

Oxidative stress induces the acquisition of cancer stem-like phenotype in breast cancer detectable by using a Sox2 regulatory region-2 (SRR2) reporter

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ABSTRACT

We have previously identified a novel intra-tumoral dichotomy in breast cancer based on the differential responsiveness to a Sox2 reporter (SRR2), with cells responsive to SRR2 (RR) being more stem-like than unresponsive cells (RU). Here, we report that RR cells derived from MCF7 and ZR751 displayed a higher tolerance to oxidative stress than their RU counterparts, supporting the concept that the RR phenotype correlates with cancer stemness. Sox2 is directly implicated in this differential H₂O₂ tolerance, since siRNA knockdown of Sox2 in RR cells leveled this difference. Interestingly, H₂O₂ converted a proportion of RU cells into RR cells, as evidenced by their expression of *Luciferase* and *GFP*, markers of SRR2 activity. Compared to RU cells, converted RR cells showed a significant increase in mammosphere formation and tolerance to H₂O₂. Converted RR cells also adopted the biochemical features of RR cells, as evidenced by their substantial increase in Sox2-SRR2 binding and the expression of 3 signature genes of RR cells (*CD133*, *GPR49* and *MUC15*). Lastly, the H₂O₂-induced RU/RR conversion was detectable in a SCID mouse xenograft model and primary tumor cells. To conclude, the H₂O₂-induced RU/RR conversion has provided a novel model to study the acquisition of cancer stemness and plasticity.

INTRODUCTION

Similar to many other types of solid tumors, breast cancer (BC) contains a small subset of cells carrying stem-like features, which are commonly labeled cancer stem cells (CSCs) [1]. In triple-negative BC, CSCs are enriched in the CD44^{high}/CD24^{low} subpopulation [2]. In these tumors, CSCs have been shown to contribute to metastasis, chemoresistance and a worse clinical outcome [3-5]. In estrogen receptor-positive BC, the identity of the CSCs is more controversial. While a few

studies have shown that the CD44^{high}/CD24^{low} is also a marker of CSCs in this subtype of BC, other studies did not find the association between this immunophenotype and cancer stemness [6, 7]. In recent years, the biology of CSCs has become an active area of research, with the hope that the combination of conventional chemotherapy with specific CSC inhibitors can dramatically improve the clinical outcome of BC patients [8]. One of the important features of CSCs is plasticity. Specifically, a few recent studies have demonstrated that the status of CSCs can be acquired [9-11]. Thus, mammary epithelial cells can

be de-differentiated into stem-like cells upon telomerase transfection [12]. Cancer stemness also can be acquired in response to adversity such as oxidative stress. In one study, hypoxia, which is a cause of oxidative stress, was found to increase the expression of stem cell-associated genes and tumorigenic potential in non-stem cells [13]. In another study, it was found that hypoxia results in the acquisition of stemness in glioma cells [14]. Likewise, oxidative stress induced by H₂O₂ has been shown to enhance the stem-like properties in human mesothelioma cells and primary brain-derived neural progenitors cells [15, 16]. Lastly, hypoxia in conjunction with H₂O₂ has been reported to enhance tumor stemness by increasing the fractions of side cell population, which is highly migratory, invasive, and tumorigenic in a variety of solid tumor cell lines [17].

Sox2 (sex determining region Y-box protein 2) is a transcription factor that plays a major role in maintaining the pluripotency of embryonic stem cells and induced pluripotent stem (iPS) cells [18, 19]. Recent studies have shown that Sox2 is aberrantly expressed in several types of solid tumors including breast cancer, lung cancer, prostate cancer, glioblastomas and melanomas [20-24]. Furthermore, Sox2 has been found to correlate with a worse prognosis in cancer patients, including those with BC [25-27]. Our previous studies using a Sox2 reporter construct (SRR2) have revealed a novel dichotomy in BC cells [28]. Specifically, using two estrogen receptor-positive BC cell lines, MCF7 and ZR751, we have found that the vast majority of these cells were reporter unresponsive (RU), despite the fact that these cells strongly expressed the Sox2 protein. In contrast, a relatively small cell subset were reporter responsive (RR), and RR cells were more stem-like than RU cells in terms of phenotype and gene expression patterns [28, 29]. Nonetheless, the relationship between RU and RR cells needs to be further defined. For instance, it will be of interest to determine if RU cells can be induced to acquire the phenotype of RR cells, analogous to the above-mentioned examples in which non-stem cells were found to acquire cancer stemness.

Our first study objective is to assess if RR cells are more tolerant to oxidative stress, a phenotype known to be associated with cancer stemness, than RU cells. During the course of our studies, we also found that RU cells can be induced in response to oxidative stress to acquire SRR2 responsiveness (i.e. to become converted RR cells). In view of these findings, the second objective of this study is to understand the nature of this RU/RR conversion, by performing comparative studies of RU, converted RR cells and native RR cells. This study not only has provided additional support of the biological significance of the RU/RR dichotomy in BC, it also has generated a novel experimental model to study cancer cell plasticity.

RESULTS

RR cells are more tolerant to oxidative stress than RU cells

We first asked if RR cells differ from RU cells with regards to their sensitivity to oxidative stress. We subjected RR and RU cells derived from MCF7 to varying concentrations of H₂O₂ in serum free media. As shown in Figure 1A, the number of viable cells at 2 hours, assessed by using the MTS assay, decreased in a dose-dependent manner in both RR and RU cells, although the IC₅₀ (inhibitory concentration at 50%) was significantly higher in RR cells as compared to RU cells (4.6 mM versus 1.2 mM, $p < 0.01$). The same phenomenon was observed when we used RR and RU cells derived from ZR751, another estrogen receptor-positive BC cell line (Figure 1B).

Sox2 directly contributes to the high tolerance to oxidative stress in BC cells

As we have previously shown that siRNA knockdown of Sox2 can abrogate the SRR2 reporter activity in RR cells derived from MCF7 [28], we asked if siRNA knockdown of Sox2 can result in any significant change to their tolerance to H₂O₂. As shown in Figure 1C, siRNA significantly decreased the IC₅₀ of RR cells in response to H₂O₂ to a level similar to that of RU cells. In comparison, siRNA knockdown of Sox2 did not significantly change the IC₅₀ of RU cells. Thus, Sox2 is directly responsible for the relative high tolerance to oxidative stress in RR cells.

Oxidative stress can induce a conversion of RU cells to RR cells

Our previous studies have suggested that RR cells derived from MCF7 and ZR751 have more stem-like features and tumorigenicity than their RU counterparts [28]. Furthermore, previous studies have shown that cancer stemness can be acquired in response to oxidative stress [15-17]. Thus, we asked if oxidative stress can convert RU to RR cells, a phenomenon that might represent the acquisition of cancer stemness and exemplify the concept of cancer cell plasticity. We tested this possibility by using purified RU cells derived from MCF7. As illustrated in Figure 2A, addition of H₂O₂ to RU cells increased the proportion of GFP-positive cells (i.e. a surrogate marker of the RR phenotype) as early as 1 hour. Specifically, 1 mM of H₂O₂ increased the GFP-positive cells from 3.0% (background level) to 5.4% whereas 5 mM of H₂O₂ increased to 17.3%. As shown in Figure 2B, the proportions of converted RR cells (or

GFP-positive) significantly increased in a time- and dose-dependent fashion. Details of the flow cytometry study results are included in Supplemental Figure 1A. In the same experiment, the cell viability also decreased in a time- and dose-dependent fashion (Figure 2C).

To ensure that the expression of GFP induced by H_2O_2 was genuine, we assessed if the converted RR cells also express luciferase, another readout marker included in the SRR2 reporter. As shown in Figure 2D, the luciferase activity in RU cells treated with H_2O_2 significantly increased in a dose-dependent manner. To highlight the magnitude of this biological change, RU cells treated with 5 mM H_2O_2 for 2 hours showed a 51-fold increase as compared to the negative control. No substantial change

in the GFP expression or luciferase activity was observed in RR cells treated with H_2O_2 (Supplemental Figure 1A and 1B).

