Wnt/β-catenin mediates radiation resistance of Sca1$^+$ progenitors in an immortalized mammary gland cell line

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Summary

The COMMA-Dβ-geo cell line has been shown to contain a permanent subpopulation of progenitor cells that are enriched in outgrowth potential. Using the COMMA-Dβ-geo cell line as a model, we sought to study the radioresistance of mammary progenitor cells. Using the putative progenitor cell marker stem cell antigen 1 (Sca1), we were able to isolate a discrete subpopulation of Sca1$^+$ multipotent cells from the immortalized COMMA-Dβ-geo murine mammary cell line. At a clinically relevant dose, the Sca1$^+$ cells were resistant to radiation (2 Gy). Sca1$^+$ cells contained fewer γ-H2AX$^+$ DNA damage foci following irradiation, displayed higher levels of endogenous β-catenin, and selectively upregulated survivin after radiation. Expression of active β-catenin enhanced self-renewal preferentially in the Sca1$^+$ cells, whereas suppressing β-catenin with a dominant negative, β-engrailed, decreased self-renewal of the Sca1$^+$ cells. Understanding the radioresistance of progenitor cells may be an important factor in improving the treatment of cancer. The COMMA-Dβ-geo cell line may provide a useful model to study the signaling pathways that control mammary progenitor cell regulation.

Key words: β-catenin, Mammary gland, Stem, Progenitor, Cell line, COMMA-D, β-geo, Sca1

Introduction

Breast epithelium is capable of completely and functionally regenerating upon transplantation. This impressive renewal capacity has been ascribed to the function of a multipotent mammary gland stem/progenitor cell population that resides and persists throughout the mammary parenchyma. Elucidating the role of stem/progenitor cells in preneoplasia may be crucial to understanding the etiology of breast cancer, and may lead to better chemopreventative strategies. Mutations or epigenetic changes either in long-term stem cells or their immediate progeny, the transit amplifying multipotent progenitors, have been suggested to be the foundation of malignancy (Clarke and Fuller, 2006). However, functional studies of progenitor cells in a preneoplastic model have not been explored.

In normal development, the Wnt pathway has been shown to be important in stem cell survival and maintenance in early embryogenesis (Wang and Wynshaw-Boris, 2004), in maintaining the stem cell pool in the adult skin (Silva-Vargas et al., 2005) and intestinal epithelium (Pinto and Clevers, 2005b; Pinto et al., 2003), and in regulating hematopoietic stem cells in their niche environment (Rattis et al., 2004). Dysregulation of the Wnt/β-catenin pathways in stem cell regulation has been proposed to be one of the signaling pathways responsible for carcinogenesis in the hematopoietic system, the intestine and the epidermis (Reya and Clevers, 2005). Studies in chronic myelogenous leukemia indicate that the elevated levels of nuclear β-catenin exist in a minor population of progenitor cells, resulting in their enhanced capacity for self-renewal and increased leukemic potential (Jamieson et al., 2004). Further evidence of dysregulation of stem/progenitor cell self-renewal and maintenance by the Wnt/β-catenin pathway have been demonstrated in lung cancer, colorectal cancer and gastrointestinal cancer (Brabletz et al., 2005; He et al., 2005; Mishra et al., 2005; Reya and Clevers, 2005; Yardy and Brewster, 2005).

Stem cell antigen 1 (Sca1), a marker of hematopoietic stem cells, is one of the putative markers used to isolate and enrich for mammary gland progenitors. Previous studies by our laboratory have demonstrated that primary mammary gland progenitor cells isolated by using Sca1 lack differentiation markers, are enriched in label-retaining, relatively quiescent cells, and have enhanced outgrowth activity compared to the Sca1$^-$ cells (Welm et al., 2003).

The COMMA-D β-geo (CDβgeo) cell line is an immortalized cell line isolated from the parent COMMA-D (CD), preneoplastic, mouse mammary cell line by both transfection and selection using a dominant-selectable gene transfer vector and by limiting dilution (Deugnier et al., 2006). Recent studies by Deugnier et al. have demonstrated that the CDβgeo cells contain a permanent population of mouse mammary epithelial progenitor cells with basal characteristics,

which express putative stem cell markers such as stem cell antigen 1 (Sca1), CD24 and CD49f, among others. These investigators demonstrated that Sca1$^+$ cells have at least fourfold enrichment in outgrowth potential compared with Sca1$^-$ cells. In addition, they have observed that Sca1$^+$ cells were able to form 3D acini on Matrigel, whereas Sca1$^-$ cells grew poorly. Staining for cell-type-specific markers indicated that the 3D-acini contained keratin 5$^+$ (K5$^+$) basal cells, K8$^+$ luminal and α-SMA$^+$ myoepithelial cells within the acinus (Deugnier et al., 2006).

Herein we report that CD β-geo cells contain a minor population of Sca1$^+$ cells that are able to self-renew, and differentiate asymmetrically. In addition, we report that Sca1$^+$ cells sustain less DNA damage as indicated by the formation of γ-H2AX foci, contain higher levels of non-phosphorylated or active β-catenin, and are more resistant to radiation than Sca1$^-$ cells. To date, this is the first report where functional studies of progenitor cells have been carried out in a preneoplastic model to explore mechanisms of therapeutic resistance in the mammary gland.

Results

COMMA-D β-geo Sca1$^+$ cells are capable of self-renewal and expansion

The CDβgeo cell line, originally isolated from the midpregnant mammary gland of BALB/c mice, has been shown to retain stem and multipotent progenitor cell characteristics (Danielson et al., 1984; Deugnier et al., 2006). Because CDβgeo cells retain stem/progenitor cell properties in vivo, we sought to distinguish self-renewing cells from cells lacking this capacity. The CDβgeo cells are heterogeneous, containing Sca1$^+$ and Sca1$^-$ populations. Routinely 13-20% Sca1$^+$ cells were observed in culture (Fig. 1a). Clonogenic assays were used to determine the replicative competence of CDβgeo subpopulations. Sca1$^+$ cells form 47-fold more colonies than Sca1$^-$ cells (P<0.00002, Fig. 1b). Consistent with these data, Deugnier et al. have shown that the Sca1$^-$ cells are also unable to form attachments in Matrigel, or give rise to an organized spheroid structure (Deugnier et al., 2006).

The hallmark of stem and multipotent progenitor cells is the ability to asymmetrically self-renew thereby maintaining the progenitor pool and giving rise to differentiated daughter cells. To examine whether CDβgeo cells were able to divide asymmetrically, cells were first sorted into Sca1$^+$ and Sca1$^-$ subpopulations. The original Sca1$^+$ cells were able to give rise to both Sca1$^+$ (75±4.2%) and Sca1$^-$ cells (24±4.1%) (Fig. 1c). By contrast, the Sca1$^-$ cells were less bipotent, giving rise to predominantly Sca1$^-$ cells (94±1.5%) and very few Sca1$^+$ cells (6±1.45%) (Fig. 1d).

CDβgeo cells can self-renew in suspension culture

To further explore the self-renewing potential of CDβgeo progenitor cells, we used the mammosphere in vitro self-renewal assay described by Dontu et al. (Dontu et al., 2003), which is based upon the hypothesis that progenitor cells are able to survive in anchorage-independent conditions. By retroviral tagging experiments, Dontu et al. showed previously that mammospheres are clonally derived, and not a result of aggregation (Dontu et al., 2003). In addition, mammospheres are now known to comprise a heterogeneous population of cells, with multipotent mammary stem cells within the core,

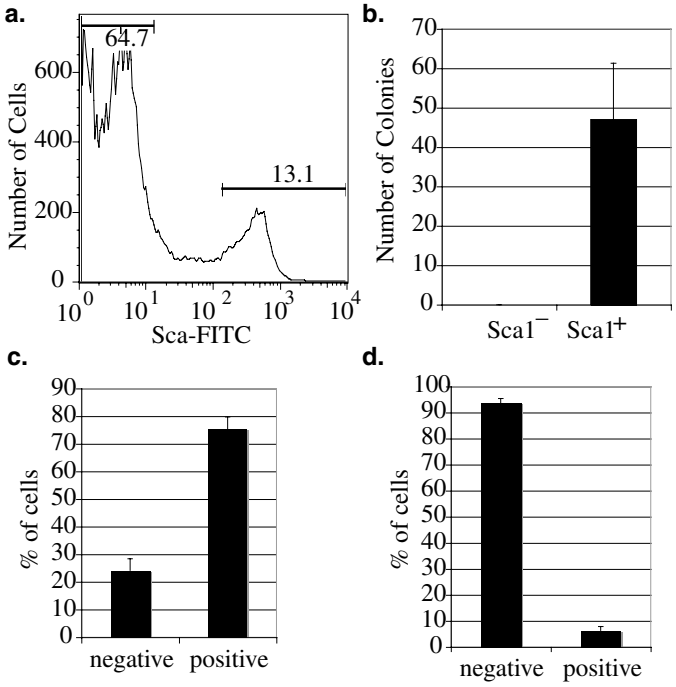

Fig. 1. CDβgeo Sca1$^+$ cells are capable of self-renewal and expansion. (a) CDβgeo cells were stained with FITC-conjugated antibody against Sca1, and analyzed by flow cytometry. Two populations can be distinguished based on Sca1 fluorescence: Sca1$^+$ 13.1%; and Sca1$^-$ 67.4%. (b) Cells were sorted into Sca1$^+$ and Sca1$^-$ populations directly into 96-well plates containing growth-factor-reduced Matrigel at 500 cells/well, and clones were counted after 10 days. The Sca1$^+$ cells gave rise to 47±14 colonies in Matrigel, whereas the Sca1$^-$ produced no colonies. Colonies were stained with Crystal Violet and counted by two independent researchers. (c) CDβgeo Sca1$^+$ population give rise to 75±4.2% Sca1$^+$ and 24±4.1% Sca1$^-$ cells. (d) CDβgeo Sca1$^-$ cells remained mostly Sca1$^-$ after culture, and gave rise to 94±1.5% Sca1$^-$ cells and only 6±1.45% Sca1$^+$ cells.

