ras transformation is associated with decreased expression of the brm/SNF2α ATPase from the mammalian SWI–SNF complex

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The brm and BRG-1 proteins are mutually exclusive subunits of the mammalian SWI–SNF complex. Within this complex, they provide the ATPase activity necessary for transcriptional regulation by nucleosome disruption. Both proteins were recently found to interact with the p105Rb tumor suppressor gene product, suggesting a role for the mammalian SWI–SNF complex in the control of cell growth. We show here that the expression of brm, but not BRG-1, is negatively regulated by mitogenic stimulation, and that growth arrest of mouse fibroblasts leads to increased accumulation of the brm protein. The expression of this protein is also down-regulated upon transformation by the ras oncogene. Re-introduction of brm into ras transformed cells leads to partial reversion of the transformed phenotype by a mechanism that depends on the ATPase domain of the protein. Our data suggest that increased levels of brm protein favour the withdrawal of the cell from the cycle whereas decreased expression of the brm gene may facilitate cellular transformation by various oncogenes.

Keywords: Brahma/cell cycle/chromatin/transcription

Introduction

The SWI–SNF genes were first characterized in Saccharomyces cerevisiae, where their products were defined as transcriptional activators, involved in the regulation of a subset of inducible genes. In this organism, the 11 SWI–SNF proteins identified so far are assembled in a 2 MDa complex that affects transcriptional activity by modifying the chromatin structure in the vicinity of target promoters (for review, see Carlson and Laurent, 1994; Peterson, 1996; Burns and Peterson, 1997a). The molecular mechanism by which the SWI–SNF complex facilitates the assembly of the pre-initiation complex has not yet been fully elucidated. However, it is likely to create stable openings in the nucleosomal array, which help general transcription factors in competing with histones for DNA binding (Owen-Hughes et al., 1996; Burns and Peterson, 1997b). A direct interaction of the SWI–SNF complex with the RNA polymerase II holoenzyme has also been suggested (Wilson et al., 1996).

Several members of the yeast SWI–SNF complex have now identified counterparts in higher eucaryotes. In Droso-
SWI2/SNF2 proteins may be necessary for the tumor suppressing activity of p105Rb. Besides, in SW13 cells that contain wild type p105Rb but low levels of endogenous SWI2/SNF2 proteins, transiently transfected hbrm or BRG-1 expression vectors induce the formation of flat, growth-arrested cells. This flat cell inducing activity is reduced either by the deletion of the pRb interaction domain of the two proteins or by the co-transfection of E1a (Dunaief et al., 1994; Strober et al., 1996). Finally, co-transfection of hbrm and p105Rb expression vectors leads to increased E2F repression as compared with p105Rb alone (Trouche et al., 1997). These observations raise the possibility that the mammalian SWI–SNF complex may co-operate with p105Rb to induce growth-arrest.

In an earlier study, we have shown that in human cells, hbrm and BRG-1 are phosphorylated at the G2/M transition, leading to decreased affinity of these proteins for their nuclear attachment sites, and resulting in their chromosomal exclusion upon mitosis. During this phase of the cell cycle, the hbrm protein is also partially degraded, only to be resynthesized in a dephosphorylated form after cytokenesis (Muchardt et al., 1996). These observations suggest that the mammalian SWI–SNF complex is re-activated during the G1-phase, at a time when the cell can either exit from the cycle or continue into S phase. This prompted us to investigate the effect of growth conditions on the cellular pools of mammalian brm and BRG-1. We will show that in mouse fibroblasts, mbrm but not mBRG-1, accumulates to high levels in growth-arrested cells, but is down regulated upon serum induced re-entry into the cell cycle. In a similar way, transformation of fibroblasts with a constitutively expressed hbrm cDNA into ras transformed cells leads to strongly decreased levels of mbrm, as compared with the parental non-transformed cells. Again, the level of mBRG-1 is not affected by ras transformation. Re-introduction of a constitutively expressed hbrm cDNA into ras transformed cells leads to partial reversion of the ras transformed phenotype. This effect of hbrm was abolished by a mutation in the ATP-binding site of its helicase-like domain. Screening of other rodent cell lines revealed that a mutation in the ATP-binding site of hbrm leads to partial reversion of the transformed phenotype. This effect of hbrm was abolished by decreased expression of the mbrm protein.

