The American Journal of Pathology, Vol. 174, No. 1, January 2009

DOI: 10.2353/ajpath.2009.080576

Musculoskeletal Pathology

β-Catenin Is a Mediator of the Response of Fibroblasts to Irradiation

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Radiation causes soft tissue complications that include fibrosis and deficient wound healing. β-Catenin, a key component in the canonical Wnt-signaling pathway, is activated in fibrotic processes and wound repair and, as such, could play a role in mediating cellular responses to irradiation. β-Catenin can form a transcriptionally active complex with members of the Tcf family. A reporter mouse model, in addition to human cell cultures, was used to demonstrate that ionizing radiation activates β-catenin-mediated, Tcf-dependent transcription both *in vitro* and *in vivo*. Furthermore, radiation activates β-catenin via a Wnt-mediated mechanism, as in the presence of dickkopf-1, an inhibitor of Wnt receptor activation, β-catenin levels did not increase after irradiation. Fibroblast cell cultures were derived from mice expressing either null or stabilized β-catenin alleles. Cells expressing stabilized β-catenin alleles had a higher proliferation rate and formed more colony-forming units than wild-type or null cells after irradiation. Wound healing was studied in these same mice after irradiation. There was a positive correlation between the tensile strength of the wound, the expression levels of type 1 collagen in the skin, and β-catenin levels. Mice treated with lithium showed increased β-catenin levels and increased wound strength. β-Catenin mediates the effects of ionizing radiation in fibroblasts, and its modulation has the potential to decrease the severity of radiation-induced soft tissue complications. ***(Am J Pathol 2009, 174:248–255; DOI: 10.2353/ajpath.2009.080576)***

Fibroblast dysfunction related to irradiation is a common occurrence and can be a limiting factor in the success of radiotherapy. For instance, wound healing is often impaired in an irradiated field. Fibroblasts proliferate to re-establish the mechanical properties of the wounded skin during wound repair. Radiation affects cell-cycle progression in fibroblasts, resulting in a delay in the entry of cells into S phase and mitosis, and ultimately death of some cells.[1] This causes a decreased number of fibroblasts in the wound, resulting in insufficient mechanical strength.[2] In contrast, radiation can also induce differentiation of fibroblasts and increased collagen production, which may contribute to an increase in wound strength and play a role in the induction of fibrosis in the soft tissues surrounding a primary radiation focal point.[3] Fibrosis can limit the amount of radiation applied to a given anatomical region. The mechanism by which irradiation causes tissue fibrosis is not fully elucidated, but is thought to involve the expression of cytokines and growth factors resulting in excessive collagen deposition, fibroblast proliferation, and abnormal remodeling of the extracellular matrix.[4]

β-Catenin-mediated Tcf-dependent transcription is activated during wound repair, regulating wound size primarily by increasing the size of and number of cells in the fibroblast compartment. β-Catenin is a key mediator in the canonical Wnt signaling pathway. In the absence of an appropriate Wnt ligand, β-catenin is phosphorylated by a complex consisting of multiple proteins including glycogen synthase kinase-3β (GSK-3β), targeting the protein for ubiquitin-mediated degradation. In the presence of an appropriate Wnt ligand, however, stabilized β-catenin can translocate to the nucleus and bind to the members of the Tcf-Lef family of transcription factors to form a transcriptional activation complex, inducing the expression of target genes.[5–7] Some of these target genes in mesenchymal cells encode for extracellular

Supported by the Canadian Institutes for Health Research (MOP 15136) and the Canada Research Chair Program (to B.A.A.).

Accepted for publication September 22, 2008.

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structural proteins and enzymes that play a role in extracellular matrix remodeling.[8] Indeed, hyperplastic wounds and fibroproliferative tumors, such as aggressive fibromatosis or desmoid tumors, are associated with elevated β-catenin protein levels.[9–11] This signaling pathway is also activated in soft tissue fibrosis associated with aging.[12]

The regulation of β-catenin by irradiation has been demonstrated in epithelial cells, where the intracellular localization is altered in a way that inhibits β-catenin-mediated signaling.[13–15] In epithelial cells, activation of β-catenin-mediated signaling usually results in improved cell survival after irradiation.[13,15–17] Because canonical Wnt signaling is activated in fibrotic processes and plays a crucial role in normal wound repair, it is possible that β-catenin signaling is a key component mediating the effect of irradiation on fibroblast dysregulation. Therefore, in this study we tested the hypothesis that β-catenin partially mediates the response of fibroblasts to ionizing radiation.

