INTRODUCTION
Ionizing radiation is used therapeutically to induce cancer cell death, decrease distant metastasis rates, and to increase overall patient survival (Darby et al., 2011). However, radiotherapy does not efficiently target all cells of the tumor mass. Tumor cell re-population and activation of DNA repair mechanisms (ATM, H2AX, and p53) are key components of tumor cell radiosensitivity (Hazen and Chen, 2008). Cells in the G2/M and G1 phases of the cell cycle have been shown to be the most radiosensitive, while cells in the S phase are radioresistant (Hwang et al., 2000; Pawlik and Keyomarsi, 2004). The latter is attributed to DNA double strand breaks (DSBs) repair systems such as homologous recombination that occur in the S phase (Kastan and Bartek, 2004; Jackson and Bartek, 2009). In the 1950s, several groups established the connection between hypoxia and radioresistance in mammalian tumors (Duschner and Gray, 1959; Dewey, 1960) and oxygen levels remain the major cell radiosensitizer known to date. In well-oxygenated conditions, the free radicals generated by ionizing radiation interact with O2 to form peroxyl radicals that damage DNA much more efficiently than reduced free radicals (Brown, 2007). Consequently, the poorly oxygenated (hypoxic) cells of tumors are more radioresistant (Gray, 1953). These studies led to the general hypothesis that oxygen acts at a physicochemical level to improve radiation induced damage as a consequence of the high affinity between the oxygen molecule and the unpaired electron on the free radical produced by radiation.

In addition to low oxygen, increased acidification is also a hallmark of hypoxic tumors and it has been suggested to play an indirect role in the poor radioreponse of hypoxic tumors (Vause, 2004). In contrast, another report indicates that extracellular acidosis may enhance radiosensitivity in combination with lactate accumulation for certain cell lines (Grotius et al., 2009). However, lactate accumulation alone (in the absence of pH disruption) has also been suggested to reduce radiosensitivity of tumor cells (Quennet et al., 2006). Furthermore, the effect of intracellular pH (pHi) and extracellular pH (pHo) regulation on the efficacy of radiotherapy remains to be clarified.

Despite the fact that all mammalian cells are capable of protecting their cytosol from acidification through expression of membrane located transporters and exchangers including the Na+/H+ exchanger 1 (NHE-1; Pouyssegur et al., 1985) and the monocarboxylate transporter 1 (MCT1; Halestrap and Price,
hypoxic tumor cells have developed additional mechanisms to regulate their pH (Chiche et al., 2010b). In solid tumors, membrane-bound carbonic anhydrases (CA) IX and XII are controlled by oxygen levels via the hypoxia-inducible factor (HIF-1; Wykoff et al., 2000) and catalyze at the cell surface the reversible hydration of carbon dioxide (CO₂) into a proton (H⁺) and bicarbonate (HCO₃⁻). Once generated, HCO₃⁻ is proposed to be rapidly taken up into the cell through the Na⁺-/HCO₃⁻ cotransporters (NBC, Romero et al., 2004; Purks et al., 2001) to sustain a slightly alkaline pH, compatible with cell survival (Morgan et al., 2007, Swietach et al., 2009; Chiche et al., 2010a). Many reports correlate CAIX expression with poor patient survival in a variety of cancers (see review Supuran, 2010; Chiche et al., 2010a). The extracellular location of the CAIX active site together with its overexpression in hypoxic cancer cells compared to minimal expression in healthy cells, except in the gastro-intestinal tract and the stomach (Paszkiewicz et al., 1997) makes hypoxia-induced CAIX an accessible target for new anti-cancer therapy (Supuran, 2008; Morris et al., 2011). CAIX function has been clearly established to contribute to extracellular acidification (Swiatlova et al., 2004). In addition, studies in our laboratory have characterized CAIX and CAXII as robust pH-regulating enzymes and have provided evidence that both CAIX and CAXII hold potential as new anti-cancer targets (Chiche et al., 2010a).

We analyzed the downstream effects of CAIX and CAXII activity on radiation-induced cell death to determine whether a combined therapy of irradiation and down-regulation of CAIX and CAXII would sensitize hypoxic cells to ionizing radiation. An alteration in pH regulation (either by inhibition of NHE-1 or expression of CAIX) revealed a decreased percentage in cells found in the radiosensitive S phase and an increase in radiosensitization that correlated with an increase in cell death. Gene silencing of ca9 and cat12 in vitro and in vivo radiosensitization as a consequence of a reduction of cells in the S phase and a decrease in the pH-regulating capacity of the cell.

