Interleukin-24 Induces Expression of β4 Integrin but Suppresses Anchorage-Independent Growth of Rat Mammary Tumor Cells by a Mechanism That Is Independent of β4

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Abstract

Wistar-Furth rats develop multiple mammary adenocarcinomas following initiation with methylglyoxal, whereas Copenhagen rats are resistant to the development of mammary tumors. We have previously isolated cell lines from tumors induced in resistant Copenhagen × Wistar-Furth F1 rats by infusion of a retrovirus harboring v-Ha-ras directly into the main mammary ducts. Some of the cell lines were able to grow in soft agar, but a significant number did not display anchorage-independent growth. Here, we compared by microarray analysis genes that are differentially expressed in these cell lines. The expression of interleukin-24 (IL-24) and β4 integrin was highly correlated with the inability of cells to grow in soft agar. Ectopic expression of IL-24 in anchorage-independent cells inhibited their growth in monolayer culture, in soft agar, and in nude mice in vivo and inhibited their ability to migrate and invade in in vitro assays. Furthermore, growth suppression by IL-24 was associated with the transcriptional up-regulation of p27Kip1 via the activation of Stat3. We showed, for the first time, that β4 integrin is a downstream target of IL-24. However, β4 does not play a direct role in regulating the proliferative capacity of rat mammary tumor cells. Our results show that IL-24 suppresses the growth of rat mammary carcinoma cells and may play a role in the resistance of Copenhagen rats to mammary carcinogenesis. (Mol Cancer Res 2009;7(3):433–42)

Introduction

Although many strains of rats develop multiple mammary adenocarcinomas when initiated with carcinogens such as methylglyoxal, several strains are resistant. For example, Wistar-Furth rats develop multiple mammary tumors following a single injection of methylglyoxal, whereas Copenhagen rats develop few, if any, tumors (1). To identify genes associated with resistance, we infused a retrovirus containing v-Ha-ras directly into the main mammary ducts of resistant F1 rats from a Copenhagen × Wistar-Furth cross (2). We reasoned that retroviral insertional inactivation of a resistance gene or activation of a susceptibility gene in only one allele of the F1s may be sufficient to confer susceptibility. Approximately 50% of infused glands developed tumors that were typical of rat mammary adenocarcinomas described previously in detail by Russo and Russo (3). Cell lines derived from these tumors displayed epithelial-like morphology and were clonal although there was no common viral integration site (2). This suggested that resistance in the F1 rats was overcome by a high level of v-Ha-ras expression.

Because it is well established that expression of v-Ha-ras in epithelial cells can overcome anchorage-dependent growth by inhibiting suspension-induced apoptosis (anoikis; ref. 4), we decided to investigate the growth properties of our cell lines. To our surprise, whereas some of the cell lines were able to grow in soft agar, a significant number did not display anchorage-independent growth, but these properties did not correlate with the level of v-Ha-ras expression (2). The growth characteristics were also reproduced when selected clones were injected into nude mice. We hypothesized that our anchorage-dependent and anchorage-independent cell lines may recapitulate the resistance and susceptibility of Copenhagen and Wistar-Furth rats, respectively, to mammary carcinogenesis that could facilitate the identification of breast cancer susceptibility genes.

Furthermore, the ability of clones to grow in soft agar showed a striking positive correlation with the expression of cyclin D1, a gene that has been shown previously to stimulate anchorage-independent growth of a human mammary epithelial cell line (5). Transfection of some clones with cyclin D1 fully overcame resistance to anchorage-independent growth, whereas the effect was partial in other clones. Transfection of cells with β-catenin produced a similar pattern of cyclin D1 up-regulation and growth in soft agar. Somatic fusion of an anchorage-dependent to an anchorage-independent clone produced a hybrid that did not grow in soft agar. These various results suggested that a high level of cyclin D1 expression is necessary, but not sufficient, for full anchorage-independent growth capacity in the mammary tumor cell lines. This raises the possibility that loss of a tumor
Expression of genes that are up-regulated in anchorage-dependent and anchorage-independent cell lines. Of several genes that are highly overexpressed in anchorage-dependent cells, interleukin-24 (IL-24), a known tumor suppressor gene is required to fully confer anchorage-independent growth. Here, we have compared by microarray analysis genes that are differentially expressed in anchorage-dependent and anchorage-independent cell lines. Of several genes in the table have higher expression levels in FE1.3 than FE1.2 cells. Only one gene under these conditions was overexpressed in FE1.2 cells (enpp1).

