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SG2NA enhances cancer cell survival by stabilizing DJ-1 and thus activating Akt

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ABSTRACT

SG2NA in association with striatin and zinedin forms a striatin family of WD-40 repeat proteins. This family of proteins functions as scaffold in different signal transduction pathways. They also act as a regulatory subunit of protein phosphatase 2A. We have shown that SG2NA which evolved first in the metazoan evolution among the striatin family members expresses different isoforms generated out of alternative splicing. We have also shown that SG2NA protects cells from oxidative stress by recruiting DJ-1 and Akt to mitochondria and membrane in the post-mitotic neuronal cells. DJ-1 is both cancer and Parkinson's disease related protein. In the present study we have shown that SG2NA protects DJ-1 from proteasomal degradation in cancer cells. Hence, downregulation of SG2NA reduces DJ-1/Akt colocalization in cancer cells resulting in the reduction of anchorage dependent and independent growth. Thus SG2NA enhances cancer cell survival. Reactive oxygen species enhances SG2NA, DJ-1 and Akt trimerization. Removal of the reactive oxygen species by N-acetyl-cysteine thus reduces cancer cell growth.

1. Introduction

SG2NA was initially characterized as a nuclear localized tumor antigen whose expression is augmented during S to G2 phases of cell cycle [1,2]. Together with striatin and zinedin it forms striatin sub-family of WD-40 repeat superfamily [3]. Among these three members, SG2NA was the earliest to evolve [4]. Striatin family members act as the B‴ subunit of the heterotrimeric protein phosphatase 2A complex [5,6]. We have reported earlier that SG2NA has several isoforms generated out of alternative splicing [7]. Expression profile of SG2NAs in tissues changes with stages of embryonic development and post-natal aging [7,8,9]. Expression of SG2NA is epigenetically regulated by Brg-1 [10]. Recently, we found that SG2NA recruits DJ-1 and Akt to plasma membrane and mitochondria to protect cells from oxidative stress [11].

Like SG2NA, DJ-1 was also discovered as an oncogene that in cooperation with ras transforms NIH3T3 cells [12]. Its expression is augmented in prostate, lung, breast, renal, hepatocellular, ovarian, acute leukemia, cervical, papillary, thyroid cancer and squamous cell carcinomas [13,14]. It transforms NIH3T3 cells by directly interacting with the SV-40 large T antigen [15]. DJ-1 activates Akt signaling by direct interaction and inhibition of PTEN [16,17]. It also can sense increased generation of reactive oxygen species (ROS); thus it acts as redox-sensitive chaperone and scavenge excess ROS, enhancing resistance against oxidative stress [18].

Akt is activated by several extra cellular signals including growth factors and by oncogenic mutations [19]. Activated Akt plays crucial physiological functions in cell proliferation, metabolism and stress responses. Its activity is tightly regulated by different combinations of kinases and phosphatases [19]. It remains in an inactive state due to an intramolecular interaction between the PH and the kinase domain. Various growth signals activate Phosphatidylinositol-3-kinases (PI3Ks) that in turn phosphorylate phosphatidylinositol-4, 5-bisphosphate (PIP2) to phosphatidylinositol-3, 4, 5-bisphosphate (PIP3) [20]. Akt simultaneously interacts with PIP3 and PDK-1, changing its conformation. PDK-1 then phsophorylates Akt at T308, inducing its activity [20]. However, for complete activation, it also needs to be phosphorylated at S473 though the mechanisms are not fully understood yet. A number of recent studies have shown

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PI3K independent activation of Akt, especially in cancer cells [20].

Cellular redox plays an essential role in maintaining the functions of the cell. Reactive oxygen species (ROS) like hydrogen peroxide and superoxide regulate many signaling pathways associated with cell growth, proliferation, survival, motility and transformation [21]. At a moderate level, ROS act as second messenger, while excessive generation is harmful as it leads to cell death.

Previously, we have shown that SG2NA acts as a scaffold for DJ-1-Akt interaction under moderate oxidative stress [11]. As cancer cells have enhanced generation of ROS [21], it is relevant to study whether SG2NA-DJ-1-Akt interaction plays a role in cancer cell survival.

2. Materials and methods

All the chemicals are purchased from Sigma–Aldrich, USA unless noted otherwise.

2.1. Cell culture

NIH3T3, H9c2, HEK293, HEK293T, DU145, HepG2 and HeLa cells were procured from NCCS, Pune. These cells were cultured in DMEM containing 10% fetal bovine serum (FBS), 1% penicillin-streptomycin-amphotericin cocktail at 37 °C in the presence of 5% CO_2.

