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The Role of Extracellular Signal-Regulated Protein Kinase in Transcriptional Regulation of the Hypoxia Marker Carbonic Anhydrase IX

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Abstract In the present study, we investigated the role of the extracellular signal-regulated protein kinase (ERK) in regulation of the hypoxia marker, carbonic anhydrase IX (CAIX). U0126, a specific inhibitor of MEK1/2, downregulated CAIX expression induced by true hypoxia and cell density. *CA9* promoter activity was similarly affected. Mapping of the U0126 effect revealed that both critical elements within the *CA9* promoter, the hypoxia response element and the juxtaposed SP1-binding PR1, were inhibited. This confirmed that ERK signaling modulates *CA9* promoter activity via its effects on hypoxia inducible factor-1 (HIF-1) and SP1. Further analysis of the U0126 effect on HIF-1-dependent transcription in MCF-7 cells identified p300, a transcriptional co-activator of HIF-1, as the target of ERK. Constitutively increased ERK activity in isogenic fibrosarcoma cell lines did not cause increased cell density-dependent CAIX expression/*CA9* promoter activity. In HeLa cells, an inverse correlation between cell density-induced CAIX expression and ERK activation was observed: sparse cultures did not express CAIX and displayed high ERK activation, whereas CAIX expression in dense cultures was associated with low ERK activation. Collectively, our data do not support any quantitative relationship between ERK activation and CAIX expression. Thus, although ERK signaling is required for optimal CAIX expression, our data are consistent with a model in which constitutive basal ERK activity plays an auxiliary role in *CA9* promoter transactivation by modulating activity of the transcription factor SP1 and the transcriptional co-activator p300. J. Cell. Biochem. 97: 207–216, 2006.

Key words: carbonic anhydrase IX; extracellular signal-regulated protein kinase; hypoxia; cell density

Carbonic anhydrase IX (CAIX) (previously known as MN) was identified in a large number of carcinomas but not in the corresponding normal tissues [Závada et al., 1993; Ivanov et al., 2001 and references therein]. Because CAIX expression correlates with lowered O_2 tension in tumors, CAIX was recently proposed as an intrinsic marker of hypoxia [Ivanov et al., 2001; Loncaster et al., 2001]. CAIX expression appears to be controlled by two separate but interdependent mechanisms, activated by lowered O_2 tension. O_2 tension $\leq 1\%$ (termed true hypoxia) positively regulates expression of CAIX via the hypoxia-response element (HRE) in the *CA9* promoter immediately upstream of the transcription start [Wykoff et al., 2000; Kaluz et al., 2003]. Heterodimeric transcription factor, hypoxia-inducible factor 1 (HIF-1), that binds to HRE consists of the regulated HIF-1α and constitutive HIF-1β subunits [Wang et al., 1995]. Under true hypoxic conditions, HIF-1 activity is increased through post-translational stabilization of the α subunit [Maxwell et al., 1997] and enhanced interaction of the C-terminal transcriptional activation domain

Abbreviations used: CAD, C-terminal transcriptional activation domain; CAIX, carbonic anhydrase IX; ERK, extracellular signal-regulated protein kinase; HIF-1, hypoxia-inducible factor 1; HRE, hypoxia-response element; PI3-K, phosphatidylinositol 3′-kinase; VEGF, vascular endothelial growth factor.

Stefan Kaluz and Milota Kaluzová contributed equally to this work.

While this manuscript was under review, another study on contribution of the MAP kinase pathway to the regulation of CAIX expression was published [J. Kopacek et al., Biochim Biophys Acta 1729: 41–49, 2005].

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(CAD) of HIF-1α with the transcriptional co-activator p300/CBP [Lando et al., 2002]. Alternatively, inactivation of the von Hippel-Lindau (VHL) tumor suppressor, which targets the HIF-1α subunit for oxygen-dependent proteolysis, leads to constitutive HIF-1 α activity [Maxwell et al., 1999].

Although there is clear evidence of co-expression of HIF-1α and CAIX in regions of hypoxia in solid tumors, it was also noted that the expression of CAIX occurred in adjacent regions that did not express HIF-1 α [Olive et al., 2001]. One possible explanation for this is that the transcriptional regulation of the *CA9* may involve factors other than HIF-1 and may reflect induction of CAIX expression in response to mild hypoxic conditions [Kaluz et al., 2002].

We have developed an in vitro model of mild hypoxic conditions (<5% and >1%), using human cancer cells grown at high cell densities. In dense cultures, a different mechanism, triggered by an intermediate decrease of O_2 tension (also termed pericellular hypoxia) due to increased O_2 consumption, seems to control CAIX expression [Kaluz et al., 2002]. O_2 tension in dense culture is too high for stabilization of HIF-1α but sufficient for activation of a phosphatidylinositol 3′-kinase (PI3-K)-dependent pathway that increased HIF-1 transactivation activity [Kaluz et al., 2002]. Although we showed that a minimal level of HIF-1 activity was necessary [Kaluz et al., 2002], a cooperation with SP1/SP3 factors binding the juxtaposed PR1 was also required for induction of *CA9* promoter activity in dense cultures [Kaluz et al., 2003].

HIF activity can be also upregulated by multiple oncogenic pathways, apparently in the absence of hypoxic stimulus [for review see Semenza, 2002]. Particularly, extracellular signal-regulated kinases (ERK)1/2, two kinases of the mitogen-activated protein kinase family, have been implicated in HIF activation [Salceda et al., 1997; Conrad et al., 1999; Richard et al., 1999]. ERK1/2 are activated by extracellular proliferative signaling triggered by membrane-tyrosine kinases and transduced through the Ras-Raf-MEK pathway by a cascade of phosphorylation events [Seger and Krebs, 1995]. Enhanced ERK signaling is a common event in tumors and may play an essential role in transformation as well as in the process of tumor growth, angiogenesis, and metastasis [Folkman, 1995].

In this study, we examined the role of the ERK pathway in regulation of expression of the hypoxia marker CAIX. We studied the effect of pharmacological inhibitors of the ERK pathway on CAIX expression induced by true hypoxia and cell density. Also, we analyzed in detail the mechanism of this action on the *CA9* promoter level as well as its critical regulatory elements. Studies, in which levels of ERK activation were related to CAIX expression, were also performed.