Materials and Methods

Transgenic Mice

Tcf reporter mice express a transgene containing three consensus Tcf-binding motifs and a c-fos minimal promoter linked to a lacZ reporter.[10] β-Catenin deficiency was studied using *Catnb*^{floxdel} mice.[18] These mice have loxP sites flanking exons 2 and 6 of β-catenin. Recombination on exposure to cre-recombinase gives rise to a null allele for β-catenin. β-Catenin stabilization was investigated using *Catnb*$^{lox(ex3)}$ mice, which contain loxP sequences flanking exon 3. Deletion by cre-recombinase gives rise to a mutant β-catenin lacking the phosphorylation sites required for degradation but otherwise exhibiting normal biological function.[19] Recombination was accomplished *in vivo* or *in vitro* using an adenovirus-expressing cre-recombinase at a concentration of 1×10^8 pfu in 50 μl injected both intraperitoneally and subcutaneously into mice 4 days before wounding, or in cell culture media 24 hours before study as previously reported.[10,11] The expected changes in β-catenin after recombination were verified using Western blot analysis.

Cell Cultures and in Vitro Irradiation

Primary fibroblast or keratinocyte cell cultures were established using previously described techniques.[9,20–22] Cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA). A normal human lung fibroblast cell line (MRC-5), normal breast fibroblast cell line (HTB-125), breast carcinoma epithelial cell line (HTB-126), a hepatocarcinoma epithelial cell line (HepG), and a keratinocyte cell line (CCD 1106 KERTr) were cultured under recommended conditions. A gammacell 40 irradiator (MDS Nordion, Ottawa, Canada) was used to irradiate cell cultures with a single 5Gy dose of Cs-gamma radiation, a dose previously shown to cause radiation-induced changes in fibroblasts.[16,23–26]

Lithium, Cycloheximide, and Dickkopf-1 Treatments

To activate β-catenin-mediated signaling, cells were treated with 50 mmol/L LiCl (Sigma, St. Louis, MO), a dose known to activate β-catenin.[9,27] Fifty mmol/L NaCl was used as a control. Dickkopf-1 (Dkk-1) was used to inhibit Wnt receptor activation. Cells were treated with 400 multiplicity of infection Ad Dkk1-HA,[28] or a control empty virus that did not express Dkk-1. Only small numbers of cells require infection to cause effects as Dkk1 is secreted. Identical techniques as previously reported were used.[9,27] Twenty mg/ml of cycloheximide (Sigma) was used to block translational activity. Western analysis was used to measure changes in β-catenin protein levels.

Mouse Wound Studies

The hind limbs of mice were exposed to 10 Gy of irradiation through a 100-kVp X-ray source collimated by a 2.5-cm-diameter lead ring. In mice examined for the effects on wound repair, 3 weeks after irradiation, either a 1-cm-long full-thickness skin incision was made in the center of the irradiated site and closed with a single surgical staple left in place for 1 week, or a 4-mm-diameter full-thickness punch biopsy was performed. Three weeks after wounding, the skin was harvested. In the case of the linear incision, the wound was divided into three equal width longitudinal strips perpendicular to the direction of the incision. The center strip was used for mechanical testing. Work to failure was measured using a standard mechanical testing machine with a custom-designed jig to hold the tissue strip as previously reported.[29] The full-thickness wound was harvested and processed as previously reported for protein analysis.[11] A dissecting microscope was used to ensure that only the actual wound tissue was used for this purpose. For mice treated with lithium, sterile 0.6 mol/L LiCl (or 0.6 mol/L NaCl as a control) was added to the drinking water at a final concentration of 0.02 mol/L. This oral dosage (200 mg/kg per day, a dose giving plasma levels comparable with the level used to treat humans with bipolar illness), was previously reported to be effective in mice.[5]

Protein and RNA Analysis

Protein extracts were separated by electrophoresis on a polyacrylamide gel and transferred onto a nitrocellulose membrane. The membranes were probed with a primary antibody that was detected using a secondary antibody and ECL reagents (Amersham, Piscataway, NJ). Primary antibodies included a rabbit polyclonal anti-β-catenin IgG (Upstate Cell Signaling Solutions, Charlottesville, VA), a goat-polyclonal anti-p53 IgG (Santa Cruz Biotechnology, Santa Cruz, CA), a mouse-monoclonal total and phosphorylated GSK3β (BD Transduction Laboratories, Lexington, KY), and actin as a loading control. Densitometry was performed on the developed films using Alpha-EaseFC Software Version 4 (Alpha Innotech Corp., San Leandro, CA), and band intensities for β-catenin were

normalized to actin as a loading control. To quantify Tcf-dependent transcriptional activation, β-gal assays were performed using a colorimetric assay on protein lysates that were extracted as described previously.[30] A 96-well enzyme-linked immunosorbent assay plate reader was used to measure β-gal activity. The normalized value was then graphed as relative β-gal activity. RNA was extracted from cryopreserved tissues using Trizol reagents. Reverse transcriptase-polymerase chain reaction (RT-PCR) was performed using primers and conditions as previously reported.[31–33]

