LGII, a Putative Tumor Metastasis Suppressor Gene, Controls in Vitro Invasiveness and Expression of Matrix Metalloproteinases in Glioma Cells through the ERK1/2 Pathway*

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Gliomas take a number of different genetic routes in the progression to glioblastoma multiforme, a highly invasive variant that is mostly unresponsive to current therapies. Gliomas express elevated levels of matrix metalloproteinases (MMPs), which have been implicated in the control of proliferation and invasion as well as neovascularization. Progressive loss of LGII expression has been associated with the development of high grade gliomas. We have shown previously that the forced re-expression of LGII in different glioma cells inhibits proliferation, invasiveness, and anchorage-independent growth in cells null for its expression. Here, using Affymetrix gene chip analysis, we show that re-expression of LGII in T98G cells results in the down-regulation of several MMP genes, in particular MMP1 and MMP3. LGII expression also results in the inhibition of ERK1/2 phosphorylation but not p38 phosphorylation. Inhibition of the MAPK pathway using the pharmacological inhibitors PD98059, U0126, and SB203580 in T98G LGII-null cells inhibits MMP1 and MMP3 production in an ERK1/2-dependent manner. Treatment of LGII-expressing cells with phorbol myristate acetate prevents the inhibition of MMP1/3 and restores invasiveness and ERK1/2 phosphorylation, suggesting that LGII acts through the ERK/MAPK pathway. Furthermore, LGII expression promotes phosphorylation of AKT, which leads to phosphorylation of Raf1Ser-259, an event shown previously to negatively regulate ERK1/2 signaling. These data suggest that LGII plays a major role in suppressing the production of MMP1/3 through the phosphatidylinositol 3-kinase/ERK pathway. Loss of LGII expression, therefore, may be an important event in the progression of gliomas that leads to a more invasive phenotype in these cells.

Glioblastoma multiforme is the most common malignant tumor of the adult central nervous system and has a median survival time of less than 12 months (1–2). The highly lethal nature of this tumor results from the acquisition of an invasive phenotype that allows the tumor cells to infiltrate surrounding brain tissue. Despite considerable heterogeneity in the genetic abnormalities detected in the various etiologies and histopathologies of these tumors (3), 90% of glioblastoma multi-formes share losses of regions on chromosome 10 (4–8). Using a positional cloning strategy, Chernova et al. (9) identified the LGII gene associated with an apparently reciprocal t(10, 19)(q24;q13) chromosome translocation in the T98G glioma cell line. LGII is expressed in low grade tumors but not in most of the high grade gliomas or permanent cell lines tested (9–10). The coincident loss of LGII expression with loss of chromosome 10 suggested that it might be important in the malignant progression of gliomas.

LGII carries a leucine-rich repeat (LRR)1 motif (9) that places it in the F20 family of LRR genes. Members of this family are predominantly involved in either receptor functions or attachment to the extracellular matrix (11). The strongest homology of the LGII LRR is with the slit2 genes (9), which are involved in the control of axonal movement in Drosophila. The loss of LGII function in glial cells at the transition from low grade to high grade tumors also suggested a possible role for this gene in controlling the invasive/migratory potential of these cells. To investigate this possible function in glioma cells, we recently expressed an exogenous copy of the wild type LGII gene in glioma cells that do not express detectable levels of LGII endogenously (12). Re-expression of LGII resulted in a significant reduction in growth potential but, significantly, almost completely suppressed the ability of these cells to invade an extracellular matrix or to form colonies in soft agar. Both of these in vitro phenotypes are indicative of a loss of malignant potential. We suggest, therefore (12), that LGII may be a member of the emerging family of genes referred to as “tumor metastasis suppressors” (13).

To determine whether LGII exerts its effect by regulating specific signaling pathways, we used oligonucleotide microarray analysis to compare the gene expression profiles from T98G cells that do not express LGII with those that are forced to re-express it. The re-expression of LGII induced the suppression of a number of extracellular matrix genes, including members of the matrix metalloproteinase (MMP) family, in particular MMP1 and MMP3. High levels of the expression of MMPs have been reported in high grade gliomas, where it is proposed that their action serves to facilitate invasion and metastasis (14, 15). Recently, Mercapide et al. (16) showed that MMP3 is responsible for the invasive phenotype in astrocytomas. Matrix metalloproteinases are a family of structurally related zinc-depen-dent neutral peptidases collectively capable of degrading essentially all components of the extracellular matrix (17).

