

NGAL is downregulated in oral squamous cell carcinoma and leads to increased survival, proliferation, migration, and chemoresistance

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Abstract

Oral cancer is a major public health burden worldwide. The lack of biomarkers for early diagnosis has increased the difficulty in managing this disease. Recent studies have reported that neutrophil gelatinase-associated lipocalin (NGAL), a secreted glycoprotein, is upregulated in various tumors. In our study we found that NGAL was significantly downregulated in primary malignant and metastatic tissues of oral cancer compared to normal tissues. The downregulation of NGAL was strongly correlated with the degree of differentiation and stage (I-IV), and can serve as a prognostic biomarker for oral cancer. Tobacco carcinogens were also found to be involved in the downregulation of NGAL. Mechanistic studies revealed that knockdown of NGAL increased oral cancer cell proliferation, survival, and migration, and also induced resistance against cisplatin. Silencing of NGAL activated mTOR signaling and reduced autophagy by the LKB1-AMPK-p53-Redd1 signaling axis. Moreover, cyclin-D1, Bcl-2, and MMP-9 were upregulated, and caspase-9 was downregulated, suggesting that silencing of NGAL increases oral cancer cell proliferation, survival, and migration. Thus, from our study it is evident that downregulation of NGAL activates the mTOR pathway and helps in the progression of oral cancer.

1. Introduction

Despite significant advancements in the management of oral cancer, it is still a major concern worldwide, accounting for approximately 128,000 deaths annually [1,2]. The five year survival rate of oral cancer is 62.1% (2003-2009), nevertheless, the survival rates worsen with advancement in clinical stages (SEER 2003-2009) [3]. Regardless of the unquestionable benefits from the available therapeutic modalities, chemoresistance and recurrence are the major complications that reduce the quality of life of patients. This demands the need for developing novel biomarkers for its early diagnosis and novel targets for discovering more potent chemotherapeutic agents for this disease.

Over the past two decades, neutrophil gelatinase-associated lipocalin (NGAL) has received enormous attention in the clinic as a biomarker of kidney injury, cardiovascular injuries, and also cancer [4-8]. NGAL, also known as Lipocalin-2 (LCN2), is a 24 kDa glycoprotein in humans encoded by the *LCN2* gene located on chromosome 9 at the locus 3p11. In recent years, it has emerged as a biomarker for several benign and malignant diseases. Upregulation of NGAL is known to increase the invasiveness of breast, bladder, gastric, gynecological, thyroid, lung, esophageal, colon cancer, and chronic myelogenous leukaemia, while in pancreatic and oral cancer, it decreases the invasiveness [9-11]. Upregulation of NGAL increased cell proliferation of cervical and lung cancer cells, while downregulation reduced cell proliferation [12,13]. NGAL is a well-known modulator of epithelial to mesenchymal transition (EMT), invasion and migration. Overexpression of NGAL persuades EMT via activation of snail, twist, N-cadherin, fibronectin, MMP-9, and NF- κ B, subsequently upregulating the genes associated with stemness, adhesion, motility, and drug efflux [14-16]. Likewise, silencing of NGAL reduced migration and invasion via downregulation of vimentin, MMP-2, and MMP-9, and increased expression of E-

cadherin [11]. These findings suggest that NGAL plays a key role in the development and progression of cancer. However, the role of NGAL in oral cancer has not been well established thus far. Although a few studies have shown that NGAL is downregulated in oral cancer, its expression and role in different types and different process of oral cancer development have not been studied thoroughly [17,18]. Therefore, studying the expression of NGAL in different processes of development of oral cancer would help us to comprehend whether NGAL can serve as a diagnostic and prognostic biomarker for oral cancer.

In the present study we have examined the expression of NGAL in different stages, grades, tumours from different tissues, degree of differentiation, and different processes of development of oral cancer. We found that NGAL plays a pivotal role in different processes of oral cancer development such as survival, proliferation, invasion, migration, and resistance to chemotherapeutic agents.

2. Materials and Methods

2.1 Tissue microarray

Tissue microarray (TMA) slides for head and neck squamous cell carcinoma (Cat no: HN803b) and oral squamous cell carcinoma (Cat no: OR802) were purchased from US Biomax, Derwood, USA.

2.2 Immunohistochemistry (IHC)

Expression of NGAL was determined by immunohistochemical analysis. Histostain plus kit (Cat no: 859043, Life technologies, California, USA) was used according to the manufacturer's protocol. Anti-hNGAL monoclonal antibody was purchased from (Cat no: ab23477, Abcam, Cambridge, USA). The TMAs were deparaffinised and rehydrated using xylene and ethanol and were blocked with 3% hydrogen peroxide in methanol for 30 minutes. After the antigen retrieval, the sections were incubated in blocking solution for 30 min and then were incubated with primary antibody (1:10 dilution) at 4 °C for overnight. The following day the sections were incubated with secondary antibody for 1 h at room temperature, stained with DAB, counter stained with haematoxylin, and were mounted using DPX.

2.3 Scoring

All slides were observed under a Nikon Eclipse Ti-E microscope, and the intensity of immunoreactivity for NGAL was examined. The staining intensity was graded on a scale of 0 to 3+ (0 for no staining; 1+ for weak immunoreactivity; 2+ for moderate immunoreactivity; and 3+ for strong immunoreactivity). The percentage of cells positive for NGAL were graded by the following protocol: grade 0 intensity (<10% positive cells); grade 1+ intensity (10-25% positive cells), grade 2+ intensity (25-50%), grade 3 intensity (50-75% positive cells), and grade 4

intensity (75-100% positive cells). The staining intensity score and the percent immunoreactivity score were then multiplied to obtain a composite score.

2.4 Materials

4-(Methylnitrosoamino)-1-(3-pyridinyl)-1-butanone (NNK, Cat No. 78013), N'-Nitrosonornicotine (NNN, Cat No. 75285), 4-Nitroquinoline N-oxide (4-NQO, Cat No. N8141), Cisplatin (PHR1624), and 5-Flurouracil (5-FU) (F6627) were purchased from Sigma- Aldrich, Missouri, USA.

2.5 Cell culture

Poorly differentiated human squamous cell carcinoma of the tongue SAS cells were procured from Rajiv Gandhi Centre for Biotechnology (RGCB), Trivandrum, India. These cells were maintained in Dulbecco's Modified Eagle Medium (DMEM; GibcoTM; Life Technologies, NY, USA) supplemented with 10% foetal bovine serum (FBS; Gibco[®], NY, USA) and 1X Penstrep (Invitrogen, CA, USA). The cells were cultured and maintained at 37°C in a 5% CO₂ and 95% humidity.

