Nerve growth factor-induced migration in oral and salivary gland tumour cells utilises the PI3K/Akt signalling pathway

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Title: Nerve growth factor-induced migration in oral and salivary gland tumour cells utilizes the PI3K/Akt signalling pathway: is there a link to perineural invasion?

Running title: Nerve growth factor utilizes PI3K/Akt pathway

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Abstract

Objectives: The aims of this study were to investigate the role of nerve growth factor on perineural invasion in oral and salivary gland tumour cell lines and whether there is an involvement of PI3K/Akt pathway.

Materials and Methods: Four cell lines were investigated: HSG and TYS (salivary gland tumours), SAS-H1 (oral squamous cell carcinoma) and HaCaT (human skin keratinocyte). Initially, Boyden chamber assay was done to examine the effect of different concentration of nerve growth factor on cell migration. Western blot/ immunofluorescence techniques were used to investigate the phosphorylation status of the Akt pathway within the cells in response to nerve growth factor. The effect of this growth factor and the addition of an Akt inhibitor on cell morphology and migration were also examined using scatter/scratch assays.

Results: Nerve growth factor triggered the PI3K/Akt pathway in oral and salivary tumour cells and induced oral and salivary tumour cells scattering and migration. Inhibitor assays confirmed that oral and salivary gland tumours cells scattering and migration is Akt dependent.

Conclusions: Nerve growth factor can stimulate scattering and migration in cells derived from oral and salivary gland tumours therefore potentially enhancing perineural invasion.

Phosphorylated Akt controls cancer cells migration and scattering. Blocking the Akt pathway may inhibit cells migration and therefore perineural invasion and metastasis.

Keywords: NGF, Akt, OSCC, salivary gland tumour, PNI

Introduction

Perineural invasion (PNI) is a form of metastatic tumour spread when cancer cells travel along nerves far from the primary lesion [1]. PNI is associated with an increased probability of metastasis and decrease survival in oral squamous cell carcinoma (OSCC) [2] and in salivary gland malignancies [3]. Cancer cell migration towards nerves requires the activation of numerous signalling pathways involving many growth factors [4]. Neurotrophins such as nerve growth factor (NGF) contribute to survival, proliferation and migration signalling in breast, prostate and pancreatic cancer [5-7]. Depending on the tumour origin, the signalling can be mediated by tropomyosin related kinase A (TrkA) receptor or p75 neurotrophin receptor ($p75^{NTR}$) [8]. NGF binding to tropomyosin related kinases (TrkA) receptor mediates proliferation and survival via activation of PI3K/Akt, Ras/MAPK and PLCγ pathways [9]. Serine/threonine kinase Akt is activated by phosphorylation at S473 and T308 might be involved in cancer metastasis [10]. NGF has been shown to play a role in tumour proliferation in OSCC [11-13] and salivary adenoid cystic carcinoma (AdCC) [14] which might be a reason for the cancer cells to migrate and invade the nerves. *Ex-vivo* studies show that Akt is phosphorylated in AdCC suggesting that PI3K/Akt signalling pathway has an important role in salivary gland carcinogenesis [15 16]. In this study we aimed to investigate the role of NGF-induced signalling pathway on PNI of oral and salivary gland tumours *in-vitro* and to confirm the involvement of PI3K/Akt pathway in cellular scattering and migration via the use of MK2206 (Akt inhibitor).

Materials and methods

I. Cell lines and culture

Human adenosquamous cell line derived from minor salivary gland (TYS) [17], OSCC line with high invasive potential (SAS-H1) derived from tongue [18] and the neoplastic duct cell line derived from submandibular salivary gland (HSG) [19] were gifted from Dr. Koji Harada, University of Tokushima, Japan. Human adult keratinocyte cell line (HaCaT) was gifted from

Professor S.L. Schor (late) [20], Dundee Dental School, University of Dundee, UK. All cells were cultured at 37ºC and 5% CO_2 in minimum essential medium (MEM) supplemented with 10% (v/v) heat-inactivated foetal calf serum (FCS) and 200mM glutamine. Ethical approval was granted by Dundee University and NHS Tayside – Scotland: (Ref: 14/ES/005).