surrounded by progenitor cells in various stages of differentiation. To determine whether CDβgeo cells have mammosphere-forming ability, we plated ten thousand cells on low adherence plates in serum free medium supplemented with EGF and bFGF as growth stimulants. Approximately 0.1-0.6% of the CDβgeo cells were able to form mammospheres. Next we examined the expression patterns of putative stem cell markers in the secondary mammospheres, such as CD49f, TIE2, keratin 6 (K6), and epithelial-specific markers keratin 14 (K14) and keratin 18 (K18). In the secondary mammospheres examined, CD49f was expressed mainly in the center of the mammosphere (Fig. 2b). K14 staining was also localized in the center of the mammosphere (Fig. 2d). Both TIE2 and K6 were randomly distributed throughout the mammospheres (Fig. 2c,e) and luminal K18$^+$ cells were not detected (data not shown). In previous studies, CD49f has been associated with multi-potent mammary gland progenitors (Stingl et al., 2005), TIE2 has been shown to identify quiescent hematopoietic stem cells and is thought to function by maintaining these cells in the bone marrow niche (Arai et al., 2004). K6 is expressed within the body cells of the developing mammary terminal end

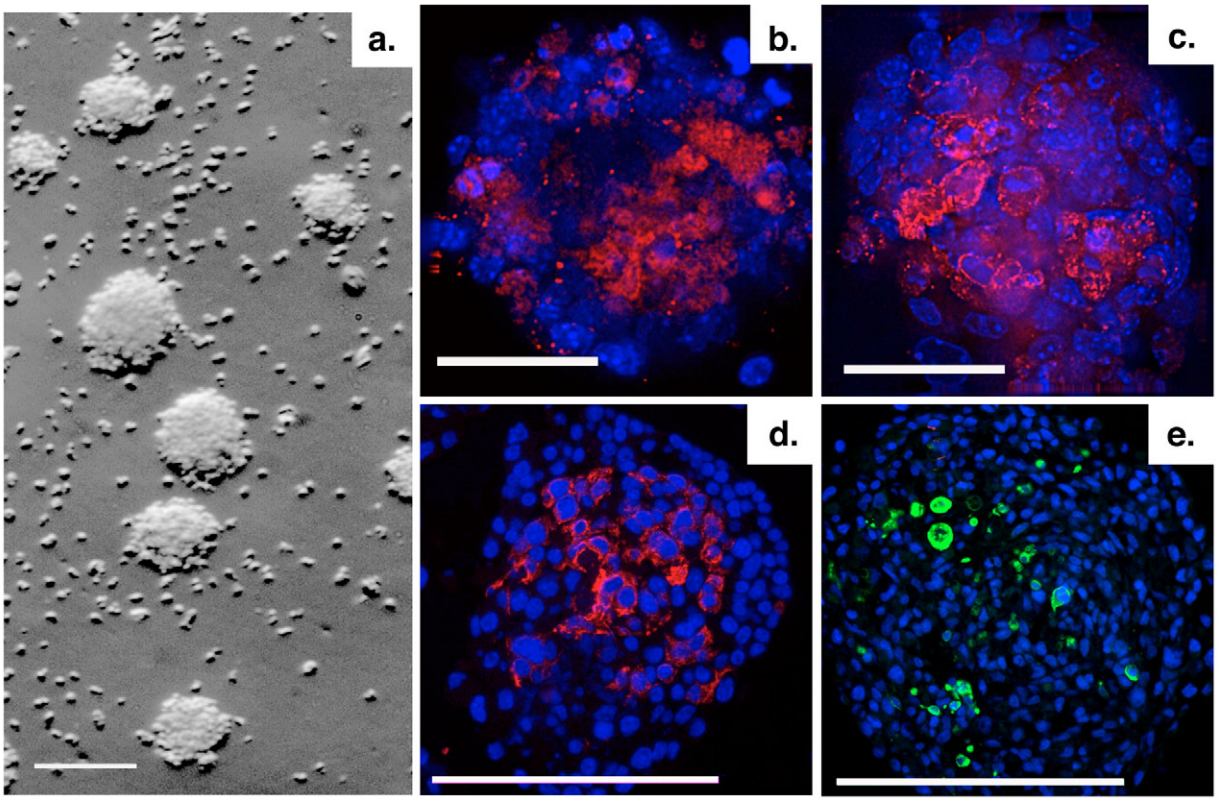

Fig. 2. CDβgeo cells can self-renew in suspension culture and express putative stem cell markers. (a) Bright-field image. Approximately 1 in 600-1000 CDβgeo cells forms a mammosphere. Bar, 100 μm. (b-e) CDβgeo mammospheres were cytospun onto glass slides, and immunostained for CD49f (b, red. Bar, 20 μm), TIE-2 (c, red. Bar, 20 μm), K14 (d, red. Bar, 50 μm), and keratin 6 (e, green. Bar, 50 μm). All nuclei were counterstained with DAPI (blue).

buds, and overexpressed in Wnt1 murine mammary tumors together with Sca1 (Grimm et al., 2006; Li et al., 2003). K14 is an epithelial-specific marker used to identify basal epithelial cells whereas K18 identifies luminal epithelial cells.

β-catenin enhances self-renewal

Studies using transgenic mice expressing Wnt1 or stabilized β-catenin, such as the MMTV-Wnt1 and MMTV-DN89β-catenin, have indicated that hyperplasias and tumors rapidly develop and that they are enriched in cells expressing stem and/or progenitor markers (Imbert et al., 2001; Li et al., 2003). Additional studies from our laboratory using a dominant negative chimera, β-engrailed, which specifically suppressed β-catenin signaling without affecting its cell-cell adhesion function, inhibited cell survival in lobuloalveolar progenitors (Tepera et al., 2003). These studies suggested that β-catenin plays a critical role in stem or multipotent progenitor cell self-renewal.

To determine whether there is any difference in self-renewing efficiency between Sca$^+$ and Sca1$^-$ subpopulations, and to examine whether β-catenin is required for self-renewal, we first transduced the CDβgeo cells with control (MSCV-IRES-GFP; murine stem cell virus – internal ribosomal entry site – green fluorescent protein), stabilized β-catenin (MSCV-β-catenin-IRES-GFP), or the dominant-negative chimera, β-engrailed (MSCV-β-engrailed-IRES-GFP), and then sorted into Sca1$^+$ and Sca1$^-$ populations. The efficiency of secondary mammosphere formation (the number of mammospheres per the number of seeded cells) generated by both the Sca1$^+$ and Sca1$^-$ populations was quantified as described in Materials and Methods. The control Sca1$^+$ population demonstrated a significant increase in the efficiency for mammosphere formation compared to the control Sca1$^-$ population (GFP control Sca1$^+$ vs Sca1$^-$; $*P<0.02$, Fig. 3). In addition, transduction with stabilized β-catenin enhanced Sca1$^+$ mammosphere formation compared to the GFP control (GFP Sca1$^+$ vs β-catenin Sca1$^+$, $**P<0.008$; Fig. 3), whereas β-engrailed decreased the number of mammospheres in both the Sca1$^+$ and the Sca1$^-$ populations. These data indicate that stabilized β-catenin selectively enhances the mammosphere-forming capacity in the Sca1$^+$ cells whereas the dominant-negative β-engrailed depletes the mammosphere-forming capacity.

Sca1$^+$ cells are resistant to clinically relevant doses of radiation

To determine whether Sca1$^+$ progenitor cells may be resistant to clinically relevant doses of radiation, we examined the replicative competence of CDβgeo progenitors after radiation and then compared the clonogenic potential of Sca1$^+$ and Sca1$^-$ cells. Clonogenic assays are classically used to demonstrate radioresistance, since cells that can form colonies after radiation are clearly still competent to reproduce and, therefore, represent a fraction of cells that persist and may lead

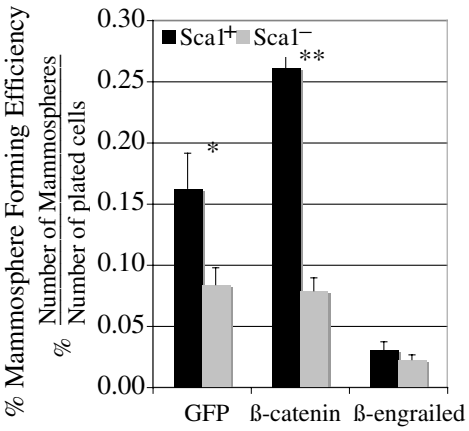

Fig. 3. Stabilized β-catenin expression enriches for stem/progenitor cells by increasing the number of mammospheres. CDβgeo cells transduced with GFP (control), β-catenin, or β-engrailed were FACS sorted into Sca1$^+$ and Sca1$^-$ populations and grown in suspension at a density of 20,000 cells per well for 14 days. The mammospheres were passaged once after 7 days, and the secondary mammospheres were counted using a Leica dissecting microscope. GFP control Sca1$^+$ vs Sca1$^-$ *P<0.02; GFP Sca1$^+$ vs β-catenin Sca1$^+$, **P<0.008. The efficiency of mammosphere formation is calculated as the number of mammospheres per the number of plated cells. Data were collected from three individual experiments performed in triplicate.

to tumor recurrence (Pawlik and Keyomarsi, 2004). Sca1$^+$ and Sca1$^-$ CDβgeo cells were sorted into 96-well plates containing growth-factor-reduced Matrigel at 500 cells per well. Plating efficiency (number of colonies/number of cells plated) for colony formation from Sca1$^-$ cells was 0% whereas plating efficiency for colony formation from Sca1$^+$ cells was 2-4% at 500-cell density (Fig. 4a), therefore, Sca1$^-$ cells were unable to form colonies. The Sca1$^+$ fraction surviving after 2 Gy was >100% suggesting resistance and proliferation, although the colony size after 2 Gy was reduced by 33% (P<0.0001).

