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RESEARCH

Silencing Poly (ADP-Ribose) Glycohydrolase (PARG) Expression Inhibits Growth of Human Colon Cancer Cells In Vitro via PI3K/Akt/NFκ-B Pathway

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Abstract Poly ADP-ribose polymerase (PARP) which is closely related to Poly ADP-ribose glycohydrolase (PARG) has already been thoroughly investigated in both experimental and clinical cancer trials compared to the latter. Nevertheless, in this experiment the importance of PARG expression was highlighted; whereby it is being silenced via lentivirus vector-mediated short hairpin RNA (shRNA). MTT assay showed that there was an inhibition in human Lovo colon cancer cell growth and flow cytometry demonstrated an increase in the population of cells in G_0/G_1 phase with a decrease in the S phase in transfected Lovo cells. Furthermore, our results suggested that the effect of silencing PARG leads to the inhibition of PARP expression; related to a decrease in the expression of Nuclear Factor Kappa-B (NFκ-B) with an increase in Akt^{473} phosphorylation; suggesting that the Phosphoinositol 3-kinase (PI3K)/Akt/NFκ-B pathway is important for cellular growth and proliferation. Hence, this study emphasizes and converges on the relevance of silencing PARG which inhibits growth of human colonic cancer cells via PI3K/Akt/NFκ-B pathway; as colon carcinoma remains to be amongst one of the commonest cancers throughout the world with high morbidity and mortality rates.

Keywords PARG · PARP · PI3K/Akt pathway · NFκ-B

Abbreviations

PARG	Poly ADP-ribose glycohydrolase
PARP	Poly ADP-ribose polymerase
shRNA	Short hairpin RNA
PI3K	Phosphoinositide 3- kinase/Phosphoinositol 3-kinase
NFκ-B	Nuclear Factor Kappa-B

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Introduction

PARG (Poly ADP-ribose glycohydrolase) being an important specific enzyme in PAR (Poly ADP-ribose) catabolism, DNA repair, gene expression, angiogenesis, metastasis, cell proliferation and death; has been discovered more than 30 years ago [1] with various isoforms and different subcellular localisations [2, 3]. Several aspects regarding its structure, substrate specificity, mechanisms involved in various processes such as inflammation, intestinal injuries, stress challenges both in vivo and in vitro have been delineated [4]. Correlations between PARP (Poly ADP-ribose polymerase) and PARG with genetic disruption of both, has led to serious consequences in some models [2, 5, 6]; therefore emphazising that PARG with PARP act in an agonist way rather than antagonistically. Over the years, more focus was drawn onto the biological roles of PAR (Poly ADP-ribose) synthesizing enzymes (PARPs) instead of PAR decomposition (PARGs). Studies have shown that PARP inhibitors are reliable therapeutic targets with strong prospects not only in cancer treatment but including a whole spectrum of other diseases; nevertheless, PARG inhibitors due to lack of specificity and membrane permeability has made it more difficult to thread a path in experimental trials. However, some PARG knockout models have thrown light onto various pathways concerning embryogenesis, single-strand break repair and transcriptional regulation [2, 5, 7]. Besides, several regulatory pathways namely protein kinase C (PKC), Erk 1/2,

mitogen-activated protein kinase (MAPK), calmodulin kinase (CaMK), casein kinase 2 (CKII) [8] have shown that PARP-1 may be regulated by phosphorylation; meanwhile PARP-1 inhibition promoting Akt pathway has been outlined in these particular models [9–11]. Akt itself is known to be a valuable therapeutic target in cancer treatment and its phosphorylation at the Serine^{473} has been detected in numerous types of cancers, including colon carcinoma [12]. On the other hand, Phosphatidylinositol-3 kinase (PI3K) is a lipid kinase whose major effector is Akt (Protein kinase B) and the PI3K/Akt pathway is responsible for a large number of cell functions including survival, proliferation, invasion, migration [13] of various cancer cell lines [14, 15] and controlling regulation of several transcription factors like Nuclear Factor Kappa-B (NFκ-B) [16, 17] which in turn intimidates inflammatory cytokines, chemokines, adhesion molecules and mediators important in growth, apoptosis, angiogenesis and metastasis. NFκ-B has also been found to be regulated by PARP [18]; hence, concluding that this pathway may be associated with tumorigenesis. Since our previous studies have demonstrated a close relationship of the inhibitory role of PARP with NFκ-B [19] as well as with PARG [20]; therefore, here by silencing PARG expression with shRNA in human Lovo cancer cell we have tried to find the correlation of knockout PARG in relation to growth and the growth promoting pathway of PI3K/Akt/NFκ-B.

Materials and Methods

Cell Culture

Human Lovo colon cancer cell line was cultured in RPMI-1640 medium supplemented with 10% Fetal Bovine Serum (FBS), 100 U/ml Penicillin and 100 μg/ml Streptomycin (Sigma-Aldrich) at 37°C in a 5% CO_2 incubator.