MATERIALS AND METHODS

Sequences are written in the 5′-3′ direction, kits, enzymes, antibodies, and reagents were used according to the manufacturers' recommendations.

Plasmid Constructions

The (−173;+31) (in respect to the transcription start) *CA9* promoter fragment was cloned in pGL2 basic vector (Promega). The 2xHRE-Luc and 3xPR1-Luc constructs were prepared by cloning two copies of the TACGTGCAT *CA9* HIF-binding sequence [Wykoff et al., 2000] and three copies of the TGGGTGGGGGAGGAGC-AAGCC *CA9* PR1 sequence [Kaluz et al., 1999], respectively, into pLuc-MCS vector (Stratagene). The Gal4-HIFCAD 786-826 (expressing the Gal4 DNA-binding domain fused to the HIF-1α CAD) and pFR-Luc (a reporter vector in which the luciferase gene is under the control of a minimal E1B promoter and upstream four copies of a Gal4 binding site) constructs [Sang et al., 2003] were kind gifts from Dr. Nianli Sang (Thomas Jefferson University). The Gal4-p300 construct was kindly provided by Dr. Antonio Giordano (Temple University). The pRL-CMV construct was purchased from Promega. The MEK1ΔED construct encodes a constitutively active double-mutant (E^{218}, D^{222}) MEK1 with a deletion of the nuclear export signal (residues 31–52) [Mansour et al., 1994]. ERK1/2 phosphatase MKP1 [Sun et al., 1993] was cloned into the pCEP4 vector.

Cell Lines and Culture

The following human cell lines were used in this study: breast carcinoma MCF-7, osteosarcoma Saos-2, cervical carcinoma HeLa, fibrosarcoma HT1080 (containing an endogenous mutated allele of the N-*ras* gene), and its derivatives MCH603c8 (lacking the mutated

N-*ras* gene) and 603 MEK^{act} (MCH603c8 stably transfected with the constitutively active MEK1ΔED) [Gupta et al., 2000]. All cells were maintained in Dulbecco's modified Eagle's medium (BioWhittaker), supplemented with 10% fetal calf serum (FCS-Life Technologies), 1.10^2 U/ml penicillin (Sigma), 1.10^2 μg/ml streptomycin (ICN), and 125 ng/ml amphotericin B (Sigma). HT1080, MCH603c8, and 603 MEK^{act} were grown in medium containing 0.25% serum 24 h before harvesting. The effect of the MEK1/2 inhibitor U0126 (Cell Signaling Technology) on endogenous CAIX expression was tested on cells that had been seeded at 10,000/cm^2 and grown for 3 days. The cells were plated at indicated densities, allowed to adhere for 5 h, pretreated with the inhibitor for 1 h (control DMSO), and exposed (in the presence of inhibitor) for 24 h to normoxia or 0.5% O_2 environment in a PROOX In Vitro Chamber (BioSpherix), controlled by the PROOX, model 110 (BioSpherix).

Western Blot Analysis

Western blot analysis of CAIX, ERK1/2, phospho-ERK1/2, HIF-1α, and β-actin expression was performed with anti-CAIX [Pastoreková et al., 1992], anti-ERK1/2, anti-active MAPK (both Promega), anti-HIF-1α (BD Bioscience), and anti-β-actin (Sigma) antibodies, respectively. Briefly, the cells were lysed in 20 mM Tris-Cl, pH 7.5, 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 25 mM NaF, 1 mM PMSF) for 20 min on ice. After centrifugation (13,000*g*, 10 min, 4°C), the protein concentration in each supernatant was measured with the BCA protein Assay kit (Pierce). The rest of the procedure was as described previously [Kaluz et al., 2002].

Transient Transfection Assay

Cells were co-transfected with a firefly luciferase reporter construct, a Gal4 or MKP1 expression construct (where appropriate), and pRL-CMV expressing *Renilla* luciferase (internal control for transfection efficiency) as described previously [Kaluz et al., 2002]. After exposure to the transfection mixture for 16 h, the cells were trypsinized, plated at 20,000/cm^2 or 160,000/cm^2, and allowed to adhere for 3 h. The cells were then pretreated with U0126 for 1 h and exposed to normoxia or 0.5 % O_2 for 24 h in the presence of the inhibitor. Reporter assays were performed as described [Kaluz et al., 2002] and measured in the Sirius luminometer (Berthold Detection Systems). Promoter activities were expressed as the ratio of firefly to *Renilla* luciferase activities from at least three independent experiments.

Statistics

Values are expressed as mean ± SD of at least three experiments. Statistical analysis was performed by the two-tailed Student's test, with *P*-values <0.05 considered statistically significant.

RESULTS

The Effect of the MEK1/2 Inhibitor U0126 on CAIX Expression in MCF-7 and Saos-2 Cells

Initially, we investigated the effect of ERK inhibition on true hypoxia- and cell density-induced CAIX expression. Neither MCF-7 nor Saos-2 cells expressed detectable levels of CAIX in the condition of sparse culture/normoxia but CAIX expression was induced in both cell lines upon exposure to 0.5% O_2, plating at high density, or in combination, with the combination having an additive effect (Fig. 1). Treating cells with 10 μM U0126 significantly inhibited ERK activation (measured by levels of phospho-ERK1/2) and decreased CAIX expression under all conditions. The decrease in CAIX expression was more pronounced for conditions of hypoxia and cell density alone than for the combination, presumably this is due to the additive effect of the two conditions, noted previously [Kaluz et al., 2002] and correspondingly higher levels of CAIX (Fig. 1). Similar observations were made also in a number of other cell lines (e.g., HT1080, HeLa; data not shown). Although levels of phospho-ERK1/2 in MCF-7 cells were considerably higher than in Saos-2 cells, steady-state CAIX levels in both cell lines were comparable. Hypoxia evidently decreased ERK1/2 activation in MCF-7 cells. Induction of CAIX in dense MCF-7 cultures was not accompanied with increased HIF-1α levels, confirming our previous results that cell density does not stabilize HIF-1α [Kaluz et al., 2002]. We conclude that inhibition of ERK activity by U0126 is correlated with downregulation of CAIX expression, however, ERK activation per se does not correlate with CAIX levels in MCF-7 and Saos-2 cells.