2.2. ShRNA constructs

The shRNA expression cassettes against SG2NA were constructed to pLKO.1 vector. HEK293T, DU145 and HepG2 cells were stably transfected with those shRNA constructs. The transfected cells were selected and maintained by adding 2.5 mg/ml and 0.5 mg/ml of puromycin to standard growth media.

2.3. Cell lysis and immunoblotting

The cells were treated as mentioned in the figures, lysed and western analysis was carried out as mentioned earlier [11].

2.4. Immunoprecipitation

Cells were lysed in buffer containing 50 mM Tris, pH 7.6, 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% NP-40, 1 mM sodium orthovanadate, 10 mM sodium fluoride, protease inhibitors and 1 mM PMSF on ice and centrifuged at 10,000 rpm for 10 min. One mg of the supernatant was pre-cleared with equilibrated protein A agarose beads by incubating for 2 h at 4 °C and then was incubated with 4 μg of primary antibody overnight at 4 °C in a rotator. Thirty μl of equilibrated protein A agarose beads (50/50 slurry) was added to lysate antibody complex and incubated for 3–5 h. The beads were washed with the lysis buffer for 3 times at 4 °C for 5 min each and then directly loaded on SDS-PAGE followed by immunoblotting.

2.5. Measurement of intracellular reactive oxygen species (ROS)

Intracellular ROS were measured in different cells with the fluorescent probe DCFH-DA. Briefly, DCFH-DA was dissolved in dimethyl sulphoxide (DMSO) and the cells were incubated with 5 μM DCFH-DA at 37 °C for 20 min. Images were captured under fluorescence microscope (Nikon) with excitation wavelength at 488 nm and emission wavelength at 530 nm.

2.6. Immunofluorescence analysis

Cells were grown on cover slips. After treatment, the cells were then fixed with 3.7% formaldehyde for 15 min, permealized with 0.1% triton-X-100 and blocked with 1% BSA prepared in 1× PBS for 1 h. The cells were incubated with primary antibody for 1 h followed by the incubation with secondary antibody for 1 h. Cover slips were mounted on slides over 10% glycerol solution, and sealed from the edges. The images were captured using confocal microscope of Olympus Fluoview 1000.

2.7. Proliferation assay

Cell proliferation assay was carried out HEK293T, HepG2 and DU145 cells stably transfected with shRNA against SG2NA and scrambled oligos. 2×10^4 cells were plated into the 48 well plates. After 12, 24, 48 and 72 h of incubation in 5% CO_2 at 37 °C, the cells were trypsinized and viable cells were counted using Trypan Blue staining method. To study anchorage independent growth by colony forming assay with soft agar method, ~0.3×10^6 cells were plated in triplicate in 0.35% (w/v) agar in DMEM supplemented with 10% FBS on the top of 0.7% (w/v) agar in 6 well plate. Cells were incubated in 5% CO_2 at 37 °C for 14 days with refeeding every alternate day. Colonies were stained with 0.005% crystal violet and images were captured with a Nikon microscope.

2.8. Statistical analysis

The extent of co-localization was quantified using Image Pro Plus 6 software. Statistical analysis was carried out from the quantified data and expressed in terms of Mean ± SEM and p value was calculated from student's t test with two tailed distribution considering two sample equal variance. Significance was considered as follows * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

