Normal Cells Control the Growth of Neighboring Transformed Cells Independent of Gap Junctional Communication and Src Activity

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ABSTRACT

The growth of many types of cancer cells can be controlled by surrounding normal cells. However, mechanisms underlying this phenomenon have not been defined. We used a layered culture system to investigate how nontransformed cells suppress the growth of neighboring transformed cells. Direct physical contact between transformed and nontransformed cells was required for growth suppression of transformed cells in this system; communication by diffusible factors was not sufficient. However, significant gap junctional communication was not required, indicating that other intercellular junctions mediated this growth regulatory response. We also report that the Src kinase activity in transformed cells was not directly inhibited by contact with nontransformed cells. Instead, nontransformed cells increased the expression of serum deprivation-response protein and the transcription factor four and a half LIM domain 1 in tumor cells. In addition, these results suggest mechanisms by which normal cells may block Wnt signaling, inhibit insulin-like growth factor activity, and promote host recognition of neighboring tumor cells.

INTRODUCTION

In 1966, Stoker et al. (1) reported that normal cells can inhibit the growth of neighboring polyoma-transformed cells. Subsequent work has shown this to be a general phenomenon. Cells transformed by a variety of agents can be normalized by contact with normal cells (2, 3). However, mechanisms to explain this phenomenon, called “heterologous growth control,” are not understood.

In the same year Stoker et al. first described heterologous growth control, Loewenstein and Kanno (4) reported that cell transformation can result in decreased gap junctional communication. Gap junctions form aqueous channels that enable cells to communicate with each other by directly sharing intracellular signals. Gap junctions are formed by integral membrane proteins called connexins, which have evolved into a family of at least 20 vertebrate members, commonly named by their predicted molecular weights (5).

A role for gap junctions in cell growth control has been supported by the identification of connexins as tumor suppressor genes. For example, expression of Cx43 and Cx26 is robust in normal glial and mammary tumor cells. Moreover, restoration of Cx26 or Cx43 expression can normalize the growth of human glioma and mammary carcinoma cells (6–8).

Many reports suggest that gap junctional communication is required for normal cells to control the growth of neighboring tumor cells (see Ref. 9 for review). In general, levels of gap junctional communication between nontransformed and transformed cells have been correlated with their ability to exhibit heterologous growth control in coculture (10). In addition, inhibiting gap junctional communication with retinoic acid (11) or connexin antisense expression vectors (12) curtailed the ability of normal cells to inhibit neighboring transformed cell growth.

However, investigations into the role of connexins in heterologous growth control have not been definitive. In particular, to date, heterologous growth control has not been examined in cells that do not exhibit a significant degree of gap junctional communication. In addition, molecular pathways that underlie growth suppression of tumor cells by neighboring nontransformed cells have not been rigorously examined.

We have used homozygous null connexin knockout (KO) mice to address the need for gap junctional communication for nontransformed cells to control the growth of neighboring cells transformed by the Src kinase. We report here that although direct contact was required for nontransformed cells to normalize the growth of neighboring tumor cells, gap junctional communication was not. Also, Src kinase activity in transformed cells was not affected during heterologous growth control, indicating that tumor cells were normalized in the face of transforming kinase activity. We also found that about 0.2% of the transcriptome of Src-transformed cells was effectively modified by contact with nontransformed cells. In general, the expression of a limited set of genes involved in growth arrest and inhibition of growth factor signaling in transformed cells was induced by contact with nontransformed cells.

MATERIALS AND METHODS

Isolation, Transfection, and Maintenance of Cells. Stage E18 fetuses were taken from Cx43 KO mice (13) by caesarean section as described previously (14). After removal of meninges, spinal column, and olfactory bulbs, forebrain, hindbrain, and midbrain were chopped, triturated, and passed through an 80-µm Nitex mesh (15). After 20 passages, single cell clones were obtained by dilution in medium supplemented with 10% colostrum (16). Twenty-four colonies derived from single cells were taken with cotton swabs for further expansion. Only two of these survived crisis and are called KoA and KoB. For all analyses, cells were maintained in MEM supplemented with 10% fetal bovine serum at 37°C in 100% humidity. KoA and KoB cells were both transformed with the v-Src kinase as previously described for other cell lines (17).

Evaluation of Cell Growth. To measure anchored and nonanchored growth, 20,000 cells were plated on each well of 12-well cluster tissue culture dishes that were uncoated or coated with poly(2-hydroxyethyl methacrylate).

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respective, and counted with a Coulter counter at appropriate time points as described previously (17, 20, 21).

To analyze cells in standard coculture, 50,000 Src-transformed and 600,000 nontransformed cells were plated alone or mixed together in each well of 6-well cluster tissue culture plates. After allowing time for foci to appear (about 9 days), nontransformed cells in some wells were killed by exposure to puromycin (an additional 3–4 days; Ref. 12). Cells were finally stained with Giemsa. Extended growth periods were also performed to demonstrate the long-term efficacy of heterologous growth control in coculture.

A layered culture system was developed using techniques similar to those described previously (22). Nontransformed cells (600,000) were plated onto inverted inserts containing porous membranes suitable for cell culture (polyester membranes 3542; Costar) or into each well of a six well cluster plate to act in control cultures. After adhering to the membranes, the inserts containing nontransformed cells were turned right side up and placed into wells of 6-well plates. Src-transformed cells (50,000) were added to each insert over nontransformed cells. Src-transformed cells (50,000) were also added to a separate set of inserts so that they adhered 1 mm above nontransformed cells in control cultures. Src-transformed cells (50,000) were also plated on inserts in wells without any nontransformed cells.

