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Nrf3 Functions Reversely as a Tumorigenic to an Antitumorigenic Transcription Factor in Obese Mice

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Tumor tissue includes cancer cells and their associated stromal cells, such as adipocytes, myocytes, and immune cells. Obesity modulates tumor microenvironment through the secretion of several inflammatory mediators by inducing adipogenesis and myogenesis. Previously, we indicated that tumor growth is promoted by a transcription factor nuclear factor erythroid 2-related factor 3 (NRF3) in human cancer cells. However, the impact of obesity on NRF3-mediated tumorigenesis remains unknown. Here we show that obesity reprograms the tumorigenic to the antitumorigenic function of Nrf3 using a diet-induced obese mouse model. Nrf3 knockdown decreased tumor growth in mice fed a normal diet (ND), whereas it reversely increased tumor growth in mice fed a high-fat diet (HFD). Then, the tumor tissues derived from Nrf3 knockdown or control cancer cells in ND- or HFD-fed mice were subjected to a DNA microarray-based analysis. Similar to the tumor formation results, the expressions of genes related to adipogenesis, myogenesis, and interferon-alpha response were reversed by obesity, implying an increase or recruitment (or both combined) of adipocytes, myocytes, and immune cells. Among these gene sets, we focused on adipocytes. We showed that Nrf3 knockdown reduced cancer cell growth in the preadipocyte culture medium, while the growth inhibitory effect of Nrf3 knockdown on cancer cells was abolished in the adipocyte culture medium. These results suggest the possibility that cancer-associated adipocytes secrete the potential reprogramming factor from the tumorigenic to the antitumorigenic function of Nrf3 in cancer cells.

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Introduction

The tumor microenvironment consists of cellular and noncellular components. Cellular components include cancer cells, tumor stroma, blood vessels, infiltrating inflammatory cells, and several associated tissue cells. The noncellular components include small secretory proteins, such as cytokines, and extracellular matrix proteins, such as collagen. Cancer cells control the function of these tumor microenvironment components through complex signaling networks to make nonmalignant cells work for their benefit. The crosstalk between cancer and other cells is consequently reflected in tumor formation and development (Baghban et al. 2020).

Obesity is not only a complex disease involving an excessive amount of fat in the body, but also a significant risk factor for several chronic diseases, such as diabetes and cardiovascular diseases (Piché et al. 2020). Moreover, obesity drives the progression of several cancers, including endometrial, breast, ovarian, prostate, liver, gallbladder, kidney, and colon (Calle and Kaaks 2004). Obesity is associated with an increase in cancer-associated adipocytes, one

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of the primary stromal cells in tumor tissues. Adipocytes contribute to promoting tumor growth through releasing adipokines, such as interferons (IFNs), interleukins (ILs), tumor necrosis factor-alpha (TNF-α), transforming growth factor-beta (TGF-β) (Trayhurn and Wood 2004).

Nuclear factor erythroid 2-related factor 3 (NRF3) belongs to the cap'n'collar family of transcription factors (Kobayashi et al. 1999). In our previous studies, we have shown the high expression of *NRF3* genes in various cancer tissues and revealed that NRF3 promotes tumor growth through protein degradation of tumor suppressors, including p53 and retinoblastoma (Aono et al. 2019; Waku et al. 2020b). Recently, we found that NRF3 activates sterol-responsive element-binding protein 2 (SREBP2), a master regulator of cholesterol biosynthesis, and reprograms cholesterol and fatty acid biosynthesis in cancer cells (Waku et al. 2021). These results imply a correlation between NRF3 and lipid metabolism (Waku and Kobayashi 2021). However, the impact of obesity on NRF3-mediated tumorigenesis remains unclear.

In this study, we showed that obesity reprograms from tumorigenic to the antitumorigenic function of Nrf3. Using a diet-induced obese mouse model, we revealed that Nrf3 knockdown decreased tumor growth in mice fed a normal diet (ND), whereas it reversely increased tumor growth in mice fed a high-fat diet (HFD). We obtained a DNA microarray dataset using these tumor tissues to identify regulatory factors. Then, we performed a Gene Set Enrichment Analysis (GSEA). Similarly, the enrichment plots of gene sets related to adipogenesis, myogenesis, and IFNα response were altered from a positive to a negative correlation with Nrf3 knockdown in the tumor tissues of HFD-fed mice. This resulted in the increase or invasion (or both combined) of stromal cells, including adipocytes, myocytes, and immune cells, into the tumor. Among these gene sets, we focused on adipocytes. We showed that cancer cell growth reduction by Nrf3 knockdown is abolished in the culture medium of adipocyte 3T3-L1 cells, but not in the culture medium of preadipocyte 3T3-L1 cells or the fresh medium containing adipocyte differentiation stimuli. These results imply the possibility that obesity leads to an increase or invasion (or both combined) of several stromal cells in the tumor. Then, tumor-associated adipocytes secrete potential reprogramming factors from the tumorigenic to the antitumorigenic function of Nrf3 in cancer cells.