**FIGURE 1.** Expression of genes that are up-regulated in anchorage-dependent cells compared with anchorage-independent cells that were identified by microarray analysis. A. Northern blot analysis of IL-24, ptpn6, msln, and β4 integrin in FE1.3 and FE1.2 cells. B and C. Expression of IL-24 (by Northern blot) and β4 integrin (by RT-PCR) in several anchorage-independent (asterisk) and anchorage-dependent cell lines.

### Table 1. Microarray Analysis of Differential Gene Expression in FE1.2 and FE1.3 Cells

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<td>Protein tyrosine phosphatase, receptor type F</td>
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<td>65.60</td>
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<td>AGR_L_202q, partial (7%) [TC558064]</td>
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<td>63.64</td>
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**NOTE:** The analysis was done for five replicates using 44K Agilent Whole Rat Genome Arrays. Only values with a fold change >5 and t-test P < 0.0005 are reported. All of the genes in the table have higher expression levels in FE1.3 than FE1.2 cells. Only one gene under these conditions was overexpressed in FE1.2 cells (enpp1).

**Results**

**Identification of Genes Differentially Expressed in Breast Cancer Cell Lines**

We performed differential microarray analysis to identify genes responsible for anchorage-independent and anchorage-dependent growth of the cell lines that we established from the mammary carcinomas induced by injection of a retrovirus containing v-Ha-ras directly into the main mammary ducts of resistant F1 (Copenhagen × Wistar-Furth) rats (2). Five independent RNA isolates from the anchorage-dependent cell line FE1.3 and anchorage-independent cell line FE1.2 were subjected to microarray analysis using 44K Whole Rat Genome arrays (Agilent Technologies). This analysis resulted in the identification of 31 genes that are up-regulated at least 4-fold with t test P < 0.0005 in FE1.3 compared with FE1.2 cells (Table 1). Interestingly, three genes were in the keratin family, confirming our previous observations by immunohistochemistry that FE1.2 and subclones stained negatively or only weakly for keratin, whereas FE1.3 cells stained positively (2). Surprisingly, only one gene, enpp1, was significantly down-regulated (3-fold) in FE1.3 cells (Table 1).

We next performed Northern blot or reverse transcription-PCR (RT-PCR) analyses of four candidate genes that were...
identified in the microarray analysis that have been shown to have diverse effects on cancer development. *mda-7*, cloned by subtractive hybridization from terminally differentiated melanoma cells, is a tumor suppressor gene that selectively induces growth suppression and apoptosis in human cancer cells but not in normal cells (7, 8). *mda-7* is now recognized as *IL-24*, a member of the *IL-10* gene family (8).

*b4 integrin* associates with *a6 integrin* to form a laminin/kalinin receptor that is a component of the hemidesmosome (9, 10). There is evidence that *b4 integrin* may function as a suppressor gene for epithelial tumors (11).

*msln* is a differentiation antigen that is overexpressed in several human cancers, including pancreatic and ovarian cancers and mesotheliomas (12), as well as methylnitrosourea-induced mammary tumors in Lewis rats (13). *ptpn6*, also known as *shp-1*, is primarily expressed in hematopoietic cells and has been suggested to be a candidate tumor suppressor gene in lymphomas, leukemias, and breast and other cancers through suppression of Jak kinases (14, 15).

Northern blot analysis confirmed the microarray data showing that *IL-24* and *b4 integrin* were highly expressed in FE1.3 cells with negligible expression in FE1.2 cells (Fig. 1A). Our microarray analysis did not identify *a6 integrin* as a gene that was differentially expressed in FE1.2 and FE1.3 cells. We confirmed this by RT-PCR, which showed that both cell lines do express *a6 integrin* but at approximately the same level (data not shown). *msln* and *ptpn6* were both clearly expressed in FE1.3 cells, although there was some expression in FE1.2 cells (Fig. 1A).

