Green Fluorescent Protein Tagging of Extracellular Signal-Regulated Kinase and p38 Pathways Reveals Novel Dynamics of Pathway Activation during Primary and Metastatic Growth

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ABSTRACT

We describe a novel approach that allows detection of primary and metastatic cells in vivo in which either the extracellular signal-regulated kinase (ERK) or the p38 pathway is activated. Our recent findings showed that ERK and p38 kinases regulate, respectively, programs dictating cell proliferation (high ERK-to-p38 ratio) or growth arrest and dormancy (low ERK-to-p38 ratio) in vivo. Thus, we were able to use live green fluorescent protein (GFP) to reflect ERK and p38 activities and, consequently, the proliferative state of cancer cells. This was accomplished by transfected human metastatic carcinoma cells, HEp3, which can be experimentally maintained in tumorigenic state or forced into dormancy (9, 10). The tumorigenic cells express high level of urokinase-type plasminogen activator/urokinase-type plasminogen activator/receptor (uPA/uPAR) complex that, by interacting with and activating fibronectin-binding α5β1 integrin, initiates a signaling pathway through FAK, epidermal growth factor receptor (EGFR), and extracellular signal-regulated kinase (ERK) leading to high ERK-to-p38 ratio and rapid tumor growth in vivo (9). Genetic or pharmacological disruption of the uPAR-α5β1 complex reverses the ERK-to-p38 ratio, favoring p38 activation and forcing these cells into growth arrest and dormancy in vivo (10–12). On the strength of these findings, we hypothesized that a reporter system in which GFP will be placed under the control of ERK and p38 signaling for proliferative success. This approach allows isolation and further characterization of metastatic cells with specific signaling signatures indicative of their phenotypes.

INTRODUCTION

Primary tumor development results from the accumulation of genetic and epigenetic changes that favor the selection of tumor cells with growth and survival advantages (1). Overwhelming clinical evidence indicates that, after dissemination and lodging in distant organs, some of these cells resume growth immediately, whereas others remain lodged in a dormant state, often for longer than a decade (2, 3). Because dormant cells are difficult to detect and isolate, the molecular mechanisms that determine dormancy remain largely unknown. A clue that points to organ microenvironment as a determinant of metastatic fate is organ specificity of metastases (2, 3). Because dormant cells are difficult to detect and isolate, the molecular mechanisms that determine dormancy remain largely unknown. A clue that points to organ microenvironment as a determinant of metastatic fate is organ specificity of metastases (2, 3). This, in turn, implies that the repertoire of cancer cell surface proteins may be critical for the ability of tumor cells to receive, transmit, and “interpret” clues from growth-permissive or -nonpermissive microenvironments, and to initiate signaling programs that, respectively, favor growth or dormancy. The fact that certain recently identified signal regulator genes are also metastasis suppressors (3), and that they are epigenetically silenced, and not deleted, supports the possibility of a switch between dormant and overt metastasis.

Efforts to analyze the growth of disseminated cells through GFP cell tagging (4, 5) and/or Alu sequence amplification (6, 7), provided positional information on tumor cells (5, 7, 8), but not qualitative or quantitative information on changes in the molecular-genetic programs that define their in vivo behavior. Immunostaining of these cells for molecular markers linked to proliferation was used to obtain additional insight into the proliferative state of these cells. However, this approach provided only static information and precluded recovery of living cells for further analysis (5). It appeared inevitable, therefore, that to monitor spatiotemporal activation of signaling pathways in living tumor cells in metastatic sites, with the goal of their subsequent isolation and characterization, will require the development of a new approach.

For that purpose, we used our well-characterized model system of human metastatic carcinoma cells, HEp3, which can be experimentally maintained in tumorigenic state or forced into dormancy (9, 10). The tumorigenic cells express high level of urokinase-type plasminogen activator/urokinase-type plasminogen activator/receptor (uPA/uPAR) complex that, by interacting with and activating fibronectin-binding α5β1 integrin, initiates a signaling pathway through FAK, epidermal growth factor receptor (EGFR), and extracellular signal-regulated kinase (ERK) leading to high ERK-to-p38 ratio and rapid tumor growth in vivo (9). Genetic or pharmacological disruption of the uPAR-α5β1 complex reverses the ERK-to-p38 ratio, favoring p38 activation and forcing these cells into growth arrest and dormancy in vivo (10–12). On the strength of these findings, we hypothesized that a reporter system in which GFP will be placed under the control of ERK or p38 signaling would allow monitoring of these pathways in vivo.

Here we present evidence showing that ERK- or p38-dependent induction of humanized renilla green fluorescent protein (hrGFP) allows monitoring of these pathways in tumor cells within primary and metastatic tumor tissues. The use of these GFP-reporter cells provided the first insight into the kinetics of ERK and p38 modulation in response to a changing microenvironment.

