3
Gp78 Involvement in Cellular Proliferation: Can Act as a Promising Modulator of Cell Cycle Regulatory Proteins

Cell cycle progression needs tight control by a highly conserved group of proteins and deregulation in cell cycle or improper cellular proliferation may cause various kinds of cancers. Cyclin-dependent kinase inhibitor 1B (p27Kip1) is a cell cycle regulatory protein, which inhibits Cyclin-dependent kinases (CDKs) in cells [Lloyd *et al.*, 1999]. Various reports suggest that p27 acts as a chief regulator in the suppression of tumorigenesis in different types of cancers and p27 knock-out mice remain more tumor prone and exhibit enhanced growth and multiple organ hyperplasia [Kiyokawa *et al.*, 1996; Nakayama *et al.*, 1996; Slingerland and Pagano, 2000]. It is well-known that expression of p27 is tightly regulated in cells and its deregulation may make cells more prone to uncontrolled proliferation, when exposed to carcinogenic events; and further UPS promotes its intracellular degradation with the help of selective E3 ubiquitin ligases [Pagano *et al.*, 1995; Slingerland and Pagano, 2000]. An overwhelming number of studies have shown that various E3 ubiquitin ligases such as SCF skp2, KPC (kip1 ubiquitination promoting complex), RING-H2-type ubiquitin ligase, Pirh2, E6-AP (E6-Associated Protein) and WWP1 (WW domain-containing E3 ubiquitin protein ligase 1) are involved in proteasomal degradation of p27 [Cao *et al.*, 2011; Carrano *et al.*, 1999; Hattori *et al.*, 2007; Kamura *et al.*, 2004; Mishra *et al.*, 2009; Sutterluty *et al.*, 1999]. In present study, the role of Gp78 has been evaluated in cellular proliferation, mediated by p27, which is putative inhibitor of Cyclins. It has been observed that overexpression of Gp78 increases the endogenous levels of p27 in cells. Massive accumulation of p27-positive inclusion like structures in cells has been seen after Gp78 overexpression. It also stabilized the steady state levels of intracellular p27. Overall these findings suggest valuable insights into the regulatory mechanism of p27 linked with UPS.

3.1 RESULTS

To find the role of Gp78 on regulation of various cell cycle regulatory proteins under stress conditions multiple experiments were performed. Results obtained by these experiments are described in following sections.

3.1.1 Gp78 Overexpression Elevates Levels of p27 in Cells

E3 ubiquitin ligase Gp78 is known for the degradation of misfolded proteins, such as, SOD1, ataxin-3 and mutant huntingtin (htt) [Yang *et al.*, 2010; Ying *et al.*, 2009]. It also retains ability to target cystic fibrosis transmembrane conductance regulator (CFTR), and transmembrane metastasis suppressor, KAI1 (also known as CD82) [Morito *et al.*, 2008; Tsai *et al.*, 2007]. Therefore, the precise role of Gp78 has been evaluated in regulation of crucial cell cycle regulatory proteins. In preliminarily experiment, the expression profile of Gp78 protein tagged with GFP was established with the help of Gp78-EGFP expression constructs as shown in Figure 3.1 (a-b). The transient expression of Gp78-EGFP was also confirmed by immunoblot analysis by using anti-GFP antibody Figure 3.1 c.

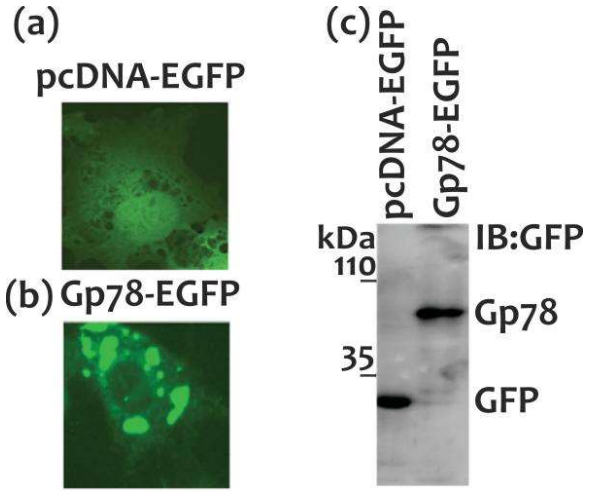

Figure 3.1 : *Expression profile of Gp78:* (a, b) Cos-7 cells were plated in two-well chamber slide. Next day, the cells were transfected with empty vector pcDNA-EGFP and Gp78-EGFP constructs. After 48 Hrs, slide was analyzed under a fluorescence microscope to confirm the expression, as well as cellular localization of the Gp78 protein. (c) Cell lysates of above-transfected cells were subjected to immunoblot analysis with anti-GFP antibody confirms overexpression of Gp78. Published as Joshi and Mishra et al., 2018.

A similar set of cells were used for assessment of endogenous levels Figure 3.2 a of Gp78 probed with Gp78 antibody in comparison to exogenously expressed Gp78-EGFP Figure 3.2 b by fluorescence micrograph analysis. As shown in Figure 3.2 c, immunoblots developed with Gp78 and β-actin antibodies confirmed overexpression of Gp78-EGFP protein in cells.

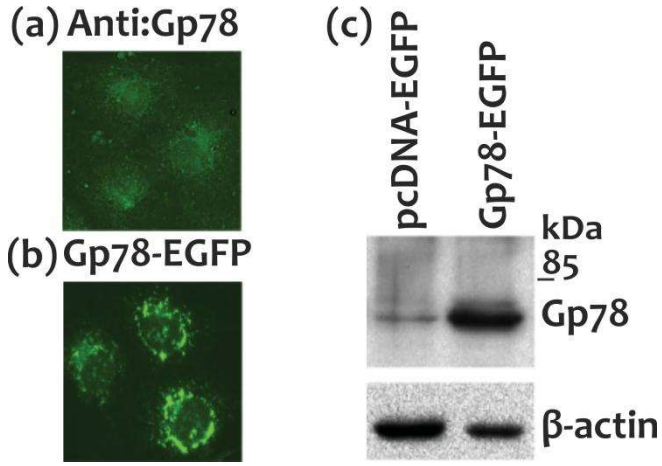

Figure 3.2 : *Comparison of exogenous and endogenous expression of Gp78:* (a) Endogenous expression of Gp78 in Cos-7 cells was observed by immunofluorescence staining with the Gp78 antibody. The FITC-conjugated secondary antibody was used to label Gp78 primary antibody. (b) Fluorescence microscope was used to visualize the exogenously expressed Gp78 in Cos-7 cells. (c) Exogenous levels of Gp78 in Cos-7 cells were analyzed by immunobloting with the Gp78 antibody; β-actin was used as loading control.