### IL-24 and *b4 Integrin Expression Are Correlated with Growth in Soft Agar

To examine whether *ptpn6*, *msln*, *IL-24*, or *b4 integrin* plays a role in anchorage-dependent growth, we examined the expression of these genes in several of our previously characterized anchorage-dependent and anchorage-independent cancer cell lines (2). The expression of both *IL-24* and *b4 integrin* directly correlated with the inability of the cell lines to grow in soft agar. As shown in Fig. 1B and C, the anchorage-dependent cell lines FE1.3, FB0.1, FB1.1, and FB2.1 express high levels of *IL-24* and *b4 integrin*, whereas a negligible level of expression of these genes was detected in the anchorage-independent cell lines FE1.2, FB2.4, and FA2.1. The expression of *msln* and *ptpn6*, however, did not correlate with the ability of these cells to grow in soft agar (data not shown).

In view of these results, we decided to investigate a possible role for *IL-24* and *b4 integrin* in anchorage-dependent and anchorage-independent growth of our rat mammary tumor cell lines, although we recognize that several other genes in Table 1 may also be involved in regulating cell growth and perhaps carcinogenesis.

### IL-24 Inhibits Growth in Soft Agar

The virtually exclusive expression of *IL-24* in our anchorage-dependent cells suggested a role for this cytokine in determining their growth characteristics. For this reason, and because *IL-24* has been shown previously to suppress the
anchorage-independent growth of DU-145 human prostate carcinoma cells (16), we decided to investigate in more detail a possible role for IL-24 in regulating anoikis in our cell lines. First, we cultured anchorage-independent FE1.2 cells in soft agar in the presence or absence of recombinant IL-24. The addition of this protein to the culture medium of the FE1.2 cells significantly suppressed the number and size of the colonies (Fig. 2A). FE1.3 cells express high levels of IL-24, and we showed that medium in which these cells had been cultured also suppressed the growth of FE1.2 cells in soft agar (data not shown).

To examine further the importance of IL-24 in the suppression of growth in soft agar, FE1.2 cells were infected with a retrovirus (MSCV2.1) expressing this gene (Fig. 2B). As shown in Fig. 2C, two clones isolated from FE1.2 cells that overexpressed IL-24 following infection with the retrovirus containing IL-24 in the sense orientation gave rise to significantly fewer colonies than cells infected with the retrovirus containing IL-24 in the antisense orientation. We showed previously that the ability of cells to grow in soft agar was positively associated with the expression of cyclin D1 (2). Here, we showed that the expression of cyclin D1 was not affected by the expression of IL-24 in FE1.2 cells (Fig. 2D).

**IL-24 Expression Inhibits Growth but Does Not Induce Apoptosis in Monolayer Culture**

Retroviral expression of IL-24 in FE1.2 cells reduced the ability of these cells to proliferate in monolayer culture compared with cells infected with the retrovirus containing IL-24 in the antisense orientation (Fig. 3A). FE1.2 cells expressing IL-24 also became more rounded, although they did not become morphologically identical to the epithelial-like FE1.3 cells (data not shown). This indicates that more than one gene may be responsible for anchorage-dependent/independent growth. Expression of IL-24, however, did not induce apoptosis in FE1.2 cells (data not shown).

**IL-24 Inhibits Cell Migration and Invasion In vitro**

As illustrated in Fig. 3B, FE1.2 cells infected with the retrovirus containing IL-24 in the sense orientation were significantly less able to migrate through polycarbonate filters compared with the same cells expressing antisense IL-24 or the parental FE1.2 cells. Similarly, using polycarbonate filters coated with Matrigel, the invasive ability of cells expressing IL-24 was reduced by 80% to 90% compared with those not expressing the gene (Fig. 3C).

**IL-24 Expression Suppresses the Growth of Breast Cancer Cells In vivo**

Because our previous studies showed a correlation between the ability of cells to grow in soft agar and to form tumors in nude mice (2), we next examined the growth in nude mice of FE1.2 cells infected with the retrovirus expressing IL-24 in the sense or antisense orientation. Figure 3D shows that tumor growth was significantly inhibited in cells expressing IL-24 compared with cells expressing antisense IL-24 or the parental cell line.