MATERIALS AND METHODS

Cells, cDNA Transfections, and Cell Culture Conditions. Tumorigenic HEp3 (T-HEp3), dormant HEp3 (D-HEp3), and tumorigenic HT1080 cells (13), were grown and transfected, as described previously (13) with pFA-Elk or pFA-CHOP (Stratagene, La Jolla, CA; G418r) and pFR-hrGFP plasmids (hygromycinb, Stratagene, La Jolla, CA). HT1080 cells were transfected with pFA-CHOP and pFR-hrGFP plasmids. Stable clones obtained after selection with G418 (400 µg/ml) that were GFP positive (5 per cell line), were subjected...
to a second selection with Hygromycin (10 μg/ml) and G418 (400 μg/ml, Invitrogen, Carlsbad, CA). Transfectants with pFR-GFP alone never developed into G418-resistant colonies. In all clones, the Elk- or CHOP-induced GFP intensity matched the profile of ERK and p38 activities determined for parental cells (13). One highly metastatic clone was further characterized. Stable transfectants were routinely cultured with G418 (400 μg/ml). In some experiments, cells growing in culture, or cells obtained from mouse or chorioallantoic membrane (CAM) tissues, were treated for 6 to 24 hours with 25–50 μmol/L As2O3 or were irradiated with 23 to 40 μJ/cm2 UV light and were cultured for an additional 6 to 24 hours in 10% fetal bovine serum (FBS)-DMEM in the absence or presence of 5 μmol/L SB203580 (Calbiochem, San Diego, CA). To modulate ERK activity, cells were starved for 24 to 48 hours and were incubated with or without 25 to 40 μmol/L PD98059 (Calbiochem, San Diego, CA).

Growth of Tumors and Spontaneous Metastases in Chick Embryo and in Nude Mice. Cells were grown on CAM as described previously (13, 14). Briefly, cells were detached with 2 mmol/L EDTA in PBS, were washed, and were inoculated on the CAMs of 9- to 10-day-old chick embryos (SPAFAS, North Franklin, CT). Quantitation of cell numbers and of tumor growth was determined as described previously (13). For metastasis studies in the chick embryo, lungs were excised, minced, and inoculated onto a new CAM for expansion of cells lodged in the lung (14). Cells were grown in nude mice as described previously (15). Briefly, 1 × 106 cells in 100 μL of PBS were inoculated subcutaneously into the interscpecular region of BALB/c nu/nu mice (Charles River, Wilmington, MA, or Taconic Farms, Germantown, NY). When tumors measured ∼1.5 g, either (a) mice were euthanized and tumors, lungs, lymph nodes, and livers were excised and placed on ice in plates containing PBS, or (b) tumors were surgically removed under full anesthesia. Tumor incidence was 100% (9 of 9) for all cell lines. The incidence of spontaneous metastases in lungs was 100% (3 of 3) for HT-CHOP cells, and 66% (4 of 6) for T-Elk cells as detected by tissue culture of excised organs and/or visual inspection of lungs with fluorescent microscope and/or histology.

Fluorescence Microscopy and Fluorescence-Activated Cell Sorting Analysis. Fluorescence microscopy was performed with an inverted Nikon (Tokyo, Japan), Eclipse TS100-F microscope coupled to a Nikon Coolpix 990 digital camera. Images used for comparison were captured with a fixed aperture time. Images were visualized and analyzed with Adobe Photoshop 6.0 and ImageJ software (http://rnb.nih.gov/ij), respectively.1 Cells in culture or mouse- or CAM-excised tissues were washed in PBSCa2+/Mg2+ containing penicillin and streptomycin and were placed in PBSCa2+/Mg2+ to photograph. Fluorescence-activated cell sorting (FACS) analysis was performed in the Flow Cytometry Shared Research Facility, Mount Sinai School of Medicine (MSSM), New York, NY. Cells detached with trypsin (for all experiments) or 2 mmol/L EDTA (for UV and arsenic experiments), or cells recovered from mouse or CAM tissues after collagenase treatment, were washed in DMEM and resuspended in PBS. hrGFP fluorescence (488 nm laser) was quantitated with a FACS-SCAN machine (Becton Dickinson, San Jose, CA). Data analysis was performed with Cell Quest software. In most experiments, a total of 5 × 106 events were analyzed. Two variables were quantitated as a measure of signaling activity: mean GFP fluorescence in the GFP-positive population and the number of GFP-positive events in different ranges of fluorescence intensity. Regions gating FLH1 from 50% to 51%, 102 to 103, 103 to 104, or 104 to 105 were selected and analyzed. Determinations were done in triplicate. Cell sorting was performed on a FACS-Vantage SE high-speed cell sorter with Turbosort option and DIVA electronics (Becton Dickinson, San Jose, CA).

Luciferase Assays and Western Blot. Luciferase assays were performed as described previously (13). Cells transfected with pFA-Elk1 or pFA-CHOP and pD700 plasmid (luciferase) or DN-p38 mutant (9) and a Renilla luciferase (Clontech, Palo Alto, CA) plasmids were inoculated (2.5 × 106 per CAM) onto 9- to 10-day-old CAMs 24 hours posttransfection and, at 48 hours postinoculation, excised CAM tissues were homogenized with the passive lysis buffer from Promega (Madison, WI). Firefly luciferase activity was detected as described previously (13). Detection of GAL4-CHOP and GAL4-Elk fusion proteins (Matchmaker, anti GAL4DBD monoclonal antibody, BD Biosciences, Palo Alto, CA), was carried out by Western blotting, as indicated elsewhere (10). Detection of phosphorylated and total levels of both ERK and p38 and Flag-tagged dominant-negative p38 (DN-p38) was performed by Western blot as described previously (9).

