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Caspase 3 promotes surviving melanoma tumor cell growth after cytotoxic therapy

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Abstract

Metastatic melanoma often relapses despite cytotoxic treatment, so the understanding of melanoma tumor repopulation is crucial to improving our current therapies. In this study, we aim to define the role of caspase 3 in melanoma tumor growth after cytotoxic therapy. We examined a paradigm-changing hypothesis that dying melanoma cells undergoing apoptosis during cytotoxic treatment activate paracrine signaling events that promote the growth of surviving tumor cells. We propose that caspase 3 plays a key role in the initiation of the release of signals from dying cells to stimulate melanoma tumor growth. We created a model for tumor cell repopulation in which a small number of luciferase-labeled, untreated melanoma cells are seeded onto a layer of a larger number of unlabeled, lethally treated melanoma cells. We found that dying melanoma cells significantly stimulate the growth of living melanoma cells *in vitro* and *in vivo*. Furthermore, we observed that caspase 3 gene knockdown attenuated the growth-stimulating effect of irradiated, dying cells on living melanoma cell growth. Finally, we showed that caspase 3-mediated dying melanoma cell stimulation of living cell growth involves secreted PGE_2. Our study therefore suggests a counterintuitive strategy to inhibit caspase 3 for therapeutic gain in melanoma treatment.

Introduction

Melanoma is a highly aggressive cancer whose incidence is increasing more dramatically than any other type of cancer (Siegel *et al.*, 2012). Metastatic melanoma portends a poor

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Conflicts of Interest
The authors state no conflict of interest.

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prognosis (Balch *et al.*, 2009), thereby suggesting that our current therapies are often ineffective in eliminating all of the melanoma tumor cells.

In order to investigate melanoma recurrence after cytotoxic therapy, one must explore the fundamental mechanism of tumor repopulation. A relevant concept is the idea of "accelerated repopulation" after radiotherapy (Trott, 1990; Tubiana, 1988). Currently, the mechanisms behind this process of melanoma tumor repopulation after cytotoxic therapy are poorly understood. Inflammation and angiogenesis have been identified as playing a role in "accelerated repopulation" (Martin *et al.*, 2003; Reuter *et al.*, 2010). However, because inflammation and angiogenesis are more likely to be secondary events, the initiating event that drives melanoma tumor repopulation remains unexplored.

Recently, our laboratory discovered the "Phoenix Rising" pathway, which may define a possible cell death-mediated mechanism that drives melanoma tumor repopulation (Huang *et al.*, 2011). We have identified caspase 3, a key molecule in the "executioner phase" of apoptosis, as playing a key role the proliferation of surrounding cells (Huang *et al.*, 2011; Li *et al.*, 2010b). Caspase 3 activates calcium-independent phospholipase A2 ($iPLA_2$) (Zhao *et al.*, 2006). Activated $iPLA_2$ increases the synthesis and release of arachidonic acid and lysophosphocholine from apoptotic cells (Akiba and Sato, 2004). Arachidonic acid is a known precursor to prostaglandin E2 (PGE_2). PGE_2 is involved in stem cell proliferation, tissue regeneration, and wound healing (Surh *et al.*, 2012). Further, PGE_2 increases tumor growth in many types of cancer such as colon (Castellone *et al.*, 2005), prostate (Vo *et al.*, 2013), breast (Zhao *et al.*, 1996), and lung (Singh and Katiyar, 2013) cancers.

In the present study, we have attempted to create a model for melanoma repopulation by combining a small number of untreated, luciferase-labeled melanoma cells with a larger number of lethally treated, unlabeled melanoma cells and measuring the luciferase activity over time. This simulates the *in vivo* treatment of a tumor in which the majority of cells are killed by the cytotoxic treatment, while only a few cells survive and go on to repopulate the tumor in the case of a relapse. We implemented this model *in vitro* using standard and transwell cell culture plates and also in mice. We used this model to study the role of caspase 3 in melanoma tumor repopulation after cytotoxic treatments.

