NRF2 / Long Noncoding RNA ROR Signaling Regulates Mammary Stem Cell Expansion and Protects against Estrogen Genotoxicity

Yongshu Zhang, Jixiang Xia, Qinglin Li, Yuan Yao, Gabriel Eades, Ramkishore Gernapudi, Nadire Duru, Thomas W. Kensler, and Qun Zhou

Department of Biochemistry and Molecular Biology, University of Maryland School of Medicine, Baltimore, Maryland, MD 21201

Department of Pharmacology & Chemical Biology, School of Medicine, University of Pittsburgh, Pittsburgh, Pennsylvania, PA 15261

Running title: NRF2 signaling regulates mammary stem cells via targeting ROR

#Address correspondence to: Qun Zhou, qzhou@som.umaryland.edu

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Background: NRF2 has recently been implicated as a hematopoietic and intestinal stem cell regulator.

Results: NRF2 knockdown increases MCF10A mammosphere formation and NRF2 binds to and suppresses transcription of embryonic factor long non-coding RNA ROR.

Conclusion: NRF2 negatively regulates mammary stem cells via lncRNA-ROR.

Significance: NRF2 regulation of stem cell renewal and cellular detoxification provides a mechanism through which NRF2 loss can lead to tumorigenesis.

Long noncoding RNAs (lncRNAs) have emerged as key regulators of gene expression in embryonic stem cell (ESC) self-renewal and differentiation. In ESCs, lncRNAs are regulated at the genetic level via transcription factor binding to lncRNA gene promoters. Here we demonstrate that the key cytoprotective transcription factor NRF2 controls lncRNA expression in mammary stem cells. By profiling lncRNAs in wild-type and NRF2 knockdown mammary stem cells, we demonstrate that the lncRNA ROR, a regulator of embryonic stem cell pluripotency, is overexpressed upon NRF2 knockdown. We performed promoter analyses and examined predicted NRF2 binding elements in the ROR promoter using luciferase reporter constructs of a ROR promoter deletion series. Our studies revealed that NRF2 binds to two specific NRF2 response elements flanking the ROR promoter and that these two NRF2 response elements are equally important to suppress ROR transcription. In addition, we identified associated H3K27me3 chromatin modification and EZH2 binding at the ROR promoter that was dependent on Nrf2 binding. We observed that NRF2 knockdown or ROR overexpression leads to increased stem cell self-renewal in mammary stem cells. Furthermore, we demonstrate Nrf2 regulation of the mammary stem cell population in vivo. These observations provide further evidence for the critical role of NRF2 in maintaining normal stem cell subpopulations in mammary epithelium.

Nuclear factor-erythroid 2-related factor (NRF2) is well established as a master regulator controlling cellular defenses against chemical carcinogenesis (1, 2). In quiescent cells, NRF2 is tightly modulated by a cytoplasmic protein, Keap1, which promotes ubiquitination and proteasomal degradation of NRF2 and results in inhibition of NRF2 signaling (3-5). Various chemopreventive agents can alter the interaction between NRF2 and Keap1 resulting in nuclear NRF2 accumulation (6). The mechanism through which this occurs is under debate but recent reports suggest that as opposed to dissociating
NRF2 from KEAP1, electrophiles may disrupt KEAP1-CUL3-mediated ubiquitination of NRF2, thereby protecting NRF2 from degradation (7, 8). Subsequently, nuclear NRF2 interacts with co-activators, binds to antioxidant response elements (ARE) in promoters of target genes, and activates transcription of NRF2-dependent genes like NQO1 and GSTs, which can prevent malignant transformation through blocking chemical-associated genotoxic effects (5, 9, 10). In addition to this well characterized chemopreventive role, NRF2 is also required to maintain homeostatic quiescence of intestinal stem cells and hematopoietic stem cells (11, 12).

NRF2 has been well characterized as an activator of gene expression for detoxifying genes. Recent reports have also found NRF2 involved in transcriptional repression of target genes although the underlying mechanisms are not understood. Kwak et al. identified Nrf2-dependent repression of 31 genes related to cholesterol and lipid biosynthesis in the liver while examining Nrf2 knockout mice (13). Similarly, Shin et al. identified NRF2-dependent repression of Fatty Acid Synthase (FASN) and Acetyl-CoA Carboxylase (ACC α & β) mRNAs (14). Furthermore, Chartoumpekis et al. identified Nrf2 repression of Fibroblast Growth Factor 21 (FGF21) in mouse serum, liver, white adipose tissue, and stromal cell line ST2 (15). They found that Nrf2 overexpression reduced FGF21 mRNA levels by 40%. Furthermore, using an FGF21 promoter luciferase reporter they found that wild-type Nrf2 but not dominant negative Nrf2 could repress promoter activity. The mechanisms and cofactors through which NRF2 acts to repress target gene transcription are currently unknown.