RESULTS

Monitoring ERK Activity through Elk-GAL4-Dependent Induction of hrGFP. To monitor ERK activation, we obtained stable clones of T-HEp3 (T-Elk) and D-HEp3 (D-Elk) cells, carrying the ElkAD-GAL4DBD and the 5X-GAL4UAS-hrGFP plasmids. Signals that activate ERK led to the downstream phosphorylation of its natural substrate Elk in the activation domain of the Elk-GAL4 fusion protein, which on binding to the GAL4UAS sequence promoted hrGFP expression. Fluorescent microscopy showed (Fig. 1A and B) that T-Elk (high phosphorylated (P)-ERK levels, Fig. 1C) cells had a greater number of highly GFP fluorescent cells than D-Elk cells (low P-ERK level, Fig. 1C). FACS analysis confirmed that the mean fluorescence was 2.5- to 4-fold greater in T-Elk cells (Fig. 1B) and the proportion of total GFP-positive cells was similar in D-Elk and T-Elk cells (~30–45%). The higher GFP signal in T-Elk cells correlated with higher P-ERK levels and was due to accumulation of GFP protein and not due to increased levels of Elk-GAL4 trans-activating protein, because these were similar in both T- and D-Elk cells (Fig. 1C). When only high fluorescence events, (>20 FLH1) were considered, the difference between T- and D-Elk cells was even more pronounced (Fig. 1D). An overnight treatment of both T- and D-Elk cells with 40 μmol/L PD98059, a Mek1/2 inhibitor, caused a ~2- to 3-fold reduction in mean GFP fluorescence and shifted the >20 FLH1 fluorescent population in T-Elk cells into low fluorescence range (Fig. 1A and D), confirming that transcriptional activity of Elk is dependent on ERK activation (Fig. 1A, B, and D). The reduction in GFP level in PD98059-treated T-Elk cells matched the >10-fold reduced level of P-ERK observed after overnight treatment of these cells with the Mek inhibitor 40 μmol/L PD98059 (Fig. 1C). Overnight treatment with 10% FBS increased the number of D-Elk GFP-positive cells in FLH1 >200 from 716 ± 92 (control) to 2134 ± 806 (10% FBS) as well as the mean fluorescence of the population (Fig. 1E). There was a 2-fold increase (30% to 60%) in the percentage of GFP-positive cells after treatment of D-Elk cells with 10% FBS. Treatment of D-Elk cells overnight with 20 ng/ml EGF resulted in a ~50% increase in the number of GFP-positive cells in FLH1 >200 (Fig. 1E). Together, these results indicated that the Elk detector system responds to established stimulators of this pathway. Treatment of T-Elk with 10% FBS or EGF caused only a very minor increase in the percentage of GFP-positive cells with no substantial change in the mean fluorescence, confirming previous findings that showed that T-HEp3 cells are refractory to further activation of ERK over the already high basal levels (data not shown and ref. 12). Experiments to sort the ~30% GFP-positive T-Elk cell population into negative (FLH1 < 103) or positive (FLH > 103) fluorescence groups, followed by a 1- to 2-week growth period, their original content of reporting cells (30–45%). These results suggest that under basal conditions, there is a steady-state reporting population of ~30% GFP-positive cells in which ERK activation is sufficient to induce detectable GFP accumulation. Because, as evidenced by resistance to 100 μg/ml Hygromycin and 400 μg/ml G418, the two constructs are present in a majority of the T-Elk cells, we suspect that the heterogeneity in reporting must be the result of regulation of the intensity of the ERK signaling pathway affecting the promoter function, some of which may produce amounts of GFP that are below the level of detection. Nevertheless, the cells in which Elk-GAL4::GFP system is activated to detectable levels, can be used to report on the state of ERK signaling. The Elk-GAL4 fusion protein does not interfere with the endogenous Elk because the rate of

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1 Internet address: http://rnb.nih.gov/ij
proliferation of cells expressing the construct is similar to that of parental cells (Fig. 3B, left panel).

**CHOP-GAL4-Induced Transcription of hrGFP As a Reporter of p38 Signaling.** To generate cells reporting for p38 signaling, a construct expressing GAL4 DBD fused to CHOP/GADD153 activation domain, a downstream target of p38 (16) was used. The number of GFP-positive events in the range of fluorescence $10^2$ was 419 and 52 for D-HEp-3-CHOP (D-CHOP) and T-HEp-3-CHOP (T-CHOP) cells, respectively (Fig. 2A). D-HEp-3-CHOP and T-HEp-3-CHOP cells retained a functional reporter system because treatment with 50 $\mu$mol/L As$_2$O$_3$, a known p38 activator (17), for 6 to 16 hours increased the number of GFP-positive cells (Fig. 2D and E). The increase in D-HEp-3 cells in response to 50 $\mu$mol/L As$_2$O$_3$ treatment was due to an induction of GFP in cells with previously undetectable levels of GFP (Fig. 2D), whereas in the HT-CHOP cells, the treatment shifted the cells to higher (700-701 and 152-10^4) fluorescence ranges (Fig. 2D and E). The effect of As$_2$O$_3$ treatment was specific for p38/CHOP activation.

