Development of Oral Squamous Cell Carcinoma with Enhanced Metastatic Potential Through Loss of NDRG2 Expression.

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ABSTRACT

Lymph node metastasis is one of the most important prognostic factors for oral squamous cell carcinoma (OSCC). We previously reported that loss of N-myc downstream-regulated gene 2 (NDRG2) expression plays an important role in the development of OSCC via enhanced activation of PI3K/AKT signalling. In addition, we recently reported NDRG2 as a candidate tumour suppressor in various types of cancer, which is one of the important negative regulators of the PI3K/AKT pathway controlling dephosphorylation of PTEN at the C-terminal S380/S382/T383 (STT). In this study, we show that the majority of OSCC tumours with lymph node metastasis exhibit high levels of phosphorylated AKT-S473 and PTEN-STT and low levels of NDRG2 expression by immunohistochemical staining. Because Ndrg2-deficient mice develop a wide range of tumours, Ndrg2-deficient mice were treated with 4-Nitroquinoline-1-oxide (4-NQO), a tobacco surrogate, to induce OSCC for studying the functional involvement of NDRG2 in OSCC. Number and size of OSCC tumours were significantly increased in Ndrg2-deficient mice. Moreover, the invasion of cervical lymph nodes by OSCC cells was frequently observed in Ndrg2-deficient mice. 4-NQO treatment of OSCC cell lines with low NDRG2 expression induced epithelial-mesenchymal transition (EMT) through the activation of NF-κB signalling, and ectopic expression of NDRG2 reversed the EMT phenotype by inhibiting NF-κB signalling via suppression of PTEN-STT and AKT-S473 phosphorylation. Thus, the loss of NDRG2 expression plays an important role in the development of OSCC with enhanced metastasis via the activation of NF-κB through the AKT pathway.

INTRODUCTION

Oral squamous cell carcinoma (OSCC) is the most common epithelial malignancy in the oral cavity and constitutes more than 90% of all oral malignancies. The numbers of OSCC patients has gradually increased as the number of elderly individuals in the Japanese population has increased. Epidemiological studies have shown that heavy tobacco smoking, high alcohol consumption and human papilloma virus infection are three major risk factors for OSCC development. Moreover, several molecular genetic changes have been reported to be associated with the development of OSCC, such as the activation of the Notch, cell cycle and mitogenic signalling pathways and the inactivation of TP53 (1). Within mitogenic signalling, high frequent activator mutations in the PI3K/AKT signalling pathway were found in EGFR, HRAS, PIK3CA, or AKT1; however, either no mutations or a very low mutation or deletion rate in PTEN as a primary negative regulator have been reported in OSCC tumours.

Although the majority of OSCCs are reported to feature constitutive activation of AKT, the main reasons for this were not known until recent findings of next generation sequence analysis became available (1). However, we have previously reported that N-myc downstream regulated gene-2 (NDRG2) is one of the important negative regulators of the PI3K/AKT signalling pathway and that the loss of NDRG2 expression is found in most cases of OSCC (2). Interestingly, positive phosphorylated-AKT staining was inversely correlated with negative NDRG2 staining in OSCC tumours with moderate to poor differentiation (2), which suggests that the loss

of NDRG2 expression might be important for the development of OSCC with poor prognosis. Moreover, we recently found that NDRG2 is a novel PTEN-binding protein that recruits protein phosphatase 2A to dephosphorylated PTEN at its C-terminus (S380/S382/T383); after binding, the lipid-phosphatase activity of PTEN is maintained (3). Therefore, we suspected that the loss of NDRG2 expression might be involved in the tumourigenesis of OSCC through constitutive activation of the AKT pathway. Therefore, we next investigated whether the loss of NDRG2 might induce OSCC in a mouse model of Ndrg2 deficiency.

To determine the function of NDRG2 in the development of OSCC, the Ndrg2-deficient mice were treated with the well-known chemical carcinogen 4-Nitroquinoline 1-oxide (4-NQO) by adding it to their drinking water. This chemical was used in this study as a surrogate for tobacco exposure to induce the development of oral cancer (4,5). The oral tumours that developed in the Ndrg2-deficient mice were larger and greater in number than those that developed in the wild-type control mice and were also a more pathologically invasive type of carcinoma. Moreover, cervical lymph node metastasis was frequently observed in Ndrg2-deficient mice, which suggests that the loss of Ndrg2 expression might be important for the development of metastasis. Therefore, using a murine model and human OSCC samples, we revealed and discussed the molecular mechanism of OSCC development and metastasis as it relates to the loss of NDRG2 expression.

MATERIALS AND METHODS

Patient samples

Primary OSCC tumour samples that were subjected to immunohistochemistry (IHC) were obtained from patients during surgery. All patient samples were embedded in paraffin for IHC analysis and were used only after permission was received from the ethics committee of the Faculty of Medicine, University of Miyazaki.

Cell lines

Eight human OSCC cell lines (Ca922, HO-1-u-1, HSC2, HSC3, HSC4, SAS, HSQ89, and Sa-3) were bought from the RIKEN BioResource Center. The HaCaT cell line, which is derived from human keratinocytes, was obtained from the Cell Line Service (Eppelheim, Germany) and the hOMK100 cell line, which is derived from human oral mucosa, was purchased from Cosmo Bio (Tokyo, Japan); both were used as controls. hOMK100 cells were maintained in EpiLife medium (Thermo Fisher Scientific, Waltham, MA USA) while the other cell lines were cultured in the appropriate media (RPMI-1640 or Dulbecco's Modified Eagle's Medium) supplemented with 10% foetal bovine serum (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin. To establish the stable knockdown of NDRG2 expression in HaCaT, we used short hairpin RNA (shRNA) expression vector (RNAi-Ready-pSIREN-RetroQ-ZnGreen vector, Clontech) against NDRG2 as described previously (3).

For more information, see Supplementary Materials and Methods.

RESULTS

Low expression of NDRG2 in OSCC enhanced the phosphorylation of AKT through PTEN phosphorylation at the C-terminus (STT)

We previously reported that expression of NDRG2 was downregulated in most OSCC tumours due to promoter methylation, which resulted in enhanced PI3K/AKT signalling in OSCC, even though PTEN expression was maintained and no somatic mutations were found (2). Recently, we found that NDRG2 is a novel PTEN-binding protein that functions as a regulator of PTEN phosphatase activity. The loss of NDRG2 expression in various cancers enhanced the activation of the PI3K/AKT signalling pathway through enhanced phosphorylation of S380T382T383 (STT) in the C-terminus region of PTEN (3). Therefore, we initially determined the expression levels of NDRG2, PTEN, and AKT along with the phosphorylation status of AKT (S473) and PTEN (STT) in OSCC cell lines and primary OSCC tumours by western blot analysis and immunohistochemistry. After testing two control cell lines (hoMK100 and HaCaT cells) and eight OSCC cell lines (Ca922, Ho1u1, HSC2, HSC3, HSC4, HSQ89, SAS and Sa3), sustained expression of PTEN with high phosphorylation at STT as well as high phosphorylation of AKT (S473) were detected in all of the OSCC cell lines by western blot analysis (**Fig. 1A**). Positive staining for phosphorylated AKT (S473) and PTEN (STT) as well as low or negative staining for NDRG2 were observed in serial sections

of a typical OSCC tumour derived from a patient with OSCC (**Fig. 1B**). After 42 tumours from patients with OSCC were examined by immunohistochemistry, high negative rates of NDRG2 staining (81%) with high positive rates of p-AKT (90.5%) and p-PTEN (76.2%) were detected in the primary OSCC tumours (**Table 1**). Since the negative staining of NDRG2 was significantly associated with the positive staining of p-AKT and p-PTEN with statistical differences ($p<0.001$) (**Supplemental Table 1**), the loss of NDRG2 expression might enhance the expression of p-AKT and p-PTEN. In addition, a group of patients with OSCC whose tumours were negative for NDRG2 staining was significantly correlated with a group of patients with OSCC with lymph node metastasis (**Table 1**). This suggests that the loss of NDRG2 expression might be involved in the enhanced metastatic potential of OSCC.