Received for publication, December 26, 2003, and in revised form, March 23, 2004
Published, JBC Papers in Press, March 26, 2004, DOI 10.1074/jbc.M314192200

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* This work was supported in part by the NCI, National Institutes of Health Grant CA108056 and National Institutes of Health Grant NS048706-01. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 The abbreviations used are: LRR, leucine-rich repeat; DME, Dulbecco’s modified Eagle’s medium; MMP, matrix metalloproteinase; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; FBS, fetal bovine serum; MEK, MAPK/ERK kinase; PBS, phosphate-buffered saline; PISK, phosphatidylinositol 3-kinase; PPK, protein kinase C; PMA, phorbol myristate acetate; TIMP, tissue inhibitor of metalloproteinase.

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These genes have been shown to play an important role in controlled tissue remodeling in physiological situations, including developmental morphogenesis, tissue repair, and angiogenesis (18–19). The increased expression of various MMPs by peripheral tumors is strongly associated with invasive phenotypes. We now demonstrate that the re-expression of LGI1 in glioma cells suppresses ERK-dependent expression of MMP1 and MMP3, and correlates with the severe loss of in vitro invasiveness. Several pathways have been reported that lead to activation of MMPs. Using a variety of pathway-specific inhibitors, we now demonstrate that LGI1 affects MMP production predominantly by inhibiting the MAPK/ERK pathway.

MATERIALS AND METHODS

Trizol was obtained from Invitrogen. Phorbol myristate acetate (PMA) and protease and phosphatase inhibitors were obtained from Sigma. PD98059, U0126, and SB203580 were from Calbiochem. Antibodies for ERK1/2 were obtained from Upstate Signaling Solutions (Charlottesville, VA). Phospho-ERK, phospho-p38, p38 MAPK, phospho-Akt, and Raf1-phosphoserine 259 antibodies were obtained from Cell Signaling (Beverly, CA). MMP1 and MMP3 antibodies were purchased from Lab Vision (Fremont, CA).

Cell Culture and Treatments—The T98G cell line was maintained in DMEM with 10% FBS under 10% CO2. The T98G LGI1 stable transfectants were maintained in DMEM plus 10% FBS and 500 μg/ml G418 sulfate. For all inhibition assays, inhibitors were added to the cells, which were then kept overnight in serum-free medium pending supernatant collection. For PMA treatment, cells were incubated with the appropriate concentration of the inhibitor for 30 min before the addition of PMA for 10 min.

Gene Expression Analysis—Total RNA was extracted from three independently isolated T98G clones that showed exogenous expression of LGI1 and was used to prepare cRNA for hybridization to the Affymetrix U133A oligonucleotide arrays as described previously (20). The gene expression profile from these cells was compared with that obtained from T98G clones that had been stably transfected with the empty pcDNA3 vector. To compare the vector-only clones with the LGI1-expressing clones, the base line-corrected data were imported into the Affymetrix Data Mining Tool (DMT 4.0) by using the publishing tool MicroDataBase (MDB 3.0). The genes were then sorted using a count and percent tool, and only those genes showing altered expression in at least two of both the vector-only clones and LGI1+ clones (two cross-comparisons) were used to define the final list. A cutoff of an average 2-fold or greater change was selected. The second analysis approach employed the dChip program developed at Harvard for analysis of GeneChip data and was available for downloading from www.dchip.org. This approach is based on the Li and Wong statistical model (21) that performs a multi-chip analysis to calculate a probe sensitivity index of GeneChip data and was available for downloading from www.dchip.org. The .DCP files were then normalized, and functional annotation and chromosomal locations were obtained using GeneNetFx (22).

RESULTS

The predicted location of the LGI1 protein at or in the cell membrane (9), together with the membership of LGI1 in the family of F20 family members (11). To test this possibility, we compared the gene expression profile of LGI1-null glioma cells carrying the pcDNA3 vector with clones expressing exogenous LGI1. The full-length LGI1 open reading frame described by Chernova et al. (9), fused to a C-terminal FLAG epitope by PCR, was cloned into pcDNA3 and then stably transfected into T98G cells (12).

