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High-Dose Radiation Increases Notch1 in Tumor Vasculature

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Abstract

Purpose: The aim of this study is to characterize the effects of high-dose radiation therapy (HDRT) on Notch signaling components of the tumor vasculature.

Methods and Materials: Human umbilical vein endothelial cells monolayers were exposed to different single fraction doses of irradiation; ribonucleic acid RNA was isolated and polymerase chain reaction was performed for Notch signaling components. The vascular response to radiation therapy was examined in a xenograft model of neuroblastoma. Tumors were treated with 0 Gy, 2 Gy, and 12 Gy single fraction doses and analyzed by double immunofluorescence staining for Notch1, Notch ligands Jagged1 and Dll4, and the endothelial cell (EC) marker endomucin. To assess the role of Notch in vivo, NGP xenograft tumors expressing Fc or Notch1-$_{1–24}$-decoy (a novel Notch inhibitor) were treated with 0 Gy and 12 Gy. Immunofluorescence staining for endomucin and endomucin/αSMA was performed to analyze the effect of combination treatment on tumor EC and endothelial-to-mesenchymal-transition (EndMT), respectively.

Results: In human umbilical vein endothelial cells monolayers doses ≥8 Gy increased expression of *NOTCH1, JAG1,* and Notch target genes *HEY1* and *HEY2* as early as 6 hours after irradiation. In vivo, 12 Gy significantly increased Notch1 and Jagged1 in tumor ECs compared with 0 Gy or 2 Gy after 72 hours. Combining HDRT with Notch inhibition using the Notch1-$_{1–24}$-decoy resulted in a greater loss of EC coverage of tumor vessels than HDRT alone at 6 hours and 72 hours post treatment. Notch inhibition reduced EndMT induced by HDRT, as indicated by diminished αSMA staining in ECs.

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Conclusions: HDRT induced Notch1 expression and increased Notch1 signaling in the endothelial component of tumor vasculature, which was not observed with lower doses. This increase in Notch1 activation might protect tumor vessels from HDRT induced damage and regulate EndMT process.

Summary

High-dose radiation (HDRT) not only has a direct cytotoxic effect on tumor cells but can also effect the tumor vasculature. Our study examined HDRT and Notch signaling in a xenograft model of neuroblastoma. We observed an increased Notch signaling in endothelial cells after HDRT. Notch1 inhibition augments the effect of HDRT on endothelial cell loss and reduces radiation-induced endothelial-to-mesenchymal transition.

Introduction

High-dose radiation therapy (HDRT), typically delivered as stereotactic radiosurgery or stereotactic ablative body radiation therapy, is increasingly used for both definitive and palliative treatment for a variety of adult and pediatric cancers.[1,2] Both stereotactic ablative body radiation therapy and stereotactic radiosurgery deliver extremely conformal high-dose radiation (≥8 Gy/fraction) in 1 to 5 fractions to targeted tumors, with rapid dose fall off to achieve acceptable radiation doses to adjacent normal tissues.[3,4] HDRT is highly effective clinically in controlling primary tumors and metastases to brain and lung by mainly targeting tumor cells; however, exact radiobiological mechanism of tumor control is still a subject of investigation.[5,6]

Although the major focus of the effects of this ablative technique has been on the tumor cell cytotoxicity, the biological impact of HDRT on the vasculature component of tumor microenvironment is not well understood. A number of studies have reported that, unlike conventional fractionated radiation doses of 1.8 to 2 Gy, HDRT with radiation doses of ≥10 Gy causes profound tumor vascular damage, resulting in reduced blood perfusion.[4] The HDRT induced vascular damage is proposed to be a result of endothelial cell (EC) apoptosis, a mechanism not observed at lower doses.[3,7] However, HDRT can also affect the tumor vasculature structure and function by other mechanisms. High-dose γ-irradiation causes a disruption of microvessels in 3-dimensional (3D) culture without appreciable EC apoptosis.[8] In vivo, treatment of xenograft tumors with 12 Gy decreases perfusion and disrupts tumor vasculature, accompanied by loss of ECs and pericytes.[9] Therefore, a better understanding of tumor vascular response is required to advance our knowledge of the biological impact of HDRT on tumors.

The Notch family of proteins consists of 4 trans-membrane receptors (Notch1–4) and 5 membrane-bound ligands (DLL1, DLL3, DLL4, JAG1, and JAG2).[10] In response to membrane-bound ligands, a Notch cell surface receptor undergoes 2 catalytic cleavages, by ADAM protease and γ-secretase, and releases its active cytoplasmic domain (NCD). NCD then translocates to the nucleus and, with the help of other coactivator proteins, induces target gene transcription.[10,11] Among Notch components, Notch1, DLL4, and JAG1 have well documented fundamental roles in the regulation of vessel formation, maturation, and stabilization.[12–14] DLL4-mediated Notch signaling inhibits the sprouting of endothelial tip

cells in growing blood vessels.[15,16] In contrast, JAG1 overexpression inhibits DLL4 signaling in ECs, increasing sprouting angiogenesis.[15] Notch1 and DLL4 are highly expressed in the tumor ECs of several cancers and have been significantly implicated in maintaining tumor vessel integrity and function by regulating EC survival.[14,17–19]

Current knowledge of the role of Notch signaling in the regulation of vascular response to HDRT is rudimentary. Conventional radiation dose fraction of 1.8 to 2 Gy has been shown to increase expression of Notch ligands JAG1 and target gene *Hey1* in microvascular ECs.[20] Additionally, Lui et al demonstrated that DLL4-Notch signaling blockade works synergistically with radiation to impair tumor growth by promoting nonfunctional tumor angiogenesis and extensive tumor necrosis, independent of tumor DLL4 expression.[21] Notch signaling has also been implicated in the regulation of the endothelial-to-mesenchymal transition (EndMT) process. Hey2 overexpression is linked with increased mesenchymal-like phenotype in human umbilical vein endothelial cells (HUVEC).[22]

In the current study, we investigated the effect of high-dose radiation on Notch signaling in the tumor vasculature. We report that HDRT induces Notch1 and Jagged1 expression in ECs and activates Notch1 signaling. Our findings demonstrate that combining Notch inhibition with HDRT significantly decreases endothelial coverage of tumor vessels and mesenchymal transition of ECs.

Methods and Materials

Cell culture

The human neuroblastoma NGP cell line was obtained from Garrett Brodeur, Children's Hospital of Philadelphia. HUVEC was purchased from Lonza.