Transfection of Lovo Parental Cells

Transfection was done with lentiviral based shRNA(short hairpin RNA) vector (Sigma-Aldrich) targeting PARG gene while for the control, non-target shRNA control transduction particles was used (Sigma-Aldrich). Transduction efficiency was optimized using pLKO.1 puro-TurboGFP (Sigma-Aldrich) and steps were carried out according to the manufacturer's instructions.

Cells were plated in a 96-well plate at 1.6×10^4 in each well; for each lentivirus construct triplicate wells were used and cells were incubated till they reached 70% confluence. After which, media was gently aspirated, 200 μl of fresh RPMI-1640 was added together with 8 μg/ml Hexadimethrine bromide per well; then different concentrations of 2, 5, 10 and 15 μl of lentivirus particles with TurboGFP were added and plate was left for incubation for 48 h. The latter was then subjected to fluorescence microscopy so as to assess the concentration of lentivirus which has resulted in optimal transduction efficiency. The 10 μl lentiviral particle concentration had displayed the maximum green fluorescein protein (TurboGFP) visible under micoscopy.

Subsequently, 10 μl of lentivirus particles of either non-target shRNA or PARG-shRNA were added to wells previously containing Hexamethrine bromide and were incubated for 48 h. Following which, media was removed and 200 μl fresh media together with Puromycin to a final concentration of 8 μg/ml (Sigma-Aldrich) were added to each well. Previous media was replaced with fresh media containing Puromycin every 2–3 days until resistant colonies could be identified.

Finally, the experimental group consisted of Lovo cells transfected with PARG-shRNA ($shRNA_{PARG}$) whereas the controls included both Lovo parental cells as well as Lovo cells transfected with non-target shRNA ($shRNA_{control}$). The samples were then assayed by RT-PCR and Western Blot analysis to detect PARG knockout in Lovo cells.

Reverse Transcriptional Polymerase Chain Reaction (RT-PCR)

Total RNAs were isolated from Lovo parent cells, $shRNA_{control}$ and $shRNA_{PARG}$ using Trizol reagent (Takara) and were reverse transcribed into cDNA. Genes were detected with a template of cDNA using oligonucleotide primers: PARG, 5′-CCA CCT CGT TTG TTT TCA-3′(forward) and 5′-CCA ACA TCT GGC AAA GGA-3′(reverse); PARP,5′-CTA GAC AAC CTC CTG GAC ATCG-3′(forward) and 5′-CTC CCA GCA TTA TTA AGC CAAT-3′(reverse); NFκB, 5′-GGG AAG GAA CGC TGT CAG AG-3′(forward), 5′-TAG CCT CAG GGT ACT CCA TCA- 3′(reverse); β-Actin,5′-GTC AAG AAA GGG TGT AAC GCA AC-3′(forward) and 5′-TCC TGT GGC ATC CAC GAA ACT-3′(reverse) was used as a control. The cycling conditions were as follows: the number of PCR cycles (94°C for 30 s, 50–58°C for 30 s, 72°C for 1 min and then 5 min for the last extension) was 35 for the amplification of reverse transcriptase products. Then finally, the PCR amplification products were separated on 1.8% agarose gel. This experiment was repeated three times.

Nuclear Extraction Preparation

Nuclear protein extracts were prepared from cells grown to 90% confluence and performed on ice with ice-cold reagents. Cells 5×10^6 were scraped into 1 ml PBS, washed twice, supernatant was discarded and the pellet was suspended into 500 μl Hypotonic Buffer and allowed to

swell on ice for 15 min. 25 μl of 10% Nonidet P_40 was added, cells were vortex for 10 s and after which, centrifuged for 10 min at 3000 rpm at 4°C. The supernatant containing cytoplasmic fraction was discarded and the pellet containing only the nuclear fraction was resuspended into 50 μl complete Cell Extraction Buffer for 30 min on ice with occasional vortexing. Nuclear extracts were recovered after centrifugation for 30 min at 14,000 g×4 at 4°C. Protein concentrations were determined by Coomassie Blue assay reagent (Pierce Biotechnology).

Western Blot Analysis

Cells were washed with PBS, scraped down into an eppendorf tube where extracts were obtained by following the protein extraction method. Protein extractions were determined using Coomassie Blue assay. Protein (20 μg/lane) was loaded onto 10% polyacrylamide gels (SDS-PAGE) which were separated by electrophoresis, transferred to nitrocellulose membranes and concerned proteins were blocked with 5% non-fat dried milk dissolved in TBST. Primary antibodies against PARG (Abcam), PARP, Akt, Phospho-Akt Serine^{473}, phospho-IκBα, NFκ-B and β-actin (Santa Cruz) 1 μg/ml concentrations were applied overnight at 4°C. The next day, membranes were washed thrice with TBST, secondary antibodies (peroxidase-conjugated goat anti-rabbit IgG) were added and incubated for 1 h. Then, blots were washed three times with TBST; enhanced chemiluminescence reagents (Pierce Biotechnology) were applied and were exposed to the gel formatter (Bio-Rad). Similar protein extraction and Western Blot steps were followed for $shRNA_{PARG}$ cells combined with LY294002 (LY) inhibitor- an inhibitor of the PI3K/Akt pathway used in a concentration of 10 μM so as to highlight the significance of the phosphorylated Akt in relation to PARG suppression. The above experiment was performed thrice.

