Multiple Aspects of the Phenotype of Mammary Epithelial Cells Transformed by Expression of Activated M-Ras Depend on an Autocrine Mechanism Mediated by Hepatocyte Growth Factor/Scatter Factor

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Abstract

Multiple aspects of the transformed phenotype induced in a murine mammary epithelial cell line scp-2 by expression of activated G22V M-Ras, including maintenance of cell number at low density, anchorage-independent growth, invasion of Matrigel, and secretion of matrix metalloproteinases (MMP) 2 and 9, were dependent on an autocrine mechanism. Conditioned medium from dense cultures of scp-2 cells expressing G22V M-Ras, but not from parental cells, induced activation of Erk and Akt in cells expressing G22V M-Ras, maintained the cell number and promoted anchorage-independent growth of cells expressing G22V M-Ras (although not the parental cells), and induced scattering of MDCK cells. The latter activities were blocked by neutralizing antibodies to hepatocyte growth factor/scatter factor (HGF/SF) and could be mimicked by HGF/SF. Anti-HGF/SF antibodies also inhibited invasion of Matrigel, and the production of MMP-2 and MMP-9, together with urokinase-type plasminogen activator, was secreted by G22V M-Ras scp-2 cells but not by parental cells. Invasion of Matrigel was blocked by an inhibitor of MMPs, BB94, and by the mitogen-activated protein kinase kinase 1/2 kinase inhibitor PD98059 but was only marginally affected by the phosphatidylinositol 3-kinase inhibitor LY294002. Autocrine HGF/SF was thus critical for expression of key features of the phenotype of mammary epithelial cells transformed by expression of activated M-Ras. (Mol Cancer Res 2004;2(4):242–55)

Introduction

M-Ras or R-Ras3 is a novel member of the Ras family of small GTPases (1–5), which shares about 50% sequence identity with the four classical p21 Ras proteins Harvey Ras (H-Ras), neuroblastoma Ras (N-Ras), and the two alternatively spliced variants of Kirsten Ras (K-Ras 4A and 4B; Ref. 6). The Ras family also includes the Rap, Ral, and R-Ras subgroups of proteins. Although the overall sequence identity of proteins in this family is only 40–55%, there is a much higher level of conservation of the regions of these proteins that undergo allosteric changes (*i.e.*, Switch-I and Switch-II). These highly conserved regions are critical in mediating interactions between positive and negative regulators, the guanine nucleotide exchange factors (GEFs) and the GTPase activating proteins, respectively, as well as with multiple effector proteins. Thus, it is not surprising that there is considerable overlap between family members both in their regulation and functions.

The p21 Ras proteins are the best studied members of the group and have been strongly implicated in oncogenesis (7), with ~25% of human cancers exhibiting activating mutations in K-Ras, N-Ras, or H-Ras (8). Although other members of the Ras subfamily have been implicated in oncogenesis (9, 10), relatively less is known about the potential molecular mechanisms involved. M-Ras is of particular interest as it is expressed ubiquitously (11). Moreover, in comparison with H-Ras, M-Ras is preferentially activated by growth factors such as epithelial growth factor, interleukin-3, and colony stimulating factor-1.[2] Signaling pathways activated by M-Ras differ from those induced by p21 Ras proteins. Thus, unlike the p21 Ras proteins, activated M-Ras interacts only weakly with Raf-1 and activates the Erk kinases poorly in comparison with activated p21 Ras proteins (5, 11, 12). Furthermore, activated M-Ras interacts only weakly with the other effector of p21 Ras that has been strongly implicated in oncogenesis (13), Ral-GDS (2, 11). Indeed, of the candidate effectors shown to interact with activated M-Ras [*i.e.*, AF-6, Nore-1, and Rin-1 (5, 11); two Rap GEFs, MR-GEF and Rap-GEF-2 (14, 15); RPM (1); and phosphatidylinositol 3-kinase (PI3K; Ref. 2)], only PI3K has a well-established role in growth, survival (16), and oncogenesis (17). Nonetheless, expression of activated mutants of M-Ras induced oncogenic transformation of fibroblasts (5, 11, 12), factor-dependent cell lines of hemopoietic origin (3, 11), normal hemopoietic stem cells,[3] and a mammary epithelial line (18). Expression of activated M-Ras, but not wild-type M-Ras, resulted in the loss of multiple epithelial features, including expression of cytokeratin, β-catenin, and E-cadherin, and loss

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[2] A. Ehrhardt *et al.*, submitted for publication.

[3] X-C. Guo and J. W. Schrader, manuscript in preparation.

of the ability to form spheres of differentiated cells (resembling mammospheres) when plated on an artificial basement membrane, Matrigel (18). The scp-2 cells lost their epithelial appearance, assumed a spindle-shaped morphology, and expressed the mesenchymal marker vimentin. They also gained the capacities for anchorage-independent growth and tumor formation in mice. M-Ras is expressed in two human mammary carcinoma cell lines (11) and M-Ras cDNA occurs in libraries prepared from normal human mammary tissues (Cancer Genome Anatomy Project; http://www.ncbi.nih.gov). Analyses of gene expression in normal and cancerous mammary tissue using microarrays (19) or serial analysis of gene expression (20) have likewise detected expression of M-Ras. With some evidence that expression of M-Ras is higher in mammary carcinomas (18), there would seem to be a case for determining whether activating mutations of M-Ras occur in human mammary cancer. In addition, given the weak ability of activated M-Ras to activate both of the major pathways downstream of p21 Ras that have been implicated in oncogenesis (*i.e.*, the Raf-1 and Ral-GDS pathways), the question arises as to the molecular mechanisms through which activated M-Ras triggers transformation.

To address this issue, we have examined the molecular mechanisms involved in the transformation of the murine breast epithelial cell line scp-2 by activated M-Ras. Here, we report that the key features of the transformed phenotype induced by expression of activated M-Ras in scp-2 cells depend on an autocrine mechanism involving hepatocyte growth factor/scatter factor (HGF/SF). We observed that multiple aspects of phenotype of scp-2 cells transformed by activated M-Ras were dependent on the cell density at which they were plated. These included survival in the absence of exogenous growth factors, anchorage-independent growth in soft agar, and invasion of Matrigel, all of which could be enhanced by the medium conditioned by scp-2 cells expressing activated M-Ras.

Results

Scp-2 Cells Expressing G22V M-Ras Exhibit Density Dependence in the Absence of Serum and Growth Factors

We used a bicistronic vector to express activated G22V M-Ras and green fluorescent protein (GFP) or, as a control, GFP alone in scp-2 cells (18). Parental scp-2 cells or scp-2 cells expressing GFP alone (GFP scp-2 cells) decreased in number when cultured in the absence of serum and insulin and detached from the plastic culture dish. In contrast, scp-2 cells expressing G22V M-Ras (G22V M-Ras scp-2 cells) remained viable and adherent to the plastic. However, more detailed analysis indicated that the decline in cell number was dependent on the density at which they were plated. Thus, at cell densities of 5×10^4 cells/ml or higher, whereas parental cells or GFP scp-2 cells rounded up and became detached from the plastic, cells expressing activated G22V M-Ras remained adherent and well spread for 4 days (Fig. 1A). Moreover, whereas control GFP scp-2 cells had decreased in number by about 70%, numbers of viable G22V M-Ras scp-2 cells had hardly changed (Fig. 1B). However, when the density of cells plated was reduced 2-fold, more of the G22V M-Ras scp-2

cells rounded up and their number fell by 50% by day 4. We made similar observations with clones of G22V M-Ras scp-2 cells or with polyclonal populations of G22V M-Ras scp-2 cells from independent retroviral infections (data not shown). This density dependence of cells expressing activated M-Ras was reproducible and suggested that expression of activated M-Ras resulted in the release of factor(s) that maintain the number of cells. To test this hypothesis, we collected medium that had been conditioned under serum-free conditions by G22V M-Ras scp-2 cells or by control GFP scp-2 cells. The conditioned medium (CM) was tested for its ability to preserve the viability and adherent morphology of G22V M-Ras scp-2 or GFP scp-2 cells cultured at a low cell density of 1.25×10^4 cells/ml in the absence of serum and insulin. Counts of viable cells at day 4 (Fig. 2) indicated that CM from G22V M-Ras scp-2 cells maintained numbers of the G22V M-Ras scp-2 cells but was much less effective in maintaining the numbers of GFP scp-2 cells. Moreover, CM from GFP scp-2 cells did not display this activity (data not shown). These observations suggested that the expression of activated M-Ras not only resulted in the secretion of autostimulatory factor(s) but also enabled G22V M-Ras scp-2 cells to respond to these autostimulatory factor(s).

Anchorage-Independent Growth of scp-2 Cells Expressing G22V M-Ras Is Density Dependent and Is Enhanced by CM

We next examined whether similar autocrine mechanisms were involved in the ability of G22V M-Ras expressing scp-2 cells to grow as anchorage-independent colonies in soft agar. We observed that both the cloning efficiency of G22V M-Ras scp-2 cells and the size of colonies were dependent on the density at which cells were plated. Thus, as shown in Fig. 3, a 10-fold increase in the density of cells plated was associated with a 3-fold increase in cloning efficiency. This was consistent with the notion that G22V M-Ras scp-2 cells secreted factor(s) that enhanced their anchorage-independent growth.