Results

Cytotoxic treatments activate caspase 3 in melanoma cells

To observe caspase 3 activation in dying melanoma cells, we treated A375 melanoma cells with radiation or vemurafenib and examined caspase 3 activation using western blot analysis and an activated caspase 3 reporter. Western blots for activated caspase 3 showed an increase in protein expression for 1, 2, and 3 days after irradiation with 10 Gy or treatment with vemurafenib 20 μM in A375 cells (Figure 1a). We created a caspase 3 reporter gene containing a polyubiquinated region, a firefly luciferase gene fused with a GFP gene (GFP-Luc), and a caspase 3 cleavage site (Figure 1b). In normal melanoma cells, the polyubiquitin tag remains attached to the reporter construct, so the fusion GFP-Luc reporter protein will be rapidly degraded by the proteasome. When caspase 3 is activated in dying melanoma cells, activated caspase 3 acts as a protease and cleaves off the polyubiqutin domain so that the

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GFP-Luc reporter becomes stabilized in cells and can be measured using bioluminescence. Our results illustrate a significant increase in luciferase activity and GFP expression in both the irradiated (> 40-fold increase) and vemurafenib-treated (> 6-fold increase) A375 caspase 3 reporter cells (Figure 1c & 1d). Since these results indicated that cytotoxic melanoma treatment activates caspase 3, we proceeded to investigate the evidence for a role for caspase 3 in cell death stimulation of melanoma cell growth.

Dying melanoma cells promote the growth of living melanoma cells *in vitro* and *in vivo*

To validate a role for cell death and caspase 3 in melanoma tumor repopulation, we tested the ability of dying melanoma cells to induce the growth of living melanoma cells *in vitro* and *in vivo*. Our *in vitro* model of melanoma tumor repopulation included a small number (200-500) of untreated, luciferase reporter melanoma (A375Fluc or A2508Fluc) cells seeded onto a large number (1×10^5) of A375 or A2508 melanoma cells lethally treated with cytotoxic therapy. In order to validate our model, we confirmed that luminescence was linearly correlated with A375Fluc and A2508Fluc cell number (supplementary Figure S1 & S2). Our results show that lethally irradiated (10 Gy) or vemurafenib-treated A375 and A2508 melanoma cells significantly ($p< 0.05$, ANOVA) stimulate the growth of living reporter cells compared with no feeder and untreated controls (Figure 2a-d). Remarkably, when compared with no feeder controls, after two weeks there was over a 110-fold difference in reporter cell luciferase activity when co-cultured with 10 Gy-irradiated A375 cells (Figure 2a) and over a 137-fold difference in reporter cell luciferase activity when co-cultured with 10 Gy-irradiated A2508 cells (Figure 2b). In addition, the growth-stimulating effect of dying melanoma cells on living melanoma cells was also observed in transwell plates, thereby providing definitive evidence that a secreted factor is involved in this process (Figure 2e & 2f). Furthermore, we have evidence that dying A375 could stimulate the growth of untreated A2508Fluc cells and vice versa (supplementary Figure S3). Therefore, the cytotoxic treatment of melanoma cells *in vitro* greatly enhances the growth of living melanoma tumors cells and this effect involves secreted factors from the dying cells.

Next, we studied whether or not cell death-mediated melanoma tumor repopulation occurred *in vivo.* A375 cells were treated with 10 Gy radiation or vemurafenib 20 μM for 3 days. A large number of the lethally treated A375 cells (2×10^5) were injected with a small number (1×10^4) of untreated, luciferase-labeled A375Fluc cells and bioluminescence was monitored over 2 weeks as a measure of A375Fluc growth. Consistent with our *in vitro* observations, we found that dying A375 cells treated with either radiation or vemurafenib significantly (at 13 days, a 62-fold increase for radiation ($p<0.05$) (Figure 3a & 3b) and a 114-fold increase for vemurafenib ($p<0.05$) (Figure 3c & 3d)) stimulated the growth of the small number of living A375Fluc cells compared with untreated A375 feeder controls. Thus, our *in vitro* and *in vivo* results establish the importance of dying cells to melanoma tumor repopulation with two possible melanoma cytotoxic treatments.