MTT Assay

All three groups of Lovo cells were grown to confluence and seeded in a 96-well plate (Costar) at 3×10^4 cells/ml. Each group was plated in triplicate and each individual well contained 100 μl of cell suspension. Blank wells were also included. Cells were allowed to grow for a maximum of 7 days (168 h). Starting from day 0, every 24 hourly 10 μl of MTT [3- (4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (5 mg/ml diluted in PBS, Sigma-Aldrich) was added to respective wells and incubated for 4 h. After which, careful aspiration of media was done with addition of 100 μl DMSO to each well. Absorbance (Optical Density) was read with a universal microplate reader (Bio-Tek) at 490 nm with 630 nm as reference wavelength. MTT Assay was performed thrice and results were plotted on a graph of Absorbance at 490 nm (A490) against number of hours. Each mark represents the mean of the collected readings.

Flow Cytometry Analysis

The cells were grown in 100 ml culture flasks initially at 3×10^4 cells/ml for 24 h; all three groups of cells were trypsinised, suspended in culture medium, centrifuged and cell count was done with a hemocytometer to achieve a cell population of 5×10^5 which was washed thrice and resuspended in cold PBS. Fixation was done using with 70% absolute ice-cold ethanol; cells were kept at -20°C overnight, were stained with a mixture of PI-Propidium, RNase A and analysed with Becton Dickison flow cytometer for cell cycle analysis (FACScan). This procedure was done thrice.

Statistical Analysis

The quantitative data were expressed as a mean of ± standard deviation (SD). Statistical analysis was performed by one-way ANOVA or Student's *t* test using SPSS 11.5 software package. A '*P'value of less than 0.05 was considered to be statistically significant.

Results

Effect of Lentivirus Transfection on Lovo Cells

The efficiency of the Lentivirus vector-mediated shRNA interference in suppressing PARG in Lovo cells was determined through RT-PCR and Western Blot analysis. Both showed that cells transfected with PARG-shRNA demonstrated a complete successful inhibition of PARG expression [Fig. 1a, b & c] compared to both parent cells and Lovo $shRNA_{control}$.

Relationship of PARG and PARP Expressions

PARP mRNA levels were detected via RT-PCR while its respective protein levels were confirmed through Western Blotting. It was seen that in both $shRNA_{PARG}$ lovo cells and $shRNA_{PARG}$ cells with LY inhibitor there was a similar decrease in PARP expression [Fig. 2a, b, c & d] compared to both controls; showing that suppression of PARG diminishes PARP expression.

Intranuclear Expression of NFκ-B in $ShRNA_{PARG}$ Lovo Cells

Western Blot analysis of cytoplamic extraction fraction showed that in $ShRNA_{PARG}$ cells, expression of phos-