β-Catenin Immunofluorescence

Fibroblasts were grown overnight on glass coverslips in 35-mm six-well cultured plates. Cells were irradiated, treated with 50 mmol/L LiCl, or kept under control conditions. They were fixed in methanol 6 hours after treatment. Cells were incubated with mouse monoclonal anti-β-catenin IgM (1:100, BD Biosciences, San Jose, CA), which was detected using anti-mouse fluorescein IgM (Vector Laboratories, Burlingame, CA). Fluorescence microscopy was used to detect the intracellular location of β-catenin.

Proliferation Assay and Colony-Forming Unit Assay

After exposure to irradiation, bromodeoxyuridine (BrdU, 1:100; Roche, Indianapolis, IN) was added overnight. Cells were washed with phosphate-buffered saline, fixed with 95% ethanol and then with 4% paraformaldehyde, and incubated with anti-BrdU antibody (1:80; DAKO, Carpinteria, CA) overnight at 4°C. BrdU incorporation was then detected using Alkaline Phosphatase ABC reagent (SK-6100, Vector) and by addition of the Vector substrate (Vector substrate kit SK-5200). The percentage of BrdU-positive cells was identified using microscopy and comparing the BrdU-positive cells with the total number of cells. Ten high-powered fields from one end to the other of the slide were observed. Colony-forming assays were performed by plating 500 fibroblasts overnight in 35-mm six-well plates. They were irradiated the following day and kept in a cell culture incubator for 12 days. Cells were stained with 0.1% methylene blue, and colonies were identified as containing a cluster of at least 50 cells. The total number of colonies in each well was counted.

Statistical Analysis

Each *in vitro* experiment was performed at least five times, and each animal experiment at least six times. The means, SD, and 95% confidence intervals were calculated. The Student's *t*-test was used to compare results between samples.

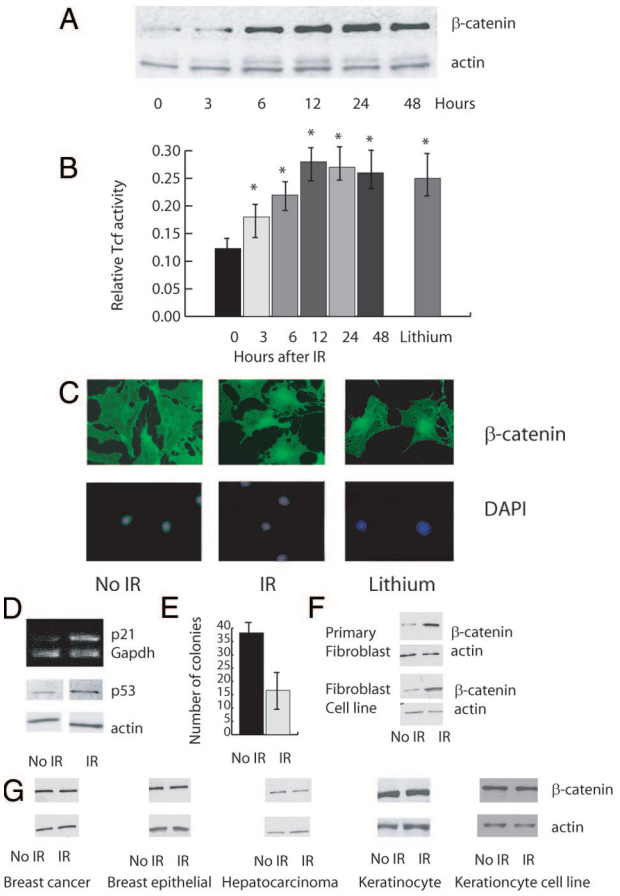

Figure 1. β-Catenin-mediated Tcf-dependent transcription is activated after irradiation of fibroblasts. **A:** A representative Western analysis showing an increase in β-catenin protein level starting 1 hour after irradiation. **B:** β-Galactosidase assays showing an increase in Tcf-dependent transcriptional activation with irradiation. Data are given as means and 95% confidence intervals for relative Tcf activity. $^*P < 0.05$ compared to the zero time point. **C:** Subcellular location of β-catenin as examined using immunohistochemistry. There is nuclear localization of β-catenin after irradiation or with lithium treatment as a positive control. **Top:** Cells stained with an antibody to β-catenin. **Bottom:** Cells stained with DAPI to identify the nuclei. **D:** The expression of both p21 and p53 increases after irradiation. p21 data are given as RT-PCR and p53 as a Western blot. Gapdh and actin are shown as loading controls. **E:** There is a decrease in the number of colony-forming units after irradiation. Data are given as means and 95% confidence intervals. **F:** Primary human skin fibroblast cell cultures, as well as a primary human fibroblast cell line, show an elevated β-catenin protein level. **G:** Epithelial cell lines (labeled breast cancer, breast epithelial, and hepatocarcinoma) and a keratinocyte cell line (labeled keratinocyte cell line) show no substantial changes in β-catenin after exposure to irradiation. A primary keratinocyte cell culture (labeled keratinocyte) also showed no substantial change in β-catenin protein level after irradiation.