2.6 Antibodies

S6 Ribosomal protein (dilution 1:2000; Cat No. 2317S), Phospho- S6 Ribosomal protein (Ser235/236) (dilution 1:2000; Cat No. 4858T), LC3B (dilution 1:1000; Cat No. 2775S), Caspase-9 (dilution 1:1000; Cat No. 9508T), Bcl-2 (dilution 1:1000; Cat No. 15071), MMP-9 (dilution 1:1000; Cat No. 13667P), and cyclin D1 (dilution 1:1000; Cat No. 2978BC), GAPDH (dilution 1:2000; Cat No. 2118S) were purchased from Cell Signaling Technology, Massachusetts, USA. Antibodies against NGAL (dilution 1:3000; Cat No. ab23477), anti-mouse

secondary antibody (dilution 1:6000; Cat No. ab97040), and anti-rabbit secondary antibody (dilution 1:6000; Cat No. ab97080) were purchased from Abcam, Cambridge, USA.

2.7 shNGAL stable knockdown

shRNA mediated knockdown of NGAL was carried out in the SAS cell line. Human shNGAL plasmids (Table 1) and puromycin (Cat No. P8833, Sigma-Aldrich, Missouri, USA) were purchased from Sigma. SAS cells were seeded at a concentration of 25×10^4 cells/well in 1ml of medium in a 24-well plate. The next day, cells were transfected with shRNA control and shNGAL plasmids (2 μ g of DNA) using X-treme gene 9 DNA transfection reagent (Cat No. 06365787001, Sigma-Aldrich, Missouri, USA) for 48h. The medium containing transfection reagent was replaced with fresh DMEM medium and the cells were allowed to recover for 24h. Then SAS cells were selected with 1 μ g/ml puromycin and stable clones were established.

2.8 Cell viability

Briefly, 2×10^3 cells/well were seeded in 96-well plates in sextuplicate and incubated for 24h and 48h time points. After each time point, 10 μ l of 3-(4,5-dimethylthiazol-2,5-diphenyl tetrazolium bromide (MTT) (5mg/ml; Cat No. M2128, Sigma- Aldrich, Missouri, USA) was added to the cells and was further incubated for 2h at 37°C. The MTT solution was removed and 100 μ l of DMSO (Cat No. 1.16743.0521, Merck, Darmstadt, Germany) was added to each well and the absorbance was then measured at 570nm using an Infinite M200 Pro (Tecan Group Ltd., Männedorf, Switzerland) after 1h.

2.9 Cell cycle analysis

Control shRNA and shNGAL cells were plated at a density of 1×10^5 cells/well, and after 24h cells were trypsinized, washed with phosphate-buffered saline (PBS), and fixed with 75%

ethanol at -20°C overnight. The following day, cells were washed with PBS, treated with PI/RNase solution (Cat No. A35126, Invitrogen, CA, USA) for 20min in the dark, and analyzed using a flow cytometer (FACS Calibur, Becton-Dickinson, New Jersey, USA). 25,000 cells in each sample were analyzed to obtain a measurable signal. The data were analyzed on FCS express6.

2.10 Cell survival assay

Control shRNA and shNGAL cells were seeded in a 6-well plate at a density of 1×10^3 cells/well. The cells were grown for fifteen days, then colonies were fixed with 70% ethanol and were stained with crystal violet. Pictures of individual wells were taken and were analyzed using imageJ software, and the surviving fraction was calculated

2.11 *In vitro* wound closure assay

Control shRNA and shNGAL cells were seeded in 6-well plates and were allowed to grow till confluence, and then serum starved for 8h. Confluent monolayers were scratched with a pipette tip. Plates were washed with PBS to remove non-adherent cells, and the wound was photographed at regular time intervals. The percentage of wound area was calculated compared to control.

2.12 Cell invasion and migration assay

Control shRNA and shNGAL cells were serum starved for 18h before seeding onto transwell migration chambers that were 24-well plates with 8mm pore transwell inserts (Cat No. 3422, Corning, New York, USA) pre-coated with matrigel. Post serum starvation, the cells were trypsinized and were seeded at a concentration of 5×10^4 cells in the upper chamber of the transwell insert, and in the lower chamber, medium containing 10% FBS was added as a chemo-

attractant. Cells were then incubated for another 24h at 37°C. The migrated cells at the bottom of the transwell insert were fixed in 70% ethanol and were stained with crystal violet solution. Stained cells were visualized under an inverted microscope and photographs were taken using a Nikon 500 camera. After the photographs were taken, the membrane was dissolved in 1% sodium dodecyl sulphate (SDS) [Cat. No. 436143, Sigma- Aldrich, Missouri, USA] solution at 37°C for 1h and absorbance was read at 595nm in a Tecan plate reader.

2.13 RNA isolation and Reverse transcriptase PCR

Total RNA was extracted using TRIzol reagent (Invitrogen), and cDNA synthesis was carried out using High-Capacity cDNA Reverse Transcription Kit (LifeTechnologies). PCR was then performed with 1µl of cDNA as a template. Primer sequences and amplicon lengths are listed in Table 2

2.14 Western blot analysis

Whole cell lysates were prepared by lysing the cells in whole cell lysis buffer (20mM HEPES, 2mM EDTA, 250mM NaCl, 0.1% NP-40) in the presence of protease inhibitors (2µg/ml Leupeptin hemisulfate, 2µg/ml Aprotinin, 1mM PMSF, 1mM DTT). The protein concentration of the lysates were measured using the Bradford assay (Cat No. 500-0205; Bio rad, California, USA) and 50µg of protein was mixed with 5X Laemmli Buffer (250mM TrisHCl, 10% SDS, 30% Glycerol, 5% β-mercaptoethanol, 0.02% Bromophenol blue), electrophoresed in a 12% SDS-acrylamide gel, and transferred to nitrocellulose transfer membrane (Bio rad, California, USA). The membranes were blocked with 5% non-fat milk in tris-buffered saline (TBS: 0.2M Tris base, 1.5M NaCl, H₂O) containing 1% tween 20 (TBST). The blots were probed with appropriate primary antibodies overnight. The following day the blots were washed with TBST and were incubated in appropriate horseradish peroxidase-conjugated secondary antibody and

were visualized using an Optiblot ECL Detection Kit (Cat No. ab133406, Abcam, Cambridge, USA). β -actin/GAPDH was used as the loading control.

2.15 PI/FACS

The cell death induced by chemotherapeutic agents was determined by staining with propidium iodide (PI) (conct. 1mg/ml; Cat No. P4170, Sigma-Aldrich, Missouri, USA). Control shRNA and shNGAL cells were seeded in a 6-well plate at a density of 5×10^4 cells/well. After 24h, the cells were treated with different concentrations of cisplatin and 5-Flourouracil for 48h. After 48h, the cells were harvested and were washed with PBS twice. 10 μ l of PI was added and was analyzed by flow cytometry (FACSCalibur, Becton-Dickinson, New Jersey, USA). The data were analyzed using FCS Express 6 software.

2.16 Statistical analysis

All the statistical analysis was carried out using Student's *t*-test or one-way ANOVA followed by Tukey test. *p*-value less than 0.05 was considered as statistically significant.