Caspase 3 inhibition attenuates the growth-stimulating effect of dying melanoma cells on living melanoma cells *in vitro*

Based on our previous studies of caspase 3 in breast cancer cells, (Huang *et al.*, 2011). we postulated that caspase 3 plays a pivotal role in the pathway of dying melanoma cell

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promotion of living melanoma cell growth. To attenuate caspase 3 activities, we created two A375 melanoma cell lines expressing two different casapse 3 shRNAs (Figure 4a & 4b) and two melanoma cell lines (A375 and A2508) constitutively expressing a dominant negative caspase 3 gene (Figure 4c & 4d). Caspase 3 knockdown was confirmed in our caspase 3 shRNA A375 melanoma cell lines (Figure 4a). We showed that in both caspase 3 shRNA and dominant negative caspase 3 expressing A375 cells, apoptosis (as measured by TUNEL assay, or terminal deoxynucleotidyl transferase dUTP nick end labeling assay) was significantly reduced (supplementary Figure S4). Additionally, we used two genetically identical mouse embryonic fibroblasts (MEF) cell lines that are wild type or genetically deficient of the Casp3 gene (Kuida *et al.*, 1996; Lakhani *et al.*, 2006) (Figure 4e & 4f). When we carried out the repopulation assay, we found that each of the aforementioned methods of caspsase 3 inhibition in feeder cells significantly decreased the growth-stimulating effect of dying cells on living melanoma cells (Figure 4b – 4f). In an experiment comparing the two A375 caspase 3 shRNA cell lines (15044 and 15045 seen in Figure 4b) with a scramble cell line control, reporter cells seeded onto irradiated caspase 3 shRNA cell lines grew approximately 60% that of cells seeded onto irradiated scramble cells after 9 days ($p<0.05$) (Figure 4b). In addition, the growth of living melanoma cells seeded onto a layer of irradiated caspase 3 knockout MEF cells was 22% that of cells seeded onto a layer of irradiated wild-type MEF cells after 9 days ($p<0.05$) (Figure 4e). These results further suggest that irradiated stromal cells may also promote melanoma cell repopulation.

Therefore by utilizing three different methodologies for attenuating caspase 3 activities, we have obtained data that strongly support a role for caspase 3 in melanoma tumor repopulation during cytotoxic therapy.

Caspase 3 mediated dying cell stimulation of living melanoma tumor cell growth occurs through a mechanism involving secreted PGE_2

Given that we previously identified PGE_2 as the downstream secreted factor from dying cell stimulated living cell growth in breast cancer cells (Huang *et al.*, 2011), we sought to determine if there is a role for PGE_2 in a caspase 3-mediated mechanism of melanoma tumor repopulation. We measured PGE_2 production induced by irradiation of melanoma cells in the supernatant of cells and found that PGE_2 levels are increased after radiation of melanoma cells (Figure 5a & 5b). In comparison with the supernatant of irradiated wild-type A375 cells, PGE_2 levels were significantly lower in irradiated A375 caspase 3 shRNA cells (Figure 5a) or after treatment with indomethacin (a cyclooxygenase I and II inhibitor that should significantly reduce PGE_2 production) (Figure 5b). Further, treatment of irradiated A375 cells with 100 μM indomethacin in a transwell plate decreased the growth-stimulating effect of dying melanoma cells on living melanoma cells by greater than 32-fold (Figure 5c). Finally, we also obtained evidence that PGE_2 was induced by radiation in A2508 cells and its production was decreased with indomethacin (supplementary Figure S5a). The importance of PGE_2 was again demonstrated by that fact that indomethacin significantly reduced cell death stimulated A2508Fluc repopulation (supplementary Figure S5b). Therefore, we conclude that PGE_2 secretion is crucial to caspase 3-mediated dying melanoma cell stimulation of living cell growth.