It is known that quiescent cells are more resistant to radiation than cycling cells. To determine if Sca1$^+$ radioresistance is secondary to quiescence of these cells, cell cycle analysis of Sca1$^+$ and Sca1$^-$ subpopulations was performed using 7-AAD and pyronin Y, which stain DNA and RNA, respectively, to discriminate G0 from different stages within G1 as shown in previous studies (Schmid et al., 2000; Xin et al., 2005). Although the presence of a minor quiescent subpopulation within either sample cannot be excluded by this method, there were no significant differences in the cell cycle profiles between Sca1$^+$ and Sca1$^-$ cells 24 hours following radiation. Both populations were actively cycling, and had cells in G0, G1 and S/G2/M (Fig. 4b). Cellular proliferation was compared between the Sca1$^+$ and Sca1$^-$ cell populations using a MTT cell proliferation assay. Sca1$^+$ cells demonstrated increased proliferation after irradiation at 2 and 4 Gy, over time in culture (*P<0.0001, **P<0.04, Fig. 4c). The rate of apoptosis was similar between the two populations by using annexin V staining. There was also no significant difference in senescence between the Sca1$^+$ and Sca1$^-$ cells (data not shown).

In addition, we examined the proliferation rates of Sca1$^+$ and Sca1$^-$ cells by generating growth curves over a period of 6 days. The CDβgeo cells were irradiated at 0 Gy (sham irradiation) and 4 Gy, then separated into Sca1$^+$ and Sca1$^-$ populations by FACS sorting. The cells were then plated at sub-confluent density, and cell number was determined every 48 hours. The Sca1$^-$ cells showed decreased proliferation compared to the Sca1$^+$ cells at 0 Gy. Following irradiation at 4 Gy, the Sca1$^+$ cells showed a decrease in proliferation rate from 48 to 96 hours, but their proliferation was nearly identical to the control 0 Gy Sca1$^+$ cells by 144 hours. By contrast, Sca1$^-$ cells exhibited a much lower proliferation rate at 4 Gy through 144 hours. Following irradiation at 4 Gy, the Sca1$^+$ cells exhibited a lag in proliferation as compared to the 0 Gy control Sca1$^+$ cells, but recovered and matched the control Sca1$^+$ cells at 144 hours. By contrast, following irradiation, the Sca1$^-$ cells not only exhibited decreased proliferation following irradiation, but also by 144 hours had not recovered as had the 0 Gy control (Fig. 4d).

To determine whether there are differences in radiation-induced DNA damage between the Sca1$^+$ and Sca1$^-$ cells, we examined DNA-damage foci using γ-H2AX as a DNA-damage marker. Immunostaining of γ-H2AX immediately following irradiation at 4 Gy showed that the Sca1$^-$ cells contained more γ-H2AX foci than the Sca1$^+$ cells (Fig. 5a). Quantification of γ-H2AX foci confirmed that Sca1$^-$ cells contained 60% more foci than the Sca1$^+$ cells at 2 hours following irradiation (*P<0.0001, Fig. 5b).

Alteration in β-catenin localization and upregulation of survivin expression following irradiation

To determine the impact of the Wnt/β-catenin signaling pathway on radioresistance, we examined whether there is any intrinsic difference between the Sca1$^+$ and Sca1$^-$ cells. First we compared the level of β-catenin in these two subpopulations using an antibody specific for the non-phosphorylated (or active) form of β-catenin. Flow cytometry analysis revealed that the level of non-phosphorylated β-catenin in the Sca1$^+$ cells differs significantly from that in the Sca1$^-$ cells. The Sca1$^+$ cells have an increased level of β-catenin over the IgG control whereas the Sca1$^-$ cells have about the same level as the control (Fig. 6a). Deconvolution microscopy revealed that non-phosphorylated β-catenin is mainly localized around the cell membrane in the Sca1$^+$ cells, whereas it is difficult to detect β-catenin in the Sca1$^-$ cells. Following irradiation at 4 Gy, the non-phosphorylated β-catenin is found mainly in and around the nucleus rather than around the cell membrane. It appears that Sca1$^+$ and Sca1$^-$ cells are intrinsically different with respect to the level of non-phosphorylated β-catenin. Interestingly, the localization of β-catenin was altered dramatically following irradiation in the Sca1$^+$ cells (Fig. 6b).

Survivin, a bifunctional member of the inhibitor of apoptosis family, has been shown to be a direct target of TCF/β-catenin signaling. In addition, studies in pancreatic cancer, brain tumors, as well as rectal cancer (Kami et al., 2004; Rodel et al., 2005; Zhen et al., 2005), indicate that survivin may play a role in radioresistance. To determine whether survivin is differentially regulated in response to radiation, we examined survivin expression in both Sca1$^+$ and Sca1$^-$ cells using real-time PCR. CDβgeo cells were transduced with control (β-galactosidase; β-gal), β-catenin (β-cat), and β-engrailed (β-eng), and FACS sorted into Sca1$^+$ and Sca1$^-$ populations. Radiation selectively enhanced survivin expression in Sca1$^+$ cells at 2 Gy (β-gal control Sca1$^+$, 0 Gy vs 2 Gy, *P<0.02; Fig. 6c). Since it is known that β-catenin directly activates survivin,

