Calpain-dependent clearance of the autophagy protein p62/SQSTM1 is a contributor to ΔPK oncolytic activity in melanoma

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INTRODUCTION

Oncolytic virotherapy is a promising strategy for reducing tumor burden through selective virus replication in rapidly proliferating cells. However, the lysis of slowly replicating cancer stem cells (CSCs), which maintain neoplastic clonality, is relatively modest and the potential contribution of programmed cell death pathways to oncolytic activity is still poorly understood. We show that the oncolytic virus ΔPK lyases CSC-enriched breast cancer and melanoma 3D spheroid cultures at low titers (0.1 pfu/cell) without resistance development and it inhibits the 3D growth potential (spheroids and agarose colonies) of melanoma and breast cancer cells. ΔPK induces calpain activation in both melanoma and breast cancer 3D cultures as determined by the loss of the p28 regulatory subunit, and 3D growth is restored by treatment with the calpain inhibitor PD150606. In melanoma, ΔPK infection also induces light chain 3 (LC3)-II accumulation and p62/SQSTM1 clearance, both markers of autophagy, and 3D growth is restored by treatment with the autophagy inhibitor chloroquine (CQ). However, expression of the autophagy-required protein Atg5 is not altered and CQ does not restore p62/SQSTM1 expression, suggesting that the CQ effect may be autophagy-independent. PD150606 restores expression of p62/SQSTM1 in ΔPK-infected melanoma cultures, suggesting that calpain activation induces anti-tumor activity through p62/SQSTM1 clearance.

Keywords: oncolytic virotherapy; CSC; spheroid cultures; melanoma; breast cancer

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ApK is an HSV-2 mutant that is only deleted in the R1 protein kinase domain (known as ICP10PK), which is not conserved in HSV-1. ICP10PK activates the Ras signaling pathways and is required for virus growth in slowly replicating normal cells, which unlike tumor cells have low levels of Ras activity. $^{21,22}$ The loss of ICP10PK imparts tumor selectivity and favors virus induction of multiple death pathways, $^{18}$ thereby presumably increasing the clinical efficacy of ApK as an oncolytic agent. $^{19,20}$ ApK is therapeutically promising because, in addition to being safe in intracranially and intraperitoneally injected animals, $^{18,21,24}$ it was well tolerated in phase I/II human clinical trials. $^{25}$ We have previously shown that ApK has strong in vivo oncolytic activity for melanoma, with xenograft-bearing animals remaining tumor-free for at least 1 year after treatment. $^{18}$ Whereas this implies that CSCs are not resistant to ApK, their lysis has not been documented and the role of virus-induced PCD pathways and tumor cell type, if any, are still poorly understood. These questions are clinically relevant because some pathways that may function in cell death, notably autophagy, are critically involved in maintaining normal physiological processes that counteract oncolytic activity in the CSCs. $^{26}$

RESULTS

ApK lyases CSC-enriched breast cancer and melanoma cultures Two series of experiments were performed in order to examine the ability of ApK to lyse cells with CSC-like properties. In the first series of experiments, we used breast cancer (HS578T) and melanoma (A2058 and A375) cells grown as 3-dimensional (3D) multicellular tumor spheroids. These cultures include a gradient of proliferating cells similar to those found in tumor microregions; they reflect the tumor microenvironment and are often used as in vitro surrogates of tumorigenesis. $^{5,27-30}$ Although the heterogeneity of CSC phenotypic markers is being increasingly recognized, $^{5,6,31}$ our cultures are significantly enriched for cells with the widely distributed markers CD44 $^+$ CD24 $^-$ low (breast cancer) and CD271 (melanoma) $^{5,31-33}$ (99.8% and 86.3% positive cells, respectively) (Supplementary Material Supplementary Figure 2S). The 3D cultures were infected with ApK (multiplicity of infection (moi) = 1) or mock-infected with phosphate-buffered saline (PBS) and examined for cell death by regular microscopy and staining with the cell death dye propidium iodide (PI) followed by flow cytometry (FCM). The ApK, but not mock-infected, spheroids were largely reduced to debris (Figure 1a) and most of the cells were stained with PI, confirming cell death (Figure 1b).