To optimize the yield of viable H_2O_2 -induced converted RR cells, we attempted different H_2O_2 treatment protocols. Our optimized protocol involved treatment of RU cells with 0.5 mM H_2O_2 in complete growth media for 6 hours, followed by washing and a 3-day routine culture (also see Methods and Materials). The yield was consistently in the approximate of 4,000 viable converted RR cells per 10,000 RU cells used at the beginning of the experiments. If these cells were cultured for another 4 days (or 7 days in total), the number of viable converted RR cells was found to decrease to approximately 2,000

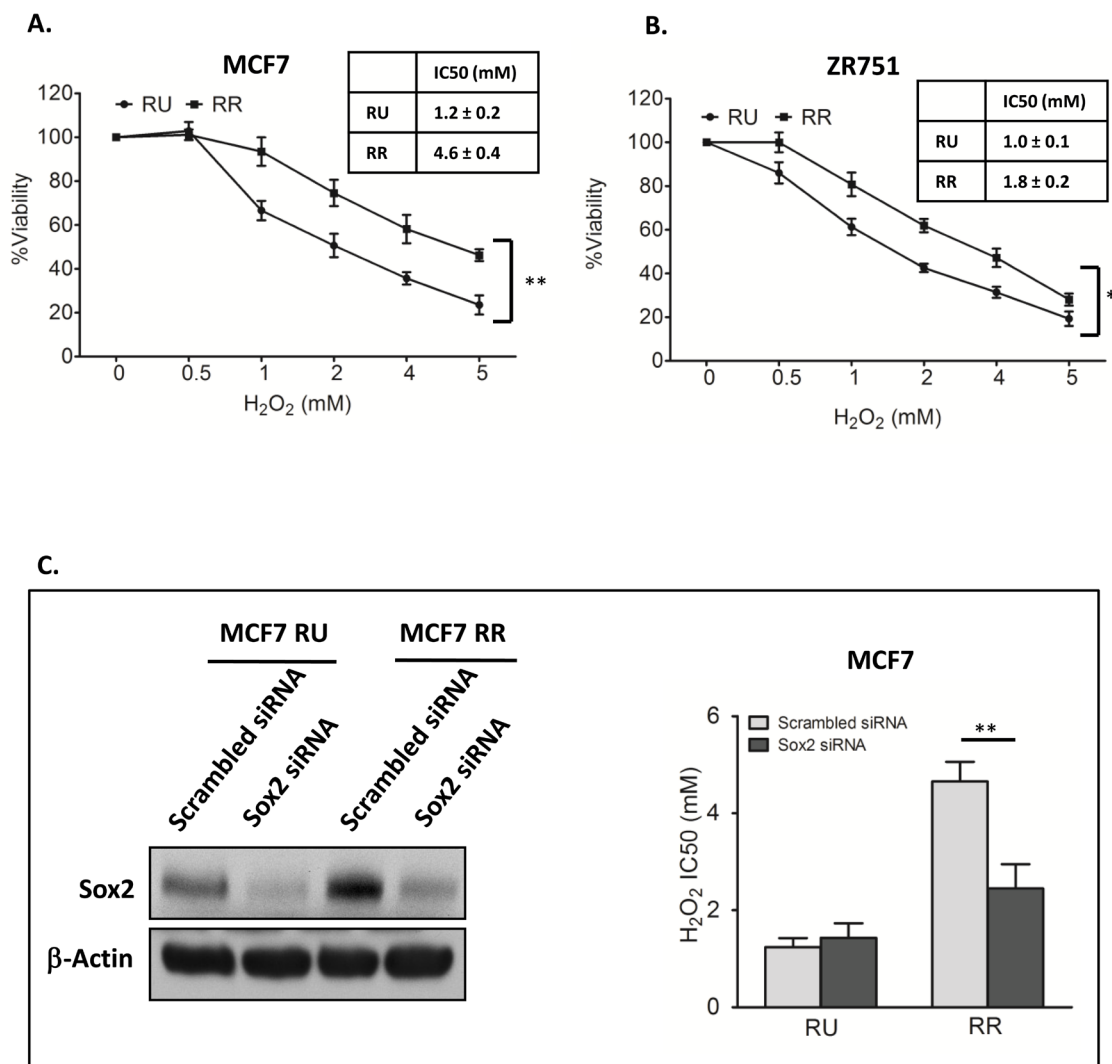


Figure 1: Sox2 activity increased H_2O_2 resistance in BC cells. **A.** RU and RR cells derived from MCF7 were exposed to varying doses of H_2O_2 for 2 hours in serum free media. MTS assay was used to assess the cell viability at the end of the experiments. Data is expressed as percentages of the negative control cells, which were set as 100%. RR cells were significantly more resistant than RU cells (4.6 mM versus 1.2 mM, $p < 0.01$). **B.** The same experiment was repeated using ZR751, which showed similar results (1.8 mM versus 1.0 mM, $p < 0.05$). **C.** RU and RR cells derived from MCF7 cells were transfected with Sox2 siRNA for 48 hours, western blots was done to confirm the knockdown efficiency, as compared to the scrambled siRNA negative control. β -actin serves as a loading control (left panel). These cells were then exposed to varying doses of H_2O_2 for 2 hours in serum free media. Knockdown of Sox2 significantly decreased the IC50 of RR cells, which was at a level similar to that of RU cells.

cells, probably due to the expansion of the RU cells and/or a conversion back to RU cells. These results are illustrated in Figure 3A. With the addition of a ‘booster’ H₂O₂ treatment (0.5 mM) on day 3, the number of viable converted RR cells was significantly increased on

day 7; an approximate of 5,000 cells for every 10,000 RU cells used at the beginning of the experiment was obtained (Figure 3B). Using these protocols, we obtained sufficient numbers of viable converted RR cells for further characterization, to be detailed below.