Lentiviral transfection

NGP was stably transfected with Fc or $N1_{1–24}$-decoy lentiviral particles as described before.[23]

Xenograft model

All animal experiments were approved by the Institutional Animal Care and Use Committee. For the formation of intrarenal human xenograft tumors, 4 to 6-week-old female nude mice (Taconic) were anesthetized with ketamine (50–80 mg/kg) and xylazine (5–10 mg/kg), an incision made at the left flank, and 10^6 cells injected into the renal parenchyma.[14,23] Tumor growth was monitored by bioluminescence and ultrasound imaging.[14,22]

Tumor irradiation

Tumors (~1 gram) were randomly assigned to 0-Gy and 12-Gy groups. Mice were irradiated to the abdomen with a cesium 137ebased g-ray irradiator (JL Shepherd and Associates) with a dose rate of 1.28 Gy/min using a custom-built lead box for shielding other body parts.[8]

Double immunofluorescence staining and quantification

Paraffin tumor sections were subjected to double immunofluorescence staining by sequential method.[14] Primary antibodies used were endomucin (1:100; Santa Cruz Biotechnology), α-smooth muscle actin (αSMA; 1:100; Thermo Scientific), Notch1 (1:50; R&D Systems), Dll4 (1:50; R&D Systems), and Jagged1 (1:50; R&D Systems). AlexaFluor 488 and AlexaFluor 568 (Thermo Scientific) conjugates were used for fluorescence staining. For quantification, an average of 8 to 10 images, taken under 20x magnification, and 3 tumors per group were analyzed. Area (%) was determined using Fiji[24] using an arbitrary threshold applied to all images.

Polymerase chain reaction

Total ribonucleic acid (RNA) was isolated from HUVEC, after irradiation, using RNeasy Mini Kit (Qiagen). Complementary deoxyribonucleic acid was made using Super-ScriptTM III First Strand RT-PCR kit (Thermo Scientific) according to the manufacturer's protocol. Complementary deoxyribonucleic acids were amplified using primers specific for Notch components.[23] The primer sequences are given as follows:

Notch1 (5' GCA GAC TAT GCC TGC AGC TG 3' and 5' GCC ACA CTC GTT GAC ATC CTG 3');

Notch 3 (5' CGC CTG AGA ATG ATC ACT GCT TC 3' and 5' TCA CCC TTG GCC ATG TTC TTC 3')

Dll4 (5' CGG GTC ATC TGC AGT GAC AAC 3' and 5' AGT TGA GAT CTT GGT CAC AAA ACA G 3')

Jag 1 (5' GCT TGG ATC TGT TGC TTG GTG AC 3' and 5' ACT TTC CAA GTC TCT GTT GTC CTG 3')

ACTB (5' CGA GGC CCA GAG CAA GAG AG 3' and 5' CTC GTA GAT GGG CAC AGT GTG 3')

Immunoblot analysis

Immunoblot analyses were performed as previously described[25] using VE-Cadherin (1:500, R&D Systems), cleaved Notch1 (1:1000, Cell Signaling Technology), Jagged1 (1: 1000, R&D Systems), Hey2 (1:1000, Abcam), and β-Actin (1:1000, Cell Signaling Technology) antibodies.

Three-dimensional contrast-enhanced ultrasonography (3D-CEUS)

Three-dimensional microbubble contrast-enhanced ultrasonography (3D-CEUS) was performed[8] using a VEVO2100 imaging system (VisualSonics) with a 30-MHz transducer before and 6 hours after irradiation. Fifty mL of microbubbles between 3 and 4 mm (Advanced Microbubbles Laboratories, LLC) at a concentration of 2×10^9 MB/mL was injected into mice tail vein. Tumor enhancement before and after microbubbles injection was measured and the percentage of agent was calculated as a measure of tumor relative blood volume (rBV). For each mouse percentage (%) change in tumor rBV was calculated at each time point.

Cell viability assay

HUVEC viability was measured using CCK-8 assay kit (Dojindo Molecular Technologies). Briefly, 1×10^4 cells were seeded per well of a 96-well plate in 100 uL growth media and then irradiated with 0 Gy, 2 Gy, and 12 Gy single fraction doses. Seventy-two hours after irradiation 10 uL of CCK-8 solution was added and incubated for 2 hours. Absorbance was measured at 450 nm using a microplate reader.

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay

Deoxyribonucleic acid (DNA) fragmentation during apoptotic cell death was evaluated by TUNEL assay using HT Titer TACS Apoptosis Detection Kit (R&D System) following manufacturer's instructions. Briefly, 72 hours after irradiation, HUVECs, in 96 well plate, were fixed with 3.7% buffered formaldehyde and were permeabilized with 100% methanol for 20 minutes. Cells were then incubated with terminal deoxynucleotidyl transferase labeling reaction mix for 1 hour and stopped with terminal deoxynucleotidyl transferase stop buffer. Strep-HRP solution was added for 30 minutes and the reaction was stopped with 2 N HCl. Absorbance was measured at 450 nm.

Statistical analysis

All statistical analysis was performed using Prism5 software (GraphPad). Normal data were analyzed by unpaired *t* test or analysis of variation with post hoc analysis by Tukey's multiple comparison test. Graphs represent mean and standard deviation in all cases. The level of significance was considered at $P \leq .05$.

Results

HDRT activates Notch1 and increases Jagged1 in ECs in vitro

HUVECs were grown in monolayers and irradiated with 0 to 12 Gy single fraction doses. Twelve Gy reduced cell viability by 30% (Fig. E1A, available online at https://doi.org/10.1016/j.ijrobp.2019.11.010) and increased apoptotic cell death by 26% (Fig. E1B, available online at https://doi.org/10.1016/j.ijrobp.2019.11.010), compared with 0 Gy, at 72 hours after irradiation. Irradiated HUVECs were analyzed for changes in the expression of Notch signaling components by reverse transcriptase-polymerase chain reaction. We found that 6 hours after irradiation, with doses 8 Gy and greater, there was increased expression of *NOTCH1* messenger ribonucleic acid (mRNA) (Fig. 1A), but low-dose irradiation (0–6 Gy) had no effect on *NOTCH1* mRNA (Fig. 1A). Irradiation had no effect on the mRNA expression of *NOTCH2* and *NOTCH4* (data not shown) or *NOTCH3* (Fig. 1A). *JAGGED1* mRNA expression was found be to slightly increased at 6 hours and markedly increased at 72 hours after irradiation with doses ≥8 Gy, whereas *DLL4* showed no change at either time points (Fig. 1A). Concomitant with increased *NOTCH1* and *JAG1,* elevated levels of Notch target genes *HEY1* and *HEY2* were also observed in response to HDRT single fraction doses ≥8 Gy. We further performed immunoblot analysis to confirm HDRT effect on protein level. Consistent with mRNA expression pattern, doses 8 Gy and greater increased NOTCH1 protein level at 6 hours and 72 hours (Fig. 1B). No change in JAGGED1 level was noted at 6 hours after both low-dose and high-dose irradiation (Fig. 1B). JAGGED1 was highly

elevated at 72 hours after 12 Gy (Fig. 1B). Increased HEY2 protein level was observed after 12 Gy, at 6 hours, and doses ≥8 Gy (Fig. 1B) at 72 hours. These results demonstrate that HDRT single fraction doses ≤8 Gy rapidly increased expression of NOTCH1 and JAG1, at both mRNA and protein level, and Notch signaling in HUVECs.