In the second series of experiments, we examined the ability of ApK to prevent growth under 3D conditions that include spheroid growth and colony formation in soft agar. 2D cultures of HS578T, A2058 and A375 cells were infected with ApK (moi = 1) or mock-infected with PBS and examined for spheroid and colony formation at 7 and 14 days post infection (p.i.), respectively. The results, expressed as spheroids or colonies $\pm$ s.d., indicate that ApK-infected cells do not grow under these conditions (Figure 1c and Supplementary Material Supplementary Figure 2S). Collectively, the data confirm that ApK lyases melanoma and breast cancer cultures that are CSC-enriched and abrogates 3D, CSC-like growth potential.

Low ApK titers lyse 3D spheroid cultures without resistance development

Modest clinical efficacy of oncolytic virotherapy was attributed to poor tumor penetration due to low levels of virus replication and the presence of cell subpopulations with innate or acquired resistance. $^{3,34}$ To examine the effect of virus titers and resistance on the ability of ApK to lyse 3D tumor-like cultures, spheroids were infected with ApK at different moi (0.1–10 pfu/cell) and examined for cell death by PI staining and by the failure to establish fresh spheroid cultures when subcultured in a virus-free medium. As shown in Figure 2 for A2058 cultures, virtually all the cells (95–99%) in the spheroids infected with 10 pfu/cell of ApK stained with PI at 48 h p.i. and the cells collected at 4–5 days p.i. failed to establish new cultures. Penetration was poorer in the cultures given 0.1 pfu/cell of ApK, with PI staining at 48 h p.i., seen only at the spheroid periphery. However, at 10 days p.i., the cultures were fully disrupted and $\geq$95% of the cells were stained with PI. Cultures established from the few remaining clusters of live (PI-negative) cells (Figure 2a arrow) were equally susceptible to ApK-mediated lysis and all the cells were lost through four iterations of infection with 0.1 pfu/cell. Similar results were obtained for A375 and HS578T cells. The data indicate that low titers of ApK can penetrate and lyse spheroids without resistance development.

ApK-infected 3D cultures evidence extensive cell lysis in the presence of low virus titers

Deletions that impart tumor selectivity are known to reduce virus growth. $^{1-3}$ This was also shown for ApK, the titers of which in 2D cultures are 600–1000-fold lower than those of the wild-type virus

Figure 1. ApK has oncolytic activity in breast cancer and melanoma spheroid cultures. (a) HS578T spheroid cultures were mock- or ApK-infected (moi = 1) and examined at 48 h p.i. (x 20). Similar results were obtained for A2058 and A375 cells. (b) Mock- or ApK-infected A2058 spheroid cultures were dissociated with accutase, stained with PI and analyzed by FCM. Similar results were obtained for A375 and HS578T cells. (c) HS578T and A2058 cells mock-infected or infected with ApK (moi = 1; 48 h) were assayed for growth in spheroid culture and the results are expressed as number of spheroids $\times 10^6$ cells $\pm$ s.d. Similar results were obtained for A375 cells and for colony forming potential in soft agar.
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Figure 2. Low titers of ΔPK penetrate and lyse 3D spheroids without resistance development. (a) A2058 spheroid cultures were infected with ΔPK at moi = 10, 1 or 0.1, virus was removed by centrifugation and the spheroids were replated in fresh, virus-free medium and counted as described in the Materials and Methods section over a period of 10 days p.i. Spheroid counts decreased with time p.i. and were no longer seen by day 10 p.i. (arrow) (b) Spheroids mock-infected or infected with ΔPK at moi = 10 and 0.1 were stained with PI at the listed times p.i. and imaged under phase contrast (top panels) and red fluorescence microscopy (bottom panels). Representative images are shown and similar results were obtained for A375 and HS578T cells. Arrow indicates a remaining viable cell.