**Evaluation of Living Cells by Fluorescence Microscopy.** Src-transformed cells were visualized by techniques similar to those described previously (12). Src-transformed cells were labeled with 1,1'-dioctadecyl-3,3',3' ,tetramethylindocarbocyanine, 4-chlorobenzene sulfonate (DiD) [Di-307; Molecular Probes] for 15 min in isotonic glucose, washed thrice with PBS, and trypsinized. These fluorescently labeled transformed cells were then used for standard or layered culture system as described above. Prior to microscopic analysis, cell nuclei were labeled with Hoechst 33342 (H-1399; Molecular Probes). Cells were viewed on a Nikon Eclipse E800 microscope equipped with a 60 × 1.4 numerical aperture oil objective. Images were taken with a Princeton MicroMax-cooled charge-coupled device (CCD) at 15–18°C and WinView 32 software for epifluorescence [rhodamine-isothiocyanate excitation (ex.) 535/50 detection mode 500 barrier acquisition 675/75, fluorescein isothiocyanate ex. 460–500 DM 505 BA 510–560, 4',6-diamidino-2-phenylindole ex. 360–370 DM 400 BA 400].

**Fluorescence (FL)-Activated Cell Sorting.** Cell sorting was used to confirm that transformed cells and nontransformed cells did not cross the membrane and mix with each other during 48-h growth in the layered culture system. Src-transformed cells were labeled with DiD as described above. Before analysis, cells were labeled with Hoechst 33342 for 15 min, washed thrice with PBS, and trypsinized to create single cell suspensions. Cells were analyzed in a Becton Dickinson fluorescence-activated cell sorter (Vantage). Single cells were gated by forward scatter and side scatter. Src-transformed cells were detected by DiD fluorescence excited with a HeNe laser at 633 nm and quantitated in FL4 at 660 nm. DNA content was measured by detection of Hoechst stain, which was excited with a UV laser at 360 nm and quantitated in FL5 at 424 nm. Thus, events were collected on at least three criterion: forward scatter, side scatter, and FL parameters, with Cell Quest software version 3.2.

**Evaluation of Gap Junctional Communication.** Src-transformed cells were identified in coculture by staining with 1,1'-dioctadecyl-3,3',3',tetramethylindocarbocyanine perchlorate or DiD as described above, although nontransformed cells were stained with cell-tracker green (5-chloromethyl-fluorescein diacetate; Molecular Probes; Refs. 23 and 24). To examine the effects of 18α-carbenoxolone (ACO) on gap junctional communication, the chemical was added to cell culture medium at the time of plating (25). Intercellular dye transfer was evaluated by microinjection, preloading and scrape loading with Lucifer Yellow, calcein, and carboxyfluorescein, respectively, and quantitated in FL5 at 424 nm. Thus, events were collected on at least three criterion: Hoechst stain, which was excited with a UV laser at 360 nm and quantitated in FL4 at 660 nm. DNA content was measured by detection of Hoechst stain, which was excited with a UV laser at 360 nm and quantitated in FL5 at 424 nm. Thus, events were collected on at least three criterion: forward scatter, side scatter, and FL parameters, with Cell Quest software version 3.2.

**Western Blot Analysis and Immunofluorescence Microscopy.** The membrane in the layered culture system allows transformed and nontransformed cells to be instantly separated from each other for comparative biochemical analysis (22). Western blot analysis was performed on cells grown in the layered culture system 26 h after plating. Cells were quickly aspirated, washed with PBS, scraped from the membranes with rubber policeman, transferred to microcentrifuge tubes, pelleted by a brief spin (1 min at 8000 × g), aspirated, lysed in SDS-PAGE sample buffer [2% SDS, 10% glycerol, 50 mM DTT, and 62.5 mM tris-HCl (pH 6.8)], sheared with five passages through a 26-gauge needle, boiled 5 min, and cooled on ice. Bromphenol blue was added to 0.01% before protein (2 μl/g) was resolved on 10% gels and transferred to Immobilon-P membranes (Millipore). For immunofluorescence microscopy, 50,000 Src-transformed cells (KoA src) were plated alone, or with 600,000 nontransformed cells (KoA), on cover slips in 35-mm tissue culture dishes, grown overnight, and fixed in methanol.

Antiseria were used to detect total pp60v-Src kinase (antivanilin clone E10 monoclonal, 05-185; Upstate Biotechnology), active pp60 kinase (rabbit anti-serum specific for Src phosphorylated at Tyr416, 2101; Cell Signaling Technologies), total mitogen-activated protein kinase (MAPK; rabbit antiserum against the p44 and p42 MAPK proteins, 9102; Cell Signaling Technologies), active MAPK (E10 monoclonal antibody specific for p42 and p44 MAPK protein phosphorylated at Thr286 and Tyr287, 9106; Cell Signaling Technologies), other proteins phosphorylated on tyrosine residues (P-Tyr100 monoclonal, 9411; Cell Signaling Technologies), and β-actin (monoclonal antibody AC15, A5411; Sigma). The utilization of these reagents to study Src kinase signaling has been well established (29–33). Immunofluorescence was detected with goat antimouse IgG conjugated to Alexa Fluor 568 or goat antirabbit Alexa Fluor 488 (Molecular Probes). Cells were viewed on an Olympus Fluoview FV300 confocal microscope (IX70) with a 60× objective and Fluoview software (version 3.1 with TIEMPO). Argon (ex. 488 nm) and helium neon (ex. 564 nm) lasers were used to detect Alexa Fluor 488 (emission 495/519 nm) and Alexa Fluor 568 (emission 556/575 nm), respectively. Western blot reactions were detected by chemiluminescence with Amersham enhanced chemiluminescence plus.