Comparative Gene Expression Analysis—RNA isolated from three independently derived T98G clones that showed exogenous expression of LGI1 (12) was used to prepare cRNA for hybridization to the Affymetrix U133A arrays. The gene expression profiles from these cells were compared with those produced from T98G clones transfected with the empty pcDNA3 vector. The data from the three clones was then filtered to display only those genes that were at least 2-fold up-regulated or 2-fold down-regulated in all three independent clones compared with the control. Of a total of 22,214 genes and
expressed sequence tags analyzed, 614 genes were up-regulated and 475 were down-regulated using the Affymetrix Data Mining Tool analysis alone. However, combining this analysis with dChip, only 189 genes were up-regulated and 15 were down-regulated. Within this series of genes it was striking that a significant number of them were associated with the extra-cellular matrix (Table I). In addition, several members of the MMP gene family also showed down-regulation. This gene family has been associated with invasion characteristics in brain tumors (23, 24). MMP1 and MMP3 were the most consistently down-regulated in all of the clones in our analysis. Only one clone showed down-regulation of MMP11 and MMP12. The up-regulation of TIMP3, a known antagonist of MMP action, was also seen in all of the clones expressing LGI1.

Effect of LGI1 on MMP1 and MMP3 Expression—Because MMP1 and MMP3 were the most consistently and severely down-regulated MMP genes in the T98G/LGI1 cells, we concentrated our subsequent analysis on these two family members (see discussion). Using reverse transcription PCR, we demonstrated that MMP1 and MMP3 are expressed endogenously in the T98G clones that carried the empty vector (Fig. 1A). In contrast, MMP1 and MMP3 transcripts were below detectable levels in cells forced to re-express LGI1.

The MMPs are structurally and functionally related zinc-dependent endopeptidases belonging to four different subfamilies (17), namely collagenases, gelatinases, stromelysins, and membrane-type MMPs (MT-MMPs). These proteolytic enzymes are secreted as inactive proenzymes (pro-MMPs), which are subsequently activated extracellularly by proteolytic cleavage. To determine whether MMP1 and MMP3 proteins were secreted from the T98G cells, supernatants were harvested from cultures grown overnight in the absence of serum and then analyzed by Western blotting. Analysis using anti-MMP1 and anti-MMP3 antibodies demonstrated high levels of these proteins in the supernatant of the cells carrying the empty vector (Fig. 1B) but not in any of the T98G cell clones expressing LGI1, confirming the LGI1-induced suppression of MMP1/3 mRNA.

To determine that the MMPs seen in the parental T98G cells were active, 10 and 20 μl of the supernatants from T98G cells carrying the empty vector were size fractionated on Novex zymogram gels (Fig. 1) and compared with supernatants from T98G clones carrying the exogenous LGI1 gene. MMP activity was clearly seen in cells that did not express LGI1 but was absent in clones that expressed it. These results demonstrate that the presence of LGI1 suppresses MMP activity.

Analysis of the ERK and p38 MAPK Pathways—Earlier reports have shown that expression and activation of the MMP1 and MMP3 gene products are coordinately controlled by the activation of various extracellular, signal-related protein kinases, including ERK1/2 and p38. Several reports also indicate that the enhancement of human MMP1 and MMP3 transcription involves the activation of the AP-1 elements in the 5'-flanking regulatory regions of these genes through distinct MAPK pathways (25, 26). It has also been shown that activation of ERK1/2 plays a major role in MMP1/3 expression, whereas the phosphorylation of p38 apparently has little effect on the transcriptional activity of the MMP1 gene (25).

To determine whether activation of these signaling pathways controls MMP1 and MMP3 production in the LGI1-expressing T98G cells, we analyzed the ERK proteins in these cells using Western blotting. T98G/vector clones show high levels of phosphorylated ERK1/2 (Fig. 2). In contrast, T98G cells expressing LGI1 contained low levels of phosphorylated ERK1/2 proteins. Total levels of ERK proteins were unaffected by the re-expression of LGI1 (Fig. 2). In contrast, neither p38 phosphorylation nor total p38 protein levels were affected by LGI1 re-expression (Fig. 2). These results suggest that LGI1 may be controlling the expression of MMP1/3 transcription through the ERK1/2 pathway.