**Fig. 1.** Elk-GAL4::GFP system as an indicator of ERK activity. A, expression of hrGFP in living T-Elk (top left panel) and D-Elk (top middle panel) cells grown in serum-free medium for 24 hours, or treated overnight with 40 $\mu$mol/L PD98059 MeK1/2 inhibitor (top right panel); left, middle and right bottom panels, phase contrast images of the same fields. B, FACS analysis (mean GFP fluorescence) of T-Elk and D-Elk cells, grown and treated as in A (for details, see Materials and Methods). The graph shows mean ± SE of three experiments. C, Western blot of total (ERK) and phosphorylated (P-ERK) levels of ERK1/2 and total levels of the Elk-GAL4 trans-activator in T- and D-Elk cells (left panel); right panels, the same in T-Elk cells, control (−) or treated overnight (+) with 50 $\mu$mol/L PD98059 (PD 50 $\mu$mol/L). D, FACS analysis of GFP-positive cells (FLH1 > 20) grown as in A. (Mean ± SE of three experiments). E, FACS analysis of serum-starved D-Elk::GFP cells treated with EGF or 10% FBS for 16 hours. Shown are the number of events in FLH1 > 200. Total events analyzed per treatment, $1 \times 10^5$.
because ERK/Elk activity was unaffected in D-Elk cells (Fig. 2F). Stress-inducing treatments did not significantly increase the number of D-CHOP cells already in high FLH1 range (Fig. 2E), probably because these cells were already maximally activated and prone to die, making them less responsive to treatment.

Similarly to T-Elk cells, HT-CHOP cells sorted into GFP-negative and -positive populations and regrown, re-established their presorting profile but with slower kinetics (data not shown), probably because high p38 activation is not advantageous for growth (13, 17). UV irradiation induced GFP expression in sorted GFP-negative cells, indicating that the reporter constructs were still present but not activated (data not shown). Although the mechanisms governing the heterogeneity of GFP expression are not fully understood, because as with Elk-GFP, GFP here is under the control of specifically activated signaling pathways, this heterogeneity is not unexpected. Nevertheless, even if only a subpopulation, they can serve as a reliable reporter of p38 signaling because the cells that express CHOP-GAL4-induced GFP respond well to changes in p38 activity such as those induced by arsenic treatment or by p38 inhibitors (ref. 13; and see Fig. 5 and Fig. 6).

