) had increased expression of v-Src above baseline levels of c-Src detected in the parental wt cells (Fig. 1*A*). The antibody recognizes both c-Src and v-Src. Although the overall increase in src levels was found to be only 1.6- and 1.8-fold, levels of activated src (Fig. 1*B*) were substantially elevated in both src clones (8.1- and 2.9-fold). Treatment with the src kinase inhibitor PP2 decreased the levels of activated src in both Src1 and Src2 cells to levels below those in wt MEFs (Fig. 1*B*).

To examine the effect of activated src expression on GJIC, we tested for phosphorylation of cx43, which has two potential src phosphorylation targets at tyrosine 247 and 265. Using an antibody specific for tyrosine-phosphorylated cx43, we detected 2- to 3-fold higher phosphorylation of cx43 in the 2 subclones expressing v-Src (Fig. 1*C*). We also immunoprecipitated cx43 from wt, Src1, and Src2 cells using anti-cx43 antibody and performed immunoblot analysis of the samples using phospho-tyrosine (Fig. 1*D, top*) or cx43 antibodies (Fig. 1*D, bottom*). The novel bands detected by the anti–phospho-tyrosine antibody in cx43 immunoprecipitation samples from Src1 and Src2 cells provide further evidence of increased cx43 phosphorylation in the presence of v-Src.

Impact of v-Src on GJIC. Lucifer yellow is a fluorescent dye that is able to pass from cell to cell through gap junction channels. By creating a scrape in a cell monolayer, the Lucifer yellow dye can enter cells along the scrape line. If GJIC is present, the dye will spread through cells outward, away from the scrape line. Visualization of GJIC using Lucifer yellow dye transfer in cell monolayers showed a decrease in GJIC in v-Src–expressing clones compared with wt cells because in wt cells, there is a greater amount of dye transfer away from the scrape line (Fig. 2*A* shows data for Src1 compared with wt). To confirm and quantify changes in GJIC caused by v-Src expression, we used a flow cytometry–based assay to assess transfer of calcein dye from cells preloaded with calcein to a population of unloaded cells. As a control, DiI, a fluorescent dye that cannot pass through gap junctions, was also preloaded into the initial cells along with calcein. The preloaded cells were then washed and mixed with unloaded cells for 3 hours, followed by fluorescence-activated cell sorting (FACS) analysis of calcein and DiI content in the mixed population. In the FACS plot (Fig. 2*B, bottom*), cells that are positive for both dyes (circled cell population) represent preloaded cells, and cells that are positive for only calcein are those that received the calcein dye through GJIC. As a control, unstained cells were analyzed by FACS (Fig. 2*B, top*). The apparently higher calcein staining in Src1 cells is due to the decreased GJIC; less neighboring cells take up the dye so it is more highly concentrated in the few cells it enters. In the wt population, more cells take up the dye so it is diluted as it moves through the

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cell monolayer. The expression of v-Src in preloaded cells led to a 40% decrease in calcein dye transfer, consistent with decreased GJIC (Fig. 2*C*).

v-Src expression abrogates the cell density dependence of the survival response to cisplatin. As expected, we found that wt MEFs show decreased cisplatin survival at increasing cell densities (Fig. 2*D*), demonstrating the ability of high-density cells to engage in cytotoxic cell-to-cell signaling after cisplatin damage (3). The addition of a GJIC inhibitor, lindane, abrogated the density dependence of wt cells, illustrating the role of GJIC in the density effect. With expression of v-Src in MEFs (Src1 and Src2 cells), the density dependence of cisplatin response was also eliminated, correlating with the effect of v-Src on GJIC in these cells (Fig. 2*A–C*). Interestingly, the baseline resistance of Src1 and Src2 cells at low density (where there is minimal cell-to-cell communication) was seen to be elevated compared with that of the parental wt MEFs at low density (Fig. 2*D*). This increased survival represents cell autonomous resistance and can be attributed to the effect of activated src on regulatory pathways acting within individual cells to influence cell growth and damage responses.