Recently, long noncoding RNA (lncRNAs; >200 nucleotides) have been found to be critical in development, differentiation and homeostasis (16). Most lncRNAs are transcribed by polymerase II, capped, and polyadenylated similar to mRNAs. Some are in close proximity or overlapping protein coding transcripts, while others are intergenic with no nearby protein coding genes. Although the sequence of lncRNAs is not heavily conserved, the promoter regions of lncRNAs were recently reported to have higher sequence conservation than that of promoters for protein coding genes.

lncRNAs are emerging as important players in stem cell biology. Recent studies have employed powerful sequencing technologies and bioinformatics tools to identify lncRNAs that regulate pluripotency and differentiation in embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs). One of the findings from profiling experiments revealed that the lncRNA ROR (Regulator of Reprogramming) was upregulated in embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) (17, 18). Studies further revealed that overexpression of ROR can enhance iPSC colony formation whereas ROR knockdown results in a remarkable decrease of iPSC colony formation. Similarly, ROR was found to be a critical regulator of ESC pluripotency and self-renewal. This evidence suggests that ROR is a powerful regulator of stem cell self-renewal for controlling reprogramming efficiency and pluripotency. The molecular mechanisms underlying ROR actions in ESCs and iPSCs are largely unknown. It is likely that ROR is required for maintenance of the specific genetic program associated with the self-renewal state and the repression of the specific genes involved in lineage differentiation. This hypothesis is supported by recent work showing that cytoplasmic ROR may serve as a competitive endogenous RNA (sponge) for miR-145, thus releasing miR-145 repression of target genes including OCT4, SOX2, and KLF4 in ESCs (18).

It remains unclear if ROR may contribute to adult stem cell programming.

Human mammary stem cells are characterized by their ability to self-renew and differentiate into lineage-restricted luminal and myoepithelial cells. Human breast stem cells have an intermediate or suprabasal position in the epithelium (19). The subpopulation with a CD44-high/CD24-low/ESA+ phenotype (20) from primary human breast epithelial cells is able to form branching structures in three-dimensional cultures in Matrigel and displays mammosphere-initiating capacity (19, 21). An important function of mammary stem cells is maintaining homeostasis in response to environmental stress. Notably, the discovery of NRF2 regulation of hematopoietic stem cell function highlights the potential role of NRF2 in regulating adult stem cell self-renewal and differentiation. Here, we demonstrate that the presence of NRF2 contributes to the growth and
survival of mammary stem cells through a unique mechanism of action involving the IncRNA ROR.

Experimental Procedures

Cell Culture, Mammosphere and Progenitor Differentiation Assay MCF10A immortalized human mammary epithelial cells were obtained from ATCC (Rockville, MD) and were grown as described previously (22). HEK-293T cells were obtained from ATCC and were grown in Dulbecco’s modified Eagle’s medium (DMEM) with 5% fetal bovine serum (FBS, HyClone; Rockford, IL) and 1% L-glutamine. All cells were incubated in 5% CO₂ at 37°C. Mammosphere and progenitor differentiation assays were performed as previously described (23).

Plasmid Constructs and Luciferase Assay pROR is an ROR promoter luciferase reporter. DNA fragments from the human ROR promoter region were amplified by PCR and cloned into KpnI and BglII sites of pGL3 basic vector using the genomic DNA of MCF10A cells as templates. The following primers were used: pROR-R 5’-CATGGGAGATCTAGCAAAGAGCAGGAGGACAG-3’, pROR-L1 5’-catgggGGTACCttctcccacattccctcaag-3’, pROR-L2 5’-CATGGGGGTACCAAAGACCACAACACACACA-3’, pROR-L3 5’-CATGGGGGTACCAGAAGTGAAAATGCCCTGCC-3’. The human ROR-expressing plasmids were constructed as previously described. ROR cDNA was cloned into pBabe-puro and resulting pBabe-lncRNA-ROR clones were sequenced for verification. Luciferase reporter transfection and dual luciferase assay were performed as previously described (24).

Viral constructs of FUW-teto-miR140 and FUW-teto-ROR. The ROR cDNA was amplified by PCR using primers 5’-ACATGGGAGATCTAGCAAAGAGCAGGAGGA CAG-3’ and 5’-catgggGGTACCttctcccacattccctcaag-3’, respectively. The resulting cDNA was then used for a 96-well IncRNA SYBR green qPCR array (System Biosciences, catalog number CRA920D-1) following the manufacturer’s instructions. qPCR was carried out using the Light Cycler 480II instrument (Roche, Indianapolis, IN, USA) and data analysis was performed using the online software (http://www.systembio.com/lncrna-research/disease-long-non-coding-rna/literature). A four-fold threshold was used to select the genes differentially expressed. Real time qRT-PCR was performed as previously described (24). Briefly, total RNA was treated with DNase I to remove genomic DNA and then used to synthesize cDNA. ROR was analyzed using the following primers: F: 5’-CTCAGTGGGGGAAGACTCCAG-3’, R: 5’-AGGAAGCCTGAAGTTCGG-3’. Data were normalized to GAPDH using the following primers: F: GAAGGTGAAGGTCGGAGTC, R: GAAGATGTTGATGGGATTTC.