II. Reagents, proteins, antibodies and inhibitors

Recombinant Human beta NGF (11050-HNAC) (Sino, Biological Inc. Beijing. China). The PI3K/Akt pathway inhibitor MK2206 (S1078) (Selleckchem, Houston, TX, USA). The primary antibodies used were: rabbit monoclonal anti-pAkt S473 (4060), anti-pAkt T308 (2965), and anti-pan Akt (4691) (Cell Signalling Technology Inc, Danvers, MA, USA). The secondary antibodies used were goat anti-rabbit HRP conjugated (7074) and goat anti-rabbit Alexa flour 488 conjugated (4412) (Cell Signalling Technology Inc, Danvers, MA, USA).

III. Modified Boyden chamber assay

A 48-well Boyden chamber (Neuroprobe, Inc, MD. USA) was used for the *in-vitro* migration assays as described previously [21]. Cell suspended in serum-free MEM with bovine serum albumin (2µg/ml) (SF-BSA) seeded into the upper compartment of the camber. The lower compartment was filled with different concentration of NGF diluted with SF-BSA. The two compartments were separated by porous membrane filter (8µm, Costar, UK) coated with type I native collagen. The chamber assay was done in duplicate for each cell line, one chamber incubated for 6-hour and other for 24-hour at 37ºC. The filter was then washed twice in PBS, fixed in cold methanol for 5 minutes and stained with Mayer's haematoxylin (MHS16, Sigma-Aldrich, St. Louis, MO, USA) overnight. The cells on the upper surface of the filter were scraped off with a cotton swab. The membrane was then mounted onto a glass slide and examined under bright field illumination at magnification x400. Six replicate wells used per variable. The number of migrated cells adherent to the lower surface of the membrane was counted in 3 random fields per well. Data expressed as mean number of cell migrated ± SD per field. Figures of the stained cell migrated were captured by Leica DC100 mounted digital camera attached to microscope.

IV. Cell lysis, BCA Protein assay, SDS-PAGE and Western Blot

Cells were grown on 60mm plastic culture dishes (around 80% confluent) and then serum starved overnight. They were then transferred to the test conditions (NGF ± inhibitor) for 24-hour and SF used as negative control. Cells were then washed with ice cold PBS and lysed on ice with RIPA buffer (50mM Tris HCl, 150mMNaCl, pH 7.4; 0.1% w/v SDS, 1% v/v Triton x-100, 1% w/v sodium deoxycholate and 5mM EDTA) containing protease and phosphatase inhibitors (Roche applied science, Bavaria, Germany) for 10 minutes, then scrapped off the dishes and cell lysates collected into Eppendorf tubes and frozen at -20°C. Prior to SDS-PAGE, protein assay was performed for the colorimetric detection and quantitation of total protein using Micro BCA Protein Assay Kit (Thermo Scientific, USA). Cell lysates were thawed and spun at 13,000 rpm in a micro centrifuge for 5 minutes, then mixed with a specific volume of Laemmli sample loading buffer (Bio-Rad Herules, CA, USA) including 5% (v/v) 2- Mercaptoethanol. Samples were then heated at 95ºC in a water bath for 5 minutes and loaded onto 10% or any kD Biorad TGX precast gels (Bio-Rad Herules, CA, USA). Gel images were captured using Gel Doc Imaging System (BioRad) for total protein. After completion of SDS-PAGE, proteins from the gels were electro-transferred onto PVDF membranes (Biorad) and then immunoblotted with anti-pAkt T308 (1:1000), anti-pAkt S473 (1:2000), anti-pan Akt (1:1000) and goat anti-rabbit HRP conjugated secondary (1:2000). Immunoblots were then developed using a WesternC substrate kit (BioRad) and chemiluminescence was detected using a ChemiDoc imaging system. Total protein gel images were used to normalise and quantity the respective blot by Image lab software (BioRad).