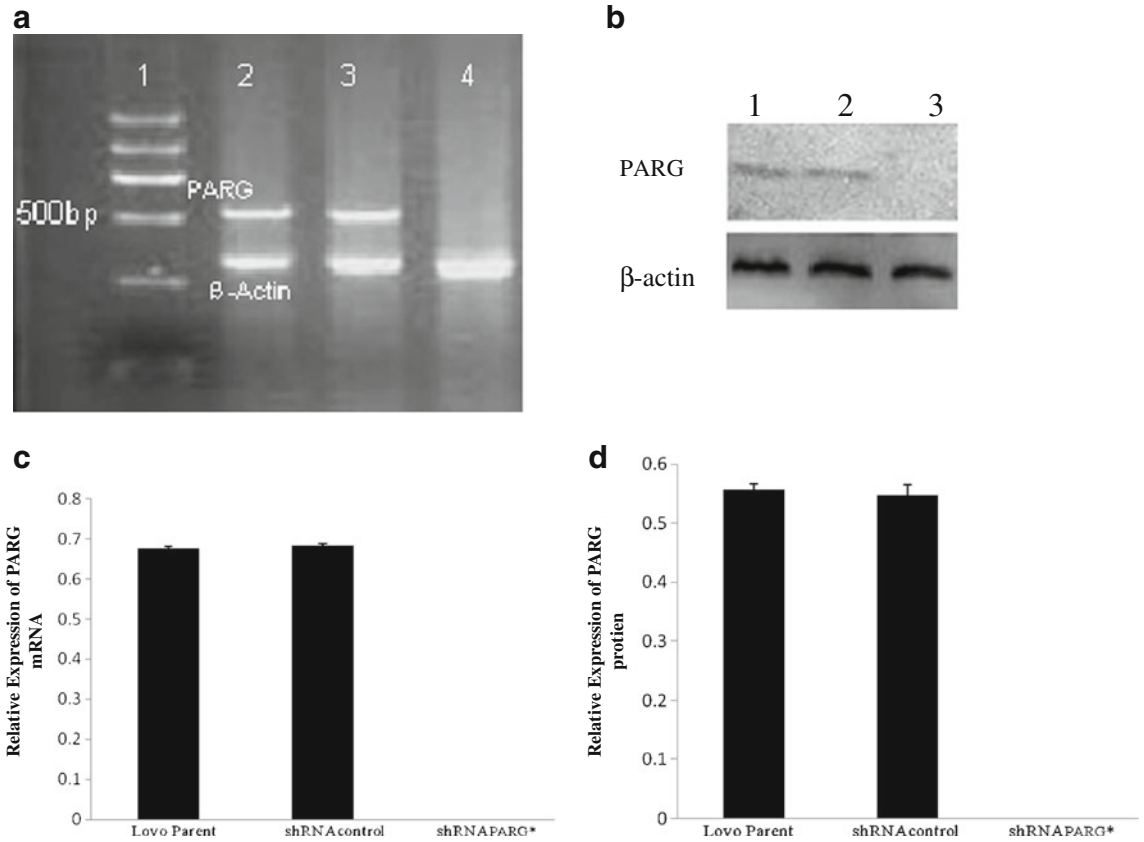

Fig. 1 **a** Marker-1, Lovo parent cells-2, Lovo $shRNA_{control}$-3, Lovo $shRNA_{PARG}$-4; **b** Lovo parent cells-1, Lovo $shRNA_{control}$-2, Lovo $shRNA_{PARG}$-3. RT- PCR (left) and Western Blot analysis (right) showing successful inhibition of PARG in PARG-shRNA transfected cells (*$P<0.05$) supported by **c & d** showing the relative expression of PARG by both methods. β-actin acted as an internal loading control

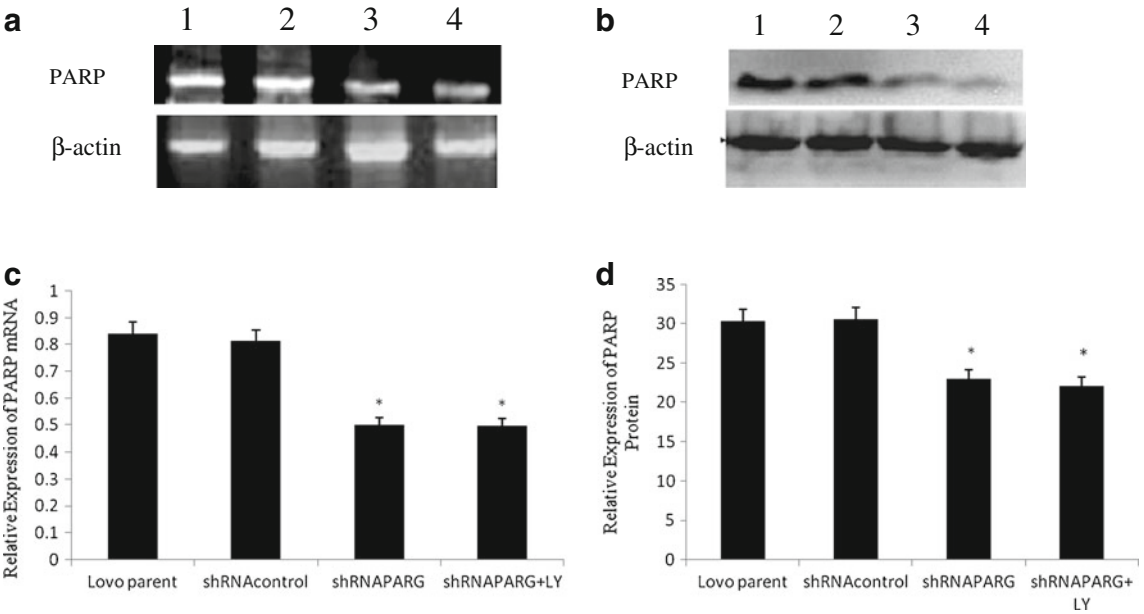

Fig. 2 **a** & **b**. Lovo parental cells-1, Lovo $shRNA_{control}$-2, Lovo $shRNA_{PARG}$-3, Lovo $shRNA_{PARG}$ with LY294002 (inhibitor of PI3K/Akt pathway); (**a**) & (**c**) Both RT-PCR and (**b**) & (**d**) Western Blotting showing suppression of PARG leading to decreased expression levels of PARP (*$P<0.05$, *$P< 0.05$) with no further change in expression of PARP with the addition of LY inhibitor. β-actin was used as a loading control

phorylated IκBα (p-IκBα) [Fig. 3a & b] was decreased as well as the relative intranuclear protein expression of NFκ-B [Fig. 3c]. Therefore, suggesting that in cells where PARG was silenced there was a decrease in intracytoplasmic expression of phosphorylated IκBα resulting in an intranuclear decrease of NFκ-B.