Chromatin Immunoprecipitation (ChIP) The transcription factor binding sites were predicted using the Patch program (www.gene-regulation.com). Two NRF2 binding sites (-1624 to -1616 and -329 to -321) in the ROR promoter region were identified. ChIP was performed as described previously (24). Cells were cross-linked with 1% formaldehyde and sonicated. Chromatin was incubated with antibodies against NRF2 (H300, Santa Cruz Biotechnology, Santa Cruz, CA) or H3K27me3 (Active Motif 39155) overnight at 4°C for immunoprecipitation. Rabbit IgG was used as the negative control. Immunoprecipitated chromatin was analyzed by Real Time qPCR using two sets of primers for the ROR promoter, the NRF2 binding site1Region 1 (-1624 to -1616 CATGCGCA, CHIPL1 5’-cttgggatatagataaaac-3’ and CHIPR1 5’-ttgctgaggtttatatag-3’) and the NRF2 binding site2 (-329 to -321 CATGCCAC, CHIPL2 5’-gcaaaaccttcttggagggag-3’ and 5’-ggaagagcagcagctaca-3’), respectively. Results are presented as the percent of input.

Mutation Assay Wild-type MCF-10A and NRF2 deficient MCF-10A cells were treated twice a week for two weeks with 2-hydroxyestradiol (2-OHE2) or 4-hydroxyestradiol (4-OHE2) at final concentrations of 100 nM and 1 µM, respectively. The treated cells were passaged three times in DMEM:F12 containing 5% horse serum, 10 µg/ml insulin (Invitrogen), 20 ng/ml EGF (Sigma), 500
ng/ml hydrocortisone (Sigma) and 100 ng/ml cholera toxin (Sigma). Cells (2 x 10⁶) were then treated with 1 µM of shikonin for 24 hours. After the treatment, the medium was changed and cells were treated with 8 µg/ml of 6-thioguanine. After 14 days, the resulting cell colonies were fixed, stained and counted as described previously (25).

**Stem Cell Isolation and Immunofluorescence**

Stem cell subpopulations were isolated using bead-conjugated CD44-APC, CD24-PE and EpCAM antibodies (BD Pharmingen, San Diego, CA) in addition to CELLection™ Pan Mouse IgG beads (Invitrogen, Carlsbad, CA). Immunostaining of breast cancer cells was performed using anti-Ki-67 and K18 (Santa Cruz) antibodies. Immunofluorescent staining was performed using Alexa Fluor 488/555 conjugated secondary antibodies (Invitrogen) with DAPI counterstaining. Fluorescent staining was visualized using an Olympus IX81 spinning disk confocal microscope.

**Fluorescence In situ Hybridization of ROR**

Cyanine dye (Cy3)-labeled ROR probes were purchased from Exiqon (Woburn, MA). Cells were fixed with 4% formaldehyde, incubated with permeabilization buffer (1xPBS/0.5% Triton X-100) followed by blocking in prehybridization buffer (3% BSA in 4x saline-sodium citrate buffer (SSC)). Cells were hybridized 1h at 60°C with ROR probes at 2 ng/ml dilution in hybridization buffer (10% dextran sulfate in 4xSSC). Cells were washed in 4x, 2x and 1x SSC. The slides were washed in 4x, 2x and 1x SSC. The slides were mounted in Prolong Gold. The Cy3-ROR results were scored based on the percentage of positive cells.

**Animals and Flow Cytometry Assay**

C57BL/6 wild-type and Nrff2 knockout mice were used. All animal experiments were approved by the Committee of Institutional Animal Care and Use according to the guidelines of the National Institutes of Health. The mouse mammary glands were removed from 6-8 wk-old female mice under sterile conditions and chopped into very fine bits with a razor blade. The isolation of mouse mammary epithelial cells was performed according to the protocol provided by the Stemcell Technologies. Mouse mammary epithelial cells were suspended in cold PBS and then incubated with anti-CD49f-APC (eBioscience clone goH3) antibody and anti-CD24-PE (BD Pharmingen 555428) antibody at 4°C for 30 minutes. A total of 100,000 viable cells were analyzed by FACSCalibur (BD Bioscience) with CellQuest software. Gates were determined with the use of appropriate isotype controls. Results are given as the positive percentage minus background from appropriate isotype control.