**Materials and Methods**

**Cell Culture and Hypoxic Exposure**

Chinese hamster lung CCL39 fibroblasts (ATCC), CCL39-derived PS120 cells lacking NHE-1, and CAIX and CAXII were cultured as described. Colon adenocarcinoma LS174T cells expressing the tetracycline (Tet) repressor were maintained in Dulbecco’s modified Eagle’s Medium (DMEM) supplemented with 10% fetal calf serum (FCS) and blastidin (10 μg/ml, Invitrogen). Incubation in hypoxia at 1% O₂ was carried out at 37°C in 95% humidity and 5% CO₂/94% N₂ in a sealed anaerobic workstation (Ruskinn). 1% CO₂ gas was delivered continuously to the workstation to maintain a slightly alkaline pH. Pipettes were changed from an extracellular pH of 7.2 to 7.6 to maintain the HCO₃⁻ concentration in the extracellular environment. Cells were plated at 2 × 10⁵ cells per dish 48 h before hypoxic exposure. Cells were allowed to attach overnight and then suspended in fresh medium containing 1% O₂ for 4 days, and freshly refreshed daily under hypoxic conditions.

**Cell Irradiation**

Irradiation of normoxic cells was performed in 25 cm² ventilated flasks (Nunc), while irradiation of hypoxic cells was performed in 95% humidity and 5% CO₂/94% N₂ in a sealed anaerobic workstation (Ruskinn). Cells were irradiated with the same schedule but with an anterior field and a bolus placed at the top of the dishes.

**Measurement of Radiation-Induced Cell Death**

Fibroblasts (1 × 10⁶) were seeded onto 60 mm dishes. Once attached the medium was replaced by either HCO₃⁻-free or 10 mM HCO₃⁻-containing DMEM buffered at an extracellular pH (also named outside pH, pHe) of 7.0 (30 mM MES) or at pH 7.5 (30 mM HEPES), supplemented with 10% dialyzed serum, hypoxanthine 0.1 mM, and uridine triphosphate 0.1 mM for growth in the absence of CO₂/HCO₃⁻ and transferred to a CO₂-free atmosphere for 24 h in the presence or absence of inhibitors (NHE-1 inhibitor HOE694 (Hoechst) 100 μM). Cells were then irradiated (0, 2, 4, 6, 8, and 10 Gy). After irradiation, dishes were returned to 3% CO₂ in regular NaHCO₃-containing medium for 5 days. Cells were then trypsinized and the percentage of cell death was determined with trypan blue.

**Stable Transgenic Cells**

PS120 cells were transfected with pcDNA as described (Chiche et al., 2010a). Tet (10 μg/ml)-inducible LS174T cells silenced for ca9 (LS-shca9/ctl) combined with a constitutive silencing of cat12 (LS-shca9/cat12) were obtained as described (Chiche et al., 2010a).

**Immunoblotting**

Cells were lysed in 1.5 × SDS sample buffer. Proteins (40 μg) were separated on 7.5% polyacrylamide gels and transferred onto polyvinylidene difluoride membranes (Millipore). Membranes were blocked with the M75 antibody to CAIX (Bayer), a polyclonal antibody to recombinant CAXII (Sigma), p21 (Santa Cruz), β-tubulin (Becton Dickinson). The proportion of G1, G2/M peaks, and the 5 phase plateau were calculated with WinMD2 software.

**Cell Cycle Analysis**

Cells (3 × 10⁵) exposed to normoxia or hypoxia were washed in PBS and fixed in ice-cold 70% ethanol for a minimum of 30 min. Cells were centrifuged, washed in 20 ml of 70% ethanol with 5 μg/ml RNase A (Sigma) and stained for 30 min at 37°C with 50 μg/ml propidium iodide (PI). DNA was analyzed for hypoxic and normoxic cell lines using a FACSscan calibur (Becton Dickinson). The proportion of G1, G2/M peaks, and the 5 phase plateau were calculated with WinMD2 software.