Materials and Methods

Cell lines and the generation of Nrf3 knockdown cells

Mouse renal carcinoma Renca cells were cultured in Roswell Park Memorial Institute (RPMI)-1640 medium (Nacalai Tesque, Kyoto, Japan). Mouse preadipocyte 3T3-L1 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM)/low glucose medium or DMEM/high glucose medium (Wako Pure Chemical Industries, Kyoto, Japan). All media were supplemented with 10% fetal bovine serum (FBS) (Nichirei Biosciences, Tokyo, Japan), 40 μg/mL streptomycin, and 40 units/mL penicillin (Wako Pure Chemical Industries). Renca cells were transfected with the piGENE hU6 plasmid (iGENE Therapeutics, Tsukuba, Japan) containing mouse *Nrf3* target sequence to generate Nrf3 knockdown cells (sense; CACCCTGTCGTA AATGTAAACTTGAGTGTGCTGTCCTCTAGTTTACGTT TACGGCAGTTTTTT, anti-sense; GCATAAAAAACTGCC GTAAACGTAAACTAGAGGACAGCACAC TCAAGTT TACATTTACGACAG) or control sequence (sense; TCGAGGTTCTCCGAACGTGTCACGTTTCAAGAGA ACGTGACACGTTCGGAGAATTTTTTACGCGTA, anti-sense; AGCTTACGCGTAAAAAATTCTCCGAACGTGT CACGTTCTCTTGAAACGTGACACGTTCGGAGA ACC). A puromycin-resistant vector was co-transfected with piGENE plasmid for drug selection. The cell growth rate (%) for 3 days was calculated using the following formula: $\frac{shNrf3\ (Final\ cell\ numbers - Initial\ cell\ numbers)}{shCtrl\ (Final\ cell\ numbers - Initial\ cell\ numbers)} \times 100$

Adipocyte differentiation of 3T3L1 and Nile Red staining

The differentiation of 3T3-L1 preadipocyte cells into adipocyte-like cells was conducted using AdipoInducer Reagent (AIR), involving 0.5 mM 3-isobutyl-1-methylxanthine (IBMX), 2.5 μM dexamethasone, and 10 μg/ml insulin (MK429 for animal cells, Takara Bio, Kusatsu, Shiga, Japan), according to the manufacturer's instructions. 3T3-L1 preadipocyte cells were cultured in the DMEM/high glucose medium with 10% FBS, 40 μg/mL streptomycin, and 40 units/mL penicillin (DMEM/high). After achieving 100% confluence, the cells were then cultured in the DMEM/low glucose medium with 10% FBS, 40 μg/mL streptomycin, and 40 units/mL penicillin. After two days, the medium was changed to the DMEM/high medium with the AIR. The DMEM/high medium with AIR was used as a control medium and is represented as AIR-M in Fig. 3B. After 2 more days, the cells were cultured in the DMEM/high medium with 10 μg/ml insulin. The cells were stained with Nile Red, and the intracellular lipid levels were measured using a flow cytometer, as described previously (Waku et al. 2021). Briefly, the cells were stained with 10 μM Nile Red (Sigma, Kyoto, Japan) for 5 h, after which they were washed twice with fluorescence-activated cell sorting (FACS) buffer [0.1% (w/v) sodium azide and 2% FBS in cold phosphate-buffered saline (PBS)]. The samples were then subjected to flow cytometry (FACSAriaII, BD Biosciences, CA, USA).