To further examine the roles of ERK1/2 in the regulation of MMP1 and MMP3 production by LGI1 (Fig. 3A), we treated the different T98G cell clones with the pharmacological MEK1 inhibitors PD98059 (50 μM) and U0126 (20 μM). Both inhibitors severely diminished relative ERK1/2 phosphorylation levels in the T98G/vector cells (data not shown). In these cells, PD98059 causes complete suppression of MMP1/3 production (Fig. 3A), and U0126 causes significant down-regulation of protein production (Fig. 3A), which is consistent with observations in other cell systems.

To investigate the relative role of the p38 pathway in MMP1/3 production, T98G/vector cells were treated with SB203580, a specific inhibitor of the p38 MAPK pathway. In these experiments, expression of the MMP1/3 was suppressed (Fig. 3B). These data suggest that p38 MAPK is also involved in MMP1/3 production in these cells. Despite the fact that ERK1/2 and p38 MAPK seem to be involved in MMP1/3 production in T98G/vector cells, because the phosphorylation status of p38 in T98G/LGI1 clones showed only a marginal decrease, it is apparently not the pathway through which LGI1 exerts its biological effect.

Effect of PMA on ERK1/2 Activation and MMP Production—In some cell systems, activation of protein kinase C (PKC) activates the RAF1-MEK-ERK signaling pathway (27). It has been shown that PKC also activates MMP1 production in malignant glioma cells (28). To determine whether PKC activation affects MMP production in T98G/vector and T98G/LGI1 cells that express exogenous LGI1, we treated these cells with PMA, a pharmacological activator of PKC. This treatment resulted in a further increase in the normal steady state expression levels of MMP1 and MMP3 in T98G/vector cells (Fig. 3). Interestingly, the inhibitory effects of LGI1 on MMP expression was abolished in T98G/LGI1 cells (Fig. 3), demonstrating that the LG11-mediated inhibition of MMPs is sensitive to the activation of PKC. The PMA-induced reactivation of MMP1/3 in T98G/LGI1 was accompanied by phosphorylation of ERK1/2 (Fig. 4) This result confirms the earlier suggestion (Fig. 2) that MMP1/3 production is regulated through the ERK pathway. To assess the migration potential of the T98G cells following treatment with PMA, we performed Matrigel matrix invasion assays (Fig. 5). T98G cells transfected with pcDNA3 migrate...
freely through these substrates, whereas T98G cells expressing exogenous LGI1 do not (Fig. 5). However, when T98G/LGI1 cells were treated with PMA, they were able to migrate through the Matrigel matrix membrane (Fig. 5). In contrast, when these cells were treated with PD98059 alone and in combination with PMA, they were no longer able to pass through the matrix, thus behaving in a similar manner to cells forced to re-express LGI1 (Fig. 5). These data demonstrate that LGI1-mediated inhibition of invasiveness can be reversed as a result of activating PKC.

When T98G/vector and T98G/LGI1 cells were co-treated with the either U0126 or PD98059, inhibitors of the ERK1/2 pathway, together with PMA, the expression of MMPs induced by LGI1 were not affected (Fig. 5). In contrast, when these cells were treated with PD98059 alone and in combination with PMA, they were no longer able to pass through the matrix, thus behaving in a similar manner to cells forced to re-express LGI1 (Fig. 5). These data demonstrate that LGI1-mediated inhibition of invasiveness can be reversed as a result of activating PKC.

**Effect of AKT Phosphorylation on the MAPK Pathway**—In some circumstances, control of cell migration has been shown to operate through the PI3K/AKT signaling pathway (29). To investigate whether LGI1 has an effect on signaling through this pathway, we analyzed the phosphorylation status of AktSer-473 in the presence and absence of LGI1. In T98G/vector cells, the level of Akt phosphorylation was barely detectable (Fig. 6A). In contrast, in cells expressing exogenous LGI1, higher levels of phosphorylated Akt were seen (Fig. 6A).