densed chromosomes. However, the decreased level of the protein during mitosis was less prominent in NIH3T3 than in HeLa cells (data not shown). The NIH3T3 cells were cultured for 72 h in medium containing either 5, 10, 15 or 20% fetal calf serum (FCS). Phase contrast microscopy showed that the cells grown in 5% FCS were flat, whereas in 20% FCS, they were rounder and more refringent, somewhat resembling transformed cells. Western blot analysis of total extracts revealed that the level of mbrm was lowest in cells grown in 20% FCS, and highest in cells grown in 5% FCS (Figure 1A, lanes 1 and 4). Quantitative RT–PCR showed that decreased levels of mbrm protein could be correlated with decreased levels of mbrm mRNA (Figure 1B). On the contrary, the level of mBRG-1 mRNA or protein was either unchanged or moderately increased when cells were grown at 20% FCS, as compared with 5% FCS (Figure 1C and D). To further characterize the effect of the serum concentration on cellular levels of mbrm and mBRG-1, NIH3T3 cells grown in medium containing 20% FCS were shifted to medium containing 0.25% FCS for 24 h. Western blot analysis of extracts from these serum-starved cells revealed a strong increase in the level of mbrm, as compared with the cells grown in 20% FCS (Figure 1E). Again, serum starvation had no effect on the level of mBRG-1 (Figure 1F). The reciprocal experiment was performed by serum starving cells for 48 h and then stimulating them with medium containing 20% FCS. As shown Figure 1G, Western blot analysis of cellular extracts, performed at different time points after stimulation, showed a marked decrease in the level of mbrm protein, first visible 10 h after the addition of serum. Quantitative RT–PCR showed that the decreased level of mbrm protein could be correlated with a decrease in the level of mbrm messenger RNA (Figure 1H). At the same time, we did not observe any significant variations in the level of mBRG-1 protein that apparently was not affected by the serum stimulation (Figures 1I and 1J). To compare the decrease in mbrm expression with the induction of an immediate early gene, we followed the levels of Jun-B protein in the serum stimulated cells (Figure 1K). The level of this protein was already high 1 h after serum stimulation and then decreased between 10 and 20 h, in agreement with previous publications (Lallemand et al., 1997). Since the decrease in mbrm levels is relatively late compared with Jun-B induction, it may be considered as a delayed early or late event in the G0/G1 transition. This prompted us to verify that the decrease in mbrm expression occurred at the G0/G1 and not at the G1/S transition or later, and we performed flow cytometric analysis of exponentially growing NIH3T3 cells indirectly labelled with anti-brm antibodies. In this experiment, we observed no variation in the level of mbrm protein between cells in G1 and S phase, confirming that the expression of mbrm was primarily regulated at the exit from quiescence (data not shown).

Results

Mitogenic stimulation of NIH3T3 cells leads to decreased expression of the mbrm protein

To determine the effect of growth factors on brm and BRG-1 expression, we cultured cells in medium containing increasing serum concentrations. For this experiment, we used NIH3T3 mouse fibroblasts, because their growth is highly dependent on the presence of mitogens in the medium and they can be efficiently arrested in G0 by serum starvation. The mouse mbrm protein present in NIH3T3 cells appeared to be regulated in a way very similar to the human hbrm present in Hela cells (Muchardt et al., 1996). As determined by Western blotting and immunofluorescent staining, the mbrm protein was phosphorylated during mitosis and excluded from the concent
transformed by the incorporation of two copies of a Ki-ras gene (Noda et al., 1983). Western blot analysis showed that DT cells contained normal levels of mBRG-1, but undetectable levels of mbrm (Figure 2A and B, lane 4). To determine if mbrm expression was reproducibly down-regulated upon ras transformation, NIH3T3 cells were transfected with an activated Ha-ras gene and a plasmid conferring neomycin resistance. After neomycin selection, several resistant clones were isolated and expanded for further analysis. Among these clones, some were morphologically similar to the initial NIH3T3 cells, while others resembled the highly tumorigenic DT cells mentioned above, with a characteristic rounded shape. The extent of transformation of these clones was evaluated by scoring colony formation in soft agar. The ability of these clones to form colonies could be roughly correlated with the level of accumulated ras protein, as estimated by Western blotting. These clones were further described elsewhere (Pfarr et al., 1994; Mechta et al., 1997). Two clones, named R2 and R3, were selected as representative of high and low degrees of transformation. R2 had a high level of ras expression and a 50% plating efficiency in soft agar, whereas R3 had a lower level of ras expression and only ~30% plating efficiency. Western blot analysis showed that mbrm was not expressed in the R2 clone, but could still be detected in the R3 clone, albeit at a reduced level (Figure 2A, lanes 2 and 3). Again, mBRG-1 accumulation was not affected. These observations suggested that, as in serum stimulated NIH3T3 cells, the level of mbrm, but not mBRG-1, is dependent on the level of mitogenic signal received by the cell. Southern blot analysis of genomic DNA from the various ras transformed cell lines showed no obvious rearrangement of the mbrm gene in the ras transformed cell lines (data not shown).