v-Src increases cisplatin survival of neighboring cells not expressing the oncoprotein. To further dissect the *trans*-acting effect of v-Src expression on neighboring cells in high-density populations, we took advantage of a previously established cell mixing assay. We had found that although Ku80−/− cells cannot initiate or send the cell death signal to neighboring cells after treatment with cisplatin, they are able to receive it (3). This finding allowed us to create a system where we can use Ku80−/− cells as a recipient cell population to test the ability of selected MEFs and other defined cell types to send a cytotoxic signal to neighboring cells after cisplatin damage. To specifically measure survival of Ku80−/− cells in mixed populations, a line of Ku80−/− cells was made puro resistant by gene transfer, creating Ku80−/−puro+ receiver cells.

To test the influence of activated src on cell-to-cell signaling in mixed populations, Ku80−/−, wt, Src1, or Src2 cells as "sender cells" were mixed at high or low density with Ku80−/−puro+ receiver cells, and the mixed populations were treated with cisplatin. After cisplatin treatment, puro was added to kill all the sender cells and allow for growth of the receiver cell population only. Visualization of monolayer growth of Ku80−/−puro+ receiver cells showed that in the high density cultures, the presence of wt sender cells caused greater cisplatin-mediated killing of Ku80−/−puro+ receiver cells compared with the result in the mixture of Ku80−/− sender cells and Ku80−/−puro+ receiver cells (Fig. 3*A*). In comparison, the expression of v-Src in wt sender cells (Src1 cells) reduced the cisplatin killing of (and so conferred better survival on) the neighboring Ku80−/−puro+ receiver cells (Fig. 3*A*). Control experiments established that all sender cells were killed by puro selection and that the visualized monolayer represents only the growth of Ku80−/−puro+ receiver cells (data not shown).

To confirm that the cells need to be in close contact for v-Src expression in the sender cell population to have an effect on the survival of Ku80−/−puro+ receiver cells, we repeated the monolayer growth assay with mixed populations of cells seeded at low density (<500 cell/cm^2) at the time of cisplatin treatment. At this low density, cells are so spread out on the dish that there is little or no direct contact between neighboring cells and thus gap