3. Results

To understand the role of NGAL in oral cancer, we carried out immunohistochemical analysis in oral cancer patient tissues. The tissue microarray contained tissues of different premalignant lesions, stages, grades, tissues, and degree of differentiation of oral cancer. Next, we have examined the effect of potent tobacco carcinogens such as NNK, NNN, and the synthetic oral carcinogen 4-NQO on the expression of NGAL in oral cancer cells. Then we have established the role of NGAL on different hallmarks of cancer and elucidated the mechanisms involved.

3.1 NGAL expression was found to be downregulated in oral cancer

To understand the role of NGAL in oral cancer, we first determined the expression of NGAL in oral cancer tissues. Our results showed moderate expression of NGAL in normal tissues compared to weak to moderate expression in malignant tissues (Fig. 1A). Intriguingly, the majority of the well differentiated epithelial cells of both malignant and normal tissues showed moderate expression of NGAL. Expression of NGAL was observed in all the tumours arising from the oral cavity including mandible, cheek, gingiva, lip, palate, parotid gland, tongue, lymph node, and larynx, and was found to be downregulated (Fig. 1B). Weak to moderate staining of NGAL was observed in the above mentioned tissues, except the nose where it was negative. Moreover, the expression of NGAL was inversely associated with the degree of differentiation of tumours. Normal and well differentiated tongue tissues showed positive staining of NGAL compared to very weak positive staining observed in moderately differentiated and poorly differentiated tongue tissues (Fig. 1C). However, no positive expression was observed in the undifferentiated tongue cancer tissues. This suggests that NGAL can serve as a prognostic biomarker for oral cancer. The expression of NGAL correlated with different stages of tongue cancer tissues where stage I showed high expression and stage IV negative expression compared to normal tissues. Similarly, the expression of NGAL was downregulated significantly with increase in grade of oral cancer compared to normal tissues. Furthermore, NGAL was also downregulated in different processes and pathological types of oral cancer and was strongly associated with lymph node metastases (Figs. 1D and 1E).

3.2 Tobacco components downregulated the expression of NGAL

NGAL is downregulated in oral cancer tissues and it is well established that tobacco is the prime risk factor for oral cancer. Therefore, we determined whether tobacco carcinogens are involved in the downregulation of NGAL. We treated SAS cells with different concentrations of NNK (Fig 2A), NNN (Fig. 2B), and the synthetic carcinogen 4-NQO (Fig. 2C) and observed that these tobacco components downregulated the expression of NGAL in a dose-dependent manner. This suggests that tobacco carcinogens play a key role in regulating the expression of NGAL.

3.3 Silencing of NGAL increased proliferation and survival of oral cancer cells

The fundamental property of cancer cells is to sustain cell survival and proliferation. Therefore, we sought to study the effect of silencing of NGAL on the proliferation and survival of oral cancer cells. To study the role of NGAL in oral cancer cell proliferation and survival, we silenced the expression of NGAL (Fig. 3A). We carried out MTT assay and observed that knockdown of NGAL increased cell viability in a time-dependent manner (Fig. 3B). To confirm that knockdown of NGAL increases cell viability, we studied its effect on different phases of the cell cycle. We found that silencing of NGAL lead to an increase in the number of cells in S-phase and reduced the number of cells in G2/M phase compared to control shRNA (Fig. 3C). The increase in number of cells in S-phase suggests that NGAL knockdown allows cancer cells to proliferate uninterrupted and pass through the G2/M check point. In addition, in NGAL deficient cells we observed that the expression of cyclin D1 is upregulated, which is regulated by the NF- κ B/PI3K-mTOR pathways. We also assessed if knockdown of NGAL increases oral cancer cell survival by using a clonogenicity assay (Fig. 3D). We observed a two fold increase in number of colonies in the shNGAL group compared to control shRNA group.

3.4 Silencing of NGAL increases invasion and migration of oral cancer cells

Our IHC results advocate that downregulation of NGAL is strongly associated with metastases, so we hypothesized that knockdown of NGAL may induce invasion and migration of oral cancer cells. To confirm this, we performed *in vitro* invasion and migration assays using NGAL knockdown cells. Results from the transwell migration assay suggested that the NGAL knockdown cells possessed higher invasive ability than shRNA control cells. It was found that the number of cells that invaded the lower part of the transwell insert was higher in the case of shNGAL cells compared to control cells (Fig. 4A). Similarly, in the *in vitro* wound healing assay, the wound was healed within 8h in the case of shNGAL cells compared to control cells. This indicates that shNGAL cells have higher migratory potential. In line with this, in NGAL silenced cells, MMP-9 was found to be upregulated, which might be responsible for the increase in cell motility.

3.5 Silencing of NGAL activates mTOR signalling and suppresses autophagy

Our previous results suggest that tobacco components downregulated the expression of NGAL, and loss of NGAL increases oral cancer cell proliferation, survival, invasion, and migration. However, the underlying mechanism is not clear. Increasing lines of evidence suggest that tobacco components play a key role in the development of oral cancer and are known to regulate the Akt/mTOR pathway. Therefore, we studied the effect of silencing of NGAL on the activation of S6, a well-established marker of the mTOR pathway. We observed that knockdown of NGAL activated S6 (serine 235/236) (Figs. 5C and 5D). Recently, Dowling *et al.*, 2007 reported that metformin inhibited the activation of S6 via AMP activated protein kinase (AMPK) pathway [19]. Hence, we studied the expression of AMPK in NGAL silenced cells and observed that the

expression of AMPK was downregulated, indicating that AMPK is the intermediate link between NGAL and S6. As it is well established that LKB1 is upstream of AMPK, and as AMPK is the only substrate, we analyzed the expression of LKB1 and found that it was downregulated [20]. Thus, knockdown of NGAL activates mTOR signalling via the AMPK-LKB pathway. Reports suggest that during hypoxia or energy stress, in head and neck squamous cell carcinoma (HNSCC) cells, regulated in development and DNA damage responses 1 (Redd1) inhibits mTOR signalling by upregulating AMPK [21,22]. Hence, we studied the expression of Redd1 in NGAL knockdown cells and found that the expression of Redd1 is completely inhibited (Fig 5A and 5B). Besides Redd1, AMPK is also known to regulate activate p53 during metabolic stress [23]. Thus, p53 serves as a downstream target of AMPK, and we found that in NGAL knockdown cells the expression of p53 was found to be downregulated (Figs. 5A and 5B).

Moreover, the promoter region of Redd1 is known to possess the consensus p53 family binding element that is required for regulation of Redd1 by p53 [21,24]. This suggests that Redd1 is a direct transcriptional target of p53 and can be a connecting link between AMPK and Redd1. Therefore, it can be concluded that silencing of NGAL increases survival, proliferation, invasion, and migration of oral cancer cells via the LKB1-AMPK-p53-Redd1-mTOR axis (Fig 5A and 5B).. We observed that knockdown of NGAL upregulated cyclin D1, Bcl-2, and MMP-9, and downregulated caspase-9, confirming the same (Fig. 5C). Apart from the significant role of mTOR in cancer progression, activation of mTOR downregulates autophagy [25-27]. Thus, we studied the expression of LC3B, an autophagy marker, and observed that the expression of LC3B was found to be downregulated. This suggests that NGAL silenced cells are more resistant to autophagy induced cell death, and decreased autophagy provides a survival advantage. Overall,

our results suggest that NGAL knockdown cells were more resistant to autophagy, which was mediated via the LKB1-AMPK-p53-Redd1 axis and activation of mTOR signalling.