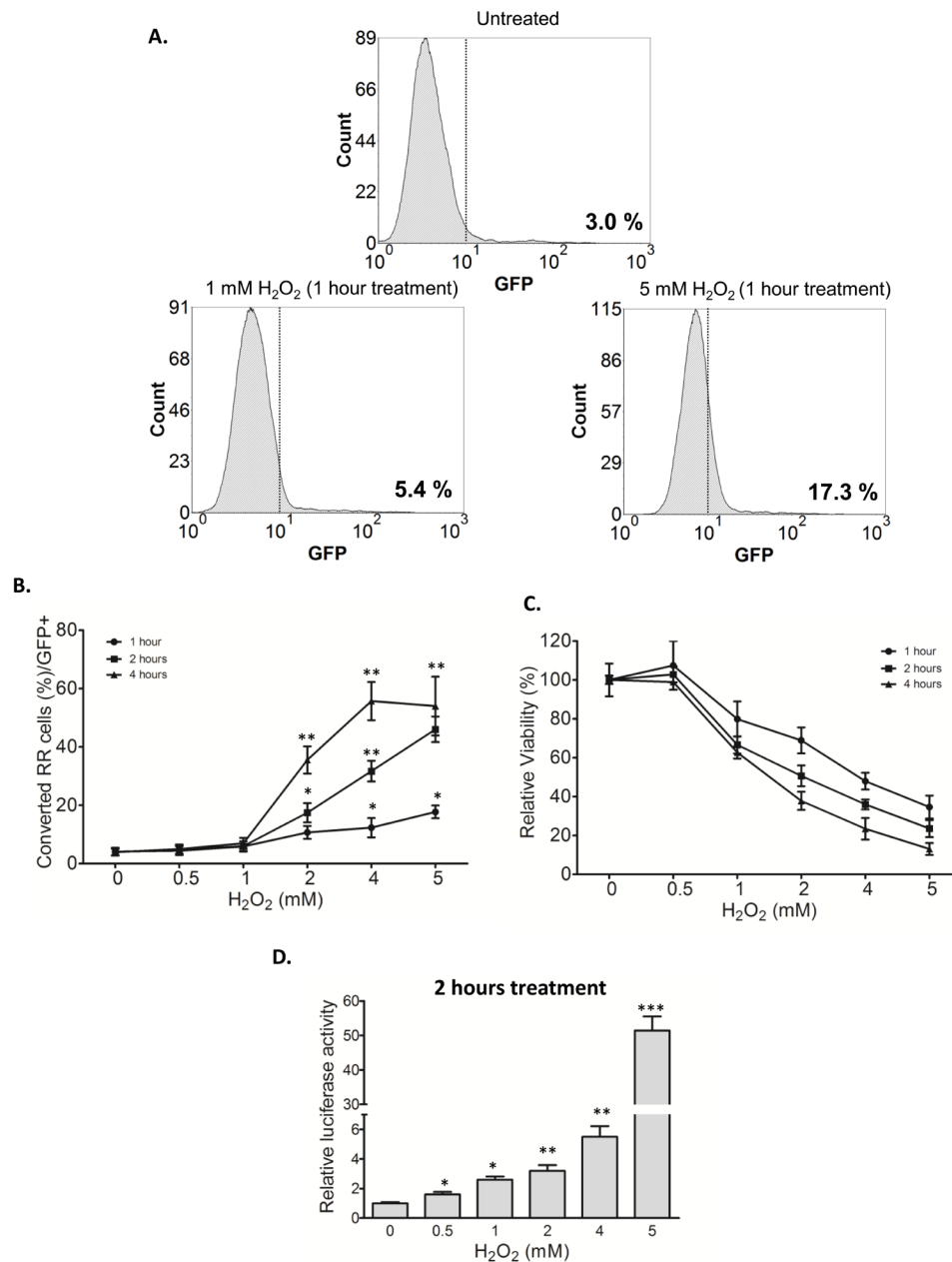


Figure 2: RU cells converted to RR cells upon H₂O₂ challenge. A. RU cells derived from MCF7 were exposed to varying doses of H₂O₂ for 1 hour in serum free media. Flow cytometry was used to assess the expression of GFP in the viable cell populations. Data is expressed relative to untreated negative control cells and the values represent the GFP positive cells. Addition of H₂O₂ to RU cells increased the proportion of GFP-positive cells (from 3.0%, background level to 17.3%). B. Data is expressed as percent of cells with higher GFP expression relative to untreated negative control detected by flow cytometry (called converted RR cells/GFP+) after exposure to varying doses of H₂O₂ for different time points in serum free media. The proportions of converted RR cells (or GFP-positive) significantly increased in a time- and dose-dependent fashion. C. Cells from above experiments were subjected to MTS assay to assess the cell viability at the end of experiments. Data is expressed as percentages of the negative control cells, which were set as 100%. The cell viability decreased in a time- and dose-dependent fashion. D. RU cells derived from MCF7 were exposed to varying doses of H₂O₂ for 2 hour in serum free media. Data is expressed as luciferase activity relative to untreated negative control. RU cells treated with H₂O₂ significantly increased the luciferase activity in a dose-dependent manner.

H₂O₂-induced RR conversion is dependent on the anti-oxidant scavenger pathway

To test that the RR conversion induced by H₂O₂ is directly related to the cellular response to oxidative stress, we experimentally manipulated the anti-oxidant scavenger pathway using N-acetyl-L-cysteine (NAC) and buthionine-sulfoximine (BSO), which can increase or deplete glutathione, respectively [30-32]. As shown in

Figure 4A, NAC pretreatment of RU cells derived from MCF7 significantly attenuated H₂O₂-induced conversion to RR. In comparison, BSO pretreatment increased the RR conversion, as evidenced by the increases in GFP expression ($p = 0.07$) and luciferase ($p < 0.05$). Similar results were obtained with RU cells derived from ZR751 (Figure 4B). The lack of a statistical significance in the change in GFP is likely due to the fact that the GFP read-out is not as sensitive as luciferase.

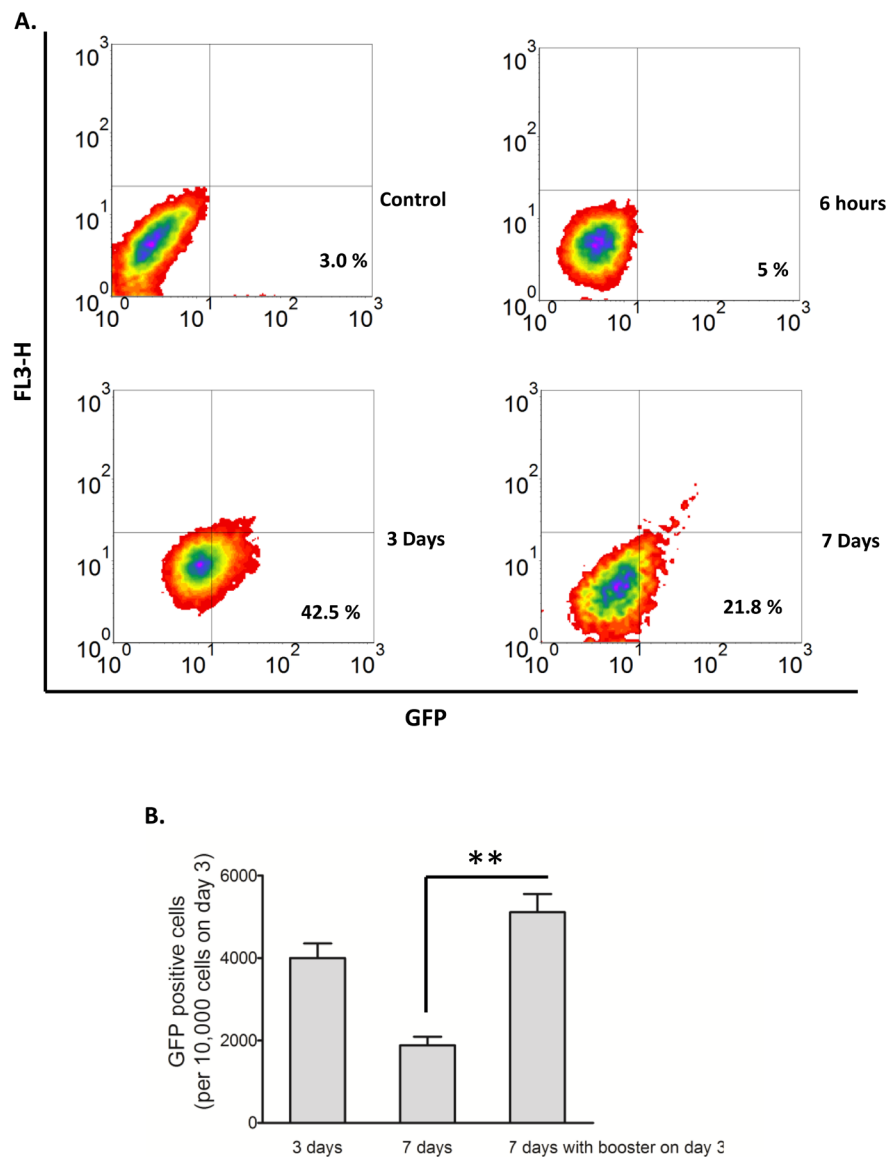


Figure 3: Conversion of RU to RR stayed longer with modified culture conditions. **A.** RU cells derived from MCF7 were exposed to 0.5 mM H₂O₂ for 6 hours in culture media supplemented with 10% FBS and further cultured in fresh media. Flow cytometry was used to assess the expression of GFP in the viable cell populations. Data is expressed relative to untreated negative control cells and the values represent the GFP positive cells. Addition of H₂O₂ to RU cells increased the proportion of GFP-positive cells from 3.0%, background level to 42.5% on day 3, which later decreased to 21.8% on day 7. **B.** The converted RR cells on day 3 of above experiment without separating them from RU cells were re-exposed to 0.5 mM H₂O₂ for 6 hours and further cultured in fresh media. Data were plotted as number of converted RR cells normalized to 10,000 cells at the beginning of re-exposure. With the addition of a 'booster' H₂O₂ treatment (0.5 mM) on day 3, the number of viable converted RR cells was significantly increased on day 7 (5000 cells versus 2000 cells, $p < 0.01$).

Converted RR cells are phenotypically similar to native RR cells

As mentioned, we have previously shown that RR cells are more efficient in forming mammospheres compared to RU cells, a finding that is in keeping with their higher stem-like properties [28]. To evaluate the biological significance of the H₂O₂-induced RR conversion, we tested if converted RR cells are phenotypically similar to the native RR cells with regards to mammosphere formation. As shown in Figure 5A, converted RR cells derived from MCF7 harvested on day 3 were subjected to mammosphere formation assay, and they formed a significantly higher number of

mammospheres than untreated cells ($p < 0.01$). Of note, since only approximately 2/3 of all viable cells on day 3 were converted RR cells (i.e. 1/3 was viable RU cells), the actual increase in the mammosphere formation ability of converted RR cells probably is substantially higher than the observed changes. Similar results were obtained with ZR751 cells (Figure 5B).

To further assess the stemness of converted RR cells, we performed the limiting dilution assay, which has been widely used to quantify the number of cancer stem-like cells. [33, 34]. As shown in Figure 5C, converted RR cells had a mammosphere-forming cell frequency of 0.025, which is significantly higher than that of native RU cells (0.014, $p < 0.05$).