Effect of Silencing PARG on Phosphorylated Akt and Total Expression of NFκ-B

Here, LY294002 (LY) which is a known cytotoxic inhibitor of PI3K/Akt pathway was used on $shRNA_{PARG}$ transfected Lovo cells in a concentration of 10 μM. In Lovo cells where PARG was suppressed ($shRNA_{PARG}$) there was an increase in the level of phosphorylated Akt [Fig. 4a] and there was an inhibition in the total expression of NFκ-B [Fig. 4c]. Whereas, when the inhibitor (LY) was applied together with the $shRNA_{PARG}$ cells there was complete inhibition of phosphorylated Akt $Serine^{473}$($Phos\text{-}Akt^{473}$) [Fig. 4a & b] compared with Akt acting as a control and the comparison of expression of NFκ-B [Fig. 4c & d] was similar to that of the Lovo parental cells and $shRNA_{control}$ NFκ-B expressions. Therefore, suggesting that PARG suppression decreases the level of NFκ-B via up-regulation of Akt phosphorylation which is an important pathway in cell growth, proliferation and invasion.

Silenced PARG on Growth of Colon Cancer Cells

The MTT colorimetric Assay was used to determine the relationship of knockout PARG on growth of human Lovo colon carcinoma cell line in vitro. The graph plotted showed that there was a much rapid increase in the Absorbance at 490 nm which is directly proportional to the number of living and proliferating cells from day 0 to day 7 (0 h–168 h) [Fig. 5] in both Lovo parent cells and $shRNA_{control;}$ while in $shRNA_{PARG}$ the anticipated growth of cells was lower over the same period. There was no significant difference between the A490 values of untransfected and empty vector expressing cells ($P>0.05$). Thus, showing that suppression of PARG does have an important inhibitory role in the growth and proliferation of Lovo cells.

PARG Knockout on Cell Cycle

The effect of PARG knockout on human Lovo colon cancer cells was found to affect the various phases of cell cycle which was determined by flow cytometry

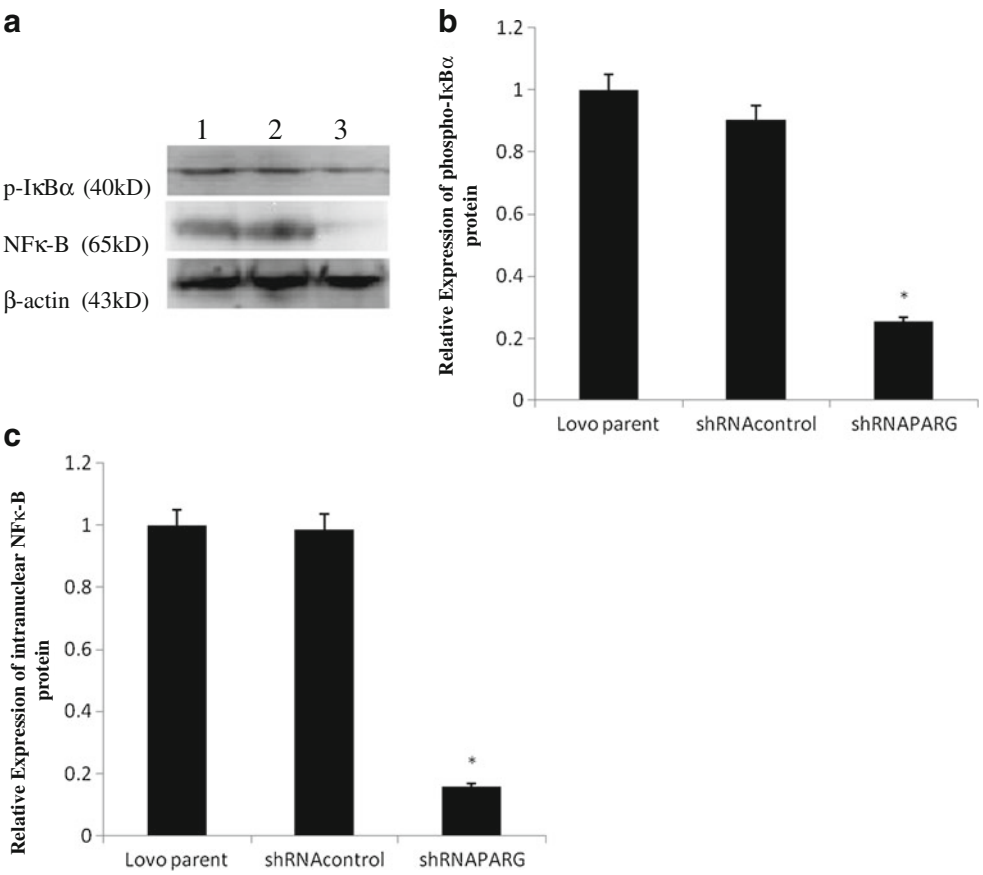

Fig. 3 **a** Lovo parent cells-1, Lovo $shRNA_{control}$-2. Lovo $shRNA_{PARG}$-3; Western Blot analysis **a** & **b** shows decrease in expression of cytoplasmic phosphorylated IκBα as well as **a** & **c** in nuclear extraction fraction of NFκ-B expression in $ShRNA_{PARG}$ cells compared to both controls (*P<0.05)