**IL-24 Induces Expression of β₄ Integrin**

The strong correlation we observed between expression of β₄ integrin and IL-24 raised the possibility that these genes are coregulated in our cell lines (Fig. 1A). Indeed, when FE1.2 cells that expressed no β₄ integrin (Fig. 1A) were infected with the
retrovirus expressing IL-24, $\beta_4$ integrin was significantly upregulated as determined by RT-PCR (Fig. 4A). The expression of $\alpha_6$ integrin, however, was not different in FE1.2 cells infected with IL-24 in the sense or antisense orientations (data not shown). Up-regulation of $\beta_4$ integrin by IL-24 was reproduced in three different isolates of these cells (FE1.2+IL-24 S1-S4) and was confirmed by Western analysis (Fig. 4B). Western analysis of $\beta_4$ integrin in indicated cells. C. Western analysis of IL-24 in FE1.3 cells, two isolates of FE1.3 cells infected with retrovirus expressing antisense IL-24 (FE1.3 + IL-24 AS1 and AS2), or FE1.3 cells infected with empty retrovirus (vector). D. Western analysis of $\beta_4$ integrin in indicated cells. E. Growth in soft agar of cells described herein. Mean ± SE. *, P < 0.05, compared with FE1.2 cells.

### IL-24 Induces p27Kip1 Transcriptional Up-Regulation through Activation of Stat3

Inhibition of growth by IL-24 in our breast cancer cell lines suggested an alteration in the cell cycle machinery. Because a recent report showed that treatment of human breast cancer cells with IL-24 led to the up-regulation of cell cycle inhibitor p27Kip1 (17), we investigated the expression of this protein in our cell lines. First, we showed that the levels of p27Kip1 were higher in the anchorage-dependent cell lines compared with those that grew in soft agar (Fig. 5A). To examine whether up-regulation of p27Kip1 is induced by IL-24, we examined the status of this cell cycle inhibitor in the anchorage-independent cell line FE1-2 infected with the retrovirus expressing IL-24. It is clear that two of the clones overexpressing IL-24 in the sense orientation expressed higher levels of p27Kip1 than control cells infected with antisense IL-24 (Fig. 5B). RT-PCR analysis showed that up-regulation of p27Kip1 by IL-24 occurred at the level of transcription (Fig. 5C). Furthermore, infection of anchorage-dependent FE1-3 cells with antisense IL-24 resulted in down-regulation p27Kip1 compared with control cells infected with the vector alone (Fig. 5D).

There are several reports that IL-24 activates Stat3 in a variety of cell types including breast cancer cells (6, 17-20). Because activation of Stat3 has been shown to induce p27Kip1 expression, we investigated Stat3 activation at both Tyr705 and Ser727 in our cell lines (21, 22). As shown in Fig. 6A to C, Stat3 was phosphorylated at Tyr705 and Ser727 in our cell lines, although growth was not as robust as FE1.2 cells.
tyrosine kinase (AG9 and AG490; refs. 23, 24) were added to the medium of FE1.2 cells infected with IL-24. Both compounds completely inhibited Stat3 phosphorylation expression and down-regulated p27Kip1 (Fig. 6A and B). Incubation of the same cells with the mitogen-activated protein kinase phosphorylation inhibitor PD98059 that blocks phosphorylation of Ser727 in Stat3 (25) also resulted in a down-regulation of p27Kip1 (Fig. 6C). Therefore, phosphorylation of both serine and tyrosine sites in Stat3 is required for p27Kip1 regulation by IL-24. Interestingly, inhibition of Stat3 tyrosine and serine kinase with AG9 had no effect on β4 integrin expression (Fig. 6D), suggesting that induction of β4 expression by IL-24 is independent of Stat3 up-regulation.