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Discussion

Our results illustrate that dying cells promote the growth of living melanoma tumor cells after cytotoxic treatment via a caspase 3-mediated mechanism. Given that conventional cancer studies often target the enhancement of apoptosis in treatment, this report presents data which suggest a very counterintuitive idea in cancer cell biology. In our study, we confirmed that radiation and vemurafenib activated caspase 3 in melanoma cells. Importantly, we demonstrated that dying melanoma cells treated with radiation or vemurafenib stimulate the growth of living tumor cells *in vitro* and *in vivo*. Furthermore, when we attenuated the activities of caspase 3 with caspase 3 shRNA, a caspase 3 dominant negative gene, or genetic deletion of caspase 3 in MEF cells, the growth stimulation of irradiated, dying cells on living cells was substantially decreased. Therefore, we propose a role for the “Phoenix Rising” pathway in melanoma tumor repopulation that to our knowledge is previously unreported. In this pathway, dying melanoma cells activate caspase 3, which results in the ultimate effect of increased surviving tumor cell growth through a mechanism involving secretion of growth-stimulating factors such as PGE_2.

The current literature contains several examples of caspases acting in pathways other than apoptosis. For instance, caspases are involved in cell differentiation (Fujita *et al.*, 2008; Kang *et al.*, 2004; Szymczyk *et al.*, 2006), dedifferentiation of fibroblasts into pluripotent stem cells (Li *et al.*, 2010a), and osteoclast differentiation (Szymczyk *et al.*, 2006). Caspases may activate T cells, which could further influence tumor biology through inflammatory factors and immune evasion (Kennedy *et al.*, 1999). In melanoma, basal caspase 3 expression correlates with invasion of melanoma cells (Liu *et al.*, 2013). Essentially, the involvement of caspase 3 in melanoma tumor repopulation is consistent with the established multifunctional nature of caspases.

Overall, we propose a counterintuitive strategy to inhibit caspase 3 for therapeutic gain in melanoma treatment. Based on our results, the combination of radiation with a topical caspase 3 inhibitor could serve as a localized treatment for superficial melanomas. For metastatic tumors, combining caspase 3 inhibitors with radiation and/or vemurafenib treatment may also be beneficial. Interestingly, caspase 3 knockout mice have a normal lifespan and only have issues with female fertility (Matikainen *et al.*, 2001) and eye and ear development (Kuida *et al.*, 1996; Makishima *et al.*, 2011). Currently, pan caspase inhibitor drugs are in clinical trials for hepatitis C, nonalcoholic steatohepatitis, and liver reperfusion injury with promising results (Baskin-Bey *et al.*, 2007; Ratziu *et al.*, 2012; Shiffman *et al.*, 2010). In essence, we believe that our finding of a caspase 3-mediated mechanism of melanoma tumor repopulation after cytotoxic therapy suggests a major rethinking of our understanding of melanoma and cancer biology that could potentially improve our limited available treatments for patients with melanoma.

Materials and Methods

Cell Lines, Drugs, and Irradiation

A375 melanoma cells and MEF cells were obtained from American Type Tissue Culture (Manassas, VA, USA). A2508 melanoma cells were provided by Dr. Jennifer Zhang from

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Duke University (Durham, NC). DMEM (Invitrogen, Carlsbad, CA) supplemented with 10% FBS was used to culture the cells at 37 °C under 5% CO_2-95% air. Vemurafenib was purchased from Selleck Chemicals (Houston, TX) and indomethacin was purchased from Sigma-Aldrich (St. Louis, MO). Drugs were dissolved in DMSO and diluted with culture medium. Cells were irradiated using the X-Rad320 X-ray machine at Duke University Medical Center (Durham, NC). The dose rate was 4.50 cGy/s.