3.6 Silencing of NGAL selectively induces resistance against cisplatin

Development of resistance is the major reason for the failure of chemotherapeutic agents in the clinic. Therefore, we studied the role of NGAL in development of resistance against the first line therapeutic agents cisplatin and 5-FU. We observed that knockdown of NGAL selectively induced resistance against cisplatin, while both control shRNA and shNGAL cells were sensitive to 5-FU (Figs. 6A and 6B). Upregulation of cyclin D1 and Bcl-2, and downregulation of caspase-9 might be the reason for the development of chemoresistance. However, the mechanism has to be studied further.

4. Discussion

We studied the expression of NGAL in oral cancer tissues and found that NGAL was downregulated in primary tumour and metastatic tissues. Our results were consistent with previous studies where NGAL was found to be downregulated in oral cancer tissues [17,18]. Downregulation of NGAL was found to be strongly correlated with the degree of differentiation and stage of oral cancer. Similarly, the study carried out by Hiromoto *et al.*, 2011, showed that the downregulation of NGAL was associated with the degree of differentiation of tumours. Thus, NGAL can serve as biomarker for identifying the degree of differentiation, prognosis, and severity of the disease. However, there are no reports about the expression of NGAL with respect to age, tissues, stages, grades etc. in oral cancer. The expression of NGAL was found to be downregulated in malignant tongue, larynx, lip, cheek, gingiva, and palatal tissues of the oral

cavity. Moreover, downregulation of NGAL was evident in all the stages (stage I-IV) and grades (grade I-III) of oral squamous cell carcinoma (OSCC).

Since tobacco is a well characterized risk factor for oral cancer, we investigated whether NGAL is regulated by tobacco carcinogens. The main carcinogens characterized from tobacco smoke include benzo[a]pyrene, nicotine, NNK, NNN, dibenzo[a]pyrene, benzene, nitrobenzene, 2-toluidine, and 2-6-dimethylaniline. Upon activation, NNK and NNN induce mutations in tumour suppressor genes and oncogenes, and form DNA adducts that result in tumour initiation [28-32]. 4-NQO is a synthetic tobacco carcinogen used to induce oral cancer in mouse that mimics the oral cancer development in humans [33,34]. In our study, it was found that tobacco carcinogens NNK, NNN, and 4-NQO downregulated the expression of NGAL in a dose-dependent manner. This indicates that NGAL plays a key role in tobacco induced carcinogenesis.

Next, we found that downregulation of NGAL induced oral cancer cell proliferation, survival, invasion, and migration. Many studies report that NGAL plays a key role in invasion and migration of oral cancer and other cancers. Recently, Lin *et al.*, 2016 reported that knockdown of NGAL increased *in vitro* cell motility and *in vivo* metastases [18]. However, this is the first study that shows that knockdown of NGAL increases *in vitro* cell viability and survival in oral cancer. Similar to our findings, a recent study in colorectal cancer showed that knockdown of NGAL increased cell proliferation and survival and induced EMT [35]. Presently, the mechanism involved has to be studied further. Our study shows that knockdown of NGAL activated mTOR signalling and reduced autophagy via the LKB1-AMPK-p53-Redd1 signalling axis. Aberrant activation of mTOR is seen in OSCC and is associated with poor prognosis [36-40]. Phosphorylated S6, the downstream target of mTOR, is found to be upregulated in epithelial

dysplasia and OSCC and can serve as a potent diagnostic biomarker for oral cancer [41]. mTOR signalling can be activated by various stimuli. During hypoxia or energy starvation, LKB1 is activated, which in turn phosphorylates AMPK. Thus, the activated AMPK phosphorylates TSC2, which results in switching off mTOR signalling [42,43]. It is well established that activation of mTOR signalling inhibits autophagy, and studies also suggest that Redd1 regulates autophagy [44]. A similar mechanism was observed in our study, indicating that silencing of NGAL mediates autophagy via Redd1. Moreover, as mentioned earlier, p53 was found to be downregulated in NGAL knockdown cells. Our results were similar to previous studies where NGAL was shown to regulate the expression of p53 [45,46].

Furthermore, AMPK activates p53 during metabolic stress by phosphorylating MDMX on serine 34, resulting in inhibition of p53 ubiquitylation [23]. Moreover, in cells lacking p53, ectopic expression of p53 induced the endogenous activity of Redd1; and the promoter region of Redd1 comprises of p53 binding sites, indicating that Redd1 is a direct transcriptional target of p53 [21]. Thus, our study demonstrates that knockdown of NGAL activates the mTOR pathway via the LKB1-AMPK-p53-Redd1 signalling axis. Moreover, the expression of cyclin-D1, Bcl-2, and MMP-9 were upregulated and caspase-9 was downregulated, which are the key molecules involved in oral cancer cell proliferation, survival, invasion, and migration. In addition to promoting mTOR signalling, knockdown of NGAL decreased autophagy. Activation of autophagy by many chemotherapeutic agents in HNSCC induced apoptosis and downregulated the mTOR pathway. Many small molecule tyrosine kinase inhibitors such as gefitinib, erlotinib, and dasatinib induced autophagy and suppressed mTOR signalling, indicating that an increase in autophagy suppresses tumour growth *in vitro* and *in vivo* [47]. These studies indicate that autophagy serves as a tumour suppressor. Thus, our study

clearly demonstrates that knockdown of NGAL increases oral cancer cell proliferation, survival, invasion, and migration by upregulating mTOR signalling and suppressing autophagy.

5. Conclusion

Our results suggest that NGAL is downregulated in oral cancer tissues and is strongly associated with degree of differentiation, stage of the tumour, and lymph node metastases. The tobacco components primarily NNK, NNN, and the synthetic carcinogen 4-NQO were implicated in the downregulation of NGAL. Mechanistic studies revealed that knockdown of NGAL augmented cell survival, invasion, and migration by activating the mTOR pathway, and downregulated autophagy via the LKB1-AMPK-p53-Redd1 signalling axis (Fig.7). This suggests the NGAL is one of the key molecule involved in oral cancer tumorigenesis. Therefore, agents that can restore the expression of NGAL would be advantageous in developing effective therapies against this dreadful disease.