Next, a detailed immunoblot analysis was performed and compared the endogenous levels of few major tumor suppressors or cell cycle regulatory proteins under the overexpression of Gp78 protein in cells. As shown in Figure 3.3 a, overexpression of Gp78 chiefly increased the steady-state levels of cellular p27, in comparison to other tumor suppressor proteins, such as, p53 and p21; β-actin was used as loading control. The quantitative analysis of p27 protein levels intensity in the presence of overexpressed Gp78 Figure 3.3 a was performed by using blots shown in Figures 3.2 b.

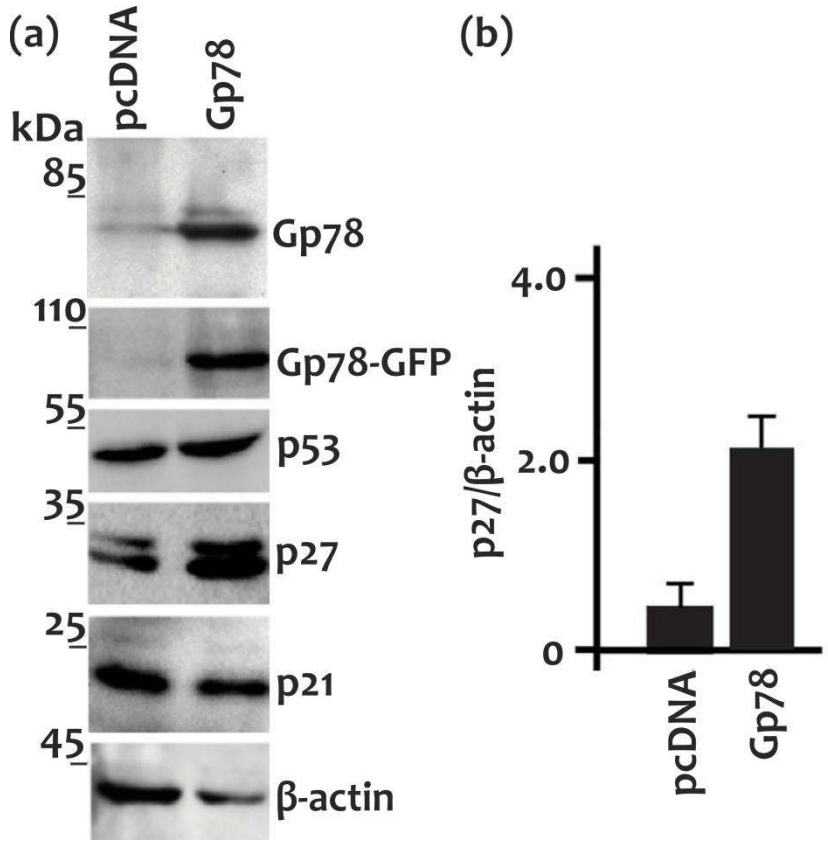

Figure 3.3 : *Gp78 overexpression increases the endogenous levels of p27:* (a) Cos-7 cells were transfected with pcDNA-EGFP and Gp78-EGFP plasmids. After 48 Hrs of transfection, cell lysates were collected and subjected to immunoblotting with Gp78, GFP, p53, p27, p21, and loading control β-actin antibodies. (b) Quantitation of p27/β-actin band intensities of Figures 3.3 (a) was done by densitometric analysis with NIH image analysis software and represented as graph. Values presented are means ± S.D. of three independent experiments.

Now, it was important to further confirm the effect of Gp78 on p27 endogenous levels, because previous finding represented that E6-AP E3 ubiquitin ligase induces the degradation of p53 and p27, therefore, E6-AP used as control for all preliminarily results [Mishra *et al.*, 2009; Mishra and Jana, 2008]. As expected, E6-AP overexpression reduced the endogenous levels of p53, p27 and p21 proteins as shown in Figure 3.4 a. The quantitative analysis of p27 protein levels intensity in the presence of overexpressed E6-AP Figure 3.4 a was performed by using blots shown in Figures 3.4 b.

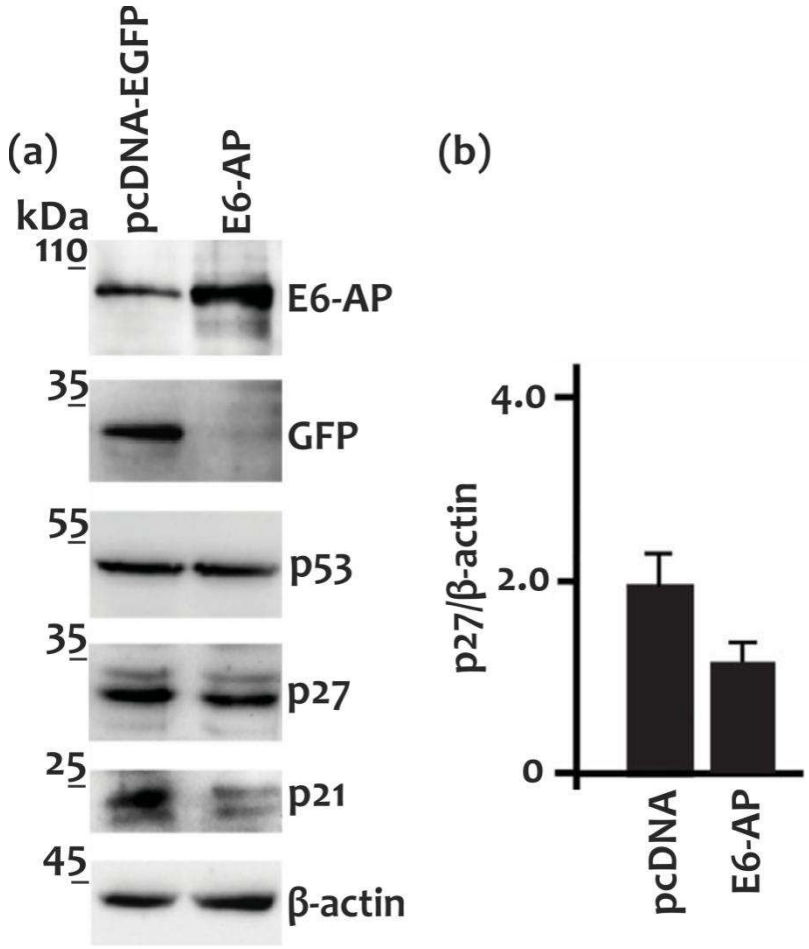

Figure 3.4 : *E6-AP as ligase control for p27:* (a) E6-AP and pcDNA-EGFP plasmids transfected Cos-7 cells were collected after 48 Hrs, and over expression of E6-AP was confirmed by using anti-E6-AP antibody. The same cell lysates were used to analyze expression levels of GFP, p53, p27, p21, and β-actin by western blotting. (b) Quantitation of p27/β-actin band intensities of Figures 3.4 (a) was done by densitometric analysis with NIH image analysis software and represented as graph. Values presented are means ± S.D. of three independent experiments.