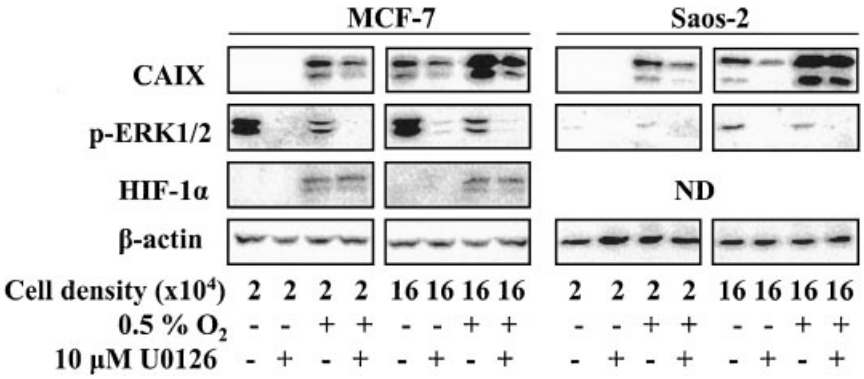

Fig. 1. The effect of U0126 treatment on CAIX expression in MCF-7 and Saos-2 cells. Cells were seeded at the indicated density, allowed to attach for 5 h, pretreated with 10 μM U0126 for 1 h (controls DMSO), and exposed to normoxia or 0.5% O_2 (hypoxia) for 24 h in the presence of U0126. Total protein lysates (30 μg) were analyzed by Western blotting.

The Effect of the MEK1/2 Inhibitor U0126 on the *CA9* Promoter and Its Critical Regulatory Elements

Next, we probed the effect of U0126 on activity of the (−173;+31) *CA9* promoter construct in MCF-7 cells. No inhibition was seen in sparse culture/normoxia (Fig. 2). This result is consistent with our previous observation that the *CA9* promoter is tightly controlled under these conditions, and its basal activity is very low [Kaluz et al., 2003]. U0126 suppressed promoter activity induced by true hypoxia and cell density (Fig. 2), confirming that the ERK pathway contributes to *CA9* promoter activation. Two critical regulatory elements were previously identified in the *CA9* promoter, the HRE [Wykoff et al., 2000] and the SP1/SP3-binding PR1 [Kaluzová et al., 2001; Kaluz et al., 2003]. We, therefore, wished to investigate the contribution of the ERK pathway for activation of these regulatory elements. For this purpose, we used reporter constructs with multimerized HRE and PR1 upstream of the heterologous TATA-box. Activity of the empty vector under the experimental conditions used varied ±10% of activity in sparse culture/normoxia (data not shown). For the 3xPR1-Luc construct, the basal and moderately induced activities under conditions of true hypoxia and cell density were inhibited in the presence of U0126 by ~50% (Fig. 2). With the 2xHRE-Luc construct, a higher induction by true hypoxia and cell density was observed, which was also down-regulated by U0126 (Fig. 2). The combination of dense culture and true hypoxia led to an additive activation with a considerably higher residual activity in the presence of U0126 (Fig. 2). Together, these results indicate a role of ERK signaling in HIF-1 and SP1/SP3 activity in MCF-7 cells. Thus, the negative effect of ERK inhibition on *CA9* promoter activity can be attributed in part to inhibition involving the PR1 region and, more prominently, involvement of the HRE.

The Effect of the MEK1/2 Inhibitor U0126 on Activity of the HIFCAD 786-826 and p300

The negative effect of U0126 on HRE function in MCF-7 cells prompted us to further investigate the corresponding mechanism. The HIF-1α CAD, responsible for the recruitment of p300/CBP transcriptional co-activator, is essential for transcriptional activation of HIF-1 target genes [Huang et al., 1998]. The role of p300/CBP in the regulation of *CA9* in renal cell carcinoma was established previously [Grabmaier et al., 2004] and also confirmed in MCF-7 cells [Kaluz, unpublished]. For dissecting the mechanism of regulation of the HRE function by the ERK pathway in MCF-7 cells, we utilized Gal4 fusion constructs. Cell density and true hypoxia induced activity of p300 (twofold) and HIF CAD (five- to sevenfold) (Fig. 3). The higher HIF CAD induction is presumably the result of the combined effect of p300 activation and inhibition of hydroxylation of N^{803} under hypoxic conditions. Hydroxylation of N^{803} by the factor inhibiting HIF decreases HIF CAD–p300 interaction under normoxic conditions and therefore it is responsible for inhibiting HIF-1 function [Lando et al., 2002]. More importantly, however, basal and induced activities of both