Examination of the mRNA levels by quantitative RT-PCR on RNA from DT, R2 or R3 cells showed a direct correlation between the level of RNA and the amount of protein detected in the different cell lines (Figure 2, panels C and D), indicating that transformation, like serum stimulation, down-regulates mbrm levels by affecting transcription efficiency or mRNA stability.

**Expression of hbrm leads to partial reversion of the ras transformed phenotype**

To evaluate the significance of reduced mbrm expression in the ras transformation process, we re-introduced a constitutively expressed hbrm cDNA into DT cells. As a full-length mouse brm cDNA has not been cloned, we
used a construct of human origin containing an HA-tagged version of the hbrm protein, expressed from an MoMuLV LTR (schematically shown in Figure 3A). The function of the human and the murine proteins is likely to be conserved, since fragments sequenced in both species have revealed an overall identity of about 98%. Two of these clones (DT19 and DT21) contained levels of hbrm similar to, or moderately higher than, the normal NIH3T3 cells, while two others (DT24 and DT27) contained lower than normal levels of the protein. The original DT cells, which are flat when first plated, rapidly become round as confluency increases. At a density of $\sim 2 \times 10^6$ cells per 60 mm plate, the cells lose adherence to the plate, most probably because of accumulation in the medium of matrix degrading proteinases (Spinucci et al., 1988). The cells then die within 24 h if they are not replated in fresh medium (Pfarr et al., 1994). Several of these growth characteristics were modified in the DT derived clones expressing hbrm. These cells remained flat, even at high confluency (compare panels E and F in Figure 3) and had a reduced growth rate compared with the parental DT cells (Figure 3G). These modified growth characteristics of the DT clones expressing hbrm could not be related to decreased ras expression, since DT cells and the derived clones all accumulated similar levels of ras protein (Figure 3B and C). Still, these observations suggested that the hbrm-expressing DT cells might be affected in their degree of transformation, as compared with the original DT cells. Therefore, we subjected the different clones expressing hbrm to standard assays to monitor cellular transformation. Three out of four clones were unable to grow in medium containing a reduced serum concentration (0.25% serum; Figure 4A). Flow cytometric analysis showed that in this medium, cells remained viable but were arrested in the G0/G1 phase of the cell cycle (data not shown). The clones were also tested for their ability to form colonies in soft agar. All were found to have a strongly reduced plating efficiency, as compared with the original DT cells (Figure 4B). Two colonies, obtained after plating the DT21 cells in soft agar (DT21.1 and DT21.2), were expanded and re-assayed for hbrm expression. These clones were both found to be revertants containing undetectable levels of hbrm protein (Figure 4C, lane 8 and 9). To further determine the effect of hbrm on DT cell growth, the DT19 and DT21 cells were tested for their ability to form tumors in nude mice. After 10 days, no tumors could be detected in mice injected with DT21 cells. Injection of DT19 cells produced tumors with an average mass of one fifth of the mass of the DT or DT plus neo tumors (Figure 4D). The modified growth characteristics of the DT cells upon re-introduction of an exogenous hbrm gene, suggest that the loss of normal mbrm expression upon transformation by ras is at least partially responsible for the transformed phenotype of these cells.

To determine if the effect of hbrm on DT cell growth required the DNA dependent ATPase activity, which has been associated with this protein (Wang et al., 1996a), we constructed DT cell lines constitutively expressing an
hbrm protein, mutated in the ATP-binding site of the helicase-like domain (ATPmut; Figure 3A and D). A similar mutation has previously been shown to convert BRG-1 into a dominant, negative transcriptional activator (Khavari et al., 1993). As shown in Figure 4B, the plating efficiency in soft agar of DT clones expressing ATPmut protein (A2 and A26), was similar to that of the initial DT cells. Again, we checked if the colonies growing in soft agar would still express the transgene. As shown in Figure 4C, the tested clones derived from A2 (A2.1 and A2.2) expressed similar or higher levels of ATPmut protein, as compared with the initial A2 cells. Thus, expression of an hbrm protein, mutated in its ATPase domain, did not appear to affect DT cell growth.