To further test this hypothesis, we investigated the effect of adding CM obtained from G22V M-Ras scp-2 cells to cultures of G22V M-Ras scp-2 cells plated in agar at either low (10^3 cells/ml) or high (10^4 cells/ml) cell densities. We observed that the CM stimulated an increase in the mean size of the colonies, especially in cultures plated at the lower cell density (Fig. 3A). Moreover, in the presence of CM, there was a 10-fold increase in the cloning efficiency of cells plated at the low cell density (10^3 cells/ml). At the higher cell density (10^4 cells/ml), the cloning efficiency was increased only 4-fold (Fig. 3B). Thus, in the presence of medium conditioned by G22V M-Ras scp-2 cells, cloning efficiencies were identical in cultures plated at low (10^3 cells/ml) or high (10^4 cells/ml) cell densities. Parental or GFP scp-2 cells failed to grow as colonies whether CM was present or absent (data not shown). These results indicate clearly that scp-2 cells expressing activated M-Ras secreted factor(s) that enhance anchorage-independent growth as assessed by colony size or the cloning efficiency. In contrast to the survival assay (Figs. 1 and 2), the anchorage-independent growth assay was carried out in the presence of 5% FCS and insulin. Thus, the experiments
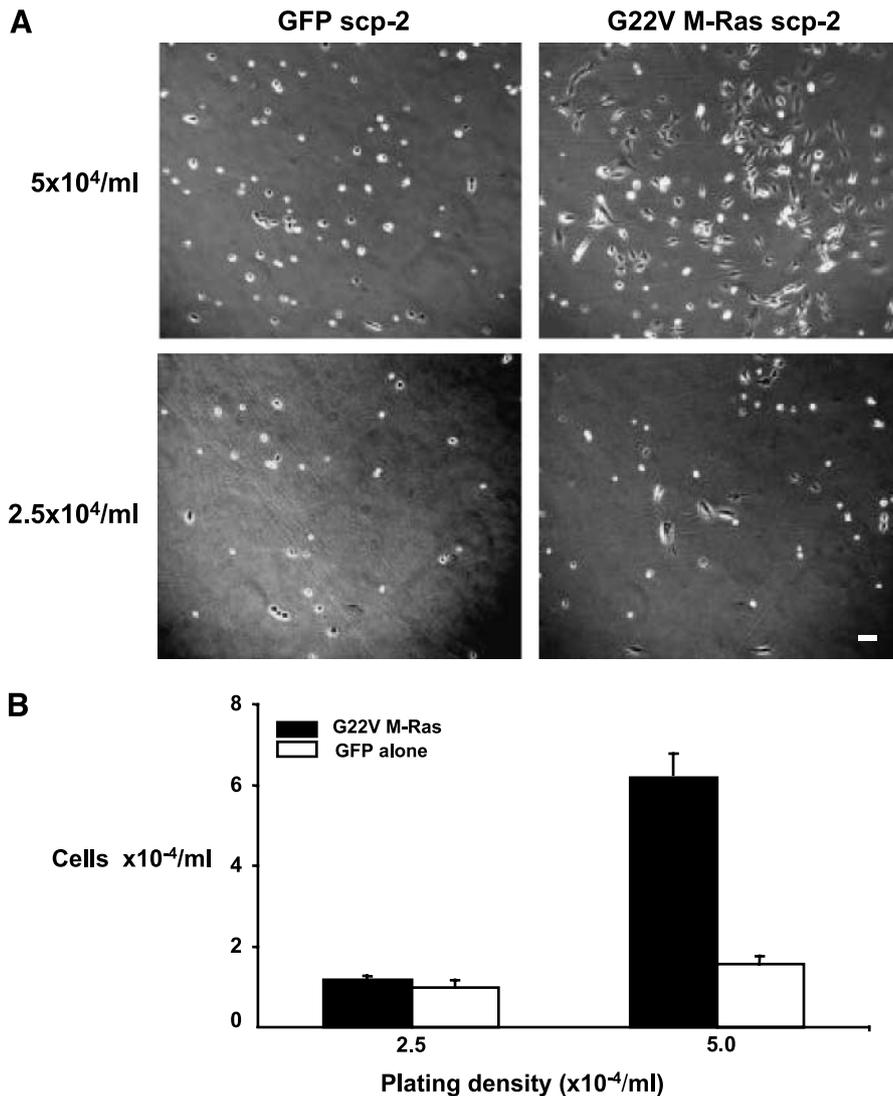
A

FIGURE 1. Density dependence of scp-2 cells expressing activated M-Ras plated in serum-free medium. GFP scp-2 or G22V M-Ras scp-2 cells were plated at 2.5×10^4 or 5×10^4 cells/ml in DMEM with no FBS or exogenous growth factors and cultured for 4 days. **A.** Photomicrographs of cultures at day 4. *Bar,* 40 μm. **B.** Counts of viable cells. *Columns,* mean; *bars,* SD. Results are representative of four experiments.

on anchorage-independent growth indicated that the factor(s) in the CM from G22V M-Ras scp-2 cells that were required for anchorage-independent growth could not be replaced by FCS or insulin.

Invasion of the Matrigel by scp-2 Cells Expressing G22V M-Ras Depended on Cell Density and the Presence of CM

Whereas parental cells or GFP scp-2 cells formed small spherical aggregates or mammospheres 24–48 h after plating on the artificial basement membrane, Matrigel, the behavior of G22V M-Ras scp-2 cells was quite different. Thus, instead of forming spherical aggregates, these cells assembled into large aggregates of elongated cells that rapidly became interconnected by bundles of cells to form a network. Cells migrated from these aggregates and bundles and invaded through the Matrigel to reach the bottom of the tissue culture dishes. This invasive behavior of G22V M-Ras scp-2 cells was also den-sity dependent. Thus, when plated at low cell densities, G22V M-Ras scp-2 cells failed to migrate through the Matrigel. In one instructive experimental design, we plated parental cells at a density of 1.25×10^5 cells/ml either alone or together with increased numbers of G22V M-Ras scp-2 cells. Because we used bicistronic vectors, cells that expressed activated M-Ras also expressed GFP and were readily distinguished from the parental cells by fluorescence microscopy. By observing the behavior of the fluorescent cells, we noted that, when plated at low cell density (1.25×10^4 cells/ml), G22V M-Ras scp-2 cells remained round and did not invade the Matrigel (Fig. 4A). In contrast, in cultures in which the number of G22V M-Ras scp-2 cells was 10-fold higher, we saw that the fluorescent G22V M-Ras scp-2 cells had invaded the Matrigel and had reached the bottom of the dish (Fig. 4B). This invasive behavior thus depended on the density of G22V M-Ras scp-2 cells.

To investigate the contribution of autostimulatory factor(s) to invasive behavior, we added CM from G22V M-Ras scp-2 cells to Matrigel cultures containing a high density of parental
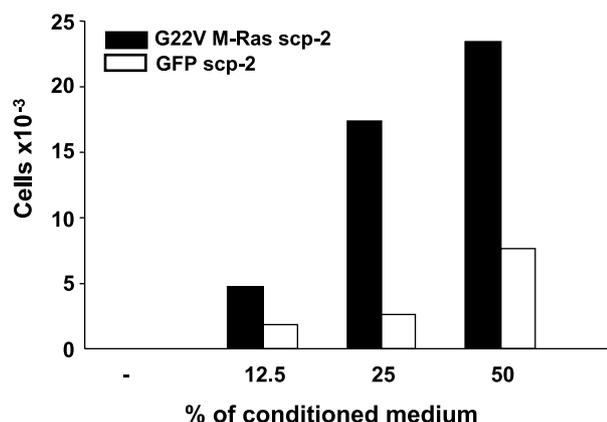
FIGURE 2. Medium conditioned by scp-2 cells expressing activated G22V M-Ras maintained numbers of viable G22V M-Ras scp-2 cells. GFP scp-2 cells (*open bars*) or G22V M-Ras scp-2 cells (*solid bars*) were plated at the low cell density of 1.25×10^4 cells/ml in 5 ml DMEM in the absence of FBS and exogenous growth factors, with or without the addition to the final percentages shown, of medium conditioned by G22V M-Ras scp-2 cells. At day 4, cells were harvested and viable cells were counted. Results are representative of two experiments.

cells but only a low density of G22V M-Ras scp-2 cells. We observed that inclusion of the CM resulted in the invasion of fluorescent G22V M-Ras scp-2 cells down to the bottom of the dish (Fig. 4C). In contrast, the parental (nongreen) scp-2 cells did not invade the Matrigel even in the presence of a high density of G22V M-Ras scp-2 cells and/or CM from G22V M-Ras scp-2 cells. This result was the further evidence that the phenotype of the G22V M-Ras scp-2 cells depended not only on the presence of autostimulatory factors but also on an altered ability of scp-2 cells expressing G22V M-Ras to respond to them.