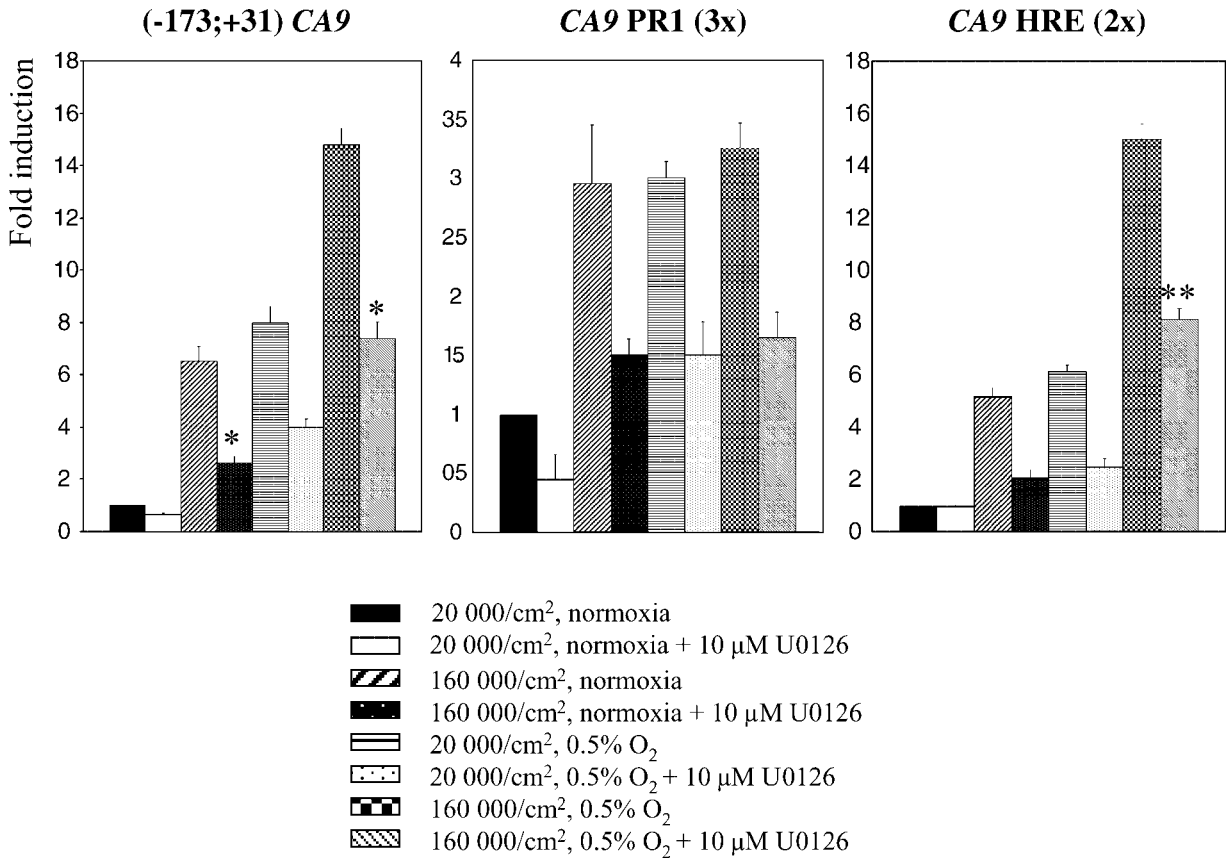

Fig. 2. The effect of U0126 treatment on activity of the *CA9* promoter, 3xPR1-Luc, and 2xHRE-Luc constructs in MCF-7 cells. Cells were co-transfected with the indicated reporter construct and pRL-CMV plasmid for 16 h, trypsinized, seeded at the indicated density, allowed to attach for 5 h, pretreated with 10 μM U0126 for 1 h (controls DMSO), and exposed to normoxia or 0.5% O_2 for 24 h in the presence of U0126. Promoter activity was expressed as the ratio of firefly activity to *Renilla* activity and the value in 20,000/cm^2/normoxia was set as 1. Activities under various conditions are expressed as the level of induction relative to the control, and each of the bars represents the mean value (±SD) from at least three individual experiments. $^*P < 0.05$, $^{**}P < 0.01$ versus DMSO-treated control.

constructs are inhibited by ~50% in the presence of U0126 (Fig. 3). It should also be mentioned that activity of the pFR-Luc in the absence of the Gal4 expression construct did not change significantly (±10% of control activity in sparse culture/normoxia; data not shown), confirming that the observed effects were indeed specific to HIF CAD and p300. These data support the notion that ERK inhibition affects the HRE function by suppressing the transactivation potential of p300.

Modulation of ERK Activity Does Not Increase Cell Density-Induced CAIX Expression/*CA9* Promoter Activity in Fibrosarcoma Cell Lines

Modulation of ERK activity via estradiol-inducible Raf-1:ER construct in CCL39 (Chinese hamster lung fibroblast)-derived cell line upregulated expression of vascular endothelial growth factor (VEGF), another hypoxia-inducible gene [Milanini et al., 1998]. As VEGF appears to be regulated in a way similar to *CA9* [Kaluz et al., 2003], we wished to investigate the effect of upregulated ERK activity on CAIX expression/*CA9* promoter activity in isogenic fibrosarcoma cell lines derived from HT1080. The parental HT 1080 cells, harboring an endogenous mutated allele of the N-ras gene, MCH603c8 with the deletion of this mutated N-ras gene, and 603 MEK^{act} (MCH603c8 stably transfected with the constitutively active MEK1ΔED) have different levels of activated MEK: the level of MEK activity in 603 MEK^{act} cells is approximately three- and tenfold higher than in HT1080 and MCH603c8 cell lines, respectively [Gupta et al., 2000]. No correlation between CAIX expression and ERK activity was observed in these fibrosarcoma cell lines, as MCH603c8 cells with the lowest phospho-ERK1/2 levels contained the highest level of