HDRT induces Notch1 in tumor ECs in vivo

In an NGP xenograft model of neuroblastoma, a single 12 Gy fraction rapidly reduces tumor perfusion and the amount of endomucin (+) vessels.[8] To assess the effect of HDRT on Notch1 in tumor vasculature, we analyzed tumor tissues with double immunofluorescence staining for Notch1 and an EC marker endomucin. Twelve Gy single fraction dose significantly reduced amount of endomucin (+) EC, compared with 0 Gy ($P < .01$) and 2 Gy ($P < .01$), at 6 hours (Fig. 2A, 2C). However, in comparison to 0 Gy and 2 Gy, 12 Gy induced a marked increase in Notch1 in surviving ECs (Fig. 2A). Quantification of Notch1(+) vessels, normalized by total number of endomucin(+) vessels, showed that 66% of vessels were Notch1(+), 6 hours after treatment with 12 Gy, in contrast to only 18% and 12% of after 0 Gy and 2 Gy, respectively (Fig. 2B). Similarly, at 72 hours, 12 Gy significantly decreased amount of EC (Fig. 2D, 2F) and increased Notch1 expression (Fig. 2D), with 85% of the surviving tumor vessels were Notch1(+), in comparison to 9% and 8% with 0 and 2 Gy, respectively (Fig. 2D, 2E). These results show that HDRT diminished tumor vascularity and increased Notch1 expression in the remaining vessels.

HDRT increases tumor endothelial Jagged1 in vivo

We also analyzed the expression of 2 Notch ligands, Dll4 and Jagged1, in tumor vasculature. Dll4 and Jagged1 have been well documented for their roles in tumor vessel growth, maturation, and stabilization.[12–14] There was no change in Jagged1 staining observed at 6 hours after 2-Gy or 12-Gy doses (Fig. E2, available online at https://doi.org/10.1016/j.ijrobp.2019.11.010). However, Jagged1 staining was significantly elevated and colocalized with endomucin (+) staining 72 hours after 12-Gy irradiation (Fig. 3A); ~24% of the remaining endomucin (+) vessels colocalized with Jagged1 (+) vessels after 12 Gy compared with 0 Gy (~0.05%, $P < .05$) and 2 Gy (~0.06%, $P < .05$) at 72 hours (Fig. 3B). There was no change in Dll4 staining observed among treatment groups both at 6 hours (data not shown) and 72 hours (Fig. E3, available online at https://doi.org/10.1016/j.ijrobp.2019.11.010). These data showed that HDRT single fraction dose induced Jagged1 expression in tumor ECs while Dll4 remained unchanged.

HDRT induces Notch effector Hey2 expression in tumor ECs

We immunostained for Hey2 transcription factor, a down-stream effector of Notch signaling, in the irradiated tumor to assess Notch signaling activity. Hey2 staining was not detected in the nuclei of endomucin (+) tumor ECs irradiated with 0 Gy and 2 Gy at 6 hours (Fig. E4A, available online at https://doi.org/10.1016/j.ijrobp.2019.11.010) and 72 hours (Fig. 4A), respectively. An increased nuclear Hey2 staining was observed in the endomucin (+) vessels at 6 hours (Fig. E4A, available online at https://doi.org/10.1016/j.ijrobp.2019.11.010) and 72 hours (Fig. 4A) post 12 Gy. We also noticed an increased tumor cell nuclear Hey2 staining at both time points. Quantification of Hey2(+) EC (endomucin +) nuclei, normalized by total number of EC (endomucin +) nuclei in the vessels demonstrated that ~40% of EC nuclei

showed increased Hey2 staining, 6 hours post 12 Gy (Fig. E4B, available online at https://doi.org/10.1016/j.ijrobp.2019.11.010), in contrast to only ~7% and ~9% after 0 Gy and 2 Gy, respectively. Quantification further showed, by 72 hours, nuclear Hey2 staining was observed in ~60% of EC in the vessel after irradiation with 12 Gy (Fig. 4B). These data demonstrated that HDRT single fraction dose increased endothelial Hey2 expression and induced endothelial Notch signaling.

Notch1 inhibition augments effect of HDRT on endothelial cell loss

We have previously demonstrated that Notch blockade disrupted tumor vasculature and inhibited endothelial-pericyte interaction.[14] Given the critical effect of HDRT on tumor vessels and increased Notch1 expression in remaining vessels, we hypothesize that combining Notch inhibition with HDRT would lead to greater inhibition of tumor vasculature than either treatment alone. To test this hypothesis the neuroblastoma cell line NGP expressing $N1_{1–24}$-decoy or Fc (control) was implanted into the left kidney of nude mice and treated with 0 Gy and 12 Gy HDRT. $N1_{1–24}$-decoy, a soluble pan-Notch antagonist, is composed of epidermal growth factor repeats 1 to 24 and blocks autocrine or paracrine Notch activation by both DLL and JAGGED ligands.[14,23] Single or combination treatments had no effect on tumor weight (data not shown). To investigate the effect of combination treatments on vessel functionality, we measured tumor blood volume by 3D-CEUS before and 6 hours after irradiation. Although $N1_{1–24}$-decoy treatment had no effect on rBV, Fc + 12 Gy tumors exhibited 40% decrease of rBV compared with 0 Gy treatment. There was a 68% decrease in rBV in $N1_{1–24}$-decoy + 12 Gy tumors at 6 hours post irradiation (Fig. E5, available online at https://doi.org/10.1016/j.ijrobp.2019.11.010). Therefore, combining Notch with HDRT showed greatest reduction of tumor perfusion and inhibited vessel functionality.