V. Scatter assay

Scatter assay was performed as described earlier [22]. Cells were seeded in a density of $5x10^4$ cells/ml per 35mm dish in 10%FCS MEM and grown until well-defined colonies were visible. The cells were washed twice and serum starved overnight, then medium was changed into test condition (NGF ± inhibitor) and SF used as negative control. The cultures were assessed for the scattering of the cellular colonies under the effect of the test conditions for 24-hour and 48-hour. To test if the changes were permanent, the overlay of the cells changed to 10%FCS MEM media and the morphology was monitored for 24 and 48-hour. Scattering of the cells was observed by at least two individuals before images were taken, images were captured by Olympus SC35 and Olympus XM10 digital cameras attached to Olympus IX70 inverted microscope.

VI. Wound-healing and immunofluorescence assays

A directional *in-vitro* 2D wound-healing assay, was performed as describe earlier [23]. A cell monolayer was serum starved overnight and then a wound was made in the monolayer using 100 µl pipette tip. The assay was then incubated in test conditions (NGF ± inhibitor) for 24-hour and SF used as negative control. Images were captured at the starting point to record the initial area of the wound and the recovery of the wounded monolayer due to cell migration toward the denuded area was evaluated after 24-hour. The images were captured by Olympus IX70 inverted microscope attached with Olympus SC35 and XM10 digital cameras.

For immunofluorescence assay, after 24-hour of the wound-healing assay, the cells were fixed with cold methanol for 15 minutes and then washed with phosphate-buffered saline (PBS). Cells were treated with 0.2% Triton X-100 in PBS for 5 minutes, the area into which cells migrated ringed with Immunopen (Dako, Cambridgeshire, UK) and blocked with 5% (v/v) normal goat serum (NGS, Vector Laboratories, Burlingame, CA, USA) in PBST (phosphate buffered solution with 0.1%Tween 20) for 30 minutes. The cells were then washed with PBS and incubated with anti-pAkt T308 (1:1600) or anti-pAkt S473 (1:2000) or anti-pan Akt (1:400) antibodies diluted in 5% (v/v) NGS in PBST at 4ºC overnight. They were washed twice with PBST, once with PBS and incubated with secondary antibody (1:1000) conjugated with Alexa Flour 488 for 30 minutes at room temperature. After washing twice with PBST and once with PBS, sections were cover slipped with aqueous mounting medium (Sigma-Aldrich, St Louis, MO, USA). Sections were viewed with an Olympus IX70 inverted fluorescent microscope. Images were collected using an Olympus SC35 and XM10 digital cameras. All devices were controlled and processed through Metamorph v6.1 software (Molecular Devices) and CellSense software (Olympus).

VII. Statistical analysis

The data was analysed using the statistical package IBM SPSS 22. All experiments were done in triplicate. Differences in mean of cell migration and percentage of the mean quantification of the Akt phosphorylation between negative control and different concentration of NGF was carried out using Kruskal-Wallis test with Bonferroni post-test, the differences defined at 95% level of confidence and considered significant when the p-value was less than 0.05.

Results

NGF stimulated oral and salivary gland tumours cells to migrate

Different concentration of NGF were used to investigate the role of this growth factor on cells migration in TYS, SAS-H1, HSG and HaCaT cells. Due to the unresponsiveness effect of HSG cells upon NGF treatment after 6-hour chamber incubation (data not shown), the results presented here are for 24-hour incubation. NGF stimulated the migration of TYS and SAS-H1 cells compared with SF (0ng/ml NGF) **(Figure 1. A&B).** However, HSG and HaCaT cells were not stimulated to migrate in response to NGF **(Figure 1. C&D).**