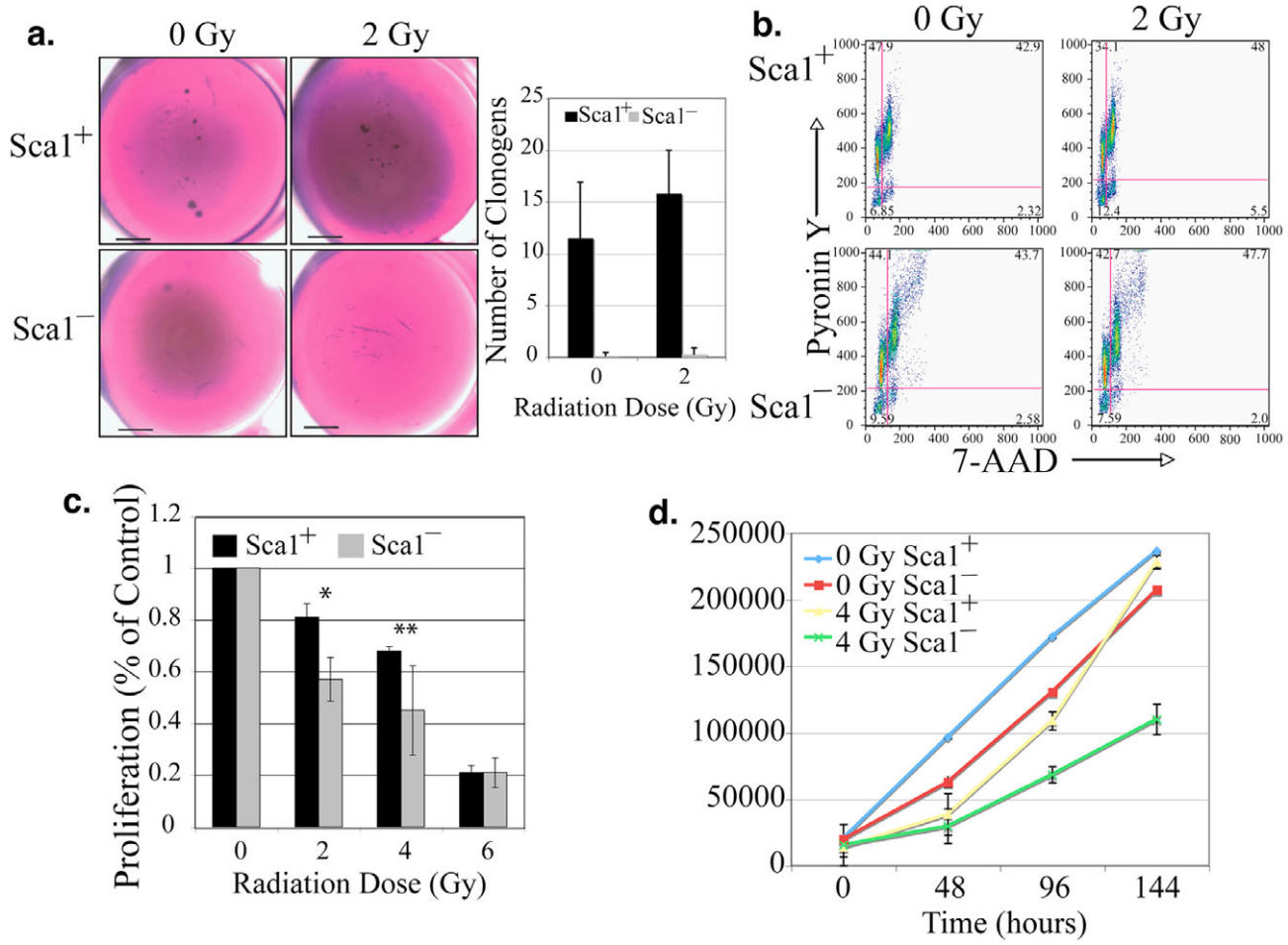

Fig. 4. Radiation does not decrease colony formation from Sca1$^+$ cells in Matrigel. (a) 500 Sca1$^-$ or Sca1$^+$ cells treated with either 0 or 2 Gy were sorted using flow cytometry into 96-well plates containing 10 μl of Matrigel. Representative images of wells containing 500 cells are shown. Bars, 2 mm. Sca1$^+$ 0 Gy vs 2 Gy, P<0.07 by two-tailed t-test. (b) CDβgeo cells were irradiated at either 0 or 2Gy, sorted into Sca1$^+$ and Sca1$^-$ populations. Sorted cells were stained with 7-AAD and pyronin Y to discriminate G0 from different stages within G1(see Materials and Methods). There is no obvious difference in the cell cycle profiles between the Sca1$^+$ and Sca1$^-$ population, at either 0 or 2 Gy. Cell cycle was analyzed using flow cytometry. Statistics were assessed on FlowJo version 4, Tree Star, Inc. (c) Significant differences in proliferation 144 hours following irradiation. MTT assay was performed on the sorted Sca1$^+$ and Sca1$^-$ cells after irradiation for 144 hours at 0 (sham), 2, 4 and 6 Gy. Following irradiation, the sorted cells were serum starved (0.1% adult bovine serum) for 48 hours, and then stimulated to proliferate with serum (5% adult bovine serum). See Materials and Methods for details. The bars indicate average OD (absorbance) and the error bars are the standard deviation of three replicate OD measurements within each group. There were statistically significant differences in growth rate between the Sca1$^+$ and Sca1$^-$ cells at 2 Gy and 4 Gy, *P<0.0001, **P<0.04, two-tailed t-test. (d) Growth properties of Sca1$^+$ and Sca1$^-$ cells at 0 and 4 Gy. Cells were directly sorted into 6-well plates at 50,000 cells per well, and the medium was replaced every 48 hours with fresh medium (plus 5% adult bovine serum). Cells were counted every 48 hours using a hemocytometer. The graph shows the growth curve of 0 Gy Sca1$^+$ (blue), 0 Gy Sca1$^-$ (red), 4 Gy Sca1$^+$ (yellow), 4 Gy Sca1$^-$ (green). Each data point represents the mean ± s.e.m. of three experiments in triplicate. The different cell culture conditions may account for the differences in the kinetics of recovery.

transduction with stabilized β-catenin significantly increased survivin expression (**P<0.03; Fig. 6c). The survivin level increased further following irradiation in the β-catenin-transduced cells (β-cat Sca1$^+$, 0 Gy vs 2 Gy, ***P<0.04, Fig. 6c). Interestingly, transduction with β-engrailed, the dominant-negative variant, did not decrease the basal level of survivin. However, radiation did not elevate survivin level in the β-engrailed-transduced cells.

Discussion

Deciphering the biological and molecular events in mammary preneoplasia will provide potential means of blocking progression to malignancy. To date, studies using several immortalized cancer cell lines, including the human breast cell line MCF-7, have indicated that they contain a subpopulation of highly tumorigenic cells, which retain stem/progenitor-like properties (Kondo et al., 2004; Patrawala et al., 2005; Ponti et al., 2005). However, functional studies of progenitor cells in a preneoplastic model have not been explored.

The parental COMMA-D cell line originally derived from primary mammary epithelial cells of mid-pregnant BALB/c mice was shown previously by transplantation into cleared fat pads of syngenic mice to result in outgrowths containing all of the epithelial cell types normally present in the mammary

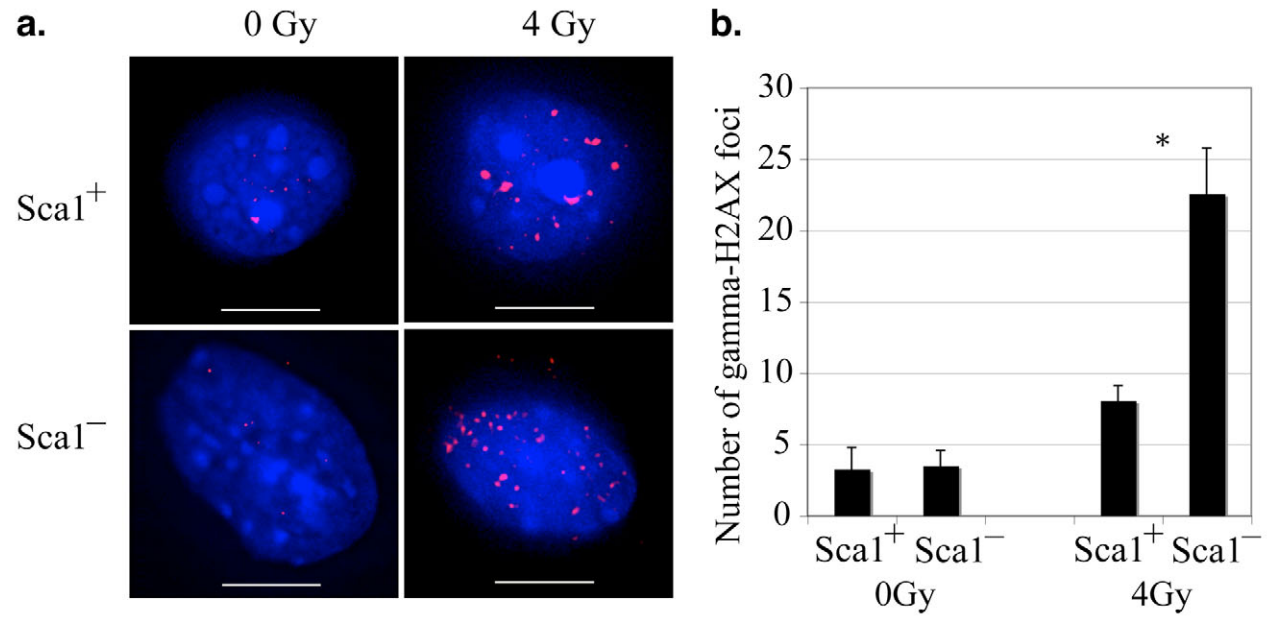

Fig. 5. Radiation induces more DNA damage foci in Sca1⁻ cells. (a) Sca1⁺ and Sca1⁻ cells were sorted directly onto glass slides following irradiation at 4 Gy and immunostained with anti-γ-H2AX (red). Nuclei were stained with DAPI (blue). Bars, 5 μm. (b) There were significantly more DNA-damage foci in the Sca1⁻ population (4 Gy Sca1⁺ vs Sca1⁻, **P*<0.0001). A minimum of 200–300 cells were counted in each group.

gland (Danielson et al., 1984). Whereas the CD cell line is known to have mutations in both p53 alleles (Jerry et al., 1994), the derivative CDβgeo cell line still provides a suitable model to study radioresistance of mammary progenitor cells for the following reason. These cells are unique in that they contain a permanent population of progenitor cells, and are able to give rise to outgrowths that contain both mammary-specific luminal and myoepithelial cell types. Furthermore, comparable results have been obtained in primary mammary epithelial cell progenitors with wild-type p53 (Woodward et al., 2007). Recent studies by Deugnier et al. have clearly demonstrated that the CDβgeo cells contain a permanent population of mouse mammary epithelial progenitor cells with basal characteristics and that express putative stem cell markers such as Sca1, CD24 and CD49f, among others. CDβgeo cells at passage 18 were reported to generate 'normal appearing mammary outgrowth' upon in vivo transplantation, and appear to be more genomically stable than the CD cells.

It is somewhat surprising to find a subpopulation of progenitor cells that are Sca1⁺ within an immortalized mammary cell line. Similar to the findings of Deugnier et al. (Deugnier et al., 2006), we observed a consistent subpopulation (13-20%) of Sca1⁺ cells within the CDβgeo cell line, which is comparable to previous data from our laboratory demonstrating a 15-20% Sca1⁺ subpopulation in primary mammary epithelial cells. Not only is the Sca1⁺ subpopulation from the primary mammary epithelial cells enriched in long term label-retaining cells and side population cells that efflux Hoechst dye, they are also enriched in transplant potential compared to the Sca1⁻ cells, suggesting that Sca1 is a marker of progenitor cells (Welm et al., 2003). By contrast, a recent report by Shackleton et al. has demonstrated that whereas they are able to isolate primitive mammary stem cells using CD49f/CD29, cells expressing high Sca1 are not enriched in CD49f/CD29 population. In addition, they found that the Sca1hi cells did not enrich for outgrowth frequency, whereas CD49f/CD29⁺ cells with increased outgrowth potential were Sca1lo and not Sca1⁻. However, in our previous transplantation studies (Welm et al., 2002) the entire Sca1⁺ population was separated from the Sca1⁻ population by magnetic bead separation or using a Sca1⁻ EGFP knockout mouse and sorting for EGFP⁺ and EGFP⁻ cells. Thus, these two results are actually consistent and suggest that the Sca1⁺ population is heterogeneous and contains within it a subpopulation with increased outgrowth potential. A comparison between the percentage Sca1 found in our laboratory and these recent studies (Shackleton et al., 2006; Stingl et al., 2006) indicates that the percentage Sca1 reported by Shackleton et al. is threefold higher than reported in our previous studies. It is probable that different cell preparation and FACS gating protocols may account for such a large discrepancy. These discrepant results may in fact represent differences in nomenclature, highlighting the importance of careful discussion and presentation of flow output and gates. Interestingly, Shackleton et al. report that Sca1 is not enriched in the CD49f/CD29 population. This again suggests that within the mammary gland, there could be a number of subpopulations of stem and/or progenitor cells, each with overlapping or distinct characteristics. Whereas CD49f/CD29 may represent a more primitive stem cell population, the Sca1 subpopulation may encompass a more committed progenitor population (Woodward et al., 2005). In both cases, the majority of these cells are in S/G2/M and only a minority of these populations contain label-retaining cells, or LRCs, thus, additional markers will be required to identify the quiescent stem cell population. Furthermore, whereas these antigens are putative progenitor cell markers, there are no functional data suggesting that either CD49f or CD29 is necessary for stem