Calpain activation contributes to the ΔPK-mediated inhibition of 3D growth

Activated PCD pathways increase the efficacy of oncolytic virotherapy. To better understand the contribution of death pathways to the oncolytic activity of ΔPK for CSC-enriched 3D cultures, A2058, A375 and HS578T cells were mock-infected with PBS or infected with ΔPK (moi = 1; 48 h) in the absence or presence of the calpain-specific inhibitor PD150606 (100 μM) or the pancaspase inhibitor ZVAD-fmk (20 μM) and assayed for growth in soft agar or spheroid culture. We focused on these two PCD pathways because they are involved in the ΔPK-mediated killing of 2D melanoma cultures. Results are expressed as % colonies or spheroids (+ s.d. calculated relative to the mock-infected cells) and are shown in Figure 4 for A2058 colony formation. ΔPK-infected cells failed to grow in 3D culture, as also shown in Figure 1c. The calpain-specific inhibitor PD150606 restored 3D growth, but growth was not restored by treatment with the pancaspase inhibitor ZVAD-fmk, and the inhibitors did not affect the 3D growth of the mock-infected cultures. Similar results were obtained for spheroid growth and in all the cell types. The data indicate that calpain, but not caspase activation, contributes to the ability of ΔPK to inhibit 3D growth in both melanoma and breast cancer. Interestingly, however, the restoration of 3D growth was approximately twofold higher in melanoma than in breast cancer cultures (52.4 ± 6.2% for A2058, 67.8 ± 4.7% for A375 and 30.5 ± 3.8% for HS578T) (Figures 4a–c), suggesting that the contribution of the death pathways to the oncolytic activity of ΔPK is malignant cell type specific.

Chloroquine (CQ) restores the 3D growth of ΔPK-infected melanoma, but not of breast cancer cells

Having previously shown that ΔPK induces the expression of the autophagy protein beclin-1 in melanoma xenografts, we wanted to know whether autophagy is also associated with the ΔPK-infected cell lysis seen in our 3D cultures. A2058, A375 and HS578T cells were mock-infected with PBS or infected with ΔPK (moi = 1; 48 h) in the absence or presence of CQ (10 μM), which is an established autophagy inhibitor, and assayed for growth in soft agar or under spheroid conditions. CQ did not affect the 3D growth potential of the uninfected cultures, but it rescued the 3D growth of the ΔPK-infected melanoma cells.
PK infection induces LC3-II accumulation but does not alter Atg5 expression in melanoma spheroids.

To further examine the relationship between PK-induced cell lysis and autophagy, we asked whether infection induces autophagy markers. We focused on the membrane-bound phosphatidyl-ethanolamine-conjugated form of microtubule-associated protein 1 light chain 3 (LC3II), which binds autophagosomal membranes, and Atg5, which conjugates with Atg12 to generate an E3 ubiquitin ligase-like enzyme required for autophagy. Both of these are widely used markers to identify autophagy.38–40 In the first series of experiments, protein extracts from A2058 and A375 spheroids mock-infected with PBS or infected with PK (MOI = 1) were immunoblotted with LC3 antibody and examined for the conversion of LC3I to LC3II. The stripped blots were probed with antibody to actin (loading control) and the results were quantified by densitometric scanning. PK caused a significant increase (34-fold) in the LC3-II/LC3-I ratio relative to that seen in the mock-infected cultures in both melanoma cultures. LC3-II increase was seen as early as 1 h p.i. and it was still present at 24 h p.i. (Figure 5a). In the second series of experiments, A2058 spheroids were mock-infected or infected with PK (MOI = 1; 24 h) in the absence or presence of CQ (10 μM) and protein extracts were immunoblotted with antibody to Atg5. Atg5 was strongly expressed in the mock-infected cultures and in cultures infected with PK in the absence of CQ (Figure 5b). Collectively, the data indicate that PK modulates some (that is, LC3II), but not other (that is, Atg5), markers of autophagy.