Results

Irradiation of Fibroblasts Activates β-Catenin-Mediated Tcf-Dependent Transcription

To determine the effect of irradiation on β-catenin-mediated Tcf-dependent transcription in fibroblasts, we initially examined primary cell cultures established from Tcf reporter mice.[10] After 5 Gy of irradiation, Western analysis showed an increase in β-catenin protein level starting a few hours after irradiation (Figure 1A). β-Galactosidase assays were performed to measure the level of Tcf-dependent transcriptional activation, and this correlated with the elevated

β-catenin levels (Figure 1B). The increase in protein level and Tcf transcriptional activity was close to that which occurred when cells were treated with lithium, an agent known to activate β-catenin-mediated signaling in mesenchymal cells.[34] The subcellular location of β-catenin was examined using immunohistochemistry, and nuclear localization of β-catenin was observed in irradiated cells (Figure 1C).

To verify that the cells were responding as expected to irradiation, parameters that are known to change in fibroblasts after irradiation were examined. The expression of both p21 and p53 increased after irradiation (Figure 1D).[35] The dose of 5 Gy irradiation also resulted in a decrease in the formation of colonies when plated as 500 cells in 100-cm^2 culture flasks (Figure 1E).

Primary human skin fibroblast cell cultures, as well as a primary human fibroblast cell line (CRL 1502, ATCC), were irradiated in an identical manner and examined for β-catenin protein. Similar to our observations in murine fibroblasts, an elevated β-catenin protein level was observed after irradiation (Figure 1F). A number of epithelial cell lines were also subjected to irradiation and analyzed for β-catenin protein levels: a breast epithelial cell line (HTB-125, ATTC), a breast carcinoma epithelial cell line (HTB-126, ATCC), a hepatocarcinoma epithelial cell line (HepG2, ATCC), and a keratinocyte cell line (CCD 1106 KERTr, ATCC). In addition, we observed the effect of irradiation in a primary keratinocyte cell culture established from wild-type mice as previously reported.[9] We observed no substantial changes in β-catenin after exposure to irradiation in these cell types (Figure 1G). These results, in concert with previous studies on the effect of radiation on epithelial cells,[13,36] suggest that although β-catenin-mediated Tcf-dependent transcription is regulated by irradiation in only select epithelial cell types, its activation is a common finding in fibroblast cell cultures.

β-Catenin Protein Level and Tcf-Dependent Transcriptional Activation Increases after Irradiation during Wound Repair in Mice

During the proliferative phase of wound repair, β-catenin-mediated Tcf-dependent transcription is activated.[9,37] To examine if irradiation alters β-catenin-mediated Tcf-dependent transcription *in vivo*, full thickness skin wounds in the hind limbs of Tcf reporter mice were examined 3 weeks after irradiation using previously reported techniques.[29] Radiation increased β-catenin-mediated Tcf-dependent transcription in healing wounds 3 weeks after injury, during the proliferative phase of healing (Figure 2, A and B). This demonstrates that irradiation will also activate β-catenin-mediated Tcf-dependent transcription *in vivo*.

Irradiation Modulates β-Catenin through a Wnt-Dependent Mechanism

To determine whether the increase in β-catenin protein was attributable to an increase in expression of β-catenin itself, we examined its expression at the mRNA level. The level of expression of the gene was lower in irradiated cells, and as such, the elevation in β-catenin protein after irradiation is not attributable to an increase in β-catenin gene expression (Figure 3A). To examine if the elevation