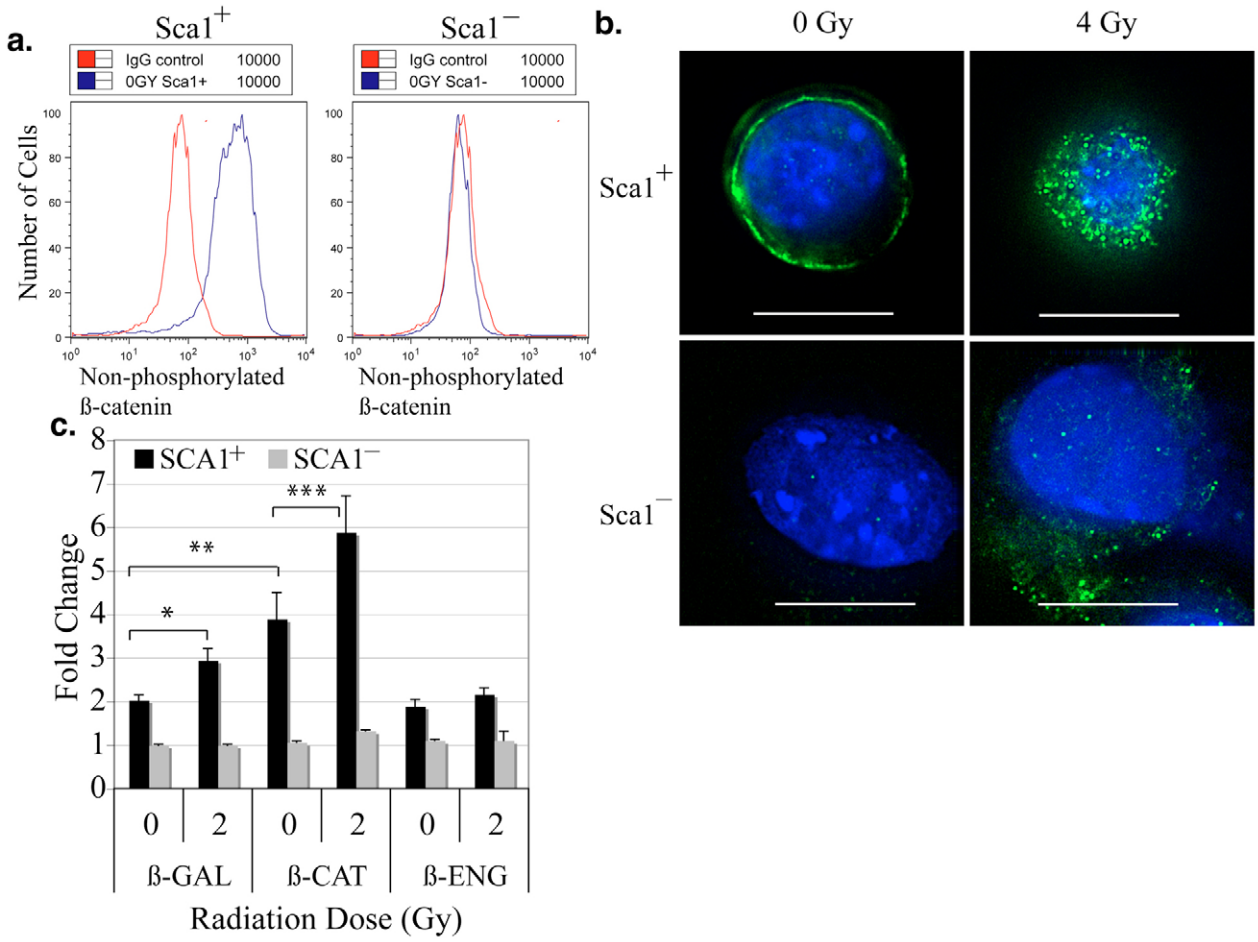

Fig. 6. Differences in β-catenin level and localization between Sca1$^+$ and Sca1$^-$ subpopulations. (a) Sca1$^+$ and Sca1$^-$ subpopulations were stained with non-phosphorylated β-catenin antibody and analyzed by flow cytometry with an Alexa Fluor 488 secondary antibody against β-catenin. Comparison of the fluorescence intensity of Alexa Fluor 488 from Sca1$^+$ and Sca1$^-$ cells (in blue) with that from IgG control (in red). (b) Immunostaining for non-phosphorylated β-catenin in Sca1$^+$ and Sca1$^-$ cells after 0 Gy (sham irradiation) and 4 Gy. β-catenin is visualized in green, and the nuclei are stained with DAPI (blue). Bars, 5 μm. Images were captured by deconvolution microscopy using a Zeiss AxioVert S100 TV microscope and a DeltaVision restoration microscopy system (Applied Precision, Inc.). For high-resolution deconvolved images, captured raw images were deconvolved with the DeltaVision constrained iterative algorithm. (c) β-Catenin regulates survivin expression following irradiation. CDβgeo cells were transduced with control vector, β-catenin, or β-engrailed, and then irradiated at 0 or 2 Gy. Cells were harvested after 24 hours, FACS sorted into Sca1$^+$ and Sca1$^-$ subpopulations, and RT-PCR was performed using the ABI real-time PCR system. β-gal control Sca1$^+$, 0 Gy vs 2 Gy, *P<0.02; 0 Gy GFP Sca1$^+$ vs 0 Gy β-cat Sca1$^+$, **P<0.03, β-cat Sca1$^+$, 0 Gy vs 2 Gy, ***P<0.04. Data were obtained from five individual experiments performed in triplicate.

cell fate. In fact, the CD49f mammary-specific knockout displayed no overt phenotype, whereas the CD29 conditional knockout exhibited only a limited lactation defect (Klinowska et al., 2001; Li et al., 2005).

The mammosphere assay (free floating spherical aggregates with the potential to self-renew and to differentiate into all cell types of the mammary gland), analogous to neurospheres (Reynolds and Weiss, 1996), is an assay for self-renewal, based on the idea that stem cells may survive in anchorage-independent conditions whereas differentiated cells need attachment to survive, and die by anoikis when they lose contact with extracellular matrix. Dontu et al. (Dontu et al., 2003) reported that when grown in conditions similar to neurospheres, four in 1000 cells isolated from a normal reduction mammoplasty survive in anchorage-independent conditions, and were able to form mammospheres with only bFGF and EGF as growth stimuli. In addition, secondary and tertiary mammospheres have been shown to be more homogeneous and are further enriched in self-renewal capacity (Dontu et al., 2003). We have observed that the CDβgeo cells display a similar ratio: approximately 1 out of 1000 CDβgeo cells gives rise to a mammosphere. Consistent with the idea that multipotent stem cells are within the core of the mammosphere, we have observed that CD49f is found in cells at the center. However, TIE2 and K6 expression appeared to be random, suggesting that perhaps these are not markers exclusively for putative stem cells, are associated with differentiated cells, and perhaps are staining for cells at various stages of differentiation. We have not observed significant mammosphere enrichment following secondary or tertiary passaging. Moreover, when we compared the self-renewal between the Sca1$^+$ and Sca1$^-$ subpopulations, the Sca1$^+$ cells

showed increased mammosphere-forming efficiency. Whereas overexpression of β-catenin increased the mammosphere-forming efficiency further, depleting endogenous β-catenin by using the dominant negative chimera, β-engrailed, decreased mammosphere-forming efficiency dramatically, suggesting that β-catenin is required in the self-renewal process. This observation is consistent with a number of reports indicating that β-catenin is important in regulating stem/progenitor cell maintenance in the intestinal epithelium, in neurogenesis and in the cilliary margin of the eye, to list just a few examples (Inoue et al., 2005; Lie et al., 2005; Pinto and Clevers, 2005a). Activation of the Wnt/β-catenin pathway in Wnt1 transgenic mice induces an enrichment of side population progenitor cells both in vitro and in vivo (Liu et al., 2004). In addition, caveolin-deficient mice have been reported to display an increase in progenitor cells, due to an indirect activation of the Wnt/β-catenin pathway (Sotgia et al., 2005).