6. Author Contributions

Conceptualization, Ajaikumar Kunnumakkara; Data curation, Javadi Monisha and Nand Roy; Formal analysis, Ganesan Padmavathi, Kishore Banik and Devivasha Bordoloi; Investigation, Javadi Monisha, Nand Roy, Ganesan Padmavathi, Devivasha Bordoloi and Amrita Khwairakpam; Methodology, Kishore Banik; Project administration, Javadi Monisha, Nand Roy and Gautam Sethi; Resources, Tahani Alahmadi and Alan Kumar; Supervision, Ajaikumar Kunnumakkara; Validation, Javadi Monisha; Visualization, Gautam Sethi, Alan Kumar and Ajaikumar Kunnumakkara; Writing – original draft, Gautam Sethi, Alan Kumar and Ajaikumar

Kunnumakkara; Writing – review & editing, Frank Arfuso, Arunachalam Chinnathambi and Sulaiman Alharbi.

7. Conflict of Interest

The authors declare no conflict of interest.

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Figure legends

Figure 1: Expression of NGAL in oral cancer. A) Representative images of expression of NGAL in oral cancer (left panel). Expression of NGAL in normal vs malignant oral cancer tissues (right panel). B) Expression of NGAL in different tissues of oral cancer. Lar: Larynx, Nos: Nose, Ton: Tongue, Che: Cheek, Gin: Gingiva, Lym: Lymph node, Man: Mandible, Par: Parotid gland, Pal: Palate. C) Expression of NGAL with degree of differentiation of oral cancer. D) Expression of NGAL in different stages of oral tongue cancer tissues. E) Expression of NGAL in different grades of oral tongue cancer tissues. Data are mean \pm SE. * $=p<0.05$.

Figure 2: Tobacco components downregulated the expression of NGAL in oral cancer cell line SAS. A) Structure of NNK (left panel). Western blot analysis of expression of NGAL after treatment with NNK for 48h in SAS cells (right panel). B) Structure of NNN (left panel).

Western blot analysis of expression of NGAL after treatment with NNN for 48h in SAS cells (right panel). C) Structure of 4-NQO (left panel). Western blot analysis of expression of NGAL after treatment with 4-NQO for 48h in SAS cells (right panel).

Figure 3: Silencing of NGAL in oral cancer cells. A) qRT-PCR showing the mRNA expression of NGAL in SAS cells post knockdown (left panel). Western blot analysis showing the NGAL in SAS cells post knockdown (right panel). B) Percentage increase in cell viability of control shRNA and shNGAL cells, determined by MTT assay. C) Cell cycle distribution was determined by flow cytometric analysis in control shRNA and shNGAL cells. D) Clonogenic assay showing an increase in number of colonies (left panel). Graphical representation of increase in number of colonies in NGAL knockdown cells (right panel). Data are means \pm SE. *= p<0.05.

Figure 4: Silencing of NGAL increased the invasion and migration of oral cancer cells. A) Cell invasion was determined by a transwell invasion assay. Cells invading through the matrigel were fixed, stained, and photographed under an inverted microscope at a 20x magnification. Graphical representation of increase in cells invading the lower surface of transwell insert (right panel). B) Cell migration was detected by scratch wound healing assay. Photographs were taken at 10x magnification. Graphical representation of decrease in wound area (right panel). Data are means \pm SE. *= p<0.05.

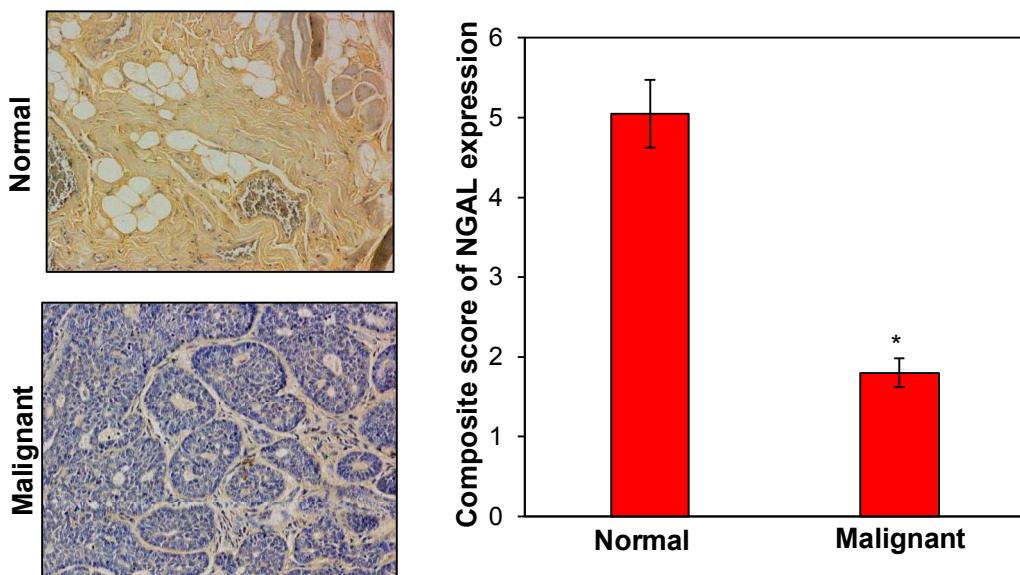
Figure 5: Silencing of NGAL activated mTOR signalling and induced autophagy. A) mRNA expression of LKB1-AMPK-P53-Redd1 in NGAL knockdown cells. B) Fold change in mRNA expression as analyzed by image lab software. C) Expression of proteins involved in mTOR

signalling and autophagy. D) Fold change in expression of proteins as analyzed by image lab software. Data are means \pm SE. *= p<0.05.

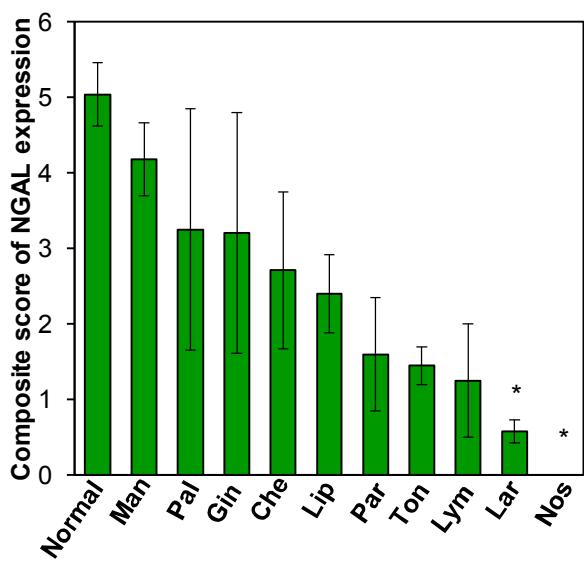
Figure 6: Silencing of NGAL selectively induces resistance against cisplatin. A) Cells were treated with cisplatin and 5-FU, and percentage of cell death was measured by staining with propidium iodide on flow cytometry at 48h. B) Graphical representation of percentage of cell death. Data are means \pm SE. *= p<0.05.

Figure 7: Downregulation of NGAL activates mTOR signalling via LKB1-AMPK-p53-Redd1 and decreases autophagy.