Medium Conditioned by scp-2 Cells Expressing G22V M-Ras Induced Activation of Erk and Akt

To investigate the molecular mechanisms through which the CM was promoting the growth and survival of G22V M-Ras scp-2 cells, we tested the ability of the CM from G22V M-Ras scp-2 cells to activate the Erk or the PI3K/Akt signaling pathways, both of which have been associated with invasion, survival, and growth. We have observed previously that G22V M-Ras scp-2 cells growing as tumors *in vivo* exhibited activation of both Erk and Akt (18). To determine the role of autocrine
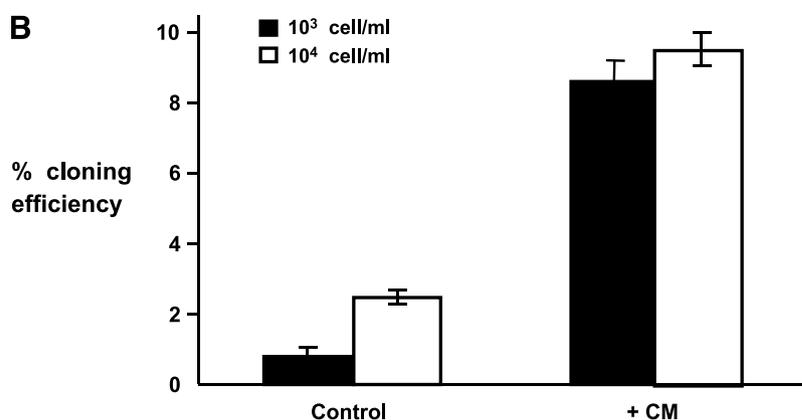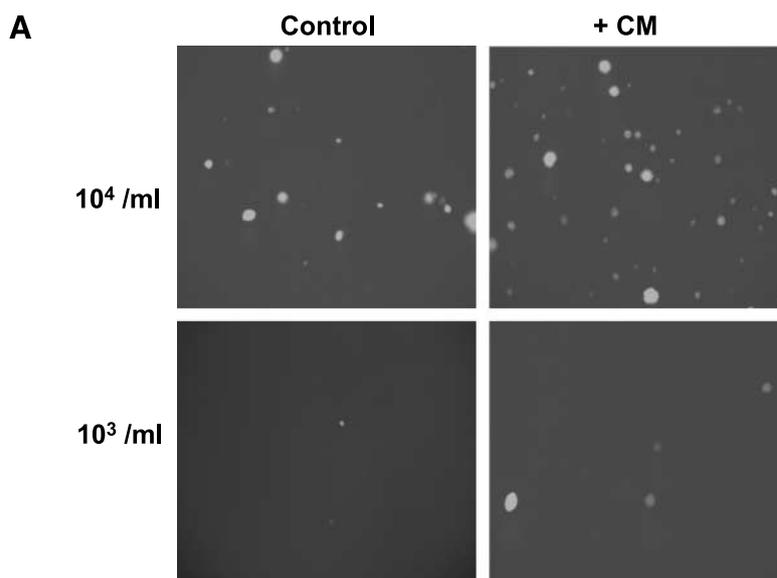
FIGURE 3. Density dependence of anchorage-independent growth of scp-2 cells expressing activated G22V M-Ras. G22V M-Ras scp-2 cells were cultured at either 10^3 or 10^4 cells/ml as indicated in 1 ml DMEM supplemented with 0.3% agar and 5% FBS with or without the addition of CM from G22V M-Ras scp-2 cells adjusted to a final concentration of 50%. At day 14, the colonies were photographed (**A**) or counted (**B**). **A.** Photomicrographs of colonies grown at the indicated cell densities with or without CM from G22V M-Ras scp-2 cells. **B.** Cloning efficiency is defined as the number of colonies formed as a percentage of the total number of cells plated. *Columns,* mean; *bars,* SD. Results are representative of three experiments.

Parental scp-2 (1.25x10^5/ml)
+
G22V M-Ras scp-2 at :

1.25 x 10^4/ml

1.25 x 10^5/ml

1.25 x 10^4/ml
+
CM

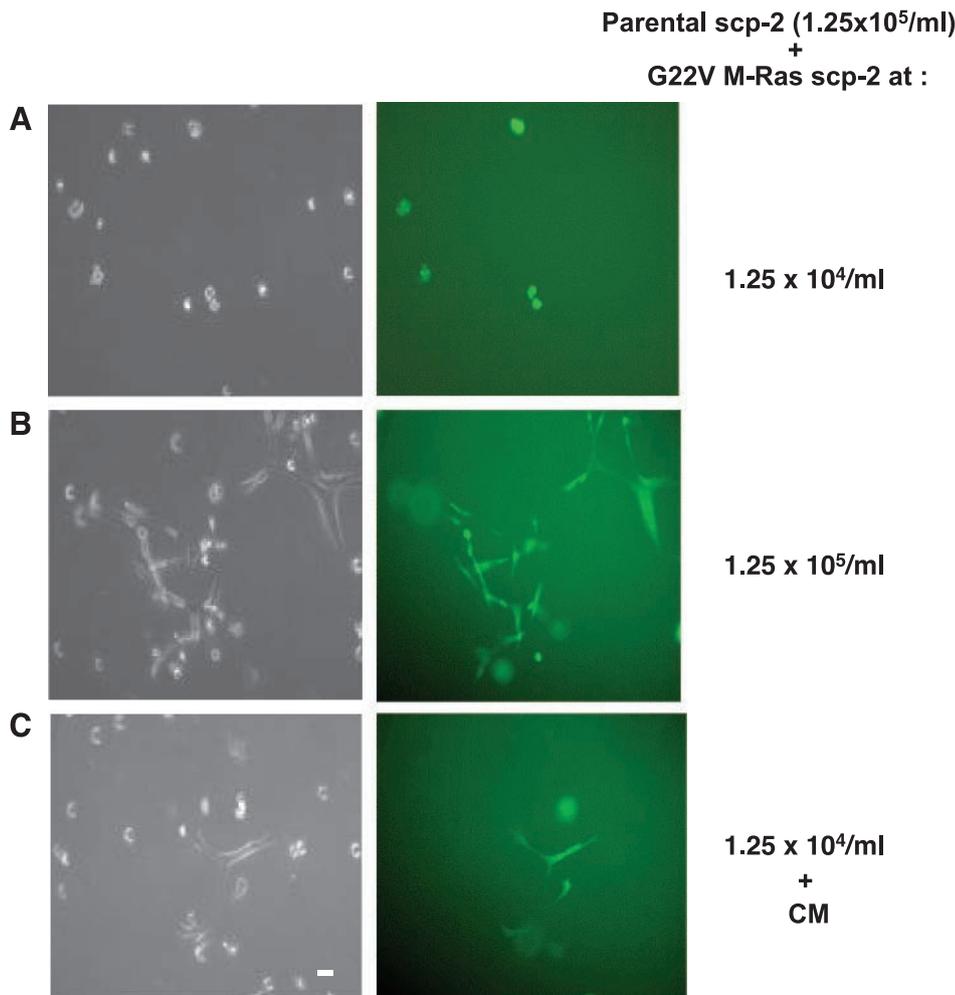**FIGURE 4.** Invasion of Matrigel by scp-2 cells expressing activated M-Ras depends on the cell density or the presence of the CM. Parental cells that did not express GFP (1.25 $\times$ 10^5) were mixed with scp-2 cells expressing G22V M-Ras and GFP at either a low density 1.25 $\times$ 10^4 (**A** and **C**) or a high density 1.25 $\times$ 10^5 (**B**) in a final volume of 1 ml and plated on Matrigel in the absence (**A** and **B**) or presence (**C**) of a 25% dilution of 50-fold concentrated medium conditioned by G22V M-Ras scp-2 cells. After 24 h in culture, the bottom of the dish was photographed to show the cells that had invaded through the Matrigel layer. *Bar,* 20 μm.

factors in activation of these pathways, we investigated the effect of CM from G22V M-Ras scp-2 cells on activation of Erk and Akt. We first cultured G22V M-Ras scp-2 cells in fresh serum-free medium for 6 h to determine whether there was activation of Erk or Akt in the absence of FBS or insulin. Using antibodies specific for activating phosphorylations of Erk or Akt, we observed that levels of phospho-Erk or phospho-Akt dropped to very low or undetectable levels in the absence of serum and insulin. We then tested the ability of CM from G22V M-Ras scp-2 to activate Erk or Akt in cells that had been cultured in serum-free DMEM for 6 h. As a positive control, we also included samples of cells that were stimulated with either 5% FBS or 12-*O*-tetradecanoylphorbol-13-acetate (TPA). Stimulation with CM from G22V M-Ras scp-2 cells induced a rapid activation of Erk (Fig. 5A). This was comparable in magnitude with that induced by FBS and was sustained for more than 1 h. Activation of Akt was also observed, although the signal was much weaker than that induced by FBS (Fig. 5B).