Does Persistent ERK/Elk Activation Distinguish Proliferative from Dormant Cell Behavior? By monitoring ERK activation through GFP expression, it was possible to assess simultaneously ERK signaling in vivo and tumor growth. We found that, although T-Elk cells inoculated on CAMs of chick embryos formed rapidly growing tumors within 7 days, low ERK activity D-Elk cells formed only small nodules that did not increase in size for up to 8 weeks (Fig. 3 A and B, right panel). This is in direct contrast to the indistinguishable in vitro proliferating capacity that these cells display (Fig. 3 A and B, left panel). Monitoring of GFP to image ERK/Elk activity showed that T-Elk cells maintained persistent and strong ERK activation 24 hours after inoculation on CAMs, activation that was further induced in growing tumors (mean, 126 mm³) after 4 days on CAM, or after subcutaneous inoculation in nude mice (Fig. 3 A and D). The percentage of GFP-
Fig. 3. Activation of ERK/Elk signaling in primary tumors. A, T- and D-Elk cells were inoculated on CAMs (5 × 10^5 cells/CAM); the CAM tumors were excised and photographed 24 to 96 hours later. Cells in culture (A-a and A-f), 24 hours on CAMs (A-b and A-g) and 4 days on CAMs (A-c-e and A-h-j). Tumor nodules captured in bright fields (A-c and A-h) and GFP channel (A-d and A-i) at ×40. A ×200 magnification of the same tumor areas are shown in A-e and A-j; blood vessels (red in bright field channel and dark in the GFP channel) close to the proliferating high ERK/Elk cells (arrow). Scale bars: A-a, -b, -f, -g, 40 μm; A-c, -d, -i, -h, 0.4 mm; A-e, -j, 160 μm. B, left panel, growth of T-Elk, T-HEp3, D-Elk, and D-HEp3 cells in culture over 4 days; all of the cells lines have a similar proliferation rate. Right panel, tumor growth of T-Elk (n = 6), D-Elk, and HT-CHOP (n = 3) cells on chick embryo CAMs or in nude mice examined as indicated in Materials and Methods. Data points, mean ± SD. C, FACS analysis of mean GFP fluorescence in T- and D-Elk cells derived from CAM nodules at 7 days (7d), dissected and dissociated by collagenase treatment (total events in FLH1 > 3 × 10^5); each bar, mean ± SE (n = 7); * P < 0.001, Mann-Whitney test. D, in situ detection of ERK/Elk signaling in 14-day-old primary tumors from nude mice injected with T-Elk (top row) or D-Elk (bottom row) cells (1 × 10^6 cells/mouse). Tumors were excised and inspected as in A. D-a, a large field of a 400-mm^3 tumor with blood vessels (BV, arrow), surrounding a tumor mass containing cells with various degrees of ERK/Elk activity (D-a and -b, dotted line); D-c, a larger magnification of a different T-Elk tumor that also shows strong GFP signal. Scale bar, 80 μm. A D-Elk 4 × 4-mm tumor after 2 weeks in the subcutaneous tissue of nude mice (D-d) was flat but vascularized. D-e, a ×200 magnification of the dotted line square in D-d; this area was negative for GFP-derived fluorescence (D-f). The green fluorescence observed is tissue autofluorescence that could be also detected in the CFP channel (D-g). The rich but immature vasculature in these dormant nodules appears black in the GFP and CFP channels.
positive cells remained similar to that of the original population (30–45%; Fig. 3A). In contrast, the smaller D-Elk nodules (mean, 40 mm³), showed very low GFP fluorescence and a reduced (<20%) proportion of detectable GFP cells on CAMs (Figs. 3A and 5D) as compared with the 45% in cells in culture (Fig. 2F). After 2 weeks on CAMs, the small dormant tumor nodules formed by D-Elk cells contained less than 5% of GFP-positive cells, and those that were positive displayed only slightly above background GFP fluorescence. A rich vasculature of the CAM tissue surrounded both proliferating tumors and dormant nodules (blood vessels appear dark in the GFP channel; Fig. 3A). FACS analysis that directly compared the mean GFP fluorescence of T-Elk cells derived from large (300 mm³) tumors with D-Elk cells derived from small (50 mm³) nodules, both after 7 days on CAMs, showed that the large tumors had 3-fold higher ERK/Elk activity as determined by mean GFP-fluorescence (Fig. 3C). Furthermore, subcutaneous tumors produced by inoculation of 10⁶ T-Elk cells in nude mice showed that, 14 to 18 days after inoculation, 100% (6 of 6) of primary tumors weighing more than 1 g contained numerous strongly GFP-positive cells, readily detected within the undisrupted tumor tissue and in a proportion similar to that in cells in culture (~30%; Fig. 3D). Areas of cells with active ERK/Elk (Fig. 3D) neighbor large and small blood vessels (Fig. 3D, BV; appear dark in the GFP channel). This is in contrast to D-Elk cells (1 × 10⁶ cells per mouse) injected in the nude mice, which had almost undetectable GFP signal in the tumor nodule or in the minces of excised tumors 1 or 14 days after subcutaneous injection (<1% GFP positive), (Fig. 3D and data not shown). In contrast to large tumors (larger than 1000 mm³) generated by T-Elk cells in nude mice after 14 to 18 days (Fig. 3B, right panel), similarly to those on CAMs, the D-Elk-produced nodules were still at 42-mm³ volume after 3 weeks (data not shown).

Together, these results show that, unlike the dormant cells, T-Elk cells that rapidly produce large tumors on CAMs and in nude mice maintain a strong ERK/Elk signaling throughout the period of tumor growth and establish this reporter system as a valid tool to monitor tumor cell populations competent for proliferation.

**Suppression of p38/CHOP Signaling in Continuously Growing Primary Tumors.** Because p38 activation antagonizes ERK activity and favors G₀-G₁ arrest of dormant cells (9, 13), we hypothesized that to proliferate, tumorigenic cells will have to silence p38 signaling. Thus, we monitored p38/CHOP activation in HT-CHOP tumor cells growing on CAMs and nude mice. Surprisingly we found that, compared with cells in culture, within 6 hours postinoculation onto the CAMs, there was a strong activation of p38/CHOP signaling in vivo, which peaked at 24 hours (Fig. 4A and D; and data not shown) and was inhibited by treatment with SB203580 (data not shown). How-

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**Fig. 4. p38/CHOP signaling during primary tumor growth.** HT-CHOP tumorigenic (A) or D-CHOP dormant (B) cells grown in culture (dish) or for 1 to 6 days on CAMs (5 × 10⁶ cells per CAM, A- and B-d1, d4, d6) or 1 and 25 days in nude mice (B-d1 and A-d25, 1 × 10⁶ cells subcutaneously), A- and B-d1 to d25, lower left corner, tumor volumes. B-d1, tumor at day 1 in nude mice was measured after opening a flap of skin over the injection site. A and B, numbers above panels at the far left (culture) and far right (B-d1 and A-d25), the percentage of GFP-positive cells in the tumor cell suspension; scale bars, 80 μm. C, CHOP-GAL4::luciferase activity in parental T-HEp3 cells transiently cotransfected with, or without, a DN-p38 plasmid, inoculated on CAMs and assayed for luciferase activity (at least four per transfection) 48 hours after inoculation; bottom panel, a Western blot with anti-Flag antibodies used to control for expression of the Flag-tagged-DN-p38 expressed in parental HEp3 cells. D, the percentage of HT-CHOP- and D-CHOP GFP-positive cells in at least three primary tumors per cell line grown on CAMs and excised, enzymatically dissociated, and quantitated for GFP-positive cells at the indicated time points.