NGF triggered P13K/AKT pathway in oral and salivary gland cancer cells

All the cell lines were treated with different concentrations of NGF for 24-hour and analysed for phosphorylation of Akt at T308, S473 and pan Akt (60 kDa). Phosphorylation of Akt at T308 and S473 was increased in NGF-induced TYS and SAS-H1 cells compared with the negative control ($p<0.05$) **(Figure 2. A&B) (supplementary Table 1. A&B).** There was no Akt phosphorylation at T308 and S473 in HSG cells in SF and in NGF. However, Akt phosphorylation at T308 and S473 was constant in HaCaT cells compared to the SF and with increasing NGF concentration. Total Akt protein level (pan-Akt) was not upregulated in all cell lines **(Figure 2C).** MK2206 is a selective inhibitor of PI3K/AKT pathway, to elucidate the effect of MK2206 on the phosphorylation of Akt, TYS and SAS-H1 cells were treated with different concentrations of MK2206 (10μM, 5 μM, 1 μM) alone and with 10ng/ml NGF for 24-hour. Akt phosphorylation at T308 and S473 was effectively blocked by MK2206 alone or in combination with NGF in both cells **(Figure 2. D&E).** The total Akt (pan Akt) was consistent in both cells treated with Akt inhibitor with and without NGF **(Figure 2F).**

NGF-induced scattering in oral and salivary cancer cells

Cell scattering is a technique used to describe the dispersion of compact colonies of each cell lines induced by certain soluble factors such as growth factors [24]. After 24-hour incubation of TYS and SAS-H1 cells treated with 10ng/ml NGF, partial scattering was noticed where some of the cells within the colony began to detach from their neighbouring cells and exhibit a shape resembling that of spindle-like fibroblastic morphology and scattered across the plate compared with non-treated cells. After 48-hour incubation, a complete scattering was recorded where some of the cells lose cell to cell junctions and single cells can be seen. These cells have an elongated phenotype and are scattered across the plates when treated with NGF compared with non-treated

cells **(Figure 3A).** These changes in morphology in both TYS and SAS-H1 cells were reversible and a noticeable change in morphology was observed in TYS and SAS-H1 cells where the cells regained back their flattened morphology with compact colonies after changing the overlay to 10%FCS MEM for 48-hour **(Figure 3B)**. In contrast, the NGF-treated HSG and HaCaT cells did not dissociate from each other and showed flattened morphology with or without NGF **(Figure 3C).** To confirm the involvement of Akt pathway in cellular scattering via the use of an Akt inhibitor, TYS and SAS-H1 cells were treated with 5μM concentration of MK2206 alone and with 10ng/ml NGF for 24 and 48-hour. MK2206 alone or in combination with NGF effectively blocked the scattering of both cells compared to SF **(Figure 3. D&E).**

NGF-induced oral and salivary gland cancer cells migration is Akt dependant

24-hour observation of cell migration in the wound-healing assay showed that NGF stimulated the migration of TYS and SAS-H1 cells, whereas HSG and HaCaT cells were not stimulated to migrate in response to the NGF **(Figure 4A).** MK2206 alone and with 10ng/ml of NGF were used to confirm the role of this growth factor in the cell migration in TYS and SAS-H1 cells via wound-healing assay for 24-hour. MK2206 alone or in combination with NGF effectively blocked the migration of both cells compared to SF **(Figure 4B).**

NGF-induced oral and salivary gland cells migration with positive Akt phosphorylation

Immunocytochemistry of the fixed cells showed the expression and localisation of phosphorylated Akt. NGF induced TYS and SAS-H1 cells showed a cytoplasmic expression of pAkt T308 and pAkt S473 **(Figure 5 A&B).** To elucidate the effect of PI3K inhibitor on the phosphorylation of Akt, TYS and SAS-H1 cells were treated 5μM MK2206 alone and with 10ng/ml NGF for 24-hour. MK2206 alone or in combination with NGF effectively blocks the phosphorylation of Akt T308 and Akt S473 in oral and salivary gland cancer cells (data not shown)**.**