Loss of Ndrg2 expression was involved in the development of SCC in Ndrg2-deficient mice after treatment with 4-Nitroquinoline 1-oxide (4-NQO)

Because the majority of OSCC tumours lose expression of NDRG2 but feature activation of the PI3K/AKT pathway, we next investigated whether the loss of NDRG2 expression might be involved in the development of OSCC tumourigenesis in vivo using Ndrg2-deficient mice. Because 4-Nitroquinoline 1-oxide (4-NQO) is one of many well-known chemical carcinogens that serves as a surrogate for tobacco exposure (4,5). Sixteen wild-type mice and Ndrg2-deficient mice (21 Ndrg2+/- and 10 Ndrg2-/- mice) were treated with 50 μg/mL 4-NQO in their drinking water for 16 weeks after which the mice were given normal water for four weeks (**Fig. 2A**). Then, 20 weeks after treatment

with 4-NQO, oral tumours developed in approximately 50% of wild-type mice. The incidence of tumour formation in both the Ndrg2+/- and Ndrg2-/- mice was significantly increased and the development of tumours in Ndrg2-deficient mice was faster than that in wild-type mice (**Fig. 2B and 2C**). The number of tumours on the tongue and the tumour volumes in Ndrg2-deficient mice were also higher than those in wild-type mice; these differences were statistically significant (**Fig. 2D**). Based on the pathological findings, the incidence of all tumours, including papilloma and squamous cell carcinoma (SCC), in Ndrg2-deficient mice was higher than that of wild-type mice. (**Supplemental Table 2**). In addition, the tumour incidence and multiplicity of invasive type-SCC in Ndrg2-deficient mice were significantly higher than those in wild-type mice (**Supplemental Table 3**). Furthermore, high expression of p-Akt and p-Pten was observed in the tumours that formed in Ndrg2-deficient mice as confirmed by low expression of Ndrg2 by immunohistochemistry using the specific antibodies described earlier (**Fig. 2E**). We also determined the lymph node metastasis rates in the mice that were treated with 4-NQO; however, metastasis to the submandibular lymph nodes, as indicated by immunohistochemical staining for keratin, was not observed in this experiment (**Supplemental Fig. 1**). Thus, these data suggest that the loss of NDRG2 expression might be involved in the development of OSCC and may particularly enhance the invasive capacity of OSCC cells.

A high incidence of cervical lymph node metastasis was observed in Ndrg2-deficient mice treated with 4-NQO for an extended 30 weeks.

Cervical lymph node metastases are very common in patients with OSCC and the lymphatic spread is associated with increased risk of loco-regional recurrence. This significantly impairs the quality-of-life (QOL) of the patients due to lymph node dissection and can also alter the prognosis of the patients. Therefore, to determine whether the loss of Ndrg2 expression might be involved in cervical lymph node metastases, the duration of 4-NQO treatment of the Ndrg2-deficient mice was extended for 30 weeks, which is a timeline based on previous reports (6) (**Fig. 3A**). The numbers of total tumours and SCC tumours in Ndrg2-/- mice were significantly higher than those in wild-type mice (**Supplemental Table 4**), and moreover, the number of invasive tumours in Ndrg2-/- mice was also higher than that in wild-type mice; these differences were statistically significant (**Supplemental Table 5**). Because the tumour burden of the Ndrg2-/- mice was not sufficient for the tumours to invade the oral cavity including the lip, gingiva and oral palate (**Fig. 3B**), we precisely assessed the tumour invasion of the cervical lymph nodes (**Fig. 3C**). In terms of cancer invasion to the cervical lymph nodes, 7 out of 12 Ndrg2-/- mice (58%) demonstrated metastasis to the cervical lymph nodes, although cervical lymph node metastasis was found in only three out of nine wild-type mice (33%). Moreover, the most of lymph nodes (27 out of 34 (79%)) in total 7 Ndrg2-/- mice contained tumour metastasis, although more than half of the lymph nodes showed no metastasis in wild-type mice (**Fig. 3D**), In Ndrg2-/- mice, the average number of metastasis lymph nodes per mouse was significantly increased compared to normal lymph nodes (**Supplemental Table 6**). Therefore, the potential metastatic ability to the cervical lymph nodes was possibly enhanced by the loss of Ndrg2 expression in

OSCC.

Enhanced activation of the PI3K/AKT signalling pathway by 4-NQO treatment in the development of SCC.

Tobacco exposure is one of most important aetiological factors in oral cancer tumourigenesis, and the tobacco carcinogens 4-NQO or NNK activate several signal transduction pathways, including AKT, during the early premalignancy stage of oral cancer development (7). Because mTOR inhibition by rapamycin has been shown to prevent early onset of 4-NQO-induced SCC in a mouse model (8), we next investigated whether treatment with 4-NQO affects tumourigenesis and cell invasion capacity in OSCC cells through the activation of the PI3K/mTOR signalling pathways. After the treatment of the SAS/OSCC cell line with 1 μM of 4-NQO, various types of signalling molecules involved in the PI3K/AKT pathway were determined at each indicated time point by the specific antibodies discussed earlier (**Supplemental Fig. 2A**). Five to ten min after 4-NQO treatment, the levels of phosphorylated PTEN (STT), AKT (S473), GSK3β (S9) and S6 (S240/244) were gradually increased until 6 hours after treatment. Moreover, to investigate whether 4-NQO treatment affects the cell migration capacity, we measured the distance between the scratch edges, 12 hours after a scratch was generated on the glass slide containing the SAS/OSCC cells that were treated with or without 4-NQO. The 4-NQO treatment enhanced the cell migration capacity in a dose-dependent manner, and SAS/OSCC cells treated with the highest dose of 4-NQO migrated to fill the gap of the scratch (**Supplemental Fig. 2B**). Therefore, activation of

the AKT signalling pathway and the cell migration capacity were enhanced after the treatment of OSCC cells with 4-NQO.

Because NDRG2 suppresses the AKT signalling pathway through the activation of PTEN phosphatase activity by dephosphorylation, we next determined whether forced NDRG2 expression suppresses the enhanced activation of the AKT signalling pathway induced by the treatment of OSCC cells with 4-NQO. Two cell lines, SAS/OSCC and HeLa/cervical cancer cell lines presented low NDRG2 expression with sustained wild-type PTEN expression and were stably transfected with NDRG2 expression vector (2, 3). These cell lines were analysed for the expression levels and the phosphorylation status of various AKT signalling molecules at each time point after treatment with 1 μM 4-NQO (**Fig. 4A**). In mock control cells, the phosphorylation level of PTEN at STT was enhanced after 5 min of treatment with 4-NQO while the phosphorylation of AKT, GSK3β and S6 was enhanced after 5 to 10 min of treatment. On the contrary, neither the phosphorylation status of PTEN, AKT, GSK3β and S6 was different before and after treatment with 4-NQO in the cell lines expressing NDRG2.

Moreover, to determine whether NDRG2 expression suppresses the enhanced cell migration capacity induced by the 4-NQO treatment, a cell scratch test was performed in SAS/OSCC cells with (SAS/NDRG2^{high}) or without NDRG2 expression (SAS/NDRG2^{low}). After treatment with 4-NQO, SAS/NDRG2^{low} cells with mock transfection migrated to almost completely fill the scratched gap after 12 hours; however, SAS/NDRG2^{high} cells could not move inside the gap within 12 hours after treatment with 4-NQO (**Fig. 4B**). To confirm whether cell invasion capacity in OSCC

cells is also suppressed by NDRG2 expression, we next used a Boyden chamber invasion assay. After SAS/NDRG2^{low} cells were treated with 4-NQO, the number of invading cells increased to approximately two times higher than that under no treatment with 4-NQO; however, the numbers of the invading cells were not different before and after treatment of SAS/NDRG2^{high} cells with 4-NQO (**Fig. 4C**). We also investigated the reverse experiment through the suppression of NDRG2 expression in HaCaT cell line. We established stable HaCaT cell lines expressing short hairpin RNA against NDRG2 (shNDRG2) or luciferase (shluc) as a control. The level of NDRG2 protein expression was significantly lower in HaCaT/shNDRG2 than in HaCaT/shluc. The phosphorylation rates of PI3K/AKT signaling pathway (PTEN, AKT, GSK3β and S6) were remarkably increased in HaCaT/shNDRG2 with the treatment of 4-NQO compared to HaCaT/shluc (**Supplemental Fig. 2C**). A wound-healing assay revealed that after treatment of 4-NQO, HaCaT/shluc recovered about 50% of the wound area, whereas HaCaT/shNDRG2 covered nearly 100% of the wound area (**Supplemental Fig. 2D)**. Furthermore, the number of invading cells with 4-NQO treatment was increased in HaCaT/shNDRG2 compared with HaCaT/shluc (**Supplemental Fig. 2E**).

Because the level of phosphorylation of PI3K under the EGF receptor was enhanced by 4-NQO treatment (8), we used LY294002 as a specific inhibitor for PI3K to determine whether the activation status of the AKT signalling pathway induced by 4-NQO might be suppressed by LY294002 treatment in two OSCC (SAS and HSC3) cell lines and one cervical cancer (HeLa) cell line (**Fig. 4D**). Although 4-NQO treatment enhanced the phosphorylation status of AKT, GSK3β and S6, treatment with 4-NQO

with LY294002 suppressed the phosphorylation of AKT, GSK3β and S6 in three cancer cell lines. Along with suppression of AKT signalling, the cell migration ability of SAS/NDRG2^{low} cells was also suppressed by LY294002 treatment (**Fig. 4E**). Therefore, the activation of PI3K by EGF or other receptors is important in the ability of 4-NQO to activate the AKT signalling pathway, which results in enhanced cell migration and invasiveness of OSCC. The phosphorylation levels of the AKT signalling pathway components were significantly enhanced in mouse embryonic fibroblasts (MEFs) derived from Ndrg2-deficient mice after 4-NQO treatment **(Supplemental Fig. 2F**). Additionally, forced NDRG2 expression in HSC3/OSCC cells suppressed their cell migration and invasion abilities after 4-NQO treatment (**Supplemental Figs. 2G and 2H**), which suggests that enhanced activation of PI3K by 4-NQO treatment and the loss of NDRG2 expression cooperate in the development of OSCC with enhanced metastasis.