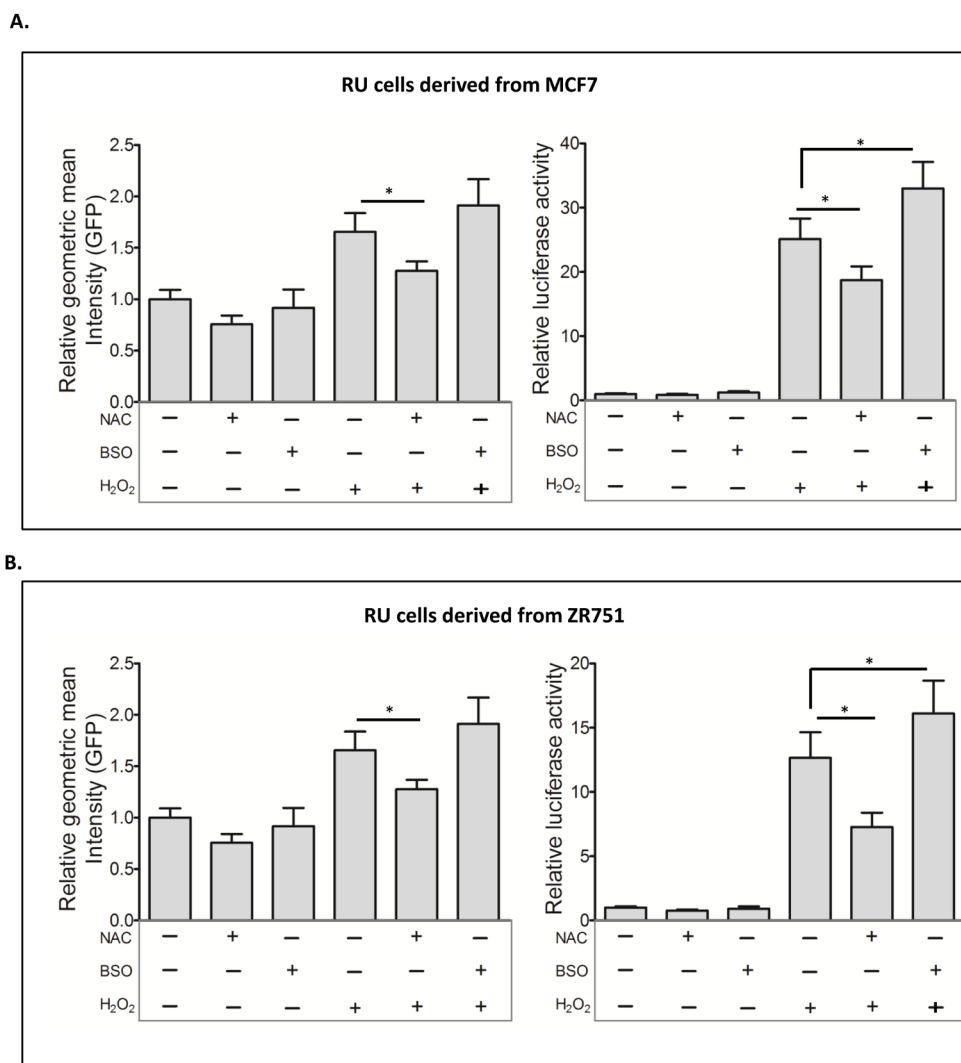


Figure 4: Glutathione modulations affected the RU to RR conversion. **A.** RU cells derived from MCF7 were exposed to NAC and BSO prior to H₂O₂ treatment. Flow cytometry was used to assess the expression of GFP in the viable cell populations. Data is expressed as mean \pm standard error of geometric mean intensity of GFP relative to untreated negative control. NAC pretreatment significantly reduced the H₂O₂ induced GFP expression (2.5 versus 1.7, $p < 0.05$) (left panel). The cells from same experiments were subjected to luciferase assay. Data is expressed as luciferase activity relative to untreated negative control. NAC and BSO pre-treatment significantly attenuated (18.7 versus 25.1, $p < 0.05$) and enhanced (32.9 versus 25.1, $p < 0.05$) the H₂O₂-induced luciferase activity, respectively (right panel). **B.** The same experiment was repeated using RU cells derived from ZR751, which showed similar results.

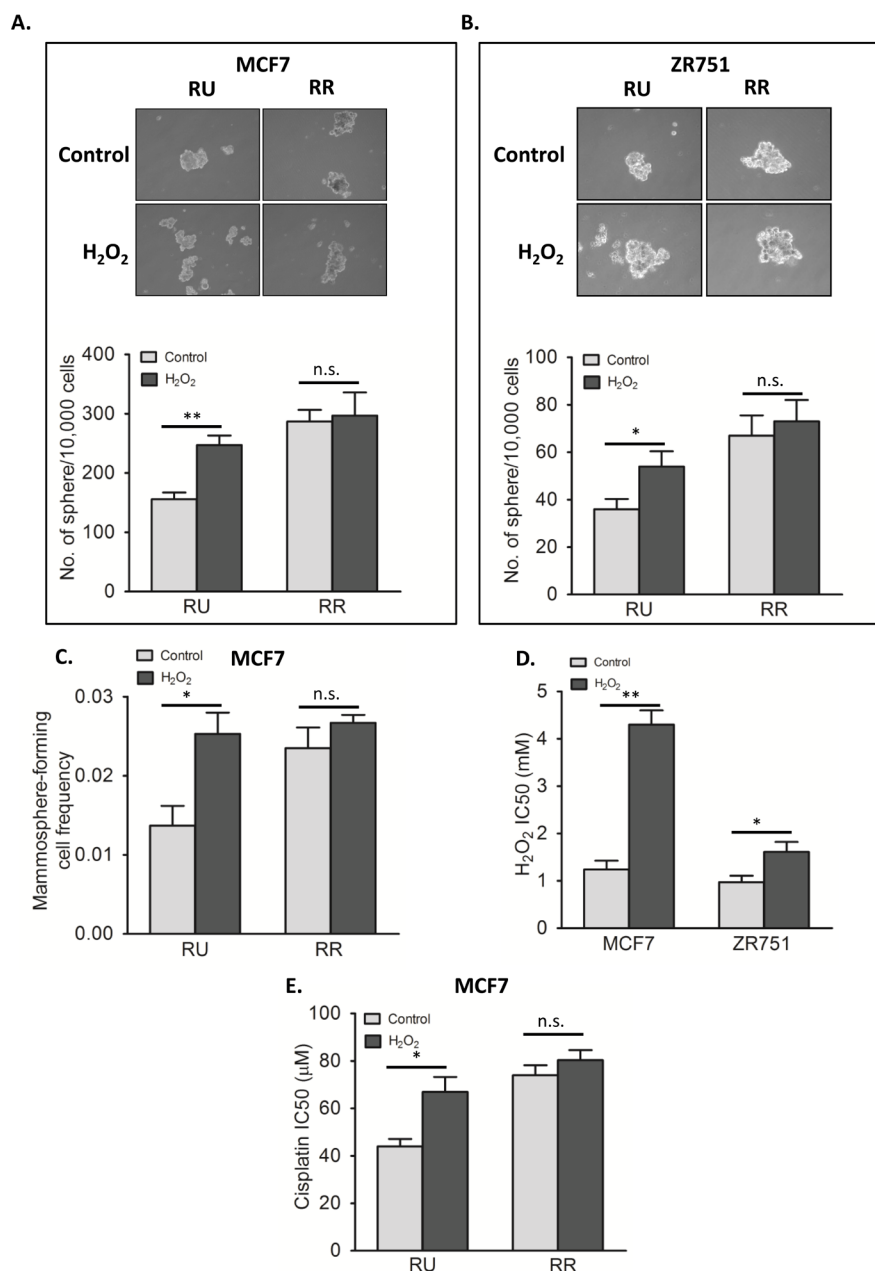


Figure 5: Converted RR cells acquired phenotypes of RR cells. **A.** RU and RR cells derived from MCF7 were exposed to 0.5 mM H₂O₂ for 6 hours in complete culture media and further cultured for 3 days in fresh media. The harvested cells were subjected to mammosphere formation assay. Data is expressed as mean \pm standard error of number of spheres per 10000 cells. RU cells upon H₂O₂ treatment (converted RR cells) showed significantly higher number of sphere as compared to untreated control (247 versus 156 sphere, $p < 0.01$). RR cells did not show any significant change in sphere formation upon H₂O₂ treatment. The representative mammosphere pictures are shown (upper panel). **B.** The same experiment was repeated using RU and RR cells derived from ZR751, which showed similar results (54 versus 36 sphere, $p < 0.05$ in case of RU cells). **C.** RU and RR cells derived from MCF7 were exposed to 0.5 mM H₂O₂ for 6 hours in complete culture media and further cultured for 3 days in fresh media. The harvested cells were seeded in 10 seeding densities ranging from 1 to 400 cells/well in 96-well plate in 6 replicates each. Data is expressed as mammosphere-forming cell frequency obtained by using extreme limiting dilution analyses. RU cells upon H₂O₂ treatment (converted RR cells) showed significant increase in mammosphere formation frequency (0.025 versus 0.014, $p < 0.05$). RR cells did not show any significant change. **D.** Converted RR cells and RU cells derived from MCF7 and ZR751 were exposed to varying doses of H₂O₂ for 2 hours with serum free media followed by assessment of cell viability by MTS assay. Data is expressed as mean \pm standard errors of IC50 values calculated by using Graphpad Prism software. Converted RR cells were significantly more resistant than RU cells (in-case of MCF7, 4.3 mM versus 1.2 mM, $p < 0.01$; in-case of ZR751, 1.6 mM versus 1.0 mM, $p < 0.05$). **E.** The same experiment was performed with varying doses of cisplatin for 24 hours with RU and RR cells derived from MCF7, which showed similar results. Converted RR cells were significantly more resistant to cisplatin than RU cells (66.9 μ M versus 44 μ M, $p < 0.05$). RR cells did not show any significant change upon H₂O₂ exposure.