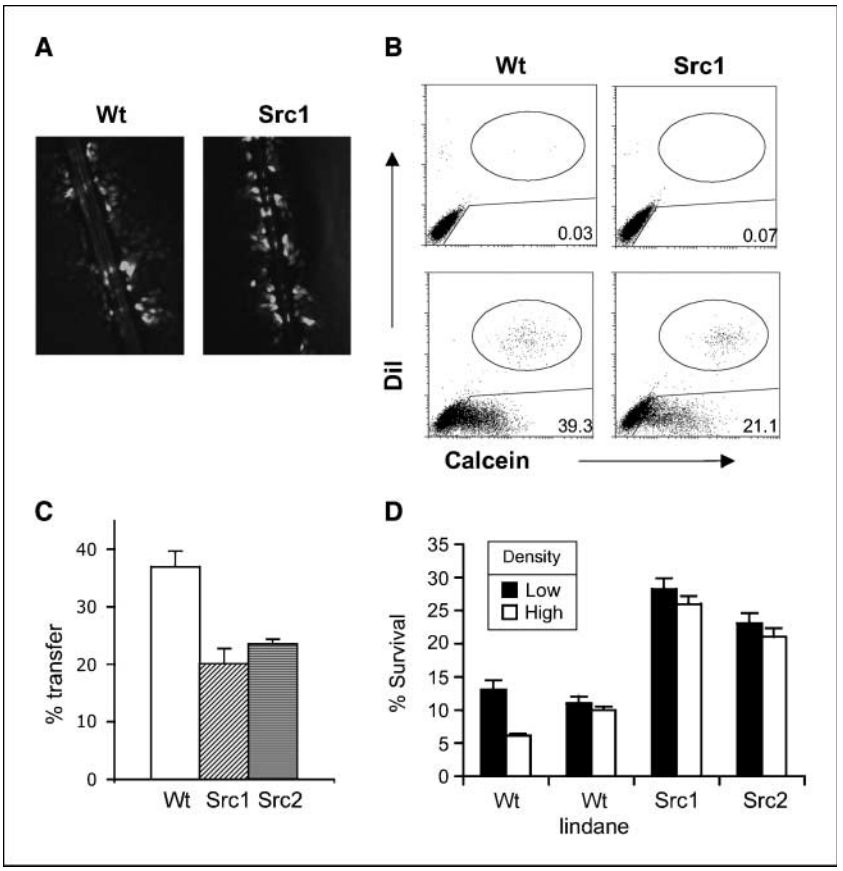

Figure 2. Decreased GJIC and increased survival in v-Src–expressing cells. *A,* visualization of Lucifer yellow dye transfer after scrape loading in wt and Src1 cells. *B,* FACS assay for GJIC. Cells were preloaded with two fluorescent dyes: calcein, which passes through gap junctions, and DiI, which cannot. The preloaded cells were then overlayed on a monolayer of unstained cells and incubated to allow time for calcein dye transfer via gap junctions. After 3 h, the mixed population was analyzed for calcein and DiI content by FACS. Cells containing both calcein and DiI represent the preloaded population (gated population in top right quadrant, *circled*). Cells containing only calcein (*bottom right quadrant*) were deemed to have received the dye by GJIC. *Top,* wt and Src1 cells alone. *Bottom,* wt and Src1 cells mixed with cells preloaded with calcein and DiI. *C,* quantification of the percentage of unloaded cells that acquired calcein, indicative of gap junction transmission. Percentages of calcein-positive cells were normalized to the percent of DiI and calcein double-positive cells that were preloaded with dye. *D,* clonogenic survival measured by colony formation of wt and v-src–expressing cells in response to cisplatin. wt, Src1, or Src2 cells were treated at either high (30,000 cell/cm^2) or low (500 cells/cm^2) density, as indicated, with 2.5 μg/mL of cisplatin, and clonogenic survival was quantified. One set of wt cells was also treated with lindane at 50mg/mL as indicated. For clonogenic survival of cells treated at high density, cells were treated for 1 h at high density, trypsinized, and then serially diluted for colony formation analysis.

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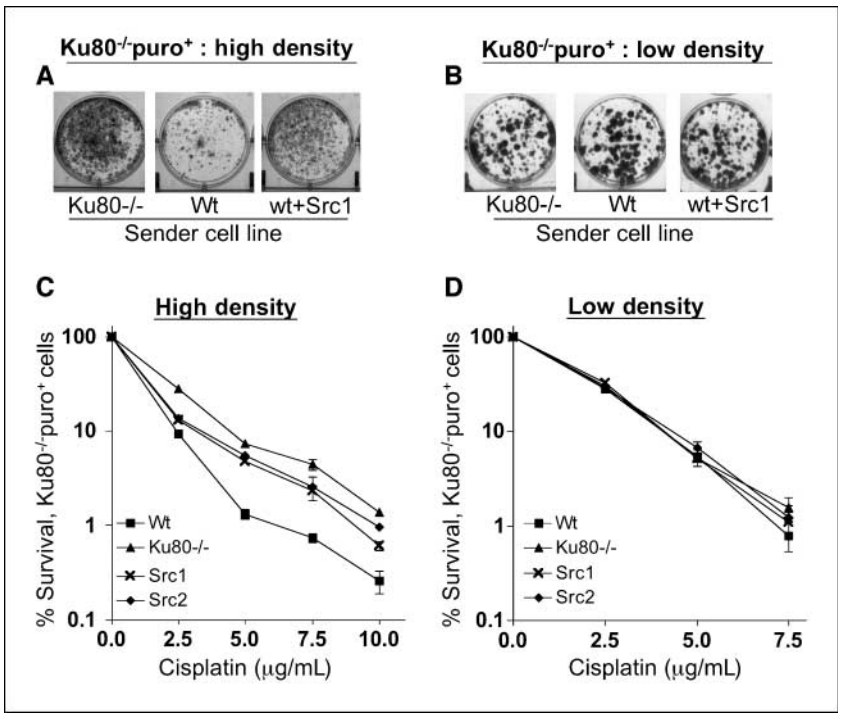