Discussion

The infiltration of cancer cells into the neural tissue is a key step in the process of PNI, and NGF might play a role in the process of PNI in oral and salivary gland tumours [14]. In this study, we have investigated the role of NGF-induced PI3K/Akt signalling pathway on PNI behaviour of oral and salivary gland tumour cells. The data presented here is evidence that NGF can induce PI3K/AKT pathway and that NGF phosphorylates Akt at T308 and S473 in oral and salivary cancer cells. Our findings also indicate that NGF stimulates a migration and scattering effect of OSCC and salivary gland tumour cells and these cells undergo a morphological change from epithelial to mesenchymal-like phenotype upon treatment with NGF. This may suggest that NGF is able to induce migratory behaviour and therefore enhance PNI. These results are in agreement with previously published study reported that NGF pre-treatment of breast cancer cells promoted epithelial to epithelial-mesenchymal transition (EMT) [25] and activation of PI3K/Akt signalling pathway can affect EMT to influence tumour aggressiveness [26]. The reversibility of EMT-like changes induced in OSCC and salivary gland cancer cells presented here might be due to reoxygenation of the cells [27] and this raises a question whether EMT promotes cancer cells metastasis.

The Akt inhibitor (MK2206) was found to block the stimulation of NGF induced scattering and migration as well as the phosphorylation of Akt at S473 and T308 in OSCC and salivary gland cancer cells. These results support data published previously reported that MK2206 inhibited the proliferation of human cancer cells in combination with anticancer agents *in-vitro* [28]. Despite the preclinical studies supporting the antitumor effect of MK2206 when combined with endocrine therapy, drugs combined with MK2206 had no significant clinical effect in patients with breast cancer in phase I trials. Administration of MK2206 caused the development of a rash and

hyperglycaemia resulting in some patients discontinuing the treatment [29]. HSG cells showed no scattering or migration changes upon NGF treatment, therefore, these cells have not shown any expression of Akt phosphorylation at T308 or S473 residues. It should be noted that HSG cells used here were grown on plastic culture dishes and had not differentiated into the "acinar state" cell line described below. A study investigated the effect of several growth factors involved in cell differentiation on the activity of salivary proteins such as amylase and kallikrein in HSG cells found that NGF had a very little effect when the HSG cells were grown on plastic culture plates [30], however, HSG cells differentiates into an acinar phenotype along with the change in morphology when the cells are grown only on Matrigel and laminin-1. Taking into consideration the previous published study and our data, we suggest that HSG require a pre-treatment condition involving addition of extracellular matrix proteins and growth factors in order to differentiate into acini and acquire malignant phenotype.

Conclusion

NGF can stimulate scattering and migration in OSCC and salivary gland cancer cells, therefore potentially enhance PNI and possibly facilitating metastasis. Activation of Akt by NGF mediate cancer cells migration is summarised in **Figure 6**. Akt is phosphorylated at both residues controlling OSCC and salivary gland cancer cells migration and the addition of an Akt pathway inhibitor, blocks the NGF-induced OSCC scattering and migration and therefore PNI which may offer a therapeutic approach in the future.

Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and /or publication of this article.

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Figure legends

Figure 1. Variable migratory responses of different cell lines treated with NGF obtained by modified Boyden chamber migration assay (24-hour incubation) compared with negative control (0ng/ml NGF as baseline)

A. TYS (mn=29.4±2) and B. OSCC (SAS-H1) (mn=22.8±3.1) were stimulated to migrated through the filter by 100ng/ml NGF compared to baseline (mn=18.4±2 & 17.5±2.3 respectively). C. HSG (mn=18.1±1.5) and D. HaCaT (mn=12.6±1.7) were not stimulated to migrate by NGF compared to baseline (mn17.2±2, 10.7±0.9 receptively). The effect of NGF on cells migration were plotted as mean number of cell migrated. The graph results are expressed as mean with differences defined at 95% level of confidence * $p<0.05$, relative to negative control. mn= mean numbers of cells migrated. The images below each graph represent the cells stained with Mayer's haematoxylin migrated from 0ng/ml NGF to 10ng/ml NGF (Images were captured by Leica DC100 mounted digital camera attached to microscope at x400 magnification).

Figure 2. Western blot experiments of Akt phosphorylation in four cell lines with a spectrum of NGF concentrations and inhibitor.