Down-regulation of mbrm expression is observed in rodent fibroblasts transformed by other oncogenes

The possibility that down-regulation of mbrm could be part of the mechanism leading to cellular transformation, prompted us to investigate the levels of mbrm and mBRG-1 expression in cell lines transformed by oncogenes other than ras. First, we tested E1Hneo cells that are derived from NIH3T3 cells by infection with a retrovirus carrying the raf oncogene, acting downstream of ras. Like the DT cells, these cells were found to have normal levels of mBRG-1 protein, but levels of mbrm significantly lower than those observed in NIH3T3 cells (Figure 5, lane 2). This observation clearly confirms that the signal transduction pathway activated by ras and raf is responsible for the down-modulation of mbrm synthesis. We next tested a rat fibroblast cell line, PYF 3.2, transformed by Polyoma middle T (Gorga et al., 1990). This oncogene activates several growth promoting signals, including the ras pathway. Again, we found that the PYF 3.2 cell line showed reduced mbrm expression, as compared with the parental immortalized rat fibroblast cell line F111 (compare lanes 3 and 4 of Figure 5). This observation indicates that down-regulation of mbrm expression can also be induced by viral oncogenes. To verify that reduced levels of mbrm were not specific for in vitro transformation, we tested a cell line derived from a methylcholantrene-induced fibrosarcoma, known as MCO-1 (Figure 5, lane 6). These cells also had a reduced level of mbrm protein.

Discussion

The brm gene is down-regulated by the ras signal transduction pathway

Recent studies have shown that at least two versions of the SWI–SNF complex coexist in mammalian cells, containing either brm or BRG-1 and several common subunits (Wang et al., 1996a,b). Initial studies suggested that brm and BRG-1 proteins share very similar properties. Both proteins were shown to stimulate the activity of nuclear hormone receptors in transient transfection studies. Furthermore, yeast two-hybrid and GST pull-down experiments revealed that both proteins interact with pRb family members via an LXCXE motif, also present in several viral oncogenes. This interaction was essential for the formation of flat G0-arrested cells, upon transient transfection of expression vectors for both proteins (Dunaief et al., 1994; Singh et al., 1995; Strober et al., 1996). The interaction of the mammalian SWI2/SNF2 homologues with pRb, and their negative effect on cell growth, was somewhat unexpected for components of a nuclear complex that is involved in facilitating transcription. Recent observations bring a possible explanation to this paradox by demonstrating that hbrm co-operates with p105Rb in repressing E2F-1-dependent transcriptional activation (Trouche et al., 1997). The inhibition of E2F-
1-activated transcription may explain the flat cell forming activity of hbrm and BRG-1.

Previous studies did not clearly distinguish between brm and BRG-1, and raised the possibility that the two proteins fulfil identical functions. At present, we clearly demonstrate that this is not the case. We show that whereas the level of BRG-1 does not vary with the growth rate of the cells, mbrm levels are highly variable. The mbrm protein accumulates in quiescent mouse fibroblasts and strongly decreases after mitogenic stimulation, which activates the ras/raf/ERK pathway. In agreement with these observations, transformation of fibroblasts by ras or raf almost completely inhibits the accumulation of mbrm mRNA and protein, with no effect on BRG-1 protein or mRNA. Re-expression of hbrm from a constitutive promoter reverses the transformed phenotype of the cells, without directly blocking the ras/raf pathway. This last conclusion is based on the finding that the typical pattern of expression and phosphorylation of Jun and Fos family members induced by ras transformation is not altered after re-introduction of hbrm (Mechta et al., 1997; C.Muchardt and B.Bourachot, unpublished observations). The stable re-introduction of a constitutively expressed hbrm gene into DT cells is the first reported experiment allowing an assay of the effect of this protein on cells in which it is not aberrantly overproduced. In our system, expression of hbrm leads the ras transformed DT cells to recover a morphology and growth rate closer to those of normal NIH3T3 cells but, unlike hbrm transfected SW13 cells, growth arrest was only observed when cells were serum-starved. It is possible that the almost physiological levels of hbrm, which we obtain in the DT cells, are not
The mbrm protein is down-regulated in several transformed cell lines. Extracts from either NIH3T3 (lanes 1 and 5), EHneo (lane 2), F111 (lane 3), PYF3.2 (lane 4) and MCO-1 (lane 6) cells were analyzed by SDS–PAGE and Western blotting with either anti-hbrm (top panel) or anti-BRG-1 (bottom panel) antibodies.