**Evaluation of Gene Expression by reverse transcription (RT)-PCR.** Connexin mRNA expression was analyzed by RT-PCR as described previously (34). Briefly, RNA was extracted from cells by the phenol-chloroform-isoamyl alcohol method, pretreated with DNase, and 200 ng was reverse transcribed in a thermal cycler (Perkin-Elmer, Norwalk, CT) using SuperScript II (18064-014; Invitrogen) and oligodeoxynucleotide primers according to the manufacturer’s instructions for 1 h at 42°C in a 20-μl reaction. Resulting cDNA (0.2 μl) was amplified with Taq polymerase and one set of oligonucleotide primers. Samples were denatured for 5 min at 95°C and then amplified for 30 cycles at 94°C for 45 s, 58°C for 1 min, and 72°C for 1 min. Aliquots (2 μl) from each PCR were then analyzed by agarose gel electrophoresis. Forward and reverse primer sequences were as follows: for Cx45 (5'-TTCAAGTTCACACCCATTGTATTAT, 5'-ATGTTCTCGAGGACTTCTGTA); Cx40 (5'-CTGTGGCCACCATGCAACT, 5'-CCGGTTGCATCATTG-TAGC); Cx30 (5'-AATGTTGGCGGATTGTTGTA, 5'-CCAAAGGGCCGTG-TGTCAC); and Cx43 (5'-CCCCACACTCCTACCTGTTCCT, 5'-ACTTGTGGC-GCGCTGATCTTCA). Superscript II was omitted from some cDNA preparations to rule out false-positive reactions. DNA from brain tissue (cortex and cerebellum) isolated from an adult CD-1 mouse was used as a positive control.

To analyze expression of Sdpr, Vcam1, Jgbfp2, β-actin, mRNA was purified from cells as described below for microarray analysis. cDNA was produced from mRNA using oligo(dt)12–18 primers and Superscript II in a Bio-Rad iycler. Subsequent PCR using ExTaq polymerase (RR0101; Takara) was carried out using the following forward and reverse primer sequences: for β-actin (5'-TGTTTCACTGGAGGAGGACA, 5'-AAGGAAAGGCTGGAA-AAGAGG); Sdpr (5'-ACCAGGCTCTGTGTTCTCTA, 5'-TGTTCTAGTT- GTGCTGTGT); Vcam1 (5'-CTGTTACTCTGGCTCTGTC, 5'-CTTATTG- CAGTTGCTGCG); and Jgbfp2 (5'-CAGACGAGTGGTAAACAGGTTA, 5'-GTCTTCAACGACCCACT). PCR reactions were stopped while the gene products from the Src-transformed cells grown in contact with normal cells were in the log linear phase of amplification. Aliquots from each PCR sample were then analyzed by agarose gel electrophoresis.
Microarray Analysis. For gene expression analysis, we used GeneChip Murine Genome U74Av2, U74Bv2, and U74Cv2 oligonucleotide microarrays (Affymetrix, Santa Clara, CA), which contained 36,701 probe sets combined (excluding controls). Target cRNA for microarray hybridization was prepared as follows. Total RNA was isolated using RNeasy columns (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. From 2 µg of the total RNA, first strand cDNA was synthesized by reverse transcription with 100 pmol of T7-(dT)24 primer [5′-GGCCAGTTAATCAGACTCAG- TATAGGGAGGCGG-(T)24-3′] and 200 units of Superscript II at 42°C for 1 h in 20 µl of 1× first strand buffer [50 mM Tris/HCl (pH 8.3), 75 mM KCl, and 3 mM MgCl2] containing 10 mM DTT and 500 µM each deoxynucleoside triphosphate. Then, the second strand was synthesized with 40 units of Escherichia coli DNA polymerase I (Invitrogen), 10 units of E. coli DNA ligase (Invitrogen), and 2 units of RNase H (Invitrogen) at 16°C for 2 h in 150 µl of 1× second strand buffer [20 mM Tris-HCl (pH 6.9), 90 mM KCl, 4.6 mM MgCl2, 0.15 mM β-NAD+, and 10 mM (NH4)2SO4] containing 200 µM each deoxynucleoside triphosphate, followed by incubation at 16°C for 5 min with 10 units of T4 DNA polymerase (Invitrogen). The resulting double strand cDNA was purified by phenol-chloroform extraction and ethanol precipitation. Biotin-labeled cRNA was synthesized from the double strand cDNA by in vitro transcription using a BioArray RNA transcript-labeling kit (Enzo Diagnostics, Farmingdale, NY) and purified using RNeasy columns. Twenty µg of the biotin-labeled cRNA was fragmented at 94°C for 35 min in 40 µl of 1× RNA fragmentation buffer [40 mM Tris/acetate (pH 8.1), 100 mM NaCl, and 30 mM Mg acetate], and used for microarray hybridization.

Hybridization, washing, staining, and scanning were carried out according to the manufacturer’s instructions. Briefly, 10 µg of the biotin-labeled and fragmented cRNA was hybridized to the microarray in 200 µl of 1× 4-morpholinepropanesulfonic acid (MES) hybridization buffer [100 mM Na2HPO4/NaH2PO4 (pH 7.4), 894 mM NaCl, 6 mM EDTA, and 0.01% Tween 20] at 25°C and then with stringent wash buffer [100 mM MES/Na-MES (pH 6.6), 890 mM NaCl, 20 mM EDTA, 0.01% Tween 20] containing 0.1 mg/ml herring sperm DNA (Promega, Madison, WI) and 0.5 mg/ml acetylated BSA (Invitrogen) at 45°C for 1 h with rotation. Subsequently, the microarrays were washed with nonstringent wash buffer [60 mM Na2HPO4/NaH2PO4 (pH 7.4), 894 mM NaCl, 6 mM EDTA, and 0.01% Tween 20] at 25°C and then with stringent wash buffer [100 mM MES/Na-MES (pH 6.6), 26 mM NaCl, and 0.01% Tween 20] at 50°C, stained with streptavidin phycoerythrin (Molecular Probes) and biotinylated antistreptavidin (Vector Laboratories, Burlingame, CA) and scanned with a GeneArray scanner (Hewlett-Packard, Santa Clara, CA). The expression value (Signal), change value (signal log ratio), presence call (presence or absence), and change call (increase, no change, or decrease) for each probe set were calculated using Affymetrix Microarray Suite software (version 5.0 software). Fold change values were recalculated from signal log ratio values.