EMT was important for enhanced metastasis after 4-NQO treatment and the loss of NDRG2 expression in OSCC.

Epithelial mesenchymal transition (EMT) is one of the important molecular mechanisms of metastasis, and the expression of NDRG2 was reported to abrogate EMT, which inhibits the invasion and migration of colorectal cancer cells (9). For these reasons, we further investigated whether the loss of NDRG2 expression in OSCC cells contributes to their metastatic potential through enhancement of EMT. Therefore, we initially treated the SAS/OSCC cells with 4-NQO to determine whether the EMT

phenotype is enhanced by 4-NQO and whether its effect is suppressed by NDRG2 expression in OSCC cell lines. After treatment with 4-NQO for 24 hours, the classical epithelial morphology of SAS/NDRG2^{low} cells, which formed densely packed colonies, changed to a mesenchymal, spindle-shaped morphology with more dispersed cell aggregates. However, the morphology of SAS/NDRG2^{high} cells maintained their epithelial morphology and continued to form densely packed colonies (**Fig. 5A**). To confirm this result, we further investigated changes in the expression of genes related to EMT, such as the mesenchymal markers Snail, Vimentin, and N-cadherin, and the epithelial marker E-cadherin. This was accomplished by semiquantitative RT-PCR, real-time PCR and immunofluorescence staining using antibodies specific to Vimentin, E-cadherin and NDRG2 (**Figs. 5B to 5C, Supplemental Figs. 3A and 3B**). The expression of mesenchymal markers (Snail, Vimentin, and N-cadherin) was significantly enhanced and the expression of E-cadherin was decreased in SAS/NDRG2^{low} cells at 24 hours after 4-NQO treatment. Moreover, the enhanced expression of Snail, Vimentin, and N-cadherin along with suppression of E-cadherin expression after 4-NQO treatment was almost completely abrogated in SAS/NDRG2^{high} cells. Conversely, EMT phenotype was enhanced with 4-NQO treatment in HaCaT/shNDRG2 compared with HaCaT/shluc (**Supplemental Fig. 3C**). We also confirmed that the expression of mesenchymal markers such as Snail, Vimentin, and N-cadherin was significantly enhanced and the epithelial marker, E-cadherin was decreased in HaCaT/shNDRG2 after the treatment of 4-NQO compared with HaCaT/shluc by semiquantitative RT-PCR, and real-time PCR (**Supplemental Figs. 3D**

and 3E). Therefore, NDRG2 expression might suppress metastatic events of OSCC through the inhibition of EMT progression.

NDRG2 suppressed EMT through the inhibition of the AKT and NF-κB signalling pathways.

Although transforming growth factor-β (TGFβ) signalling has a predominant role in EMT, the convergence of other signalling pathways such as PI3K/AKT and NF-κB is also essential for EMT (10-12). Therefore, we next determined whether the 4-NQO treatment could modulate EMT signalling through the NF-κB and/or PI3K/AKT pathways. To investigate whether 4-NQO treatment modulates the NF-κB signalling pathway, SAS/OSCC and HSC3/OSCC cell lines were treated with 4-NQO and/or LY294002, a PI3K inhibitor, to determine the effects on the NF-κB pathway. The levels of phosphorylated IKKα/β and IκBα were increased by the 4-NQO treatments in the two cell lines, while the total IκBα protein was decreased. However, the enhanced level of phosphorylation induced by 4-NQO was almost completely suppressed by LY294002 treatment (**Fig. 6A**). Therefore, the activating effects of 4-NQO on NF-κB signalling were derived from the activation of PI3K/AKT. Moreover, forced expression of NDRG2 abrogated the effects of 4-NQO treatment, which increased the phosphorylation of IKKα/β and IκBα but decreased the levels of total IkBα protein (**Fig. 6B**). To confirm these results, we determined the level of active NF-κB according to the nuclear localization and expression level of the p65 protein, which is a transcription factor of the canonical NF-κB pathway, after 4-NQO treatment with or without forced expression

of NDRG2. The level of p65 protein was increased in the nucleus at 2 hours after 4-NQO treatment along with increased NF-κB transcriptional activity; however, the forced-expression of NDRG2 in OSCC cells abrogated the nuclear localization of p65 and the activation of NF-κB-mediated transcription (**Supplementary Fig. 4A and Fig. 6C**). Using MEFs derived from Ndgr2-deficient mice, we investigated the relationship between Ndrg2 expression and the effect of 4-NQO treatment on the NF-κB signalling pathway. The 4-NQO treatment enhanced NF-κB activity with increased phosphorylation of Ikkα/β and IκBα, suppressed total protein of IκBα, and increased NF-κB transcription activity in MEFs derived from Ndrg2-deficient mice compared with MEFs derived from control wild-type mice (**Figs. 6D and E)**. Furthermore, the HaCaT/shNDRG2 cell line showed increased phosphorylation of IKKα/β and IκBα, decreased total IκBα levels, increased nuclear translocation of p65, and increased NF-κB transcriptional activity as compared with the HaCaT/shluc cell line (**Supplementary Figs. 4B to 4D**).

To confirm whether activation of NF-κB signalling is important for the enhanced expression of mesenchymal markers (TWIST, Snail, Vimentin, and N-cadherin) and the downregulation of E-cadherin, BAY 11-7082, which is an NF-κB inhibitor, was administered to SAS/OSCC cells with or without forced NDRG2 expression. 4-NQO-induced EMT phenotype was suppressed by the treatment of BAY 11-7082 in SAS/NDRG2^{low} cells (**Supplementary Fig. 4E)**. The 4-NQO-induced enhancement of expression of mesenchymal markers (Snail, Vimentin, and N-cadherin) and the downregulation of E-cadherin expression in SAS/NDRG2^{low} cells were abrogated by

the treatment of BAY 11-7082 (**Figs. 6F and 6G**). The suppressive effect of BAY 11-7082 on the action of 4-NQO in SAS/control cells was abrogated by the forced-expression of NDRG2. Therefore, NF-κB activation might be an important signalling pathway in EMT progression and the expression level of NDRG2 may be one of the key factors in the progression of metastasis in OSCC.

DISCUSSION

In this study, the loss of NDRG2 expression not only promoted oral carcinogenesis in a chemically induced-OSCC mouse model using 4-NQO, it also promoted metastasis of human OSCC. High expression of NDRG2 suppresses the PI3K/AKT and NF-κB signalling pathways through the dephosphorylation of PTEN at the C-terminal domain, which results in the suppression of EMT phenotypes in OSCC cells. Because a group of OSCC patients whose tumours were negative for NDRG2 according to immunohistochemistry was significantly correlated with a group of OSCC patients with lymph node metastasis, a low level of NDRG2 expression may contribute to enhanced tumour development with cervical metastasis in OSCC.

For metastasis to occur, cancer cells frequently change their gene expression programmes to those of embryonic cells during foetal development, such as EMT and/or various signalling pathways that are induced by TGF-β signalling (13). On the contrary because local inflammation, hypoxia, and/or infection are the major activators of metastasis and invasion, cigarette smoking, alcohol addiction, and/or viral infections such as HPV are the major risk factors for OSCC. Importantly, NF-κB signalling is

established as a critical mediator in the response to the inflammation and infections in cases of OSCC (14). Because activation of the NF-κB pathway is required for the induction and maintenance of Ras-/PI3K/AKT- and TGF-β-dependent EMT (10, 11), in this manuscript, we show that activation of the NF-κB pathway through the loss of NDRG2 expression is crucial for the activation of EMT in OSCC; this process might also be involved in the lymph node metastasis observed in OSCC. Therefore, both the 4-NQO treatment as an activator of PI3K/AKT and the loss of NDRG2 expression as an inhibitor of PTEN activation co-ordinately enhance the activation of the NF-κB pathway through PI3K/AKT, which results in enhanced carcinogenesis of OSCC with cervical metastasis. Because the loss of expression of NDRG2 might be induced by promoter methylation through activators of the NF-κB pathway (e.g., chronic inflammation and infection) (paper in preparation), this suggests that constitutive activators of the NF-κB pathway, such as heavy tobacco use, alcoholism and chronic infection, might be important risk factors for the development and metastasis of OSCC.