PK infection induces LC3-II accumulation but does not alter Atg5 expression in melanoma spheroids.

PK-induced cell lysis with PK-infected melanoma spheroids was fully restored in the PD150606-treated cultures, indicating that its clearance is calpain-dependent. The loss of which documents calpain activation, or apoptosis-inducing factor (AIF), which is an apoptosis-associated calpain target.46 p62/SQSTM1 is cleared from PK-infected melanoma spheroids p62/SQSTM1 is a stress-inducible protein that interacts with LC3. It has a crucial role as an assembly factor for ubiquitinated proteins and organelles and is ultimately degraded by autophagy.47 Having seen that PK modulates some but not other autophagy markers, we wanted to better understand its effect on p62/SQSTM1, which also functions as a signaling hub in the life and death pathways and is implicated in tumorigenesis.42–44 A2058 and A375 spheroids were mock-infected or infected with PK in the absence or presence of CQ (10 μM) and immunoblotted with antibody to p62/SQSTM1. The blots were stripped and reblotted with antibody to GAPDH and the levels of p62/SQSTM1 determined by densitometric scanning and analyzed relative to GAPDH. As shown in Figure 5b for A2058 cells, p62/SQSTM1 was expressed in the mock, but not PK-infected, cultures, indicating that PK induces p62/SQSTM1 clearance. Significantly, however, expression was not restored by treatment of the infected cells with the autophagy inhibitor CQ (Figures 5b and c), suggesting that the PK-mediated clearance of p62/SQSTM1 is through a mechanism other than autophagy. Similar results were obtained for A375 spheroids. This is particularly relevant from the standpoint of virus oncolysis because, in addition to being implicated in tumorigenesis,43 high levels of p62/SQSTM1 were also associated with significantly worse prognosis and tumor progression, at least in lung and breast cancer.44,45

PK-mediated p62/SQSTM1 clearance is calpain-dependent.

To better understand the mechanism responsible for the clearance of p62/SQSTM1, duplicate spheroid cultures were infected with PK (MOI = 1; 24 h) in the absence or presence of PD150606 (100 μM), and protein extracts were immunoblotted with antibody to p62/SQSTM1 followed by immunoblotting of the sequentially stripped blots with antibodies to the calpain regulatory subunit p28, the loss of which documents calpain activation, or apoptosis-inducing factor (AIF), which is an apoptosis-associated calpain target.46 p62/SQSTM1 expression was fully restored in the PD150606-treated PK-infected spheroid cultures, indicating that its clearance is calpain-dependent (Figures 5b and c). Indeed, PK induced calpain activation as evidenced by i) loss of the p28 regulatory subunit in PK-infected cells and ii) restored expression in infected cells treated with the calpain inhibitor PD150606 (Figures 5b and c). The levels of AIF were similar under all conditions (Figures 5b and c),
Figure 5. ΔPK induces LC3-II accumulation and calpain-dependent clearance of p62/SQSTM1. (a) A2058 and A375 spheroid cultures were dissociated into single suspensions and mock- or ΔPK-infected (moi = 1) for 1, 4 or 24 h and protein extracts were immunoblotted with antibodies to LC3 followed by actin (loading control). Data were quantified by densitometric scanning and the results obtained for three replicate experiments are expressed as LC3-II/LC3-I ratio. (b) A2058 spheroid cultures dissociated into single suspensions were mock- or ΔPK-infected (moi = 1; 24 h) in the absence or presence of PD150606 (100 μM) or CQ (10 μM) and protein extracts were immunoblotted with antibodies to Atg5, p62/SQSTM1, the calpain p28 regulatory subunit, AIF, or GAPDH (loading control). The blots were stripped between antibodies and representatives of three replicate experiments are shown. Similar results were obtained in A375 spheroids. (c) Data were quantified by densitometric scanning and the results obtained for three replicate experiments are expressed as densitometric units ± s.d. for Atg5, p62/SQSTM1 and p28 (calpain).

indicating that calpain does not function at this level. Collectively, the data indicate that calpain-mediated p62/SQSTM1 degradation is a key contributor to the ΔPK oncolytic activity in CSC-enriched 3D melanoma cultures.