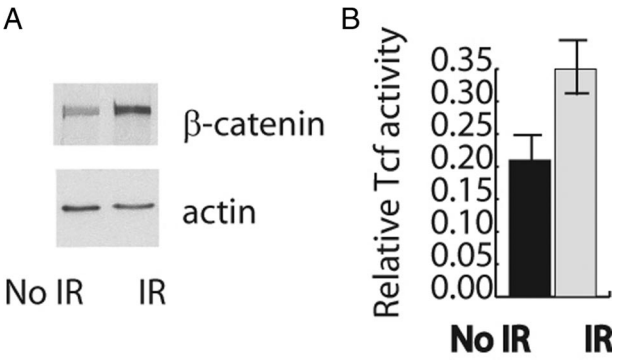

Figure 2. β-Catenin protein level and Tcf-dependent transcriptional activation during wound repair in mice after irradiation. **A:** Western analysis from mouse wound tissues. IR is from irradiated tissues and non-IR is from wounds that were not radiated. There is an increase in β-catenin protein level after irradiation. The blots were stripped and reprobed using an antibody for actin as a loading control. **B:** β-Galactosidase assays show an increase in Tcf-dependent transcriptional activation with irradiation. Data are given as means and 95% confidence intervals for relative activity. There is a significant ($P < 0.05$) difference between cells after irradiation and control cells.

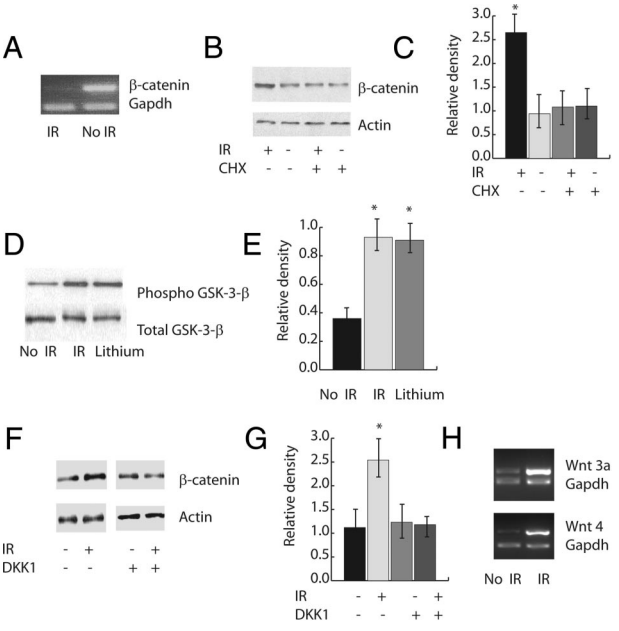

Figure 3. Irradiation modulates β-catenin through a Wnt-dependent mechanism. **A:** The level of expression of β-catenin is lower in irradiated cells, as detected using RT-PCR (lanes labeled IR are from irradiated cells). **B** and **C:** Treatment with cycloheximide inhibits the elevation of β-catenin after irradiation. **B:** Representative Western analysis. **C:** Relative density of the β-catenin compared to control protein as detected using densitometry. *$P < 0.05$ compared to nonirradiated cells without cycloheximide treatment. **D** and **E:** After irradiation there is an increase in phospho-Ser-9-GSK3β levels similar to that seen in cells treated with lithium. Western analysis using a phospho-Ser-9-GSK3β antibody is shown in **D**. The blot was stripped and reprobed with an antibody to total GSK3β. **E:** Relative level of Ser-9-GSK3β to total GSK3β as measured using densitometry. *$P < 0.05$ compared to nonirradiated cells. **F** and **G:** Treatment with Dkk-1 abolishes the increase in β-catenin protein levels. **F:** Representative Western analysis. **G:** Relative level of β-catenin compared to control protein as determined using densitometry. *$P < 0.05$ compared to nonirradiated cells without Dkk-1 treatment. **H:** There is a higher level of expression of Wnt3a and Wnt 4 after irradiation in fibroblasts as detected using RT-PCR.

in β-catenin protein was attributable to a translation-dependent mechanism, primary Tcf reporter fibroblasts were irradiated in the presence of the protein synthesis inhibitor cycloheximide. Cells were irradiated, and protein was analyzed by Western analysis for β-catenin. Cycloheximide suppressed the elevation in β-catenin after irradiation (Figure 3, B and C).

One family of proteins that can regulate β-catenin-mediated Tcf-dependent transcription is Wnt ligands. They act through a multiprotein complex that regulates phosphorylation of serine and threonine residues near the amino terminus of β-catenin.[7] GSK-3β acts to regulate serine and threonine β-catenin phosphorylation in this process, and GSK-3β phosphorylation was recently shown to be regulated by irradiation.[38] To examine this possibility, we first observed the phosphorylation status of GSK-3β. After irradiation, we found an increase in phospho-Ser-9-GSK3β levels similar to that seen in cells treated with 50 mmol/L LiCl (Figure 3, D and E). We then infected cells with an adenovirus construct containing Dickkopf-1 (Dkk-1), which will inhibit Wnt ligands from activating the canonical Wnt pathway.[28] Primary fibroblasts from Tcf reporter mice were treated with the adenovirus expressing Dkk-1 or a control virus.[27] Treatment with Dkk-1 abolished the observed increase in β-catenin-mediated signaling after irradiation (Figure 3, F and G). Treatment with Wnt3A-conditioned media was used as a control to verify that Dkk-1 treatment would inhibit canonical Wnt signaling activation. Taken together, these results suggest that the increase in β-catenin after irradiation is caused by elevated expression of Wnt ligands leading to reduced protein degradation. To determine whether irradiation would activate Wnt expression, RT-PCR was used to determine the level of expression of Wnt ligands. There was a more than a threefold increase in expression of Wnt3a and Wnt4 after irradiation in the fibroblast cell cultures (Figure 3H).