We further assessed the effect of combining Notch inhibition with HDRT on tumor EC component of vasculature by IHC for endomucin. At 6 hours single $N1_{1–24}$-decoy and 12 Gy treatments significantly reduced ECs compared with control (Fc + 0Gy) and there was a paucity of ECs in the $N1_{1–24}$-decoy + 12 Gy treated tumors (Fig. 5A). Quantification of area fraction of ECs further showed there was a significant reduction of ECs in $N1_{1–24}$-decoy + 12 Gy tumors (mean ± SD, 0.3875 ± 0.3465) compared with Fc + 0 Gy (mean 1.65 ± SD, ± 1.28), $N1_{1–24}$-decoy + 0 Gy (mean ± SD, 1.07 ± 0.73) and Fc + 12 Gy (mean ± SD, 0.97 ± 0.79) tumors (Fig. 5B). By 72 hours significantly fewer ECs were also noted after $N1_{1–24}$-decoy 12 Gy (% endomucin, mean ± SD, 0.24 ± 0.21) than Fc + 0Gy (mean ± SD, 1.43 ± 0.94), $N1_{1–24}$-decoy + 0 Gy (mean ± SD, 0.70 ± 0.58), and Fc + 12 Gy (mean ± SD, 0.67 ± 0.44) (Fig. 5C, 5D). Therefore, a greater loss of tumor ECs where observed in combining Notch inhibition with HDRT suggesting that Notch inhibition radio sensitized tumor vasculature.

Notch inhibition reduces radiation-induced endothelial-to-mesenchymal transition (EndMT)

HDRT have been previously implicated in EndMT process of tumor vasculature after radiation therapy.[22,26] To investigate whether HDRT induces EndMT, HUVECs monolayers were exposed to 0, 2, 8, and 12 Gy irradiation and cell lysates were analyzed for VE-

Cadherin (an endothelial marker) by immunoblot analyses. We detected no changes in VE-Cadherin at 72 hours after 2 Gy; however, reduced VE-Cadherin was observed after 8 Gy and 12 Gy (Fig. 6A). Concomitant with VE-Cadherin reduction, elevated level of cleaved NOTCH1 expression, an indicator of activated Notch1 signaling, after 8 and 12 Gy was noticed (Fig. 6A). These suggested EndMT was associated with increased Notch1 signaling activation in irradiated ECs in vitro.

Inspired by our findings in vitro, we next analyzed EndMT in NGP tumor vasculature by coimmunostaining for endomucin and αSMA (mesenchymal marker). Increased colocalization of endomucin and αSMA was observed at 72 hours 12 Gy postirradiation (Fig. 6B), compared with nonirradiated tumors, indicating transition of ECs. Interestingly, Notch inhibition reduced colocalizing αSMA (+) endomucin (+) staining in $N1_{1–24}$-decoy + 12 Gy tumors. Quantification showed that whereas ~4% and ~3% of endomucin (+) vessels expressed αSMA, fafter Fc + 0Gy and $N1_{1–24}$-decoy + 0 Gy treatments, respectively, ~30% of the surviving vessels were αSMA (+) after Fc 12 Gy (Fig. 6C). Notch inhibition reduced αSMA staining in surviving vessels as ~4% of the vessels were αSMA (+) in $N1_{1–24}$-decoy + 12 Gy tumors (Fig. 6C). Collectively, these data indicated HDRT induced EndMT and Notch blockade inhibited this process.

Discussion

Our understanding of tumor vascular Notch response to HDRT is unclear. In the current study employing in vivo mice model and in vitro EC culture model we have shown that HDRT single fraction doses, 8 Gy and higher, resulted in induction of Notch1 and Jagged 1 expression and activation of Notch1 signaling in ECs. Our results are consistent with prior findings of increased Notch1 signaling in microvascular ECs after 5-Gy and 10-Gy single fraction doses.[27] Notch signaling is known to play an important role in the regulation of vessel integrity and function.[12,13] However, Notch response to irradiation in the vascular component of tumor microenvironment is not well documented. Here we document that Notch1 and Jagged1 in tumor ECs mediate early vascular response to HDRT.

We observe similar HDRT effect on endothelial Notch in HUVECs, in vitro, and tumor ECs, in vivo. Like HUVECs, tumor ECs exhibited increased Notch1 expression at 6 hours and 72 hours and increased Jagged1 expression at 72 hours after single fraction high-dose irradiation. Increased Hey2 expression, indicative of increased Notch activation, was also observed in HUVECs and tumor ECs at both time points after 12 Gy. Low-dose irradiation (2 Gy) had no effect on Notch in both experimental settings. Therefore, our in vitro findings correspond with in vivo results and demonstrate a novel function of endothelial Notch.

An important finding of our study is that remaining tumor vessels, survived post 12-Gy treatments, had an increased endothelial Notch1 and Jagged1 expression in vivo. These suggest Notch signaling is involved during recovery and regrowth period after radiation therapy. Notch1 had been implicated in the regulation of EC viability.[14] There is also increasing evidence that the Notch signaling pathway plays a critical role in radiation resistance of cells. HDRT has been shown to upregulate Notch2, Notch3, and Jagged1 in breast cancer cells, and Notch inhibition after radiation therapy resulted in the reduction of

breast cancer stem cells.[28] Notch signaling has also been implicated in DNA damage response. Notch1 inactivated ataxia-telengiectasia mutated kinase and inhibited radiation induced DNA damage response.[29,30] Notch inhibition had also been shown to enhance the radiation response by decreasing cancer stem cells proliferation and self-renewal in tumor explants in the presence of endothelial stroma suggesting a critical role of Notch signaling linking endothelial cells to cancer cells.[30] Lui et al demonstrated that DLL4-Notch signaling blockade works synergistically with radiation to impair tumor growth by promoting nonfunctional tumor angiogenesis and extensive tumor necrosis.[21] Our study demonstrated that combining Notch inhibition with HDRT decreased tumor perfusion and EC coverage more than HDRT alone. Therefore, we speculate that increased Notch protects ECs from HDRT-induced damages and vascular dysfunction. It remains to be illustrated the mechanism of EC loss after combined treatment of Notch inhibition and HDRT.

It has previously been shown that 20 Gy dose induces EndMT, in lung tumors in mice, which leads to abnormal vasculature development during tumor regrowth after radiation therapy.[26] EndMT promotes formation of cancer-associated fibroblast which facilitates tumor cell extravasation.[31] HDRT-induced EndMT also reduces radiotherapeutic efficacy by stimulating the proliferation of dormant hypoxic CD44v6 + cancer-stem cells and affecting immune response by modulating M1/M2 tumor-associated macrophage polarization.[26] We demonstrated that 12 Gy induced EndMT in tumor ECs and inhibition of Notch reduced this transition. Thus, targeting tumor EndMT by anti-Notch therapy may enhance radiation therapy efficacy.

In the present study, we have used $N1_{1–24}$-decoy that is a secreted molecule and can act as antagonists of both DLL and JAGGED ligands in the neighboring cells.[14,23] We observed endothelial Jagged1 expression is increased after HDRT in cell culture in vitro and in tumors in vivo; however, no change in Dll4 expression is observed. Over-expression of activated Notch1 domain and Jagged1 induces EndMT in adult human macrovascular and microvascular cells.[32] Given that increased Notch1 and Jagged1 expression were associated with decreased VE-Cadherin at 72 hours post treatment, we speculate that Jagged1-Notch1 signaling axis regulates radiation-induced tumor EndMT.