Possible functional differences between brm and BRG-1

The studies cited above clearly demonstrate that brm and BRG-1 may have both common and distinct roles. Both affect the activity of nuclear hormone receptors, interact with pRB and may be important for controlling G1 progression. However, only brm is down regulated by ras. Another difference between brm and BRG-1 was observed during the study of embryonic carcinoma (F9) and embryonic stem (ES) cells. While non-differentiated cells contain predominantly BRG-1, brm accumulated upon in vitro differentiation (C.Muchardt and J.-C.Reyes, unpublished observations). One has to recall that undifferentiated ES or EC cells have a very short G1 phase, and that F9 embryonic carcinoma cells are tumorigenic before, but not after, differentiation (Dony et al., 1986; Savatier et al., 1994). In this respect, a recent study by Sumi-Ichinose et al. (1997) has shown that inactivation of one of the two BRG-1 genes by homologous recombination in F9 cells, affected their capacity to proliferate, but not to differentiate. No viable cells lacking both copies of BRG-1 were obtained after drug selection. On the contrary, we have isolated viable ES cells that lack both copies of mbrm (J.-C.Reyes, unpublished results). These studies strongly suggest that BRG-1, but not brm, is essential for viability of early embryonic cells. The persistence of BRG-1 in faster growing cells, and the accumulation of brm in cells that become slow growing or arrested, raise the possibility that a partial switch from BRG-1 to brm-containing complexes occurs when cells slow their growth. Part of the chromatin sites occupied by BRG-1-containing complexes may be replaced by brm-containing complexes. In addition, new sites may be occupied by the brm complex. This differential targeting could be mediated by the structure of promoters or by DNA-binding proteins with affinity for either brm or BRG-1. This may facilitate the expression of genes that are important for the maintenance of the quiescent or terminally differentiated state of cells. SWI–SNF complexes containing brm may be important for the activation of growth arrest related genes, as well as genes that promote the formation of cell–cell or cell–matrix contacts and the stabilization of the cytoskeletal network. Studies of mutant mice lacking brm or BRG-1, and eventually of cells isolated from such mice, will permit us to test our hypothesis. A switch of protein isoforms between rapid early embryonic cell divisions and later slower somatic divisions has frequently been reported. Besides, it is well documented that several species, including Xenopus laevis or sea urchin, use different repertoires of histones H2A and H2B and linker histone genes during development, leading to changes in the degree of DNA compaction (Wolfe, 1995).

Chromatin associated factors and cellular transformation

In Drosophila, the polycomb group of proteins, involved in homeotic gene regulation, has been shown to create local heterochromatin-like structures, leading to transcriptional repression. This repression is antagonized by members of the trithorax family of proteins, by a mechanism that is not yet understood (for review, see Orlando and Paro, 1995). The bmi-1 protein was the first mammalian homologue of a member of the polycomb group to be isolated. This protein is functionally related to its Drosophila homologue, Psc, and was initially identified as a proto-oncogene, able to accelerate lymphomagenesis in Eμ-myc transgenic mice (Haupt et al., 1991; Alkema et al., 1995). In parallel, mutations of the Drosophila brm gene can antagonize Polycmb mutations, and brm is considered to be a member of the trithorax group of genes (Brizuela et al., 1994; Tamkun, 1995). In the present study, we provide evidence that the mammalian homologue of Drosophila brm is involved in growth control, and that deregulation of this protein may be one of the events leading to cellular transformation. bmi-1 and mammalian brm may not directly antagonize the effect of each other, but they provide evidence that proteins involved in transcriptional regulation at the level of chromatin may be important regulators of cell growth.