3.1.2 Upregulation of p27 Protein Level in Gp78 Overexpressed Cells

Next, the effect of overexpression of both, Gp78 and its mutant form Gp78-R2M was checked, on p27 endogenous levels. Gp78 mutant R2m (RING 2 mutant) is a double mutant of residues C356G and H362A. This mutant form of Gp78 shows disturbed function of the RING domain of Gp78 E3 ubiquitin ligase [Fang *et al.*, 2001]. Cells were then transfected with Gp78-EGFP and Gp78-R2m plasmids and few sets of cells were also exposed to putative proteasomal inhibitor MG132. Post-transfected cells were used for lysate preparation and endogenous levels of p27, p53 and p21 proteins were observed by immunoblot analysis, using their respective antibodies. Overexpression of Gp78 was confirmed by GFP antibody and β-actin was used as a loading control, as shown in Figure 3.5. p27 levels were observed high in Gp78 overexpressed cells and as expected a more additive accumulation of p27 was noticed in MG132 treated cells coexpressing Gp78 protein.

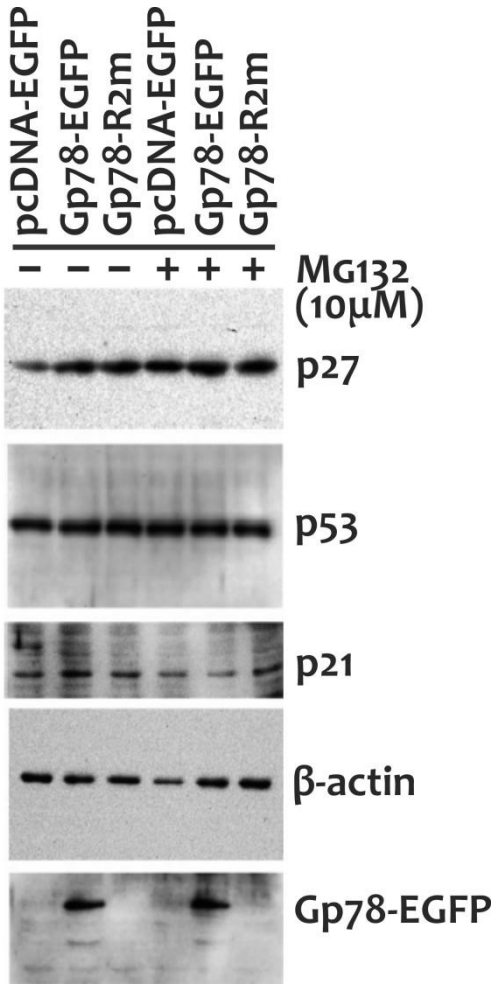

Figure 3.5 : *Effect of Gp78 mutation on p27 accumulation:* Cos-7 cells were plated in six-well tissue culture plate and transfected with pcDNA-EGFP, Gp78-EGFP, and mutant Gp78-R2m plasmids with or without MG132 (10 μM for 12 Hrs). Transfected cells were collected after 48 Hrs and cell lysates were immunoblotted with antibodies for p27, p53, p21, GFP, and β-actin.

As shown in Figures 3.5 and 3.6 a; Gp78 might be involved in the elevation of endogenous levels of p27 protein. Few sets of samples confirmed the proteasomal inhibitory effect of MG132 on p27 protein levels, were exposure of MG132 causes the accumulation of p27 levels as represented in Figure 3.6 b.

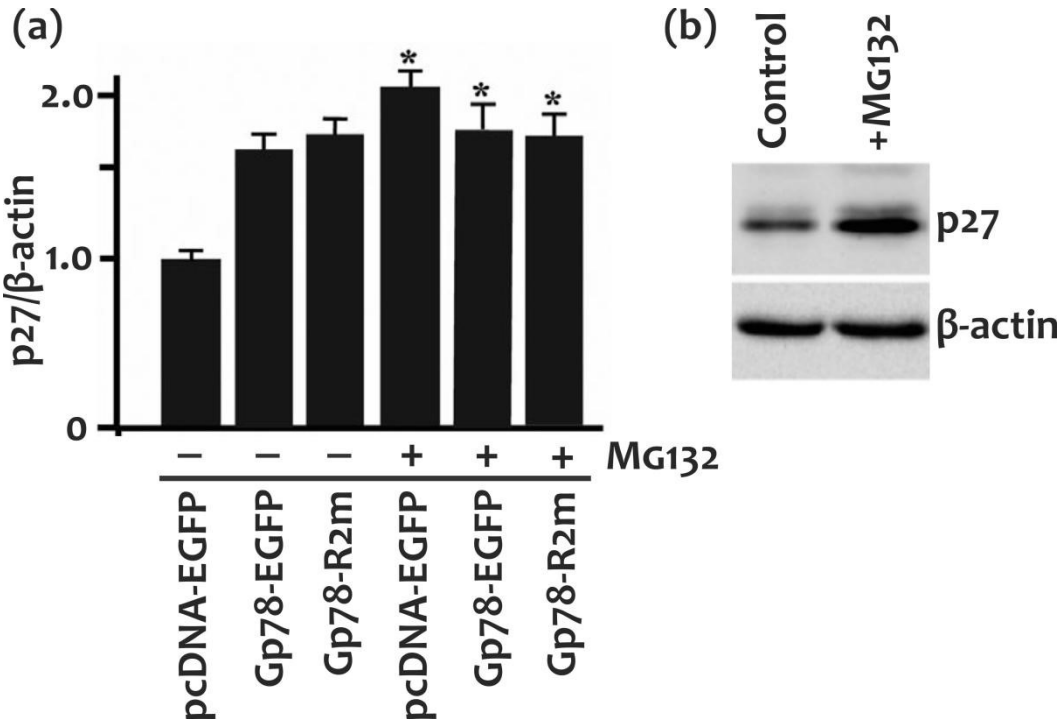

Figure 3.6 : *p27 is degraded by proteasome:* (a) Quantitation of p27/β-actin band intensities as shown in Figure 3.5 obtained from three different experiments via using NIH image analysis software. Values are means ± S.D obtained. *$p < 0.05$ compared with untreated pcDNA-EGFP samples. (b) Cos-7 cells were treated with and without MG132 (10 μM for 12 Hrs), and post-treated cells were collected and cell lysates were used for immunoblot using p27 and β-actin antibodies.