Obesity mouse model, cholesterol measurement, and tumor formation assay

This assay was based on a previous method (Ringel et al. 2020). Four-to-six-week old BALB/cCrSlc male mice were purchased from Shimizu Laboratory Supplies (Kyoto, Japan) and assigned to *ad libitum* feeding of ND (CE-2, CREA Japan, Tokyo, Japan) or HFD (HFD-60, Oriental Yeast, Tokyo, Japan) for 17 weeks. Then, the serum cholesterol levels were measured using the LabAssay

Cholesterol kit (Cholesterol Oxidase DAOS method), according to the manufacturer's instructions (#LABCHO-M1, Wako Pure Chemical Industries). The tumor formation assay was based on a previous method (Waku et al. 2020b). Renca-shCtrl or shNrf3 cells were subcutaneously injected into the ND or HFD-fed mice flanks. After 2 weeks, the tumor tissues were removed and weighed. All animal experiments were performed following the guidelines for the care and use of laboratory animals at Doshisha University, Japan.

RNA extraction and real-time quantitative PCR (RT-qPCR)

This assay was based on a previous method (Waku et al. 2020b). Total RNA was extracted and purified using ISOGEN II (NIPPON GENE, Toyama, Japan) according to the manufacturer's instructions. Aliquots of total RNA (1 μg) were reverse transcribed using pd (N)6 random primer (Takara Bio) and Moloney murine leukemia virus reverse transcriptase (Invitrogen,Waltham, MA, USA) with a 250 μM deoxy nucleoside triphosphate (Takara Bio) concentration, according to the manufacturer's instructions. RT-qPCR was also performed using SYBR Premix Ex Taq II (Takara Bio), and gene primers were conducted using a Thermal Cycler Dice Real-Time System (Takara Bio). The *Nrf3* gene expression level was also normalized to the mRNA levels of the *β-actin* gene using the following qPCR primers: *Nrf3* forward primer: ACCGAGGCTAGGAACGAGA, *Nrf3* reverse primer: CACTGAGATGCCCTCCAGA, *β-actin* forward primer: TGTCCACCTTCCAGCAGATGT, *β-actin* reverse primer: AGCTCAGTAACAGTCCGCCTAG.

DNA microarray analysis and GSEA

DNA microarray data of tumor tissues derived from Renca-shCtrl or shNrf3 cells in ND or HFD-fed mice, as described in our previous study (Waku et al. 2020a). Briefly, total RNA was processed with the Ambion WT Expression Kit (Affymetrix, Santa Clara, CA, USA) according to the manufacturer's instructions. cRNA was then fragmented, labeled, and hybridized to the Affymetrix Clariom S mouse arrays using the GeneChip WT Terminal Labeling and Hybridization Kit (Affymetrix). The GeneChip fluidics station 450 was used for processing the arrays. Fluorescent signals were detected using the GeneChip scanner 3000 7G. The expression data of all genes in these DNA microarrays were obtained using the Transcription Analysis Console (Affymetrix). They were then subjected to GSEA using open-source software v.3.0 (Subramanian et al. 2005). The gene sets used in this study were also downloaded from the Molecular Signatures Database v7.2. The DNA microarray data presented in this study have been deposited in the Gene Expression Omnibus from NCBI and are accessible through GEO Series accession number GSE213208.

Statistics

Data are reported as mean ± standard deviation (SD). Welch's *t*-test was used to compare the two groups.

Results

Obesity reprograms from the tumorigenic to the antitumorigenic function of Nrf3

We investigated the impact of Nrf3 knockdown on tumor growth in obese mice. We generated mouse renal carcinoma cells with Renca-shRNA-mediated Nrf3 knockdown (Fig. 1A) to address this issue. We then used a diet-induced obese model in which BALB/cCrSlc mice were fed *ad libitum* either a ND or a HFD. After 17 weeks of feeding, the HFD-fed mice gained significantly more weight (Fig. 1B) and exhibited systemic obesity-associated hypercholesterolemia (Fig. 1C). shNrf3 or control shRNA (shCtrl) cells were subcutaneously injected into one frank of ND- or HFD-fed mice. Compared with shCtrl, shNrf3 decreased tumor weight in ND-fed mice (Fig. 1D), whereas it reversely increased in HFD-fed mice (Fig. 1E). We also found that shCtrl cells did not alter tumor weight in ND- and HFD-fed mice (Fig. 1F). Previously, we reported the inhibitory effect of Nrf3 knockdown on tumor growth in ND-fed mice (Waku et al. 2020b). Therefore, these results indicated that Nrf3 acts reversely as a tumorigenic to antitumorigenic factor in obese mice.