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however, as the tumor mass increased during the next 4 to 7 days, both GFP fluorescence intensity and HT-CHOP GFP-positive cells decreased dramatically to levels similar to, or lower than, those observed in culture conditions (Fig. 4A and D). Similarly, T-HEp3 cells, transfected with a CHOP-GAL4 plasmid and a luciferase reporter instead of GFP, showed a very strong induction of p38/CHOP-dependent luciferase activity 48 hours after inoculation on CAM, which was curtailed by cotransfection with a dominant-negative p38 (DN-p38) construct (Fig. 4C), indicating that the increase in CHOP activity is dependent on p38 signaling. In these cells, this increased p38 activity caused a mild inhibition of ERK signaling (data not shown). In contrast, D-CHOP cells (7 ± 3.2% GFP-positive in culture) that formed very small nodules on CAMs, showed an initial strong activation of p38/CHOP signaling that reached 31 and 48% of GFP-positive cells at 24 and 96 hours of inoculation, respectively, and persisted at high levels (31–42%) for at least 2 weeks (Fig. 4B and D). A similar trend was found in D-CHOP cells injected subcutaneously into nude mice, in which ~25% of the cells became GFP positive 24 hours after injection in vivo (Fig. 4B). Monitoring of D-CHOP cells injected subcutaneously in nude mice showed that, for at least 3 weeks, their tumor volume did not exceed 60 mm³ (in contrast to T-Elk or HT-CHOP cells, which formed >600-mm³ tumors in the same time); thus, regardless of the in vivo system used, D-CHOP cells entered dormancy.

Examination of GFP expression in HT-CHOP cells that were injected into nude mice and that formed large primary tumors (Fig. 3B), showed a reduction in active p38-containing cells from 21.8 ± 5.7% in culture to 1 ± 0.6% in vivo (3/3 mice; Fig. 4A). The antibiotic-resistant but GFP-negative tumor cells retained their reporter plasmids because treatment with inducers of p38 activity (As₂O₃ or UV irradiation) produced respectively, an ~3- and 10-fold increase in the percentage of cells expressing GFP (Fig. 5A and B). This induction was p38 dependent, because the UV-induced increase in GFP-positive cells was inhibited by cotreatment with 5 μmol/L SB203580 p38 inhibitor (Fig. 5A and B). FACS analysis indicated that the UV treatment induced a 2.1-fold increase in the total number of GFP-positive cells, a 3.2-fold increase in fluorescence >50% and a 16-fold increase in fluorescence >20% (Fig. 5C, and data not shown).

Treatment of D-Elk cells (high p38) in culture for 48 hours with 5 μmol/L SB203580 caused a pronounced increase in ERK/Elk signaling, which persisted for 24–96 hours in the inoculation site in vivo both on CAMs and in nude mice (Fig. 5D), a treatment that results in increased proliferation of D-HEp3 cells (9). The larger tumor nodules produced by SB203580-pretreated cells contained a higher percentage (~45%) of GFP-positive cells than did untreated D-Elk cells, which produced small nodules containing only 18 ± 6% of GFP-positive cells. GFP signal in D-Elk nodules in nude mice was even lower (~5%) than on the CAMs (Fig. 5D). Thus, although the dormant
D-CHOP and D-Elk cells are unable to correct the negative effect of p38 on ERK on their own, when appropriately stimulated, they can reactivate ERK (Fig. 5D) and exit dormancy (9).

**Does Differential Activation of ERK/Elk and p38/CHOP Pathways Dictate Metastatic Growth?** Liver and lungs of the chick embryo are the sites of spontaneous HEp3 cell metastases (18). We tested whether differential ERK and p38 activation occurred in cells disseminated to the liver during early stages (48–50 hours) of their growth. T-HEp3 cells transiently transfected with the Elk- or CHOP-GAL4::luciferase reporter plasmids were inoculated on CAMs, and 48 hours later, luciferase activity was measured in liver lysates. We found that cells lodged in the liver activated ERK/Elk-dependent luciferase expression, whereas CHOP activity was undetectable (Fig. 6A). In the nude mouse, a permissive organ for HEp3 metastasis is the lung, in which we have shown that at the time of primary tumor removal 10% of mice have lung metastases and, 4 weeks later, 61% of the mice have lung metastases (15, 19). In contrast, the liver and spleen are sites known to not harbor overt HEp3 metastases in nude mice. Elk::GFP or CHOP::GFP tumor cells were not detected in livers and spleens with fluorescence microscopy nor identified in hematoxylin and eosin (H&E)-stained tissue sections (data not shown), which suggests that either they did not lodge in these organs or that CHOP, and thus p38 activation in nonpermissive sites, was strong enough to induce cell death and clearance of these cells. At the time of primary tumor removal, the lungs of mice had cells with active ERK/Elk::GFP activity in the pleura (~10 GFP-positive cells/lung) of 3 of 6 mice (early metastatic spread; Fig. 6B-b and -c). These were readily observed by microscopic inspection of the excised lungs or tissue minces placed in PBS-containing Petri dishes (a single-cell top panel, or 2–3 cells middle panel; Fig. 6B-b and -c). The GFP signal was specific because no fluorescence was detected in the cyan fluorescent protein (CFP) channel (Fig. 6B-d). In chick embryo, T-Elk cells metastasized with 100% efficiency to the lungs 7 days after inoculation because each of the lungs examined displayed some GFP-positive cells (Fig. 6B-a). We used the standard metastasis quantitation assay (18) in which lung minces are reincubated on fresh CAMs and examined 7 days later when the proportion of tumor/host lung cells increases because of tumor cell proliferation (18). This experiment revealed that 17.3 ± 7.2% (median, 19.2%; maximum, 28%; minimum, 8%) of the cells recovered from the lungs were positive for GFP (n = 8). These results suggest that in a growth-permissive organ, such as the mouse lung or chick embryo lungs and liver (Fig. 6A), lodged cells are able to activate mitogenic ERK/Elk signaling.