DISCUSSION

The salient feature of the data presented in this report is the finding that ΔPK has strong oncolytic activity in CSC-enriched melanoma and breast cancer 3D cultures that involves calpain activation and the clearance of p62/SQSTM1 in the melanoma cultures. The following comments seem pertinent with respect to these findings.

Direct studies of CSC lysis are relatively scant. oHSV, including those engineered to increase tumor penetration and virus spread, were shown to kill glioblastoma, neuroblastoma and rhabdomyosarcoma cells with CSC-like properties. However, tumors appear to harbor multiple phenotypically and/or genetically distinct CSCs, underscoring the therapeutic advantage of a virus that elicits the PCD pathways tailored to distinct cell types. ΔPK differs from most oHSV in that it is based on HSV-2 and is deleted in ICP10PK, which is not conserved in HSV-1, but it retains the replication-associated ICP34.5 and ribonucleotide reductase activities. ICP10PK activates the Ras signaling pathways and contributes to virus growth, such that its deletion enables tumor selectivity, reduces replicative potential and induces multiple PCD pathways. The ICP10PK-deleted virus, ΔPK, was well tolerated in phase I/II human clinical trials and it has strong in vivo oncolytic activity in melanoma, with xenograft-bearing animals remaining tumor-free for at least 1 year after treatment. Although this implies that CSCs are not resistant to ΔPK, their lysis remains to be documented.

We studied 3D melanoma and breast cancer cultures that are enriched in the widely distributed CSC phenotypic markers CD44^high CD24^low (breast cancer) and CD271 (melanoma). These cultures are enriched for slow cycling pluripotent cells with enhanced tumorigenicity and drug resistance and mimic in vivo tumors in their microenvironment, pH and oxygen gradients, growth factor distribution and interaction with the extracellular matrix. They are known to resist oncolytic virotherapy owing to reduced virus penetration and spread. Our data indicate that ΔPK effectively penetrates 3D spheroid cultures even when given at the very low titer of 0.1 pfu/cell and it lysed most of the cells by 10 days p.i. The few cells that survived this treatment were equally susceptible to lysis by these low virus titers and the entire culture was completely eradicated through four cycles of infection, indicative of a fundamental lack of resistance to ΔPK lysis. Resistance development is a major therapeutic challenge and its absence is clinically relevant, because multiple injections are typically required during a treatment regimen. While these and previous findings suggest that ΔPK has effective tumor penetration, the potential contribution of density, hypoxic regions, intratumoral heterogeneity, stromal tissue barriers and blood flow remains to be determined and studies on cancer-initiating cells from actual tumors are still needed.

In addition to lysing CSC-enriched cultures, ΔPK inhibited the 3D growth potential of both melanoma and breast cancer cells, as measured by spheroid and agarose colony formation. Growth was restored by treatment with PD150606, which is a cell-permeable, non-competitive and selective inhibitor of both calpain-1 and -2 that is directed towards the calpain Ca^2+ binding sites. The calpain specificity of PD150606 is fully established and it was used at independently established doses. Growth was not restored by PD145305, which is an inactive analog of PD150606 (data not shown). Together with the findings that (i) ΔPK caused the loss of the p28 calpain regulatory subunit and (ii) PD150606 restored its expression, the data indicate that calpain activation contributes to the oncolytic activity of ΔPK in the 3D melanoma and breast cancer cultures.
Calpains are Ca$^{2+}$-activated non-lysosomal cysteine proteases that have paradoxical functions in the perturbed cancer apoptotic pathways. Calpain activity was implicated in the prosurvival activities of the tumor suppressor protein p53 and nuclear factor-κB, but it was also shown to cause cell death by inducing the collapse of the mitochondrial membrane potential ($ΔΨ_{m}$) and the induction of both caspase-dependent and independent (AIr-dependent) death pathways.40,41 Interestingly, the pancaspase inhibitor ZVAD-fmk did not restore 3D spherical/agarose growth, although caspase activation is involved in the oncolytic activity of ΔPK in 2D melanoma cultures,18 potentially reflecting increased CSC expression of anti-apoptotic proteins (that is, Bcl-2 and survivin).52