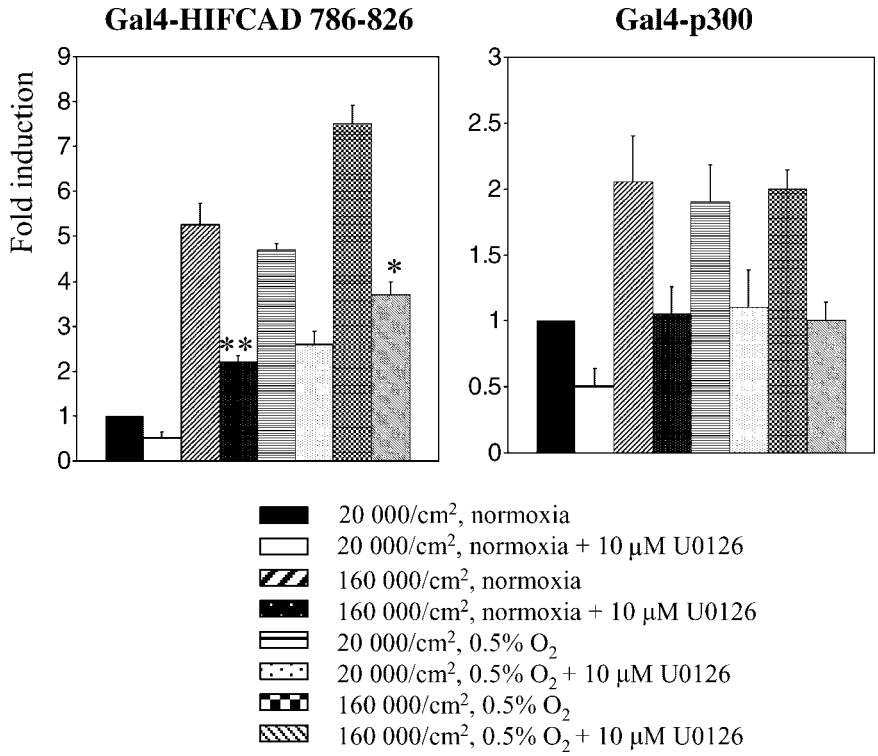

Fig. 3. The effect of U0126 treatment on activity of Gal4-HIFCAD 786-826 and Gal4-p300 fusion constructs in MCF-7 cells. Cells were co-transfected with a construct expressing Gal4-HIFCAD 786-826 or Gal4-p300, pFR-Luc (containing Gal4 binding sites), and pRL-CMV. Treatment and activities are as described in Figure 2. $^{*}P < 0.05$, $^{**}P < 0.01$ versus DMSO-treated control.

cell-density induced CAIX (Fig. 4A). In addition, transient transfections also revealed a modest increase of *CA9* promoter activity in MCH603c8 cells (~1.5-fold higher than in HT 1080 or 603 MEK^{act}; Fig. 4B). Thus, at least in these human fibrosarcoma cells, modulation of ERK activity does not increase CAIX expression/*CA9* promoter activity

ERK Activity Is Inversely Proportionate to Cell Density-Induced CAIX Expression in HeLa Cells

One of the hallmarks of CAIX expression is its striking dependence on cell culture density [Závada et al., 1993]. Having established a role for ERK signaling in CAIX activation, we asked how cell density-induced CAIX expression relates to ERK activation in HeLa cells. These cells needed to be plated at a density 80,000/cm^2 or higher in order to express detectable levels of CAIX within 24 h (Fig. 5A). Surprisingly, cells plated at these densities displayed very low levels of phospho-ERK1/2 (but detectable on overexposed blots) and, conversely, the cells at the lowest density that did not express CAIX exhibited the highest phospho-ERK1/2 levels (Fig. 5A). Yet, a dose-dependent inhibition of density-induced CAIX expression was observed in the presence of U0126 (Fig. 5B). U0126 exerted a similar dose-dependent inhibition of *CA9* promoter activity (Fig. 5C). Similar results were obtained with a different MEK1/2 inhibitor, PD 98059 (data not shown). MEK inhibitors U0126 and PD 98059 were listed among the compounds with the most impressive selectivity profiles [Davies et al., 2000], therefore it is unlikely that inhibition of CAIX expression is due to a non-specific, ERK pathway-unrelated effect. However, we provided an independent proof of ERK pathway being implicated in regulation of CAIX by overexpressing MKP1, a specific phosphatase of ERK1/2 [Sun et al., 1993]. In HeLa cells, cell density-induced *CA9* promoter activity was downregulated in the presence of MKP1 (Fig. 5D). These results in HeLa cells lend themselves to the following conclusions: (i) cell density-induced CAIX expression is inversely proportionate to ERK activation and (ii) a basal level of ERK activation suffices for density-induced CAIX expression/*CA9* promoter activity. We, therefore, conclude

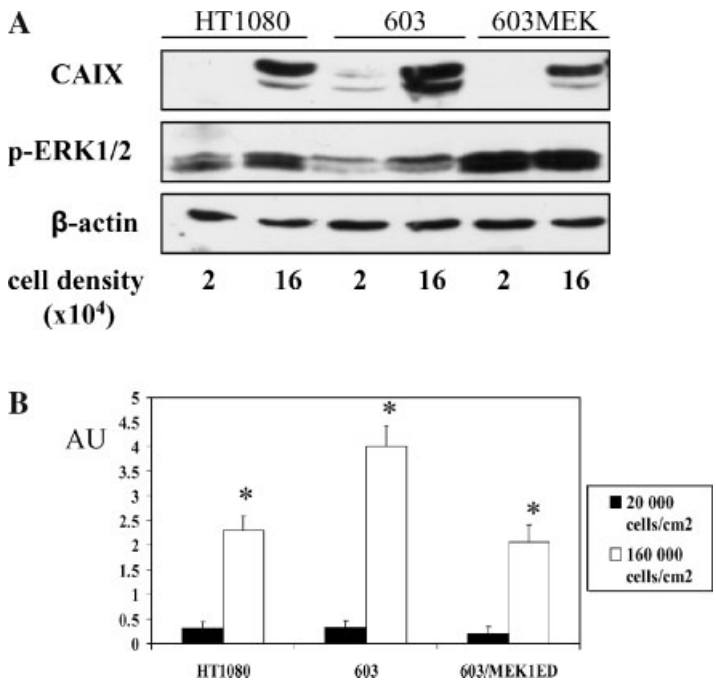

Fig. 4. The relationship between cell density-induced CAIX expression/*CA9* promoter activity and ERK activation in human fibrosarcoma cell lines. **A**: Cell-density induced CAIX expression. Cells were seeded at the indicated density, allowed to attach for 5 h, and then starved in 0.25% serum for 24 h. Total protein lysates (30 μg) were analyzed by Western blotting. **B**: Cell-density induced *CA9* promoter activity. Cells were co-transfected with the (−173;+31) *CA9* promoter construct and pRL-CMV as in Figure 2, except for being incubated the last 24 h in 0.25% serum. Promoter activities are expressed in arbitrary units. $^*P < 0.05$ versus sparse control.

that although a basal ERK activity is necessary for activation of CAIX expression, there is no correlation between overall ERK activity and CAIX expression.