Medium Conditioned by scp-2 Cells Expressing G22V M-Ras Induced Scattering of MDCK Cells

One candidate for the component of CM that enabled G22V M-Ras scp-2 cells to survive in serum-free medium, to grow as colonies in soft agar, and to migrate and invade the artificial basement membrane was HGF/SF. It was reported that HGF/SF was produced by a murine mammary carcinoma cell line SP-1 and that antibodies that neutralized HGF/SF blocked the ability of SP-1 cells to invade the Matrigel (21). To investigate whether HGF/SF was present in the CM of G22V M-Ras scp-2 cells, we used MDCK, a canine kidney epithelial cell line that responds to HGF/SF with a characteristic, dose-dependent dissociation of epithelial monolayers and the migration/scattering of individual cells. As shown in Fig. 6, CM from G22V M-Ras scp-2 cells clearly induced scattering of MDCK cells. In contrast, CM from GFP scp-2 cells had no effect on epithelial layers of MDCK cells. The scatter-inducing activity in CM from G22V M-Ras scp-2 cells was completely blocked by the inclusion of antibodies that neutralized the activity of HGF/SF. As a positive control, we showed that recombinant HGF/SF induced scattering and that its effect was similarly neutralized by the neutralizing antibodies. Control antibodies of an irrelevant specificity failed to inhibit the ability of the CM to induce scattering of MDCK cells (data not shown). We concluded that expression of activated M-Ras in scp-2 cells led to the production of biologically significant amounts of HGF/SF.

A

Time course

| | Conditioned Medium | | | | | | FBS | TPA | DMSO |
| | 0 | 3' | 10' | 30' | 1h | 2h | 2h | 10' | 10' |

◄ ERK-(p)

◄ β-actin

B

Time course

| | Conditioned Medium | | | | | FBS | TPA | DMSO |
| | 0 | 3' | 10' | 30' | 1h | 2h | 10' | 10' |

◄ PKB-(p)

◄ β-actin

FIGURE 5. Medium conditioned by scp-2 cells expressing activated M-Ras induced activation of Erk and Akt. G22V M-Ras scp-2 cells (2×10^6) plated in 10 cm dishes were starved for 6 h at 37°C in DMEM followed by stimulation with 10 ml DMEM and 50% of CM from G22V M-Ras scp-2 cells. Other aliquots of cells were treated with TPA or 5% FBS as positive controls or DMSO as a vehicle control. Shown are immunoblots with antibodies recognizing phospho-Erk (**A**) or phospho-Akt (**B**). The loading controls were performed by stripping and reblotting the same membrane with antibodies recognizing β-actin.

Control

+ CM GFP

+ CM G22V M-Ras

+ CM G22V M-Ras
+ anti-HGF (4μg/ml)

+ rHGF

+ rHGF
+ anti-HGF (2μg/ml)

FIGURE 6. Medium conditioned by scp-2 cells expressing G22V M-Ras stimulated the scattering of MDCK cells. As described above, epithelial monolayers of MDCK cells were incubated for 24 h with 1 ml of medium alone (*Control*) or 1 ml of a 25% dilution of 50-fold concentrated medium conditioned by G22V M-Ras scp-2 cells (*CM G22V M-Ras*) or GFP scp-2 cells (*CM GFP*) or 1 ml of a 25% dilution of 50-fold concentrated CM G22V M-Ras that had been premixed with 4 μg/ml antibodies to HGF/SF (*anti-HGF*). Also shown are parallel cultures in 1 ml medium containing 25 ng recombinant HGF/SF (*rHGF*) or 1 ml medium containing 25 ng recombinant HGF/SF premixed with 4 μg/ml anti-HGF/SF antibodies. Results shown are representative of three independent experiments. *Bar,* 40 μm.

HGF/SF Is Both Necessary and Sufficient to Support Multiple Aspects of the Transformed Phenotype of scp-2 Cells Expressing Activated M-Ras

We next asked whether HGF/SF present in medium conditioned by G22V M-Ras scp-2 accounted for the effects of the CM we had observed. We investigated the effect of neutralizing anti-HGF/SF antibodies on the ability of CM from G22V M-Ras scp-2 cells to maintain the numbers of G22V M-Ras scp-2 cells plated at low density. The presence of anti-HGF/SF antibodies significantly abrogated the ability of the CM to maintain the number of G22V M-Ras scp-2 cells (Fig. 7A).

Likewise, the presence of neutralizing HGF/SF with anti-HGF/SF antibodies also abolished the ability of the CM to promote anchorage-independent growth in terms of both the number and the size of the colonies that grew (Fig. 7B).

We next asked whether HGF/SF was sufficient to account for the effects of the CM. As shown in (Fig. 8A), HGF/SF was able to replace CM in maintaining the numbers of G22V M-Ras scp-2 cells plated at low density in the absence of serum and insulin and to adhere to the plastic and survive. Likewise, HGF/SF was sufficient to promote the anchorage-independent growth of G22V scp-2 cells in soft agar (Fig. 8B). As was
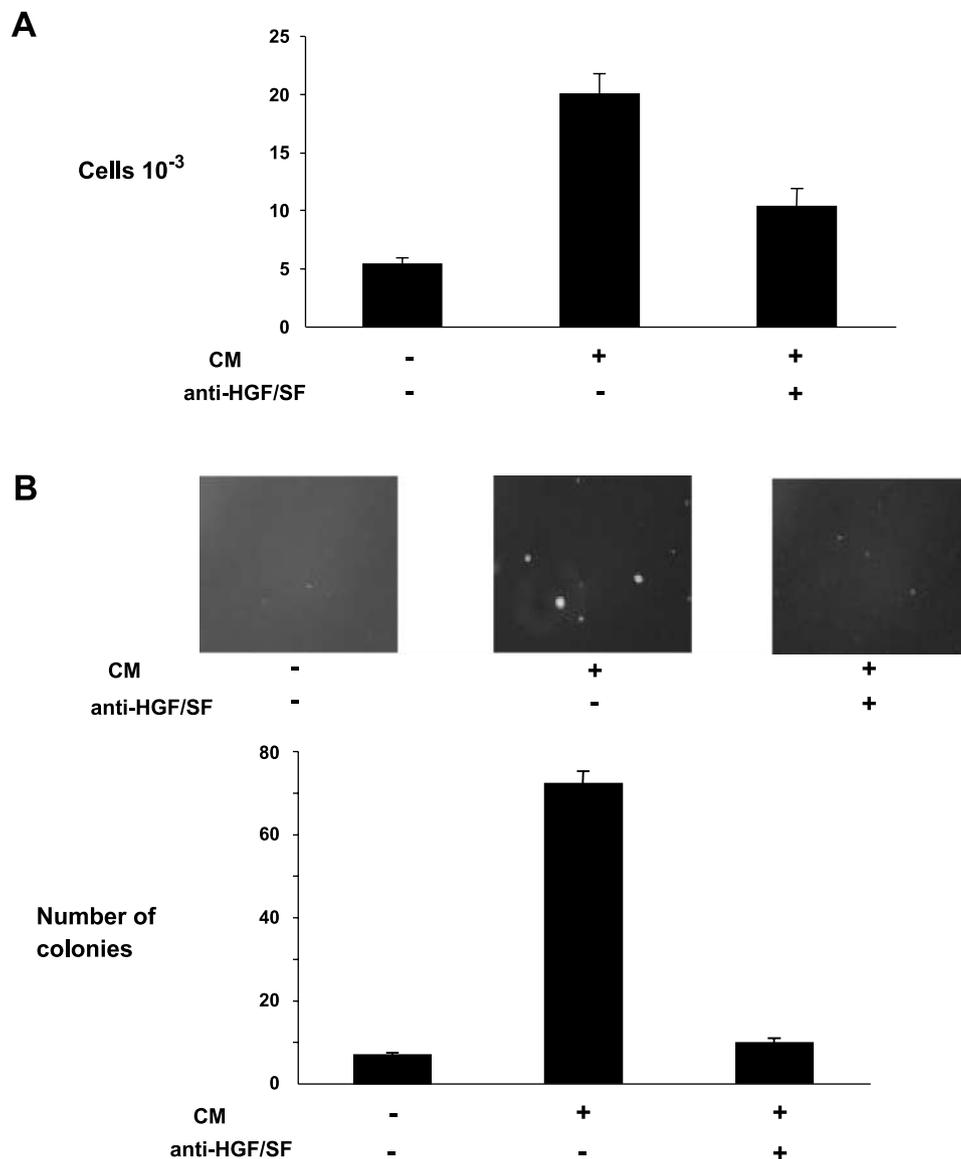

FIGURE 7.   The ability of CM to maintain the cell number or anchorage-independent growth is dependent on the activity of HGF/SF. **A.** G22V M-Ras scp-2 were cultured at 2.5×10^4/ml in a 5 ml DMEM supplemented with CM G22V M-Ras adjusted to a final concentration of 50% or 5 ml DMEM supplemented with CM G22V M-Ras adjusted to a final concentration of 50% that had been premixed with 5 μg/ml HGF/SF neutralizing antibody. After 4 days, cells were counted. **B.** Photomicrographs of colonies and the average numbers of colonies/dish grown from G22V M-Ras scp-2 cells that were plated for 14 days in 1 ml DMEM supplemented with 0.3% agar and 5% FBS with CM G22V M-Ras adjusted to 50% or 1 ml of the same medium to which HGF/SF neutralizing antibodies (5 μg/ml) had been added.