Gene transduction into A375 cells

The lentiviral vector pLEX system was purchased from Open Biosystems (Huntsville, AL) and used to introduce genes into A375 and A2508 cells. The firefly luciferase gene (Fluc) was obtained from Promega (Madison, WI) and transferred from the plasmid pGL4.31-luc2 into A375 and A2508 cells to create luciferase-labeled A375 (A375Fluc) cells and A2508 (A2508Fluc) cells, respectively. The activated caspase 3 reporter gene was cloned into the lentiviral vector pLEX. For shRNA against human caspase 3, two shRNA-encoding lentivirus vector in the GIPZ backbone were purchased from Open Biosystems (now Thermo-Fisher): Clone 1: V2LHS_15044. Clone 2:V2LHS_15045. For caspase 3 dominant negative A375 and A2508 cells, we mutated a key cysteine in the catalytic domain of murine caspase 3 (C163?A) through site directed mutagenesis. All the lentiviral vectors were packaged into live lentiviral viruses in 293T cells following manufacturer's instructions. Target A375 and A2508 cells were infected with the lentivirus vector and polybrene in culture medium. Transfected cells were selected for with puromycin 1 μg/ml in culture medium. All lentivirus vectors were made according to manufacturer's insturctions.

Bioluminescence and Fluorescence Imaging

To measure luciferase activity *in vitro,* we used the Synergy H1 Hybrid Multi-Mode Microplate Reader (BioTek). D-luciferin (0.06 mg/well) from Promega (Madison, WI) was added to wells. To image luciferase activity *in vitro* and *in vivo*, the IVIS Kinetic instrument from Caliper Life Sciences (Optical Molecular Imaging and Analysis, Durham, NC) was used. Mice were injected with 150 mg/kg body weight of D-luciferin intraperitoneally in 100 μL of deionized water and then anesthetized with continuous 2.5% isoflurane. Imaging was carried out within 10 minutes after administration of D-luciferin. For fluorescence imaging of GFP-expressing cells, the Zeiss AX10 microscope was used.

Activated Caspase 3 Reporter Experiments

A375 cells transduced with the caspase 3 reporter gene were seeded (1×10^5 cells/well) into 6-well plates and treated with vehicle, 10 Gy radiation, or vemurafenib 20 μM for 1,3, and 5 days. Luciferase activity was measured at the 3 time points and cells were counted using the BioRad cell counter (Hercules, CA) by trypan blue exclusion to calculate a luciferase activity per cell ratio. GFP images were taken on day 5.

Tumor Cell Growth *In Vitro* and *In Vivo*

In vitro, 200-500 untreated A375Fluc or A2508Fluc cells were seeded onto a larger number (1×10^5) of A375 or A2508 feeder cells within 24 hours after treatment in either 12 or 24 well plates, or 12 or 24 well transwell plates in 2% FBS culture medium. Feeder cells were

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either untreated or lethally treated with 10 Gy radiation or with vemurafenib 20 or 30 μM for 3 days. Luciferase activity was monitored every 2-3 days over a 2 week period with an IVIS Kinetic images taken at the end of 2 weeks.

Male nude mice between 6-8 weeks year old were purchased from Jackson laboratory (Bar Harbor, ME). Xenograft injections contained a small number (1×10^4) of A375Fluc cells mixed with a larger number (2×10^5) of A375 feeder cells (lethally treated with 10 Gy radiation or vemurafenib 20 μM for 3 days in the right leg or untreated in the left leg). Cells were resuspended in PBS and 50 μL was injected subcutaneously into the hind legs of the nude mice. Bioluminescence was measured 3 times over 2 weeks. Once tumors became visible, tumor volume was measured and bioluminescence was normalized to the tumor volume. All animal experiments were approved by the Duke University IACUC.