(A) pAkt T308 phosphorylation in TYS, SAS-H1, HSG and HaCaT with NGF concentrations of 0ng/ml, 500pg/ml, 1ng/ml, 10ng/ml, 100ng/ml and 200ng/ml. (B) pAkt S473 phosphorylation and (C) total Akt protein in the same cell lines with same NGF concentration as in (A). (D) pAkt T308 phosphorylation in TYS and SAS-H1 cells with MK2206 inhibitor of 10μM, 5 μM, 1 μM alone and with 10ng/ml NGF. (E) pAkt S473 phosphorylation and (C) total Akt protein in the same cell lines with same condition as in (D). All cells were treated for 24-hour and cell lysates were then analysed by SDS-APGE and WB experiments for pAkt T308, pAkt S473 and pan Akt antibodies. All the images were cropped from the original blot images. 0ng/ml NGF used as a negative control. * denote significant changes in phosphorylation.

Figure 3. Scatter Assay of TYS, SAS-H1, HSG and HaCaT cells.

(A) TYS and SAS-H1cells respectively in the absence of NGF (SF) and presence of 10ng/ml of NGF during 24 and 48-hour observation. Images illustrate the scattering as cells remain in colony in SF (red arrow). Partial scattering of the colonies where the cells exhibit an elongated phenotype (yellow

arrows) and complete scattering where some loss of cell to cell junction and single cells with an elongated migratory phenotype (green arrows). (B) NGF induced reversible morphological changes responses in TYS and SAS-H1 cells after 48-hour treatment of the cells with 10%FCS MEM, upon the removal of NGF. The cells gradually regained their original phenotype with compact colonies and established cell to cell junctions (blue arrow). (C) NGF does not mediate any morphological changes in HSG and HaCaT. The cells do not dissociate from each other and showed flattened morphology with or without NGF. (All images captured using an Olympus SC35 digital camera at x100 magnification). (D & E) TYS and SAS-H1cells respectively in presence of 10ng/ml of NGF, 10ng/ml of NGF with 5μM MK2206 and 5μM MK2206 during 24 and 48-hour observation. MK2206 alone or in combination with NGF effectively blocks the scattering of both cells during the different incubation time compered to SF. (All images were captured using an Olympus XM10 digital camera at x100 magnification).

Figure 4. Wound-healing assay. Images have shown variable migratory behaviour of different cell lines.

(A) TYS, SAS-HI, showed migration in response to 10ng/ml of NGF over 24-hour treatment. HSG and HaCaT cells showed no migratory response upon treatment with NGF. (All images were captured using an Olympus SC35 digital camera at x100 magnification). (B) wound-healing assay of TYS and SAS-H1cells with the use of 5μM MK2206 showed that MK2206 alone or in combination with 10ng/ml of NGF effectively blocks the migration of both cells compered to SF (All images were captured using an Olympus XM10 digital camera at x40 magnification)

Figure 5. Immunofluorescence staining of TYS and SAS-H1. The cells were treated for 24-hour then fixed and stained for Akt (total or phosphorylated) with a fluorescent labelled antibodies.

NGF increased cytoplasmic localisation of pAkt T308, pAkt S473 and total Akt in TYS cells (A) and in SAS-H1 cells (B). (All the fluorescent images were captured using an Olympus SC35 digital camera at x200 magnification).

Figure 6. Activation of Akt by NGF in PNI-positive OSCC.

(A) Histological example of PNI in OSCC where the tumour cells (black arrow) surrounding the nerve (in red) (haematoxylin-eosin staining x100 magnification**)**. (B) NGF binding to TrkA receptor mediates migration via activation of PI3K/Akt pathway. (C) PNI-postive OSCC showing marked immunoreactivity to NGF (black arrows) (x100 magnification) (histological images were taken from a study in progress)