**Plasmids**

Full-length human ca9 cDNA was obtained and inserted into pTREX-A (pcDNA4/TD/myc-His A; Invitrogen; psi9) as described (Chiche et al., 2010a). The short-hairpin (sh) RNA, ca9 (shca9) was obtained with oligonucleotides: forward 5′-AGTTAAGGCTAATCACAA-3′ and reverse 5′-TCTGGATAGGCTTAAC-3′ and inserted into either pTER vector (also named shca9). Lentivirus particles for two independent sequences (#1 and #2) of pLKO.1-Puro shRNA targeting ca12 (cat12) and non-target shRNA (ctrl; Sigma, TRCN0000116249, TRCN0000116251, and SHC002V) were used to constitutively silence cat12.
CLOTHING EFFICIENCY
LS174T cells were plated in clonogenic conditions (1000 cells per plate, triplicate) in 25 cm² ventilated flasks during 24 h, then exposed to hypoxia (1% O₂) for 48 h, and subsequently closed with non-ventilated flask caps before irradiation (0, 1, 2, 4, 6, and 8 Gy). Cells were then returned to normoxic conditions to allow cell recovery and determination of colony number following irradiation. PS120 cells were plated onto 60 mm dishes in clonogenic conditions (1000 cells per dish, duplicate). Once attached, cells were exposed to 30 mM HEPES, 100 μM hypoxanthine, 180 μM uridine, and 10% diazylated FCS medium adjusted to two different extracellular pH (either pH 7.0 or 7.5) in a CO₂/HCO₃⁻ free environment and subsequently irradiated (0, 1, 2, 4, 6, and 8 Gy) in these environments. Following irradiation, the medium were replaced by a regular medium. Six days (for PS120 cells) or 10 days (for LS174T cells) following irradiation, cells were fixed, stained with Giemsa, and counted using ImageJ software. Of note for both LS174T and PS120 cells, irradiation was not performed but after two or three cell divisions. This raises the possibility of microcolony formation and overestimation of the number of colonies, however, we used caution to exclude microcolonies from our final analysis. Furthermore, our calculations involve a ratio between experimental clones and control, therefore, the absolute number of clones is normalized because every condition grows with the same amplitude of error.

CELL PROLIFERATION IN THREE-DIMENSIONS AND IRRADIATION OF SPHEROIDS
To grow spheroids, 2 × 10⁶ cells were seeded in drops in 20 μl of HCO₃⁻ free DMEM buffered with 30 mM HEPES pH 7.7 containing 10% FCS. After 8 days, spheroids were irradiated (0, 2, 4, 6, and 8 Gy) as described above. intact spheroids were then transferred to polyhema-coated 96-well plates for continued growth in the same respective media for 4 days (12 days total growth time including irradiation). Spheroids were dissociated in Accutase (Life Technologies) and living and dead cells were immediately counted using trypan blue exclusion.

NUDE MICE XENOGRAFTS AND IRRADIATION OF MOUSE TUMORS
Cells (1 × 10⁶) were subcutaneously injected into the flanks of 4-week-old male athymic nude mice (Harlan) according to CNRS institutional guidelines and tumor growth was measured as reported previously (Chiche et al., 2010a). A total of 750 μg/ml doxycycline (DOX; Sigma) was added in the drinking water before the injection of tumor cells following the previously established protocol in our laboratory allowing us to obtain 90% of ca IX silencing in vivo (see Chiche et al., 2010a) for immunohistochemical analysis of inducible ca IX silencing in this model). Tumors of 4.5–5 mm were irradiated (8 Gy) with contact X-rays (Conrad et al., 2011) using a RT 50 Phillips unit delivering a 50委托V energy X-ray beam. The source–surface distance was 40 mm and the dose rate was 20 Gy/min. The X-ray tube was handled and the precision was controlled through direct vision by the radiation oncologist using a 20-mm diameter applicator. The dose was prescribed at the exit surface of the applicator. One single fraction of 8 Gy was delivered into the visible lesion.

STATISTICAL ANALYSIS
The Student’s t-test was used wherein P-values of <0.05 were considered significant.