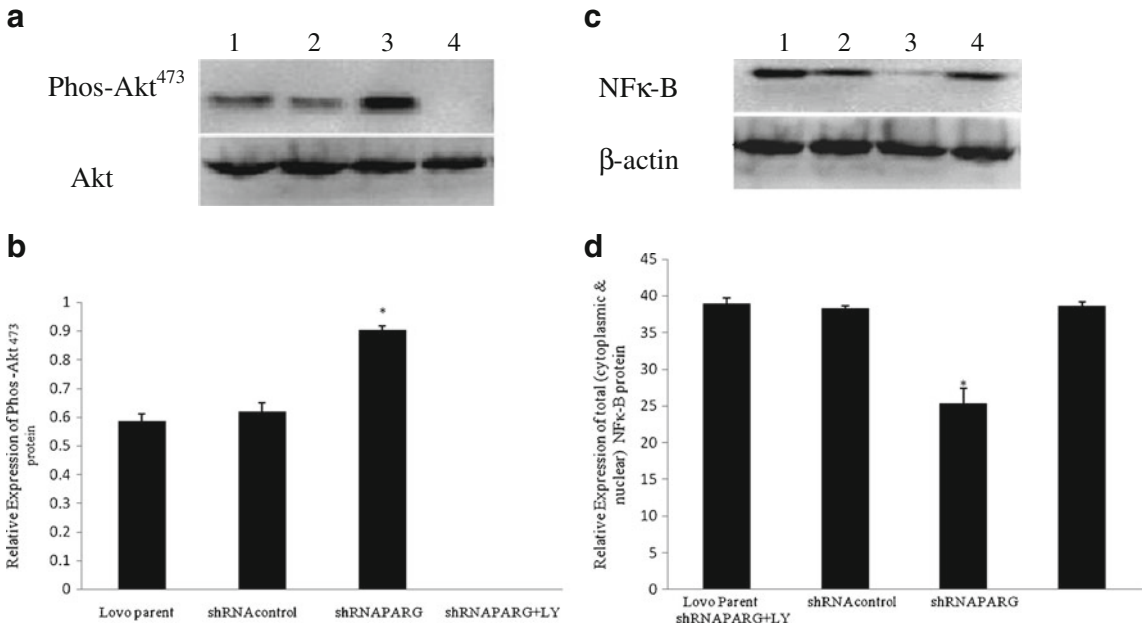

Fig. 4 **a**. Lovo parental cells-1, $shRNA_{control}$-2, $shRNA_{PARG}$-3, $shRNA_{PARG}$ with LY294002 (inhibitor of PI3K/Akt pathway) -4. Western blot analysis outlining enhanced level of phosphorylated Akt (Phos-Akt^{473}) in PARG knockout cells; **b**. Densitometric analysis of level of phosphorylated Akt band intensity normalized with the intensity of Akt is shown and the data is represented as mean of independent experiments performed which revealed a $*P<0.05$ vs. both controls. Besides, there was complete inhibition of Phos-Akt^{473} with the LY294002 compared with Akt. **c** & **d**. Moreover, suppression of NFκ-B in PARG silenced Lovo cells was seen but there was no change in expression of NFκ-B with LY294002 in comparison to the both controls. β-actin acted as a control. A $*P<0.05$ in $shRNA_{PARG}$ cells as compared to parent cells, $shRNA_{control}$ and $shRNA_{PARG}$ + LY

analysis using PI. In the Lovo parent cells [Fig. 6a] the proportion of cells in G_0/G_1 phase was of 60.63%, S phase 20.58% and G2/M was 18.79% while in $shRNA_{control}$ transfected cells [Fig. 6b] we had G_0/G_1 61.0%, S phase correlated with 20.10% and G_2/M 18.90%. Both controls did not differ substantially. However, in [Figs. 6c] $shRNA_{PARG}$ Lovo cells which were analysed, we found an increase in G_0/G_1 phase of 74.90%, with a decrease in both S phase 8.26% and G_2/M 16.84% respectively.