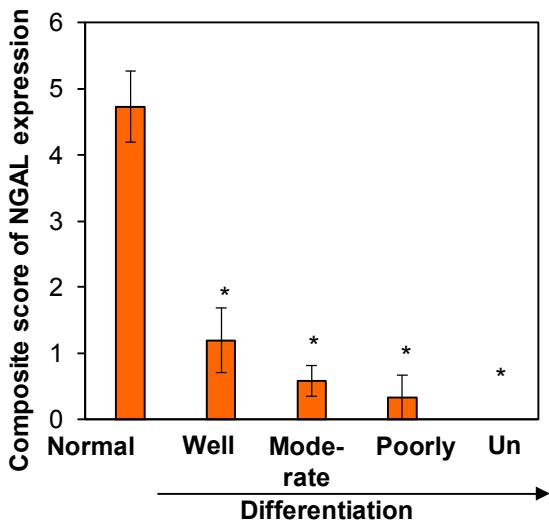
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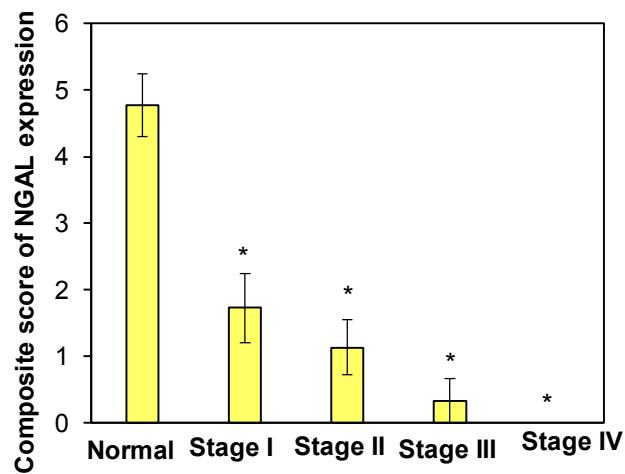
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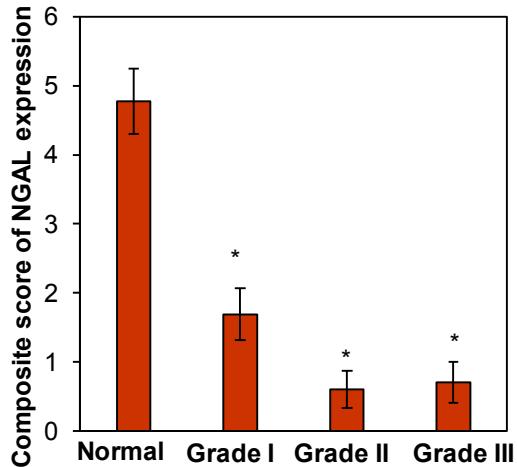
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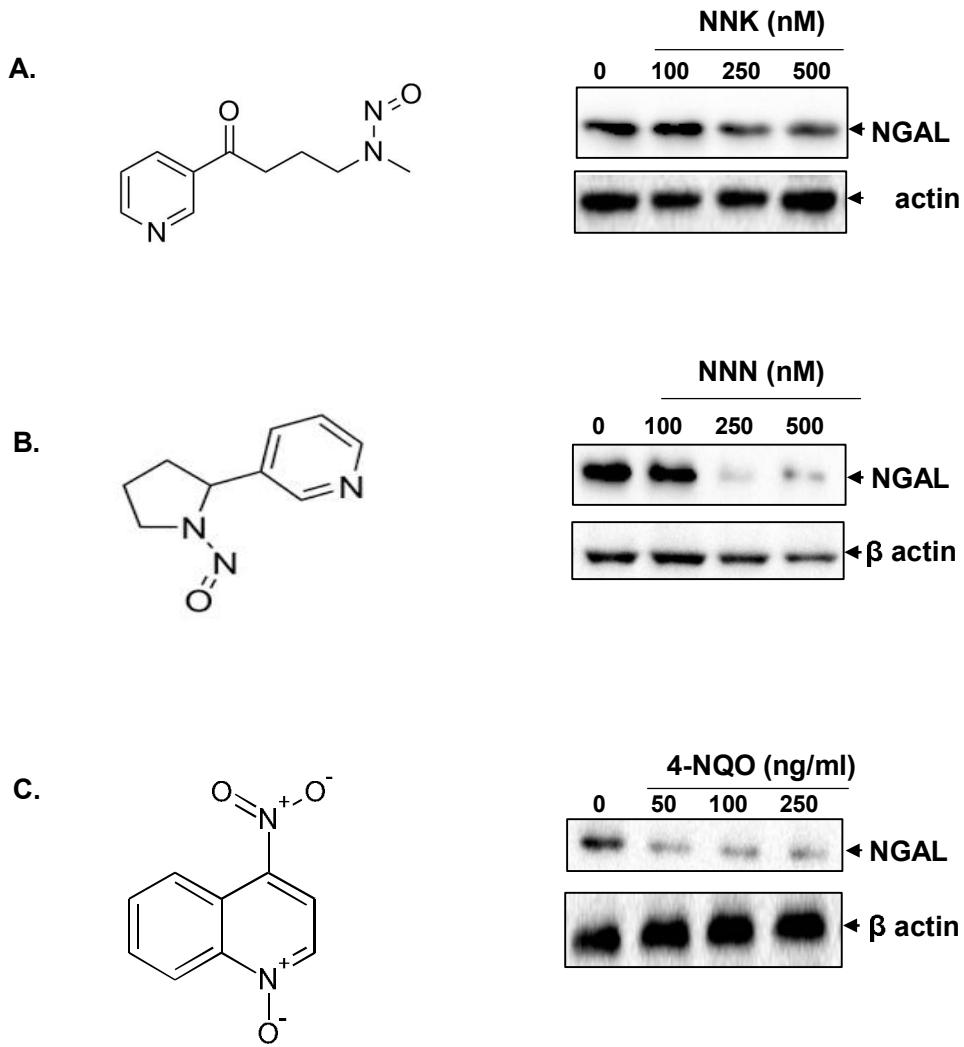
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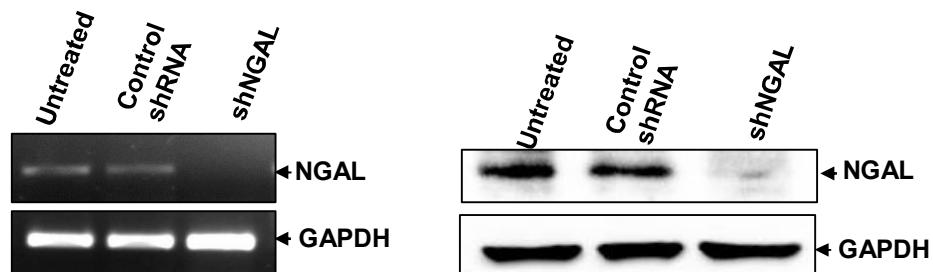