It has been reported (30) that hyperphosphorylation of Akt can down-regulate the ERK1/2 MAPK pathway by phosphorylation at the serine 259 position in Raf1. To determine whether the increased Akt phosphorylation seen in the presence of LGI1 expression operates in this way, we analyzed the phosphorylation status of AktSer-473 in the presence and absence of LGI1. In T98G/vector cells, the level of Akt phosphorylation was barely detectable (Fig. 6A). In contrast, in cells expressing exogenous LGI1, higher levels of phosphorylated Akt were seen (Fig. 6A).

![Fig. 1. Analysis of the expression of MMP1 and MMP3 in T98G/vector (T98GV) and T98G/LGI1 cells. A, using reverse transcription PCR (top two panels), the parental T98G cells, as well as T98G cells transfected with the pcDNA3 vector alone, show production of the MMP1/3 mRNAs. β-actin was used as the loading control (bottom panel). B, Western blot analysis shows the presence of the MMP1/3 proteins in the supernatants of the T98G/vector cells. In contrast, none of the six independently isolated clones from T98G/LGI1 shows the presence of the MMP1/3 proteins. Actin levels from the cells in the same cultures are shown below. C, zymogram gel assays demonstrate MMP3 activity in the vector-only transfected cells compared with those expressing exogenous LGI1 (showing T98G clone 2 as a representative).](http://www.jbc.org/)

![Fig. 2. Analysis of the phosphorylation status of ERK1/2 and p38 MAPK in T98G cells. T98G/vector cells show high levels of phospho-ERK1/2, but T98G cells (clones 2 and 5 shown as examples) expressing LGI1 show marked reduction in phospho-ERK1/2 levels. Levels of total ERK1/2 proteins in these cells were not affected by the presence of LGI1 (top two panels). In contrast, levels of phospho-p38 MAPK (bottom two panels), which is constitutively present in T98G/vector cells, are unaffected by the presence of LGI1 in T98G/LGI1 cells.](http://www.jbc.org/)

![Fig. 3. Production of MMP1 and MMP3 following treatment with inhibitors of the ERK1/2 and p38/MAPK pathways. When T98G/vector cells and T98G/LGI1 cells (clone 2 shown as an example) are treated with PD98059 (an inhibitor of the ERK1/2 pathway), expression of MMP1 and MMP3 is lost. Treatment with PMA allows expression of MMP1/3 in LGI1-expressing cells, demonstrating that LGI1 exerts its effect upstream of PKC, which acts on the Raf-MEK pathway. When the cells that do and do not express LGI1 are stimulated with PMA and, at the same time, exposed to a potent synthetic inhibitor of ERK1/2 (PD98059), MMP1/3 production is again severely down-regulated. Treatment of T98G cells with U0126, another inhibitor of the ERK1/2 pathway, demonstrates the same inhibition of MMP production with or without the inclusion of PMA. Similarly, when the same cells are treated with SB203580 (an inhibitor for the p38 MAPK pathway), loss of MMP1 and MMP3 production was seen, showing that p38 contributes to the control of MMP expression. This inhibition by SB203580 is lost in the presence of PMA.](http://www.jbc.org/)
ation status of Raf1 at the previously implicated serine 259 residue. Using an antibody that is specific for the phospho-Raf1Ser-259, we demonstrated that, in the presence of the exogenous expression of LGI1, Raf1Ser-259 is distinctly phosphorylated. In T98G/vector cells, phospho-Akt was not identified and neither was phospho-Raf1Ser-259 (Fig. 6B). These data demonstrate that re-expression of LGI1 in glioma cells can apparently influence the inactivation of the ERK/MAPK pathway through Akt phosphorylation (Fig. 6B). When these cells were treated with a PI3K inhibitor, LY294002, phosphorylation of both Akt and Raf1Ser-259 was inhibited. On the other hand, even though treatment of cells with LY294002 inhibited Akt phosphorylation, ERK1/2 phosphorylation was partially restored (Fig. 6B). This suggests that LGI1 may also regulate the ERK/MAPK pathway through other mechanisms.