Obesity alters the correlation between the expression of Nrf3 and genes related to adipogenesis, myogenesis, and IFNα response in tumors

Next, we investigated the molecular basis of how obesity reverses tumor growth through the regulation of Nrf3 function in cancer cells. Considering that the crosstalk between cancer and other cells is consequently reflected in tumor formation and development (Baghban et al. 2020), we hypothesized that NRF3 function in cancer cells affect not only gene expression in tumor tissues but also tumor cell component. To address this issue, we initially prepared four types of tumor tissues in ND- or HFD-fed mice, including shCtrl-ND, shNrf3-ND, shCtrl-HFD, and shNrf3-HFD. We obtained the expression data of all genes using a DNA microarray analysis (GSE213208). We then performed GSEA using the DNA microarray dataset. GSEA is a threshold-free analysis for all genes, based on their differential expression rank, without prior gene filtering (Fig. 2A). Interestingly, we found that the enrichment plots of gene sets related to "Adipogenesis," "Myogenesis," and "IFNα response" were positively correlated with Nrf3 knockdown in the tumor tissues of ND mice. In contrast, these plots are negatively correlated in the tumor tissues of HFD mice (Fig. 2B-D). The gene expression differences appear to reflect these cell components in tumor tissues, because the tumor contains cancer and associated cells, such as adipocytes, myocytes, and immune cells. These results imply that obesity contributes to the reprogramming of Nrf3-mediated tumorigenesis through the possible recruitment of these stroma cells.