The loss of NDRG2 expression by promoter methylation has been frequently reported to involve tumourigenesis and enhanced metastasis in breast, prostate, hepatocellular, gastric, pancreatic, and colon cancers, among others (15-20). In regards to cancer metastasis, a few papers have reported that the loss of NDRG2 expression enhanced EMT in colorectal, gallbladder and breast cancer cells (9, 20, 21); however, the direct molecular mechanism by which NDRG2 affects EMT has not yet been fully elucidated. We previously reported that NDRG2 can bind directly to PTEN and NIK to modulate their enzyme activity by phosphorylation via the recruitment of PP2A

phosphatase with NDRG2; this in turn leads to the suppression of the PI3K/AKT and NF-κB signalling pathways (3, 22). Because the PI3K/AKT and NF-κB signalling pathways, along with TGFβ signalling, are important regulators of EMT, here, we demonstrate the molecular mechanism of EMT progression in OSCC cells that have lost NDRG2 expression (Supplemental **Fig. 5**). When OSCC cells lose NDRG2 expression, PP2A cannot be recruited to PTEN, which results in a high phosphorylation status; this inactivates the lipid phosphatase activity of PTEN. Because 4-NQO enhances the phosphorylation of PI3K thorough the activation of some receptor-type tyrosine kinases, the activation of AKT is derived from the effects of both PI3K activation by 4-NQO and PTEN inactivation by loss of NDRG2 expression. Phosphorylated AKT activates the canonical NF-κB pathway and NF-κB activates the transcription of the EMT-related transcription factors TWIST and SNAIL, which inhibit the transcription of E-cadherin and activate the transcription of Vimentin and N-cadherin. Therefore, most of the published data on the regulation of EMT by NDRG2 is explained in this scheme, with the exception of STAT3 regulation. Because NDRG2 suppresses the expression of SOCS3 (suppressor of cytokine signalling), the level of phosphorylation of STAT3 is enhanced to induce the transcription of SNAIL (20, 23); however, direct evidence as to how NDRG2 regulates the transcription of SOCS and how JAK/STAT signalling regulates SNAIL expression was not found. Therefore, in the future, we need to further analyse the relationship between NDRG2 expression and the JAK/STAT signalling pathway, as well as the relationship between SNAIL transcription and JAK/STAT signalling.

NDRG2 functions as a stress-induced gene that maintains homeostasis in the cellular environment after genotoxic stress that involves p53, hypoxic stress mediated by HIF1α, or inflammation mediated by the NF-κB signalling pathway (24-26). Particularly, according to the work presented in this manuscript, the activation of NF-κB has an important function in the development of OSCC with cervical metastasis. The progression of OSCC might be dependent on the activation of a signal activator, such as 4-NQO, along with the suppression of a signal repressor, such as PTEN or NDRG2 inactivation. When we prevent the progression of OSCC carcinogenesis, the PI3K/AKT and NF-κB signalling pathways may be turned off by specific molecularly targeted drugs. Recently, we revealed that abnormal methylation of the NDRG2 promoter might be dependent on EZH1/EZH2 expression in leukaemia cells (paper in preparation). Therefore, in the near future, we plan to identify the relationship between the expression of PRC2 methylation enzymes (e.g., EZH1/EZH2) and the progression and metastasis of OSCC. When we identify the molecular mechanism of abnormal methylation in OSCC, one goal would be the development of a novel therapeutic drug targeted at PRC2 methylation enzymes for the treatment of OSCC.

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FIGURE LEGENDS

Figure 1. Identification of the expression of NDRG2, p-AKT (S473), and p-PTEN (STT indicated as Ser380/Thr382/Thr383) proteins in OSCC tissues.

(A) The protein expression of p-PTEN (STT), total PTEN, p-AKT (S473), total AKT, NDRG2 and β-actin (as a control) was determined in eight OSCC cell lines and two control cell lines (hoMK100 and HaCaT) using the specific antibodies described in the materials and methods.

(B) An immunohistochemical analysis of NDRG2, p-AKT (S473) and p-PTEN (STT) was performed in 32 human OSCC specimens using the specific antibodies described in the materials and methods (**Table 1**). H & E is indicated as haematoxylin and eosin staining.

Figure 2. Induction of OSCC tumours in Ndrg2-deficient mice by treatment with 4-NQO in the drinking water.

(A) This scheme represents the protocol for the treatment of the mice with 4-NQO for 16 weeks with an observation period of an additional four weeks.

(B) Images of the murine oral cavity show tumour formation on the tongue of each mouse after the administration of 4-NQO to wild-type (WT) and Ndrg2-deficient mice (Ndrg2+/- and -/-).

(C) Time course of tumour incidence is presented after 4-NQO treatment in each mouse group. Star (*) indicates a statistically significant difference at $p<0.01$ and double stars

(**) indicate a statistically significant difference at $p<0.005$.

(D) Comparisons of the number of tumours that developed (left) and the sizes of those tumours (right) between wild-type and Ndrg2-deficient mice are shown along with the statistical analysis. Star (*) indicates a significant difference at $p<0.01$ and double stars (**) indicate a significant difference at $p<0.005$.

(E) Tumours that developed on the tongue of each indicated mouse were immunostained using antibodies specific to Ndrg2, p-Akt (S473) and p-Pten (STT) and were also stained with haematoxylin and eosin (H & E).

Figure 3. Induction of OSCC tumours with metastasis in Ndrg2-deficient mice by treatment with 4-NQO for longer periods of time until 30 weeks.

(A) This scheme represents a protocol for the treatment of mice with 4-NQO for 30 weeks with an observation period of an additional two weeks.

(B) Images show the oral tumours on the faces of the mice in each group. Arrows indicate tumours on the tongue and the arrowhead near the Ndrg2-/- mice indicates swelling around the mouth due to tumour cell infiltration.

(C) This image shows swelling of the cervical lymph node (white arrow).

(D) The swollen lymph nodes were immunohistochemically stained with an antibody against keratin, which illustrates metastasis in two sections.

Figure 4. Treatment with 4-NQO and the loss of NDRG2 expression acted together to enhance the migration and invasion capacity of OSCC cells.

(A) Using, SAS/OSCC or HeLa cells transfected with the mock control or the NDRG2-expressing plasmid, the effect of 4-NQO treatment on the PI3K/AKT signalling pathway were determined at each time point by western blot analysis using antibodies specific to NDRG2, Flag, phosphorylated PTEN (STT) with total PTEN, phosphorylated AKT (S473) with total AKT, phosphorylated GSK3β (S9) with total GSK3β, phosphorylated S6 (S240/244) with total S6 and β-actin as a control.

(B) Cell migration ability under four different conditions with/without expression of NDRG2 and with/without 4-NQO treatment in SAS/OSCC cells was determined by in vitro scratch assays. Images on the left show SAS cells under four different conditions on the glass slide; the black lines indicate the initial edge of the cells, while the red arrows indicate the migrated cells after the scratch was made. The bar graph (right side) illustrates the relative migration rates of SAS cells under the four different conditions indicated under the bar.

(C) The images on the left show migrated SAS/OSCC cells under the four different conditions indicated in the figure; these cells were stained with crystal violet in a cell invasion assay using Boyden chambers (magnification, ×100). The bar graph on right side shows the number of migrated cells under the four different conditions. Star (*) indicates statistical significance of $p<0.05$.

(D) A PI3K inhibitor (LY294002) suppressed the 4-NQO-induced activation of AKT signalling in three cell lines (SAS/OSCC, HSC3/OSCC and HeLa cells). Using specific antibodies, the expression of phosphorylated AKT (S473) with total AKT, phosphorylated GSK3β (S9) with total GSK3β, phosphorylated S6 with total S6 and

β-actin (as a control) was determined in three cell lines cultured under the four different conditions indicated in the figure.

(E) A PI3K inhibitor (LY294002) suppressed the 4-NQO-induced migration of SAS/OSCC cells. Images on the left side show SAS cells under the four different conditions on the glass slide; the black lines indicate the initial edge of the cells, while the red arrows indicate the migrated cells after the scratch was made. The bar graph (right side) illustrates the relative migration rates of SAS cells under the four different conditions indicated under the bar. Star (*) indicates statistical significance at $p<0.05$. The data represent at least three experiments performed in triplicate.

Figure 5. 4-NQO treatment induced EMT but the activation of EMT by 4-NQO was suppressed by high NDRG2 expression.

(A) Images show the morphology of SAS cells with or without NDRG2 expression before and 24 hours after the 4-NQO treatment. 4-NQO treatment induced morphological changes in the spindle-shaped cells; however, NDRG2 expression suppressed those morphological changes induced by 4-NQO treatment in SAS cells.

(B and C) mRNA expression of the mesenchymal genes Snail, TWIST1, Vimentin, and N-Cadherin was induced by 4-NQO treatment, while the mRNA expression of the epithelial gene E-cadherin was suppressed. After the introduction of the NDRG2 expression plasmid into SAS cells, the changes in gene expression by 4-NQO treatment were significantly suppressed. Semi-quantitative RT-PCR was used to determine the expression of the indicated genes **(B)** and the relative expression rates and statistical

significance of each gene were determined by quantitative RT-PCR **(C)**. Star (*) indicates statistical significance at $p<0.05$. The data represent at least three experiments performed in triplicate.

Figure 6. 4-NQO regulated EMT through the activation of NF-κB signalling.