**Fig. 6.** ERK/Elk and p38/CHOP signaling in metastatic sites. A, Elk- or CHOP-GAL4::luciferase activity in livers of chick embryos. Transfected T-HEp3 cells, (see Materials and Methods), were inoculated on CAMs 24 hours posttransfection, and 48 hours later, the chick embryo livers were assayed for luciferase activity. Background levels of luciferase activity (below dashed line) were determined in T-HEp3 cells transfected with the luciferase plasmid alone. B, GFP-positive T-Elk cells in lungs of chick embryos 7 days after inoculation (B-a, arrows) and nude mice at the time of primary tumor removal (B-b, -c, and -d, arrows). Examples of single T-Elk cells (B-b, arrows) or small groups of cells with active ERK/Elk signaling in the GFP (B-c, arrows) or CFP (B-d). Scale bar (B-a, -b, -c, -d, 40 μm). H&E staining of histologic sections of a mouse lung 21 days after removal of an HT-CHOP primary tumor removal (B-e, arrow). p38/CHOP activity in HT-CHOP cells (B-f, control cells; Control) obtained from mice lungs (B-e) in response to UV irradiation (B-g, U.V.) or arsenic treatment for 24 hours (B-h, Ars. 25 μM/L). Scale bar (B-f, -g, -h), 80 μm. The cells isolated from the lungs are almost completely negative (B-e). C, FACS of HT-CHOP cells treated as in B for 24 hours after UV treatment (middle histogram, U.V.) or As2O3 (bottom histogram, Ars. 25 μM/L) treatment compared with control cells (top histogram, D). quantitation by FACS of GFP-positive events in metastatic HT-CHOP cells treated as in B. Below the graph, fluorescence ranges in which the events were quantitated. Total events analyzed were 5 × 10^5.
Metastasis to lung was examined in nude mice inoculated with HT-CHOP cells 17–21 days after the primary tumor excision. A comparison of H&E staining of lung sections with inspection of lung minces under fluorescent microscope revealed that, although all of the lungs had micrometastases by H&E (Fig. 6B-e), only one of three lungs showed two GFP-positive cells. However, when cultured in selection medium, these minces produced numerous antibiotic-resistant colonies (40–50 colonies) that we expanded for a week and pooled (Fig. 6B-f). When examined, similarly to their primary tumors these lung-derived metastatic cells showed only 1–2% of GFP-positive cells after 10 days in culture (Fig. 6C and D). However, treatment of these cells with 25 μmol/L As2O3 for 7 to 24 hours induced a 3- to 10-fold increase in GFP-positive cells because of conversion of undetectable GFP-p38/CHOP cells into stronger GFP expressers (Fig. 6B-h, C, and D). UV irradiation (see Materials and Methods) also generated a strong increase in p38/CHOP activity (Fig. 6B-g).

These results showed that the presence of the CHOP-GAL4 reporter system does not interfere with dissemination and growth of metastatic cells and that, similarly to the cell in the primary tumors, the disseminated cells in a growth-permissive site maintain the proliferative mode by persistently down-regulating their p38/CHOP signaling.

**DISCUSSION**

We demonstrate here that GFP expression, driven by Elk- or CHOP-GAL4 trans-activators, can specifically report for ERK or p38 activity, respectively, and that in vivo monitoring of these pathways through GFP expression, aids in elucidating tumor cell fate at primary and metastatic sites, and in their subsequent isolation. Placing GFP expression under the control of a regulated pathway provides several advantages over the tagging of tumor cells with constitutive expression of GFP (8, 20) because, in addition to positional identification of cells, it allows for monitoring of their active signaling programs. In addition, the modular properties of GAL4DBD allow different activation domains to be combined to monitor diverse signaling or protein–protein interactions (21). This, in turn, may allow the examination of in vivo effects of microenvironments or treatments on multiple signaling pathways and downstream genetic programs.