RESULTS
INHIBITION OF THE MAJOR pH-I REGULATING SYSTEM NHE-1 SENSITIZES CELLS TO RADIATION-INDUCED CELL DEATH
The contribution of intracellular acidosis to cell radiosensitivity was studied on fibroblasts growing in pH 7.5 or a more acidic pH 7.0, medium, in the presence or in the absence of NHE-1 inhibitor. We choose to work at pH 7.0 as it is low enough to reduce the pHi compared to the pHi obtained at pH 7.5 but is not low enough to prevent an observation of radiosensitization in acidic conditions due to a reduction in cell viability caused by acidosis alone. The impact of inhibiting NHE-1 on pHi regulation in these cells has been well established with NHE-1 inhibition causing a significant reduction in pHi in a pHo of 7.0 (Pouysegur et al., 1984). Prior to irradiation, we determined the effect of NHE-1 inhibition on cell cycle phase distribution. Selective inhibition of NHE-1 using HOE#694 (100 μM; see Masereel et al., 2003 for a review of NHE inhibitors and HOE#694 effectiveness) at the more acidic pH 7.0 condition reduced the percentage of CCL39 cells in the most radiosensitive S phase (34% decrease of cells in the radiosensitive S phase in the presence of HOE#694 compared to non-treated cells) while it had no effect in a more neutral pH 7.5 medium (Figure 1A). Consistent with this finding, irradiation of NHE-1-inhibited fibroblasts in a pH 7.0 medium led to an increase in cell death (37% for 10 Gy) compared to either untreated cells (33% for 10 Gy; Figure 1B) or cells exposed to a pH 7.5 medium treated or not with HOE#694 (32% for 10 Gy; Figure 1C).

EXPRESSION OF THE HYPOXIA-INDUCED pH-I REGULATING CARBONIC ANHYDRASE IX PROTECTS CELLS AGAINST IRRADIATION
We have previously demonstrated that expression of catalytically active CAIX in NHE-1-deficient CCL39 fibroblasts (PS120 cells) maintains a higher pHi compared to control PS120 cells lacking CAIX, when cells were exposed to a nominally bicarbonate free acidic medium (Chiche et al., 2010a). Here we showed that in the condition where CAIX is required for pHi regulation (pH 7.0 compared to pH 7.5 medium), expression of CAIX in PS120 cells (PS120-pCAIX) maintains the distribution of the cell cycle phases, while in the absence of CAIX, PS120-pev cells demonstrate a 35% reduction in the most radiosensitive S phase (Figure 2A). Consequently, control PS120-pev cells growing at pH 7.0 were shown to be more radiosensitive than PS120-pCAIX cells, with 78% cell death following irradiation of 10 Gy for PS120-pev cells compared to 37% for PS120-pCAIX cells (Figure 2B). Of note, PS120-pCAIX cells irradiated with 10 Gy at pH 7.0 exhibited similar cell death rates to that at pH 7.5 while PS120-pev cells had much higher cell death at low pH 7.5 (Figures 2B,C). Thus, active CAIX protects cells against ionizing irradiation at low pH. To definitively validate that the pHI regulating functions of CAIX are indeed involved in cellular radioresistance, PS120-pev and PS120-pCAIX cells were exposed to a pH 7.0 medium containing 10 mM HCO₃⁻. This
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FIGURE 1 | NHE-1 protects cells against irradiation-induced cell death in an acidic environment. (A) Cell cycle distribution of CCL39 cells treated (+) or not (−) with 100 μM of the NHE-1 inhibitor (HOE694) in normoxia in a HCO$_3^{-}$/CO$_2$-free environment at pHo 7.5 or 7.0 for 24 h. (B,C) CCL39 cells (1 × 10$^4$) were plated in 60 mm dishes. Once attached cells were incubated in a HCO$_3^{-}$-free medium adjusted to pHo 7.5 or 7.0, and treated in the presence (+) or absence (−) of 100 μM of NHE-1 inhibitor (HOE694) for 24 h in a CO$_2$-free atmosphere. Dishes were then irradiated (0, 2, 4, 6, 8, and 10 Gy) and returned to a 5% CO$_2$ incubator with fresh HCO$_3^{-}$-containing medium for 4 days. Cell death was determined by the trypan blue exclusion assay.

HCO$_3^{-}$ addition has been shown previously to maintain pH$_i$ in acidic pH$_o$ environments (Chiche et al., 2010a). Irradiation of PS120-pc9 cells in the presence of HCO$_3^{-}$ reduced the percentage of cell death to that obtained for PS120-pc9 cells in a pH$_o$ 7.0 medium (Figure 2D). Cloning efficiency experiments also confirm the capacity of irradiated cells to survive and recover following irradiation. From 4 to 8 Gy single doses of ionizing radiation of PS120-pc9 cells exposed to a pH$_o$ 7.0 medium drastically reduced the cloning efficiency, compared to that observed in a pH$_o$ 7.5 medium (Figure 2E, left panel). In contrast, PS120-pc9 cells exposed to a pH$_o$ 7.0 medium were capable to recover after irradiation, to the same extent that we observed in a pH$_o$ 7.5 (Figure 2E, right panel). Taken together these results suggest that the pH$_i$-regulating properties of NHE-1 and CAIX protect cells against irradiation.