To further confirm this notion, the concentration dependent effect of Gp78 has been checked on p27 proteins. As represented in Figure 3.7, cells were transfected with Gp78-EGFP in concentration-dependent manner and blots were detected with p27, p53, p21, β-actin and GFP antibodies. These observations again indicated that Gp78 is most likely involved in the elevation of endogenous p27 levels when compared to those that were transfected with control plasmid (pcDNA-EGFP).

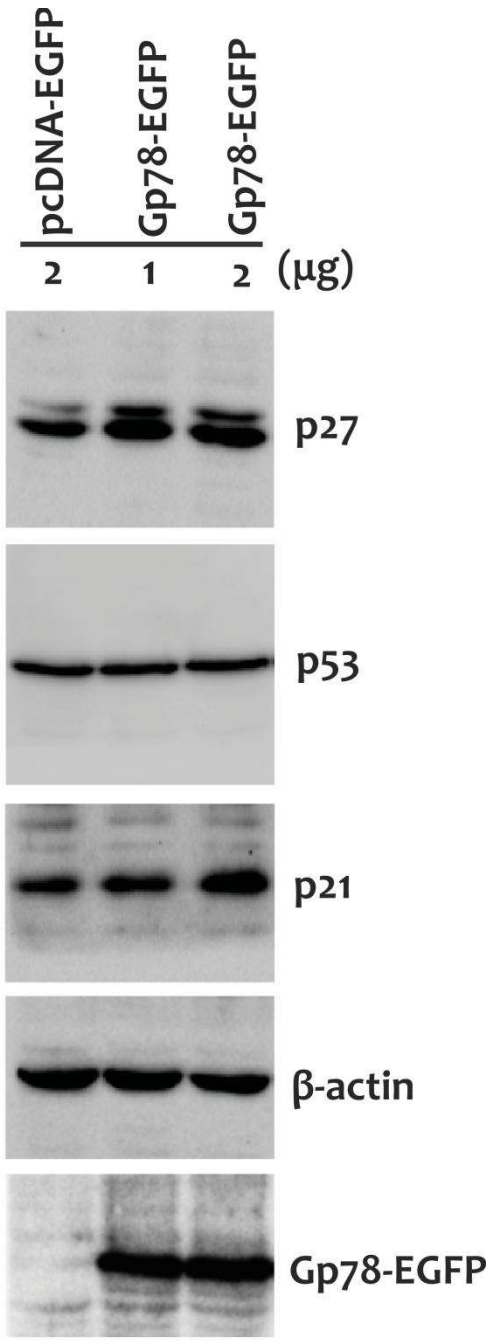

Figure 3.7 : *Upregulation of p27 following Gp78 overexpression:* Cos-7 cells were transfected with pcDNA-EGFP and Gp78-EGFP constructs in different concentration, post-transfection, cells were collected, and cell lysates were used for immunoblot analysis.

To further confirm the previous results, again cells were transiently transfected with low concentration of Gp78 and immunoblot analysis of these results show the high levels of endogenous p27, when compared with control as shown in Figure 3.8.

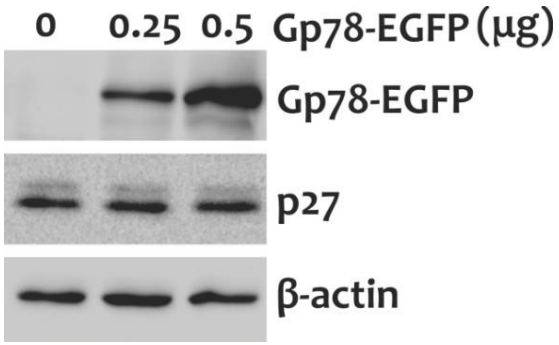

Figure 3.8 : *Observation of effect of lower doses of Gp78 on p27:* Cos-7 cells were transfected with Gp78-EGFP in different concentration (low), post-transfection cells were collected, and cell lysates were used for immunoblot analysis by GFP, p27 and β-actin antibodies.

To further confirm this hypothesis, Gp78-EGFP was expressed with increasing concentrations of wild-type form and later transfected cells were used for isolation of RNA to perform RT-PCR expression analysis as shown in Figure 3.9. A modest increase in p27 mRNA levels in the Gp78 overexpressed cells has been noticed, as compared to control cells. These results indicate that most likely Gp78 overexpression may influence both p27 mRNA and protein levels in the cells.