Conclusions

Our results show that HDRT in single fraction doses induces Notch1 in tumor endothelial cell component of tumor vasculature in a manner not observed at lower irradiation doses. Notch inhibition radiosensitizes tumor vasculature to HDRT and inhibits EndMT. Our findings will assist in expanding our current understanding of the radiobiology of tumor vascular response to HDRT and encourage the prospect of employing stereotactic body radiation therapy with anti-Notch therapy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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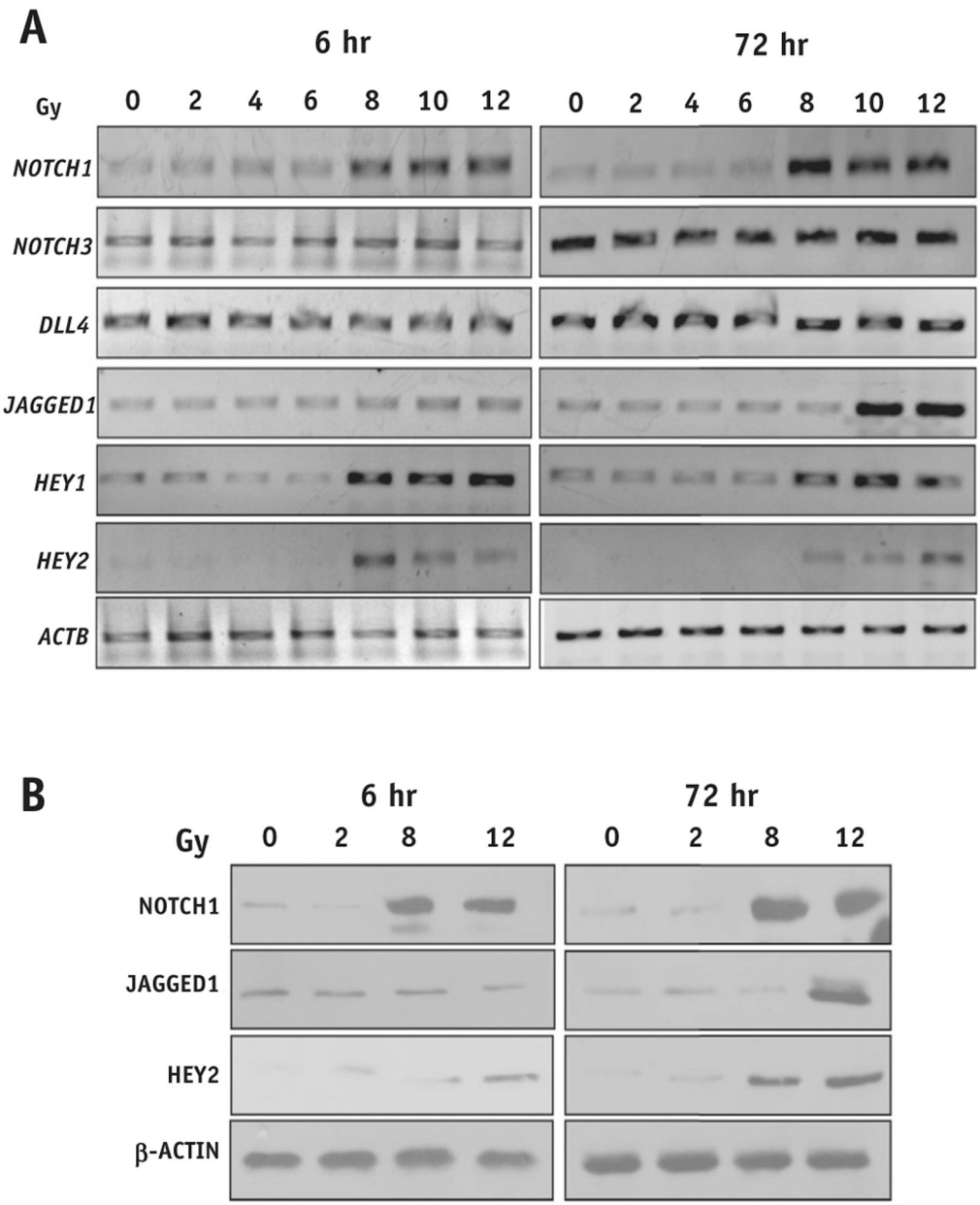

Fig. 1.

High-dose radiation therapy induces NOTCH1 and JAGGED1 signaling in human umbilical vein endothelial cells (HUVEC). (A) Reverse transcriptase-polymerase chain reaction analysis of messenger ribonucleic acid (mRNA) of Notch components after irradiation. HUVEC monolayers were treated with different single fraction irradiation doses. RNA was isolated at 6 hours and 72 hours, reverse transcribed and polymerase chain reaction was performed for Notch receptors and ligands. Increased *NOTCH1* message was observed at 6 hours and 72 hours after irradiation with doses ≥8 Gy. *JAGGED1* mRNA level was slightly increased, at 6 hours after ≥8 Gy, and markedly increased at 72 hours after ≥10 Gy doses. Concomitant with *NOTCH1* mRNA increase, target genes HEY1 and HEY2 mRNA levels were also elevated. *ACTB* (*β-Actin*) was used as internal control. (B) Immunoblot analyses

for Notch components after irradiation. HUVEC were exposed to different irradiation doses for 6 hours and 72 hours and cell lysates were immunobloted with NOTCH1, JAGGED1, HEY2, and β-Actin antibodies. Irradiation doses ≥8 Gy increased NOTCH1 protein expression at 6 hours and 72 hours. JAGGED1 level is elevated only at 72 hours after 12 Gy. HEY2 was also markedly increased at 6 hours and 72 hours after irradiation with 12 Gy.