A

+PBS   + HGF/SF 25 ng/ml

B

C

+PBS   + HGF/SF 25 ng/ml   + HGF/SF 25 ng/ml
                                                + Anti-HGF 4 µg/ml

D   0   HGF TPA DMSO

← Erk-(p)

← β-actin

FIGURE 8. Recombinant HGF/SF is sufficient to maintain number of cells, anchorage-independent growth, and invasion of Matrigel by G22V M-Ras scp-2 cells when plated at low density. **A.** G22V M-Ras scp-2 cells were cultured for 48 h at low cell density (2.5×10^4/ml) in serum- and insulin-free DMEM in the presence of HGF/SF (25 ng/ml) or PBS as a control. Note the increased numbers of cells in the presence of HGF/SF. **B.** G22V M-Ras scp-2 cells (10^3) were plated in 1 ml DMEM supplemented with 0.3% agar and 5% FBS and either PBS, HGF/SF (25 ng/ml), or HGF/SF (25 ng/ml) that had been preincubated with 4 µg/ml anti-HGF/SF antibodies for 30 min. Shown are the colonies after 14 days in culture. **C.** G22V M-Ras scp-2 cells were plated at low cell density (2×10^3/ml) on Matrigel for 24 h in 1 ml medium supplemented with 4% FBS containing PBS, HGF/SF, or HGF/SF preincubated with 4 µg/ml anti-HGF/SF antibodies. The photomicrographs of the bottom of the dishes show the cells that have invaded through the Matrigel to the bottom of the dish in the cultures supplemented with HGF/SF as well as the spherical mammospheres. **D.** Prestarved G22V M-Ras scp-2 cells were stimulated for 10 min with HGF/SF (25 ng/ml) or TPA (100 nM) and lysed in lysis buffer. WCLs (25 µl) were subjected to SDS-PAGE and immunoblotting with antibodies specific for activating phosphorylations on Erk. Loading controls were performed by stripping and reblotting of the same membrane with antibodies recognizing β-actin.
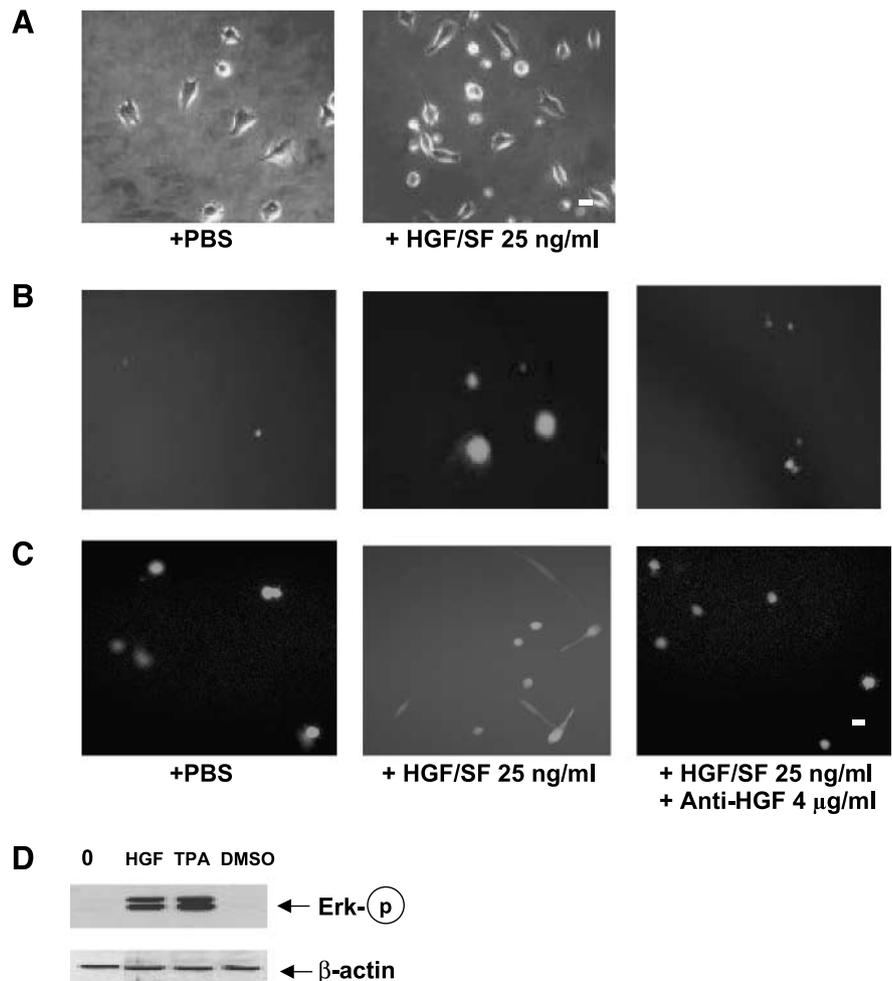
the case with CM, HGF/SF did not promote the anchorage-independent growth of parental scp-2 cells (data not shown). Finally, the addition of HGF/SF to cultures of G22V scp-2 cells plated on the Matrigel at low cell density induced the cells to migrate and invade the Matrigel (Fig. 8C). We also noted that HGF/SF strongly activated Erk mitogen-activated protein kinase in G22V scp-2 cells (Fig. 8D). Thus, HGF/SF was sufficient to replicate all of the observed effects of the CM.

Molecular Mechanisms Involved in Invasion

To investigate whether HGF/SF was involved in the invasive behavior of G22V M-Ras scp-2 cells, we plated these cells at a relatively high cell density (2.5×10^5 cells/ml) on Matrigel. In parallel, we set up cultures to which were added varying concentrations of antibodies that neutralized HGF/SF. We observed that the anti-HGF/SF antibodies inhibited both formation of the networks of cell bundles and invasion of cells through the Matrigel to the bottom of dish (Fig. 9B). Inclusion of anti-HGF/SF antibodies also partially inhibited the growth of G22V M-Ras scp-2 cells on Matrigel (Fig. 9C). Control immunoglobulins not specific for HGF/SF had no effect on invasion or growth (data not shown).

Invasion depends on the action of a variety of proteases (22, 23). To determine whether expression of activated M-Ras

induced the production of proteases, we used zymography with various protein substrates. We observed that CM from G22V M-Ras scp-2 cells, but not from GFP scp-2 cells, contained two proteases of apparent molecular mass of 92 and 72 kDa, respectively (Fig. 10A). These electrophoretic mobilities correlated with those of the uncleaved forms of matrix metalloproteinases (MMPs) 9 and 2 (pro-MMP-9 and pro-MMP-2) and low amount of activated MMP-2. Using zymography with two substrates (*i.e.*, plasminogen and gelatin), we also detected an additional enzymatic activity in CM from G22V M-Ras scp-2 cells but not from GFP scp-2 cells. This proteolytic activity had an apparent M_r of 45 kDa (Fig. 10B) and was blocked when phenylmethylsulfonyl fluoride (PMSF), an inhibitor of serine proteases, was included in the incubation buffer. Moreover, no enzymatic activity was detected if plasminogen was omitted (data not shown). By these criteria, we identified this 45-kDa band as corresponding to a serine protease of the urokinase-type plasminogen activator (uPA) family.

We used a broad-spectrum inhibitor of MMPs, BB94, to evaluate the role of MMPs in the behavior of G22V M-Ras scp-2 cells in Matrigel. BB94 induced a dose-dependent inhibition of the formation of the network of cell bundles and of the migration and invasion of cells through the Matrigel to the bottom of the dish (Fig. 10C).
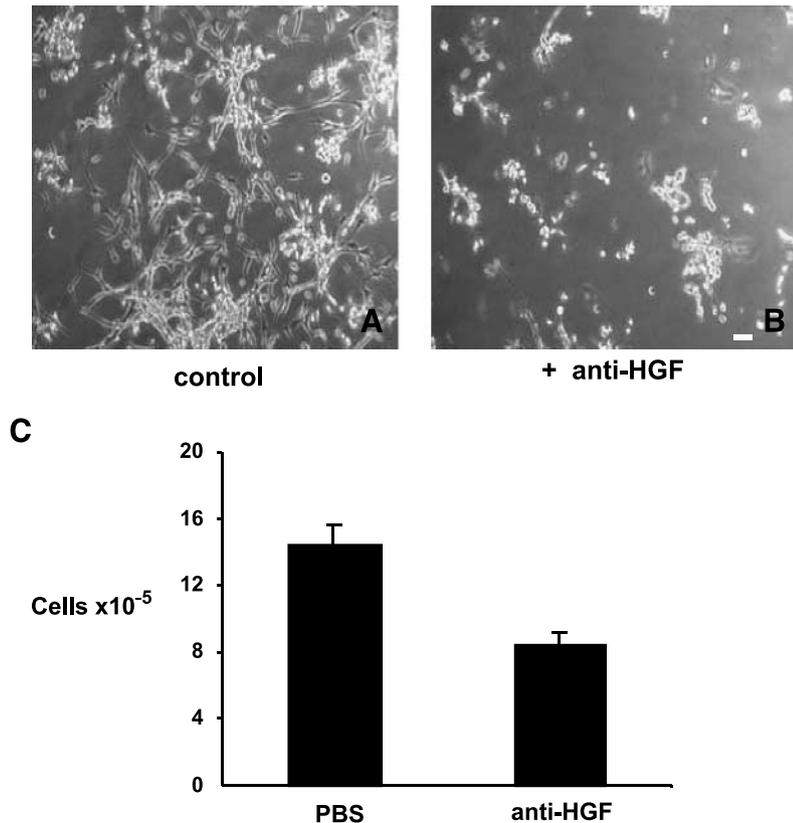
FIGURE 9. Invasive growth of scp-2 cells expressing activated M-Ras depends on the autocrine factor HGF/SF. G22V M-Ras scp-2 cells (2.5×10^5/ml) were maintained in 0.5% FBS and 5 μg/ml insulin and plated on Matrigel precoated on 35 mm dishes with PBS as control (**A**) or a final concentration of 10 μg/ml of an antibody neutralizing HGF/SF (**B**). The photographs were taken after culture for 18 h. *Bar,* 40 μm. **C.** After 72 h in culture, the cells were released from Matrigel by digestion with dispase and were counted.