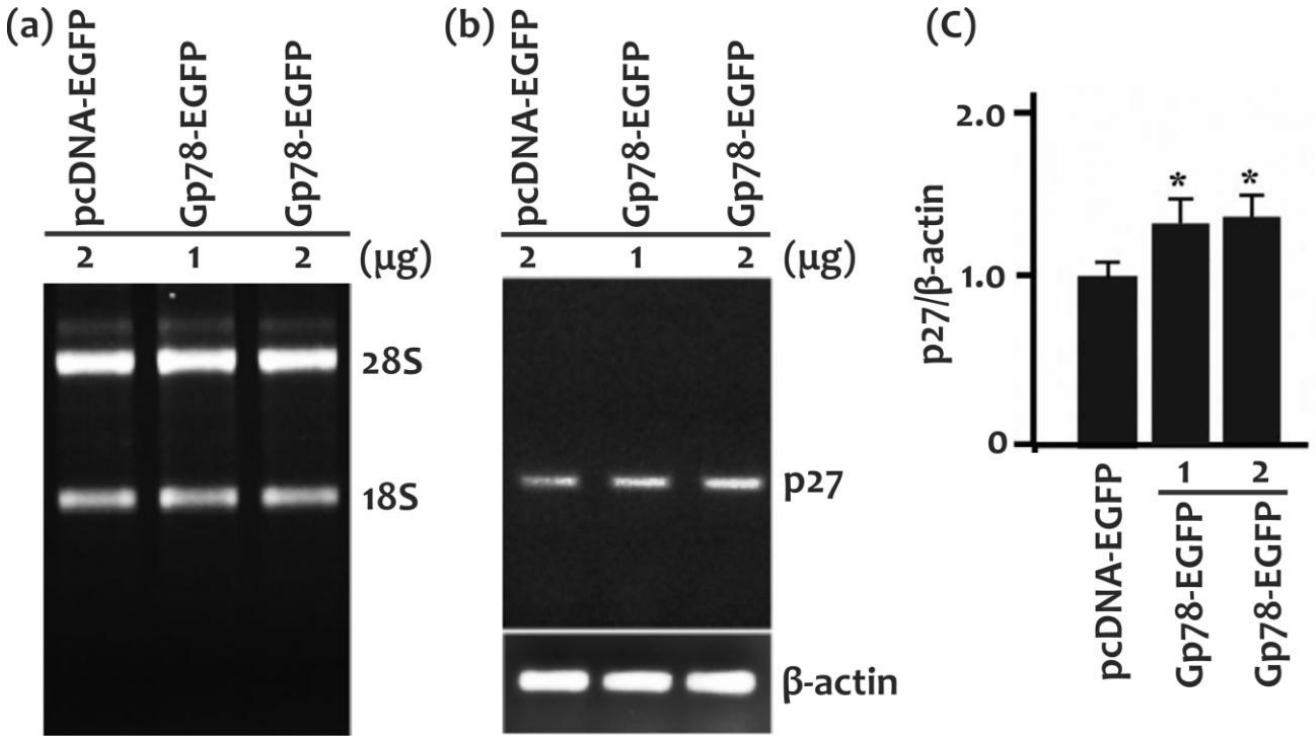

Figure 3.9 : *Change in p27 RNA by Gp78 overexpression:* (a) A549 cells transfected in concentration dependent manner with control and Gp78-EGFP plasmids, were used to isolate RNA by the TRIzol method. (b) Collected RNAs were processed for RT-PCR analysis for expression of p27 and β-actin. (c) Histogram represents quantitation of p27/β-actin bands in figure (a) and (b). Values are means ± S.D acquired from three different sets of experiments. *$p < 0.05$ with control.

3.1.3 Gp78 Overexpression Exhibits Massive Accumulation of p27-Positive Inclusion-Like Structures in Cells

As overexpression of Gp78 induces the elevation of p27 protein level in cells; in order to further confirm this observation in cells the direct effect of Gp78 overexpression on p27 subcellular localization was checked. Therefore, Cos-7 cells were transfected with control and Gp78-EGFP constructs and these cells were processed for immunofluorescence staining using p27 antibody. As depicted in Figures 3.10 a and 3.10 b, the cells overexpressing Gp78-EGFP proteins, were strongly positive for massive accumulation of p27-positive intracellular inclusion-like structures, mostly in the nucleus compared to control cells. Then performed quantification of immunofluorescence micrographs obtained from above experiments and noticed that approximately twenty percent cells massively accumulate p27-positive inclusion like structures mostly in the nucleus of Gp78-EGFP positive cells. To further ascertain above results, next staining was performed without secondary Figure 3.11 a, and primary antibodies Figure 3.11 b, respectively, as controls.

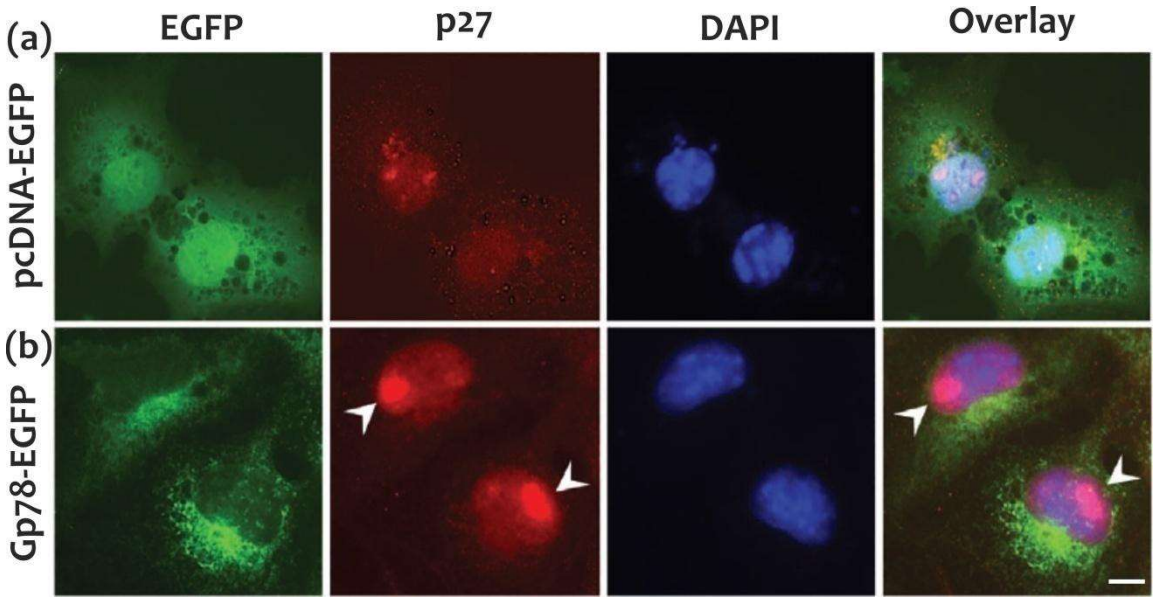

Figure 3.10 : *Accumulation of p27-positive inclusions in Gp78 overexpressed cells:* (a, b) Cos-7 cells, transfected with pcDNA-EGFP (a) and Gp78-EGFP (b) (green) plasmids, were used for immunofluorescence analysis of p27 (red). Texas red-conjugated secondary antibody was used for p27 and DAPI (blue) was used for nuclei staining. Arrows indicate the p27 inclusions formed inside the nucleus of Gp78 overexpressed cells. Scale bar 20 μm.