DUAL SILENCING OF THE HYPOXIA-INDUCED pH$_i$-REGULATING SYSTEM ca9/ca12 STRONGLY COMPROMISES IN VITRO AND IN VIVO TUMOR GROWTH WHEN COMBINED WITH IONIZING RADIATION

LS174T cells cultured in hypoxia before exposure to an increasing dose of ionizing radiation demonstrated a higher cloning efficiency than normoxic cells along with equal distribution of cell cycle phases before irradiation (data not shown). This established the classical radioresistance of LS174T cells as observed in other hypoxic cells and validated this model for our study. In a regular HCO$_3^{-}$-containing medium a higher number of cells in the radiosensitive G1/G2/M phases was observed when ca9 or both ca9 and ca12 were silenced (Figure 3A). Protein expression levels of CAIX and CAXII in the Tet-inducible silencing of ca9 in control LS174T cells (LS-shca9/ctl) or ca12 silenced cells (LS-shca9/ca12−) were confirmed for efficient knock-down (Figure 3A, inset). In the same conditions, ca9 or both ca9/ca12 silencing was accompanied by an increase in p21, E-cadherin, and β1 integrin expression, which were associated with a reduced cell proliferation (Figure 3B). To mimic both the tumor hypoxic and proton gradient observed in vivo, we grew LS174T cells in three dimensions. Spheroids were grown in nominally bicarbonate free media to enhance the pH gradients that develop during spheroid growth. Irradiation of ca9-silenced spheroids (LS-shca9/ctl + Tet, 8 Gy) revealed a cumulative decrease in the proliferation index (Figure 3C) and a twofold increase in cell death from 27.5% (0 Gy) to 51.7% (8 Gy) when compared to non-irradiated ca9-silenced spheroids (Figure 3D). While ca12 silencing alone did not alter...
FIGURE 2 | CAIX protects cells against irradiation-induced cell death in an acidic environment. (A) Cell cycle distribution of NHE-1-depleted fibroblasts PS120 cells expressing (pca9) or not (p ev) CAIX, in normoxia in a HCO$_3$-CO$_2$-free environment at pH$_o$ 7.0 or 7.5 for 24 h. (B–D) PS120-p ev and PS120-p ca9 cells (1 × 10$^4$) were plated in 60 mm dishes. Once attached cells were incubated in 30 mM HEPES-buffered HCO$_3$-free medium adjusted to pH$_o$ 7.0 in the absence (B) or in the presence of 10 mM HCO$_3$ (D) or to pH$_o$ 7.5 (C) for 24 h in a CO$_2$-free atmosphere. Dishes were then irradiated (0, 2, 4, 6, 8, and 10 Gy) and returned to a CO$_2$-containing incubator with fresh regular HCO$_3$-containing (44 mM) medium for 4 days. Cell death was determined by the trypan blue exclusion assay. Data represent the average of three independent experiments. (E) The clonogenic capacity of PS120-p ev and PS120-p ca9 cells exposed to a medium adjusted to pH$_o$ 7.0 or 7.5 was measured 10 days after irradiation (0, 1, 2, 4, 6, and 8 Gy). Dishes were stained with Giemsa (Fluka). The colonies were counted with ImageJ software according to the following parameters: particles size = 0.15–5 mm$^2$ and circularity = 0.1–1.
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FIGURE 3 | Silencing of the hypoxia-induced pH-regulating enzymes ca9 and ca12 induced in vitro cell death of LS174T cells when combined with ionizing radiation. (A) Inset: Expression of CAIX and CAXII in colon carcinoma Tet-onxylene (Tet-inducible LS-shca9/ctl + Tet) or for ca12 (LS-shca12/ctl + Tet) or both cell and ca12 (LS-shca9/12 + Tet) in hypoxia 1% O2 (Hx) for 48 h. Hsp90 was used as a loading control. The cell cycle phase distribution was determined by FACScan analysis of LS-shca9/ctl + Tet and LS-shca12/ctl + Tet cells exposed hypoxia of 1% O2 (Hx) for 24 h in a HCO3- containing medium. (B) Immunoblotting of p21, β1 integrin, and Hsp90 (loading control) in LS-shca9/ctl and LS-shca12 cells pre-incubated for 4 days in the presence (+Tet) or absence (−Tet) of Tet to silence ca9; before exposure to hypoxia of 1% O2 (Hx) for 48 h. (C) Tet-inducible LS174T cells silenced for ca9 or ca12 or both ca9 and ca12 were cultured as spheroids in a CO2 atmosphere and HEPES-buffered HCO3- free medium pH 7.7 in the absence (−Tet) or presence (+Tet) of Tet for 8 days before they were irradiated (8 Gy) or not (0 Gy). After irradiation, spheroids were transferred to polyhema-coated 96-well plates containing fresh medium for 5 days. Spheroids were then subjected to Accutase dissociation and individuated live cells (C) and dead cells (D) were counted using trypan blue. The spherical proliferation index was calculated as the ratio of the number of living cells counted at day 13 to the number of cells at day 0. Data represent the average of three independent experiments. (E) The clonogenic capacity of LS174T-shca9/ctl + Tet and LS174T-shca9/12−/−Tet cells exposed to hypoxia (1% O2) for 48 h in a regular medium, was measured 10 days after irradiation (0, 1, 2, 4, 6, and 8 Gy). Dishes were stained with Giemsa (Fluka). The colonies were counted with Image J software.
Acidosis within the tumor microenvironment arises from the hypoxia-induced metabolic shift from oxidative phosphorylation to glycolysis, along with the capacity of hypoxic tumor cells to regulate their pH, through efficient export of CO₂ and lactic acid. Consequently, targeting tumor pH-regulating systems in hypoxia holds potential as a key strategy to reduce tumor growth (Pouysségur et al., 2006). Here we have explored a combination of this pH disrupting strategy with radiotherapy as it was suggested that acidosis plays a role in tumor radioresistance (Vaspu, 2004). However, previously there was no clear demonstration whether the extracellular and/or the intracellular acidosis were responsible for the poor radioreponse of tumor cells. Dubois et al. (2011) have demonstrated that a combination of CAs inhibition with irradiation in colon HT29 tumor-bearing mice improved the anti-tumor effect compared to a single radiation dose. However, CA inhibition did not result in increased radiosensitivity in vitro and the contribution of the pH-regulating functions of CAIX in the tumor radio-response remained to be clarified. Herein, we demonstrate the importance of pH₇ regulation in radioresistance by observing an increase in radiation-induced cell death of fibroblasts inhibited for NHE-1 or lacking both NHE-1 and CAIX when they are grown in an acidic and HCO₃⁻ free medium. In contrast, ectopic expression of CAIX was able to improve cell survival following irradiation. The mechanism of CAIX-induced radioresistance was demonstrated with NHE-1-deficient CCL39 fibroblasts (PS120 cells) in a nominally CO₂/HCO₃⁻ free acidic environment by: (i) a decrease in the pH₇-regulating capacity of cells lacking CAIX (see Chiche et al., 2009) and (ii) a correlation with the positioning of these cells in the most radiosensitive G1/G2/M phases, prior to irradiation. This cell cycle data is consistent with the reduction in S phase entry as previously demonstrated for PS120 cells compared to the parental cell line (Pouysségur et al., 1985). Expression of CAIX prevents the reduction of cells in S phase as it allows cells to maintain a higher pH₇ in acidic medium (Chiche et al., 2010a). With addition of HCO₃⁻/CO₂ in low pH medium (pH₇ 7.0) we observed no difference in cell death between irradiated-PS120-por and irradiated-PS120-por cells due to the buffering ability of HCO₃⁻/CO₂ to restore alkaline pH₇ values as previously demonstrated (Chiche et al., 2010a). We conclude that the CAIX-induced protection against irradiation at pH₇ 7.0 could be explained by the capacity of CAIX to sustain an intracellular alkaline shift.