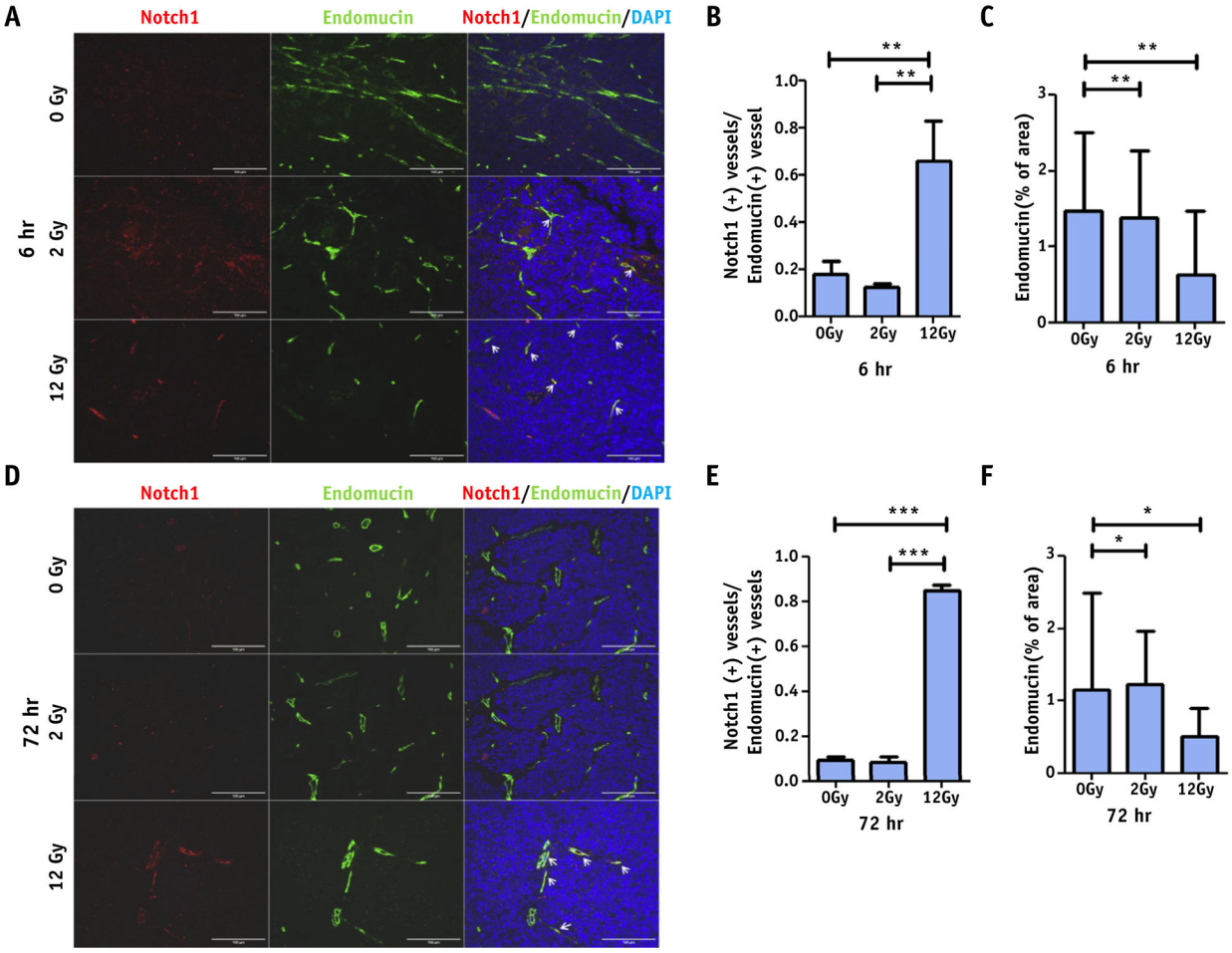

Fig. 2.

High-dose radiation increases Notch1 expression in tumor endothelial cells. Representative images double immunofluorescence staining for Notch1 (red) and endomucin (green) of NGP tumors at 6 hours (A) and 72 hours (D) after 0 Gy, 2 Gy, and 12 Gy single fraction dose treatment. Increased Notch1 immunostaining colocalizing (arrows) with endomucin staining was observed at 6 hours and 72 hours after 12 Gy. Quantification of Notch1 (+) vessels, normalized by total number of endomucin (+) vessels, per field (magnification, 20x) in NGP tumors at 6 hours (B) and 72 hours (E) after 12 Gy. Quantification of amount of endomucin, as % area, per field (magnification, 20X) at 6 hours (C) and 72 hours (F). **P* < .05, ***P* < .01, ****P* < .001. Mean ± SD. Bar, 100 μm.

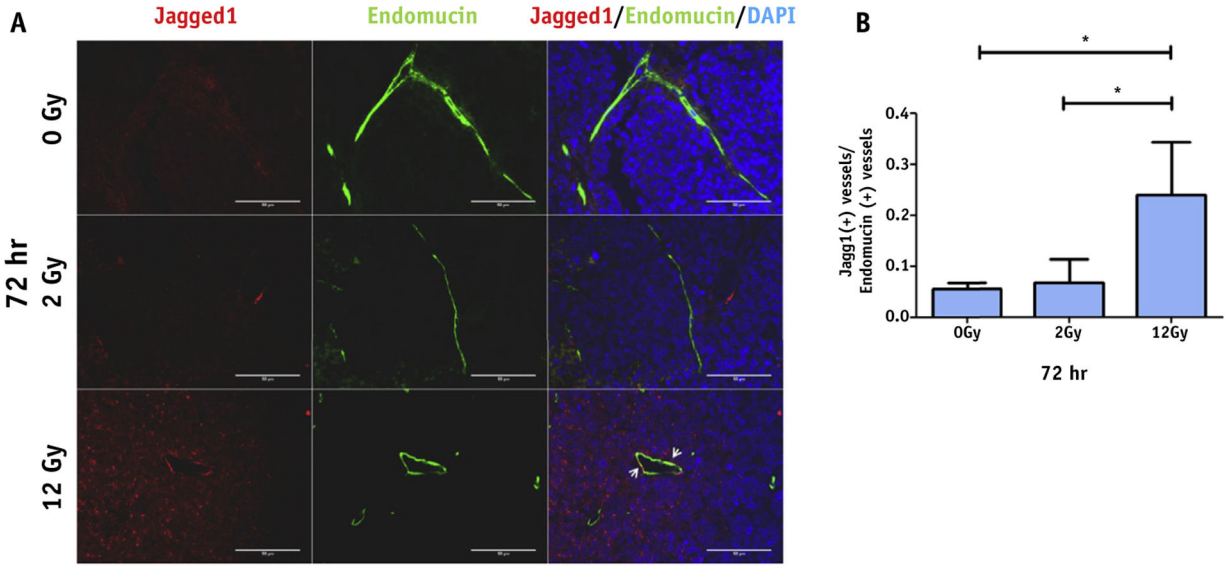

Fig. 3.

High-dose radiation induces endothelial Jagged1 expression in tumor vessel in vivo. (A) Double immunofluorescence staining of Jagged1 (red) colocalizing (arrows) with endomucin (green) in tumors at 72 hours after 0 Gy, 2 Gy, and 12 Gy. (B) Quantification of Jagged1 (+) vessels, normalized by endomucin (+) vessels, in tumors per field (magnification, 20X). Mean ± SD. *$P < .05$. Bar, 50 μm.