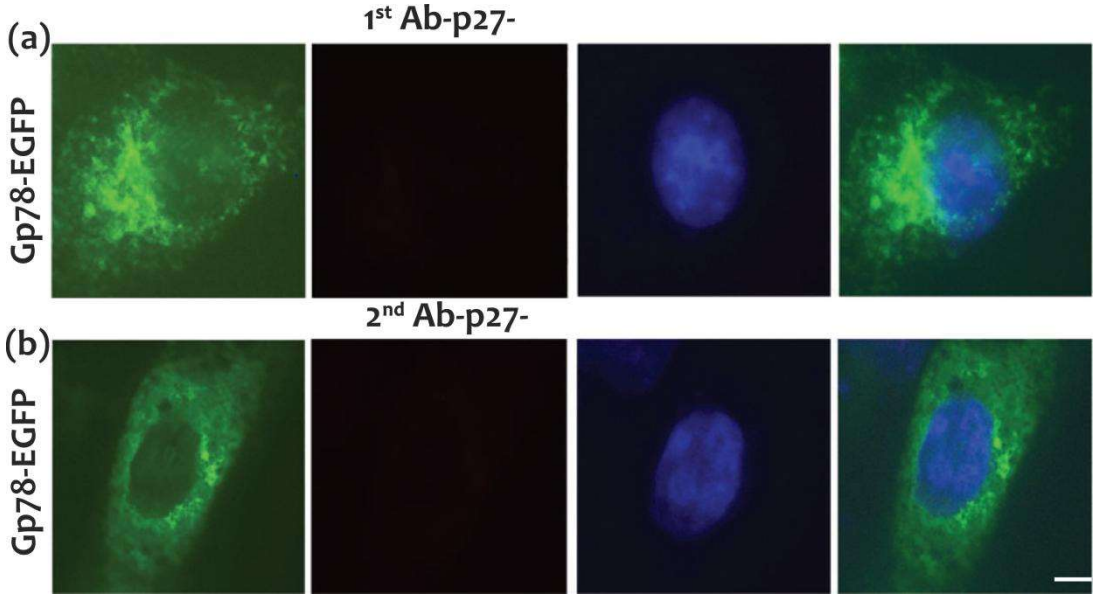

Figure 3.11 : *Control immunocytochemistry for p27 antibodies:* Gp78-EGFP transfected cells were analyzed for control staining in the absence of secondary (a) and primary antibodies (b), respectively, with DAPI staining in cells. Scale bar 20 µm.

Since p27-positive inclusion-like structures showed nuclear distribution, and are induced in the presence of exogenously expressed Gp78, then examined if p53 and p21 proteins display a similar effect with formation of inclusion-like structures in the presence of overexpressed Gp78 protein. To confirm this, cells were processed for immunofluorescence staining with similar sets of transfected cells. The cells were stained for p53 Figures 3.12 a and b and p21 Figures 3.13 a and b proteins and no significant changes in the patterns of p53 and p21 were observed, when cells were overexpressing Gp78-EGFP.

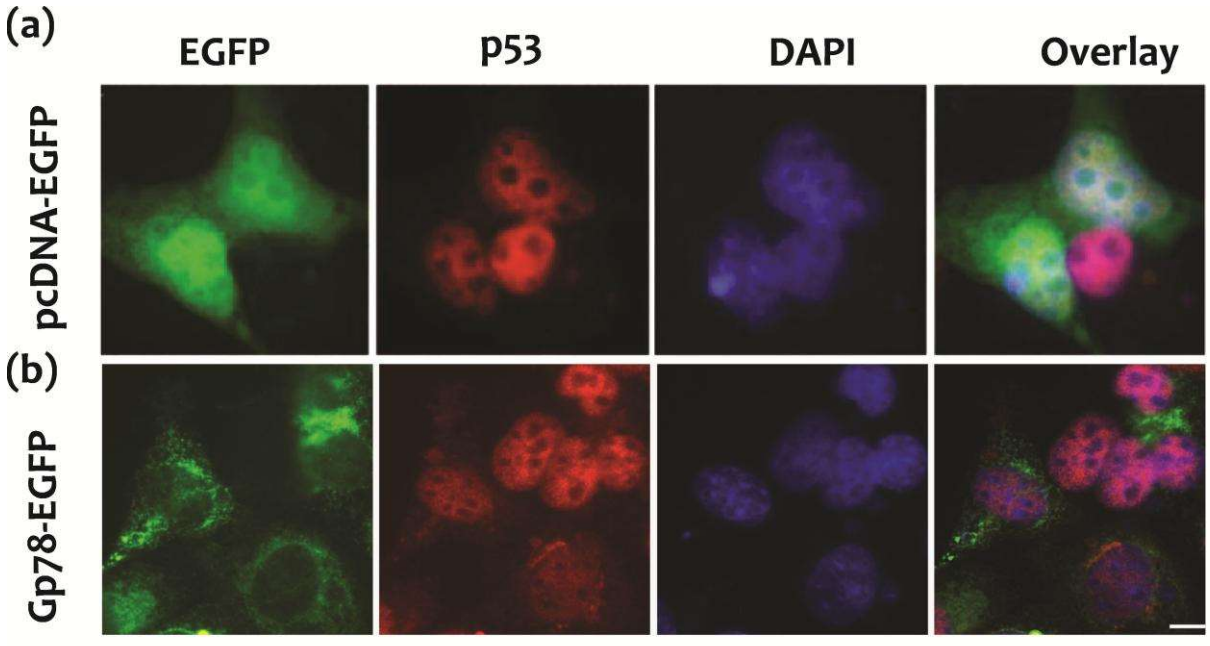

Figure 3.12 : *Immunocytochemistry of p53 in Gp78 overexpressed cell:* Cells were transfected with pcDNA and Gp78 plasmids as shown in Figures (a) and (b), and further processed for immunofluorescence staining of p53. Scale bar 20 µm.

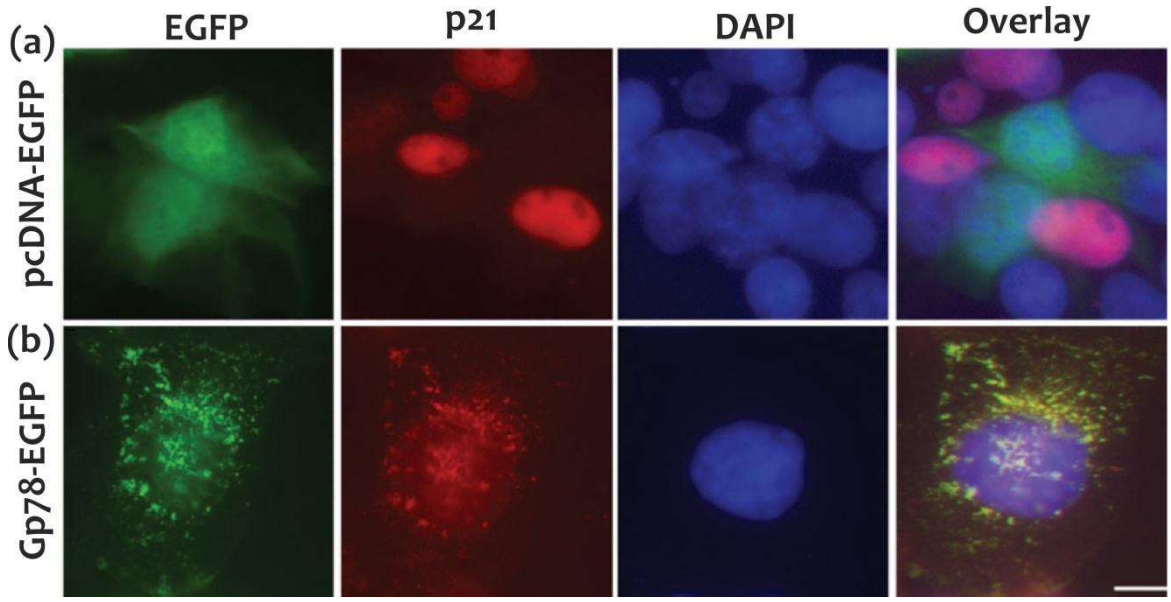

Figure 3.13 : *Immunocytochemistry of p21 in Gp78 overexpressed cells:* Cells were transfected with pcDNA-EGFP and Gp78-EGFP and 48 Hrs after transfection processed for immunofluorescence with p21 (red) primary antibody. Slides were mounted with antifade DAPI. Scale bar 20 µm.