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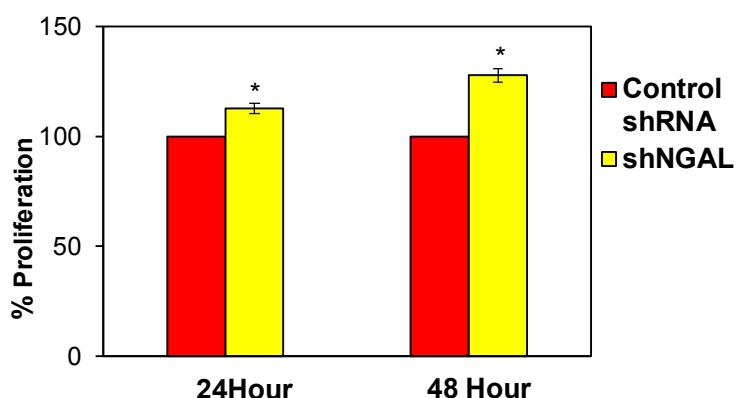


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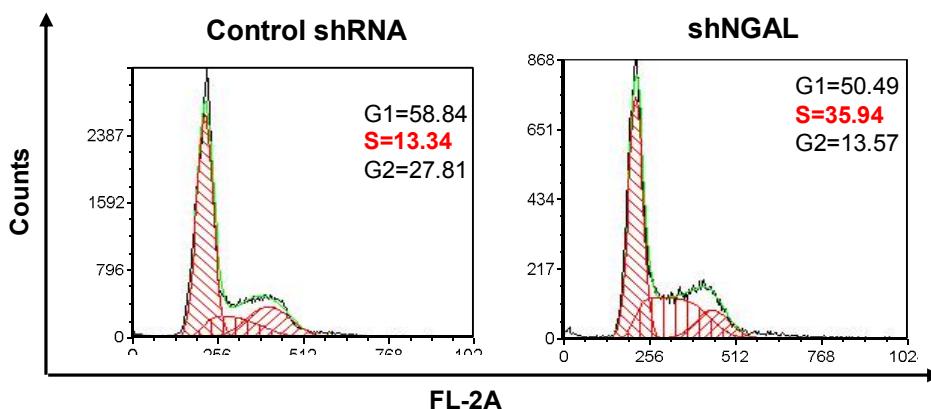
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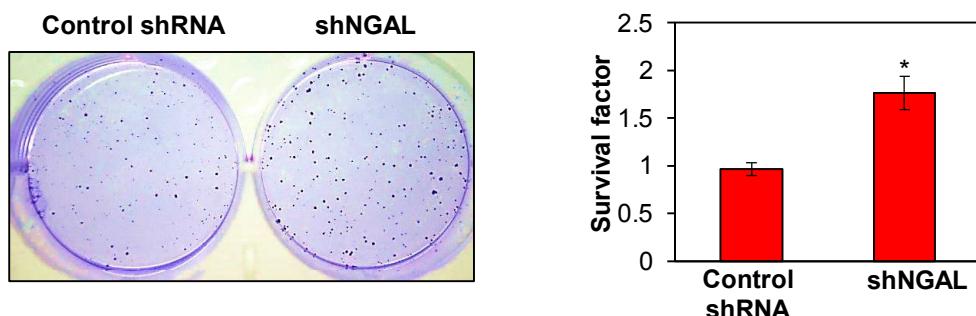
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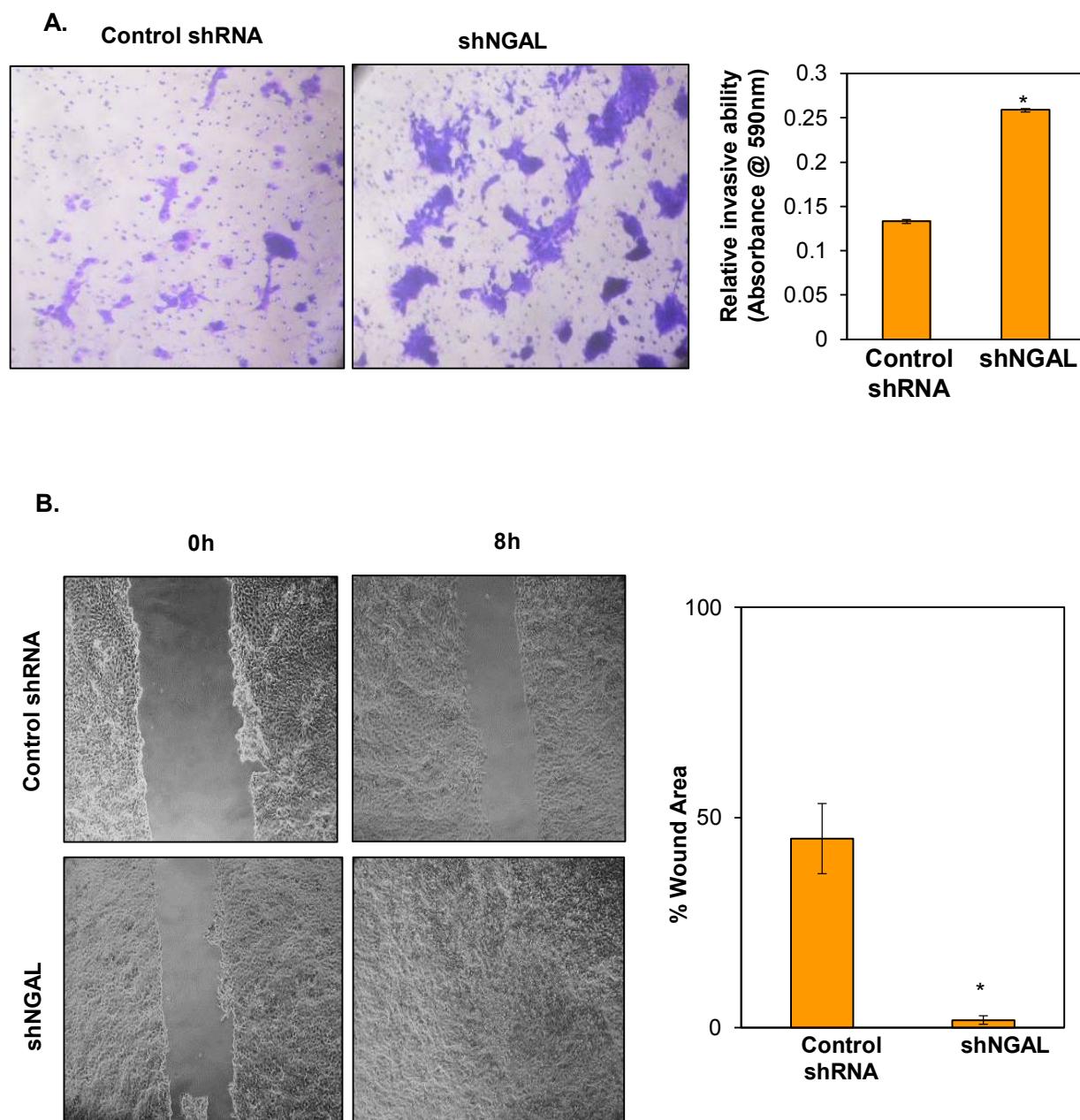
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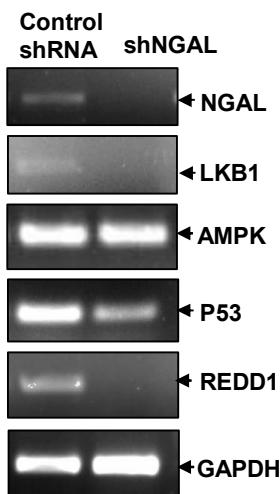
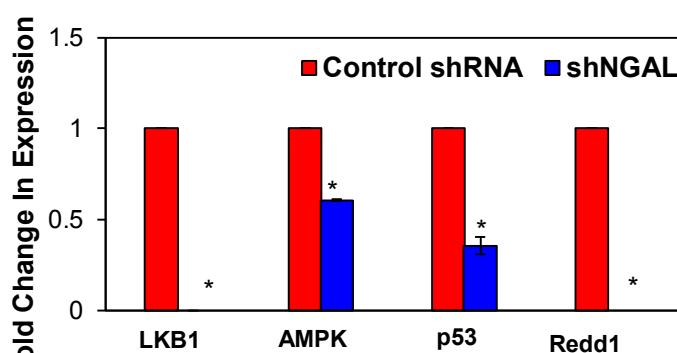
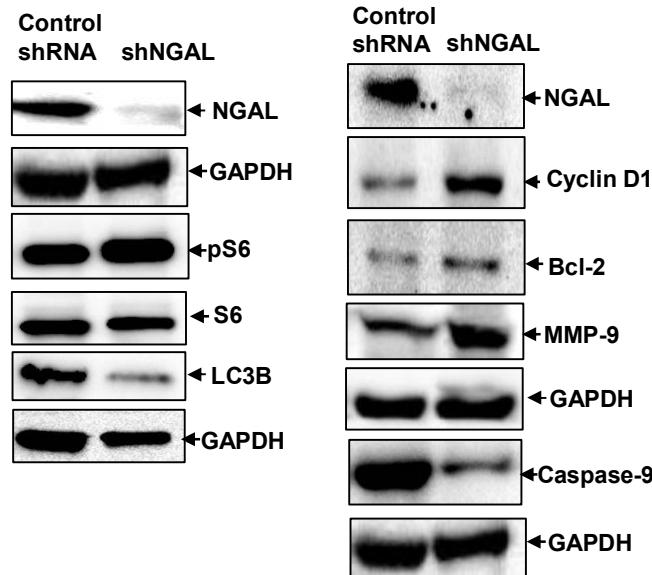
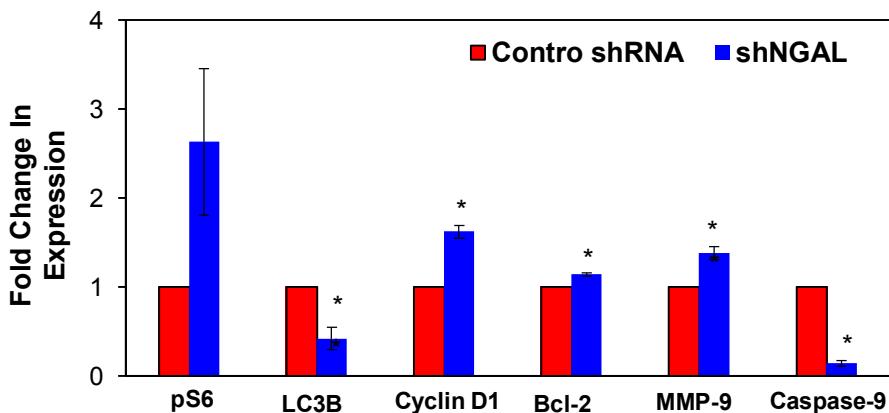