(A) 4-NQO treatment activated NF-κB signalling, and the activation of NF-κB signalling by 4-NQO was suppressed by the PI3K inhibitor LY294002 in three cell lines (SAS/OSCC and HSC3/OSCC cells). The expression of phosphorylated IKKα/β, phosphorylated IκBα (S32/36) with total IκBα and β-actin (as a control) was determined by western blot analysis using specific antibodies in two cell lines under four different conditions, as indicated.

(B) SAS/OSCC cell line was treated with 4-NQO at each time point under low (mock) or high NDRG2 expression conditions, and the activation of NF-κB was determined by western blot analysis.

(C) NF-κB transcriptional activity was determined by an NF-κB promoter assay using a luciferase reporter under the same condition as in **(Supplemental Fig. 4)**. Star (*) indicates statistical significance at $p<0.05$.

(D) Using MEF cells derived from wild-type and Ndrg2(-/-) mice, we found that 4-NQO treatment activated NF-κB signalling in MEF cells derived from the Ndrg2(-/-) mice, but not in those derived from wild-type mice; the protein level of phosphorylated IKKα/β, phosphorylated IκBα (S32/36) with total IκBα and β-actin (as a control) was determined by western blot analysis.

(E) NF-κB transcriptional activity was determined by an NF-κB promoter assay using a luciferase promoter under the same condition as in (E). Star (*) indicates statistical significance at $p<0.05$. The data represent at least three experiments performed in triplicate.

(F) mRNA expression of the mesenchymal markers Snail, Vimentin and N-cadherin and the epithelial marker E-cadherin was determined by quantitative RT-PCR in eight different combined conditions: NDRG2 (+/-), 4-NQO treatment (+/-) and administration of the NF-κB inhibitor BAY 11-7082 (+/-). mRNA expression of Snail, Vimentin and N-cadherin was induced by 4-NQO treatment; however, NDRG2 expression or BAY 11-7082 treatment inhibited the expression of Snail, Vimentin and N-cadherin mRNA. In contrast, the expression of E-cadherin mRNA was suppressed by 4-NQO treatment; however, NDRG2 expression or BAY11-7082 treatment reversed the inhibition by 4-NQO treatment. Star (*) indicates the statistical significance ($p<0.05$). The data represent at least three experiments performed in triplicate.

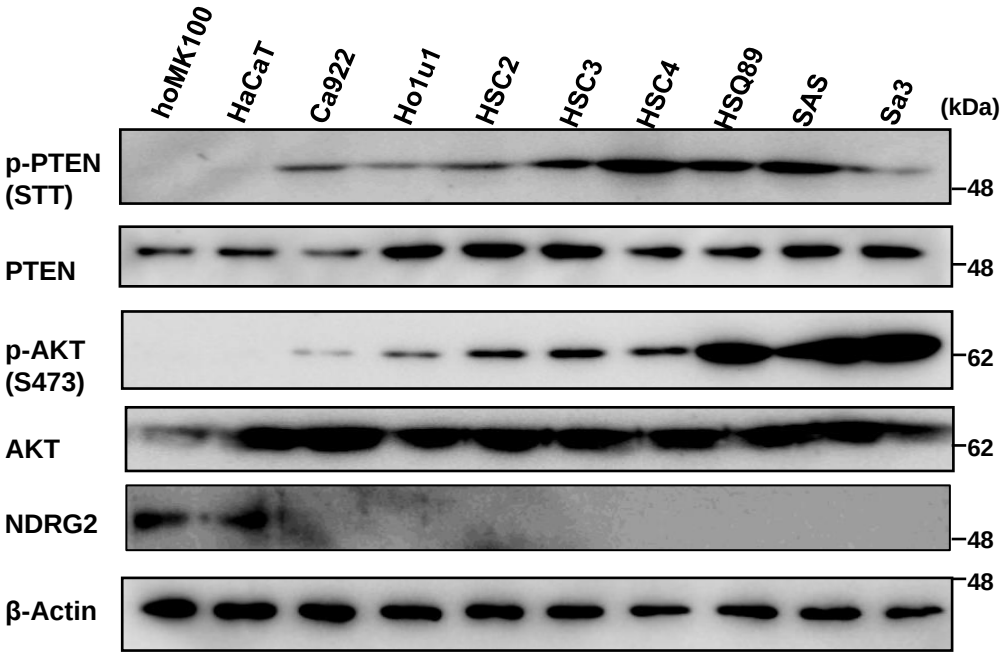
(G) The same effect of BAY11-7082 was confirmed in **(E)** cells under the same condition by western blot analysis of E-cadherin, Vimentin and β-actin (as a control).

Table 1. Expression of NDRG2, p-Akt, p-PTEN in OSCC patients

Parameter	N	NDRG2			p-Akt			p-PTEN		
		Negative (%)	Positive (%)	p value	Negative (%)	Positive (%)	p value	Negative (%)	Positive (%)	p value
Total	42	34 (81)	8 (19)	p<0.001	4 (9.5)	38 (90.5)	p<0.001	10 (23.8)	32 (76.2)	p<0.001
Gender				0.65			0.448			0.209
Male	24	20 (83.3)	4 (16.7)		3 (12.5)	21(87.5)		4 (16.7)	20(83.3)	
Female	18	14 (77.8)	4 (22.2)		1 (5.6)	17(94.4)		6 (33.3)	12(66.7)	
TNM stage				0.013			0.57			0.177
I - II	26	18 (69.2)	8 (30.8)		3 (11.5)	23(88.5)		8 (30.8)	18 (69.2)	
III - IV	16	16 (100)	0 (0)		1 (6.3)	15(93.7)		2(12.5)	14 (87.5)	
T stage				0.035			0.786			0.101
T1/T2	29	21 (72.4)	8 (27.6)		3 (10.3)	26(89.7)		9 (31)	20 (69)	
T3/T4	13	13 (100)	0 (0)		1 (7.7)	12 (92.3)		1(7.7)	12 (92.3)	
Lymph nodes				0.321			0.812			0.439
N0	17	15 (88.2)	2 (11.8)		1 (5.9)	16 (94.1)		3 (17.6)	14 (82.4)	
N+	25	19 (76)	6 (24)	p<0.001	3 (12)	22 (88)		7 (28)	18 (72)	

Figure 1

A


hoMK100
HaCaT
Ca922
Ho1u1
HSC2
HSC3
HSC4
HSQ89
SAS
Sa3
(kDa)
p-PTEN (STT)
PTEN
p-AKT (S473)
AKT
NDRG2
β-Actin
48
48
62
62
48
48