Gene expression was evaluated 26 h after plating cells. The expression profiles of genes in Src-transformed KoA cells grown on membranes were compared with genes expressed in nontransformed KoA cells grown on membranes to identify genes altered by Src transformation. Genes altered by Src were identified by the following criteria: (a) they were expressed (a presence call) in either transformed or nontransformed cells; (b) differences between signals in nontransformed and transformed cells resulted in a change call (increase or decrease) with a fold change of at least 2 in both experiments; (c) differences in signals in transformed cells grown alone; (f) there were no significant differences in signals from transformed cells grown alone, when compared with transformed cells grown over a membrane in direct contact with other transformed cells with the bottom layer acting as control for the effects of nontransformed cells.

RESULTS

Cx43 KO Cells Are Transformed by the v-Src Kinase. Two cell lines, called KoA and KoB, were obtained from the brains of homozygous null Cx43 KO mice. KoA cells appeared more trapezoidal in shape, compared with KoB cells which were more bipolar. Nonetheless, both cell lines assumed a normal cellular morphology and formed contact inhibited monolayers (Fig. 1).

Expression of the v-Src kinase caused these cells to display a typically transformed morphology. As shown in Fig. 1, the Src-transfected cells were more refractive and less adherent than the parental cells. More importantly, Src expression caused the cells to lose contact growth inhibition, allowing the transformed cells to form multilayered foci.

Loss of contact growth inhibition by Src-transformed cells was confirmed by parallel growth curve analysis of the cells. As shown in Fig. 2A, Src-transformed cells achieved a cell saturation density that was 2- to 3-fold higher than nontransformed parental cells. Nontransformed cells reached a cell saturation density of about 3,500 to 3,800 cells/mm², whereas KoASrc and KoBSrc cells reached densities of about 7,000 cells/mm² and 10,000 cells/mm², respectively.

In addition to loss of contact growth inhibition, Src transformation resulted in a loss of anchorage dependence. As shown in Fig. 2B, nontransformed cells did not grow when not anchored to substrata. In contrast, the Src-transformed cells grew in suspension. Thus, Src-transformed cells assumed a refractive and disordered morphology, lost contact growth inhibition, formed multilayered foci, and exhibited anchorage-independent growth.

![Fig. 1. Morphology of nontransformed and Src-transformed cells. Phase-contrast images of KoA and KoB cells before and after Src transformation are shown as indicated. Src-transformed cells assumed a typically transformed morphology compared with nontransformed cells. Bar = 200 µm.](image-url)
The ability of Src-transformed cells to form foci was blocked by surrounding nontransformed cells. While nontransformed cells formed foci when grown by themselves (Fig. 1 and Fig. 3), Src-transformed cells did not form foci when cocultured with nontransformed cells. Therefore, intimate contact between nontransformed and transformed cells was required for foci inhibition.

In addition to standard two-dimensional culture techniques, the suppression of Src foci formation by nontransformed cells in culture was confirmed in a layered culture system shown in Fig. 3B. In this system, Src-transformed cells and nontransformed cells are separated by a porous membrane. However, the transformed and nontransformed cells are able to contact each other through pores in the membrane.

The Layered Culture System allowed us to separate the effects of direct physical contact between cells from the effects of communication between cells by diffusible factors. In contrast to the complete growth suppression of transformed cells cultured in direct contact with nontransformed cells (Fig. 3, A and B), Src-transformed cells separated from nontransformed cells by 1 mm were able to form foci (Fig. 3C). Because diffusible factors produced by the cells could flow freely between the two cell populations, growth factor signaling by nontransformed cells was not sufficient to stop transformed cells from forming foci. Therefore, intimate contact between nontransformed and transformed cells was required for foci inhibition.

Src-Transformed Cells Revert to a Normal Phenotype but Survive and Retain Their Transformed Genotype During Growth Suppression by Neighboring Nontransformed Cells. As seen in Fig. 3, Src-transformed cells reemerged to form foci from cocultures after nontransformed cells were selectively killed by puromycin (to which Src-transformed cells were made resistant). These data indicate that transformed cells survived growth inhibition by neighboring nontransformed cells. In addition, the data indicate that transformed cells retained their transformed genotype during heterologous growth suppression.

Transformed cells were fluorescently labeled to visualize their presence in coculture with nontransformed cells. As shown in Fig. 4, transformed cells assumed a normal morphology similar to their nontransformed neighbors in coculture. This phenomenon was observed in standard coculture as well as the layered culture system. These data confirm that transformed cells survive but revert to a normal morphology when cocultured with nontransformed cells.

Robust Gap Junctional Communication Is Not Required for Normal Cells to Control Transformed Cell Growth. Gap junctional communication has long held a suspected role in the ability of normal cells to control transformed cell growth (9, 11). Connexin KO cells were used in this investigation to test this hypothesis. If nontransformed cells could control the growth of neighboring transformed cells in the absence of gap junctional communication, then connexins may not be required.

The Cx43 KO cells used in this study proved to be substantially deficient in gap junctional communication. No transfer of fluorescent dyes, including Lucifer Yellow, calcein, or carboxyfluorescein, between any of the cells could be detected (data not shown). Electrophysiological measurements, summarized in Table 1, demonstrated that only 10 out of 24 (42%) heterologous cell pairs of transformed and nontransformed KoA cells were coupled to each other. The pairs that were coupled showed total conductances of only 170 ± 50 pico Siemens (mean ± SE, n = 10).

As shown in Fig. 5A, the predominant unitary conductance of channels between transformed and nontransformed cells was about 30 pS. Therefore, only about six active channels mediated gap junctional communication between coupled transformed and nontransformed cells at any given moment. Moreover, as mentioned above, less than half of transformed cells were coupled to nontransformed cells at all. Therefore, on average, transformed cells were coupled to nontransformed cells by only about 2.5 channels.