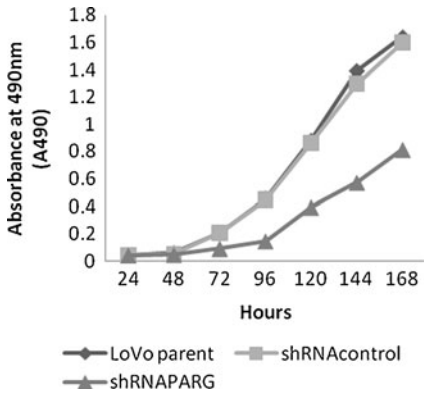

Fig. 5 Growth of viable cells was quantified using MTT Assay. Data points were expressed as mean of A490 ± SD from three separate experiments for each cell line every 24 hourly. $shRNA_{PARG}$ transfected cells had a retarded growth and proliferation compared to both controls. A $*P< 0.05$ in $shRNA_{PARG}$ cells vs. the parental cells and $shRNA_{control}$ cells

Discussion

To date only one single PARG gene has been detected in mammals whose major isoform is 110 kDa while the human PARG which is 111 kDa has been known to be confined to the nucleus [1] and is the main enzyme catabolising PAR to ADP-ribose. Both PARP and PARG play important biological functions in the body, their functional similarities have been accepted [7, 21, 22] as well as their agonistic actions. Inhibition of PARP has proved to be of valuable interest to researchers, pharmaceutical companies and clinicians in multiple fields including cancer research. Several ongoing experimental and clinical studies are on the run [23] to be able to make them available on the market; since the number of cancer patients is increasing tremendously and urge for reliable, safe with less side effect drugs is on demand. Besides, some experiments have correlated the effects of PARG inhibition to be similar to that of inhibiting PARP-1; it therefore seems plausible to test its effects onto various pathological mechanisms. Nevertheless, during the past few years a lot of attention has been concentrated towards inhibiting PARP