Figure 3. v-Src expression in one cell can influence survival of a neighboring cell not expressing the oncoprotein. *A* and *B,* the indicated sender cell line was mixed in a 1:1 ratio with puro-resistant Ku80−/−puro+ receiver cells. The mixed populations of cells were treated with cisplatin for 1 h at (*A*) high density or (*B*) low density. After cisplatin exposure, the medium was replaced with fresh medium containing puro to kill off the sender cells and allow only for growth of Ku80−/−puro+ receiver cells. Seven days after cisplatin treatment, cells were stained with crystal violet and monolayer growth was visualized. *C* and *D,* colony formation by Ku80−/−puro+ receiver cells was assayed in mixed population of cells containing Ku80−/−puro+ receiver cells and indicated sender cells: Ku80−/− MEFs (not puro resistant), wt MEFs, MEF subclones expressing v-Src (Src1 or Src2). The cisplatin treatments were performed with the indicated concentrations of drug at high (*C*) and low (*D*) overall cell density. After cisplatin treatment, cell cultures were detached and replated in the presence of puro to allow detection and quantification of colony formation specifically by the Ku80−/−puro+ receiver cells.

junction channels are not formed (3). We found that, at low density, there were no differences in survival of Ku80−/−puro+ receiver cells regardless of the nature of the comixed "sender" cell line (Fig. 3*B*), consistent with a lack of cell-to-cell signaling in these sparse cell populations.

To better quantify the above observations, we treated mixed populations at high or low density and then assayed for colony formation of Ku80−/−puro+ receiver cells after serial dilution. When cells were treated with cisplatin in high-density cultures, expression of v-Src in the sender cells (Src1 and Src2) resulted in increased survival of Ku80−/−puro+ receiver cells over a range of doses compared with when wt cells were mixed with receiver cells (Fig. 3*C*). In contrast, at low density, there was no effect of Src1 or Src2 cells on the survival of Ku80−/−puro+ receiver cells (Fig. 3*D*). These data show quantitatively that v-Src expression in wt cells can increase survival of neighboring cells to cisplatin even if the neighboring cells do not express the oncoprotein.

The effect of activated src on survival of neighboring cells is reversed by forced overexpression of cx43 or by src kinase inhibition or RNAi-mediated knockdown. To determine if forced overexpression of cx43 could overcome the effect of v-Src on GJIC and on cell-to-cell cisplatin killing, we used two methods to up-regulate cx43 in cells. First, a vector expressing human cx43 cDNA was transfected into Src1 cells, and a clone stably overexpressing cx43 was selected for further study (Src1+cx cells; Fig. 4*A*). Second, it has been reported that blocking cx43 degradation via proteasome inhibition can increase cx43 levels and gap junction formation (9). In our hands, we found that treatment of the Src1 cells for 24 hours with the proteasome inhibitor MG132 led to elevated cx43 expression (Fig. 4*A*), and we determined that cx43 levels remained high for at least 6 hours after MG132 removal (data not shown). The increased levels of cx43 protein expression conferred by either stable cx43 expression or by proteasome inhibition correlated with detectable increases in GJIC, as measured by FACS (Fig. 4*B*). Using the monolayer growth assay with Ku80−/−puro+ receiver cells in mixed populations, we found that there was increased killing of the receiver cells when they were mixed with Src1+cx sender cells or with Src1 sender cells pretreated with MG132 compared with unmodified or untreated Src1 cells (Fig. 4*C*). It is important to note that in the Src1+MG132 sample, only Src1 cells were treated with MG132; Ku80−/−puro+ receiver cells were not exposed to MG132 and were mixed with Src1 cells only after MG132 had been removed. This protocol was designed to ensure that survival of the receiver cells was not affected by direct treatment with MG132 and that the effect observed could be attributed specifically to the influence of MG132 on Src1 cells. Hence, the results show that both stable overexpression of cx43 and transient overexpression of cx43, by pharmacologic manipulation, are able to overcome the effects of v-Src on GJIC and thereby influence cisplatin response in neighboring cells.