It is widely accepted that radiation-induced cell death typically occurs via mitotic catastrophe during cell division rather than apoptosis. However, increasing evidence suggests that stem/progenitor cells evade cell death by a number of mechanisms, such as quiescence and drug-efflux conferred by expression of the ABC-family of membrane transporters (Dean et al., 2005). The cell cycle profiles of the CDβgeo $Sca1^+$ and $Sca1^-$ cells suggest that the $Sca1^+$ cells are not quiescent stem cells, but rather, are actively proliferating transit amplifying progenitor cells. In addition, the increased clonogenicity and proliferation potential of the $Sca1^+$ cells suggest that these cells have enhanced ability to sustain radiation in contrast to the $Sca1^-$ cells. Similar to our findings, using CD133 as a glioma tumor stem cell marker, recent studies by Bao et al. have demonstrated that, following irradiation, the tumor xenographs are enriched in $CD133^+$ cells compared with $CD133^-$ cells (Bao et al., 2006). The $CD133^+$ cells appear to repair DNA damage more efficiently than $CD133^-$ cells, which may account for glioma radioresistance and could become the source of recurrence. Furthermore, preliminary studies indicate that radiation does not deplete the efficiency of mammosphere formation, suggesting that radiation at 2 Gy does not affect the self-renewal capacity of $Sca1^+$ CDβgeo cells at clinically relevant doses (S. L. Poplack and J.M.R., unpublished).

The differences in the levels of DNA damage as well as differences in proliferation following irradiation between the $Sca1^+$ and $Sca1^-$ cells may be due to in part to their intrinsic chromatin structure as well as the differences in the endogenous levels and the localization of β-catenin, which has been suggested to play a role in proliferation as well as DNA damage response. Studies in human breast cancer have shown that Wnt signaling triggers DNA damage response, followed by events that result in conversion of primary human mammary epithelial cells (Ayyanan et al., 2006). In addition, studies in colorectal cancer have shown that PARP1, a DNA binding protein that plays a role in DNA repair, recombination and proliferation, binds to TCF/β-catenin, and its expression tightly correlates with overexpression of β-catenin, c-myc, and cyclin D1 in colorectal cancers (Nosho et al., 2006). Moreover, recent studies have suggested that β-catenin plays a critical role in histone modification and subsequent activation of proliferation-associated genes by binding to TRRAP/TIP60 and SET1 type chromatin modifying enzymes (Sierra et al., 2006). Taken together, these studies suggest that β-catenin plays a key role in regulating DNA damage repair and proliferation, preferentially in the $Sca1^+$ cells. Conversely $Sca1^-$ cells, which express a markedly decreased level of active β-catenin, do not show the same level of proliferation following irradiation.

The increased radioresistance observed in the CDβgeo $Sca1^+$ cells may be conferred also in part, by elevated levels of survivin, a direct target of β-catenin/Tcf signaling. Survivin was shown to be strongly expressed in human and mouse embryonic intestinal crypts, whereas its expression was absent in Tcf-4 knockout animals (Kim et al., 2003). Expression of non-destructible β-catenin mutant increased survivin expression and protected colorectal cancer cells against UV-induced apoptosis, suggesting that Tcf/β-catenin-mediated expression of survivin imparts a stem cell-like phenotype to colonic crypt epithelium coupling enhanced cell proliferation with resistance to apoptosis. The selective increase in survivin expression level in $Sca1^+$ cells expressing β-catenin suggests that survivin may be a mechanism regulated in part by the Wnt/β-catenin pathway, which increases radioresistance in the progenitor cells. Moreover, since apoptosis is a minor component of radiation-induced cell death in solid tumors at low doses (Hall, 2000), this finding potentially suggests a non-apoptosis related role for survivin in these cells. Indeed, such a role has recently been described in colon cancer cell lines where survivin was shown to assist cancer cells in escaping replicative senescence by enhancing telomerase activity (Endoh et al., 2005). However, the lack of suppression in β-eng transduced cells suggests that survivin may be regulated in part by other pathways. Notably, the stress response NF-kappaB pathway has been shown to regulate survivin expression. Kawakami et al. (Kawakami et al., 2005) have shown that inhibiting the NF-kappaB pathway resulted in suppression of survivin expression and caused apoptosis of Tax-expressing malignant CTLL-2 T-cells. Furthermore, recent studies by Vong et al. (Vong et al., 2005) provided evidence for the role of survivin in regulating mitosis via a differential ubiquitination mechanism.

This study demonstrates that the CDβgeo cell line maintains a population of self-renewing, asymmetrically dividing, progenitor cells. We were able to use the CDβgeo cells as a model to establish that the Wnt/β-catenin pathway impacts self-renewal and proliferation of progenitor cells. Finally, we provided evidence for radioresistance of progenitor cells, and more importantly, that the Wnt/β-catenin pathway enhances radioresistance. The data presented suggest that progenitor cell response to radiation can be specifically targeted. Immortalized cell lines such as CDβgeo that contain a subpopulation of multipotent progenitors may be useful initial preclinical models to identify targets for which a therapeutic window could be examined and novel targeted clinical therapies designed.

Materials and Methods

Antibodies

Anti-CD49f rat monoclonal antibody was obtained from BD Pharmingen Anti-TIE2 polyclonal antibody was obtained from Santa Cruz Biotechnologies Anti-K6 polyclonal antibody was obtained from Covance. Anti-K8 rat monoclonal antibody was obtained from Developmental Studies Hybridoma Bank, University of Iowa. Anti-non-phosphorylated β-catenin antibody, clone 8E4 and γ-H2ax antibody were obtained from Upstate Cell Signaling Solutions (Charlottesville, VA).

Immunofluorescence

Samples on glass slides: Samples were fixed with 4% paraformaldehyde in phosphate-buffered saline for 20 minutes, permeabilized with 5% Triton X-100 for 20 minutes, followed by a 1-hour blocking step in 20% goat serum/3% bovine serum albumin/0.1% Tween 20.

Paraffin-embedded sections: 3 μm sections were deparaffinized in xylene, then rehydrated through a graded ethanol series. Immunostaining was performed after microwave antigen retrieval (20 minutes) in 10 mM sodium citrate and blocking in 20% bovine serum albumin in phosphate-buffered saline containing 0.1% Tween 20. Primary antibody staining was performed either for 1 hour at room temperature or overnight at 4°C. Immunofluorescence staining was detected with the appropriate secondary antibodies conjugated with Texas Red, Alexa Fluor 568, or Alexa Fluor 488 (Molecular Probes, Eugene, OR), performed at room temperature for 45 minutes. Nuclei were counter-stained with DAPI (Vector, Berlingame, CA) and coverslips were mounted with SlowFade Light Antifade Kit (Molecular Probes). Images were captured using a Zeiss CCD camera at 100× magnification or captured by deconvolution microscopy using a Zeiss AxioVert S100 TV microscope and a DeltaVision restoration microscopy system (Applied Precision). For high-resolution deconvolved images, captured raw images were deconvolved with the DeltaVision constrained iterative algorithm. All images were digitally processed for presentation with Adobe Photoshop.

Cell line

COMMA-D β-geo cell line passages 21-27 was kindly provided by D. Medina at Baylor College of Medicine, Houston, TX. The cells are grown in DMEM/F12 (Invitrogen) at pH 7.6, with 2% adult bovine serum (Gemini Bioproducts, CA), 2.383 mg/ml Hepes (Invitrogen), 5 μg/ml gentamycin (Sigma), 10 μg/ml insulin (Invitrogen), and 5 ng/ml EGF (Invitrogen).

Retroviral transduction

Stabilized β-catenin (Montross et al., 2000) and β-engrailed constructs were cloned into the pS2 retroviral backbone [kindly provided by Aguilar-Cordova, Baylor College of Medicine, (Faustinella et al., 1994)] as described by Tepera et al. (Tepera et al., 2003). Stabilized β-catenin and β-engrailed were also cloned into the pMSCV-IRES-GFP retroviral backbone (kindly provided by Greg Hannon, Cold Spring Harbor, NY). Transduction procedures was performed as described by Tepera et al. (Tepera et al., 2003). Briefly, 293T-packaging cells (ATCC) were transiently transfected with target constructs and pCL-Eco (Imgenex) using Fugene (Roche) according to the manufacturer's guidelines. Forty-eight hours after transfection (day 3), medium was collected from transfected 293T cells, filtered through a 0.22 mm syringe filter, and applied to CDβgeo cells in a 1:1 ratio (1 plate 293T to 1 plate CDβgeo). The cells were spun at 3,000 ***g*** in a Marathon 6K clinical centrifuge (Fisher Scientific) on a swinging platform rotor for 30 minutes. The retroviral supernatant was removed from CDβgeo cells and replaced with medium.

Flow cytometry

Samples were prepared for flow cytometry using anti-Sca1 antibody conjugated to FITC or PE (BD Pharmingen). Cells were incubated with Sca1 antibody for 15 minutes on ice, resuspended in HBSS+ [HBSS (Invitrogen), 2% fetal bovine serum (JRH Biosciences) and 100 mM Hepes (Invitrogen)], and filtered through a 45 μm cell filter into polypropylene tubes containing 0.5 μg/ml propidium iodide (Sigma) to exclude dead cells. Analysis and sorting were performed on a triple laser MoFlo (Cytomation, Fort Collins, CO). Data analysis was performed on FlowJo version 4, Tree Star.

Flow cytometry analysis for non-phosphorylated β-catenin

Cells were sorted directly onto glass slides (FisherBiotech ProbeOn Plus, Hampton, NH) at 500 cells each for immunofluorescence staining. For non-phosphorylated β-catenin flow cytometry analysis, cells were directly sorted into 5 ml polypropylene tubes (Falcon), and then fixed and permeabilized using BD Cytofix/Cytoperm kit (BD Biosciences, San Jose, CA). Primary antibody was used at 1:200 dilution in 1× Cytoperm at 4°C overnight, and Alexa Fluor 488-conjugated secondary antibody was used at 1:500, at room temperature for 30 minutes. Cells were washed and resuspended in 1× Cytoperm, and strained through a 45-μm filter before flow cytometry analysis.