Autophagy has been implicated in maintaining normal physiological processes, particularly in CSC, where it enables survival in the tumor microenvironment,26 and it contributes to breast cancer tumorigenicity.53 However, a growing body of evidence supports the contribution of bona fide autophagic cell death to various pathological conditions, with various factors governing the cellular decision to die by autophagy.54 In cancer, autophagy can be pro- or anti-tumorigenic depending on the specific tissue and tumor stage.55 Currently available data for melanoma support both protective and cytotoxic activities, albeit by still poorly understood mechanisms.56,57 We found that CQ, used at a dose at which it is known to inhibit autophagy, restored the 3D growth of ΔPK-infected melanoma, but not breast cancer cultures, providing a measure of specificity. In addition, the ΔPK-infected melanoma spheroids had increased levels of LC3-II accompanied by p62/SQSTM1 clearance, an established signature of autophagy.41,42 However, the relationship between ΔPK and autophagy is confounded by the finding that ΔPK did not alter the expression of Atg5, which has a critical role in autophagy activation by participating in two essential pathways—that is, Atg12–Atg5 conjugation and LC3 lipidation.8,48–50 Moreover, the expression of p62/SQSTM1 was not restored by CQ but rather by the calpain inhibitor PD150606. A possible interpretation of our findings is that the observed LC3-II accumulation reflects a measure of specificity. In addition, the ΔPK-infected melanoma spheroids had increased levels of LC3-II accompanied by p62/SQSTM1 clearance, an established signature of autophagy.41,42

However, in the interpretation that this confirms the ability of CQ to restore 3D growth is through a prosurvival function other than autophagy that is melanoma-specific. While such a function remains elusive, and discounting the possible contribution of dose, it may be important to point out that CQ (used at a dose fivefold higher than ours), was recently shown to induce the collapse of the mitochondrial membrane potential ($ΔΨ_{m}$) and the induction of both caspase-dependent and independent (AIr-dependent) death pathways.40,41 Interestingly, the pancaspase inhibitor ZVAD-fmk did not restore 3D spherical/agarose growth, although caspase activation is involved in the oncolytic activity of ΔPK in 2D melanoma cultures,18 potentially reflecting increased CSC expression of anti-apoptotic proteins (that is, Bcl-2 and survivin).52

**Antibodies, pharmacological inhibitors and chemical reagents**

The generation and specificity of the rabbit polyclonal antibody to ICPI0, which recognizes an epitope that is retained by both ICPI0 and the PK-deleted p95 protein, have been previously described.18,21–24,46 The following antibodies were purchased and used according to the manufacturer’s instructions. FITC-conjugated anti-CD20 and APC-conjugated anti-CD133 antibodies were from Miltenyi Biotec (Auburn, CA, USA). FITC-conjugated anti-CD24, APC-conjugated anti-CD44, APC-conjugated anti-B2CD2, FITC-conjugated IgG1 isotype, APC-conjugated IgG2b isotype and APC-conjugated anti-mouse antibodies were from BD Biosciences (San Jose, CA, USA). Antibodies to AIF, LC3, calpain and GAPDH were from Santa Cruz Biotechnologies (Santa Cruz, CA, USA); antibodies to p62 and ATG5 were from Cell Signaling Technologies (Danvers, MA, USA); and antibody to VPS was from Viruses (Taneytown, MD, USA). Alexafluor 594-conjugated anti-mouse, Alexafluor 488-conjugated anti-rabbit antibody and SlowFade Gold (with 4’,6-diamidino-2-phenylindole (DAPI)) mounting medium were from Invitrogen. The calpain inhibitor PD150606 was from Calbiochem (La Jolla, CA, USA) and the pancaspase inhibitor benzoxycarbonyl-Val-Ala-Asp-fluoromethyl ketone (zVAD-fmk) from Promega (Madison, WI, USA). CQ, PI, low melting point agarose and benzoxycarbonyl-Val-Ala-Asp-fluoromethyl ketone (zVAD-fmk) from Promega (Madison, WI, USA). CQ, PI, low melting point agarose and Tween 20 were from Sigma-Aldrich (Carlsbad, CA, USA) and Accutase was from Innovative Cell Technologies, Inc (San Diego, CA, USA). All the inhibitors were selected for their target specificity and used at doses recommended by the manufacturer and confirmed in the literature.18,47–49,51