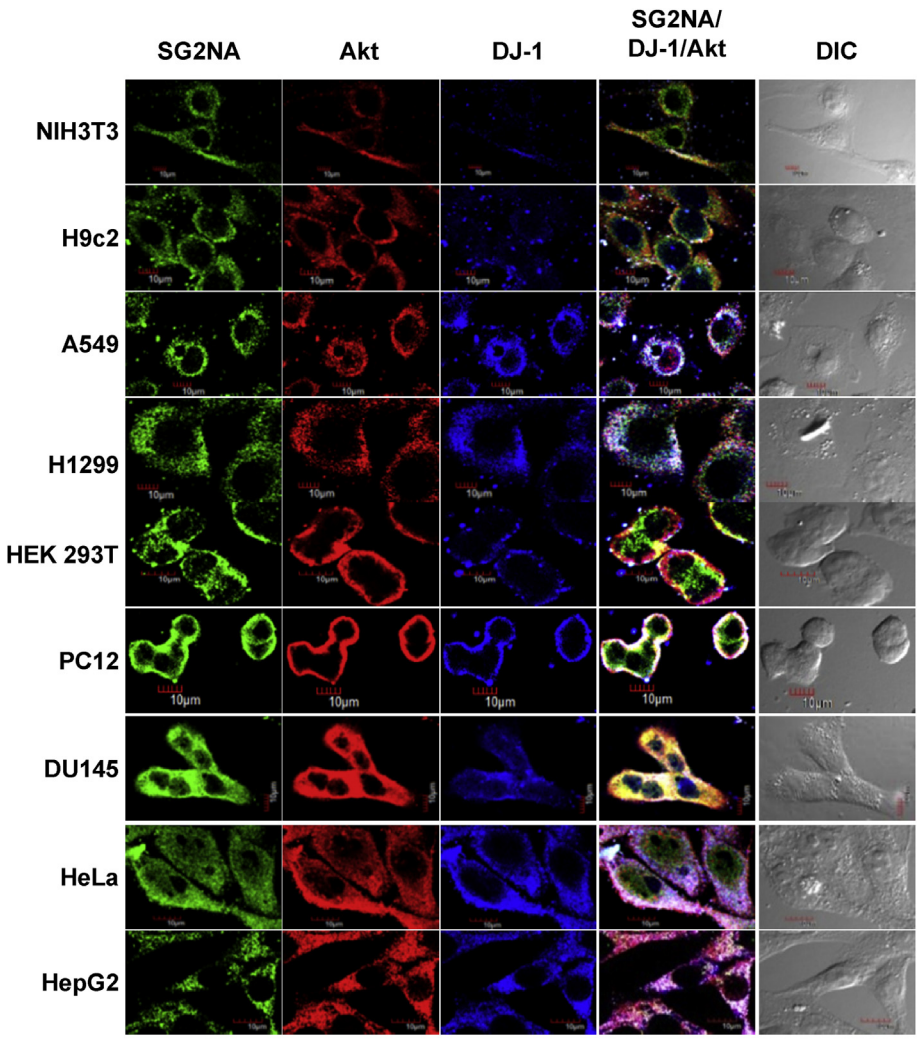
3. Results

3.1. Association of Akt, DJ-1 and SG2NA is increased in cancer cell lines

Increased DJ-1 and Akt activities have been associated with cancer development [22]. In view of our recent observation that SG2NA modulates cell cycle progression (unpublished) and it recruits Akt and DJ-1 under oxidative stress [11]; we tested whether association of SG2NA, DJ-1 and Akt is also enhanced in cancer cells. A number of established cancer cell lines viz., A549, H1299, HEK293T, DU145, HepG2, HeLa, PC12 were immunostained for SG2NA, DJ-1 and Akt. As shown in Fig. 1, association of SG2NA-DJ-1, Akt-DJ-1 and SG2NA-Akt were significantly higher in those cell lines as compared to that in a number of immortalized untransformed cell lines viz., NIH3T3 and H9c2. Hence, increased co-localization of three proteins in those cells might be due to their recruitment by SG2NA.

3.2. Ablation of SG2NA disrupts DJ-1/Akt association

In cancer cells DJ-1 activates Akt by multiple mechanisms including inhibition of PTEN [16]. We thus tested whether SG2NA, the scaffold for DJ-1 and Akt interaction, plays any role in this process. Two well characterized cancer cell lines DU145 and HepG2 and an experimentally transformed cell HEK293T were stably transfected with shRNA against SG2NA. As shown in Fig. 2, down-regulation of SG2NA decreased the level of colocalization between