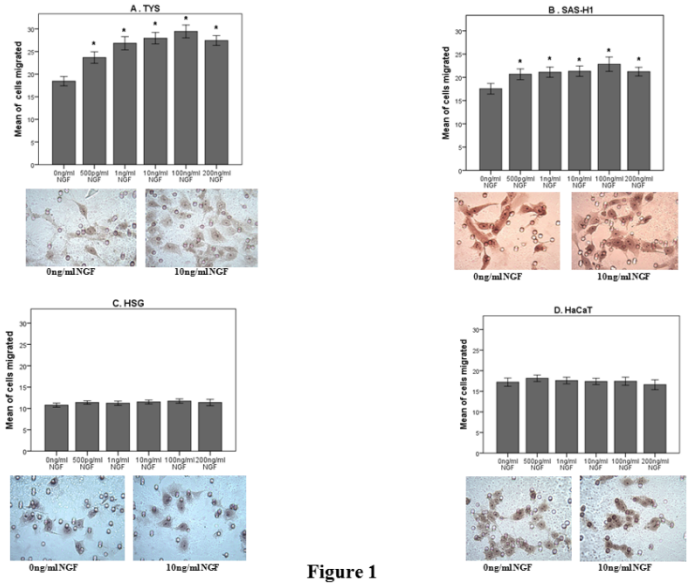

Figure 1

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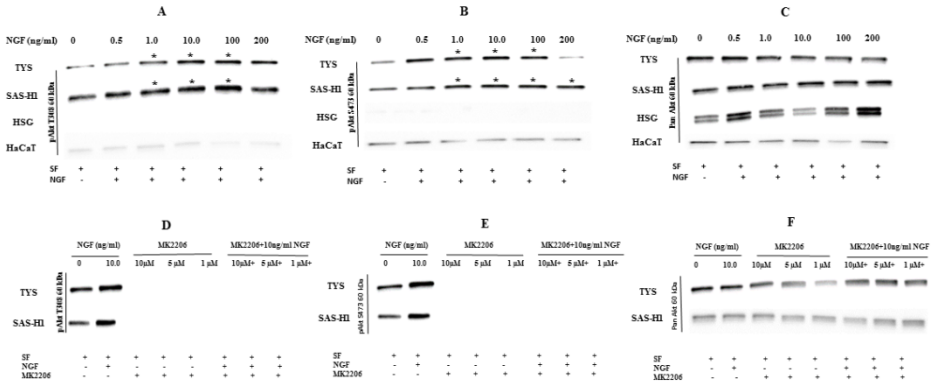

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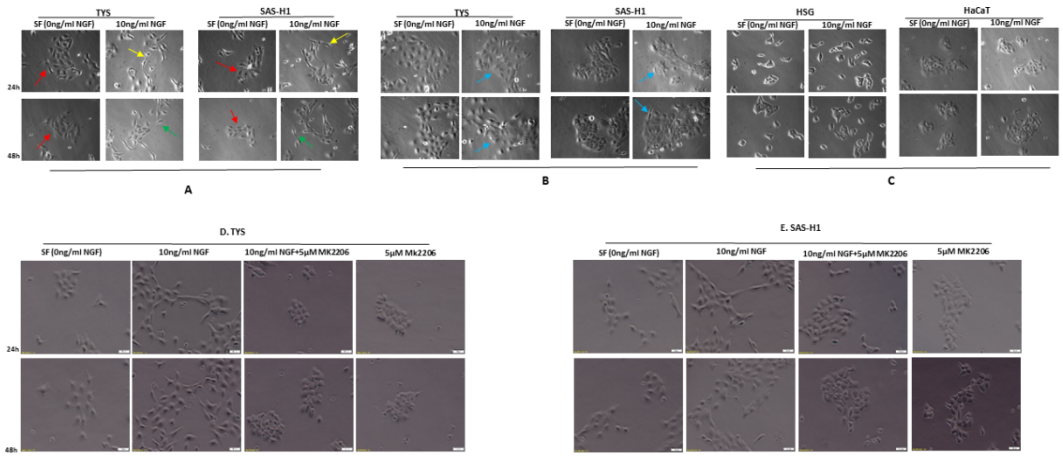