Of several connexins examined, only Cx45 was detected by RT-PCR (Fig. 5B). In addition, the channel properties exhibited by these cells, shown in Fig. 5A, were consistent with properties of gap junctions formed by Cx45 (23). Therefore, if gap junctional communication was necessary for the heterologous growth control observed in these studies, then only a few channels formed by Cx45 were sufficient.

We applied gap junction blockers to further examine whether gap junctional communication was needed for normal cells to control the growth of neighboring tumor cells. As shown in Table 1, ACO blocked gap junctional communication between these cells in a dose-dependent manner. ACO (60 μM) reduced coupling between these cells >2-fold, to an average of <1 channel per cell pair. ACO (100 μM) reduced communication even further, to only about 1 channel per 10 cell pairs. Thus, gap junctional communication between transformed and nontransformed cells was effectively blocked by 100 μM ACO.

Transformed cells were cocultured with nontransformed cells in ACO to determine whether gap junctional communication was required for nontransformed cells to normalize transformed cell growth. As shown in Fig. 6, nontransformed cells normalized the growth of neighboring transformed cells in the presence of 100 μM ACO. Thus, it appears that gap junctional communication was not required for heterologous growth control in this system.
The Src Kinase in Transformed Cells Is Not Affected by Contact with Nontransformed Cells. Because the cells used in this study were transformed by the Src kinase, we hypothesized that nontransformed cells may suppress Src kinase activity in neighboring transformed cells. The layered culture system enabled quantitative assays to be performed in each population of cells. This was done by separately collecting the transformed and nontransformed cell layers from the top and bottom of the Transwell membranes, respectively. As shown in Fig. 7, Src activity in transformed cells was not affected by communication with nontransformed cells. Moreover, the activity of MAPK, which lies downstream of Src, was also unaffected. Western blotting with antiserum against phosphotyrosine revealed at most a modest decrease in the overall extent of protein-tyrosine phosphorylation; this slight decrease is unlikely to account for the complete suppression of the transformed phenotype.

Unperturbed Src signaling in transformed cells subjected to growth suppression by neighboring nontransformed cells was confirmed by immunofluorescent microscopic analysis. As seen in Fig. 8, transformed cells grown alone displayed levels of total and active Src kinase that were similar to transformed cells grown in coculture.

Identification of Genes in Src-Transformed Cells that Are Modulated by Contact with Normal Cells. In addition to the kinase assays shown in Fig. 7, the layered culture system enabled gene expression profiles to be examined in each population of cells grown.
in coculture. Cells were examined 26 h after plating, giving them time to contact each other, but not cross the membrane, as demonstrated by fluorescent-activated cell sorting (data not shown) and previous studies (22). The efficacy of the membrane system for these methods is also demonstrated by the fact that v-Src protein was not detected in transformed and nontransformed cells, including those on the bottom side of the membrane (bottom layer) in the layered culture system. The presence of Src-transformed cells in coculture confirmed their survival during growth suppression by neighboring nontransformed cells.

We evaluated the expression profiles of approximately 30,000 genes (36,701 probe sets), thus analyzing a major portion of the transcriptomes of nontransformed cells, Src-transformed cells, Src-transformed cells exposed to direct contact with nontransformed cells, and Src-transformed cells exposed to factors produced by nontransformed cells. As an additional experimental control, we also examined Src-transformed cells exposed to direct contact with Src-transformed cells. In this way, we performed a global search for genes in transformed cells that were affected by contact with nontransformed cells.

Transformed and Nontransformed Cells Expressed Several Genes Encoding Cell Junctions. Transcripts representing about 9,000 genes (9,400 probe sets) were detected in the cells used for these studies. Because physical contact between nontransformed and transformed cells was required for heterologous growth control, we searched for the expression of cadherins, connexins, and integrins. The cells expressed cadherin1 (Cdh1), cadherin2 (Cdh2), cadherin13 (Cdh13), and protocadherin7 (Cdhp7). In addition, Src-transformed cells expressed low levels of cadherin 6 (Cdh6), which was not detected in nontransformed cells. Cells expressed integrins α3 (Itgα3), α6 (Itgα6), β1 (Itgβ1), β5 (Itgβ5), and β7 (Itgβ7). Transcripts encoding focal adhesion kinase, β-catenin, and integrin linked kinase were also detected.

Consistent with the gap junctional communication-deficient prop-

Table 1 Effects of ACO6 on gap junctional communication between Src transformed and nontransformed cells

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<td>24</td>
<td>42%</td>
<td>170 ± 50 pS</td>
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<td>10</td>
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<td>15</td>
<td>6.7%</td>
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* ACO, 18α-carbenoxolone.

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* ACO, 18α-carbenoxolone.

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in coculture. Cells were examined 26 h after plating, giving them time to contact each other, but not cross the membrane, as demonstrated by fluorescent-activated cell sorting (data not shown) and previous studies (22). The efficacy of the membrane system for these methods is also demonstrated by the fact that v-Src protein was not detected in nontransformed cells in the layered culture system. The cells expressed cadherin1 (Cdh1), cadherin2 (Cdh2), cadherin13 (Cdh13), and protocadherin7 (Cdhp7). In addition, Src-transformed cells expressed low levels of cadherin 6 (Cdh6), which was not detected in nontransformed cells. Cells expressed integrins α3 (Itgα3), α6 (Itgα6), β1 (Itgβ1), β5 (Itgβ5), and β7 (Itgβ7). Transcripts encoding focal adhesion kinase, β-catenin, and integrin linked kinase were also detected.