Under three-dimensional growth conditions that result in hypoxia (Chiche et al., 2010a, Pelleret et al., 2012) and acidosis (Bovet et al., 2010), LS174T spheroids silenced for ca9 or ca12 showed a decrease in proliferation and a cumulative increase in cell death (75%) after a single radiation dose. Double silenced cells were indeed most sensitive to irradiation, since: (i) combined silencing reduced the capacity of LS174T cells to regulate their pH, in acidic medium while single silencing of ca9 was not sufficient to do so (Chiche et al., 2010a), (ii) silencing of ca9 or ca12 increased p21 expression indicating a cell cycle arrest in G₁ along with increased levels of β₁ integrin, two key proteins involved in cell contact and adhesion which may influence proliferation (Svastová et al., 2005) and (iii) ca9 silencing led to a reduction in proliferation and a decrease in cell number in the radioresistant S phase. A single radiation dose on xenograft tumors dramatically reduced the growth rate of ca9- and ca9/12-silenced tumors. Twenty-five days after irradiation, ca9-silenced tumors recovered a growth rate that was comparable to control tumors, while ca9/12-silenced
FIGURE 4 | Combined silencing of \( \text{ca9} \) and \( \text{ca12} \) compromises in vivo LS174T cell proliferation when combined with ionizing radiation. (A,B). Anti-tumor activity of silencing \( \text{ca9} \) or \( \text{ca12} \), individually or combined, is increased in conjunction with ionizing radiation in LS174T xenograft tumors. At 4 days before injection of LS-sh\( \text{ca9} \)/ctl (A) or LS-sh\( \text{ca9}/\text{ca12} \)– cells (B), cells were incubated with or without Tet (+/−Tet) to silence \( \text{ca9} \). In vivo xenograft assays were performed by s.c injection of viable and individual tumor cells \((1 \times 10^6)\) into the flanks of athymic nude mice. To maintain cell knock-down mice received DOX in the drinking water 4 days before cell injection. Fifteen days after cell injection, when the tumor size reached 4–5 mm, a single dose (8 Gy) of irradiation (IR) was delivered to the tumors with “contact X-rays.” Xenograft growth was determined by measuring tumor volume. (C) Xenograft tumor growth of control tumors LS-sh\( \text{ca9} \)/ctl irradiated (IR) or not, in the presence (+DOX) or in the absence (−DOX) of doxycycline in the drinking water. Five mice were used per condition.