**Statistical Analysis**

Statistical analysis was performed by Student’s T test. p values of < 0.05 (*) were considered significant. Data are presented as mean ± S.D. Data were analyzed using GraphPad Prism 6.0.

**Results**

**NRF2 knockdown leads to lncRNA ROR overexpression in mammary stem cells**

Our goal was to identify novel lncRNAs involved in NRF2 signaling in normal mammary stem cells. For this purpose, we performed an lncRNA array to monitor the impact of NRF2 modulation on lncRNA expression in an in vitro model of normal mammary stem cells. MCF10A cells, non-tumorigenic immortalized human mammary epithelial cells, were stably transfected with NRF2 shRNA. The NRF2 knockdown was confirmed by real time qRT-PCR and western blotting (Fig. 1A). We further documented the impact of NRF2 knockdown on dampened expression of known downstream gene targets NQO1 and HO1 (Fig. 1A). Next, we cultured NRF2 knockdown cells in mammosphere culture, where stem-like cells remain viable and grow as non-adherent spheres. Using mammosphere culture we collected stem-like cells from parental MCF10A and NRF2 deficient MCF10A cells. To identify the impact of NRF2 deficiency on lncRNA expression in mammary stem cells, we performed PCR based lncRNA array analysis and evaluated differential expression of 90 lncRNAs that have been characterized in ESCs or in human diseases. Analysis of lncRNA arrays revealed numerous up-regulated lncRNAs in NRF2 deficient cells (MCF10A NRF2KD) compared with parental cells (MCF10A). Fig. 1B shows that among all lncRNAs examined, 60 lncRNAs were up- or down-regulated at least 4-fold in NRF2 deficient stem cells. We were particularly interested in the potential direct regulatory involvement of NRF2 in controlling lncRNA expression. One of the most promising candidates for direct NRF2 targeting was lncRNA ROR. We selected ROR for
subsequent analysis because 1) this lncRNA was known as a stem cell self-renewal regulator in ESCs and iPSCs (17, 18), and 2) the ROR promoter has potential NRF2 binding sites (see Fig. 2). To further determine if ROR represents a NRF2 target gene in mammary stem cells, we first validated our array data using individual qRT-PCR. We examined whole cell populations and stem cell subpopulations from MCF10A NRF2KD mammospheres and its parental cells (MCF10A) for ROR expression by qRT-PCR. NRF2 knockdown resulted in an increase in ROR expression in MCF10A whole cell population (Fig. 1C). We also observed a dramatic increase in ROR levels following stable knockdown of NRF2 by shRNA in MCF10A NRF2KD stem cells. After qPCR validation, we performed 3-D cell culture to further examine the impact of NRF2 on ROR in MCF10A stem cells. We choose 3-D cell culture since only mammary stem cells can grow under 3-D cell culture conditions and this assay can recapitulate mammary organogenesis in vitro. ROR was detected using fluorescence in situ hybridization. As shown in Fig. 1, endogenous ROR was weakly detected in the nucleus of MCF10A cells but NRF2 knockdown resulted in increased detection in the nucleus of MCF10A NRF2KD cells (Fig. 1D). These results indicate the important role of NRF2 in maintaining ROR expression levels in mammary stem cells.

**Identification of NRF2 binding sites in ROR promoter** The current understanding of NRF2 signaling is that the transcription factor NRF2 can shuttle from the cytoplasm to the nucleus and subsequently bind to antioxidant response elements in target gene promoters. Since we observed an increased ROR in the nucleus of MCF10A NRF2KD cells (Fig. 1), we hypothesized that NRF2 may regulate ROR promoter activity. To test this hypothesis, we investigated the potential direct regulatory involvement of NRF2 in controlling ROR expression. Analysis of the predicted transcription factor binding sites located within a 2 kb region upstream of the transcriptional start site of lncRNAs using Genomatix software identified 2 potential NRF2 binding sites in the ROR promoter (-1624/-1616, and -329/-321). To determine whether these predicted NRF2 binding sites were indeed involved in the regulation of ROR promoter activity, we cloned the ROR promoter into a luciferase reporter construct and created a series of ROR promoter reporters by deleting the NRF2 binding sites (Fig. 2A). HEK-293T cells were transfected with wild-type or mutant ROR promoter luciferase reporters in the presence or absence of NRF2. Fig. 2A shows the results of ROR promoter activity assays for wild-type and deleted ROR luciferase constructs. The co-transfected NRF2 resulted in decreased reporter activity for the wild-type promoter (Fig. 2A). We also confirmed that NRF2 suppression of ROR promoter activity was greatly diminished when the -1624/-1616 and -329/-321 NRF2 binding sites were deleted. These data indicate that the presence of these two NRF2 binding sites are critical for NRF2 induced inhibition of ROR promoter activity.