We also tested the effects of inhibitors of the Erk and PI3K pathways on G22V M-Ras scp-2 cells cultured on Matrigel. We observed that an inhibitor of Erk activation, the mitogen-activated protein kinase kinase (MEK) 1/2 inhibitor PD98059, blocked both the formation of the networks of cell bundles and the invasion of Matrigel (Fig. 11A). Nevertheless, the phenotype of G22V M-Ras scp-2 cells cultured with PD98059 did not revert to that of parental scp-2 cells in that, in the presence of PD98059, G22V M-Ras scp-2 cells formed large aggregates rather than the smaller mammospheres formed by the parental cells. The concentration of PD98059 used blocked the activation of Erk without affecting the activity of PI3K as monitored by phosphorylation of protein kinase B (Fig. 11B). The inhibitory effect of PD98059 was not due to decreased cell viability, as there was no difference in the numbers of cells that took up propidium iodide between cultures treated with PD98059 and controls. In contrast, the presence of PI3K inhibitor LY294002, at concentrations that completely blocked the phosphorylation of Akt (data not shown), had only a slight inhibitory effect on the formation of the networks of cell bundles and on invasion of Matrigel (Fig. 11A). We also tested the effect of PD98059 and anti-HGF/SF on the expression of MMPs. Inclusion of PD98059 in the culture of G22V M-Ras scp-2 slightly inhibited the expression of both pro-MMP-2 and activated MMP-2 and less so of pro-MMP-9. Inclusion of antibodies that neutralized HGF/SF significantly inhibited the secretion of pro-MMP-9 and pro-MMP-2 and its activated form in a dose-dependent manner (Fig. 11C).

Discussion

Our observations demonstrate the importance of autocrine mechanisms involving HGF/SF in multiple aspects of the transformed phenotypes of scp-2 cells expressing activated M-Ras. Anchorage-independent growth in agar, invasion of Matrigel, and maintenance of viable cell numbers under serum-free conditions were all cell density dependent and were all promoted by CM. In each case, the effects of CM were specifically inhibited by inclusion of antibodies that neutralized HGF/SF, indicating that autocrine HGF/SF was necessary for expression of these phenotypes. In addition, in all cases, recombinant HGF/SF could replace the CM, indicating that HGF/SF was sufficient to account for these phenotypes.

The fact that levels of Erk or Akt activation dropped when G22V M-Ras scp-2 cells were deprived of serum and insulin and were plated in the fresh medium (Fig. 5) indicates that stable expression of activated M-Ras was not sufficient to directly sustain high levels of activation of Erk or PI3K. Our observation that CM or HGF/SF induced a sustained activation of Erk in G22V M-Ras scp-2 cells suggests strongly that autostimulatory factors are likely to account for the elevated levels of activated Erk that we observed in lysates of tumors formed from G22V M-Ras scp-2 cells (18). The notion that levels of Erk activity are maintained by autocrine HGF/SF rather than by a direct effect of G22V M-Ras is consistent with data that G22V M-Ras interacts only weakly with Raf-1 and that overexpression of activated M-Ras is a relatively weak activator of the Erk pathway (5, 11, 12).

Our previous report that expression of activated M-Ras resulted in the induction of epithelial-mesenchymal transition (EMT;18) and, as shown here in the secretion of HGF/SF, is consistent with observations that expression of HGF/SF is tightly restricted to mesenchymal but not epithelial cells (24). Indeed, there is evidence that HGF/SF can induce EMT in some cells (25). However, in another murine mammary epithelial cell line, EpH4, HGF/SF induced only a reversible change in morphology and did not induce biochemical hallmarks of EMT such as up-regulation of vimentin (26). Secretion of HGF/SF has been reported to occur in most human mammary cancers, where its production is correlated with invasive growth and metastasis (27–29). Our observation that neutralizing antibodies to HGF/SF blocked the invasion of Matrigel by G22V M-Ras scp-2 cells (Fig. 9) is consistent with the report that a murine mammary carcinoma SP-1 secreted HGF/SF and that neutralizing antibodies to HGF/SF blocked the ability of SP-1 cells to invade (21). Our observation that an inhibitor of MMPs, BB94, blocked the invasion of Matrigel by G22V M-Ras scp-2 cells is consistent with our observation that inhibitors of Erk activation inhibited secretion of HGF/SF by G22V M-Ras scp-2 cells (Fig. 11C) as well as evidence from others that HGF/SF stimulates invasion through induction of MMPs (30, 31). Likewise, our finding that an inhibitor of the Erk pathway, PD98059, blocked invasion (Fig. 11A) may reflect, at least in part, the role of Erk in HGF/SF-mediated induction of MMPs essential for invasion of cells through Matrigel (32, 33). HGF/SF may also be responsible for the induction of uPA that we observed in G22V M-Ras cells, as this was the case in a NIH/3T3 cell line expressing human c-Met, HGF/SF receptor (34). Our findings that the ability of CM to maintain the numbers of G22V M-Ras scp-2 cells plated at low cell densities was dependent on HGF/SF (Fig. 7A) and that HGF/SF had the same activity as the CM from G22V M-Ras scp-2 cells (Fig. 8) is consistent with other reports that HGF/SF promotes the cell growth and inhibits apoptosis (35, 36). Thus, up-regulation of HGF/SF in G22V M-Ras scp-2 cells is likely to account for multiple aspects of the transformed phenotype.

The signaling mechanisms through which activation of pathways downstream of M-Ras induces the secretion of HGF/SF remain to be established. There is evidence for the involvement of Erk kinases and STAT3 in activation of HGF/SF promoter (37). However, given our observation that G22V M-Ras scp-2 cells do not exhibit marked activation of Erk when cultured at low density in the absence of serum and insulin (Fig. 5), and other evidence that activated M-Ras is a poor activator of Erk pathway (5, 11, 12), it is unlikely that expression of activated M-Ras is up-regulating HGF/SF by directly activating the Erk pathway. Apparently, HGF/SF is enhancing its own production. Alternatively, HGF/SF production may be enhanced by an as yet uncharacterized effector or combination of effectors downstream of activated M-Ras.

We noted that scp-2 cells expressing activated M-Ras were much more responsive to the stimulatory effects of CM or HGF/SF in enhancing survival at low cell density. Moreover, neither CM nor HGF/SF enabled the parental scp-2 cells to grow in agar or invade Matrigel. One possibility was that the expression of activated M-Ras resulted in increased expression of the receptor for HGF/SF, c-Met. However, we have seen no
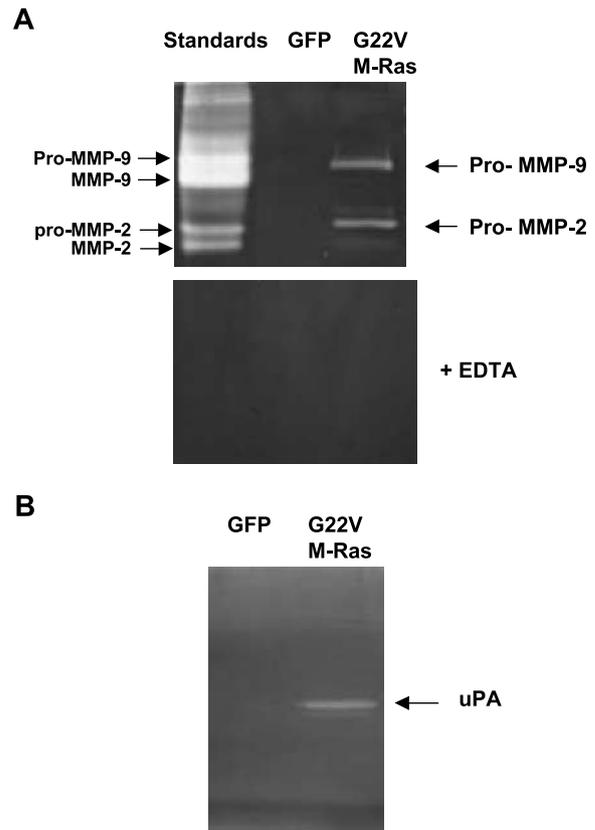

FIGURE 10. Expression of activated M-Ras in scp-2 cells results in secretion of MMPs, the activity of which is necessary for invasion of Matrigel. CM from GFP scp-2 or G22V M-Ras scp-2 cells was collected and concentrated 5-fold and CM (10 μl) was subjected to 10% SDS-PAGE under nonreducing conditions in the presence of the substrates described as follows. **A.** The gel contained 0.1% gelatin and was stained with Coomassie blue to show the electrophoretic mobility of activities that digested gelatin. Also shown is a stained control gel developed in the presence of EDTA to inhibit the activity of gelatinases like MMP-2 and MMP-9. **B.** The gel contained 0.1% gelatin and plasminogen (20 μg/ml) and was developed in the presence of 10 mM EDTA to inhibit MMPs. Coomassie staining shows an activity that cleaved plasminogen into plasmin that in turn digested the gelatin and was consistent in its M_r with uPA.