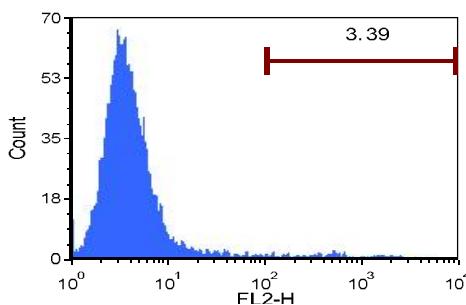
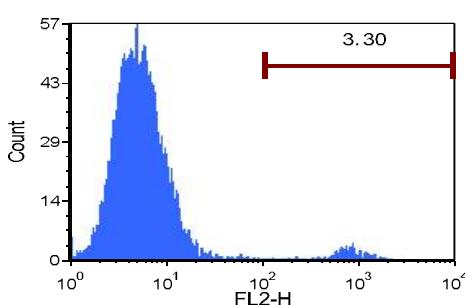
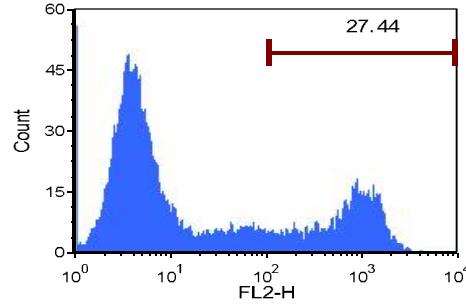
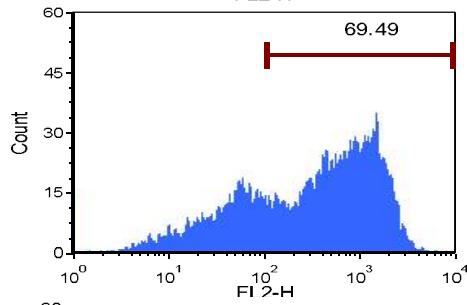
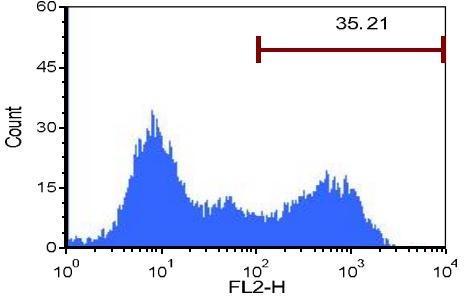
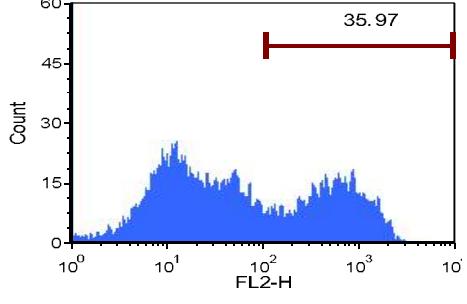
D.



F4



A.**B.****C.****D.**

A.**Control shRNA****shNGAL****Untreated****Cisplatin
50µM****5-FU
50µg/ml****B.****Control shRNA** **shNGAL**