Cell cycle analysis

Cell cycle analysis was performed according to the protocol described by Xin et al. (Xin et al., 2005). Briefly, cells were resuspended in 0.5 ml of NASS buffer (0.15 M NaCl 5 mM sodium EDTA 0.5% BSA fraction V 0.1 M phosphate citrate buffer, pH 4.8) containing 0.02% saponin and 10 μg/ml 7-aminoactinomycin D at room temperature for 20 minutes. Cells were washed with PBS and resuspended in NASS containing 0.02% saponin and 10 μg/ml actinomycin D at 4°C for 5 minutes. Pyronin Y (0.5 μl of 1 g/ml) diluted in distilled water was added, and samples were incubated at 4°C for 10 minutes before flow cytometry analysis.

Mammosphere assays

Mammosphere growth conditions were based on those of Dontu et al. (Dontu et al., 2003). Briefly, 10,000-20,000 CDβgeo cells/well were grown in 6-well Ultra Low Attachment plates (Corning, NY) in serum free DMEM/F12 (Invitrogen) supplemented with 20 ng/ml bFGF (Invitrogen), 20 ng/ml EGF (Invitrogen) and B27 (Invitrogen). The cells were fed every 3-4 days, and passaged using 0.05% trypsin, 0.53 mM EDTA-4Na (Invitrogen). Following trypsin digestion, single cells from the primary mammospheres were plated at 1000 cells/well. After passage 2, the mammospheres were counted using a Leica dissecting scope. Two researchers counted colonies independently and the numbers were averaged. In preparation for immunofluorescence staining, mammospheres were cyto-spun onto glass coverslips, fixed for 15 minutes on ice in 4% paraformaldehyde, and stored in 70% ethanol. Otherwise, paraffin-embedded sections of mammospheres were sectioned at a thickness of 3 μm.

Clonogenic assays

For clonogenic assays using sorted $Sca1^+$ or $Sca1^-$ cells, CDβgeo cells were sham irradiated or treated with 2 Gray (1 Gy=100 rads) approximately 1 hour prior to trypsinization. CDβgeo cells were trypsinized for 3 minutes, washed in HBSS+ and stained for 30 minutes with FITC-conjugated anti-Sca1-antibody (BD Pharmingen). Cells were sorted into 96-well plates (round-bottom, Costar) containing 10 μl of growth factor reduced Matrigel (Discovery Labware) per well. Plates were prepared with Matrigel while on ice and kept on ice prior to sorting. After sorting, 50-100 μl of CDβgeo medium was carefully added to each well. After 7-11 days, plates were fixed in glacial acetic acid and methanol (1:2; 10 minutes) and stained with Crystal Violet. Fixing and staining were performed with great care in order not to dislodge the Matrigel from the well. Crystal Violet was rinsed by adding water to wells overnight rather than repeat washings, to reduce perturbation of wells. Two researchers counted colonies independently and numbers were averaged.

MTT assay

CDβgeo cells were sorted by flow cytometry into $Sca1^-$ and $Sca1^+$ subpopulations as described previously. After sorting, the cells were irradiated at the indicated radiation intensity, 0, 2, 4 and 6 Gy followed by plating in 6-well plates at a density of 2500 cells per well in triplicates. Cells were serum-starved (0.1% adult bovine serum), and stimulated to proliferate in serum (5% adult bovine serum). The MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (Sigma Aldrich®) assay was performed at 24, 48 and 144 hours after serum starvation.

Growth curve

The relative proliferation between the CDβgeo $Sca1^+$ and $Sca1^-$ cells was examined by generating a growth curve. The CDβgeo cells were irradiated at 0 (sham) and 2 Gy followed by separation of the cells into $Sca1^+$ and $Sca1^-$ sub-populations by FACS sorting directly into 6-well plates at 50,000 cells per well. The cells were replenished with fresh medium containing 5% adult bovine serum every 48 hours. The cells were harvested by trypsinization every 48 hours, and the cell number was determined using a hemocytometer.

Real-time PCR

Survivin primer sequences: survivin for 5′aagaactaccgcatcgccacc and survivin rev 5′ agccagctccgccatt. Cells were harvested 24 hours after irradiation. SYBR green quantitative PCR was performed using Applied Biosystems ABI 7500 real-time PCR system.

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References

Arai, F., Hirao, A., Ohmura, M., Sato, H., Matsuoka, S., Takubo, K., Ito, K., Koh, G. Y. and Suda, T. (2004). Tie2/angiopoietin-1 signaling regulates hematopoietic stem cell quiescence in the bone marrow niche. *Cell* **118**, 149-161.

Ayyanan, A., Civenni, G., Ciarloni, L., Morel, C., Mueller, N., Lefort, K., Mandinova, A., Raffoul, W., Fiche, M., Dotto, G. P. et al. (2006). Increased Wnt signaling triggers oncogenic conversion of human breast epithelial cells by a Notch-dependent mechanism. *Proc. Natl. Acad. Sci. USA* **103**, 3799-3804.

Bao, S., Wu, Q., McLendon, R. E., Hao, Y., Shi, Q., Hjelmeland, A. B., Dewhirst, M. W., Bigner, D. D. and Rich, J. N. (2006). Glioma stem cells promote radioresistance by preferential activation of the DNA damage response. *Nature* **444**, 756-760.

Brabletz, T., Hlubek, F., Spaderna, S., Schmalhofer, O., Hiendlmeyer, E., Jung, A. and Kirchner, T. (2005). Invasion and metastasis in colorectal cancer: epithelial-mesenchymal transition, mesenchymal-epithelial transition, stem cells and beta-catenin. *Cells Tissues Organs* **179**, 56-65.

Clarke, M. F. and Fuller, M. (2006). Stem cells and cancer: two faces of eve. *Cell* **124**, 1111-1115.

Danielson, K. G., Oborn, C. J., Durban, E. M., Butel, J. S. and Medina, D. (1984). Epithelial mouse mammary cell line exhibiting normal morphogenesis in vivo and functional differentiation in vitro. *Proc. Natl. Acad. Sci. USA* **81**, 3756-3760.

Dean, M., Fojo, T. and Bates, S. (2005). Tumour stem cells and drug resistance. *Nat. Rev. Cancer* **5**, 275-284.

Deugnier, M.-A., Faraldo, M. M., Teulière, J., Thiery, J. P., Medina, D. and Glukhova, M. A. (2006). Isolation of mouse mammary epithelial progenitor cells with basal characteristics from the Comma-Dβ cell line. *Dev. Biol.* **293**, 414-425.

Dontu, G., Abdallah, W. M., Foley, J. M., Jackson, K. W., Clarke, M. F., Kawamura, M. J. and Wicha, M. S. (2003). In vitro propagation and transcriptional profiling of human mammary stem/progenitor cells. *Genes Dev.* **17**, 1253-1270.

Endoh, T., Tsuji, N., Asanuma, K., Yagihashi, A. and Watanabe, N. (2005). Survivin enhances telomerase activity via up-regulation of specificity protein 1- and c-Myc-mediated human telomerase reverse transcriptase gene transcription. *Exp. Cell Res.* **305**, 300-311.

Faustinella, F., Kwon, H., Serrano, F., Belmont, J. W., Caskey, C. T. and Aguilar-Cordova, E. (1994). A new family of murine retroviral vectors with extended multiple cloning sites for gene insertion. *Hum Gene Ther.* **5**, 307-312.

Grimm, S. L., Bu, W., Longley, M. A., Roop, D. R., Li, Y. and Rosen, J. M. (2006). Keratin 6, is not essential for mammary gland development. *Breast Cancer Res.* **8**, R29.

Hall, E. J. (2000). *Radiobiology for the Radiologist*. Philadelphia: Lippincott Williams & Wilkins.

He, B., Barg, R. N., You, L., Xu, Z., Reguart, N., Mikami, I., Batra, S., Rosell, R. and Jablons, D. M. (2005). Wnt signaling in stem cells and non-small-cell lung cancer. *Clin. Lung Cancer* **7**, 54-60.

Imbert, A., Eelkema, R., Jordan, S., Feiner, H. and Cowin, P. (2001). Delta N89 beta-catenin induces precocious development, differentiation, and neoplasia in mammary gland. *J. Cell Biol.* **153**, 555-568.

Inoue, T., Kagawa, T., Fukushima, M., Shimizu, T., Yoshinaga, Y., Takada, S., Tanihara, H. and Taga, T. (2005). Activation of canonical Wnt pathway promotes proliferation of retinal stem cells derived from adult mouse ciliary margin. *Stem Cells* **24**, 95-104.

Jamieson, C. H., Ailles, L. E., Dylla, S. J., Muijtjens, M., Jones, C., Zehnder, J. L., Gotlib, J., Li, K., Manz, M. G., Keating, A. et al. (2004). Granulocyte-macrophage progenitors as candidate leukemic stem cells in blast-crisis CML. *N. Engl. J. Med.* **351**, 657-667.

Jerry, D. J., Medina, D. and Butel, J. S. (1994). p53 mutations in COMMA-D cells. *In Vitro Cell. Dev. Biol. Anim.* **30A**, 87-89.

Kami, K., Doi, R., Koizumi, M., Toyoda, E., Mori, T., Ito, D., Fujimoto, K., Wada, M., Miyatake, S. and Imamura, M. (2004). Survivin expression is a prognostic marker in pancreatic cancer patients. *Surgery* **136**, 443-448.

Kawakami, H., Tomita, M., Matsuda, T., Ohta, T., Tanaka, Y., Fujii, M., Hatano, M., Tokuhisa, T. and Mori, N. (2005). Transcriptional activation of survivin through the NF-kappaB pathway by human T-cell leukemia virus type I tax. *Int. J. Cancer* **115**, 967-974.