Based on our analysis of the ROR promoter reporters, we hypothesized that NRF2 directly binds the ROR promoter at these NRF2 response elements and subsequently inhibits ROR transcription. To test our hypothesis, we conducted chromatin immunoprecipitation (ChIP) analysis using an anti-NRF2 antibody, followed by real-time qRT-PCR for these DNA regions in MCF10A cells. We designed primers to amplify the two putative NRF2 binding elements within the ROR promoter region. ChIP results (Fig. 2B&C) revealed NRF2 recruitment to the ROR promoter (3-6 fold that of control IgG), suggesting NRF2 directly interacts with this promoter. We also performed ChIP on MCF10A NRF2KD cells where as expected we failed to detect NRF2 binding to these promoter regions. As a positive control we examined NRF2 binding to the NQO1 promoter (Fig. 2D), where we observe a dramatic decrease in promoter occupancy following Nrf2 knockdown.

In addition to NRF2 transcription factor binding, we examined cooperative epigenetic involvement in ROR promoter silencing. Polycomb repressor complex (PRC1/2) is an important regulator of stem cell self-renewal that functions through epigenetic silencing of differentiation genes (26). We examined Histone 3 Lysine 27 trimethylation (H3K27me3) chromatin marking catalyzed by the histone methyltransferase Enhancer of Zeste Homologue 2 (EZH2) a member of PRC2 (Fig. 2E). Following knockdown of NRF2, we detected a significant
decrease in EZH2 binding and a significant decrease in the repressive mark H3K27me3, which correlated with increased ROR expression. This suggests that the Polycomb group proteins may also play an important role in regulating ROR expression.

The NRF2/ROR pathway critically regulates self-renewal in mammary stem cells

Mammosphere formation can serve as a surrogate assay of mammary stem cell self-renewal (27, 28). To study the role of the NRF2/ROR pathway in regulating mammary stem cell function, we performed a mammosphere assay to examine the impact of NRF2/ROR on mammary stem cell self-renewal in vitro. MCF10A NRF2KD cells showed a significant increase in sphere formation whereas ROR knockdown resulted in a significant decrease in sphere formation in MCF-10A cells (Fig. 3A). We next tested whether the impact of NRF2 on mammosphere is mediated through ROR. MCF10A NRF2KD cells were transfected with ROR shRNA to inhibit ROR expression. We observed that ROR knockdown resulted in a significant decrease in mammosphere formation in MCF10A NRF2KD cells. This indicates that ROR regulation of mammary stem cell signaling is downstream of NRF2. Together, these results suggest that NRF2/ROR signaling controls normal stem cell self-renewal pathways in our model system.

To examine the impact of ROR overexpression on stem cell self-renewal, we established the inducible ROR overexpression system (Fig. 3B). MDA-MB-231 breast cancer cells were stably infected with lentiviral Tet-ON linc-RNA-ROR inducible expression system. We found that upon induction of ROR overexpression following doxycycline treatment, there was a detectable increase in mammosphere formation (Fig. 3B).

Characterization of the impact of NRF2 on progenitor cells

Stem cells are characterized by their potential for self-renewal and multi-lineage differentiation. Since we found that NRF2 knockdown leads to an increase in self-renewal in normal mammary epithelial cells (MCF10A), we next examined whether NRF2 may impact the differentiation capacity of mammary stem cells. Therefore, we performed co-culture colony formation assays that promote progenitor proliferation and differentiation (23). Limiting numbers of MCF10A cells were cultured in clonal conditions atop a feeder layer of irradiated NIH-3T3 fibroblasts. Under these conditions mammary epithelial cells form differentiated colonies with distinct luminal or myoepithelial morphologies and express lineage-specific markers of differentiation. We examined colony formation by morphologic analysis and the expression of lineage-associated cytokeratin expression. MCF10A cells formed CK18+ luminal colonies with luminal epithelial morphology (Fig. 3C). We examined the impact of NRF2 on colony formation of MCF10A progenitor cells. We observed a slight increase in colony formation in MCF10A NRF2KD cells, possibly due to expanded stem/progenitor populations. In both control MCF10A and MCF10A NRF2KD cells we observed distinct luminal morphology and expression of luminal marker cytokeratin 18, indicating NRF2 was not affecting cellular differentiation of mammary stem cells.

NRF2 is required for maintaining the mammary stem cell subpopulation

In addition to examining NRF2KD we also tested the impact of NRF2 overexpression on mammary stem cell renewal. MCF10A cells were transfected with an NRF2 overexpression vector and grown in mammosphere culture. NRF2 overexpression led to a significant decrease in mammosphere number and size (Fig. 3D). We also tested these findings in another mammary epithelial cell line, MCF12A, to demonstrate that NRF2 regulation of stem cells signaling is not cell-line dependent. Again, NRF2 overexpression dramatically decreased mammosphere formation.