To further link src kinase activity in Src1 cells with the altered cisplatin survival of comixed Ku80−/−puro+ receiver cells, we compared cisplatin survival of Ku80−/−puro+ receiver cells in high-density mixed populations of Src1 and Ku80−/−puro+ with or without pretreatment with the src kinase inhibitor PP2 or with siRNA knockdown of v-Src in the Src1 cells. Western blot analysis for activated src revealed that PP2 treatment reduced src kinase activity to below wt levels (Fig. 1*B*). Survival of Ku80−/−puro+ receiver cells in this experiment was quantified by colony formation (Fig. 4*D*), revealing that PP2 completely blocks the *trans*-acting prosurvival effect of v-Src on Ku80−/−puro+ receiver cells. Note that PP2 has no effect on the survival of Ku80−/−puro+ receiver cells when they are mixed with wt cells that do not contain activated src, suggesting that cancers containing cells with activated src would be sensitized to cisplatin by src kinase inhibition but healthy tissue would be unaffected.

To confirm and extend the results with the src kinase inhibitor, siRNA oligonucleotides specific for v-Src were used to knockdown v-Src expression in Src1 cells. Knockdown of v-Src led to a significant decrease of activated src (indicated by the decrease in

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phosphorylated src by Western blot analysis) in Src1 cells (Fig. 4*E, right*) and resulted in decreased survival of comixed Ku80−/− puro+ receiver cells to cisplatin treatment in the mixed cell population [compare survival of Ku80−/−puro+ receiver cells mixed with v-Src siRNA–treated cells versus cells treated with GAPDH siRNA (Fig. 4*E, left*)]. We chose to compare v-Src siRNA knockdown in Src1 cells to GAPDH siRNA knockdown to account for nonspecific effects of siRNA transfection. These results further support our model in which v-Src kinase mediates connexin phosphorylation and reduces GJIC, thereby attenuating transmission of the cisplatin-stimulated cell-to-cell death signal.

Discussion

We have shown here that expression of v-Src in one cell population can alter the survival of neighboring cells (even those not expressing it) to the chemotherapeutic drug, cisplatin. Stable expression of v-Src in two independent clones produced increased tyrosine phosphorylation of cx43, yielded decreased GJIC, and abrogated the density dependence of cisplatin killing in cell populations. In mixed populations in which non–v-Src expressing cells were tagged by puro resistance for detection, we found that v-Src expression in one set of cells can block cell-to-cell transmission to neighboring cells of a cytotoxic signal triggered by cisplatin exposure. Inhibition of src kinase with PP2 or by siRNA knockdown of v-Src was found to restore transmission of the cell death signal and to increase the cisplatin killing of neighboring cells. In addition, forced overexpression of cx43 by either stable transfection or by pretreatment of v-Src–expressing cells with the proteasome inhibitor MG132 was also found to restore transmission of the cisplatin-induced death signal to neighboring cells. It should be noted that our work does not imply that there are no cell autonomous effects of src that can cause resistance to cytotoxic agents. Indeed, we observed this ourselves in Fig. 2*D*, where v-Src was seen to elevate the cisplatin survival of cells treated at low density compared with low density survival of wt cells. At the low density tested in these experiments, there is minimal cell-to-cell contact, and so, cell survival is determined on a cell autonomous basis.