We then asked if converted RR cells acquired the resistance towards H_2O_2 . As shown in Figure 5D, converted RR cells from both MCF7 and ZR751 showed significantly higher IC50 value as compared to untreated RU cells (MCF7; 4.3 mM versus 1.2 mM, $p < 0.01$, and ZR751; 1.6 mM versus 1.0 mM, $p < 0.05$). We also evaluated the effectiveness of cisplatin on native RU as well as converted RR cells derived from MCF7. As shown in Figure 5E, the IC50 of converted RR cells was significantly higher than RU cells (66.9 μ M versus 44.0 μ M, $p < 0.05$).

Converted RR cells are biochemically similar to RR cells

We then asked if converted RR cells are biochemically similar to native RR cells. We previously reported that the expression of several cancer stemness-related genes such as *PROM1* (*CD133*), *GPR49* (*Lgr5*) and *MUC15* was significantly higher in RR cells as compared to RU cells derived from BC cell lines as well as primary tumor samples [29]. Thus, we compared the expression of these signature 3 genes between converted RR cells and native RU cells. As shown in Figure 6A-C,

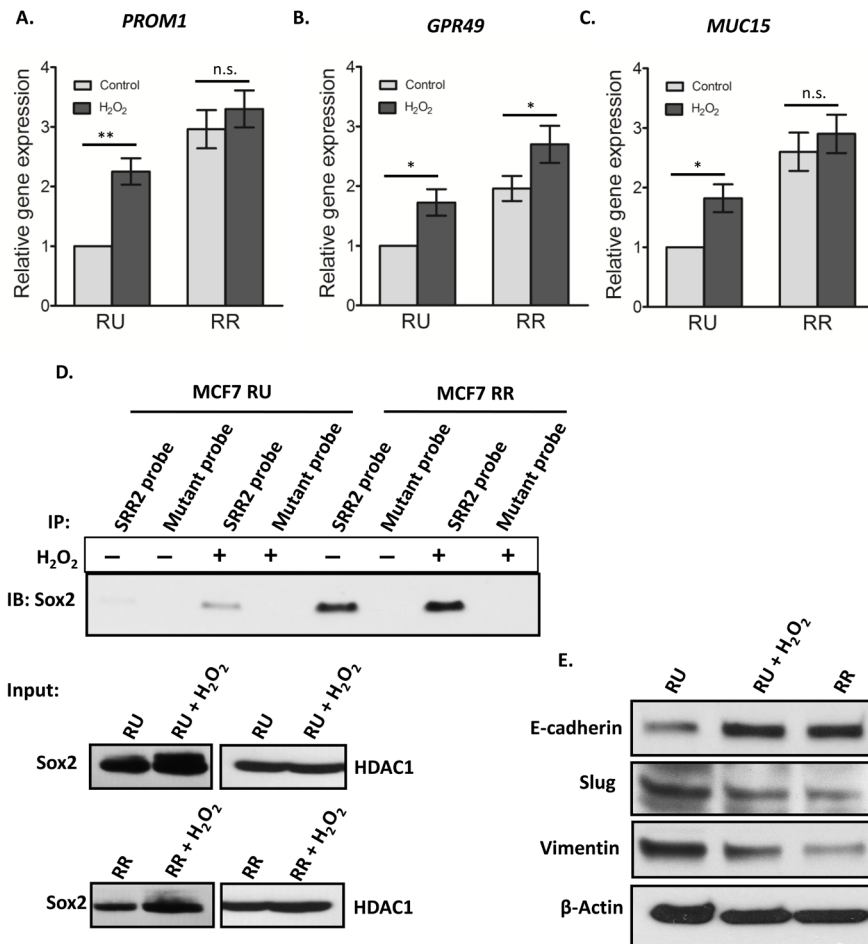


Figure 6: Converted MCF7 RR cells biochemically similar to RR cells. RU and RR cells derived from MCF7 were exposed to 0.5 mM H_2O_2 for 6 hours in complete culture media and further cultured for 3 days in fresh media. The mRNA expression levels of *PROM1* **A.**, *GPR49* **B.** and *MUC15* **C.** were measured by qRT-PCR by using specific primers. The data showed the significant increase in the mRNA expression of all three genes upon H_2O_2 treatment in the RU cells. RR cells did not show any significant change in gene expressions except in case of *GPR49*. **D.** Cell lysates derived from the nuclear fraction of H_2O_2 treated RU and RR cells were incubated with the biotinylated SRR2 probe. A similar amount of lysate proteins derived from treated and untreated RU and RR cells was used. The SRR2 probe-protein complexes were then captured by streptavidin beads. By western blots, Sox2 was prominently detectable in H_2O_2 treated RU cells contrary to very low to no detection in untreated RU cells. RR cells did not show any noticeable change in Sox2 binding to SRR2. Mutation of the SRR2 completely abrogated the binding of Sox2 to the probe (top panel). The western blot of input samples were evaluated for the quality of lysates of nuclear fractions by using an anti-Sox2 antibody. HDAC-1 was used as loading control (bottom panel). **E.** Cell lysates of H_2O_2 treated and untreated cells were analyzed by western blot. Protein level of E-cadherin was prominently increased, and Slug and vimentin were decreased after H_2O_2 treatment in RU cells in comparison to that of untreated RU cells. RR cells showed similar pattern as converted RR cells.

converted RR cells had significantly higher expressions (~2 fold) in *PROM1*, *GPR49* and *MUC15* as compared to the native RU cells ($p < 0.05$).

To further evaluate the changes in biochemical properties of Sox2, we evaluated the Sox2 binding to the SRR2 reporter. As shown by western blots, converted RR cells showed substantially higher Sox2 binding to the SRR2 as compared to native RU (Figure 6D). RR cells did not show any appreciable change in the Sox2 binding to SRR2 reporter upon H_2O_2 exposure. Western blot experiments showed that the total Sox2 protein level was increased in both converted RR cells and native RR cells treated with H_2O_2 . In parallel with the previously published results [28], which showed that enforced

expression of Sox2 in native RU cells did not induce GFP or luciferase expression, we found that transfection of Sox2 into RU cells did not result in an increase in Sox2-probe binding (not shown).

We also asked if the observed RU/RR conversion in response to oxidative stress is linked to epithelial mesenchymal transition (EMT), a process that is also known to be associated with an acquisition of cancer stemness [35]. As shown in Figure 6E, converted RR cells derived from MCF7 showed an appreciable increase in the protein level of E-cadherin and a corresponding decrease in the level of vimentin and Slug, a well-known negative regulator of E-cadherin (Figure 6E). This pattern of protein expression was similar to that of native RR cells.