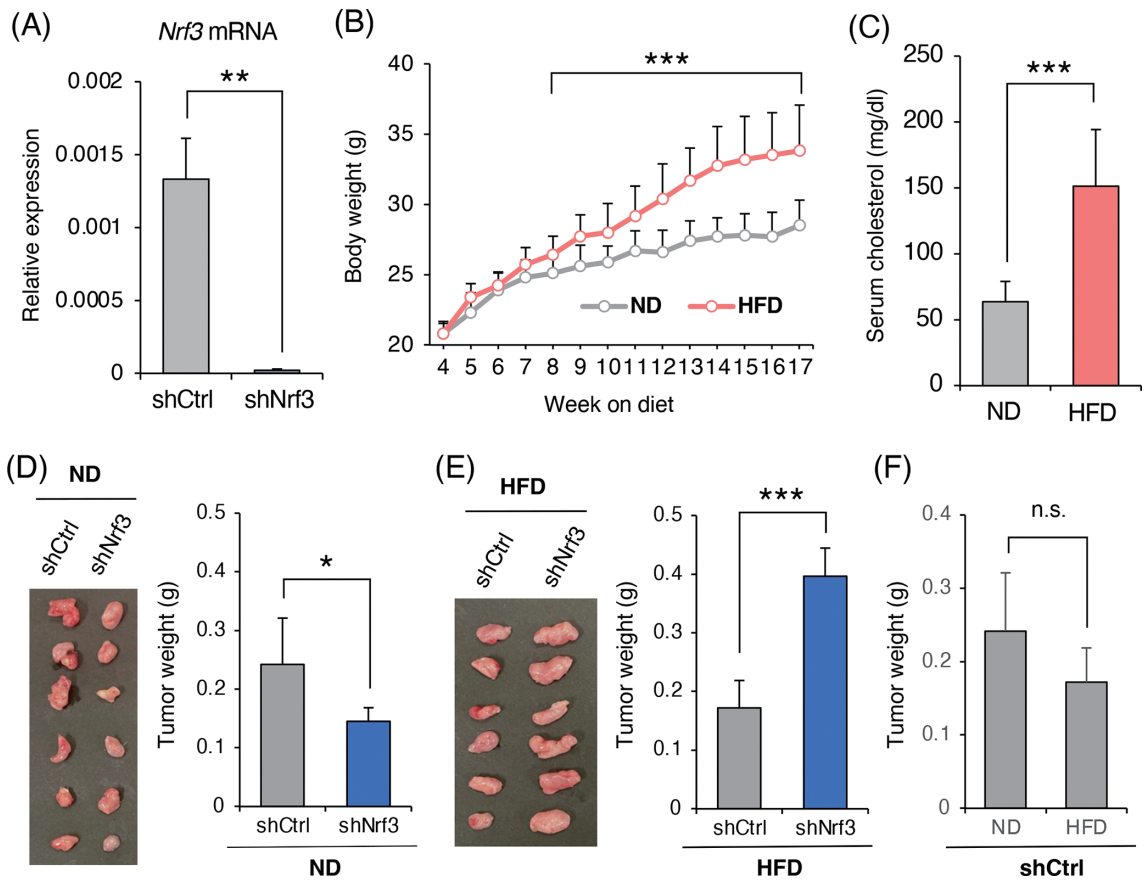

Fig. 1. Tumor growth derived from Nrf3 knockdown cells in high-fat diet (HFD)-fed mice.
(A) Knockdown efficiency of shNrf3 in Renca cells. shCtrl was represented as a control shRNA [$n = 3$, mean ± standard deviation (SD)]. (B) Body weight of BALB/cCrSlc mice fed a high-fat diet (HFD) or a normal diet (ND) at 17 weeks and weighed weekly ($n = 10$ in each group, mean ± SD). (C) The serum total cholesterol levels of BALB/cCrSlc mice fed with a HFD or a normal diet (ND) for 17 weeks. The total cholesterol levels in the serum were measured by the cholesterol oxidase/DAOS method ($n = 10$ in each group, mean ± SD). (D-F) Nrf3 knockdown or a HFD affects tumor growth. Renca-shCtrl cells were injected subcutaneously into ND-fed mice (D) or HFD-fed mice (E). Photographs and weights of tumors 4 weeks after injection are shown in the left and right panels, respectively ($n = 6$ in each group, mean ± SD). Tumor weights of Renca-shCtrl cells in ND-fed mice (D) or HFD-fed mice (E) are also compared in (F). Welch *t*-test: $***p < 0.005$; $**p < 0.01$; $*p < 0.05$. n.s., not significant.

The inhibitory effect of Nrf3 knockdown on cancer cell growth is abolished in adipocyte culture medium

Adipocytes communicate with cancer cells in tumor tissue by releasing various soluble factors. The adipocyte-cancer cell crosstalk leads to phenotypical and functional changes in both cell types, further enhancing tumor growth. Intriguingly, we found using above DNA microarray dataset that the gene expression of *peroxisome proliferator activated receptor gamma* (*Pparg*), of which protein functions as a master regulator of adipogenesis (Rosen et al. 1999), was increased by Nrf3 knockdown in the tumor tissues of ND-fed mice (fold = 2.29, vs. shCtrl). In contrast, it was decreased by Nrf3 knockdown in the tumor tissues of HFD-fed mice (fold = −2.55, vs. shCtrl). In a tumor tissue, cancer cells are associated with several types of stroma cells, such as adipocytes, myocytes, and immune cells. Thus, the distinct expression data of *Pparg* gene imply that *Nrf3* expression in cancer cells affect the crosstalk with adipocytes. Thus, we investigated the effect of adipocyte culture medium on cancer cell growth. To address this issue, we used 3T3-L1 mouse preadipocytes, which are differentiated into adipocytes in response to AIR containing IBMX, dexamethasone, and insulin (Fig. 3A). In the culture medium of preadipocyte 3T3-L1 cells, the growth rate of the Renca cells was reduced by Nrf3 knockdown compared with the control knockdown (Fig. 3B, Pre-CM). Meanwhile, the cell growth reduction by Nrf3 knockdown was abolished in the culture medium of adipocyte 3T3-L1 cells (Fig. 3B, Diff-CM). We investigated whether the difference occurred with the AIR, and confirmed that the cell growth reduction by Nrf3 knockdown was not abolished in the fresh medium containing the AIR (Fig. 3B, AIR-M). These results suggest that the potential reprogramming factor from the tumorigenic to the antitumorigenic function of Nrf3 is generated or secreted (or both combined) from adipocytes. A future study is needed to clarify the impacts of myogenesis and IFNα response on this point.