**Discussion**

We have shown by microarray analysis and confirmed by Northern and RT-PCR analyses that IL-24 is highly overexpressed in rat mammary tumor cells that are unable to grow in soft agar compared with cells that are anchorage independent. The differential expression of IL-24 in FE1.2 and FE1.3 cells was somewhat surprising because both cell lines overexpress v-Ha-ras (2) and expression of IL-24 has been shown to be induced in Rat1 cells and rat intestinal epithelial cells by oncogenic Ha-ras (26). Our results clearly show that IL-24 expression in mammary tumor cells is regulated differently. In strong support of our microarray results, we have shown that ectopic expression of IL-24 in our anchorage-independent FE1.2 cells inhibited their growth in soft agar. These results are in keeping with those of Su et al. who recently showed that immortalized normal human cells infected with a recombinant adenovirus expressing IL-24 (Ad.mda-7) secreted IL-24 that suppressed the anchorage-independent growth of DU-145 human prostate carcinoma cells (16). We have shown previously that a high expression level of cyclin D1 is necessary, although not sufficient, for anchorage-independent growth of our breast cancer cell lines (2). Here, we show that expression of cyclin D1 was not affected by the expression of IL-24, suggesting that suppression of the growth of FE1.2 cells in soft agar by IL-24 is independent of cyclin D1.

We have also shown that IL-24 expression in FE1.2 cells inhibited their growth in monolayer culture. Others have shown that ectopic expression of a transfected IL-24 gene induces growth suppression in cell lines derived from a variety of human tumors (ref. 17 and references therein). Although IL-24 expression inhibited growth of FE1.2 cells, it did not induce apoptosis. These cells clearly behave differently in this respect from the human tumors in which IL-24 expression induced apoptosis (ref. 17 and references therein), although a recent report shows that recombinant IL-24 lacks apoptosis-inducing properties in human melanoma cells (27). Ramesh et al. (28) have shown previously that ectopic production of IL-24 inhibits invasion and migration of human lung cancer cells. Likewise, we have shown that expression of IL-24 in FE1.2 cells inhibited...
both migration and invasion in in vitro assays, suggesting that in vivo expression of this gene will suppress malignancy. Consistent with the action of IL-24 to inhibit growth of FE1.2 cells in soft agar and in monolayer culture, the ability of FE1.2 cells to form tumors in nude mice in vivo was significantly inhibited in cells expressing IL-24 compared with those expressing antisense IL-24 or the parental cells. These results confirm those reported by Su et al. (29) who showed that infection of human breast cancer cells with Ad.mda-7 before injection into nude mice inhibited tumor development. Overall, our results show that the effects of IL-24 expression in rat mammary tumor cells are similar in most respects to its effects in human cancer cells.

In addition to IL-24, our microarray analysis also revealed that β4 integrin is highly overexpressed in the cells that are unable to grow in soft agar compared with the anchorage-independent cells. To our surprise, ectopic expression of IL-24 in FE1.2 cells led to a highly significant up-regulation of β4 expression. Furthermore, infection of FE1.3 cells with the antisense IL-24 construct led to a down-regulation of β4 expression and growth of the cells became partially anchorage-independent. Although there is evidence that the β4 and α6 integrin subunits can act as tumor suppressors (11, 30, 31), there is also evidence that β4 enhances anchorage-independent growth of human breast carcinoma cells and stimulates tumor growth in nude mice (ref. 32 and references therein). Our results, therefore, stand in contrast to these studies. In our rat mammary carcinoma cells, it is possible that the growth-inhibitory actions of the highly overexpressed IL-24 override the growth-enhancing actions of β4. Furthermore, although β4 was overexpressed in FE1.3 cells, the expression of α6 was similar in the anchorage-dependent and anchorage-independent cells and was not induced by IL-24. It is possible, therefore, that the growth-enhancing activity of the overexpressed β4 was limited by the expression of α6.

IL-24-induced inhibition of cell proliferation was associated with the transcriptional up-regulation of the cell cycle inhibitor p27kip1 mediated by Stat3 activation. Thus, our results confirm the association among p27kip1, Stat3, and IL-24 that was shown recently in two human breast cancer cell lines (17), and we additionally show that up-regulation of both serine and tyrosine phosphorylation of Stat3 by IL-24 is required for p27kip1 up-regulation. The inhibitory effect of p27kip1 on tumor