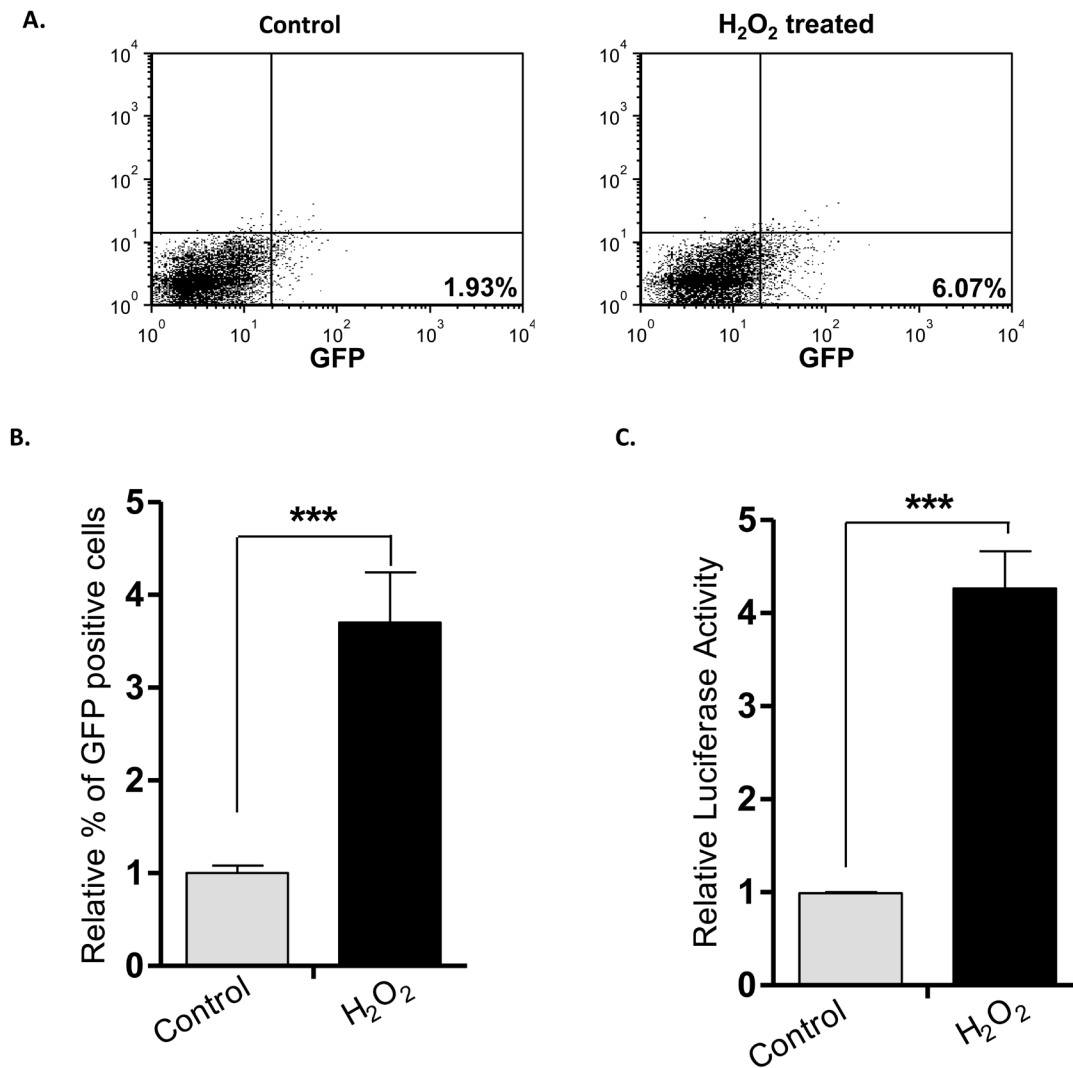


Figure 7: H_2O_2 -induced RU/RR conversion *in-vivo*. RU cells derived from MCF7 were injected subcutaneously in both flanks of SCID/Beige female mice. When tumor size reached 0.5mm, H_2O_2 (0.1mM) was injected intra-tumorally into one flank whereas xenograft on the opposite flank was left untreated. After 24 hours of H_2O_2 injection, tumors were subjected to flow cytometry and luciferase activity. (A) Flow cytometry results of a representative mouse are illustrated, and we observed a higher proportion of GFP-positive tumor cells in the xenograft treated with H_2O_2 , as compared to the untreated xenograft on the opposite flank. (B. & C.) The average relative % of GFP-positive cells and luciferase activity of the 5 H_2O_2 -treated xenografts were significantly different from those of their untreated counterparts ($***p < 0.001$).

H₂O₂-induced RR conversion in a mouse xenograft model

To address the question of whether the H₂O₂-induced RU/RR conversion occurred in an *in-vivo* experimental system, we employed a mouse xenograft model. Thus, we injected RU cells derived from MCF7 cells into the mammary fat pad of mice (n=5). As the tumor reached 0.5 mm in the greatest dimension, H₂O₂ (0.1 mM) was injected intra-tumorally. Animals were euthanized 24 hours after the injection and the harvested tumor cells were examined for GFP and luciferase expression. As illustrated in Figure 7A-7B, H₂O₂-treated xenograft tumor cells showed a significant increase in GFP-positive cells ($p < 0.001$) and luciferase activity ($p < 0.001$), compared to untreated xenograft tumor cells (Figure 7C).

H₂O₂-induced RR conversion also occurred in primary patient samples

Lastly, we examined if the H₂O₂-induced RU/RR conversion occurs in primary patient samples. We purified RU cells derived from two patient samples and treated the cells with 0.5 mM of H₂O₂. We then performed flow cytometry and luciferase assay 6 hours after H₂O₂ treatment. As illustrated in Figure 8A, flow cytometric analysis revealed an appreciable increase in GFP-positive cells (i.e. from 9% to 19%) in one patient sample. Luciferase assay in triplicate were performed for both samples, and we found a significant increase in luciferase activity in response to H₂O₂ in both samples ($p < 0.001$) (Figure 8B).

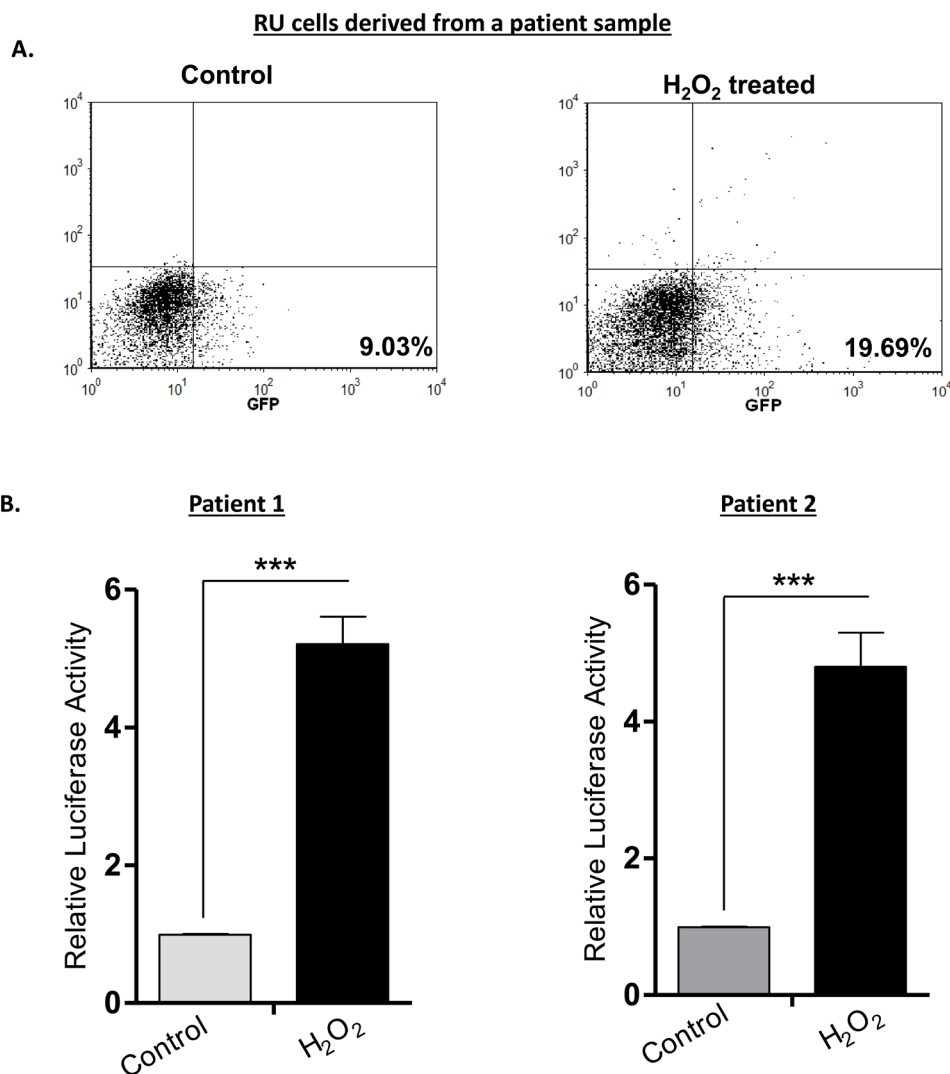


Figure 8: RU cells from patient samples can also convert to RR cells upon H₂O₂ treatment. Tumor cells from 2 primary patient breast tumor samples were infected with the lentiviral SRR2 reporter and RU cells were sorted using flow cytometry. Purified RU cells were treated with H₂O₂ (0.5 mM) for 6 hours and analyzed for GFP and luciferase activity. **A.** Flow cytometry results of a representative sample are illustrated, and we observed an increase in the proportion of GFP-positive tumor cells after H₂O₂ treatment. **B.** The luciferase activity of both tumor samples treated H₂O₂ was significantly higher than that of the negative (untreated) controls.

DISCUSSION

Oxidative stress is related to the generation of various reactive oxygen species (ROS), such as hydroxyl radical (OH^\cdot), superoxide anions (O_2^\cdot), and hydrogen peroxide (H_2O_2), all of which are produced during normal aerobic metabolism [36]. Multiple cellular mechanisms exist to minimize oxidative stress and its detrimental effects to the cells [37]. Oxidative stress and ROS are highly relevant in cancer biology. Since tumors frequently outgrow their blood supply, cancer cells are constantly subjected to glucose deprivation and hypoxia, both of which are strong inducers of ROS and oxidative stress [38, 39]. ROS and oxidative stress have been shown to increase anchorage-independent growth, motility, and cell survival in cancer [40-42]. There is also evidence that ROS and oxidative stress can increase cancer stemness [15-17]. Importantly, CSCs are known to be more tolerant to oxidative stress, which correlates well with the observation that CSCs contain a significantly lower ROS level compared to the non-stem cell population [43]. These published findings prompted us to test if RR cells, believed to be more stem-like than RU cells, are more tolerant to oxidative stress.