ELISA Assay for PGE2 Concentration

A375 or A375 C3 shRNA cells were either untreated or irradiated with 10 Gy and plated in 12-well plates (1×10^5 cells/well) in 2% FBS culture medium. Supernatant from the cells was collected 72 hours after radiation and diluted 50 fold. Cells were counted using trypan blue exclusion using the BioRad cell counter (Hercules, CA) in order to normalize PGE2 concentration to cell number. PGE2 was measured in the supernatant following a protocol from an ELISA kit purchased from Cayman Chemical Company (Ann Arbor, MI).

Western Blot Analysis

For running gel electrophoresis, 40 μg of protein per sample was loaded on a 10% polyacrylamide gel. Gels were transferred to a PVDF membrane to be blotted with antibodies for cleaved caspase 3 (rabbit) and β-actin (mouse).

Statistical Analysis

One-way ANOVA tests and student's two-tailed t-tests were used to analyze the experiment results. A p-value of 0.05 was considered statistically significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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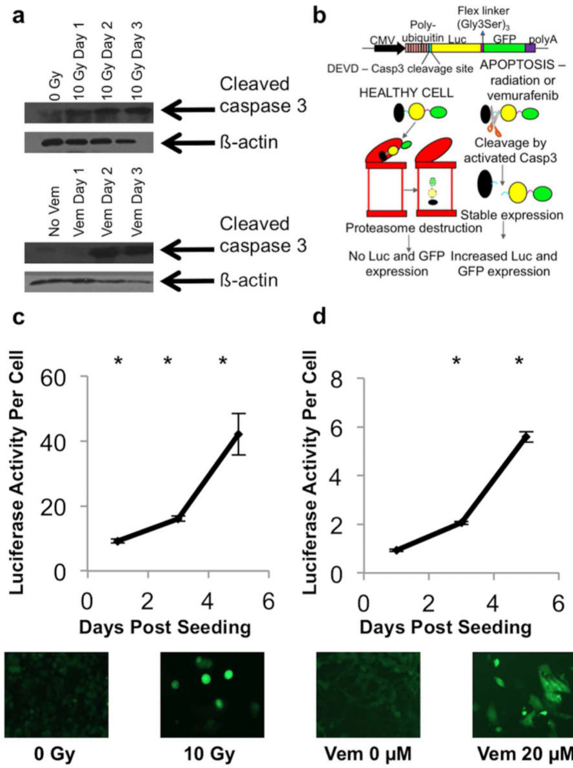

Figure 1. Cytotoxic treatment increases activated caspase 3 levels in A375 melanoma cells

A) Western blots for cleaved caspase 3 protein expression in A375 cells treated with10 Gy radiation or vemurafenib 20 μM. **B)** A schematic drawing of the activated caspase 3 reporter illustrating that luciferase and GFP expression can be quantified as a measure of activated caspase 3 activity. **C**) Graph of luciferase activity per cell vs. time and pictures of GFP expression on day 5 in irradiated A375 caspase 3 reporter melanoma cells. **D**) Graph of luciferase activity per cell vs. time and pictures of GFP expression on day 5 in vemurafenib-treated A375 caspase 3 reporter melanoma cells. Error bars are mean +/− SEM, n= 3; *p<0.05, t-test.

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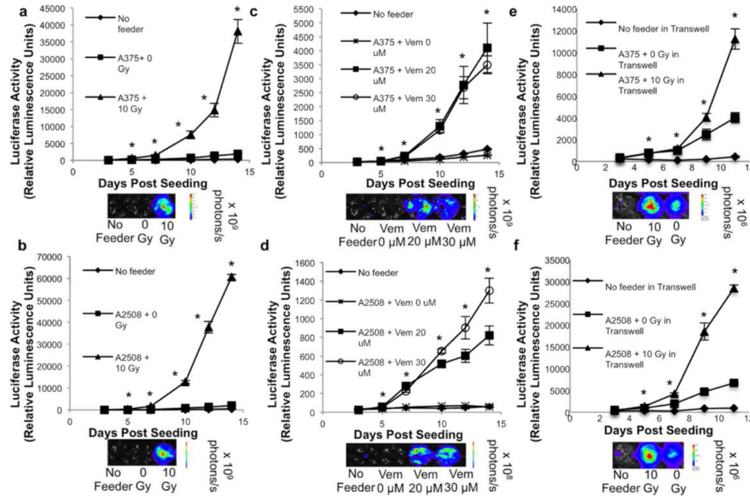