FIGURE 6. Stat3 phosphorylation at Tyr205 and Ser727 in anchorage-dependent and anchorage-independent cell lines and effects on β4 integrin expression. A and B, Western analysis of effects of inhibitors of Stat3 tyrosine kinase (AG9 and AG490) on Stat3 phosphorylation and p27kip1 levels in FE1.2 and FE1.3 cells and two isolates of FE1.2 cells infected with retrovirus expressing IL-24 in the sense orientation (FE1.2 + IL-24 S1 and S2). C, Western analysis of effect of inhibiting Stat3 serine kinase by mitogen-activated protein kinase phosphorylation inhibitor PD98059 on Stat3, Stat3 phosphorylation, and p27kip1 levels in cells described herein. D, Western analysis of effect of Stat3 tyrosine kinase inhibitor AG9 on β4 integrin expression in cells indicated as well as FE1.2 cells infected with retrovirus expressing antisense IL-24 (FE1.2 + IL-24 AS).
proliferation is mainly through its interaction with the CDK-cyclin D1 complex (33). Indeed, we have shown previously lower levels of cyclin D1 expression in anchorage-dependent versus anchorage-independent cell lines despite expressing similar levels of Ha-ras (2). Our microarray analysis showed higher levels of Cdk4 expression in anchorage-dependent than in anchorage-independent cells, although cyclin D1 expression was not affected by IL-24 expression. Interestingly, Cdkn2a (p16INK4a) was highly up-regulated in the anchorage-dependent cells (Table 1), suggesting that stimulation of cell growth caused by Cdk4 up-regulation would have been inhibited. We have recently observed overexpression of Cdk2 in the anchorage-dependent cells. Thus, inhibition of proliferation by IL-24 may also be a result of increased amounts of the Cdk2/p27Kip1 complex leading to inhibition of Cdk2 activity. Indeed, a recent study showed that oncostatin M, an IL-6 family cytokine, inhibits the growth of anchorage-dependent skeletal muscle cells through down-regulation of cyclin D1 and inhibition of Cdk2/p27Kip1 complex (34). p27Kip1 likely functions as a more potent inhibitor of cell proliferation in anchorage-dependent cell lines. Because cyclin D1 expression in FE1.3 cells is regulated through the β-catenin pathway (2), cooperation between this cyclin and IL-24/Stat3 may be necessary to confer anchorage-dependent growth.

It has been shown that IL-24 exerts its effects on cell growth in cancer cells and not in normal cells (8). Our observations raise the possibility, however, that the expression of IL-24 is activated in the preneoplastic lesions of Copenhagen rats leading to their failure to progress to cancer as we have described (35, 36). This notion is currently under investigation.

In summary, using microarray analysis, we identified several genes that are highly expressed in anchorage-dependent rat mammary tumor cells but not in anchorage-independent cells. One of these genes, IL-24, was shown to suppress proliferation, anchorage-independent growth, migration, and invasion in vitro and growth of cells in vivo. Furthermore, growth suppression by IL-24 was associated with up-regulation of p27Kip1 mediated by Stat3. These cellular and molecular effects of IL-24 in rat mammary tumor cells are similar to its effects in human cancer cells.

Materials and Methods

Cell Culture

The establishment of the rat breast cancer cell lines was described previously (2). All cell lines were maintained in α-MEM supplemented with 10% fetal bovine serum, 10 ng/mL epidermal growth factor, 1 μg/mL hydrocortisone, and 1 μg/mL 17β-estradiol. To examine growth rates, 10^4 cells were seeded per well in triplicate on 24-well plates and counted on each of 4 consecutive days.

Microarray Analysis

FE1.2 and FE1.3 cells were grown in 10 cm dishes until ~70% confluent. Total RNA was isolated using TRIzol according to the manufacturer’s instructions (Invitrogen). A Low RNA Input Fluorescent Linear Amplification Kit (Agilent Technologies) was used to amplify 200 ng RNA and label the FE1.3 cell samples with Cy5 and the FE1.2 cell samples with Cy3. The cRNA was then fragmented and hybridized using a Gene Expression Hybridization Kit (Agilent Technologies). Microarray analyses were done at the University Health Network (Toronto) Microarray Centre. Five 44K Whole Rat Genome arrays (Agilent Technologies) were hybridized with five different isolates of each of the two cell lines. The arrays were hybridized for 17 h at 60°C according to the manufacturer’s recommendations. The arrays were then washed for 1 min in 6× SSPE, 0.005% N-lauroylsarcosine and 1 min in 0.06× SSPE, 0.005% N-lauroylsarcosine. A final wash step was done for 30 s in Stabilization and Drying Solution (Agilent). The arrays were then scanned in the Agilent G2565AA Microarray scanner and quantified using Agilent Feature Extraction (version 8.1). GeneSpring (version 7.5) was used to analyze the array data using a lower signal threshold of 100. Significant differences between the two cell lines were determined by both signal fold differences of >2 and t test P values < 0.05.