Consistent with the gap junctional communication-deficient prop-

Table 1 Effects of ACO6 on gap junctional communication between Src transformed and nontransformed cells

<table>
<thead>
<tr>
<th>ACO</th>
<th>Pairs examined</th>
<th>% coupled</th>
<th>Total conductance</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 μM</td>
<td>24</td>
<td>42%</td>
<td>170 ± 50 pS</td>
</tr>
<tr>
<td>60 μM</td>
<td>10</td>
<td>40%</td>
<td>60 ± 21 pS</td>
</tr>
<tr>
<td>100 μM</td>
<td>15</td>
<td>6.7%</td>
<td>30 or 60 pS</td>
</tr>
</tbody>
</table>

* ACO, 18α-carbenoxolone.

* SEM could not be calculated in 100 μM ACO because only 1 pair of 15 examined exhibited any coupling, with conductance between this pair fluctuating between 30 and 60 pS, representing 1 or 2 channels, respectively.
Fig. 7. Effects of nontransformed cells on Src and mitogen-activated protein kinase (MAPK) activity in neighboring transformed cells. Total and active forms of Src and MAPK, and proteins containing phosphotyrosine, were assayed by Western blot analysis as indicated. β-Actin was used to verify equal loading and transfer. The activities of Src and MAPK, and levels of proteins containing phosphotyrosine, in transformed cells were not affected during their growth suppression by neighboring nontransformed cells.

Fig. 8. Src expression in transformed cells grown alone or cocultured with nontransformed cells. Total and active forms of the Src kinase in transformed cells cultured alone or with nontransformed cells were visualized by immunofluorescent confocal microscopy as indicated. Src kinase activity in transformed cells was not affected by contact with nontransformed cells. DIC, differential interference contrast.
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genes modulated by Src transformation. As shown in Table 3, the expression of these 18 genes was increased by contact with nontransformed cells.

We evaluated gene expression 26 h after plating. Thus, the genes shown in Table 3 are primary targets of nontransformed cells because they suppress the growth of transformed cells. These encode many types of proteins including growth factors (Bmp4, Clu, Cxcl12, Edn1, Plf3), growth factor-binding proteins (Igfbp2, Sfrp1, Wif1), cadherins (Celsr2), integrin receptors (Vcam1), proteases (Prss11), metabolic enzymes (Ckb), membrane channels and receptors (P2ry4), cytoskeleton modulating proteins (Cadps, Synpo), transcription factors (Fhl1), and proteins with undetermined activities but related to cell growth (Celsr2, Vcam1, and proteins with undetermined activities but related to cell growth (Cadps)).

As mentioned above, three of the five genes that were most decreased as a result of Src transformation were subsequently up-regulated by contact with normal cells (Table 3). The expression of Sdpr, Wif1, and Vcam1 in transformed cells was increased by about 20-fold by contact with nontransformed cells, which may induce cell growth arrest (38), inhibit Wnt signaling (39), and mediate T-cell recognition (40), respectively. Other genes affected by contact with nontransformed cells also play roles related to these events. For example, along with Wif1, which binds to Wnt and blocks Wnt signaling, Sfrp1, which encodes a secreted frizzled related protein that also binds Wnt (41), was induced by about 4-fold.

In addition to blocking Wnt signaling, two genes increased by contact with nontransformed cells inhibit insulin-like growth factor (IGF) signaling. These include IGF-binding protein 2 (Igfbp2; Ref. 42), which was increased by about 12-fold, and a secreted protease that targets IGF (Prrx1; Ref. 43), which was increased over 2-fold.

In contrast to IGF and Wnt signaling, specific forms of transforming growth factor β signaling in transformed cells may be augmented by contact with normal cells. For example, transcripts from genes

Table 3 Genes in Src transformed cells increased or decreased by contact with nontransformed cells

<table>
<thead>
<tr>
<th>Symbol</th>
<th>gb</th>
<th>Name</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sdpr</td>
<td>A1839175</td>
<td>Serum deprivation response</td>
<td>Cell growth arrest</td>
</tr>
<tr>
<td>AV248465</td>
<td>46</td>
<td>Wnt inhibitory factor 1</td>
<td>Inhibit Wnt signaling</td>
</tr>
<tr>
<td>AW107799</td>
<td>295</td>
<td>Vascular cell adhesion molecule 1</td>
<td>Leukocyte binding</td>
</tr>
<tr>
<td>U12884</td>
<td>295</td>
<td>Wnt inhibitory factor 1</td>
<td>Inhibit Wnt signaling</td>
</tr>
<tr>
<td>AW000475</td>
<td>207</td>
<td>Vascular cell adhesion molecule 1</td>
<td>Leukocyte binding</td>
</tr>
<tr>
<td>AW108175</td>
<td>103</td>
<td>EST from 10 day neonate head</td>
<td>Unknown</td>
</tr>
<tr>
<td>L1352</td>
<td>–46</td>
<td>Amphiregulin</td>
<td>Growth factor</td>
</tr>
<tr>
<td>AB000758</td>
<td>–51</td>
<td>Protoocadherin 7</td>
<td>Cell adhesion</td>
</tr>
<tr>
<td>AV293830</td>
<td>–68</td>
<td>Annexin VIII</td>
<td>Anticoagulant</td>
</tr>
<tr>
<td>AJ003390</td>
<td>–52</td>
<td>Annexin VIII</td>
<td>Anticoagulant</td>
</tr>
<tr>
<td>X16490</td>
<td>–68</td>
<td>Plasminogen activator inhibitor, type II</td>
<td>Protease inhibitor</td>
</tr>
<tr>
<td>U79748</td>
<td>–73</td>
<td>Mothers against decapentaplegic homolog 4</td>
<td>TGF-β signaling</td>
</tr>
</tbody>
</table>

Fold change in transformed cells in contact with nontransformed cells compared to Src transformed cells grown alone.

Fold change in nontransformed cells compared to Src transformed cells.

Three genes that are included in Table 2.

EST, expressed sequence tag for which there is little information.