β-Catenin Regulates the Proliferation Rate and Clonogenic Survival of Irradiated Fibroblasts

To determine the functional implications of β-catenin in irradiated fibroblasts, cells from mice expressing conditional stabilized and null alleles activated by expression of cre-recombinase were studied.[9] Primary fibroblast cultures were derived from mice expressing null ($Catnb^{floxdel}$) alleles[18] or stabilized ($Catnb^{lox(ex3)}$) alleles.[19] The expected changes in β-catenin levels were confirmed using Western analysis, with the expected smaller size of the protein band from cells expressing the $Catnb^{lox(ex3)}$ alleles identified (Figure 4A). Recombination of cells expressing the null alleles did not completely abolish β-catenin protein level because recombination does not occur in every cell. Previous studies show recombination not in all cells, but in more than two-thirds of cells.[9] Cells were then irradiated with 5 Gy, BrdU was added overnight, and immunohistochemistry was used to identify the proportion of cells that incorporated BrdU. Irradiated cell cultures had fewer cells staining for BrdU, and

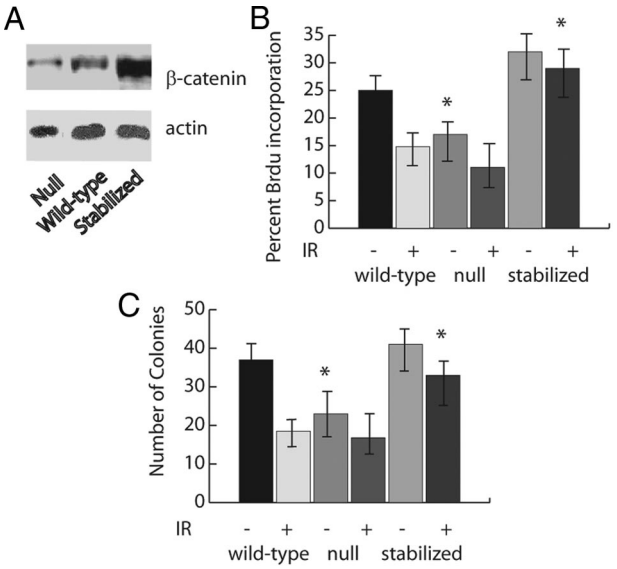

Figure 4. β-Catenin regulates the proliferation rate and clonogenic survival of irradiated fibroblasts. **A:** The expected changes in β-catenin levels are confirmed using Western analysis, with the expected smaller size of the protein band from cells expressing the $Catnb^{lox(ex3)}$ alleles identified. **B:** Proliferation as measured using BrdU incorporation. The proportion of cells that incorporate BrdU is shown as means and 95% confidence intervals. $^*P < 0.05$ from the same radiation exposure in wild-type cells. There is a significant difference between radiated and nonirradiated wild-type cells, but not between radiated and nonirradiated β-catenin-null or stabilized cells. **C:** Number of colonies formed after irradiation. Data are shown as means and 95% confidence intervals. Cell cultures from mice expressing $Catnb^{lox(ex3)}$ alleles have a greater proliferation rate and more colony-forming units than wild-type controls. $^*P < 0.05$ from the same irradiation exposure in wild-type cells. There is a significant difference between irradiated and nonirradiated wild-type cells, but not between irradiated and nonirradiated β-catenin-null or stabilized cells.

β-catenin-stabilized cells had a larger number of BrdU cells (Figure 4B).

Because radiation impairs clonogenic survival of fibroblasts,[39,40] we examined this parameter in fibroblasts expressing wild-type, $Catnb^{floxdel}$, or $Catnb^{lox(ex3)}$ alleles. There was a substantial decline in the number of colonies that developed when cells were irradiated. In cells that were not irradiated, there was only a small difference in the number of colonies between fibroblasts expressing wild-type, $Catnb^{floxdel}$, or $Catnb^{lox(ex3)}$ alleles. In contrast, β-catenin levels regulated the numbers of colonies that formed after irradiation, with fibroblasts expressing $Catnb^{lox(ex3)}$ (stabilized β-catenin) alleles forming a larger number of colonies than did fibroblasts expressing wild-type alleles (Figure 4C).