Our observation that RR cells were more tolerant to H_2O_2 than RU supports that RR cells are more stem-like than RU cells. Similar to CSCs, normal stem cells are also known to be more tolerant to oxidative damage through several mechanisms, including (a) promotion of glycolysis, (b) low oxygen consumption, (c) decreased mitochondrial mass, (d) down-regulation of ROS-producing enzymes, and (e) upregulation of ROS scavengers [7]. A recent study discovered that the CSC marker CD44, in particular the CD44v isoform, interacts and stabilizes xCT, a subunit of a glutamate-cystine transporter, to promote the uptake of cystine for the synthesis of the master antioxidant glutathione [44]. While the exact mechanism(s) underlying the oxidative stress tolerance of RR cells needs to be further investigated, our studies have provided evidence that Sox2 plays a direct role, since siRNA knockdown of this protein significantly decreased the tolerance of RR cells to the level of RU cells. At the same time, siRNA knockdown of Sox2 in RU cells, which express a similar Sox2 protein level as RR cells [28], did not result in any significant change to their tolerance to H_2O_2 . Taken together, it is highly likely that the differential sensitivity to H_2O_2 between RR and RU cells is directly attributed to their differential Sox2 transcriptional status, the defining criterion used to separate RR from RU cells.

One key observation of this study is related to the H_2O_2 -induced conversion from RU cells to RR cells (i.e. converted RR cells). As indicated in our results, this conversion is not restricted to BC cell lines used *in-vitro*, as we found a similar phenomenon in BC cell lines *in-vivo* as well as primary BC tumor cells. Considering the concept that RR cells are more stem-like than RU

cells, this observation suggests that H_2O_2 can induce the acquisition of cancer stemness, a concept that have been previously suggested. For instance, it has been found that irradiation can enrich cancer stem-like cells in MCF7 [45, 46]. Furthermore, compared to tumor samples at diagnosis, post-chemotherapy tumor samples often contained a higher proportion of cancer stem-like cells that carry enhanced mammosphere forming efficiency and metastatic potential [4, 47]. *In vitro* experiments also have shown that hypoxia, which is known to increase oxidative stress, can induce the expression of CSC markers in BC [48].

We have previously shown that Sox2 binds to largely exclusive genes sets between RU and RR cells; as a result, there are substantial differences in their gene expression patterns [29]. In the same study, we also have highlighted the differential expression of three Sox2 downstream target genes, including *PROM1* (encoding CD133), *GPR49* (encoding Lgr5) and *MUC15*, all of which are higher expressed in RR cells. These findings correlate with our observation that Sox2 binds to SRR2 in RR cells but not RU cells [28]. In the current study, we found that Sox2 in converted RR cells can bind to SRR2. Correlating with this finding, converted RR cells adopted the gene expression pattern of the three Sox2 downstream target genes of native RR cells. Specifically, the expression level of *PROM1*, *GRP49* and *MUC15* were all up-regulated in converted RR cells. Taken together, our observations support the concept that the H_2O_2 -induced RU/RR conversion involves the re-activation of the Sox2 transcriptional activity and a genuine biochemical re-programming, leading to a stem-like gene expression pattern. The significance of these findings can be better appreciated when the functions of these Sox2 downstream target genes are considered. The use of both CD133 and Lgr5 as surrogate markers for CSCs in BC and other cancer types has been demonstrated previously [49-51]. Lgr5 is a member of the G-protein-coupled receptor family of proteins, a target of Wnt signaling, and it is a marker of stem cells in various adult organs in humans [52]. CD133, a member of the prominin family, is expressed in normal progenitor cells as well as CSCs [53]. The role of CD133 in a wide range of cellular processes including self-renewal, tumorigenesis, metastasis, chemo-resistance, autophagy and apoptosis has been recently reviewed [54]. Regarding MUC15, it was recently described to be a Sox2 downstream target and a CSC marker in estrogen receptor-positive BC cell lines and primary tumors [29]. MUC15, a highly glycosylated extracellular protein, has been found to be elevated in the tumor initiating cell populations in colon cancer, hepatocellular carcinoma and papillary thyroid carcinoma [55-58].

EMT is a process by which cancer cells can acquire features of stemness. In this regard, it has been shown that EMT can be induced by a variety of stimuli including H_2O_2 . For instance, *in vitro* studies using treatment of

human mesothelioma cells using H₂O₂-induced EMT, as evidenced by the increased expression of vimentin, slug and twist1, as well as the decreased expression of E-cadherin [15]. Furthermore, ectopic expression of either Twist or Snail, both of which are key regulators of EMT, promotes the expression of CSC markers and mammosphere formation in human mammary epithelial cells [35]. With this background, we asked if our observed RU/RR conversion is related to EMT. Based on the expression pattern of E-cadherin, slug and vimentin, we concluded that the RU/RR conversion correlates with mesenchymal epithelial transition (MET) rather than EMT. This finding is somewhat surprising to us, but it is not completely contradictory to the literature. Specifically, during the reprogramming of mouse fibroblasts to iPS cells, it has been reported that Sox2 (partnered with Oct3/4) up-regulates E-cadherin by suppressing the expression of its negative regulators, Snail and Klf4 [59]. Moreover, inhibition of E-cadherin during the iPS transformation was found to decrease the yield of iPS cells [60]. While further studies need to be done in clarifying the role of EMT or MET in the context of RU/RR conversion, it is tempting to speculate that RU/RR conversion may involve some of the biochemical modulations implicated in the induction of iPS cells. In support of this concept, Sox2 is indeed one of the 4 important iPS factors initially reported by *Takahashi et. al.* [19].

The concept of “tumor cell plasticity” has been recently reviewed [61]. Sox2 has been implicated in the process in which non-stem cells convert into CSCs. In glioblastoma, Sox2 has been reported to regulate the expression of key genes involved in cancer stemness; Sox2 knockdown abolished de-differentiation and the acquisition of CSC phenotype [62]. In colorectal cancer, introduction of a set of defined factors (Oct3/4, Sox2 and Klf4) showed an enhancement in CSC properties such as sphere formation capability, expression of CSC marker genes, chemo-drug resistance, and tumorigenicity [63]. Sox2 also has been reported to be aberrantly expressed in pancreatic cancer and contributes to cell proliferation and stemness/dedifferentiation through the regulation of a set of genes controlling G1/S transition [64]. In BC, it was found that over-expression of Sox2 increased mammosphere formation whereas knockdown of Sox2 delayed tumour formation in xenograft tumour initiation models [65]. Recently, H3K9 demethylation and Sox2 gene expression have been reported to be essential for the elevation of self-renewal capability of differentiated melanoma cells [66].

From the technical point of view, we believe that our findings related to the RU/RR conversion are highly important and relevant to the future studies of CSCs, particularly with respect to the acquisition of cancer stemness in response to various stimuli. In contrast with the conventional EMT experiments, detection of increased stemness can be readily detectable by using

flow cytometry in a quantitative fashion. Since the Sox2 reporter also contains the luciferase gene, localization and tracking of stem-like cells (i.e. RR cells) in animal xenograft studies can be achieved by *ex-vivo* imaging. With this model, one can readily isolate converted RR cells and delineate the molecular mechanisms that are responsible for the conversion and acquisition of cancer stemness.

In conclusion, our findings have further supported the biological significance of the RU/RR dichotomy and the concept that RR cells are more stem-like than RU cells. We believe that the H₂O₂-induced RU/RR conversion is a valuable model to study stress-induced acquisition of cancer stemness.

MATERIALS AND METHODS

Cell lines, cell culture, and reagents

Parental ER+breast cancer cell lines MCF7 and ZR751 were purchased from American Type Culture Collection (ATCC, Rockville, USA). The RU and RR cell lines are derived as previously described [28]. The RU and RR cells have been purified based on GFP expression from the parental cells stably infected with the Sox2 reporter (SRR2). Since the SRR2 reporter carries dual reporter genes encoding luciferase and green fluorescence protein (GFP), RR cells but not RU cells stably show luciferase activity and GFP expression over time [28]. RU and RR cells are maintained and culture separately for our studies, and keep their distinct phenotypes [28]. All the above mentioned cell lines were maintained in high glucose Dulbecco's Modified Eagle Medium (DMEM) (Life Technologies, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS) (Life Technologies, Grand Island, NY, USA) and 10 µg/ml puromycin (in-case of SRR2 stable cells) (Life Technologies, Grand Island, NY, USA). H₂O₂ (Fisher scientific, ON, Canada), N-acetyl-L-cysteine (NAC) and L-buthionine-S,R-sulfoximine (BSO) (Sigma-Aldrich Canada, Oakville, ON, Canada) were freshly prepared.