B

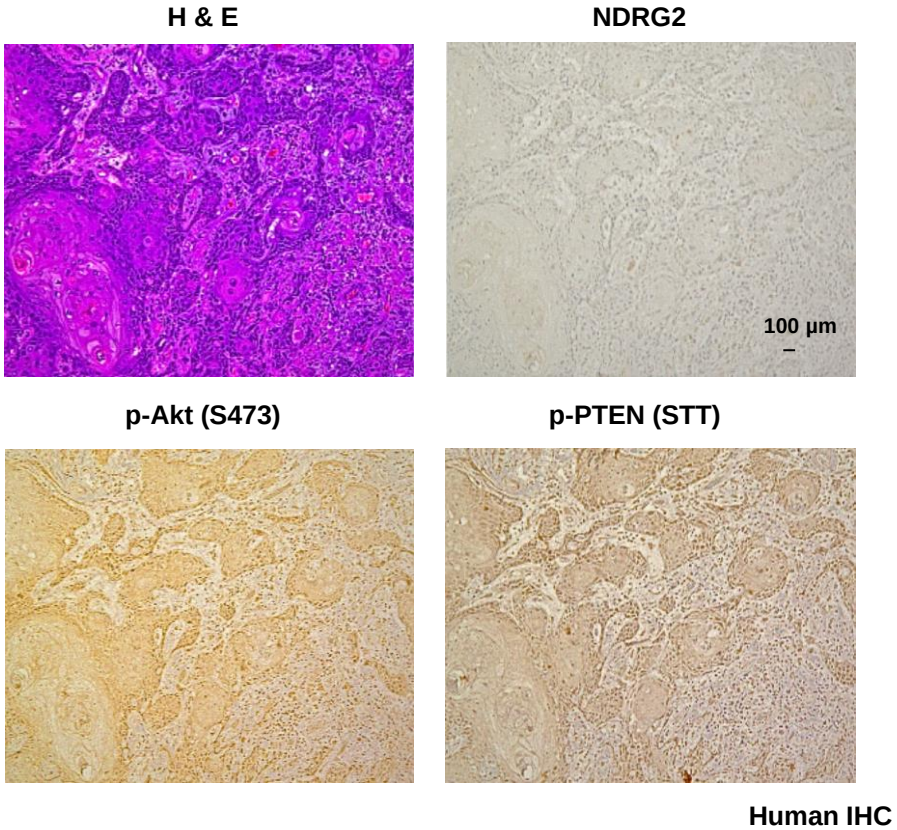
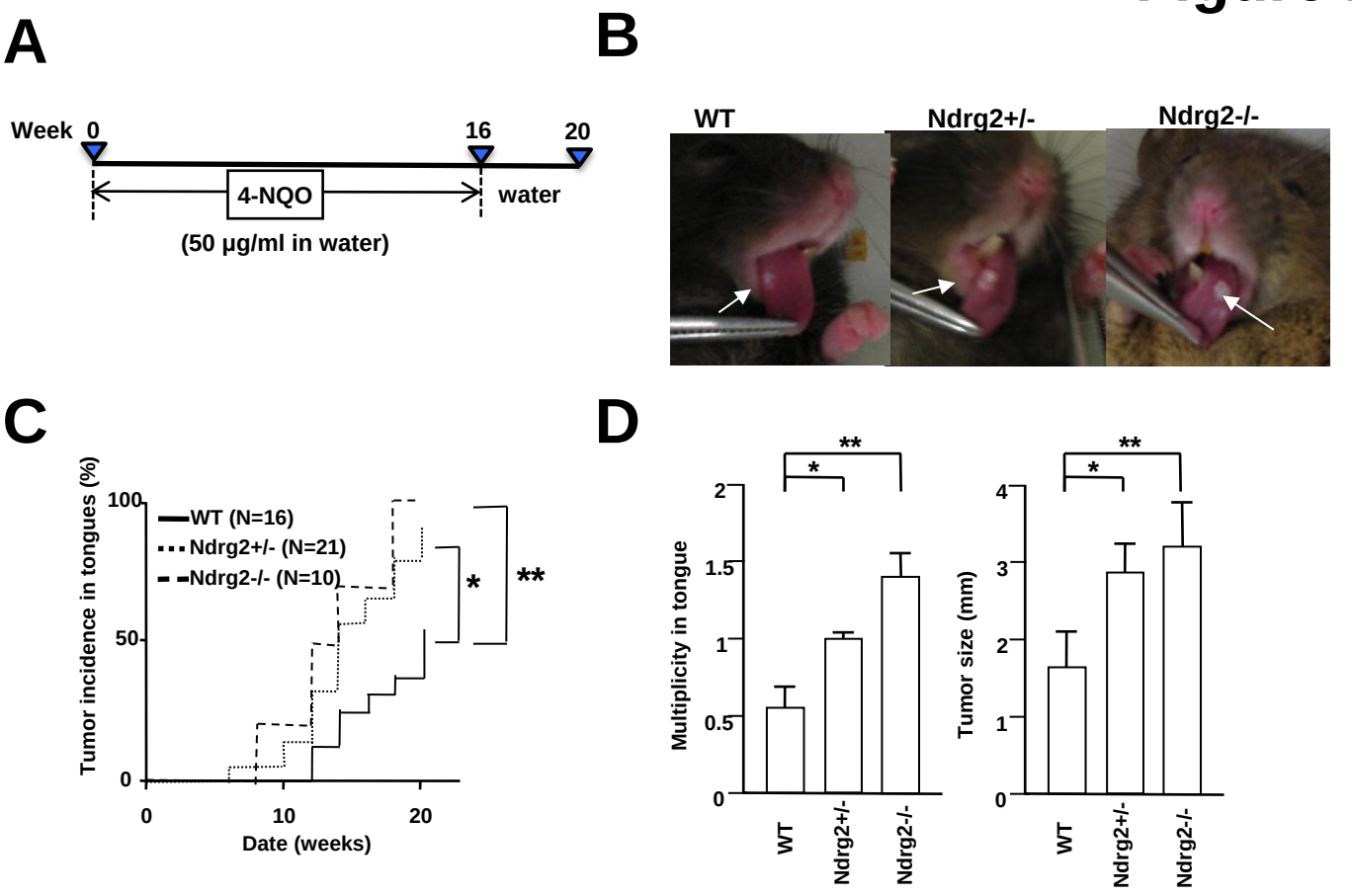

H & E
NDRG2
100 μm
p-Akt (S473)
p-PTEN (STT)
Human IHC

Figure 2


A
Week 0
16
20
4-NQO
water
(50 µg/ml in water)
B
WT
Ndrg2+/-
Ndrg2-/-
C
Tumor incidence in tongues (%)
100
50
0
WT (N=16)
Ndrg2+/- (N=21)
Ndrg2-/- (N=10)
*
**
0
10
20
Date (weeks)
D
Multiplicity in tongue
2
1.5
1
0.5
0
**
*
WT
Ndrg2+/-
Ndrg2-/-
Tumor size (mm)
4
3
2
1
0
**
*
WT
Ndrg2+/-
Ndrg2-/-

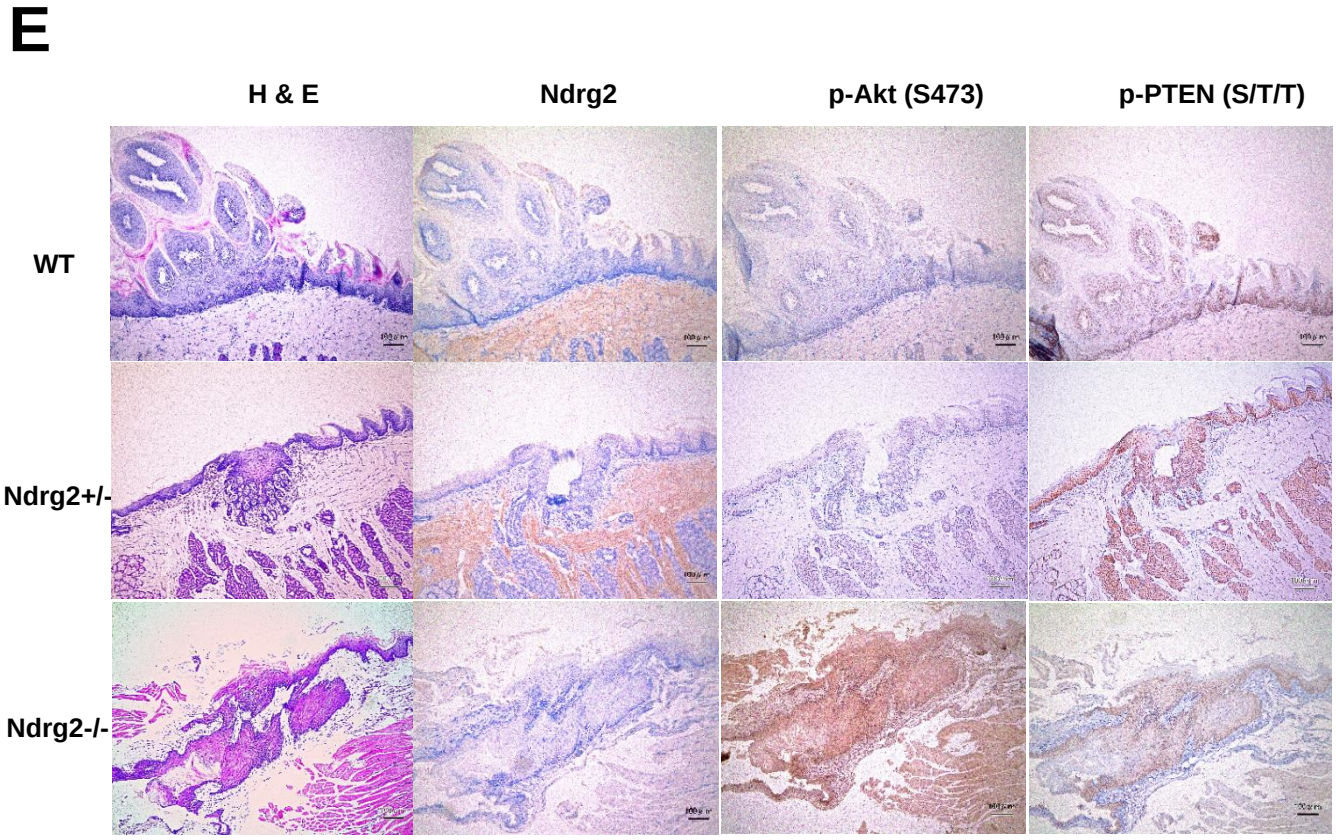
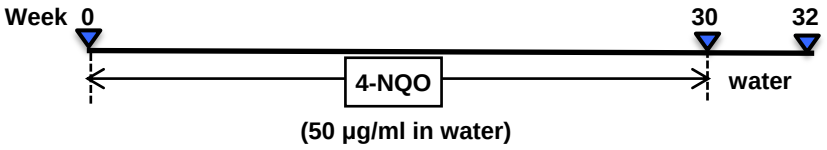