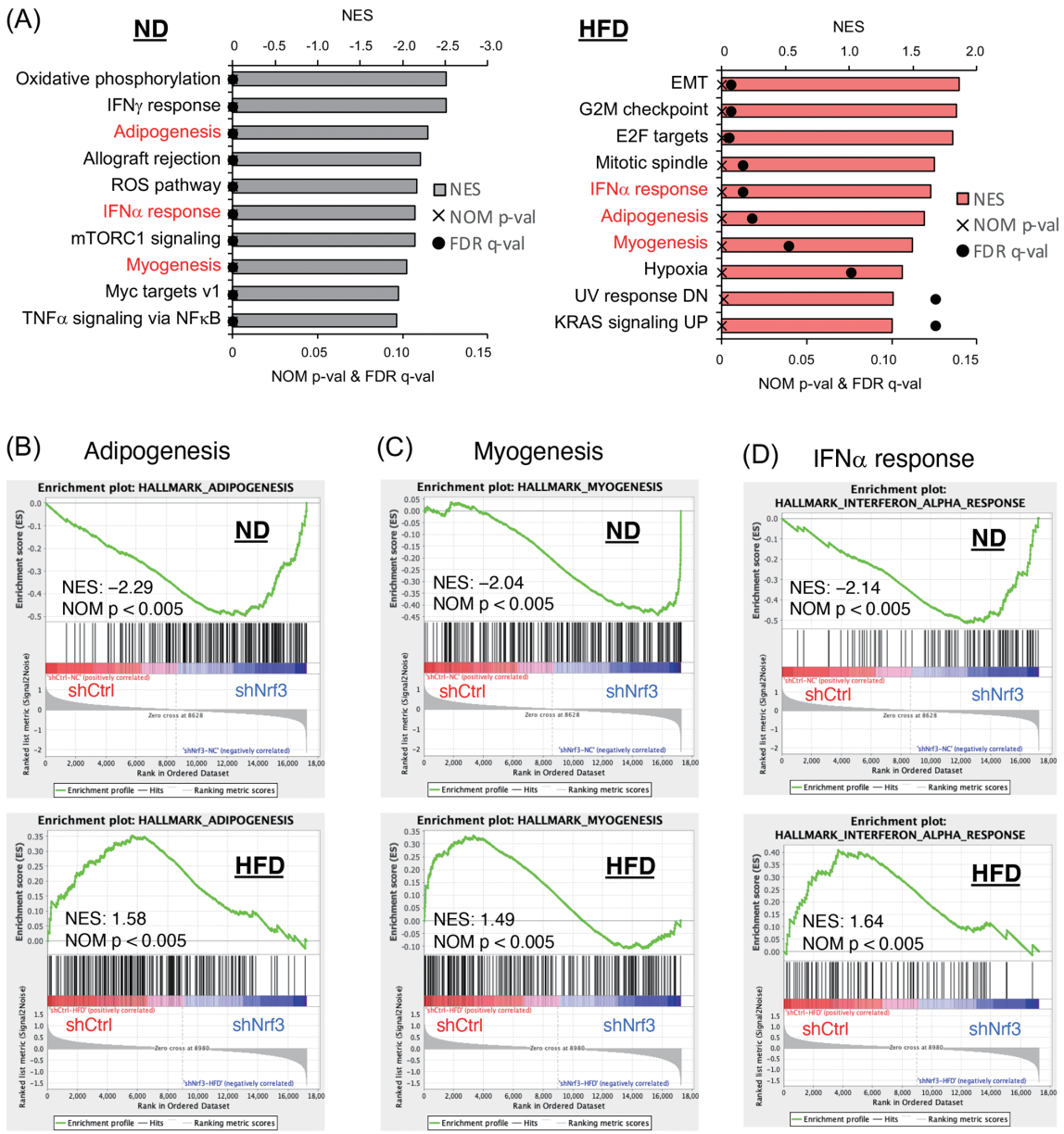

Fig. 2. Gene set enrichment analysis (GSEA) of tumors derived from Nrf3 knockdown cells in mice fed a HFD. (A) The 10 high-ranking gene sets in the tumor tissue in ND- or HFD-fed mice. A DNA microarray dataset of Renca-shCtrl or shNrf3 cells (GSE213208) was analyzed using open-source GSEA software v.3.0 (Subramanian et al. 2005). The rank of gene sets is arranged by normalized enrichment score with nominal p-value (NOM p-val) and false discovery rate q-value (FDR q-val). EMT, epithelial-mesenchymal transition; ROS, reactive oxygen species. (B-D) The enrichment plot of gene sets related to "Adipogenesis" (B), "Myogenesis" (C), or "IFNα response" (D) in the tumor tissue of ND- or HFD-fed mice. NES, normalized enrichment score.