DISSCUSSION

Protein kinase cascades play a critical role in the regulation of gene expression and understanding of which pathways that are involved in regulation of a particular gene provides a means of modulating levels of its gene product. In order to further increase our knowledge of regulation of expression of the hypoxia marker CAIX, we have studied the involvement of the ERK pathway in induction of CAIX by true hypoxia and cell density. These two modes of CAIX induction were chosen because they differ in levels of HIF-1α, a factor that plays a critical role in CAIX regulation. Regardless of the mode of activation, the specific pharmacological inhibitors of MEK1/2,U0126 and PD98059 downregulated CAIX expression in a number of cell lines. A similar inhibitory effect on the promoter construct confirmed that the ERK pathway plays a role in activation of the *CA9* promoter by true hypoxia and cell density. Activities of the HRE and PR1, two critical regulatory elements within the *CA9* promoter, were also down-regulated in the presence of U0126.

As for down-regulation of the SP1/SP3-binding PR1, there is experimental evidence that ERK signaling stimulates SP1 activity. ERK1/2 directly phosphorylates SP1 on T^{453} and T^{739} both in vitro and in vivo; mutation of these residues to alanine led to a 50% decrease of the ERK-dependent transcriptional activity of SP1 in the context of the *VEGF* promoter [Milanini-Mongiat et al., 2002]. In addition, the transactivation effect of the constitutively active MEK1 on the *VEGF* promoter was mapped to the SP1 binding GC-rich region between −88 and −66 [Milanini et al., 1998]. As SP1 activity is also critical for the *CA9* promoter function [Kaluzová et al., 2001; Kaluz et al., 2003], these data corroborate our findings that suppression of SP1 activity in the presence of the MEK1/2 inhibitor is, at least partially, responsible for downregulation of *CA9* promoter activity.

Activity of HIF-1 is modulated primarily by post-translational stabilization of HIF-1α under true hypoxic conditions, although a number of

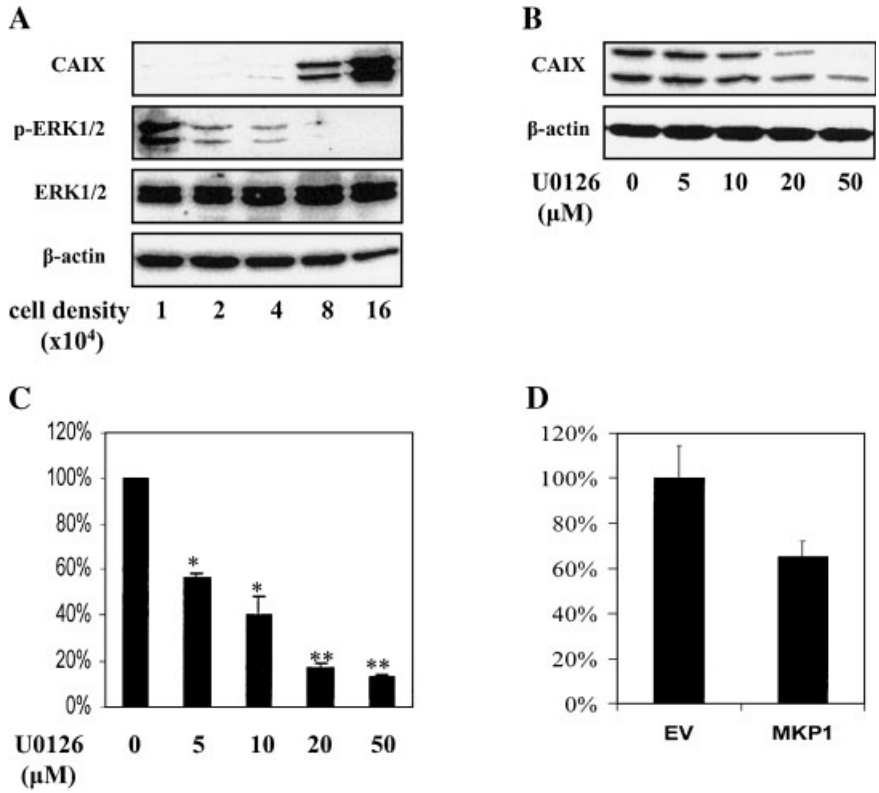

Fig. 5. The relationship between CAIX expression and ERK activation and the effect of inhibition of the ERK pathway on cell density-induced CAIX expression/*CA9* promoter activity in HeLa cells. **A**: The effect of cell density on CAIX expression and ERK activation. Cells were seeded at the indicated density and incubated for 24 h. Total protein lysates (30 μg) were analyzed by Western blotting. **B**: Dose-dependent effect of U0126 on cell density-induced CAIX. Cells were seeded at 160,000/cm^2 and treated as in Figure 1 with the indicated concentrations of U0126. **C**: Dose-dependent effect of U0126 on cell density-induced *CA9* promoter activity. Cells were co-transfected with the (−173;+31) *CA9* promoter construct and pRL-CMV as in Figure 2. Trypsinized transfectants were seeded at 160,000/cm^2 and treated with the indicated concentrations of U0126. Promoter activities are expressed as the percent of control activity in the presence of DMSO only. $^*P < 0.05$, $^{**}P < 0.01$ versus DMSO-treated control. **D**: The effect of the ERK phosphatase MKP1 on cell density-induced *CA9* promoter activity. Cells were co-transfected with the (−173;+31) *CA9* promoter construct, pRL-CMV, and MKP1 construct or empty vector (EV). Trypsinized cells were seeded at 160,000/cm^2 and harvested 24 h later. Promoter activities are expressed as the percent of activity in the presence of EV.