**Materials and methods**

**Cells and virus**

A2058 and A375 melanoma cells were obtained from the American Type Culture Collection (Manassas, VA, USA). Hs578t breast cancer cells were a gift from Dr Angela Brodie (University of Maryland, Baltimore, MD, USA). They were grown in adherent monolayers cultures in Dulbecco modified Eagle’s medium (DMEM), Invitrogen, Carlsbad, CA, USA) supplemented with l-glutamine (4 mmol/L) and 10% fetal bovine serum (FBS, Gemini Bioproducts, Calabasas, CA, USA). WI-38 cells (normal human embryonic lung fibroblasts) are an expansion from passage 9 and have a limited lifespan of 50 population doublings. They were cultured in minimal essential medium with Earle’s salts, 10% fetal bovine serum, 1 mm sodium pyruvate and 0.1 mm nonessential amino acids. The generation and properties of the HSV-2 mutant ΔPK that is deleted in the kinase domain of the large subunit of ribonucleotide reductase (R1, also known as ICPI0) were previously described.18,21–24,46

**Anchorage-independent growth**

Anchorage-independent growth was assessed by both spheroid and soft agar growth assays. For spheroid growth, cells were suspended in serum-free DMEM supplemented with 20 ng/ml basic fibroblast growth factor (bFGF, R&D Systems, Minneapolis, MN, USA) and 20 ng/ml epidermal growth factor (EGF, R&D Systems), plated (1 × 10^3 cells/well) onto ultra-low attachment plates (Corning, Corning, NY, USA) and grown at 37°C for 7 days. The assay was performed in triplicate, spheroids (>500 μm in diameter) were counted and the results are expressed as number of spheroids/10^4 cells ± s.d. Soft agar colonies were grown as previously described.59 Briefly, cells (500 or 5000/well) were suspended in 0.3% low melting temperature agarose in 1 × DMEM and overlayed onto 0.6% agarose in 1 × DMEM in 24-well plates. The solidified agarose-cell mixture was overlaid with 1 × DMEM, the plates were incubated at 37°C for 14 days, and colonies (defined as ≥50 μm diameter) were counted. The assay was performed in triplicate and the results are expressed as number of colonies/10^4 cells ± s.d.

**Virus infection of spheroid cultures**

Spheroid cultures were infected either as intact 3D cultures or after they were dissociated into single cell suspensions by treatment with accutase (37°C, 5 min). To estimate the moi=pfu/cell for the intact spheroid cultures, duplicate representative cultures were dissociated before cell counting. For all cultures, virus was adsorbed in adsorption medium (PBS with 0.2% glucose and 0.2% bovine serum albumin) for 1 h at 4°C (synchronized adsorption) and removed by centrifugation (800–1500 rpm; 5–10 min) prior to reincubation at 37°C.