Materials and methods

Cell extracts and immunoblot analysis

All cell lines were maintained at 37°C in 7% CO2. Hela, NIH3T3, EHneo, F111, PYF3.2 and MCO-1 were grown in DMEM supplemented with 7% FCS, unless otherwise indicated. Cells were lysed in 200 μl of Buffer B (8 M urea, 0.1 M Na2HPO4, 0.01 M Tris, pH 8). Protein concentrations were determined using the Bio-Rad Bradford reagent. For Western blot analysis, 20 μg of extract were fractionated by SDS-PAGE and transferred to nitro-cellulose membranes. The membranes were then blocked with PBS/0.2% Tween-20/10% horse serum, and incubated with the various antibodies. Enhanced chemiluminescence (ECL) reagents (Amersham) were used for detection. Antibodies used for detection of hbrm and BRG-1 have previously been described (Muchardt et al., 1996). Polyclonal rabbit antibodies against Jun-B were kindly provided by D.Lallemand. The ras oncogene was sufficiently to counteract the mitogenic signals induced both by serum and activated ras. Suppression of the ras transformed phenotype requires functional hbrm, since a mutant in the ATP-binding domain is quasi inactive. Deletion of the E7 homology, which abolishes pRB binding in the yeast two-hybrid system (Strober et al., 1996) reduced, but did not abolish growth in agar, raising the possibility that agar growth suppression is not exclusively dependent on pRB binding (C.Muchardt and B.Bourachot, unpublished results).
detected using a monoclonal mouse anti-ras antibody from Oncogene Science (cat# OP40). HA-tagged hbrm was detected using the 12CA5 monoclonal mouse antibody.

RNA preparation and RT–PCR
Total RNA was purified on CsCl cushion, as previously described (Sambrook et al., 1989). 2 μg of this RNA were then used for cDNA synthesis, using 200 ng of random hexamer primer and 200U of M-MLV RT in 20 μl, under conditions recommended by RT manufacturer (BRL). A negative control was made under the same conditions but without RT. One tenth of the reaction or the negative control was amplified for 13 cycles in a two step PCR reaction (1 min at 95°C and 1 min at 60°C) using Taq polymerase and mbrm, mBRG-1 or GPDH specific primers (total volume: 100 μl). Preliminary experiments have shown that the PCR reactions were within the linear range between 10 and 15 cycles of amplification. 5 μl of the PCR product was resolved on a 10% polyacrylamide gel and analyzed by Southern blotting, using a BamH1/Sac1 restriction fragment from the hbrm cDNA and a Asp718/Sac1 fragment from the BRG-1 cDNA (respectively, nucleotide 274–548 and 245–599 of the published sequences). Primer sequences: mbrm sense, CCGGCACCTCCAGGCTC; mbrm reverse, GCCAGCGAAC-GGCTCTTT; mBRG-1 sense, CACCCAGGGGCGTGGAGG; mBRG-1 reverse, TCTTGTTGGGGACACTGA.

Preparation of stable cell lines
Wild type and mutant hbrm constructs were inserted as EcoRI fragments downstream of the MoMuLV LTR of pVLMPN1 (Marty et al., 1990). hbrm WT and APmut have previously been described (Muchardt et al., 1995). The Ki-ras transformed NIH3T3 cells (DT) were seeded at a density of 5×10^5 per 100 mm plate and transfected the next day with 10 μg of plasmid DNA using the calcium phosphate coprecipitation method. After 2 days, each plate was divided in two and 500 μl of G418 was added to the medium. 18 days after the transfection, individual colonies were picked, subcloned and characterized by PCR on the genomic DNA and by immunoblotting. DT cells were also transfected with pVLMPN1 vector without insert and several neomycin clones were also picked for control purposes. These clones were visible 9–10 days after the transfection. The Ha-ras transformed clones, R2 and R3, were derived from our NIH3T3 cells. They have been previously described (Pfart et al., 1994; Mechter et al., 1997).

Transformation assays
Growth at low serum concentrations was tested by plating 10^5 cells in DMEM containing 0.25% FCS. Cells were then counted after 7 days. For assay of cell growth in soft agar, 10^3 and 10^4 cells were plated in 0.3% agar (Difco) prepared in DMEM plus 7% FCS, over a cushion of 0.3% agar in 60 mm dishes. Colonies were counted after growth for 15 days. For tumor growth assays, 10^6 cells in midlog-phase of growth were harvested, washed with PBS and resuspended in 250 μl of PBS, and injected subcutaneously into the scapular region of Swiss 3T3 nude mice. After 11 days of growth, the mice were sacrificed and the tumors were dissected out and weighted.

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References


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