Discussion

Obesity causes cancer development by altering the tumor microenvironment. Previously, we found that NRF3 contributes to tumor growth (Waku et al. 2020b), although the impact of obesity on NRF3-mediated tumorigenesis remains unknown. Here, we used a diet-induced obese mouse model to find that Nrf3 knockdown decreased tumor growth in ND-fed mice. In contrast, it reversely increased tumor growth in HFD-fed mice (Fig. 1), indicating that obesity reprograms from the tumorigenic to the antitumorigenic function of Nrf3. Numerous studies have documented the "obesity paradox," in which obese patients have a better prognosis than lean patients. For example, critical evidence of the obesity paradox in survival after renal cell carcinoma (RCC) that cancer-specific survival was higher in obese patients with RCC was provided in a previous meta-analysis study. Nonetheless, overall survival was lower in obese patients with RCC (Bagheri et al. 2016). Interestingly, in another study using a gene expression analysis linking the body mass index of RCC patients, it was suggested that the survival advantage conferred by obesity

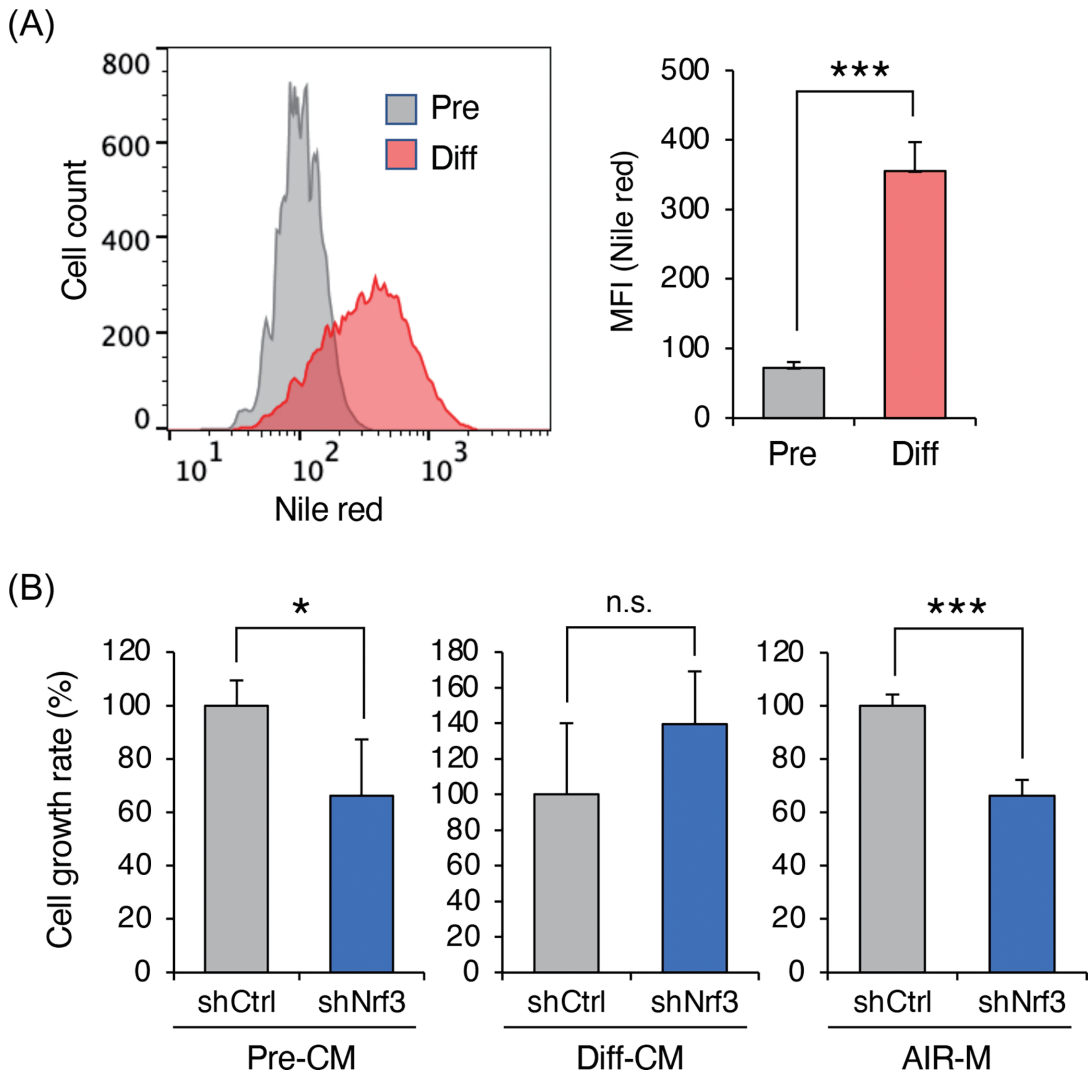

Fig. 3. Nrf3 knockdown cell growth in the adipocyte culture medium.
(A) Intracellular lipid levels in 3T3-L1 mouse preadipocytes or differentiated adipocyte cells. 3T3-L1 preadipocyte cells (Pre) were differentiated into adipocyte cells (Diff). The preadipocytes and differentiated adipocyte cells were stained with Nile Red. Then, the Nile Red fluorescence intensities in the indicated cells were measured by flow cytometry (left). Median fluorescent intensity (MFI) values of Nile Red are shown in the right graph ($n = 3$, mean ± SD). (B) The effect of preadipocyte or adipocyte culture medium on Nrf3 knockdown cell growth. The three-day culture medium of 3T3-L1 preadipocytes or adipocyte cells is represented as Pre-CM (left) and Diff-CM (middle), respectively. On the right, a fresh medium containing the AdipoInducer Reagent (AIR) was used as a control (AIR-M). Renca-shCtrl or shNrf3 cells were grown in the indicated medium for 3 days, and the cell growth rate was calculated ($n = 3$, mean ± SD). Welch *t*-test: $^{***}p < 0.005$; $^{*}p < 0.05$. n.s., not significant.

is highly correlated with gene expression related to fatty acid metabolism, such as fatty acid synthase (FASN). More importantly, the reports showed that, similar to *FASN*, *NRF3* expression is also correlated with the obesity paradox (Hakimi et al. 2013). These insights imply the potential role of NRF3 in the obesity paradox of cancer, particularly RCC, although a future study is needed to clarify this issue.