Table 2 The five genes most decreased or increased by Src transformation

<table>
<thead>
<tr>
<th>Symbol</th>
<th>gb</th>
<th>Name</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plf3</td>
<td>K03235</td>
<td>Proliferin 2</td>
<td>Angiogenesis</td>
</tr>
<tr>
<td>Plf2</td>
<td>K16009</td>
<td>Proliferin 3</td>
<td>Angiogenesis</td>
</tr>
</tbody>
</table>

Fold change in transformed cells in contact with nontransformed cells compared to Src transformed cells grown alone.
encoding BMP4 (Bmp4) and endothelin (Edn1) were increased over 2-fold. Interestingly, in addition to BMP4 signaling, induction of endothelin may decrease tumor vascularization (44).

The two genes in Src-transformed cells that were most decreased by contact with normal cells encode proliferin 2 and 3. Expression of these genes (Pfl2 and Pfl3) was decreased by nearly 2-fold (Table 3). In concert with endothelin activity, this decrease in proliferin expression could hamper angiogenesis of transformed cells (45). Interestingly, expression of proliferin 3 was increased about 45-fold by Src transformation.

We found only one transcription factor to be affected during the initial phase of heterologous growth control that was examined. The expression of the transcription factor Fbl1 in transformed cells was increased about 2-fold by contact with nontransformed cells. This family of factors is uniquely involved in mediating transcriptional activation by cyclic AMP (46) and suppressing transcription by the recombination signal sequence-binding protein-J DNA-binding protein (47).

The effects of Src transformation, and heterologous growth control of Src-transformed cells, on the expression of some genes presented in Table 3 were confirmed by RT-PCR analysis. As shown in Fig. 9, results from RT-PCR were consistent with those obtained by microarray analysis. The expression of Sdpr, Wif1, Vcam1, Igfbp2, and β-actin were analyzed by reverse transcription-PCR. The layered culture system was used to examine RNA from nontransformed cells, transformed cells, transformed cells grown in contact over transformed cells, transformed cells grown 1 mm over nontransformed cells, and transformed cells grown in contact over nontransformed cells. Transformed cells (50,000) and nontransformed cells (30,000) were plated for these assays producing a 1:6 ratio in cocultures. All genes were affected in a manner consistent with data obtained by microarray analysis shown in Table 3, although β-actin was not affected.

**DISCUSSION**

The growth of a variety of transformed cells can be controlled by surrounding normal cells (2, 3). This project was designed to elucidate mechanisms that underlie this phenomenon. Results presented here indicate that (a) direct contact between transformed and nontransformed cells is required, (b) but robust gap junctional communication is not; (c) transformed cells survive heterologous growth suppression, (d) but they retain their transformed genotype and growth potential; (e) Src kinase activity in transformed cells is not affected, (f) but expression of a limited set of genes involved with cell growth control, immunological recognition, and angiogenesis is involved.

A role for gap junctional communication has long been suspected in heterologous growth control (11). We used cells lacking Cx43 because it is a predominant gap junction protein expressed by cells grown in culture. For example, hepatocytes express Cx32 and Cx26 in animals, but they shut these off and express Cx43 in standard cell culture conditions (48, 49). We also used ACO to further suppress gap junctional communication between these cells. Nontransformed cells completely suppressed foci formation of neighboring transformed cells even though they were coupled by <1 gap junction channel per 10 cell pairs. This is very good evidence that gap junctions were not required for heterologous growth control in this cell system. Therefore, we propose that connexins did not play a critical role in heterologous growth control.

Although these data indicate that gap junctional communication was not required, they do not rule out a scenario in which gap junctions contribute to heterologous growth control. Indeed, experiments we have performed previously with Cx43 antisense constructs demonstrate that gap junctional communication augmented the ability of nontransformed cells to normalize the growth of neighboring Src-transformed cells (12). Nonetheless, the results presented here clearly demonstrate that although gap junctional communication may promote heterologous growth control, it is not required.

Data presented here indicate that direct contact between nontransformed and transformed cells was required for complete heterologous growth control. Soluble factors produced by nontransformed cells did not block foci formation of transformed cells. However, these data do not rule out a contribution of diffusible factors produced by nontransformed cells in normalizing the growth of neighboring transformed cells. In any case, even if signaling by such factors is required for heterologous growth control, it is not sufficient. These data confirmed that junctional communication (although not gap junctional communication) between transformed and nontransformed cells was required for heterologous growth control.

In the absence of gap junctional communication, the role of other intercellular junctions in heterologous growth control must be considered. The cells used in these studies expressed genes encoding E-cadherin (Cdh1) and β-catenin (Catnb). These proteins may play a part in normalization of tumor cell growth because they are targeted by Src in mediating proliferative signals (50).

In addition to cadherins, roles for integrins in heterologous growth control should also be considered. For example, some integrins may complex with cadherins of neighboring cells, as well as cellular substrata (40, 51). In addition, integrins can mediate cell adhesion and the spreading of Src-transformed cells on a fibronectin matrix (52).

Consistent with earlier studies (12), data from this work indicate that tumor cells survive during growth suppression by neighboring normal cells. Moreover, tumor cells remain genetically transformed and retain oncogenic kinase activity during heterologous growth control. In the cell system used here, Src kinase activity was not suppressed in normalized transformed cells. This is important because increased Src kinase activity has been implicated in a broad range of tumors including non-small-cell lung, ovarian, brain, and colon cancer, making Src an enticing target for anticancer drug therapy (53). Data presented here indicate that cancer cell growth may be controlled by contact with normal cells, even in the face of continued Src kinase activity.

We used DNA microarray technology to investigate how normal cells affect neighboring tumor cells. We examined gene expression relatively soon after cell contact to discover genes involved in hetero-
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Fig. 10. Genes affected by Src transformation and subsequent normalization of transformed cells by contact with nontransformed cells. Transcripts encoding Smad4 and Pcdh7 were augmented by Src, while the others were decreased by Src and increased by contact with normal cells. Putative roles of these gene products are also indicated. Data are shown quantitatively in Tables 2 and 3.

ologous growth control, as opposed to being modified as a consequence of it. Because presentation of results from the many genes we analyzed would be far beyond the scope of this report, we focus here on the genes that were most affected by Src transformation and subsequent normalization by heterologous cell contact. Despite the diverse roles assigned to genes modulated by normalization of transformed cell growth by surrounding nontransformed cells, some themes emerge that may underlie this phenomenon. Thus, these analyses have revealed genes that may act in concert to suppress transformed cell growth, as presented schematically in Fig. 10.