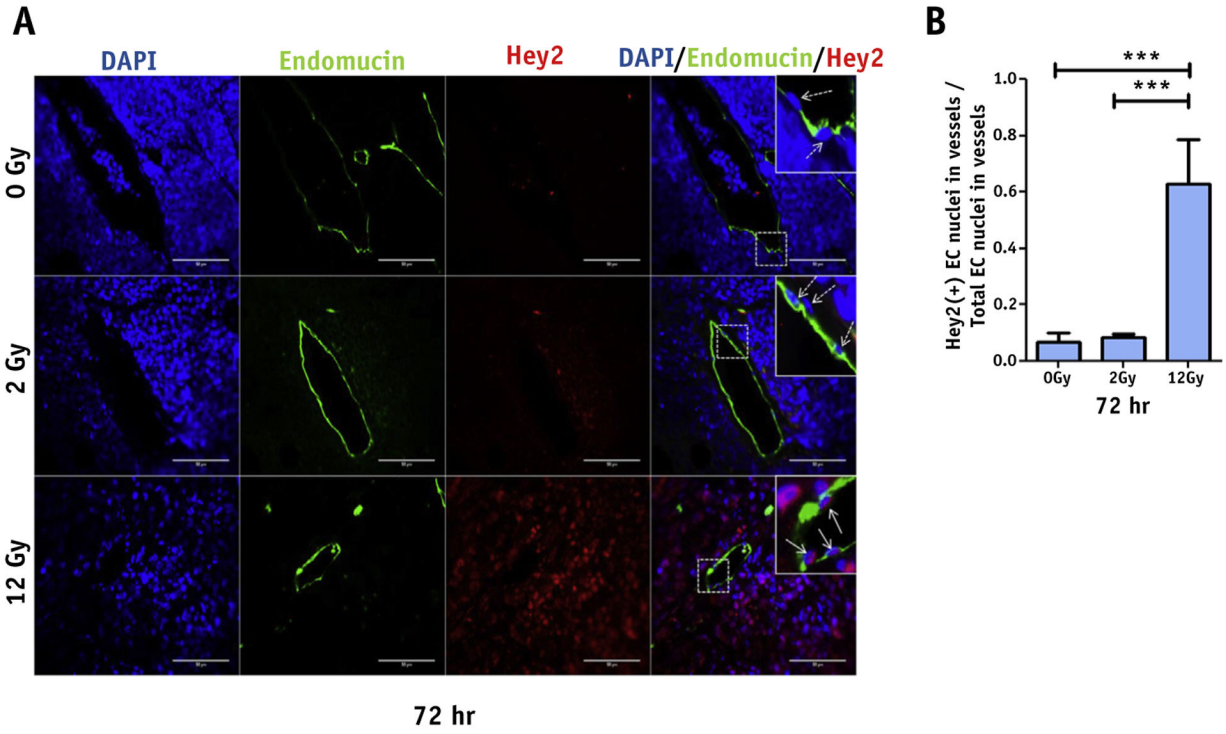

Fig. 4.

High-dose radiation increases nuclear Hey2 expression in tumor endothelial cell at 72 hours (A) Triple immunofluorescence of DAPI (blue), endomucin (green), and Hey2 (red). Very little Hey2 staining was detected in the DAPI (+) nuclei (dotted arrow) of endomucin (+) endothelial cells (ECs) at 72 hours after 0 Gy and 2 Gy. 12 Gy single fraction dose increased Hey2 staining (solid arrow) in the nuclei of endomucin (+) EC in vessel. Hey2 was localized in the nuclei and endomucin staining was observed around the nuclei. Insets represent the same portion (dotted box) of the tumor. Bar, 50μm. (B) Quantification of Hey2(+) nuclei of endomucin (+) EC in the vessels, normalized by total number of DAPI(+) nuclei in the endomucin (+) EC in vessels, in tumors per field (magnification, 40X). Mean ± SD. ***P <.001.

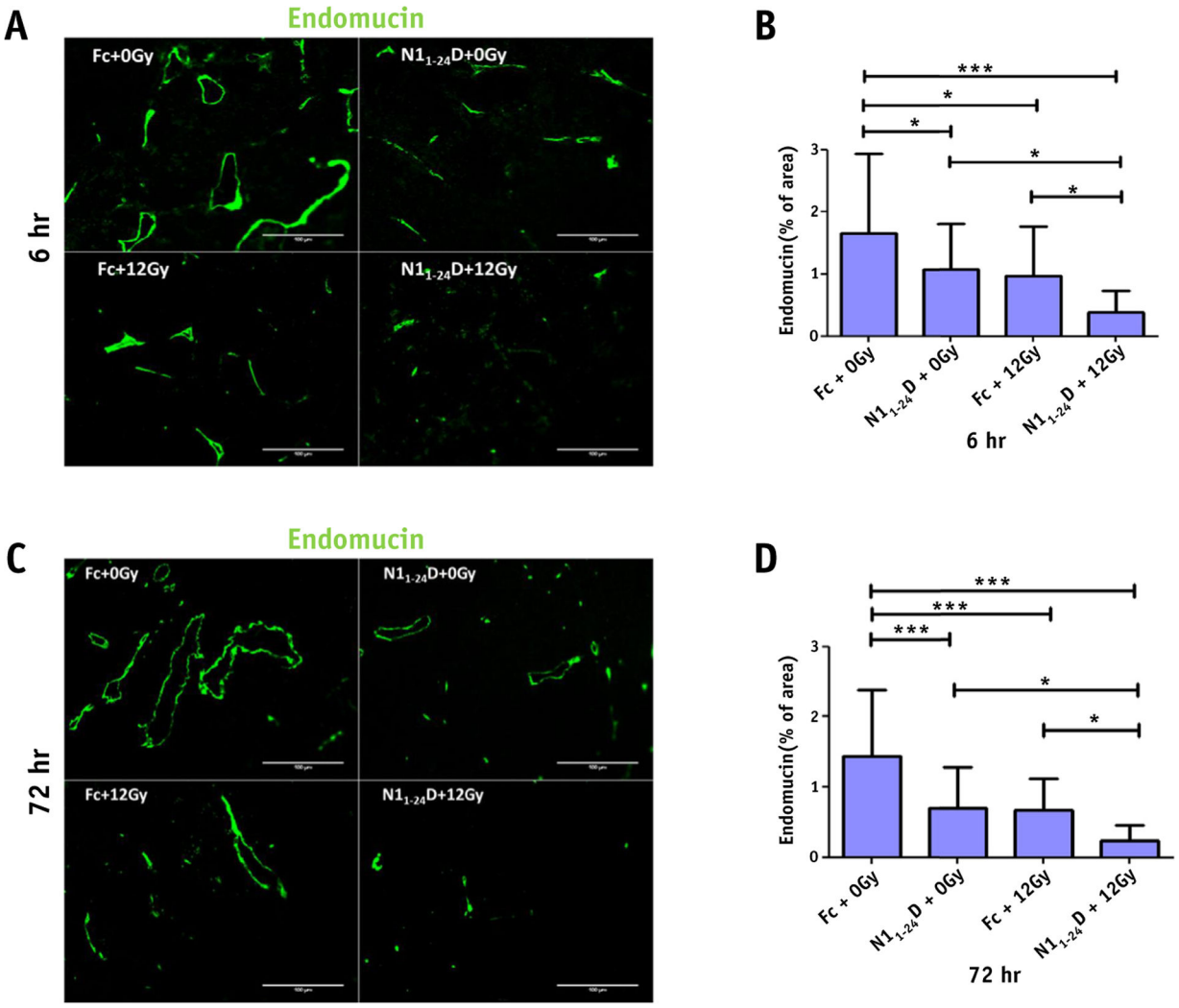

Fig. 5.