We further performed GSEA and identified the gene sets related to adipogenesis, myogenesis, and IFNα response, as the potential regulatory factors of the obesity paradox in Nrf3-mediated tumorigenesis (Fig. 2). These results imply an increase or invasion (or both combined) of tumor-associated stromal cells, including adipocytes, myocytes, and immune cells. Among these, we focused on adipogenesis. It was suggested in the cell culture results that adipocytes secrete the potential factors to abolish, but not enough to reverse, the inhibitory effect of Nrf3 knockdown on cancer cell growth. This insight implies that additional factors are needed for tumor growth derived from Nrf3 knockdown cancer cells in HFD-fed mice (Fig.1E). Adipocytes release several adipocytokines, including tumor necrosis factor-alpha (TNF-α) (Kern et al. 1995). TNF-α, as well as Nrf3, functions as tumorigenic and antitumorigenic factor in chronic inflammation and acute inflammation, respectively (Montfort et al. 2019). Furthermore, a previous study reported that TNF-α induces NRF3 expres-

sion *in vitro* culture (Bury et al. 2019). These insights suggest that TNF-α modulates Nrf3 function or expression (or both combined) and decides whether pro- or anti-tumorigenic fate. We need to verify this point. Similar to adipocytes, lymphocytes and myocytes are also associated with cancer cells and act as tumor microenvironment components (Wang et al. 2017), implying that myogenesis and IFNα response are additional potential signals for the obesity paradox in Nrf3-mediated tumorigenesis. On the other hand, several studies have shown that cancer cells exert significant effects on lipid storage and mobilization by adipocytes. For instance, cancer cells stimulate the release of fatty acids by adipocytes, and these fatty acids are transferred to cancer cells for storage and mitochondrial oxidation through triacylglycerol lipases in adipocytes (Balaban et al. 2017). In the future, we should address the molecular mechanism how Nrf3 in cancer cells coordinates a function as well as an increase or invasion (or both combined) of tumor-associated stromal cells, including adipocytes, myocytes, and immune cells.

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Author Contributions

Conceptualization, T.W.; Validation and investigation, T.I., H.M., T.W., S.H., I.A.; Writing – original draft and visualization, T.W., S.H.; Writing – review & editing, A.K.; Supervision and project administration, T.W.; Funding acquisition. T.W., A.K., S.H.

Conflict of Interest

The authors declare no conflict of interest.

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