β-Catenin Regulates Changes Associated with Irradiation in Type 1 Collagen Expression in Skin and the Ultimate Tensile Strength of Healing Wounds

To determine whether β-catenin has a functional role in fibroblasts *in vivo*, we examined its role in wound repair and the expression of type 1 collagen in skin. Radiation is responsible for decrease in wound strength. It is also causes skin fibrosis, a condition associated with increased expression of extracellular matrix components

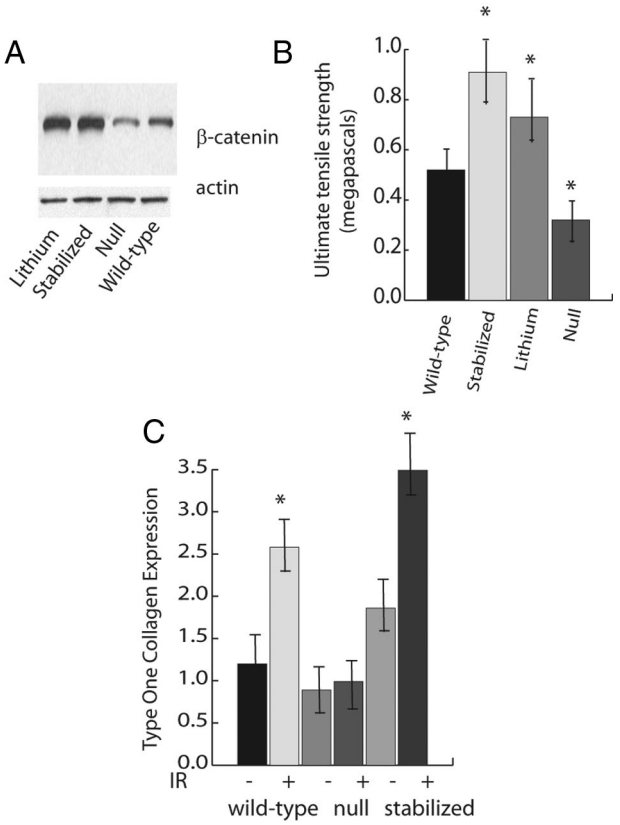

Figure 5. β-Catenin regulates the ultimate tensile strength of healing wounds in mice and the ability of irradiation to induce collagen expression. **A:** The expected changes in β-catenin levels were confirmed using Western analysis, with the expected smaller size of the protein band from cells expressing the $Catnb^{lox(ex3)}$ alleles identified. **B:** Ultimate tensile strength in megapascals shown as means and 95% confidence intervals, showing an increase in strength when β-catenin protein is stabilized and a decrease when null alleles are expressed. $^*P < 0.05$ from the nonirradiated wounds. **C:** Expression of type I collagen relative to *Gapdh* in normal unwounded skin exposed to irradiation in mice. $^*P < 0.05$ from the nonirradiated skin. There is an increase in expression of type 1 collagen in mice expressing wild-type or stabilized β-catenin alleles, but not in mice expressing null alleles.

such as type 1 collagen. β-Catenin has the potential to mediate cell functions in both these processes.[9,10,30] Wound healing was observed in mice expressing wild-type, $Catnb^{floxdel}$ (stabilized), or $Catnb^{lox(ex3)}$ (null) alleles (Figure 5A). Linear wounds were generated, and ultimate tensile strength (in megapascals, MPa) determined after irradiation when examined 3 weeks after wounding as previously reported.[29] In mice expressing $Catnb^{lox(ex3)}$ alleles, there was an increase in ultimate tensile strength after irradiation, whereas there was a decrease in strength in wounds expressing null alleles (Figure 5B). To determine whether lithium, which increases β-catenin levels in mesenchymal cells,[5,31] would have a similar effect as mice expressing the $Catnb^{lox(ex3)}$ allele, we treated mice with lithium, and examined work to failure. Lithium treatment resulted in an increase work to failure in irradiated wounds (Figure 5B). This shows a functional role for β-catenin in regulating wound strength in irradiated wounds *in vivo*. Intriguingly, this also suggests that lithium treatment, a therapeutic agent that is already in use in patients, could be used to improve the strength of wound repair in irradiated wounds.