Combining Notch inhibition with high-dose radiation therapy decreases endothelial cell coverage of tumor. (A) Representative images of endomucin immunofluorescence (green) of Fc + 0 Gy control N1$_{1–24}$-decoy + 0 Gy, Fc + 12 Gy, and N1$_{1–24}$-decoy + 12 Gy tumors (NGP xenograft) at 6 hours. Bar = 100 μm. (B) Quantification of endomucin immunofluorescence, at 6 hours, as area percentage. N1$_{1–24}$-decoy + 0 Gy and Fc + 12 Gy tumors had decreased endomucin than Fc + 0 Gy control. N1$_{1–24}$-decoy + 12 Gy tumors had an additive decrease in endomucin area fraction. (C) Immunofluorescence detection of endomucin in tumors at 72 hours. (D) Quantification of endomucin, as area percentage, at 72 hours also shows greater loss of endothelial cells post N1$_{1–24}$-decoy + 12 Gy treatments. Mean ± SD. $^{**}P < .05$, $^{***}P < .001$. Bar, 100μm.

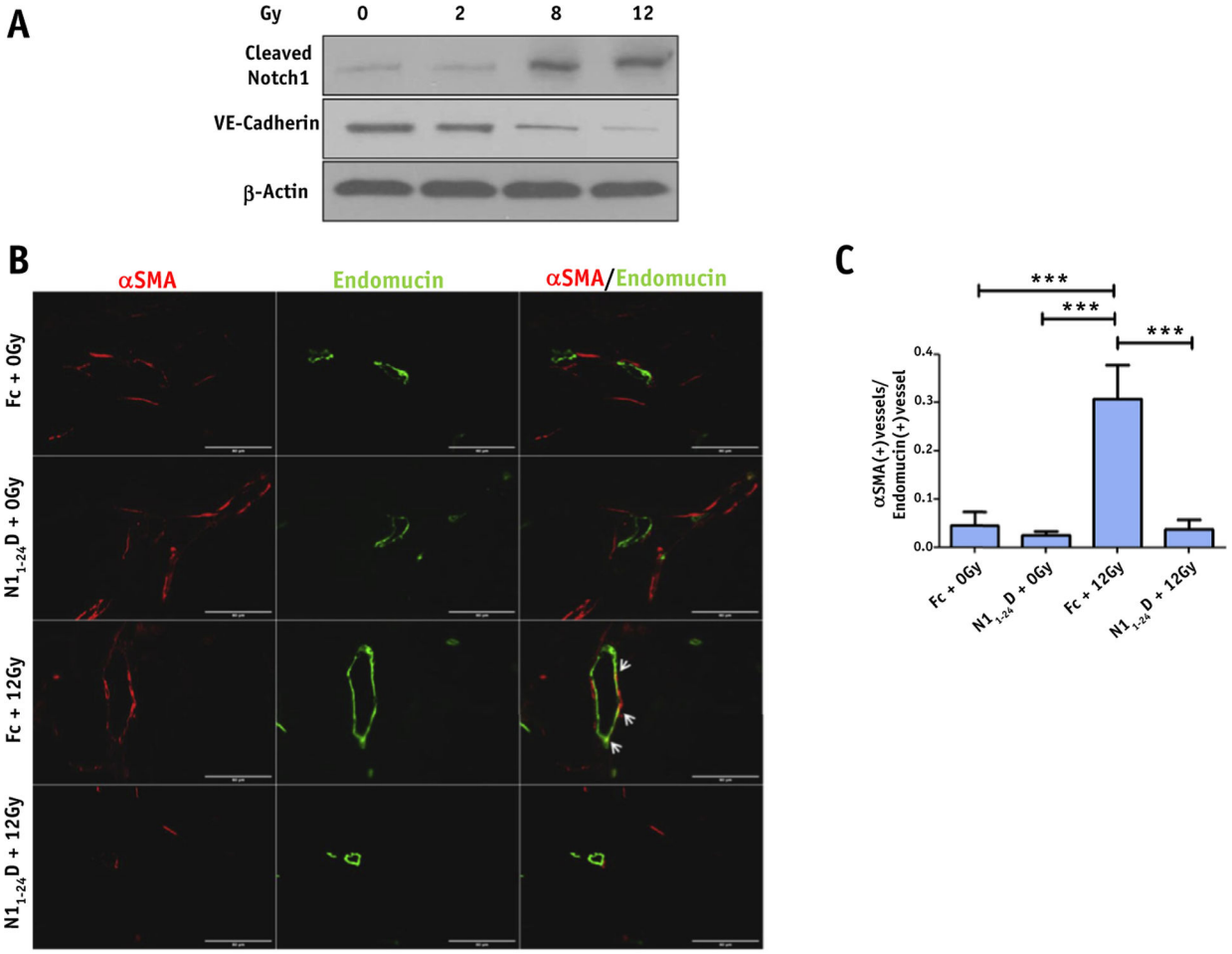

Fig. 6.

Increased Notch1 activity is associated with endothelial-to-mesenchymal transition (A) human umbilical vein endothelial cells monolayers were exposed to different doses of irradiation and cells lysate were analyzed at 72 hours by immunoblot for VE-Cadherin and cleaved NOTCH1. 8 Gy and 12 Gy doses, but not 2 Gy, increased cleaved NOTCH1 and decreased VE-Cadherin. (B) Notch inhibition reduced radiation-induced EndMT in tumor vasculature. Double immunofluorescence of endomucin and αSMA in tumors. 12 Gy increased endomucin and αSMA colocalization (arrows) in NGP tumors at 72 hours post 12 Gy. Combining high-dose radiation therapy with $N1_{1–24}$-decoy treatment decreased colocalization. Bar, 50 μm. (C) Quantification of αSMA (+) vessels, normalized by endomucin (+) vessels, in tumors per field (magnification, 20X). Mean ± SD. ***P<.001.