However, by comparing cisplatin responses at high versus low cell density and in mixed cell populations, our work reveals the novel finding that src expression in one cell can elevate survival of neighboring cells to cisplatin by disrupting cell-to-cell transmission of a death signal. This linkage of src expression to cell-to-cell communication is most clearly shown in the cell mixing experiments in which the Ku80−/−puro+ receiver cells showed better cisplatin survival when mixed with the Src1 or Src2 cells than when mixed with wt MEFs. This increased survival in the non–src-expressing Ku80−/−puro+ receiver cells was only observed when the cell populations were treated at high density,

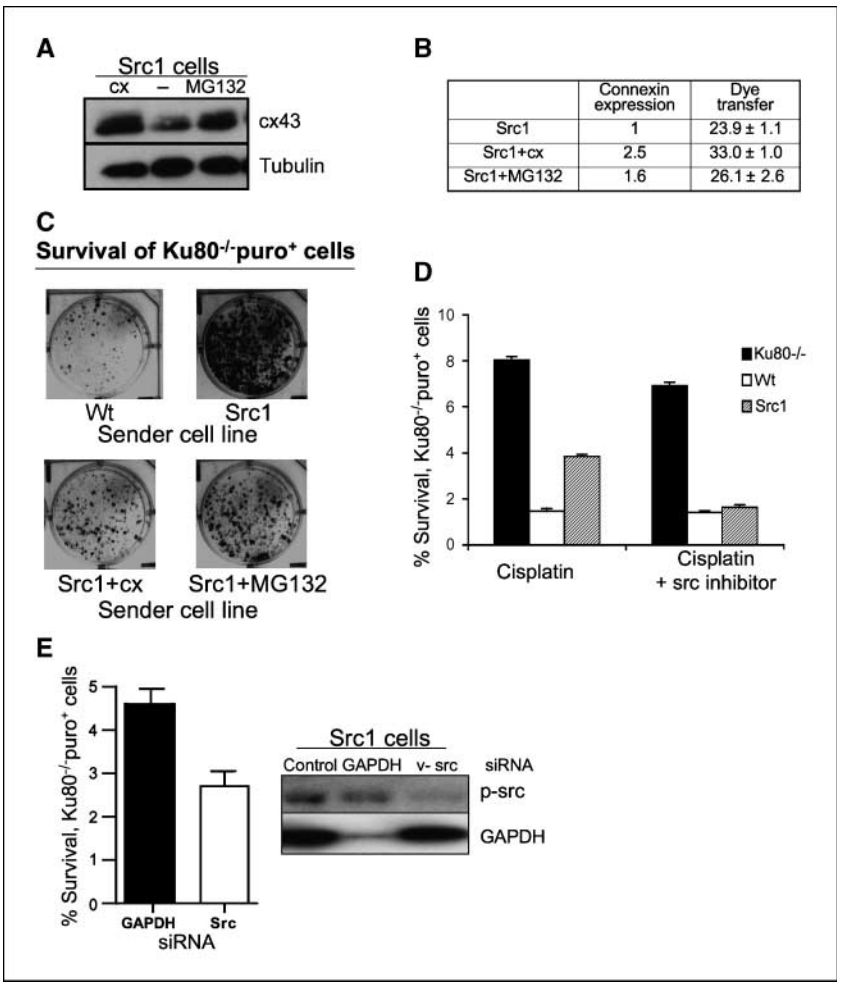

Figure 4. Inhibition of v-Src kinase activity or overexpression of connexin overcomes the increased survival conferred on neighboring cells by v-Src. *A,* Western blot analysis of cx43 expression in Src1 cells, Src1 cells transfected with a vector expressing human cx43 cDNA, or Src1 cells treated with the proteasome inhibitor MG132 (100 nmol/L for 24 h). *B,* table of relative cx43 expression levels and extent of dye transfer in the indicated Src1 cell lines. The level of cx43 expression in each sample is relative to untreated Src1 cells (normalized to tubulin), calculated from the Western blot in *A*. FACS assay for dye transfer was performed as described in Materials and Methods and quantified as in Fig. 2*C*. *C,* visualization of monolayer growth (in the presence of puro) by Ku80−/−puro+ receiver cells comixed with the indicated MEF-derived cell lines: wt MEFs, MEFs expressing v-Src (Src1), Src1 cells overexpressing cx43 by gene transfer (*Src1+cx*), and Src1 cells pretreated with the proteasome inhibitor MG132. Puro selection restricted growth only to the Ku80−/−puro+ receiver cells. *D,* clonogenic survival of Ku80−/−puro+ receiver cells comixed with the indicated sender cell line and treated with cisplatin in the presence or absence of the Src tyrosine kinase inhibitor PP2, as indicated. Mixed cell populations were seeded at high density, treated with PP2, then exposed to cisplatin. After treatment, cisplatin and PP2 were removed, cells were washed with PBS, trypsinized, and reseeded at low density in the presence of puro to specifically detect growth of Ku80−/−puro+ receiver cell colonies. *E,* siRNA-mediated knockdown of v-Src. Src1 cells were treated with siRNA oligonucleotides targeted against either v-Src or GAPDH, as indicated. Twenty-four hours later, Src1 cells were reseeded in a mixed population with Ku80−/−puro+ receiver cells, and the mixed population of cells was treated with cisplatin the following day. Clonogenic survival of the Ku80−/−puro+ receiver cells as measured by colony formation is graphed at left. *Right,* the Western blot shows the levels of phosphorylated src (*p-src*) and of GAPDH in the Src1 cells 48 h after transfection with the indicated siRNAs (the time at which the cisplatin exposure was done in the left).