We next examined the impact of Nrf2 knockout on mammary stem cells in vivo. We isolated mammary epithelial cells from wild-type and Nrf2-knockout mice and examined stem cell subpopulations using flow cytometry. CD49f has been recognized as a marker for mammary stem cells in mice, where CD49f+/CD24+(med) cells are enriched in mouse mammary stem cells (MaSC) (29). We observed a dramatic increase in CD49f-positive mammary epithelial cells in Nrf2 knockout mammary epithelial cells (Fig. 3E). Furthermore, we observed a significant increase in the MaSC-enriched population of CD49f+/CD24+ cells (0.76% to 3.76%) indicating an expansion of the mammary stem cell subpopulation.
Next, we isolated mammary epithelial cells from WT and Nrf2 knockout mice. We isolated CD49f+/CD24+ MaSC populations using FACS and cultured them in mammosphere conditions. Isolated Nrf2KO MaSC formed significantly larger and significantly more mammospheres demonstrating enhanced self-renewal properties (Fig. 3F).

It is well established that CD44high/CD24low/ESA+ subpopulations demonstrate stem cell self-renewal properties in human breast epithelium and that these surface markers can be used to enrich stem-like cells from non-stem cells (21). We examined the impact of NRF2 knockdown on the CD44high/CD24low/ESA+ subpopulation. We observed a dramatic increase in the CD44high/CD24low/ESA+ stem cell population in MCF10A NRF2KD cells compared to MCF10A cells (~30% compared to ~17% for control cells) (Fig. 4A). Isolated CD44high/CD24low/ESA+ stem cells were further examined by mammosphere culture to test for self-renewal. MCF10A NRF2KD cells showed a statistically significant increase in mammosphere formation in CD44high/CD24low/ESA+ cells. We also cultured CD44high/CD24low/ESA+ subpopulations derived from MCF-10A cells in 3-D cell culture. As shown in Fig. 4B, we found that MCF10A NRF2KD cells showed increased proliferation as evidenced by staining for the proliferative marker Ki67. These results provide further evidence that the NRF2/ROR pathway is a powerful regulator of mammary stem cell homeostasis.

**NRF2/ROR signaling can regulate the genotoxic effects of estrogen in mammary epithelial cells** It is well accepted that estrogen metabolites (e.g., 4-hydroxyestrone (4-OHE2)) can induce genotoxicity in human mammary epithelial cells (25). To test the impact of NRF2/ROR pathway on the genotoxicity of 4-OHE2, we collected 4-OHE2-treated mammospheres from MCF10A cells and measured mRNA levels of *NRF2* and *ROR*. We observed that treatment with 4-OHE2 significantly reduces *NRF2* transcripts but increases ROR levels (Fig. 4C). We next examined whether the NRF2/ROR pathway may impact the genotoxicity of 4-OHE2. The hypoxanthine phosphoribosyl transferase (*HPRT*) gene mutation assay was performed to detect 4-OHE2-induced mutations (25). This assay involves treatment of cells with toxic nucleoside analog 6-thioguanine (6-TG) in the presence or absence of chemical mutagen, in this case 4-OHE2. Cells without an HPRT mutation will die as a result of 6-TG, but those with a mutation will survive and form colonies allowing this assay to serve as a readout for mutation frequency. MCF10A cells and MCF10A NRF2KD cells were first treated with 1 µM 4-OHE2 for 14 days and then cultured in media containing 6-TG. The results showed that MCF10A cells treated with 4-OHE2 produced significantly more 6-TG-resistant cell colonies (more mutations) (Fig. 4D), which confirmed the genotoxicity of 4-OHE2 as reported previously (25). In the absence of 4-OHE2, NRF2KD cells and the parental MCF10A cells did not form colonies. NRF2KD cells treated with 4-OHE2 showed a remarkable increase in colonies compared to 4-OHE2 treated MCF10A cells, suggesting that NRF2 can protect against 4-OHE2-induced DNA damage. Next, we characterized the impact of ROR on 4-OHE2-induced genotoxicity by performing HPRT gene mutation assay with MCF10A cells overexpressing ROR. We observed an increase in colony formation in ROR overexpressing MCF10A cells compared to control MCF10A cells, in response to stimulation by 4-OHE2. The chemopreventative agent shikonin has been shown to activate the NRF2 pathway and protect against estrogen-induced DNA damage (30). We used this pharmacological agent to confirm our results regarding NRF2/ROR regulation of 4-OHE2-induced DNA damage. Consistent with previous observations, combination treatment with shikonin significantly reduced 4-OHE2-induced colonies. Taken together, our data suggest an important role for NRF2 in inhibition of estrogen genotoxicity via inhibiting ROR in human mammary stem cells.