other post-translational modifications of HIF-1α are required for optimal HIF-1 function [Semenza, 2002; Huang and Bunn, 2003]. Among others, the ERK pathway has been implicated in modulating HIF-1 function, in that activated ERK was shown to phosphorylate HIF-1α in vitro [Richard et al., 1999], transfection with active forms of ERK1/2 stimulated HIF-1 transcriptional activity without affecting HIF-1α stability [Conrad et al., 1999], and specific inhibitors of MEK1/2 suppressed HIF-1-dependent reporter gene expression [Salceda et al., 1997]. However, there have been inconsistencies between results of different groups with respect to the role of ERK-induced direct phosphorylation of HIF-1α, and no direct evidence for correlation between ERK-mediated phosphorylation of HIF-1α and its transactivation activity was found [Sang et al., 2003]. In vitro, activated ERK phosphorylated HIF-1α outside the hypoxia-responsive CAD region, and yet the hypoxia-responsive and constitutive CAD were inhibited by PD 98059 [Sang et al., 2003]. Subsequently, it was revealed that ERK signaling was required for the HIF-1α–p300 interaction and also for transactivation activity of p300 [Sang et al., 2003]. Our data in MCF-7 cells correspond well with the proposed mechanism of ERK-dependent modulation of HIF-1 activity via p300. The similar inhibition profile of the constitutive HIF CAD (786–826) and p300 Gal4-fusion constructs by U0126 indicates that ERK activity is indeed required for optimal p300 function. Upon induction, the

residual HIF CAD activity in the presence of U0126 is higher than the basal activity (sparse/normoxia), presumably due to inhibition of hydroxylation of N^{803} [Lando et al., 2002].

Despite the fact that cell density-induced pericellular hypoxia did not stabilize HIF-1α, cell-density-induced CAIX expression is still critically dependent on HIF-1α, suggesting an increase in HIF-1α transactivation capacity without its concomitant stabilization [Kaluz et al., 2002]. The previous report documenting stimulation of HIF-1α transcriptional activity by ERK without affecting HIF-1α stability [Conrad et al., 1999] prompted us to consider the role of the ERK pathway in this process. However, the observed overall pattern of inhibition of the reporter constructs driven by the *CA9* promoter, its individual regulatory elements, and HIF CAD and p300 Gal4 fusion constructs by U0126 were similar for true hypoxia and cell density conditions. This indicates a similar mechanism of ERK contribution to *CA9* promoter activation by true hypoxia and cell density (targeting SP1 and p300), rather than specifically implicating the ERK pathway in cell density-dependent induction. As we previously showed that activity of a PI3-K-dependent pathway was critical for cell density-dependent activation of HIF-1 and consequent CAIX expression, it is possible that the transactivation potential of HIF-1 can be modulated by multiple pathways, presumably in a cell-specific manner.

Inconsistencies in reports about ERK role in regulation of HIF-1-dependent transcription prompted us to analyze the quantitative relationship between ERK activity and CAIX expression. Heterologous MCF-7 and Saos-2 cells express comparable levels of CAIX despite having very different levels of phospho-ERK1/2. Modulation of ERK activity in isogenic fibrosarcoma cell lines 603 MEK^{act}, HT1080, and MCH603c8 (in the order of decreasing ERK activity) resulted in profoundly different phenotypes [Gupta et al., 2000]. In mice, 603 MEK^{act} cells produced the most aggressively growing tumors and, morphologically, 603 MEK^{act} and HT1080 had rounded appearance with disorganized actin fibers in contrast to flat-shaped MCH603c8 with well-organized cytoplasmic actin fibers [Gupta et al., 2000]. However, steady-state amounts of CAIX and *CA9* promoter activity were the highest in MCH603c8, the cell line with the lowest ERK activity. The most convincing piece of evidence against any correlation between ERK activation and CAIX expression was obtained in HeLa cells. In these cells, CAIX was detected only in dense cultures with little ERK activation and, conversely, no CAIX was observed in sparse cultures where ERK activation was the highest. ERK activity was still needed for induction of CAIX expression in dense cultures. The high level of ERK activation in rapidly proliferating sparse cells and a considerably lower level in dense, spatially constrained cultures with significantly decreased proliferation capacity, is not surprising as ERK activation directly correlates with cell proliferation [Seger and Krebs, 1995]. It should be noted, however, that this type of relationship appears to be cell type-specific, as no decrease in ERK activation in dense cultures was observed with MCF-7 and Saos-2 cells. On the basis of observations made in various cell systems, we conclude that there is no positive correlation between increased ERK activation and CAIX expression. Although for optimal CAIX expression, a basal ERK activity was required, further ERK stimulation did not increase CAIX expression.

In summary, we have shown that the ERK pathway contributes to regulation of CAIX expression by modulating the activity of the transcription factor SP1 and p300, the transcriptional co-activator of HIF-1. We have also provided evidence that although a basal ERK activation is required for optimal CAIX expression, CAIX levels generally do not correspond with increased activation of the ERK pathway. This is consistent with the notion that ERK signaling is not implicated in mechanism(s) inducing CAIX per se but plays an auxiliary role by contributing to maintaining optimal SP1 and p300 activity.

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REFERENCES

Conrad PW, Freeman TL, Beitner-Johnson D, Milhorn DE. 1999. EPAS1 trans-activation during hypoxia requires p42/p44 MAPK. J Biol Chem 274:33709–33713.

Davies SP, Reddy H, Caivano M, Cohen P. 2000. Specificity and mechanism of action of some commonly used protein kinase inhibitors. Biochem J 351:95–105.

Folkman J. 1995. Angiogenesis in cancer, vascular, rheumatoid and other disease. Nat Med 1:27–31.

Grabmaier K, de Weijert MCA, Verhaegh GW, Schalken JA, Oosterwijk E. 2004. Strict regulation of $CAIX^{G25/MN}$ by HIF-1α in clear cell renal cell carcinoma. Oncogene 23:5624–5631.

Gupta S, Plattner R, Der CJ, Stanbridge EJ. 2000. Dissection of Ras-dependent signaling pathways controlling aggressive tumor growth of human fibrosarcoma cells: Evidence for a potential novel pathway. Mol Cel Biol 20:9294–9306.

Huang LE, Bunn HF. 2003. Hypoxia-inducible factor and its biomedical relevance. J Biol Chem 278:19575–19578.

Huang LE, Gu J, Schau M, Bunn HF. 1998. Regulation of hypoxia-inducible factor 1α is mediated by an O_2-dependent degradation domain via the ubiquitin-proteasome pathway. Proc Natl Acad Sci USA 95:7987–7992.