E
H & E
Ndrg2
p-Akt (S473)
p-PTEN (S/T/T)
WT
Ndrg2+/-
Ndrg2-/-

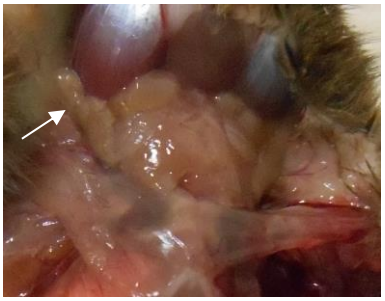
Figure 3

A



B

C



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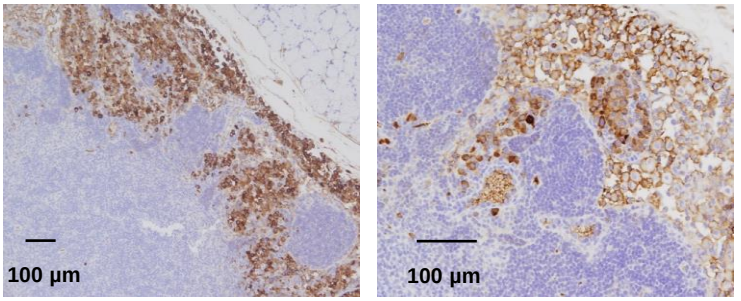
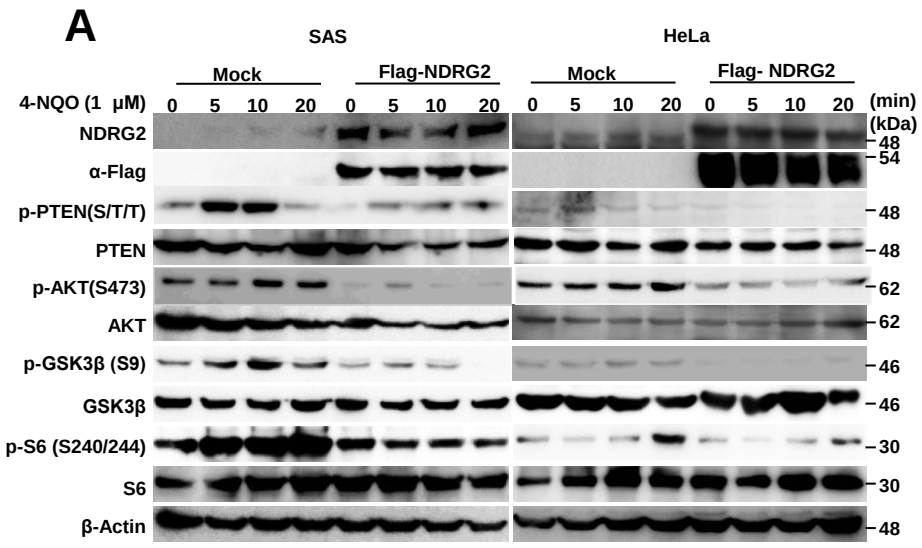
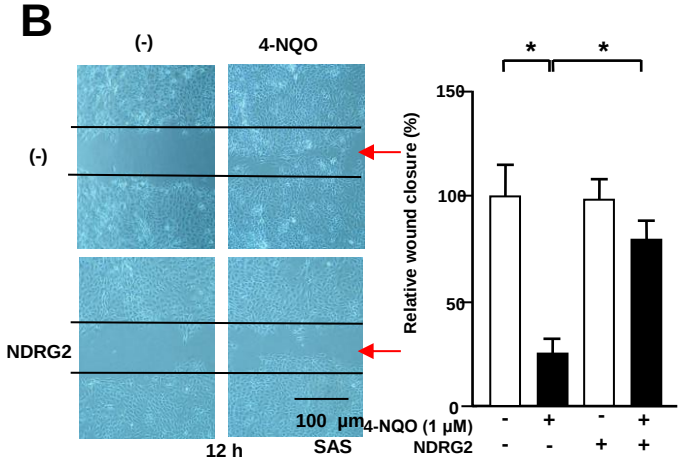
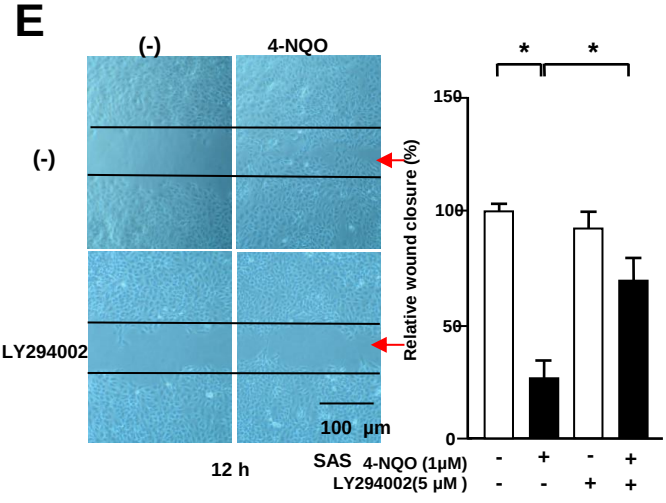
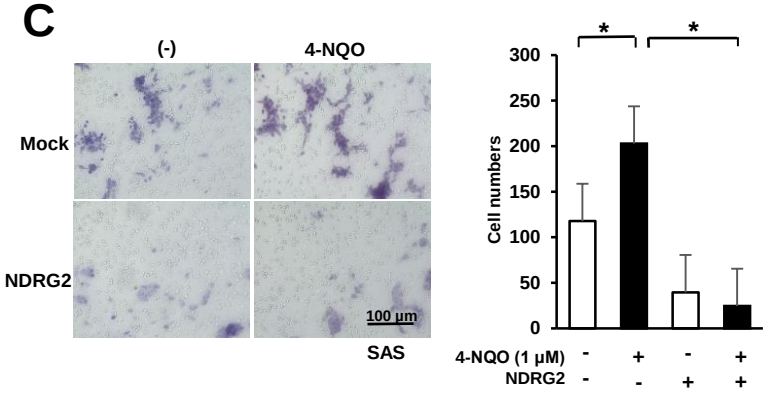


Figure 4


A
SAS
HeLa
Mock
Flag-NDRG2
Mock
Flag- NDRG2
4-NQO (1 μM)
0 5 10 20 0 5 10 20 0 5 10 20 0 5 10 20 (min)
(kDa)
NDRG2
α-Flag
p-PTEN(S/T/T)
PTEN
p-AKT(S473)
AKT
p-GSK3β (S9)
GSK3β
p-S6 (S240/244)
S6
β-Actin
48
54
48
48
62
62
46
46
30
30
48


B
(-)
4-NQO
(-)
NDRG2
100 μm
12 h
SAS
Relative wound closure (%)
150
100
50
0
*
*
4-NQO (1 μM) - + - +
NDRG2 - - + +


E
(-)
4-NQO
(-)
LY294002
100 μm
12 h
SAS
Relative wound closure (%)
150
100
50
0
*
*
4-NQO (1μM) - + - +
LY294002(5 μM) - - + +


C
(-)
4-NQO
Mock
NDRG2
100 μm
SAS
Cell numbers
300
250
200
150
100
50
0
*
*
4-NQO (1 μM) - + - +
NDRG2 - - + +

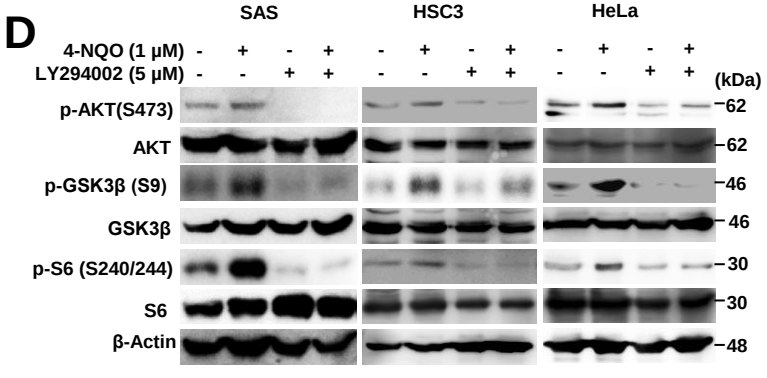

D
SAS
HSC3
HeLa
4-NQO (1 μM) - + - + - + - + - + - +
LY294002 (5 μM) - - + + - - + + - - + +
(kDa)
p-AKT(S473)
AKT
p-GSK3β (S9)
GSK3β
p-S6 (S240/244)
S6
β-Actin
62
62
46
46
30
30
48