**Discussion**

It was recently found that NRF2 is a powerful adult stem cell regulator in hematopoietic and intestinal stem cells (11, 12). Here, we report for the first time that NRF2 is a major regulator of mammary stem cell self-renewal. We find that loss of NRF2 results in increased self-renewal as evidenced by significantly increased mammosphere formation. We also established Nrf2 control of mammary stem cell renewal in...
vivo, as we observed a dramatic increase in mouse mammary stem cells in Nrf2 knockout mice. Furthermore, in human mammary epithelium we find that NRF2 knockdown results in a significant increase in the CD44+/CD24-/ESA+ subpopulation enriched in stem cell activity. Our results add further demonstration for a new function for NRF2 in addition to its roles in cellular detoxification, that of a critical stem cell regulator in adult tissues. Conceptually, under conditions in which NRF2 is activated (high cellular stress) stem cell expansion could be dangerous as genomic integrity could be compromised, as such it would seem fitting that NRF2 would inhibit stem cell expansion under these conditions.

We have identified an important molecular mechanism through which NRF2 can regulate stem cell signaling, regulation of lncRNA ROR promoter activity. ROR is a well-described stem cell regulator in ESCs and iPSCs (17, 18), however, it has never been previously implicated in regulation of adult stem cell function. Here, we demonstrate that NRF2 knockdown results in significant dysregulation of lncRNA expression. We find that ROR expression is dramatically upregulated in mammary stem cells following NRF2 loss. We identified two putative NRF2 response elements in the ROR promoter. Using a promoter reporter deletion series and chromatin immunoprecipitation we were able to demonstrate that NRF2 directly binds both sites in the ROR promoter and represses transcriptional activity. In addition to transcriptional silencing of ROR via NRF2 we identified epigenetic changes at the ROR promoter (EZH2 and H3K27me3) that were dependent on NRF2 binding, indicating that NRF2 may cooperate with chromatin modifying enzymes to silence ROR expression. We also demonstrated that ROR positively regulates mammary stem cell renewal as evidenced by decreased mammosphere formation following ROR knockdown. Next, we examined ROR overexpression in MDA-MB-231 breast cancer cells, finding that ROR induction led to an increase in mammosphere formation. These results suggest that embryonic reprogramming factor ROR is a critical regulator of stem cell activity in normal and neoplastic mammary stem cells.

Finally, we demonstrate that the genotoxic estrogen metabolite 4-OHE2 can modulate NRF2/ROR signaling. We observe decrease NRF2 transcript and increasing levels of ROR following treatment of MCF10A cells with 4-OHE2. Furthermore, we find that the loss of NRF2 and/or overexpression of ROR can actually increase the genotoxic effects of 4-OHE2. This suggests that ROR may also be involved in the cytoprotective effects of NRF2 signaling and is further evidence linking NRF2 and ROR signaling pathways. Finally, we find that treatment with the chemopreventive compound Shikonin (a known activator of NRF2), protected ROR overexpressing MCF10A cells against 4-OHE2 induced DNA damage.

It seems possible that maintenance of stem cell genomic integrity is a critical job for NRF2/ROR signaling. Numerous studies have demonstrated that Nrf2 deficient mice show significantly increased rates of carcinogen-induced tumorigenesis (31). We find that exposure of MCF10A cells to estrogen metabolites can down-regulate NRF2 expression and result in upregulation of ROR. In addition to losing the protective effects of NRF2 and elevating the risk of DNA mutation, exposure to estrogen metabolites might result in dysregulated stem cell signaling via ROR up-regulation. This is likely a dangerous combination and might be one potential mechanism behind estrogen-mediated breast tumorigenesis.

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FIGURE LEGENDS

FIGURE 1. NRF2 Knockdown Results in Significant Dysregulation of IncRNAs Expression. A, NRF2 knockdown and antioxidant response gene expression. mRNA and protein expression levels in MCF10A and MCF10A NRF2 KD cells relative to controls. B, IncRNAs dysregulated by 4 fold or greater in NRF2 knockdown stem cells. Results of IncRNA SYBR green qPCR array profiling 90 IncRNAs in MCF10A mammospheres following NRF2 loss. C, qRT-PCR validation of ROR expression, normalizing to GAPDH. Stem cell population was isolated from mammospheres. D, 3-D cell culture of MCF10A cells. Fluorescent staining for ROR was performed by in situ hybridization. N=3 ± S.E.M. p value determined by Student’s t test, * p < 0.05

FIGURE 2. NRF2 Directly Regulates IncRNA ROR Transcriptional Activity. A, Luciferase reporter deletion series for ROR promoter region. 293T cells were co-transfected with NRF2 expression vector ROR-luciferase promoters and Renilla control vector. Luciferase activity is normalized to full length ROR-luc1 activity. B & C, Chromatin immunoprecipitation (ChIP) for the NRF2 response elements in the ROR promoter. Following cross-linking and sonication in MCF10A cells and NRF2 knockdown cells, chromatin was immunoprecipitated with NRF2 antibody or control IgG. PCR / qPCR was performed to examine binding of NRF2 with these loci. Results are presented as the percent of input control and ethidium bromide stained gel. D, ChIP results for NRF2 binding at NQO1 promoter. E, ChIP results for EZH2 & H3K27me3 at or near the NRF2 response elements in the ROR promoter. Data shown for control MCF10A cells and NRF2 knockdown cells. N=3 ± S.E.M. p value determined by Student’s t test, * p < 0.05