Ivanov S, Liao SY, Ivanova A, Danilkovitch-Miagkova A, Tarasova N, Weirich G, Merril MJ, Proescholdt MA, Oldfield EH, Lee J, Závada J, Waheed A, Sly W, Lerman MI, Stanbridge EJ. 2001. Expression of hypoxia-inducible cell-surface transmembrane carbonic anhydrases in human cancer. Am J Pathol 158:905–919.

Kaluz S, Kaluzová M, Opavský R, Pastoreková S, Gibadulinová A, Dequiedt F, Kettmann R, Pastorek J. 1999. Transcriptional regulation of the MN/CA 9 gene coding for the tumor-associated carbonic anhydrase IX. Identification and characterization of a proximal silencer element. J Biol Chem 274:32588–32595.

Kaluz S, Kaluzová M, Chrastina A, Olive PL, Pastoreková S, Pastorek J, Lerman MI, Stanbridge EJ. 2002. Lowered oxygen tension induces expression of the hypoxia marker MN/Carbonic anhydrase IX in the absence of hypoxia-inducible factor 1α stabilization: A role for phosphatidylinositol 3′-kinase. Cancer Res 62:4469–4477.

Kaluz S, Kaluzová M, Stanbridge EJ. 2003. Expression of the hypoxia marker carbonic anhydrase IX is critically dependent on SP1 activity. Identification of a novel type of hypoxia-responsive enhancer. Cancer Res 63:917–922.

Kaluzová M, Pastoreková S, Švastová E, Pastorek J, Stanbridge EJ, Kaluz S. 2001. Characterization of the MN/CA 9 promoter proximal region—A role for SP and AP1 factors. Biochem J 359:669–677.

Lando D, Peet DJ, Whelan DA, Gorman JJ, Whitelaw ML. 2002. Asparagine hydroxylation of the HIF transactivation domain: A hypoxic switch. Science 295:858–861.

Loncaster JA, Harris AL, Davidson SE, Logue JP, Hunter RD, Wycoff CC, Pastorek J, Ratcliffe PJ, Stratford IJ, West CML. 2001. Carbonic anhydrase (CAIX) expression, a potential new intrinsic marker of hypoxia: Correlations with tumor oxygen measurements and prognosis in locally advanced carcinoma of the cervix. Cancer Res 61:6394–6399.

Mansour SJ, Matten WT, Hermann AS, Candia JM, Rong S, Fukasawa K, Vande Woude GF, Ahn NG. 1994. Transformation of mammalian cells by constitutively active MAP kinase kinase. Science 265:966–970.

Maxwell P, Dachs GU, Gleadle JM, Nicholls LG, Harris AL, Stratford IJ, Hankinson O, Pugh CW, Ratcliffe PJ. 1997. Hypoxia inducible factor-1 modulates gene expression in solid tumors and influences both angiogenesis and tumor growth. Proc Natl Acad Sci USA 94:8104–8109.

Maxwell PH, Wiesener M, Chang G-W, Clifford S, Vaux E, Cockman M, Wykoff CC, Pugh C, Maher E, Ratcliffe PJ. 1999. The tumour suppressor protein VHL targets hypoxia-inducible factors for oxygen-dependent proteolysis. Nature (Lond) 399:271–275.

Milanini J, Viñals F, Pouysségur J, Pagès G. 1998. p42/p44 MAP kinase module plays a key role in the transcriptional regulation of the vascular endothelial growth factor gene in fibroblasts. J Biol Chem 273:18165–18172.

Milanini-Mongiat J, Pouysségur J, Pagès G. 2002. Identification of two SP1 phosphorylation sites for p42/p44 mitogen-activated protein kinase. J Biol Chem 277: 20631–20639.

Olive PL, Aquino-Parsons C, MacPhail SH, Liao SY, Raleigh JA, Lerman MI, Stanbridge EJ. 2001. Carbonic anhydrase 9 as an endogenous marker for hypoxic cells in cervical cancer. Cancer Res 61:8924–8929.

Pastoreková S, Závadová Z, Koštál M, Babušíková J, Závada J. 1992. A novel quasi-viral agent, MaTu, is a two-component system. Virology 187:620–626.

Richard DE, Berra E, Gothie E, Roux D, Pouysségur J. 1999. p42/p44 mitogen-activated protein kinases phosphorylate hypoxia-inducible factor 1α (HIF-1 α) and enhance the transcriptional activity of HIF-1. J Biol Chem 274:32631–32637.

Salceda S, Beck I, Srinivas V, Caro J. 1997. Complex role of protein phosphorylation in gene activation by hypoxia. Kidney Int 51:556–559.

Sang N, Stiehl DP, Bohensky J, Leshchinsky I, Srinivas V, Caro J. 2003. MAPK signaling up-regulates the activity of hypoxia-inducible factors by its effects on p300. J Biol Chem 278:14013–14019.

Seger R, Krebs EG. 1995. The MAPK signaling cascade. FASEB J 9:726–735.

Semenza GL. 2002. Signal transduction to hypoxia-inducible factor 1. Biochem Pharmacol 64:993–998.

Sun H, Charles CH, Lau LF, Tonks NK. 1993. MKP-1 (3CH134), an immediate early gene product, is a dual specificity phosphatase that dephosphorylates MAP kinase in vivo. Cell 75:487–493.

Wang GL, Jiang BH, Rue EA, Semenza GL. 1995. Hypoxia-inducible factor 1 is a basic-helix-loop-helix-PAS heterodimer regulated by cellular O_2 tension. Proc Natl Acad Sci USA 92:5510–5514.

Wykoff CC, Beasley NJ, Watson PH, Turner KJ, Pastorek J, Sibtain A, Wilson GD, Turley H, Talks KL, Maxwell PH, Pugh CW, Ratcliffe PJ, Harris AL. 2000. Hypoxia-inducible expression of tumor-associated carbonic anhydrases. Cancer Res 60:7075–7083.

Závada J, Závadová Z, Pastoreková S, Čiampor F, Pastorek J, Zelník V. 1993. Expression of MaTu-MN protein in human tumor cultures and in clinical specimens. Int J Cancer 54:268–274.