evidence for increased expression of c-Met in cells expressing activated M-Ras (unpublished observations), raising the possibility that there is synergy between signals directly downstream of activated M-Ras and activation of c-Met. It is also possible that the difference in responsiveness to HGF/SF following expression of activated M-Ras is secondary to the induction of EMT. Our results from the treatment of G22V M-Ras scp-2 cells with the MEK-1/2 inhibitor PD90589 demonstrated the importance of the Erk pathway for invasion of Matrigel and the generation of the characteristic pattern of networks of bundles of cells. However, the fact that, even in the presence of PD90589, G22V M-Ras scp-2 cells still formed large aggregates rather than the small mammospheres formed by the parental scp-2 cells (Fig. 11A) indicates that key aspects of the phenotype are maintained in the absence of significant Erk activity. It has been reported that PD90589 blocked the induction of EMT by transforming growth factor-β
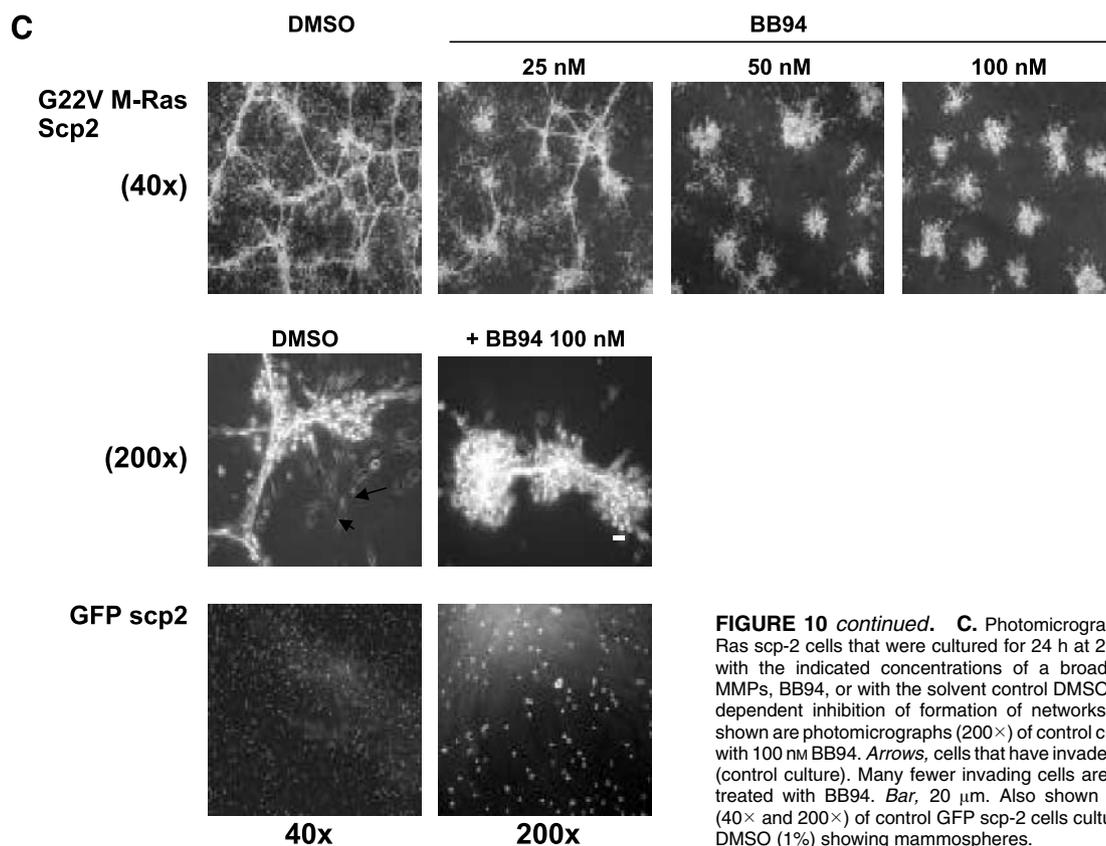
C

FIGURE 10 *continued*.   **C.** Photomicrographs (40×) of G22V M-Ras scp-2 cells that were cultured for 24 h at 2.5 × 10^5/ml in Matrigel with the indicated concentrations of a broad-spectrum inhibitor of MMPs, BB94, or with the solvent control DMSO (1%). Shown is dose-dependent inhibition of formation of networks of cell bundles. Also shown are photomicrographs (200×) of control culture or culture treated with 100 nM BB94. *Arrows,* cells that have invaded the bottom of the dish (control culture). Many fewer invading cells are present in the culture treated with BB94. *Bar,* 20 μm. Also shown are photomicrographs (40× and 200×) of control GFP scp-2 cells cultured in the presence of DMSO (1%) showing mammospheres.

in EpH4 cells that were expressing activated H-Ras (26). In this model, maintenance of EMT, while not requiring the continued presence of transforming growth factor-β, did require the constant presence of p21 Ras activity. It will be interesting in future studies with our model to determine whether suppression of M-Ras activity also results in reversion of EMT.

Materials and Methods
Materials
Antibodies recognizing phospho-Erk (Thr^{202}/Tyr^{204}) or phospho-Akt (Ser^{473}) were purchased from Cell Signaling (Beverly, MA). Antibodies against β-actin were from Sigma Chemical Co. (Sigma-Aldrich, St. Louis, MO). Inhibitors of MEK kinase (PD98059) or PI3K (LY294002) were purchased from Upstate Biotechnology, Inc. (Charlottesville, VA). An inhibitor of MMPs, BB 94, was kindly provided by Dr. Georgina Butler (Dr. Chris Overall's laboratory, University of British Columbia, Vancouver, BC, Canada).

Vectors and Cells
The retroviral vectors used were based on pMXpie and were bicistronic using an internal ribosomal entry site to express a constitutively activated mutant of murine M-Ras (G22V M-Ras) and the enhanced GFP from the same mRNA. Culture of murine scp-2 cells (kindly provided by Dr. Cal Roskelly, Department of Anatomy, University of British Columbia) and

their retroviral transduction by a constitutively activated mutant of murine M-Ras (G22V M-Ras) and GFP or, as a control, GFP alone were carried out as described (11, 38). Cells so prepared are referred as "G22V M-Ras scp-2" or "GFP scp-2," respectively.

Preparation of the CM
G22V M-Ras scp-2 cells or GFP scp-2 cells were cultured to 80–90% confluency in DMEM containing 5% FBS, 5 μg/ml insulin, and 50 μg/ml gentamicin. After washing thoroughly with serum-free DMEM, the cells were cultured in serum-free DMEM for 24 h. The medium so conditioned was collected and clarified by centrifugation at 4000 rpm for 5 min. For zymography, CM was concentrated by filtering through a 10-kDa cutoff filter (Millipore, Billerica, MA). For use in the cell scattering assay or Matrigel assays, the CM was concentrated 50-fold through a 5-kDa cutoff membrane using the Medium Concentrator (Fisher Technology, London, UK) and then dialyzed against DMEM overnight through 6–8-kDa cutoff dialysis membrane (Pierce Chemical Co., Rockford, IL).