and irradiated tumors never recovered the growth rate of control cells. In addition, the loss of radioresistant hypoxic cells due to CA9/CA12 silencing could contribute to the reduction of tumor growth in combination with their radio-sensitivity due to decreased pH\(_i\) regulation.

Hypoxia-specific cytotoxins such as tirapazamine form toxic radical species that act to kill hypoxic cells and are thus proposed to be used in combination with irradiation to create a synergistic effect (Brown, 1993). Unfortunately this treatment-strategy failed to be efficient in patients (Rischin et al., 2010). Intense research...
is ongoing in the development of small molecule inhibitors to specifically target membrane-bound CA(s) over cytosolic CAs to appraise the potential of targeting CAIX and CAXII to decrease tumor progression (Morris et al., 2011). The synthesis of new CA(s) inhibitors (Supuran, 2008; Morris et al., 2011) has also revived interest in acetazolamide (ACTZ), which has been used in the clinic for over 40 years as a CA inhibitor (Kaur et al., 2002). Recently, ACTZ was linked to a C-terminal albumin-binding peptide (Albu-ACTZ) with the aim of not only reducing blood clearance but also preventing internalization of the molecule to target more specifically membrane-bound CAIX and CAXII. This compound demonstrated its in vivo efficacy by retarding tumor growth of renal SK-RK-52 xenografts. However, it had no significant impact on highly proliferative LS174T tumors (Ahlbok et al., 2009).

In the present study, we took advantage of the expression of the hypoxia-induced pH-regulating systems CAIX and CAXII to target radiosensitive hypoxic cells. This study reinforces the notion that CAIX and CAXII represent potential targets for anti-cancer treatment. The present study also supports the use of radiotherapy in combination with CA inhibitors as a new anti-cancer strategy.

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REFERENCES

Ahlbok, J. K., Drumond, C. G., Truu, S., Mikkila, I., and Nerl, D. (2009). In vivo targeting of tumor-associated carbonic anhydrases using acetazolamide derivatives. Biol. Chem. Lett. 29, 4853–4856.

Brown, J. M. (1995). SR 425 (tra-pamaine): a new anticancer drug exploiting hypoxia in solid tumours. Br. J. Cancer 67, 1163–1170.

Brown, J. M. (2007). Tumor hypoxia in cancer therapy. Methods Enzymol. 435, 297–322.

Chiche, J., Bruguière-Horn, M. C., and Pouysségur, J. (2010a). Tumor hypoxia induces a metabolic shift causing acidosis a common feature in cancer. J. Cell Mol. Med. 14, 771–794.

Chiche, J., De, K., Bruguiere-Horn, M. C., and Pouysségur, J. (2010b). Membrane-bound carbonic anhydrase s as key pH regulators controlling tumor growth and cell migration. Adv. Enzyme Regul. 50, 20–33.