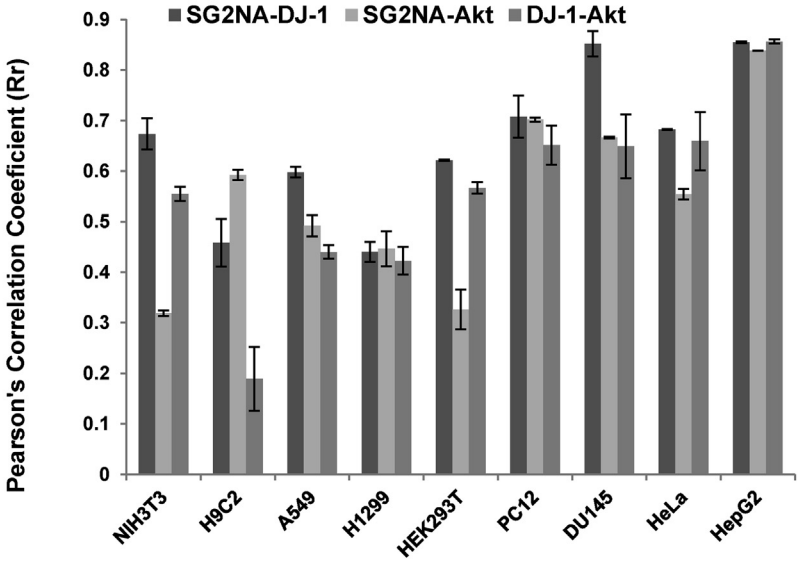


Fig. 1. Increased colocalization of SG2NA, DJ-1 and Akt in certain cancer cell lines. A number of cancer cell lines as marked above were immunostained with SG2NA, DJ-1 and Akt antibodies. The proteins were then visualized with Alexa 488 (SG2NA), Alexa 555 (Akt) and Alexa 633 (DJ-1) conjugated secondary antibodies. Images were captured by FV1000 Confocal Laser Scanning Biological Microscope from Olympus (scale bar 10 μm). The extent of colocalization between SG2NA-DJ-1, SG2NA-Akt and DJ-1-Akt was quantified by Image Pro plus which shows that in A549, H1299, HEK293T, PC12, DU145, HeLa and HepG2 cells, colocalization is higher than their normal counterparts like NIH3T3, H9c2 and HEK.