Srcdr, Wif1, and Vcam1 were among the genes most decreased by Src transformation. These genes were also the most up-regulated during heterologous growth control of transformed cells. Therefore, consequences of these effects on tumor cell growth deserve consideration.

Induction of Sdpr, which encodes serum deprivation-response protein (Sdpr), is particularly intriguing. This protein binds phosphatidylserine and is phosphorylated by protein kinase C, a process required for protein kinase C compartmentalization to caveolae. Sdpr can be induced in nontransformed cells by deprivation of serum and growth factors but not cell contact. However, Sdpr is not induced in transformed cells by serum or growth factor deprivation (38, 54). Therefore, growth control of transformed cells by contact with nontransformed cells presents a novel mode of Sdpr induction. Interestingly, a serum deprivation-response factor-related gene product that also binds to protein kinase C (Srbc) is down-regulated in many transformed cells.

Along with Sdpr, Wif1, which encodes Wnt-inhibitory factor 1 (Wif1), was highly up-regulated during heterologous growth control. Wif1 binds to Wnt, thereby blocking Wnt signaling (39). Activation of the Wnt signaling pathway can lead to a variety of tumors. For example, mutations of this pathway underlie about 90% of colorectal cancer (55).

Therefore, it is quite conceivable that inhibition of Wnt activity plays an active role in heterologous growth control. This scenario is consistent with expression of β-catenin and E-cadherin described above, because Wnt signaling would prevent β-catenin degradation and allow it to complex with T-cell factor in the nucleus to activate genes associated with cell growth (50, 57–58). This hypothesis is supported by the induction of Sfyl1 in transformed cells by contact with nontransformed cells. This gene encodes secreted frizzled-related protein 1, which, like Wif1, binds to Wnt and inhibits its activity (41).

Given its high level of induction, vascular cell adhesion molecule 1 may play an important role in heterologous growth control. The gene encoding this protein was among the most suppressed in cells transformed by Src, which is consistent with previous reports that v-Src can inhibit Vcam1 expression (59). However, the induction of Vcam1 in transformed cells by contact with nontransformed cells reported here is novel.

Vascular cell adhesion molecule 1 associates with the fibronectin receptor integrin α4 (very late antigen 4), which is expressed by lymphocytes. Therefore, the induction of Vcam1 in transformed cells may increase their detection by the immune system in vivo (40). However, transcripts encoding integrin α4 were not expressed to detectable levels by the cells used in this study. Nonetheless, potential effects of associations between vascular cell adhesion molecule 1 and other integrins expressed by the cells used in these studies, such as the fibronectin receptor integrins α3 and β1, should probably not be ruled out.

In addition to inhibiting Wnt, normal cells appear to inhibit IGF activity in neighboring tumor cells. For example, expression of Igbp2 and Prss11 were both increased during heterologous growth control. Igbp2 encodes an IGF-binding protein (Igbp2) which interferes with IGF receptor activation (60), whereas Prss11 encodes a secreted protease that targets IGF (43). Interestingly, these genes are downregulated in some glioma (42, 60) and melanoma (43) cells.

However, in addition to enhancing tumor cell mitogenesis, IGF can promote invasion and metastasis (61). For example, IGF may induce vascular endothelial growth factor expression in an Src-dependent manner (62). Therefore, induction of Igbp2 and Prss11 during heterologous growth control may decrease vascularization of tumor cells in vivo. Suppression of tumor vascularization during heterologous growth control is also inferred by activation of the gene encoding endothelin, which can inhibit angiogenesis (44), in concert with suppression of genes encoding proliferin, which increase angiogenesis (45).

Ultimately, regulation of gene expression in transformed cells during heterologous growth control should rely to some extent on the modulation of specific transcription factors. The expression of Fhl1, which encodes the transcription factor Fhl1, was increased about 2-fold in transformed cells within 26 h of their first contact with nontransformed cells. Fhl1 binds to the recombination signal sequence-binding protein-J DNA-binding protein. This association disrupts the ability of recombination signal sequence-binding protein-J to target the Epstein-Barr virus nuclear antigen 2 and the intracellular portions of Notch receptors to specific promoters. Fhl1 may use this mechanism to suppress the activation of some genes required for cell division and transformation (47).

Classic experiments by Mintz and Illmensee (63, 64) demonstrated that tumor cells injected into blastocysts can contribute to the formation of normal organs in mice. Puck et al. (65, 66) have also shown that the morphology of Src-transformed cells can be normalized by
cyclic AMP. If given the right cues, cancer cells can be controlled and normalized. This report represents a step toward elucidating some of the signals responsible for the suppression of tumor cell growth by neighboring normal cells. Contrary to many hypotheses, gap junctional communication between transformed and nontransformed cells does not appear to be critical. However, some other form of junctional communication is required. A role for cadherins may be expected, particularly because β-catenin is mobilized from cadherins junctions to participate as a transcription factor in response to Wnt signaling (67). We show here that this process appears to be suppressed by contact with normal cells. Moreover, induction of Sdpr, Vcam-1, and Fhl1, as well as potential suppression of IFG signaling, in heterologous growth control was brought to light by this study. It is possible that modulation of these activities may be used in conjunction with other approaches to help combat tumor cell growth in vivo.

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Normal Cells Control the Growth of Neighboring Transformed Cells Independent of Gap Junctional Communication and Src Activity

David B. Alexander, Hitoshi Ichikawa, John F. Bechberger, et al.

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