Chiche, J., De, K., Morris, N. M., and Pouysségur, J. (2009). Hypoxia-inducible carbonic anhydrase IX and XII promote tumor cell growth by counteracting acidosis through the regulation of the intracellular pH. Cancer Res. 69, 358–364.

Darby, S., Peto, J., Morris, N., and Pouysségur, J. (1998). Membrane-bound carbonic anhydrases as key pH regulators controlling tumor growth and cell migration. Adv. Enzyme Regul. 38, 20–33.

Darby, S., Peto, J., Morris, N. M., and Pouysségur, J. (2009). Hypoxia-inducible carbonic anhydrase IX and XII promote tumor cell growth by counteracting acidosis through the regulation of the intracellular pH. Cancer Res. 69, 358–364.

Debruyne, F., De, K., and Pouysségur, J. (2011). Role of cell cycle in mediating the G1 restriction point into S-phase: implications for fluoropyrimidine radiosensitization. Cancer Res. 69, 82–90.

Jackson, S. P., and Bartek, J. (2009). The DNA-damage response in human biology and disease. Nature 461, 1071–1078.

Kastan, M. B., and Bartk, J. (2014). Cell-cycle checkpoints and cancer. Nature 452, 516–525.

Kazer, I. P., Smitha, R., Aggarwal, D., and Kapoor, M. (2002). Acetazolamide: future perspective in topical glaucoma therapy. Int. J. Pharm. 246, 1–14.

Mammel, B., Dreyfus, L., and Lachmann, D. (2003). An overview of inhibitors of Na+/H+ -exchanger S他说steubert method of Ehrlich ascites tumor cells irradiated through the G1 restriction point into S-phase: implications for fluoropyrimidine radiosensitization. Cancer Res. 69, 82–90.

Jackson, S. P., and Bartek, J. (2009). The DNA-damage response in human biology and disease. Nature 461, 1071–1078.

Kastan, M. B., and Bartek, J. (2014). Cell-cycle checkpoints and cancer. Nature 452, 516–525.

Kazer, I. P., Smitha, R., Aggarwal, D., and Kapoor, M. (2002). Acetazolamide: future perspective in topical glaucoma therapy. Int. J. Pharm. 246, 1–14.

Mammel, B., Dreyfus, L., and Lachmann, D. (2003). An overview of inhibitors of Na+/H+ -exchanger S他说steubert method of Ehrlich ascites tumor cells irradiated through the G1 restriction point into S-phase: implications for fluoropyrimidine radiosensitization. Cancer Res. 69, 82–90.

Jackson, S. P., and Bartek, J. (2009). The DNA-damage response in human biology and disease. Nature 461, 1071–1078.
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Romero, M. F., Fulton, C. M., and Boron, W. F. (2004). The SLC4 family of HCO\(^{-3}\) transporters. Pflugers Arch. 447, 495–509.

Supuran, C. T. (2008). Carbonic anhydrases: novel therapeutic applications for inhibitors and activators. Nat. Rev. Drug Discov. 7, 158–161.

Svastová, E., Hulíková, A., Kaťáková, M., Začírová, M., Gibadulínova, A., Časni, A., et al. (2004). Hypoxia activates the capacity of tumor-associated carbonic anhydrase IX to acidify extracellular pH. FEBS Lett. 577, 439–445.

Svastová, E., Žíkka, N., Začírová, M., Gibadulínova, A., Camper, F., Pastorek, J., et al. (2003). Carbonic anhydrase IX reduces E-cadherin-mediated adhesion of MDCK cells via interaction with ß-catenin. Exp. Cell Res. 290, 332–346.

Swietach, P., Hulíková, A., Vaughan-Jones, R. D., and Harris, A. L. (2010). New insights into the physiological role of carbonic anhydrase IX in tumour pH regulation. Oncogene 29, 6309–6321.

Swietach, P., Patar, S., Supuran, C. T., Harris, A. L., and Vaughan-Jones, R. D. (2009). The role of carbonic anhydrase IX in regulating extracellular and intracellular pH in three-dimensional tumor cell growths. J. Biol. Chem. 284, 20299–20310.

Wykoff, C. C., Baseler, N. J., Watson, P. H., Turner, R. J., Pastorek, J., Sibstein, A., et al. (2000). Hypoxia-inducible expression of tumor-associated carbonic anhydrase. Cancer Res. 60, 7075–7083.

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