Kim, P. J., Plescia, J., Clevers, H., Fearon, E. R. and Altieri, D. C. (2003). Survivin and molecular pathogenesis of colorectal cancer. *Lancet* **362**, 205-209.

Klinowska, T. C., Alexander, C. M., Georges-Labouesse, E., Van der Neut, R., Kreidberg, J. A., Jones, C. J., Sonnenberg, A. and Streuli, C. H. (2001). Epithelial development and differentiation in the mammary gland is not dependent on alpha 3 or alpha 6 integrin subunits. *Dev. Biol.* **233**, 449-467.

Kondo, T., Setoguchi, T. and Taga, T. (2004). Persistence of a small subpopulation of cancer stem-like cells in the C6 glioma cell line. *Proc. Natl. Acad. Sci. USA* **101**, 781-786.

Li, N., Zhang, Y., Naylor, M. J., Schatzmann, F., Maurer, F., Wintermantel, T., Schuetz, G., Mueller, U., Streuli, C. H. and Hynes, N. E. (2005). Beta1 integrins regulate mammary gland proliferation and maintain the integrity of mammary alveoli. *EMBO J.* **24**, 1942-1953.

Li, Y., Welm, B., Podsypanina, K., Huang, S., Chamorro, M., Zhang, X., Rowlands, T., Egeblad, M., Cowin, P., Werb, Z. et al. (2003). Evidence that transgenes encoding components of the Wnt signaling pathway preferentially induce mammary cancers from progenitor cells. *Proc. Natl. Acad. Sci. USA* **100**, 15853-15858.

Lie, D. C., Colamarino, S. A., Song, H. J., Desire, L., Mira, H., Consiglio, A., Lein, E. S., Jessberger, S., Lansford, H., Dearie, A. R. et al. (2005). Wnt signalling regulates adult hippocampal neurogenesis. *Nature* **437**, 1370-1375.

Liu, B. Y., McDermott, S. P., Khwaja, S. S. and Alexander, C. M. (2004). The transforming activity of Wnt effectors correlates with their ability to induce the accumulation of mammary progenitor cells. *Proc. Natl. Acad. Sci. USA* **101**, 4158-4163.

Mishra, L., Shetty, K., Tang, Y., Stuart, A. and Byers, S. W. (2005). The role of TGF-beta and Wnt signaling in gastrointestinal stem cells and cancer. *Oncogene* **24**, 5775-5789.

Montross, W. T., Ji, H. and McCrea, P. D. (2000). A beta-catenin/engrailed chimera selectively suppresses Wnt signaling. *J. Cell Sci.* **113**, 1759-1770.

Nosho, K., Yamamoto, H., Mikami, M., Taniguchi, H., Takahashi, T., Adachi, Y., Imamura, A., Imai, K. and Shinomura, Y. (2006). Overexpression of poly(ADP-ribose) polymerase-1 (PARP-1) in the early stage of colorectal carcinogenesis. *Eur. J. Cancer* **42**, 2374-2381.

Patrawala, L., Calhoun, T., Schneider-Broussard, R., Zhou, J., Claypool, K. and Tang, D. G. (2005). Side population is enriched in tumorigenic, stem-like cancer cells, whereas ABCG2+ and ABCG2– cancer cells are similarly tumorigenic. *Cancer Res.* **65**, 6207-6219.

Pawlik, T. M. and Keyomarsi, K. (2004). Role of cell cycle in mediating sensitivity to radiotherapy. *Int. J. Radiat. Oncol. Biol. Phys.* **59**, 928-942.

Pinto, D. and Clevers, H. (2005a). Wnt control of stem cells and differentiation in the intestinal epithelium. *Exp. Cell Res.* **306**, 357-363.

Pinto, D. and Clevers, H. (2005b). Wnt, stem cells and cancer in the intestine. *Biol. Cell* **97**, 185-196.

Pinto, D., Gregorieff, A., Begthel, H. and Clevers, H. (2003). Canonical Wnt signals are essential for homeostasis of the intestinal epithelium. *Genes Dev.* **17**, 1709-1713.

Ponti, D., Costa, A., Zaffaroni, N., Pratesi, G., Petrangolini, G., Coradini, D., Pilotti, S., Pierotti, M. A. and Daidone, M. G. (2005). Isolation and in vitro propagation of tumorigenic breast cancer cells with stem/progenitor cell properties. *Cancer Res.* **65**, 5506-5511.

Rattis, F. M., Voermans, C. and Reya, T. (2004). Wnt signaling in the stem cell niche. *Curr. Opin. Hematol.* **11**, 88-94.

Reya, T. and Clevers, H. (2005). Wnt signalling in stem cells and cancer. *Nature* **434**, 843-850.

Reynolds, B. A. and Weiss, S. (1996). Clonal and population analyses demonstrate that an EGF-responsive mammalian embryonic CNS precursor is a stem cell. *Dev. Biol.* **175**, 1-13.

Rodel, F., Hoffmann, J., Distel, L., Herrmann, M., Noisternig, T., Papadopoulos, T., Sauer, R. and Rodel, C. (2005). Survivin as a radioresistance factor, and prognostic and therapeutic target for radiotherapy in rectal cancer. *Cancer Res.* **65**, 4881-4887.

Schmid, I., Cole, S. W., Korin, Y. D., Zack, J. A. and Giorgi, J. V. (2000). Detection of cell cycle subcompartments by flow cytometric estimation of DNA-RNA content in combination with dual-color immunofluorescence. *Cytometry* **39**, 108-116.

Shackleton, M., Vaillant, F., Simpson, K. J., Stingl, J., Smyth, G. K., Asselin-Labat, M. L., Wu, L., Lindeman, G. J. and Visvader, J. E. (2006). Generation of a functional mammary gland from a single stem cell. *Nature* **439**, 84-88.

Sierra, J., Yoshida, T., Joazeiro, C. A. and Jones, K. A. (2006). The APC tumor suppressor counteracts beta-catenin activation and H3K4 methylation at Wnt target genes. *Genes Dev.* **20**, 586-600.

Silva-Vargas, V., Lo Celso, C., Giangreco, A., Ofstad, T., Prowse, D. M., Braun, K. M. and Watt, F. M. (2005). Beta-catenin and Hedgehog signal strength can specify number and location of hair follicles in adult epidermis without recruitment of bulge stem cells. *Dev. Cell* **9**, 121-131.

Sotgia, F., Williams, T. M., Cohen, A. W., Minetti, C., Pestell, R. G. and Lisanti, M. P. (2005). Caveolin-1-deficient mice have an increased mammary stem cell population with upregulation of Wnt/beta-catenin signaling. *Cell Cycle* **4**, 1808-1816.

Stingl, J., Raouf, A., Emerman, J. T. and Eaves, C. J. (2005). Epithelial progenitors in the normal human mammary gland. *J. Mammary Gland Biol. Neoplasia* **10**, 49-59.

Stingl, J., Eirew, P., Ricketson, I., Shackleton, M., Vaillant, F., Choi, D., Li, H. I. and Eaves, C. J. (2006). Purification and unique properties of mammary epithelial stem cells. *Nature* **439**, 993-997.

Tepera, S. B., McCrea, P. D. and Rosen, J. M. (2003). A beta-catenin survival signal is required for normal lobular development in the mammary gland. *J. Cell Sci.* **116**, 1137-1149.

Vong, Q. P., Cao, K., Li, H. Y., Iglesias, P. A. and Zheng, Y. (2005). Chromosome alignment and segregation regulated by ubiquitination of survivin. *Science* **310**, 1499-1504.

Wang, J. and Wynshaw-Boris, A. (2004). The canonical Wnt pathway in early mammalian embryogenesis and stem cell maintenance/differentiation. *Curr. Opin. Genet. Dev.* **14**, 533-539.

Welm, B. E., Tepera, S. B., Venezia, T., Graubert, T. A., Rosen, J. M. and Goodell, M. A. (2002). Sca-1(pos) cells in the mouse mammary gland represent an enriched progenitor cell population. *Dev. Biol.* **245**, 42-56.

Welm, B., Behbod, F., Goodell, M. A. and Rosen, J. M. (2003). Isolation and characterization of functional mammary gland stem cells. *Cell Prolif.* **36** Suppl. 1, 17-32.

Woodward, W. A., Chen, M. S., Behbod, F. and Rosen, J. M. (2005). On mammary stem cells. *J. Cell Sci.* **118**, 3585-3594.

Woodward, W. A., Chen, M. S., Behbod, F., Alfaro, M. P., Buchholz, T. A. and Rosen, J. M. (2007) Wnt/β-catenin mediates radiation resistance of mouse mammary progenitor cells, *Proc. Natl. Acad. Sci. USA* **104**, 618-623.

Xin, L., Lawson, D. A. and Witte, O. N. (2005). The Sca-1 cell surface marker enriches for a prostate-regenerating cell subpopulation that can initiate prostate tumorigenesis. *Proc. Natl. Acad. Sci. USA* **102**, 6942-6947.

Yardy, G. W. and Brewster, S. F. (2005). Wnt signalling and prostate cancer. *Prostate Cancer Prostatic Dis.* **8**, 119-126.

Zhen, H. N., Zhang, X., Hu, P. Z., Yang, T. T., Fei, Z., Zhang, J. N., Fu, L. A., He, X. S., Ma, F. C. and Wang, X. L. (2005). Survivin expression and its relation with proliferation, apoptosis, and angiogenesis in brain gliomas. *Cancer* **104**, 2775-2783.

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