Overall, these results suggest that p53 and p21 showed normal distribution even when the cells were overexpressed with Gp78; however, p27-positive inclusion-like structures were found to be primarily accumulated in the nucleus. DAPI was used for nuclear staining in cells.

3.1.4 Exogenous Expression of Gp78 Increases Stability of p27 in Cells

Using immunofluorescence staining, it has been observed that overexpression of Gp78 induces the accumulation of p27-positive inclusion-like structures inside the cells. These observations indicate that Gp78 could probably be involved in the regulation of p27 proteins in cells by some unknown mechanism. To further investigate this question, it has been explored the role of Gp78 in the regulation of half-life of p27. Gp78 has earlier been demonstrated to degrade metastasis suppressor protein KAI1 and overexpression of AMFR-C also induces the expression of rho-associated coiled-coil kinase protein kinase 2 (ROCK2), which is involved in human acute monocytic leukemia cell line proliferation [Tsai *et al.*, 2007; Wang *et al.*, 2015]. Next, cycloheximide chase experiment was performed with or without MG132 to assess the effect of Gp78, on the turnover of cellular p27. Interestingly, overexpression of Gp78 results in significant increase in the half life of p27. In another similar set of cells treated with MG132 (proteasome inhibitor), Gp78 produces cumulative accumulation of p27, as shown in Figure 3.14 a-d. Results indicate a possible involvement of Gp78 in the stabilization of p27.

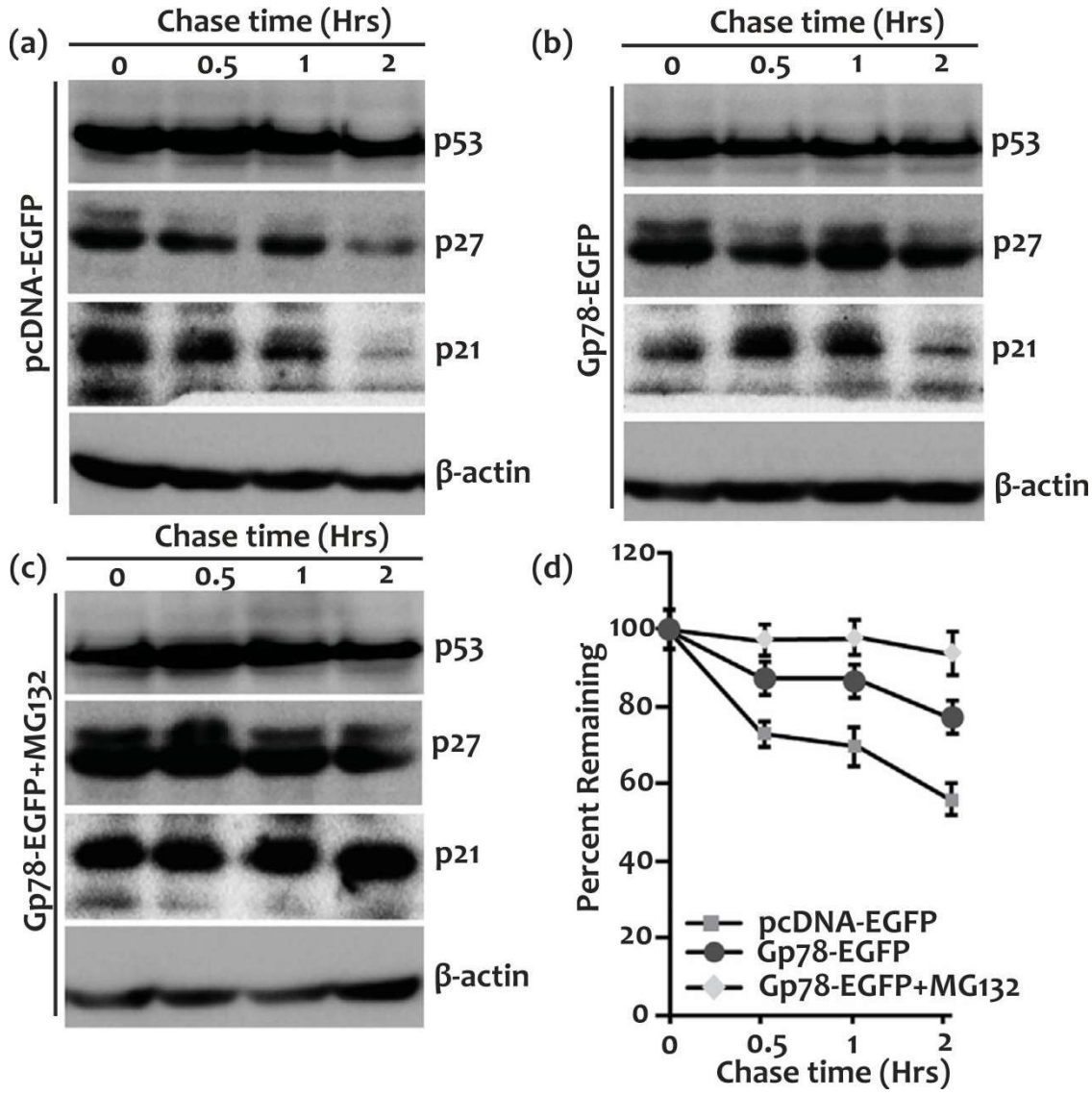

Figure 3.14 : *Gp78 overexpression stabilizes p27 in cells:* (a-b) Cos-7 cells were seeded into six well tissue culture plates, and transfected with pcDNA-EGFP or Gp78-EGFP plasmids, before treatment with cycloheximide (15 µg/ml) after 48 Hrs and later were chased for different time periods up to 2 Hrs. Lysates were used for immunobloting with p53, p27, p21, and β-actin antibodies. (c) Gp78-EGFP transfected Cos-7 cells were treated with MG132 (10 µM for 12 Hrs) and cycloheximide (15 µg/ml) and chased at different time interval and blots were developed with above described antibodies. (d) Quantitation of these experimental data of Figure a-c, with NIH image analysis software represents the stabilization in the steady-state levels of p27 protein.