FIGURE 3. NRF2/ROR Regulates Mammary Stem Cell Self-Renewal. A, Mammosphere formation following shRNA knockdown of ROR in MCF10A cells. Mammosphere formation was also examined in NRF2 knockdown cells that were transfected with ROR shRNA. N=3 ± S.E.M. p value determined by Student’s t test, * p < 0.05. B, A schematic of tetracycline (tet)-inducible expression of ROR system. MDA-MB-231 cells were transfected with the tet-inducible ROR and then treated with or without tet. Mammosphere formation was counted (n=2). C, Mammary Progenitor Colony Formation Assay. Limiting amount of control and NRF2 knockdown MCF10A cells were grown in the presence of irradiated NIH-3T3 fibroblasts. After 7 days, colonies were stained with crystal violet and quantified using light microscopy or examined by immunofluorescence for expression of cytokeratin 18 (CK18). D, Mammosphere formation of MCF10A or MCF12A mammary epithelial cells transfected with NRF2 overexpression vector E, Representative FACS plot of mouse mammary epithelial cells from wild-type
and Nrf2 knockout mice (n=6). Cells were incubated with antibodies specific for CD49f and CD24 and then assessed for the CD49fhigh/CD24high subpopulation. 

F, Isolated WT & Nrf2KO CD49fhigh/CD24high MaSC were cultured as mammospheres. N=3 ± S.E.M. p value determined by Student’s t test, * p < 0.05; ** p < 0.01.

FIGURE 4. NRF2/ROR regulates self-renewal of the CD44+/CD24- subpopulation of mammary stem cells. A, CD44+/CD24-/ESA+ subpopulations were selected using magnet bead based cells sorting. % of total population is shown. The ratio of stem cells to whole populations from both wild type and NRF2 knock down is presented. Isolated cells were grown in attachment free conditions and mammosphere formation was quantified B, CD44+/CD24-/ESA+ cells were grown in 3-D cell culture in matrigel. Immunofluorescent staining for Ki67 was performed on resulting acini. DAPI was used as a counterstain. Both Ki67+ acini and total cells per spheroid are quantified. Data are shown as the percentage of Ki67 positive cells per spheroid. C, mammosphere from MCF10A cells was treated with 4-hydroxyestrone (4-OHE2) and NRF2 mRNA and ROR expression were examined by qRT-PCR and normalized to GAPDH expression. D, HPRT gene mutation assay was performed using NRF2 knockdown and ROR overexpressing MCF10A cells with or without 4-OHE2 treatment. In addition Shikonin (SK), a known activator of NRF2, treatment was given to 10A cells overexpressing ROR. N=3 ± S.E.M. p value determined by Student’s t test, * p < 0.05.
Fig. 1

A) Nrf2 mRNA

B) Antioxidant Response Genes

| IncRNA      | Upregulated (fold change) |
|-------------|---------------------------|
| Anti-NOS2A  | 62.9                      |
| TMEMPG1     | 50.4                      |
| ZEB2NAT     | 45.1                      |
| SOX2OT      | 43.0                      |
| TU_0017829  | 39.3                      |
| PCAT-15     | 36.6                      |
| WT-1AS      | 34.0                      |
| NDM29       | 30.2                      |
| AK029348    | 28.2                      |
| 21A         | 28.0                      |
| PCGEM1      | 27.0                      |
| AAA1        | 27.6                      |
| DISC2       | 24.2                      |
| CMPO        | 23.8                      |
| KRASP1      | 22.2                      |
| PCAT-32     | 21.2                      |
| HOTAIR      | 19.8                      |
| BIC         | 18.8                      |
| PSF inhibiting RNA | 18.6                  |
| HOTTIP      | 18.2                      |
| SAF         | 17.8                      |
| Y4          | 17.1                      |
| LincRNA-SFMBT2 | 17.7                  |
| BC200       | 17.3                      |
| Y1          | 16.1                      |
| EGO         | 16.4                      |
| Itpa16      | 15.5                      |

C) Whole cell population

D) DAPI

| IncRNA | Downregulated (fold change) |
|--------|-----------------------------|
| hHIF   | 0.2                         |
| HOX1AS | 0.2                         |
| AAA489505 | 0.2                    |
| NEAT1  | 0.1                         |
| MALAT1 | 0.1                         |
| PRINS  | 0.01                        |

Merge