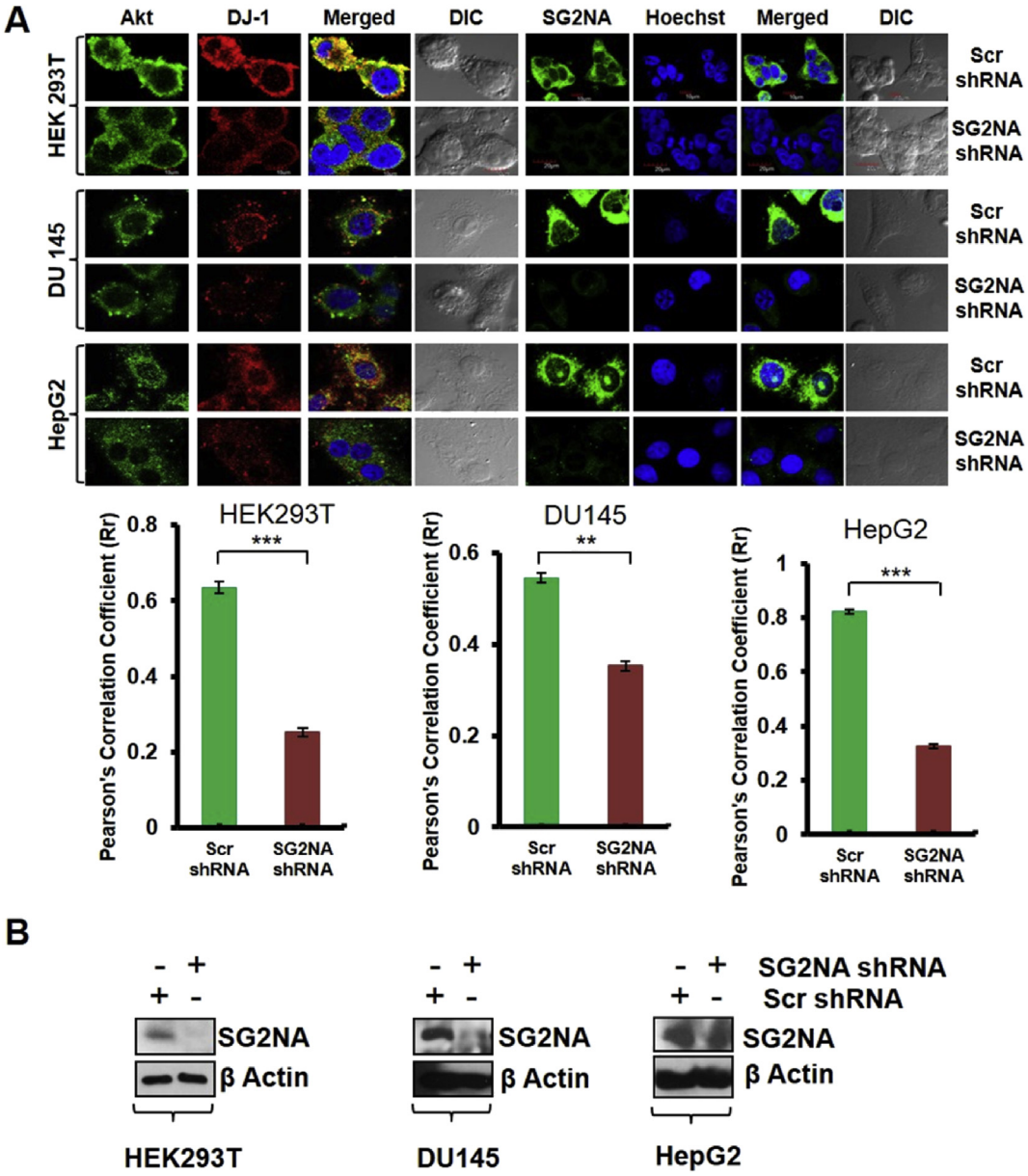


Fig. 2. Association of DJ-1 and Akt diminishes in absence of SG2NA. (A) Three different cell lines viz., HEK293T, DU145 and HepG2 depleted of SG2NA were immunostained with DJ-1 and Akt (left panel) and SG2NA (right panel). Alexa 488 (Akt and SG2NA), Alexa 555 (DJ-1) conjugated secondary antibodies were used to visualize the proteins. Images were captured by FV1000 Confocal Laser Scanning Biological Microscope from Olympus (scale bar 10 μm). The extent of colocalization between DJ-1-Akt was quantified by Image Pro plus. Quantification of the colocalization was carried out for DJ-1 and Akt and expressed as Pearson's correlation coefficient from three independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ ($n = 14–29$). (B) Down regulation of SG2NA in HEK293T, DU145 and HepG2 by the stable expression of shRNA [11] was confirmed by Western blot analysis. Equal amount of extracts (100 μg) from each cell lines were separated in SDS-PAGE and immunoblotted for SG2NA. Antibody against β actin was used as loading control.

Akt and DJ-1. The effectiveness of shRNA against SG2NA in these cells have shown in Fig. 2B.

3.3. Quenching of reactive oxygen species dislodges SG2NA, DJ-1 and Akt complex

Cancer cells are often characterized by increased levels of ROS which contribute to angiogenesis; tumor progression and suppression by p53; epithelial–mesenchymal transition, metastasis; functioning of tumor stromal cells and cancer stem cells; chemo and radio resistance [23–25]. They are also characterized by the presence of deregulated MAPK and Akt signaling pathways enhancing their survival potential [26,27]. Three transformed cell lines viz. DU145, HepG2 and HEK293T and two immortalized cell lines i.e., NIH3T3 and H9c2 were compared for endogenous ROS levels under normal growing conditions. As shown supplementary Fig. 3A, the ROS level (DCFHDA positive) was substantially higher in transformed cells as compared to their normal counterparts. Cells were then treated with N-acetyl cysteine (NAC, 10 mM), a widely used thiol-antioxidant. Noticeably, this dose of NAC suppressed ROS in DU145 and HepG2 cells but not in HEK293T cells (Fig. 3B). Upon NAC treatment, reduction in colocalization of SG2NA, DJ-1 and Akt were seen in DU145 and HepG2 cells but not in HEK293T cells (Fig. 3C). Immunoprecipitation of SG2NA followed by immunoblotting for Akt and DJ-1 also showed a reduction in their association in DU145 cells upon NAC treatment (Fig. 3D) which