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where gap junctions can be formed, and not at low density, indicating that cell-to-cell contact and intercellular communication are required.

Clinically, overexpression and activation of src is seen in many cancer types that are currently treated with cisplatin, including head and neck squamous cell carcinomas, non–small cell lung cancers, and ovarian carcinomas (10–12). In this regard, our finding that the src kinase inhibitor PP2 can reverse the effect of activated src in one cell and thereby increase the cytotoxicity of cisplatin on neighboring cells suggests a novel application for src kinase inhibitors in combination with cisplatin, i.e., that src inhibitors have the potential to sensitize tumor cells expressing activated src as well as to sensitize tumor cells without activated src that are in direct contact with src-activated cells to cisplatin. One src inhibitor, Dasatinib (sprycel), is already in clinical use to treat chronic myelogenous leukemia, and our work indicates that it could have a new therapeutic application in a strategy to enhance the effectiveness of cisplatin in many solid tumors.

The regulation of connexins in cancer is also an area of active interest because it has been shown that increased connexin expression and consequent elevated GJIC in certain cancer cell lines can reverse aspects of the malignant phenotype (13, 14). Here, we have shown that the level of the gap junction protein cx43 modulates cell-to-cell cisplatin killing and that elevated expression of cx43 by gene transfer or by up-regulation via proteasome inhibition can overcome the effects of v-Src. In this regard, because proteasome inhibitors such as bortezomib are available for clinical use, our observation that MG132 can sufficiently up-regulate cx43 expression to sensitize cell populations to cisplatin may also provide the basis for a new therapeutic strategy to combine proteasome inhibitors with cisplatin treatment.

Finally, the ability of src, as well as of other oncoproteins, to promote cell growth and survival in the face of cytotoxic agents is well-established (15–17). However, no prior studies considered or even suspected that src might act in trans to alter the response of non–src-expressing neighboring cells. In our prior work, in which we surveyed a series of cytotoxic agents for their ability to induce cell death via gap junction transmission, we found that only cisplatin triggers this pathway (3). Hence, it is likely that the effect of activated src identified here is specific for cisplatin exposure. Nonetheless, in addition to src, there is evidence that signaling by Ha-Ras (18, 19) and by the epidermal growth factor receptor (20) may also modulate GJIC, and so it is possible that these factors may therefore also influence the survival of cells to cisplatin without having to be expressed/activated in all cells in a tumor population. If so, pharmacologic targeting of these factors may provide yet another means to sensitize malignant cell populations to cisplatin.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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