Figure 5

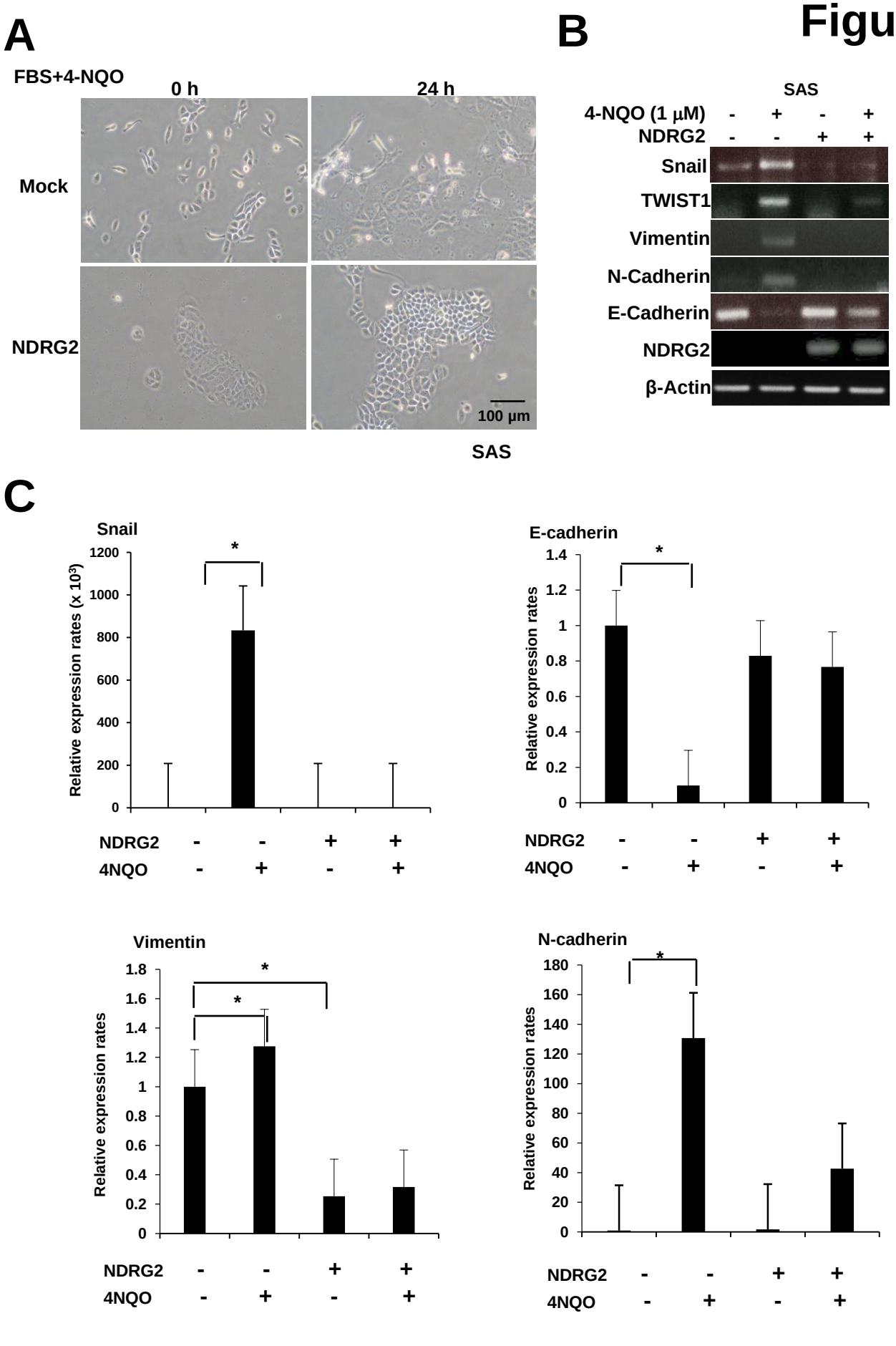
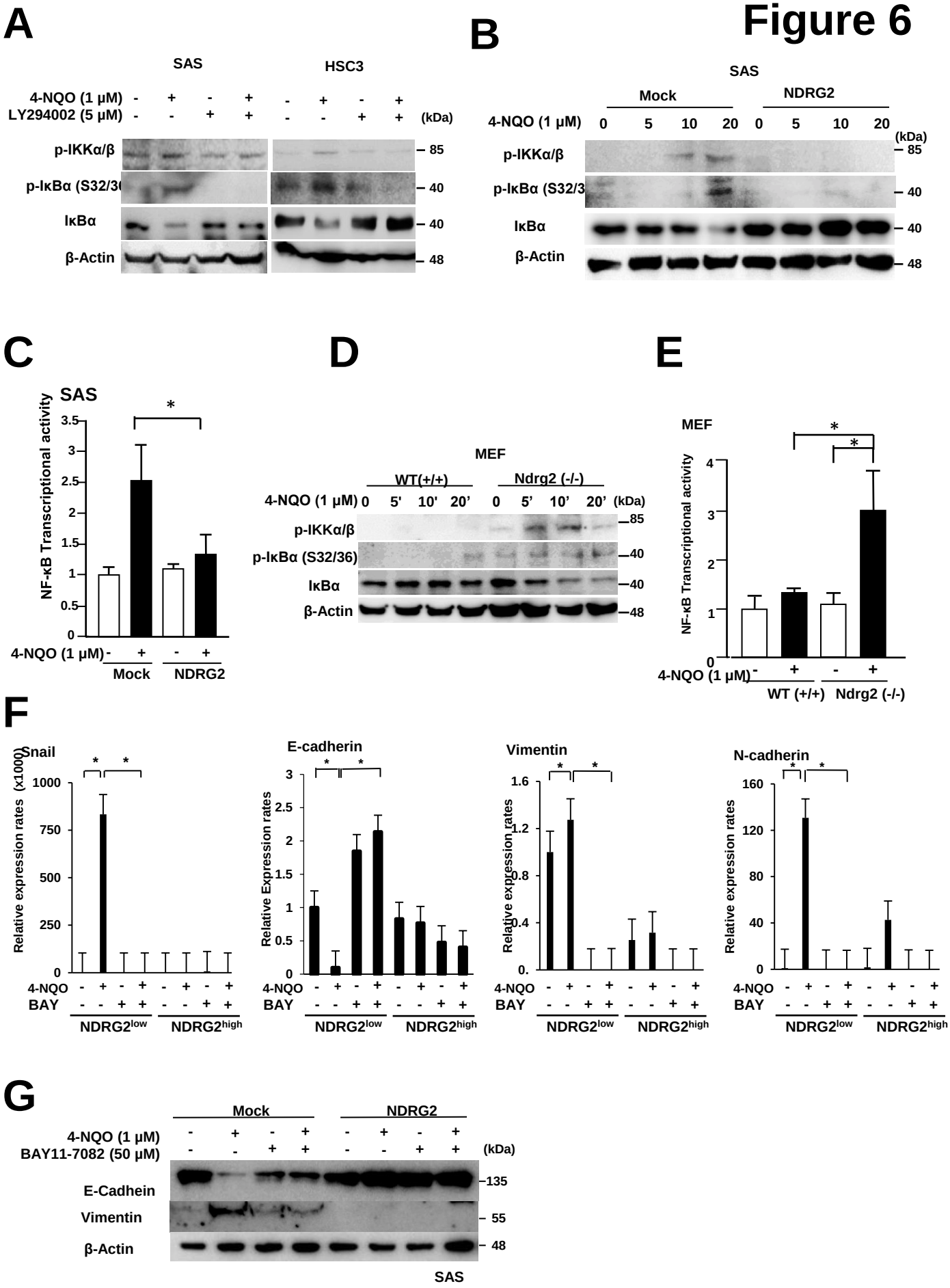

A
FBS+4-NQO
0 h
24 h
Mock
NDRG2
100 μm
SAS
B
SAS
4-NQO (1 μM) - + - +
NDRG2 - - + +
Snail
TWIST1
Vimentin
N-Cadherin
E-Cadherin
NDRG2
β-Actin
C
Snail
Relative expression rates (x 10³)
1200
1000
800
600
400
200
0
*
NDRG2 - - + +
4NQO - + - +
E-cadherin
Relative expression rates
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
NDRG2 - - + +
4NQO - + - +
Vimentin
Relative expression rates
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
*
NDRG2 - - + +
4NQO - + - +
N-cadherin
Relative expression rates
180
160
140
120
100
80
60
40
20
0
*
NDRG2 - - + +
4NQO - + - +

Figure 6


A
SAS
HSC3
4-NQO (1 μM)
LY294002 (5 μM)
(kDa)
p-IKKα/β
p-IκBα (S32/36)
IκBα
β-Actin
85
40
40
48
B
SAS
Mock
NDRG2
4-NQO (1 μM)
0 5 10 20 0 5 10 20
(kDa)
p-IKKα/β
p-IκBα (S32/36)
IκBα
β-Actin
85
40
40
48
C
SAS
NF-κB Transcriptional activity
4-NQO (1 μM)
Mock
NDRG2
D
MEF
WT(+/+)
Ndrg2 (-/-)
4-NQO (1 μM) 0 5' 10' 20' 0 5' 10' 20' (kDa)
p-IKKα/β
p-IκBα (S32/36)
IκBα
β-Actin
E
MEF
NF-κB Transcriptional activity
4-NQO (1 μM)
WT (+/+)
Ndrg2 (-/-)
F
Snail
E-cadherin
Vimentin
N-cadherin
Relative expression rates (x1000)
Relative Expression rates
Relative expression rates
4-NQO
BAY
NDRG2low
NDRG2high
G
Mock
NDRG2
4-NQO (1 μM)
BAY11-7082 (50 μM)
(kDa)
E-Cadhein
Vimentin
β-Actin
135
55
48
SAS