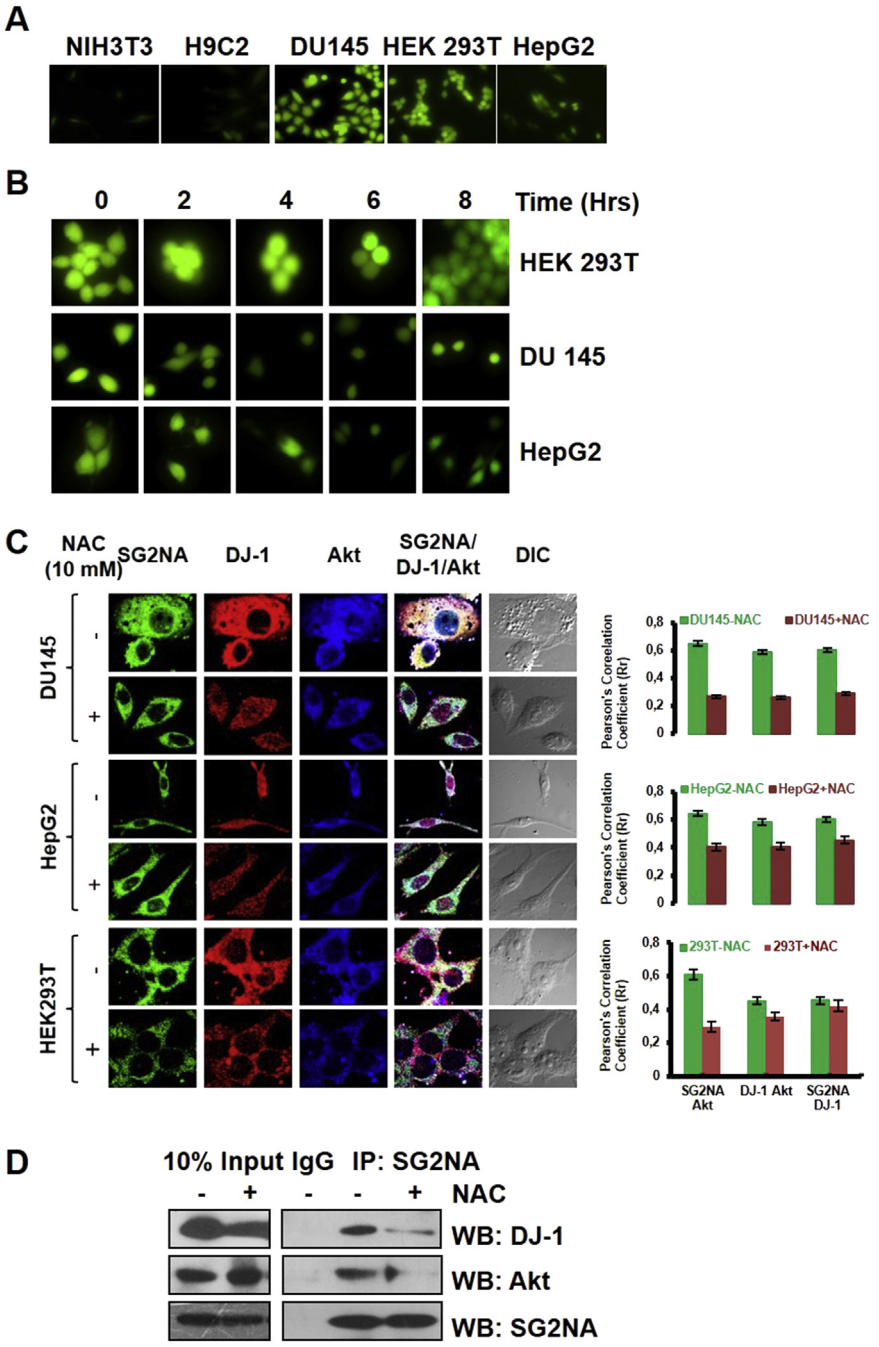


Fig. 3. Colocalization of SG2NA, DJ-1 and Akt complex is dependent on ROS. (A) NIH 3T3, H9C2, DU145, HEK293T and HepG2 cells were grown in 12 well plates and probed with DCFHDA for ROS generation under fluorescence microscope. (B) HEK293T, DU145 and HepG2 cells were treated with 10 mM NAC till 8 h and level of ROS was monitored by DCFH DA under microscope. In DU145 and HepG2 cells, NAC reduced ROS level in 4 h but in HEK293T cells there was no reduction in ROS upon treatment with NAC till 8 h, the last time point tested. (C) DU145, HepG2 and HEK293T cells was treated with 10 mM of NAC for 4 h. The cells were immunostained with SG2NA, DJ-1 and Akt antibodies. These proteins were then visualized with Alexa 488 (SG2NA), Alexa 555 (Akt) and Alexa 633 (DJ-1) conjugated secondary antibodies. Images were captured by FV1000 Confocal Laser Scanning Biological Microscope from Olympus (scale bar 10 μm). Extent of co-localization of SG2NA, DJ-1 and Akt was calculated for ~15–25 cells in three independent experiments and expressed as Pearson's correlation coefficient (Rr). *P < 0.05, **P < 0.01 and ***P < 0.001. (D) DU145 cells were treated with 10 mM of NAC for 4 h and then cell lysate was immunoprecipitated with SG2NA antibody followed by western analysis for SG2NA, DJ-1 and Akt.

further demonstrates that association of DJ-1 and Akt mediated by SG2NA is ROS dependent.

3.4. Downregulation of SG2NA decreases cancer cells growth

Activation of Akt is one of the crucial mechanisms for cancer cell survival. DJ-1 has also been associated with cancer cell survival and metastasis. Recently, it has been shown that downregulation of striatin 4/zinedin, the third member of striatin family, reduces cancer cell survival [28]. We thus asked whether SG2NA being a scaffold for both DJ-1 and Akt, promotes cancer cell proliferation. Expression of SG2NA was suppressed in DU145, HepG2 and HEK293T cells by stable transfection of SG2NA shRNA. Equal number of cells were seeded in 48 well plates and incubated for 12, 24, 48 h followed by counting the cell number in a haemocytometer using trypan blue. We observed that down regulation of SG2NA

leads to a decrease in the rate of proliferation (Fig. 4A). This result suggests that SG2NA supports cell proliferation. To check whether SG2NA is also involved in the anchorage independent growth, the cells were seeded in 0.35% agarose in DMEM supplemented with 10% FBS on top of 0.7% agarose. Cells were incubated at 37 °C and 5% (v/v) CO_2 for 2–3 weeks with refeeding every day or every alternate day. The colonies were stained with 0.005% crystal violet and image was captured by a Nikon stereo zoom microscope (1×). The colonies which appeared as round and colored spots, were counted manually. As shown in Fig. 4B, down regulation of SG2NA decreases the anchorage independent growth of the cancer cells. Taken together, these data suggest that SG2NA plays a major role in both anchorage dependent and anchorage independent growth of cancer cells.