**DISCUSSION**

Brain tumors are the third most frequent cause of cancer-related deaths in adults and the second most common cause of cancer-related deaths in children. Even though most primary brain tumors do not metastasize and rarely disseminate through the cerebrospinal fluid, they do invade the surrounding normal brain tissue. This characteristic local invasiveness of gliomas contributes substantially to the inability to achieve total resection by surgery and often results in a recurrence at the primary site and throughout the brain. Glial cell invasion is a multistep process that requires these cells to first attach to the barrier matrix and create a proteolytic defect in it. These cells then migrate through the defect. Several reports have shown that there is a strong association between expression of various proteases, such as serine proteases, metalloproteases, and the plasminogen activation/plasmin system, and the invasive behavior of gliomas (31–35). The MMPs in particular, because they are extracellular endopeptidases and thus responsible for degradation of the extracellular matrix, have been implicated in the invasion of the tumor cells. The complexity of the regulation of MMPs is only just being explored, and here we demonstrate that the LGI1 gene regulates the production of MMP1/3 through the MAPK pathway (Fig. 7). Previous studies had suggested that the proteolytic capacity of various cell types was regulated through ERK1/2 and p38 MAPK, which, in turn, regulate the activation of MMP1 and MMP3 as well as gelatinase B (MMP-9) and collagenase-3 (MMP13) (36–40). The demonstration that the biological consequences of LGI1 expression functions by regulating the ERK pathway provides a link between this cell surface effector and MMP regulation in glioma cells. Although only MMP1 and MMP3 were analyzed in T98G cells, in an independent gene expression profiling study using A172 cells that were forced to re-express LGI1 (12), we found that LGI1 also inhibited other MMPs, the most signifi-
MAPK pathway (42). Raf-1 is a serine/threonine kinase (43) shown to be a very important event in the activation of the pathway. On the members of the ERK1/2

Inhibition of MMP function has already been explored as a means to treat cancer, although most of the compounds that have been tested so far have failed (46). These approaches included the injection of TIMPs and various synthetic inhibitors targeting the active sites of MMPs. The use of TIMPs in cancer therapy, however, has been disappointing, because these protein-based treatments are difficult to administer and generally have poor pharmacokinetics. Likewise, synthetic MMP inhibitors produce indiscriminate inhibition of related proteases. It is possible, therefore, that a better understanding of the regulatory mechanisms that control MMP production may lead to more effective strategies. The demonstration by at least two groups that LGI1 is expressed in low grade tumors and is lost in invasive cells (12, 13), together with the demonstration here that LGI1 controls MMP production, raises the

![Diagrammatic representation of the effect of LGI1 expression on the members of the ERK1/2 pathway.](https://example.com/diagram.png)
possibility that the LGI1 signal transduction pathway may be one such potential target for the treatment of metastatic/invasive brain tumors.

Acknowledgments—We thank Drs. Irwin Gelman, Andrei Bakin, and Heinz Baumann for critical reading of the manuscript and Leighton Steen and Julie Platt for assistance in the Affymetrix experiments. We also thank Lisa Wylie for assistance.

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LGII Controls the MMP Production

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LGII, a Putative Tumor Metastasis Suppressor Gene, Controls in Vitro Invasiveness and Expression of Matrix Metalloproteinases in Glioma Cells through the ERK1/2 Pathway

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J. Biol. Chem. 2004, 279:23151-23157.
doi: 10.1074/jbc.M314192200 originally published online March 26, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M314192200

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**ADDITIONS AND CORRECTIONS**

**VOLUME 279 (2004) PAGES 23151–23157**

*LG11, a putative tumor metastasis suppressor gene, controls in vitro invasiveness and expression of matrix metalloproteinases in glioma cells through the ERK1/2 pathway.*

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In this article we suggested that phosphorylation of serine 259 in RAFB was part of the signaling cascade that led to down-regulation of MMP production by LG11. As a result of due diligence in our continued analysis of this system, the suggestion that AKT regulation of RAFB is part of the mechanism by which LG11 suppresses invasion in T98G cells has now been shown to be due to inadvertent contamination of the control T98G cell stocks with 293 cells. Thus, the AKT/RAFB axis should no longer be considered part of the mechanism that facilitates LG11 regulation of MMP production in T98G cells. The remaining data and conclusions in the paper, performed with uncontaminated parental T98G stocks, have been reconfirmed with independent cell stocks.

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