The relative expression of type 1 collagen expression was measured in unwounded skin in the same mice. There was an increase in expression after irradiation in wild-type mice; however, there was no change in expression in mice expressing null alleles of β-catenin (Figure 5C). Mice expressing the stabilized alleles of β-catenin showed an increase in type 1 collagen expression with irradiation, and had a higher level of expression than wild-type mice. The lack of increase in expression of type 1 collagen in mice expressing null alleles suggests that β-catenin is an important mediator in radiation-induced fibrosis.

Discussion

In this study, we investigated the role of β-catenin as a mediator of ionizing radiation in fibroblasts and during wound repair. Ionizing radiation activates β-catenin protein-mediated Tcf-dependent transcriptional activity in fibroblasts through a Wnt ligand-dependent process. Using cells from mice expressing conditional null and knockout alleles of β-catenin, we found that β-catenin acts to maintain cell viability in irradiated fibroblasts.

The response of β-catenin to irradiation has been studied in a variety of epithelial cell types, and these studies primarily show changes resulting in a decrease in β-catenin-mediated Tcf-dependent transitional activity.[17] This effect is also demonstrated *in vivo*, in which irradiation causes a drastic loss of β-catenin staining in the gastrointestinal epithelium.[41] In contrast to these studies, we found an increase in β-catenin protein abundance and transcriptional activity after irradiation in both mouse and human fibroblast cell cultures, as well as during wound healing *in vivo*. These data suggest a different response to irradiation in mesenchymal cell types than in many epithelial cell types.

β-Catenin acts to promote the survival of irradiated fibroblasts. The notion that β-catenin promotes cell survival after irradiation is supported by studies showing that Wnt ligands promote survival in other cell types after irradiation, for instance in head and neck cancers and in mammary progenitor cells.[15,17] These data suggest that the difference in regulation of β-catenin in response to irradiation between different cell types could in part be responsible for variability in the response to irradiation. Because β-catenin acts to increase proliferation rate and maintain cell viability, its activation by irradiation in fibroblasts likely acts to make this cell type relatively resistant to irradiation.

Our data suggest that Wnt ligand activation causes increased β-catenin signaling after irradiation. Because Wnt ligands regulate β-catenin via GSK3-β activity,[5] this mechanism is supported by recent data showing that irradiation can regulate GSK3β activity in mesenchymal cells,[38] and data from array analysis showing up-regulation of Wnt genes after irradiation in fibroblasts.[42] The expression of other proteins are up-regulated by irradiation, such as p21,[35] and it is possible that a similar

mechanism is responsible for the increase in expression of Wnt ligands. Although p53 can regulate β-catenin-mediated signaling, it is not likely to play a role in the up-regulation of β-catenin in fibroblasts, as it is reported to inhibit β-catenin-mediated Tcf-dependent transcriptional activity.[43–45] Because Wnt ligands are secreted proteins that have effects on surrounding cells, it is possible that the activation of Wnt signaling by fibroblasts has local effects in other situations. For instance, fibroblasts often surround neoplastic epithelial cells and Wnt ligands activated by irradiation in fibroblasts may alter the viability of such epithelial neoplastic cells during irradiation.

To determine whether the regulation of β-catenin has functional implications *in vivo*, we examined wound healing and the expression of type 1 collagen after radiotherapy. Detrimental effects of irradiation on these parameters are thought to primarily relate to fibroblast dysregulation.[4,29,46] We found that β-catenin positively regulates the strength of healing irradiated wounds, suggesting that the increased activity of β-catenin-mediated signaling alters cell viability and gene expression, perhaps of extracellular matrix components. These data suggest a positive functional role for β-catenin signaling in activation. In contrast, fibrosis after irradiation is a troublesome side effect.[4] Recent studies suggest that canonical Wnt signaling contributes to fibrosis associated with aging,[12] and in hyperplastic wounds.[11,37] β-Catenin levels correlated with the expression of type 1 collagen in skin, suggesting that it also plays a role in radiation-induced fibrosis. Although other signaling pathways, such as transforming growth factor-β, are also shown to be activated in radiation-induced fibrosis,[47] the effect of transforming growth factor-β on wound healing is mediated by β-catenin,[9,30] and a similar process might occur in radiation-induced fibrosis. Thus there might be a balance in β-catenin activation between a detrimental effect causing soft tissue fibrosis and a beneficial effect improving wound repair. This also raises the intriguing possibility that modulating β-catenin with pharmacological agents could be used to reduce the incidence of certain radiation-induced side effects. For instance, lithium can be used to activate β-catenin signaling,[9] and our data shows that it improves wound strength after irradiation. Because lithium is already approved for use in patients, this therapeutic approach could be used to improve healing when surgery is required after radiation therapy.

Acknowledgment

We thank Dr. Calvin Kuo (Stanford University, Stanford, CA) for providing us with the Dkk-1 adenovirus.

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