Figure 3

jop_12979_f3.tif

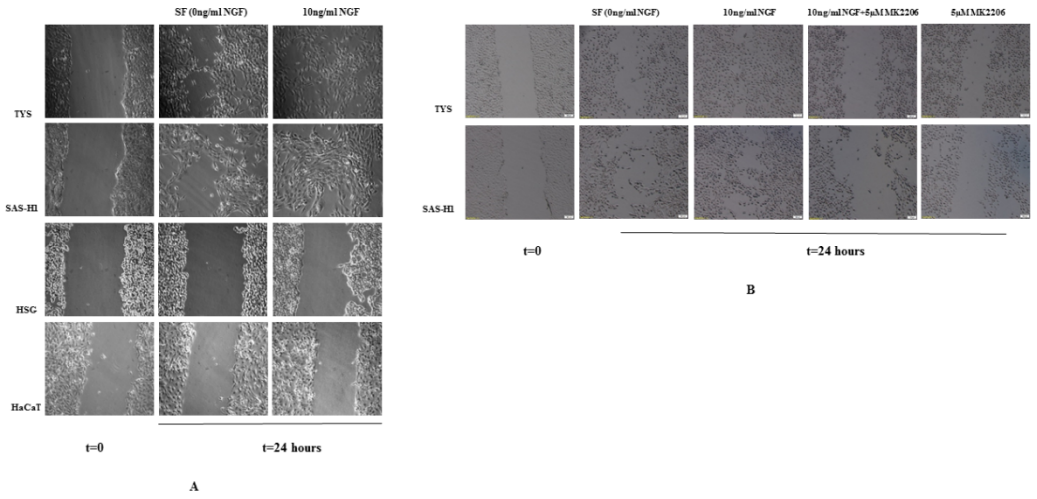

Figure 4

jop_12979_f4.tif

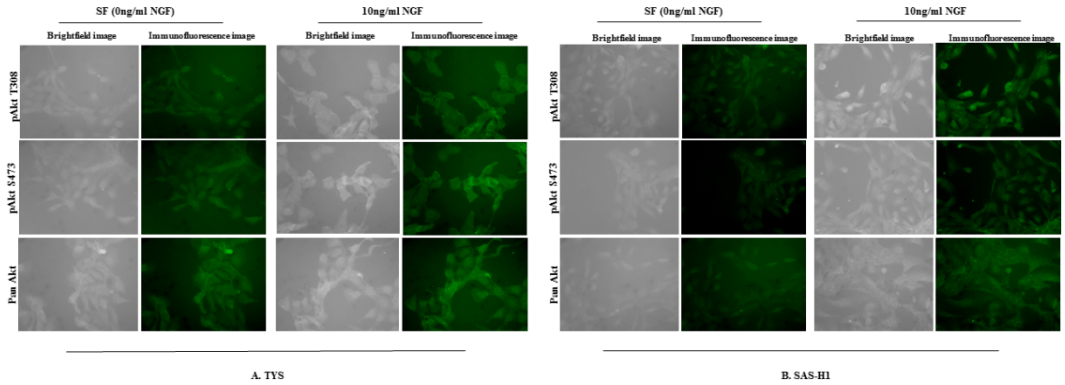

Figure 5

jop_12979_f5.tif

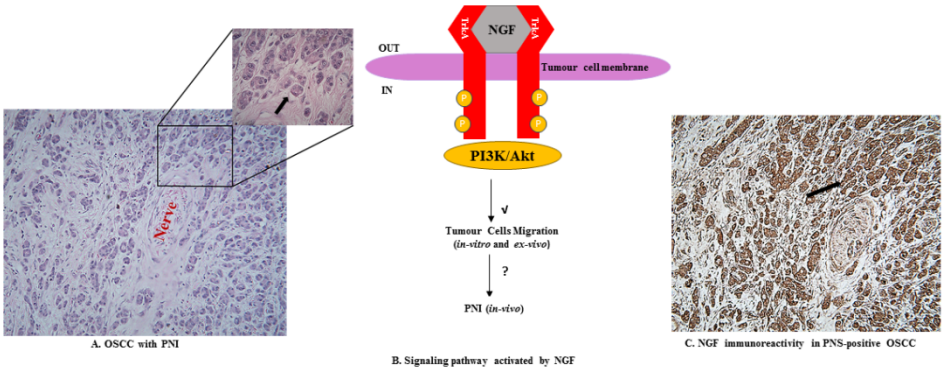

Figure 6

jop_12979_f6.tif