3.5. Depletion of SG2NA destabilizes DJ-1 and activates Akt

DJ-1 is a chaperone and its mutated versions are rapidly degraded by the proteasome [17,29]. However, whether same pathway contributes to the stability of the wild type protein is not known. We observed that upon down regulation of SG2NA, the protein level of DJ-1 also goes down (Fig. 4D) but not the transcript level (Fig. 4D). To decipher the mechanism, SG2NA down regulated cells were treated with 10 μM MG132, a proteasome inhibitor and western analysis was carried out showing that DJ-1 level is partially restored (Fig. 4E). This also restores Akt activation (Fig. 4E). These results suggest that in cancer cells SG2NA activates Akt by stabilizing DJ-1.

4. Discussion

Cancer has long been considered as a multifactorial disorder wherein genetic, environmental and life style factors contribute to the progression of the disease. However, a recent pioneering study attributed random mutations during DNA replication in noncancerous stem cells to cancers in various tissues [30]. Nonetheless, since the discovery of oncogenes, over or under activities of hundreds of regulatory molecules has been associated with the

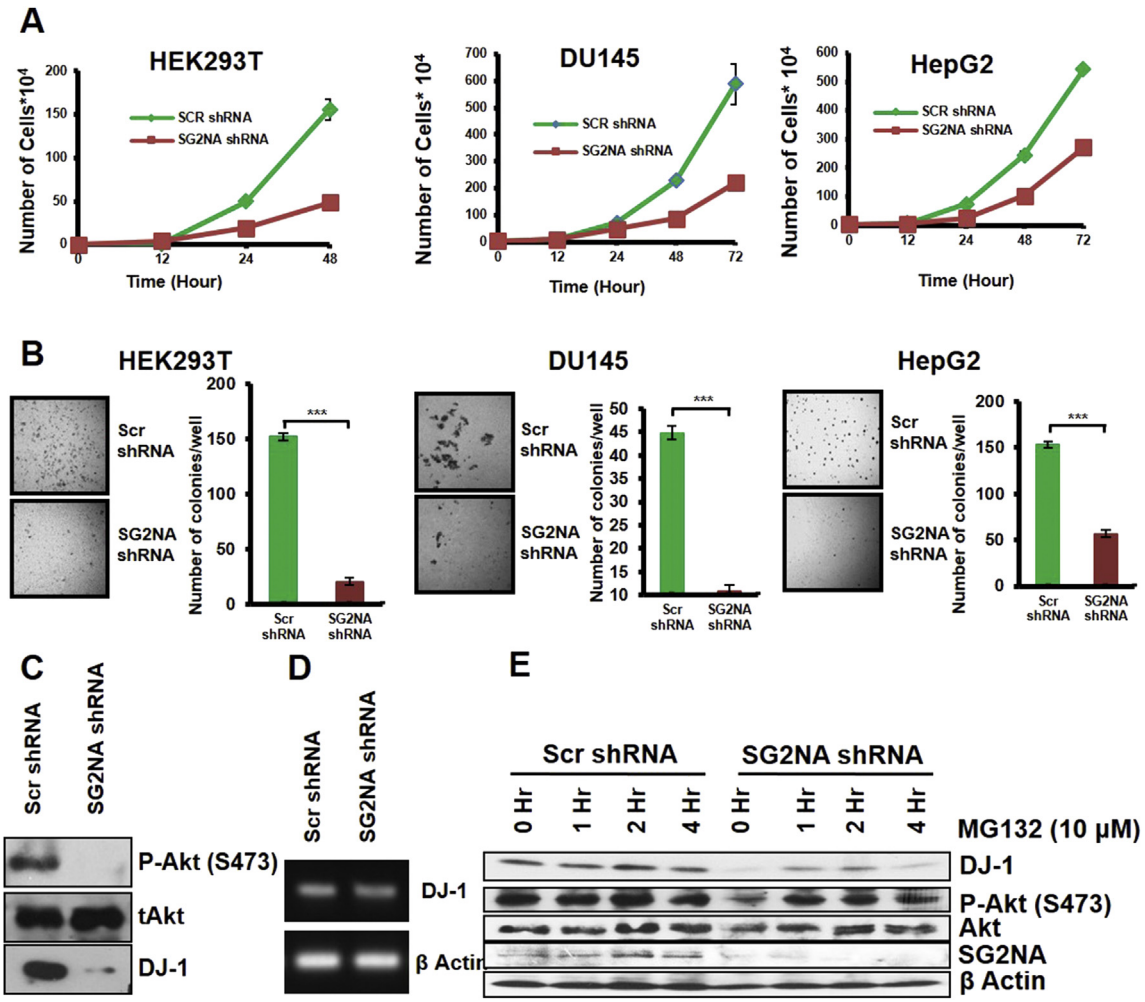


Fig. 4. SG2NA is involved in anchorage dependent growth of cancer cells. (A) HEK293T, DU145 and HepG2 cells stably depleted of SG2NA by shRNA were grown in 96 well plates overnight and synchronized with serum starvation followed by serum stimulation for 12, 24 and 48 h. The cells were counted by trypan blue staining method. Growth rates of these three different cell lines show that downregulation of SG2NA impairs the anchorage dependent growth. (B) Colony forming assay by soft agar method for the three cell lines upon down regulation of SG2NA. $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ ($n = 5$). (C) SG2NA was downregulated by the stable expression of shRNA in DU145 cells. Equal amount of extracts (100 μg) were separated in SDS-PAGE and immunoblotted with p-Akt (S473), Akt and DJ-1. Results show that upon down regulation of SG2NA, level of phosphorylated Akt decreases along with the level of DJ-1. (D) RNA was isolated from DU145 and HepG2 cells stably transfected with shRNA against SG2NA. Semi quantitative RT-PCR was carried out for DJ-1 transcripts. There is no significant decrease in DJ-1 transcripts in SG2NA depleted cells. (E) DU145 cells stably transfected with shRNA against SG2NA were treated with 10 μM of MG132 for 0–4 h. Western analysis for DJ-1, p-Akt (S473) and SG2NA shows that activation of Akt and expression of DJ-1 in SG2NA depleted cells is largely restored after treatment with MG132.

disease, it is justified to say that irrespective of the trigger, progression of cancer involves cross communications between hundreds of regulatory modules involving thousands of gene products [31].