Assay for Anchorage-Independent Growth
The assay was conducted as described (39) with some modifications. Briefly, 10^3 or 10^4 cells were suspended in 1 ml DMEM containing a final concentration of 0.3% agar and 5%
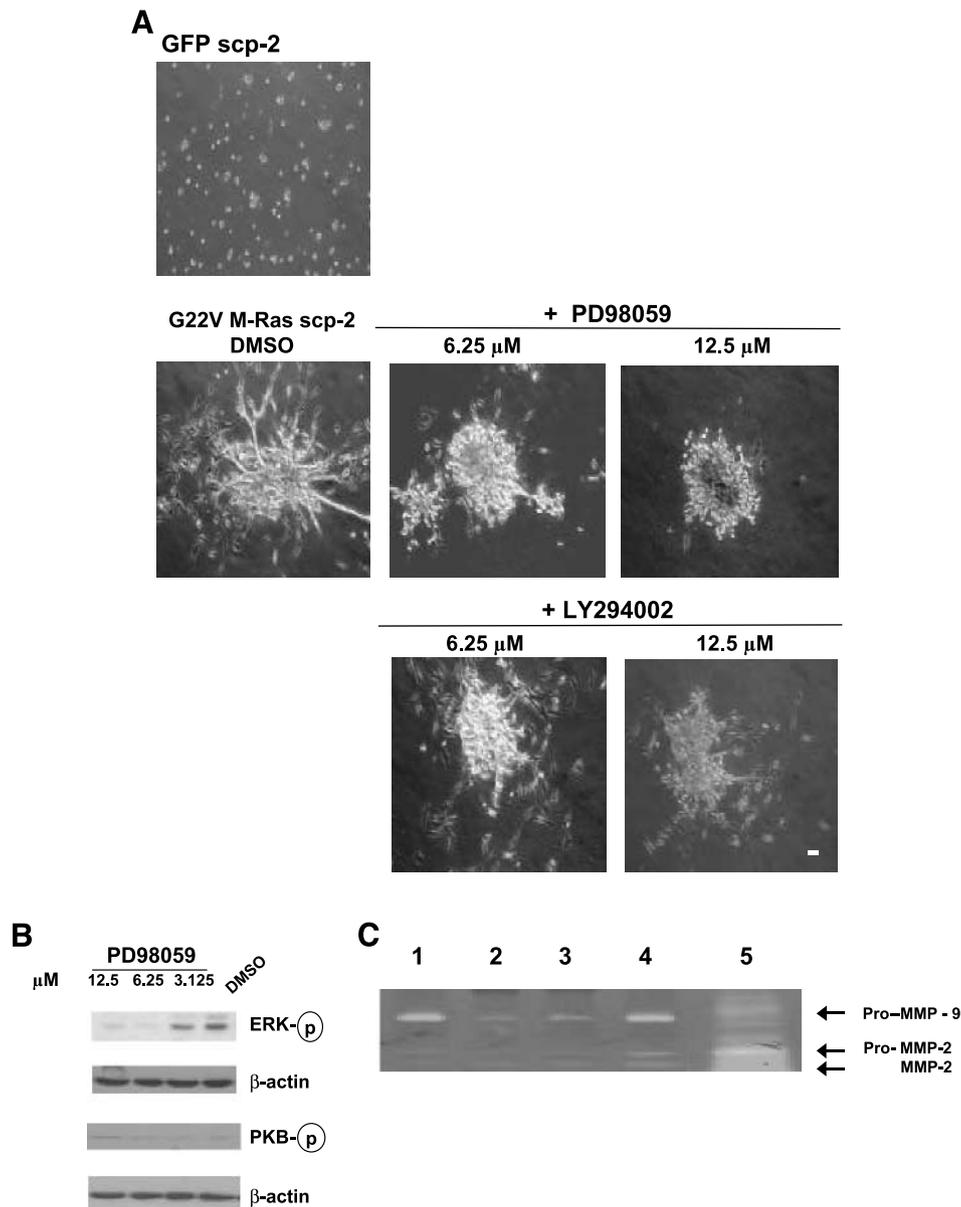
FIGURE 11. Inhibition of Erk activation inhibits invasion of Matrigel by G22V M-Ras scp-2 cells. **A.** G22V M-Ras scp-2 cells were plated on Matrigel for 24 h at 2.5 × 10⁵/ml in the presence of the indicated concentrations of the MEK-1/2 inhibitor PD98059 or the PI3K inhibitor LY294002 or a solvent control, DMSO (1%). The bottoms of the dishes were photographed after 24 h to show the numbers of cells that had invaded through the Matrigel and had spread out on the bottom of the dish. *Bar,* 20 μm. **B.** G22V M-Ras scp-2 cells were cultured for 24 h in the presence of the indicated concentrations of the MEK-1/2 inhibitor PD98059 or DMSO (1%). Shown are immunoblots of SDS-PAGE of cell lysates using antibodies recognizing phospho-Erk or phospho-Akt. The loading controls were performed by stripping and reblotting the same membrane with antibodies recognizing β-actin. **C.** CM was generated from G22V M-Ras scp-2 cells that had been starved for 6 h and were cultured at 2.5 × 10⁵/ml in DMEM containing PD98059 (12.5 μM; *1*), anti-HGF/SF antibodies at a concentration of 20 μg/ml (*2*) or 10 μg/ml (*3*), or DMSO as a control (*4*). A positive control consisting of a supernatant from a cell expressing MMP-9 and MMP-2 was included (*5*). After 24 h of culture, CM was collected and concentrated 10-fold and the numbers of cells were counted. Volumes of concentrated CM corresponding to 4 × 10⁵ cells were subjected to 10% SDS-PAGE under nonreducing conditions and zymography was performed using 0.1% gelatin as a substrate.

FBS with or without the addition of CM from G22V M-Ras scp-2 cells adjusted to a final concentration of 50%. The mixture was plated on top of a solidified layer of 0.5% agar containing the same concentration of CM. Numbers of colonies were scored at day 14 using a colony counting microscope (Olympus, Shibuya-Ku, Tokyo, Japan) and photographed using a fluorescence dissecting microscope (Leica MZ FL III, Heerbrugg, Swiss).

Matrigel Invasion Assay

The assay was carried out as described (40, 41) with some modifications. Briefly, 2×10^5 cells of polyclonal populations of scp-2 cells expressing activated M-Ras and GFP or GFP alone were plated on top of a layer of Matrigel (Collaborative Research, Bedford, MA) coated on a 35 mm dish in DMEM containing 2% FBS, 5 μg/ml insulin, and 50 μg/ml gentamicin. After incubation for 24 h, the medium was replaced with DMEM containing 0.5% FBS, 5 μg/ml insulin, and 50 μg/ml gentamicin. Formation of the mammospheres or networks was scored 24–48 h after incubation of the cultures. Invasion was assessed by observing the number of cells that had reached the focal plane of the bottom of the dish.

Cell Scattering Assay

Cell scattering assays were performed as described (42) with some modifications. Briefly, MDCK cells (kindly provided by Dr. Auersperg, Department of Obstetrics and Gynecology, University of British Columbia) were plated at the density of 10^4 cells/well of a 24-well plate (Nunc, Roskilde, Denmark) in DMEM containing 5% FBS and were incubated for 24 h to ensure the formation of well-defined patches of epithelial cells. After washing, the cells were incubated in 5% FBS in DMEM with or without 25–50 ng/ml recombinant HGF/SF (R&D Systems, Inc., Minneapolis, MN) as a positive control and a 25% dilution of 50-fold concentrated CM from G22V M-Ras scp-2 cells or GFP scp-2 cells. In other experiments, anti-HGF/SF neutralizing antibody (goat origin) or a control goat anti-mouse IgG were premixed with CM or HGF/SF as indicated and the mixture was added to MDCK cells. The effect of CM or HGF/SF was scored after incubation for 24 h.

Immunoblots

After washing thrice with cold PBS, cells were lysed in lysis buffer [50 mM Tris-HC1 (pH 7.5), 150 mM NaCl, 1% NP40, 5 mM EDTA, 10 μg/ml each of leupeptin, aprotinin, and soybean trypsin inhibitors, 0.7 μg/ml pepstatin, and 40 μg/ml PMSF]. Concentrations of proteins in the whole cell lysate (WCL) were quantified with Pierce protein assay kit (Pierce Chemical). WCLs (20–40 μg) were denatured by adding 5× Laemmli sample buffer [10% SDS, 50% glycerol, 200 mM Tris-HCl (pH 6.8), and bromophenol blue] and boiled for 3 min. The denatured proteins were then subjected to 12% SDS-PAGE. After separation, the proteins were transferred to nitrocellulose membranes. Membranes were blocked with 5% skimmed milk for 30 min, washed in 0.1% Tween 20 in TBS (TBS-T), and exposed to primary antibodies at 4°C overnight. After washing in TBS-T, membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (DAKO, Carpinteria, CA). After three more washes with TBS-T, horseradish peroxidase antibodies were detected using the enhanced chemiluminescence reagent according to the manufacturer's instructions (Amersham, Oakville, ON, Canada).

Zymography

CM from GFP scp-2 (CM GFP) or G22V M-Ras scp-2 (CM G22V M-Ras) was analyzed on SDS-PAGE containing specific substrates for different enzymes (21, 43). Briefly, to detect gelatinases, 5-fold concentrated CM from G22V M-Ras scp-2 cells or GFP scp-2 cells were mixed with nonreducing Laemmli sample buffer and were subjected to 10% SDS-PAGE containing 0.1% gelatin (Sigma Chemical, St. Louis, MO). The gel was washed for 2 h in an exchanging buffer [100 mM Tris-HCl (pH 7.4), 15 mM CaCl$_2$, and 2.5% Triton X-100]. After washing for 1 h in the incubation buffer (the same as the exchanging buffer but without Triton X-100), the gel was incubated overnight in the same buffer in the presence or absence of 10 mM EDTA (which blocked gelatinase activity). The gels were then stained with Coomassie blue for 1 h and destained with 45% methanol-12% acetic acid. Gelatinase activities were visible as clear bands on the blue background. To detect uPA, we used double-substrate zymography. Briefly, in a 10% polyacrylamide gel, we included 0.1% gelatin and 20 μg/ml plasminogen (Sigma Chemical), the latter acting as a substrate for plasminogen activator (a serine proteinase). Plasminogen activator, if present in the sample, will cleave the plasminogen into the active enzyme plasmin, which in turn will degrade the gelatin. The gel was incubated in a buffer [Triza base (pH 8.1)] containing 10 mM EDTA to inhibit the direct proteolysis of gelatin by MMPs in the sample. We performed the assay in the presence or absence of the serine protease inhibitor, PMSF, in the incubation buffer. We also ran control gels in which we omitted plasminogen.

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Multiple Aspects of the Phenotype of Mammary Epithelial Cells Transformed by Expression of Activated M-Ras Depend on an Autocrine Mechanism Mediated by Hepatocyte Growth Factor/Scatter Factor [1] 1Canadian Institute of Health Research and Canadian Breast Cancer Research Initiative.

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