Our experiments show that in vivo proliferating ERK/Elk::GFP cells express GFP and that the expression is bound to the state of activation of the ERK pathway. These results fit well with our previous prediction that cells with high ERK activity in culture will be tumorigenic in vivo (13). However, only through this new approach were we able to show that T-Elk tumor cells maintain high ERK/p38 activity throughout tumor growth, whereas the dormant D-Elk cells, which have a much lower level of ERK activity, both in culture and at 24 hours on CAM, appear to reduce it even further at 72 to 96 hours in vivo. These results provide a functional link to our previous findings showing an increased S-phase fraction of T-HEp3 cells and a rapid G0/G1 arrest of D-HEp3 cells, respectively, 48 hours after in vivo inoculation (10). These conclusions, reached through the study of a primary tumor, were also true for metastasis where we found ERK/Elk activation in growth-permissive metastatic sites at times when micrometastatic growth is known to begin (15). In both the primary and the metastatic sites, a proportion of the T-Elk cells had undetectable GFP levels, which suggested that the threshold of ERK signaling that is required to induce GFP is higher than that required to promote cell cycle progression, or indicated a more complete silencing of ERK signaling and induction of dormancy. We resolved this uncertainty by testing T-HEp3 and HT1080 cells transfected with the p38-reporting CHOP::GFP. Because we previously showed that dormant cells have high p38 activity (9, 13), we expected that cells incapable of sustaining ERK signaling after dissemination would have high p38/CHOP-induced GFP expression and would rapidly enter a dormant state. However, monitoring p38/CHOP signaling in vivo in T-HEp3 and HT1080 cells revealed a previously unrecognized p38 regulation; a rapid (24 hours of in vivo inoculation) and strong rise in p38 activity, which turned out to be transient. Unlike ERK activation, which is maintained at high levels throughout the tumor growth, the p38 peak was down-regulated within 48 hours. The rapid silencing of p38 activity appeared to have prevented a more substantial negative effect of p38 on ERK that was observed when high p38 activity was more persistent (9, 17, 22). The initial rise in p38 activity may be due to stress-activated signaling in response to interaction with the in vivo microenvironment. This transition from culture to in vivo conditions may be an exaggerated version of the changes a cancer cell encounters in vivo when in transit from a growth-permissive to a growth-restrictive milieu. Nonetheless, it shows that p38 activity remains susceptible to regulation by external cues and that, to ensure a proliferative ERK-to-p38 ratio, tumorigenic cells activate a mechanism that rapidly silences p38 signaling. Overall, these results suggest that cancer cells that are not "equipped" to correct an imbalance in ERK-to-p38 ratio to favor ERK signaling may die or enter dormancy, but that those that can curtail the stress-activated p38 may resume proliferation. This was the case for D-Elk and D-CHOP cells that, on interaction with the in vivo microenvironment, rapidly down-regulated ERK signaling and persistently activated p38 for at least the first 14 days. Our findings are further supported by studies showing that MKK4, an important upstream activator of c-Jun NH2-terminal kinase (JNK) and also p38 (3, 23), is a metastasis suppressor gene silenced in prostate cancer (23, 24). By disabling the JNK or p38 pathway, the loss of MKK4 function may allow the cells to escape growth-inhibitory effects.

Although lung tissues suspected of harboring metastasis had very few GFP-expressing HT-CHOP cells, growth and selection of lung cell suspensions in G418 produced many antibiotic-resistant colonies that retained the ability to activate CHOP signaling on treatment with As2O3 or UV irradiation. This suggests that if there was transient activation of p38 in cells lodged in metastatic sites, it was subsequently silenced and that these cells retained the ability to achieve a proliferative ERK/p38 balance. More importantly, these results indicate that the pathway and the reporter system remain intact and suggest that, at least in this case, the molecular-genetic programs activated in vivo, are maintained in culture, thus providing an opportunity for their characterization. HT1080 cells are derived from a metastatic lesion. The fact that only very few HT-CHOP::GFP-positive cells were detected in lungs, whereas explantation of these lungs in culture produced many tumor colonies, suggests that only a small proportion of these cells can maintain p38 signaling to become dormant in a growth-permissive site. Although the mechanism of p38 inhibition is unclear, the small GTPase Ras, known to favor metastatic growth (25), requires p38 suppression for transformation (26, 27). Because T-HEp3 cells display constitutive GTP-loaded Ras (11), and an N-Ras activating mutation was found in HT1080 cells (28), it is possible that unknown signals branching from the Ras-Raf module inhibit p38 activation. Our previous findings showed that p58 down-regulation and subsequent inactivation of α5β1, results in p38 activation, which in turn depends on activation of Cdc42 (13). Treatments that result in the disruption of the p58 complex stimulate p38 activation and inhibited in vivo growth (9, 10). Thus, it is possible that α5β1-dependent signals, other than those activating Ras-ERK signaling (9–13) but still emerging from the intact p58 complex, are responsible for the rapid silencing of p38 signaling in proliferating cells in primary and metastatic sites.

In summary, the use of a reporter system that allows spatial-temporal monitoring of ERK or p38 signaling through GFP induction, revealed a previously unrecognized regulation of these pathways that appears to dominate the tumor cell fate in primary and metastatic
tissues. Our findings set the stage for future efforts aimed at uncovering mechanisms of dormancy induction by identifying spontaneously dormant metastatic cell populations in vivo and recovering them for characterization.

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