Figure 2. Dying melanoma cells promote the growth of living melanoma reporter cells *in vitro*

A small number of living melanoma luciferase reporter cells were seeded onto a larger number of lethally treated unlabeled melanoma cells. **A and B)** Graph depicting reporter cell luciferase activity vs. time when seeded onto irradiated melanoma cells with luminescence images on day 13. **C and D)** Graph depicting reporter cell luciferase activity vs. time when seeded onto vemurafenib treated melanoma cells with luminescence images on day 13. **E and F)** Graph depicting reporter cell luciferase activity vs. time when seeded onto irradiated melanoma cells in transwell plates with luminescence images on day 11. Error bars are mean +/− SEM, n= 3; *p <0.05 one-way ANOVA test.

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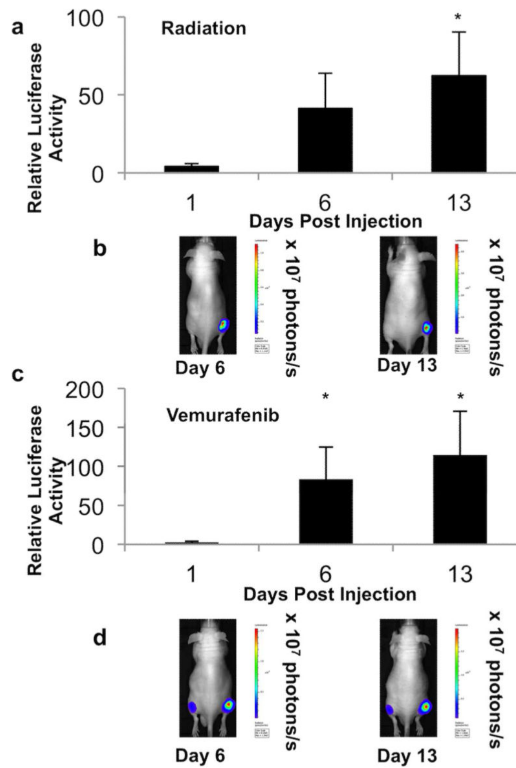

Figure 3. Cell death-stimulated growth of A375Fluc melanoma cells *in vivo*

A small number (1×10^4) of A375Fluc cells were injected with a larger number (2×10^5) of feeder cells treated with 10 Gy radiation or vemurafenib 20 μM in the right leg or untreated feeder cells on the left leg. **A**) Graph presenting fold difference in A375Fluc luciferase activity and **B)** Luminescence images of 10 Gy-irradiated feeder cells (right leg) compared with untreated feeder cells (left leg). **C**) Graph presenting fold difference in A375Fluc luciferase activity and **D)** Luminescence images of feeder cells treated with vemurafenib 20 μM in (right leg) compared with untreated feeder cells (left leg). Error bars are mean +/− SEM, n=4 radiation, n=5 vemurafenib; *$p<0.05$, t-test.