In the present study, we have demonstrated the interactions between two well established players in cancer progression i.e., Akt, DJ-1 with a third protein SG2NA. Although SG2NA (also called Striatin3 [STRN3]) was initially identified as a tumor autoantigen, its role in cancer is now established. Madsen and coworkers in an elegant study have just demonstrated that SG2NA/striatin 3 as a component of STRIPAK complex determine the mode of cancer cell migration and metastasis [32]. Among the two interactants of SG2NA, Akt has long been accepted as a nodal player in cancer cell survival [33], but the role of DJ-1 in cancer development has lately been appreciated. Available data suggest that DJ-1 is involved in multiple facets of cancer development i.e., maintenance of transformed phenotype, regulation of cell growth, survival, metastasis and resistance to chemotherapeutics [18,22,34,35]. Such wider role of DJ-1 is likely due to its diverse functions including RNA-binding, redox-regulated chaperone activity, scavenging of ROS, cysteine proteolysis and transcriptional coactivation [34,36,37].

SG2NA dependent increased association of DJ-1 and Akt in a number of cancer cell lines suggests the existence of a hitherto pathway for cancer cell survival. The role of ROS in enhancing their association further heightens its significance in the redox framework of cancer development.

Our observation that quenching of ROS ablates SG2NA-DJ-1-Akt interaction is also significant as it has already been shown that under oxidative stress, DJ-1 rapidly activates p53 and then through a negative feedback loop, p53 downregulates DJ-1 and Akt [38]. Since down regulation of SG2NA also suppresses DJ-1, we anticipate a reciprocal role of p53 and SG2NA in tumor biology. Such anticipation is further substantiated by our observation that downregulation of SG2NA decreases proliferation of a number cancer cell lines. Since SG2NA has multiple splice variants present in various cellular compartments, present study has not been able to address which among the isoforms indeed involved in recruiting DJ-1 and activating Akt. Nonetheless, the data presented in this report in association with the recent findings by Madsen et al. [32] unequivocally establishes a complex role of striatin family members in cancer cell propagation and survival.

Author contribution

GKT and SKG wrote the manuscript, GKT prepared the figures, GKT and SKG conceived the idea of the manuscript, GKT and SP carried out the experiments. All authors reviewed the manuscript.

Additional information

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Competing financial interests

The authors declare no competing financial interests.

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