Infection and Transfection Experiments

An IL-24-producing retrovirus was generated by inserting the entire 600-bp coding sequence of rat IL-24 in sense and antisense orientations into a unique EcoRI site in the retroviral expression vector MSCV2.1 (37). Replication-defective viruses were prepared by transfecting the viral plasmid into the helper-free packaging cell line GP+/- (38) as described previously (37). For viral infection, supernatants from the virus-producing cells were used to infect FE1.2 cells plated at a density of 2 × 10^6. After 48 h G418 selection was started and after 10 d, G418-resistant cells were pooled and subjected to subcloning by limiting dilution. Transfection was done using Lipofectamine 2000 according to the manufacturer’s protocol (Invitrogen).

Growth of Cells in Soft Agar

To monitor growth of cells in soft agar, two layers of agarose were used. The bottom layer contained 0.5% agarose and the top layer contained 0.3% agarose both in α-MEM. Triplicate samples of 5,000 cells were seeded on 6 cm plates and incubated for 5 to 7 days. At the end of the incubation period, colonies with >25 cells were counted. FE1.2 cells were also grown in soft agar in the presence of 5 mmol/L IL-24-alkaline phosphatase fusion protein (GenHunter).

Growth of Cells in Nude Mice

Four groups of six female athymic nude mice (CD1 nu/nu), ages 7 weeks, were purchased from Charles River Laboratories. At age 8 weeks, 10^3 FE1.2 or FE1.2 cells expressing IL-24 in the sense or antisense orientation, in a volume of 50 μL, were injected through a 27-gauge needle into the right and left thoracic fat pads. Tumor size was measured twice weekly with vernier calipers by a single blinded observer and tumors were harvested 2 weeks after the mice were injected with the cells.

4 Unpublished results.
Cell Migration and Invasion Assays

Cell migration and invasion assays were done as described by Chen and Thompson (39).

Western Blotting

Western blotting was done as we have described previously (2). Primary murine monoclonal antibodies were obtained from the following sources: cyclin D1 from Neomarkers used at a dilution of 1:50,000, Stat3 and phospho-Stat3 from Cell Signaling used at a dilution of 1:500, mitogen-activated protein kinase and phospho-mitogen-activated protein kinase also from Cell Signaling used at a dilution of 1:1,000, p27 from Santa Cruz Biotechnology used at a dilution of 1:1,000, and β4 integrin from Chemicon International used at a dilution of 1:1,000.

Stat3 tyrosine kinase inhibitors AG9 and AG490 (Calbiochem) dissolved in DMSO were added to the cells to yield final concentrations of 200 and 20 nmol/mL, respectively. Mitogen-activated protein kinase phosphorylation inhibitor PD 98059 (Sigma) dissolved in DMSO was added to cells at final concentration of 100 nmol/mL. Control cells were treated with the same volumes of DMSO alone. Cells were harvested for Western blot analysis after 1 h of treatment.

Northern Blotting and RT-PCR

Total RNA was prepared by using TRIzol. Electrophoresis on 1% formaldehyde denatured agarose gels (20 μg RNA/lane) was followed by transfer to Zeta-Probe Nylon membranes (Bio-Rad). The membranes were hybridized with 32P-labeled IL-24, msln, ptpn6, and GAPDH forward and reverse primer probes at 42°C overnight. Hybridization buffer and wash procedures were as described previously (40).

RT-PCR Analysis

The total RNA was extracted by using TRIzol. Electrophoresis on 1% formaldehyde denatured agarose gels (20 μg RNA/lane) was followed by transfer to Zeta-Probe Nylon membranes (Bio-Rad). The membranes were hybridized with 32P-labeled IL-24, msln, ptpn6, and cyclin D1 cDNA probes at 42°C overnight. Hybridization buffer and wash procedures were as described previously (40).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References


Molecular Cancer Research

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Wanli Xuan, You-Jun Li, Guodong Liu, et al.


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