H₂O₂, NAC and BSO treatment

About 5 × 10⁵ cells were seeded into a 6-well plate. Cells were treated with 0.5 mM to 5 mM H₂O₂ in serum free media for 1, 2 and 4 hours. These cells were subjected to cell proliferation assay, luciferase reporter assay and flow cytometry for assessment of GFP expression. To assess the phenotypic and biochemical changes after H₂O₂ treatment, cells were exposed to 0.5 mM H₂O₂ in DMEM (Life Technologies, Grand Island, NY, USA) supplemented with 10% FBS (Life technologies, Grand Island, NY, USA) for 6 hours and further cultured in fresh

media supplemented with FBS under an atmosphere of 5% CO₂ at 37 °C for 3 days. Cells were then harvested and subjected to cell proliferation assay, mammosphere formation assay, cisplatin as well as H₂O₂ resistance assay and biochemical characterizations. To assess if the glutathione modulation affects the H₂O₂ effect, cells were initially treated with 100 μM of BSO for 48 hours and 10 mM NAC for 24 hours, followed by exposure to 5 mM H₂O₂ for 2 hours in serum free media. These cells were subjected to luciferase assay and flow cytometry for the evaluation of GFP expression.

Nuclear cytoplasmic fractionation

Nuclear and cytoplasmic proteins of cells were extracted using the NE-PER Protein Extraction Kit (#78833, Thermo Scientific, USA) according to the manufacturer's protocol. For western blotting analyses, histone deacetylase 1 (HDAC1, Santa Cruz biotechnology Inc. USA) were used as marker for nuclear fraction.

SRR2-pull down for Sox2 binding

Biotinylated SRR2 wild type and mutant probes were synthesized from Integrated DNA Technologies, USA. SRR2 sequence is 5'-AAGAATTTCCCGGGCTCGGGCAGCCATTGTG ATGCATATAGGATTATTCACGTGGTAATG-3' in which the Sox2 consensus sequence is underlined. An equal amount of nuclear protein (300 μg) was incubated with 3 pmole of either mutant or wild-type SRR2 probe for 30 min at room temperature. Then 75 μl streptavidin beads were added and the samples were incubated overnight by rotation at 4°C. The beads were then washed 3 times with cold PBS. The bound proteins were eluted by boiling at 95°C for 5 min with 20 μL of SDS protein buffer and then processed for western blotting.

Western blotting

Western blot analyses were performed as previously described [29]. All antibodies were diluted in 5% bovine serum albumin (BSA) in Tris buffered saline and 0.1% Tween-20 (TBST): anti-Sox2 (1:1000, Cat. #3579), anti-Vimentin (1:1000, Cat. #5741), anti-Slug (1:500, Cat. #9585), and anti-E-cadherin (1:1000, Cat. #3195) were purchased from Cell Signaling Technologies, Danvers, MA, USA. Anti-β-actin (1:5000, # 47778) was purchased from Santa Cruz Biotechnology, USA. The expression of beta-actin served as the loading control for all western blots.

siRNA knockdown of Sox2

Sox2 siRNAs (SMARTpool: ON-TARGETplus Sox2 siRNA, Dharmacon, Fisher Scientific, ON, Canada) or scrambled (Scr) siRNAs (ON-TARGETplus Non-targeting Pool, #477C20, Dharmacon, Fischer Scientific, ON, Canada) at 40 pmol per rxn (20 nM final concentration) and 5 μL of Lipofectamine RNAiMAX (Life Technologies, Grand Island, NY, USA) were added to 0.5 mL of OptiMEM media (Life Technologies, Grand Island, NY, USA) and reverse transfected to 0.8x10⁶ cells in normal culture medium in a 6-well plate format. Cells were incubated with siRNAs for 48 hours before harvesting.

Cell proliferation assay, luciferase reporter assay and flow cytometry

Cell proliferation was determined using the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) assay (#G3580, Promega, Madison, WI, USA) according to the manufacturer's instructions. Luciferase reporter assay was performed using luciferase assay system kit (#E4530, Promega, Corporation, Madison, USA) according to the manufacturer's protocol, plated on Costar white polystyrene opaque 96-well plates (#3912, Corning, NY, USA) and analyzed on the FLUOstar Omega multi-mode microplate reader (BMG Labtech, Ortenburg, Germany). Flow cytometry analyses were performed as previously described [28].

Limiting dilution and mammosphere formation assay

Mammospheres were seeded and cultured as previously described [67]. Briefly, cells were trypsinized and passed through a 40 μm cell strainer (BD, Franklin Lakes, New Jersey, USA) and seeded into ultra-low adherent plates (Corning, NY, USA) in Mammocult media (StemCell Technologies, Vancouver, BC, Canada) as per manufacturer's instructions. Mammosphere larger than 60 μm were counted 5-7 days after seeding. Limiting dilution assay has been used as a gold standard for the assessment of cancer stem cells [33, 34]. To perform these experiments, cells were seeded in 96-well low-adherent plate (Corning, NY, USA) at 10 limiting dilutions ranging from 1 to 400 cells. Each dilution had 6 replicates, and each well was scored for presence or absence of mammosphere after 5-7 days. Data were analyzed using the Extreme Limiting Dilution Analysis (ELDA) software for three independent experiments [68].

RNA extraction, cDNA synthesis, quantitative reverse transcription PCR (q-RT-PCR)

Total RNA extraction was performed with the Qiagen RNeasy Kit (Qiagen, Canada) according to the manufacturer's protocol: 1 µg of RNA was reverse transcribed using oligo-dT and superscript II (Life Technologies, Grand Island, NY, USA) according to the manufacturer's protocol. 1 µL of the resulting cDNA mixture was added to the Platinum SYBR Green qPCR SuperMix-UDG with Rox (Life Technologies, Grand Island, NY, USA) and amplified with target gene-specific primers. Please see Additional file 2: Table S1 for list of primer sequences. All genes of interest are normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) transcript expression levels.

SCID mouse xenograft studies

Five SCID/Beige mice purchased from Taconic (Hudson, NY) were kept virus- and antigen-free and housed in the Alberta Institute for Viral Immunology biocontainment facility at University of Alberta. Our experimental protocols had been reviewed and approved by the institutional Animal Welfare Committee, and animals care was provided in accordance with the 1993 guidelines of the Canadian Council on Animal Care. RU cells derived from MCF7 were re-suspended in 1:1 Matrigel/PBS. All mice at 8-10 weeks old received two subcutaneous injections of 5×10^6 RU cells in 200 µL of 1:1 Matrigel/PBS solution one on either flank side. A slow release pellet of estradiol (1.7 mg, Innovative Research) was implanted subcutaneously at the nape of the neck to promote tumor growth. When tumor size reached ≥ 5 mm, H_2O_2 (0.1mM) was administered by intra-tumoral injection (50 µL) on one flank, while the tumor on the opposite flank was untreated. After the H_2O_2 injection (24 hours), animals were euthanized and tumor cells were dissociated using the Macs tumor dissociation kit (Miltenyi Biotec, Auburn, CA) as per the manufacturer's protocol. Tumors cells were then evaluated for SRR2 reporter activity by using flow cytometry and Luciferase activity.

Patient samples and experimental manipulations

Two primary patient tumors were processed using a protocol described previously [29]. Briefly, BC cells were harvested by using the Cancer Cell Isolation Kit (Panomics Solutions, Affymetrix, Santa Clara, CA) as per the manufacturer's protocol. Isolated tumor cells were infected with our modified lentiviral Sox2 GFP-RFP dual-color reporter, SRR2-mCMV-GFP-EF1-RFP. RU cells (RFP+/GFP-) were sorted using flow cytometry. Sorted RU cells were cultured for 24 hours in RPMI media

followed by treatment with H_2O_2 (0.5 mM). After 6 hours of H_2O_2 treatment, tumors cells were analyzed for GFP expression and Luciferase activity.

Statistical analyses

Data are expressed as mean \pm standard errors. IC50 was calculated by Graphpad Prism (La Jolla, CA). Paired Student's T-tests were used for statistical analyses of experiments throughout, where $p < 0.05$ is denoted by *, $p < 0.01$ is denoted by **, and $p < 0.001$ is denoted by ***. All graphs represent the average of at least 3 independent experiments with triplicates.

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Authors' contributions

KG and NG designed and performed experiments, analyzed data, and wrote the manuscript. HZ, AA and HA performed portions of the experiments. AL provided intellectual input and contributed to manuscript writing. GB contributed patient samples to this study. NG, JTL, DND and NMK designed and executed the SCID mouse xenograft experiments. RL conceived and designed the experiments and contributed to manuscript writing. All authors read and approved the final manuscript.

CONFLICTS OF INTERESTS

The authors declare no conflicts of interest.

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