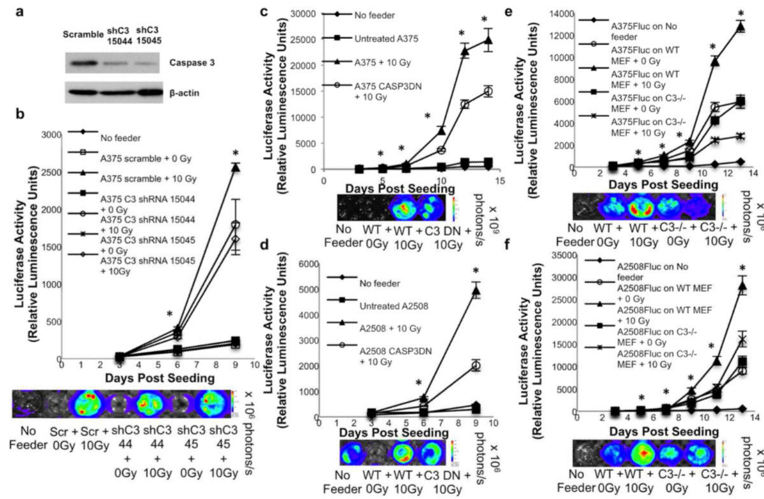

Figure 4. Inhibition of caspase 3 attenuates the growth-stimulating effect of dying cells on living melanoma cells

A) Western blot showing caspase 3 knockdown in A375 caspase 3 shRNA melanoma cells. **B**) Graph illustrating reporter luciferase activity vs. time when seeded onto irradiated caspase 3 shRNA melanoma cells or scramble control cells with luminescence images on day 9. **C and D**) Graphs illustrating reporter luciferase activity vs. time when seeded onto irradiated caspase 3 dominant negative melanoma cells with luminescence images on day 13. **E and F**) Graphs illustrating reporter luciferase activity vs. time when seeded onto irradiated caspase 3 knockout MEF cells with luminescence images on day 13. Error bars are mean +/− SEM, n= 3; *p < 0.05, one-way ANOVA test.

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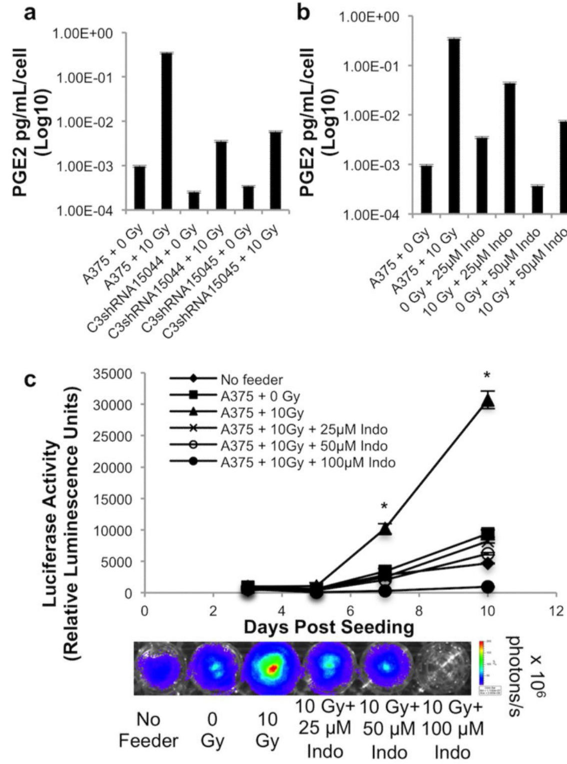

Figure 5. Caspase 3 regulates radiation-induced PGE_2 secretion and indomethacin decreases dying melanoma cell stimulated living cell growth

Melanoma cells were irradiated and PGE_2 concentrations were measured in the supernatant using an ELISA assay for PGE_2 72 hours after radiation. **A)** Graph showing PGE_2 concentration per cell number in irradiated or untreated A375 and A375 C3shRNA cells. **B)** Graph showing PGE_2 concentration per cell in irradiated or untreated A375 cells treated with indomethacin 25 or 50 μM. **C)** Graph showing reporter luciferase activity vs. time when seeded onto irradiated A375 cells treated with indomethacin 25, 50 or 100 μM with luminescence images on day 10, Error bars are mean +/− SEM, n= 3. *$p < 0.05$, one-way ANOVA test.