**Cell death**

Staining with PI, a cell impermeant red fluorescent nuclear dye used to determine cell death, was performed according to the manufacturer’s instructions and visualized by microscopy using a Nikon E400 fluorescent microscope (Melville, NY, USA) that utilizes bright-field and a Texas Red (540–580 nm) cube. Stained cells were counted in five randomly selected
3 mm² fields (≥ 250 cells each), and the % positive cells was calculated relative to total number of cells imaged by phase contrast microscopy.23,24,46

Immunofluorescence and FCM

Immunofluorescent staining was as previously described.23,24,46 Briefly, cells were fixed with 4% paraformaldehyde overnight at 4 °C, blocked with 5% normal goat serum and 5% bovine serum albumin (30 min, room temperature) and incubated with primary antibody overnight at 4 °C. They were washed in PBS with 0.1% Tween 20, exposed to fluorochrome-conjugated secondary antibodies (37 °C, 1 h) and mounted in Vectashield with DAPI (Vector Laboratories, Burlingame, CA, USA). Slides were visualized with an Olympus BX50 fluorescence microscope (Center Valley, PA, USA) utilizing FITC (330–380 nm), UV (for DAPI) (465–495 nm) and Texas red (540–580 nm) cubes. Stained cells were counted in five randomly selected 3 mm² fields (≥ 250 cells each) and the percentage of positive cells was calculated relative to total number of cells imaged by DAPI. FCM was carried out as previously described.46 Briefly, washed cells were resuspended (10⁶ cells/tube) in FCM buffer (2% fetal bovine serum in radioimmunoprecipitation buffer (RIPA; 20 mM Tris-HCl (pH 7.4), 0.15 mM NaCl, 1% Nonidet P-40, 0.1% sodium dodecyl sulfate (SDS), 0.5% sodium deoxycholate) supplemented with protease and phosphatase inhibitor cocktails (Sigma-Aldrich) and sonicated twice for 30 s at 25% output power with a Sonicator ultrasonic processor (Misonix, Inc., Farmingdale, NY, USA). Protein concentrations were determined by the bicinchoninic assay (Pierce, Rockford, IL, USA) and 100 µg protein samples were resolved by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride membranes. The membranes were blocked (1 h, room temperature) in 5% nonfat milk in TN-T buffer (0.01 M Tris-HCl pH 7.4, 0.15 M NaCl, 0.05% Tween 20), exposed (1 h) to primary antibodies, washed in TN-T buffer and incubated (1 h) in HRP-conjugated secondary antibodies. Detection was carried out using ECL reagents (Amersham, Pittsburg, PA, USA) and high-performance chemiluminescence film (Hyperfilm ECL, Amersham). Quantitation was by densitometric scanning (Hyperfilm ECL, Amersham). Analysis of variance was performed with SigmaStat version 3.1 for Windows (Systat Software, Point Richmond, CA, USA).

Immunoblotting

Preparation of protein extracts and immunoblotting were as previously described.46 Briefly, cultured cells were lysed with radioimmunoprecipitation buffer (RIPA: 20 mM Tris-HCl (pH 7.4), 0.15 mM NaCl, 1% Nonidet P-40, 0.1% sodium dodecyl sulfate (SDS), 0.5% sodium deoxycholate) supplemented with protease and phosphatase inhibitor cocktails (Sigma-Aldrich) and sonicated twice for 30 s at 25% output power with a Sonicator ultrasonic processor (Misonix, Inc., Farmingdale, NY, USA). Protein concentrations were determined by the bicinchoninic assay (Pierce, Rockford, IL, USA) and 100 µg protein samples were resolved by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride membranes. The membranes were blocked (1 h, room temperature) in 5% nonfat milk in TN-T buffer (0.01 M Tris-HCl pH 7.4, 0.15 M NaCl, 0.05% Tween 20), exposed (1 h) to primary antibodies, washed in TN-T buffer and incubated (1 h) in HRP-conjugated secondary antibodies. Detection was carried out using ECL reagents (Amersham, Pittsburg, PA, USA) and high-performance chemiluminescence film (Hyperfilm ECL, Amersham). Quantitation was by densitometric scanning (Hyperfilm ECL, Amersham). Analysis of variance was performed with SigmaStat version 3.1 for Windows (Systat Software, Point Richmond, CA, USA).

Statistical analysis

Analysis of variance was performed with SigmaStat version 3.1 for Windows (Systat Software, Point Richmond, CA, USA).

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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