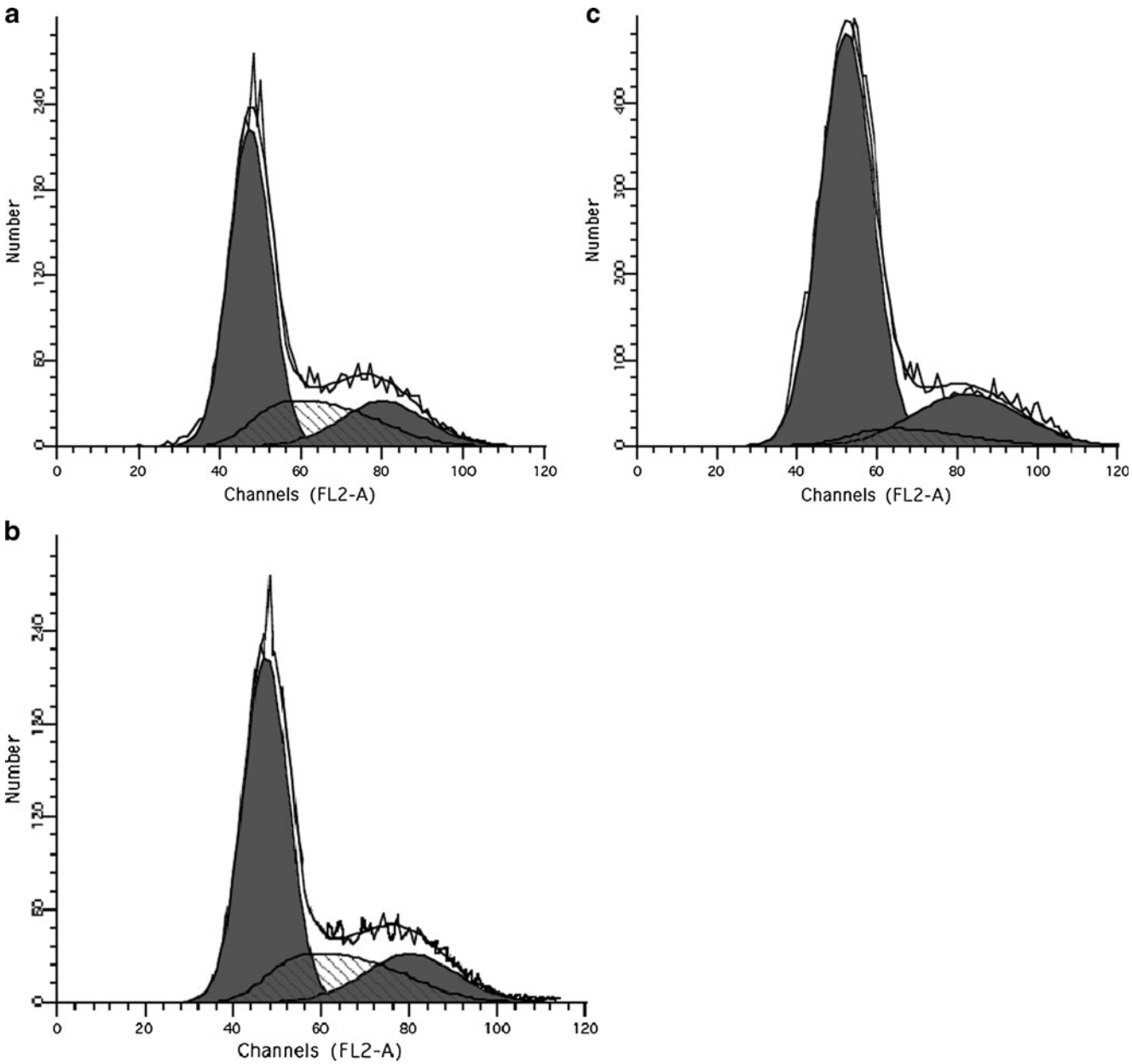

Fig. 6 **a**. Lovo parent cells, **b** $shRNA_{control}$ and **c** $shRNA_{PARG}$. The effect of knockout PARG on the cell cycle of Lovo colon cancer cells was analysed by flow cytometry analysis using channels (FL2-A) and the different phases G_0/G_1 (left peak), S and G_2/M (right peak) were analysed

while inhibition of PARG has not been thoroughly investigated in the field of cancer.

Our study reveals the effectiveness of knockout of PARG expression in inhibiting the growth of human Lovo colon cancer cell line and its correlation with PI3K/Akt/NFκ-B pathway which is known to be involved in cancer progression [13, 24]. The Lovo parent cells were successfully transfected by the lentivirus vector-mediated shRNA and the expression of PARG was completely suppressed. Upon the diminished expression of PARG, there was also a decrease in the expression level of PARP; hence, confirming the relationship of PARP with PARG which was also outlined in our previous study [20]. PARG was shown to be an important factor in the growth and proliferation of the Lovo cells in vitro as the $shRNA_{PARG}$ transfected cells had a slower growth pattern as compared to the controls over those 7 days (168 h). However, no morphological abnormalities or distortion of the cells were observed. The cell cycle showed that more cells were in the G_0/G_1 phase and fewer in the S phase in $shRNA_{PARG}$ compared to both controls, showing that there was an arrest from the transition of G_1 to S phase; therefore, PARG knockout did cause an inhibition in the proliferation

of Lovo cancer cells; showing its relevance in cell cycle control checkpoints [23]. Moreover, the absence of PARG with low expression of PARP in $shRNA_{PARG}$ cells showed an attenuation of total NFκ-B expression with an upstream regulation of phosphorylated Akt and this was sustained by a similar pathway with a PARP inhibitor found in the current studies [9, 25, 26]. Afterwards, LY294002 (LY) which is a highly efficacious inhibitor of PI3K/Akt pathway [10, 14] was used so as to highlight the relation between silenced PARG, PARP, NFκ-B and PI3K/Akt pathway. Upon the use of LY294002 on $shRNA_{PARG}$ transfected cells, it totally inhibited the phosphorylated Akt and we found out that the expression of transcription factor NFκ-B remained unchanged as compared to Lovo parent cells and $shRNA_{control}$. Akt usually activates IKK kinases; upon phosphorylation, IκBα is polyubiquitinated, degraded and enables NFκ-B nuclear translocation and transcriptional activity. This pathway is considered to be the classic (canonic) mode of activation and the kinetics of NFκ-B activation in various studies has been assessed using NFκ-B p65 subunit [27, 28]. Therefore, in our experiment we have seen that phosphorylated cytoplasmic IκBα is diminished in $ShRNA_{PARG}$ Lovo cells and consequently the intranuclear expression of NFκ-B p65 and the total protein expression of the latter are decreased; showing a sign of diminished activation as well as for its dependent genes which has already been proved in both our previous and other related studies [19, 20, 29–31] concerning growth, proliferation and metastasis. Our current findings are also in concordance that, low levels of IκBα lead to the downregulation of the classic p65 dimer of NFκ-B [27] which negatively regulated proliferation by Natural Killer (NK) cells [32]; thus, preventing its growth. Subsequently, our results imply that PI3K/Akt pathway negatively regulates NFκ-B and that without PI3K/Akt pathway upregulation, the NFκ-B expression cannot be suppressed; showing that knockout PARG which inhibited PARP expression [20] is required for the activation of PI3K/Akt pathway [10, 11] which act as a downstream suppressor of NFκ-B which will in turn probably lead to a decrease in cell proliferation, adhesion, inflammation, migration and invasion; all that is required for prevention of cancer dissemination is supported by the following studies [33–37].

The conclusion drawn is that PARG knockout can also be an important factor in the growth control mechanism of colonic cancer cells but nevertheless, PI3K/Akt/NFκ-B is not an ultimate and sole pathway in cancer progression therefore, further studies are going on to underline the importance of silencing PARG gene in angiogenesis, invasion and metastasis through other pathways; our previous studies had already highlighted similar effects of PARP inhibition in relation to colon cancer [17, 18]. Somehow to our knowledge a lot research still remains to be done in the field of cancer treatment but however, with a ray of hope in the experimental studies, a path may be carved to introduce PARG inhibitors into clinical trials in the near future.

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