Next, it was important to check the effect of Gp78 overexpression on the levels of phosphorylated form of p27 because previous reports indicate that unphosphorylated p27 binds to Cyclin/CDK complex and inhibits cell cycle progression; however, functional activation or inactivation of p27 is controlled by its cytoplasmic localization or phosphorylation [Chu *et al.*, 2008; Kossatz and Malek, 2007; Lee and Kim, 2009]. Previous finding observed that T187 P-p27 is accumulated in proliferating cancer cells [Troncone *et al.*, 2005]. To further explore the mechanism of Gp78 induced stabilization of phosphorylated form of p27; immunocytochemistry and immunoblot analyses were performed by using phospho anti-p27 antibody. As shown in Figures 3.15 and 3.16, immunofluorescence staining and immunoblot analysis, respectively revealed that phosphorylated form of p27 was also enormously accumulated in cells overexpressing Gp78–EGFP. Taken together, these results suggest that overexpression of Gp78 may induce the stabilization of p27 in cells.

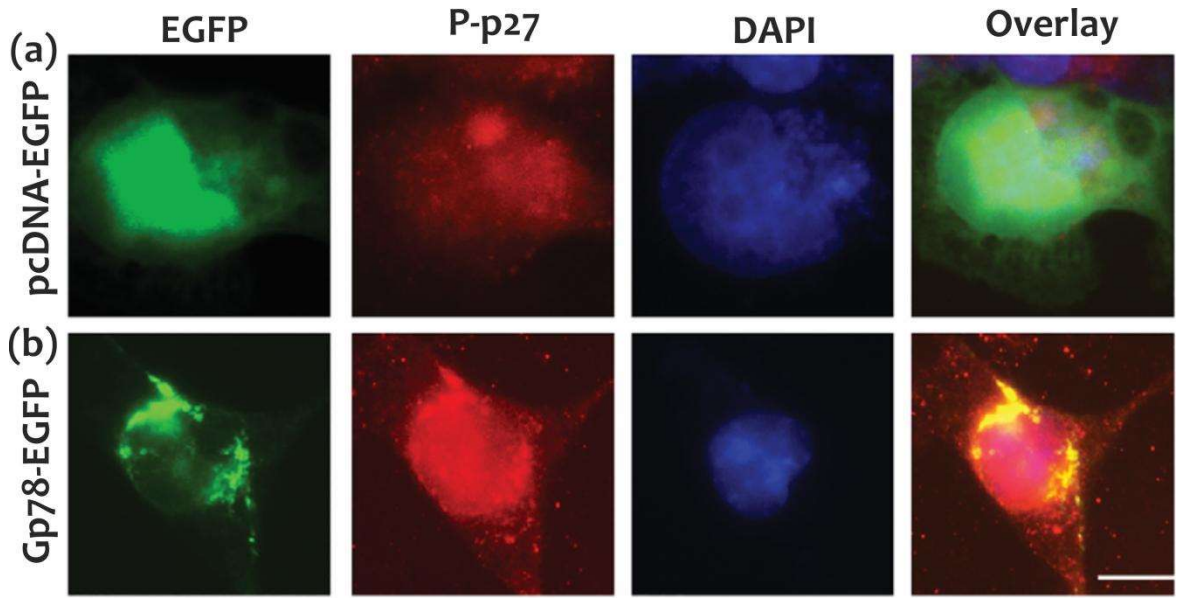

Figure 3.15 : *Expression analysis of P-p27 in Gp78 overexpressed cells:* Cos-7 cells were transiently transfected with pcDNA-EGFP and Gp78-EGFP to perform immunofluorescence staining with the phosphorylated-p27 antibody (T187P-p27) to observe phosphorylated form of p27. DAPI is used for nuclear staining. Scale bar 20 µm.

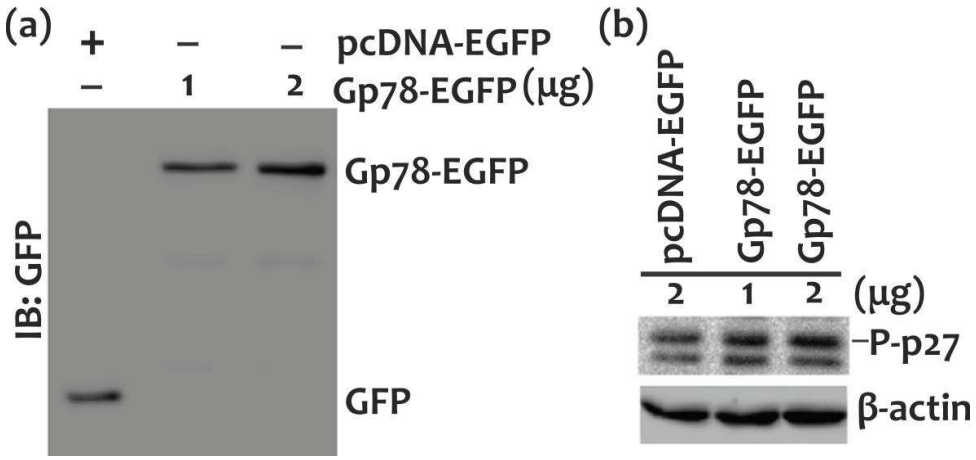

Figure 3.16 : *Immunoblot analysis of P-p27 in cells:* Cells were seeded into six-well plates and Gp78-EGFP plasmid was used for transient transfection. After 48 Hrs of transfection cells were collected and same cell lysates were used for immunoblot analysis by using GFP, phosphorylated-p27 antibody (T187P-p27) and β-actin antibodies.

3.1.5 Overexpression and Knockdown of Gp78 Affects Cellular Proliferation and Apoptosis

Previous reports indicate that p27 is a tumor suppressor protein and its deletion in mice develops more vulnerability for tumorigenesis, tempted by chemical carcinogens or irradiation [Fero *et al.*, 1996; Kiyokawa *et al.*, 1996; Nakayama *et al.*, 1996]. Interestingly, another report shows that *in vivo* expression of p27CK−protein (unable to bind and inhibit Cyclin/CDK complexes) functions as an oncogene and generates a prominent increase in spontaneous tumorigenesis in many tissues of mice linked with amplification of stem/progenitor cell populations [Besson *et al.*, 2007]. Based on earlier results showing the possible implication of Gp78 in regulation of steady state level of p27, it has been observed that the impact of depletion in the level of Gp78 through knockdown on the survival and growth of cells, transfected with

control and Gp78-siRNA. Post-transfected cells were then monitored at different time intervals Figure 3.17 a-c. The Gp78-siRNA transfected cells also exhibit increased rate of apoptosis as shown in Figure 3.17 d.

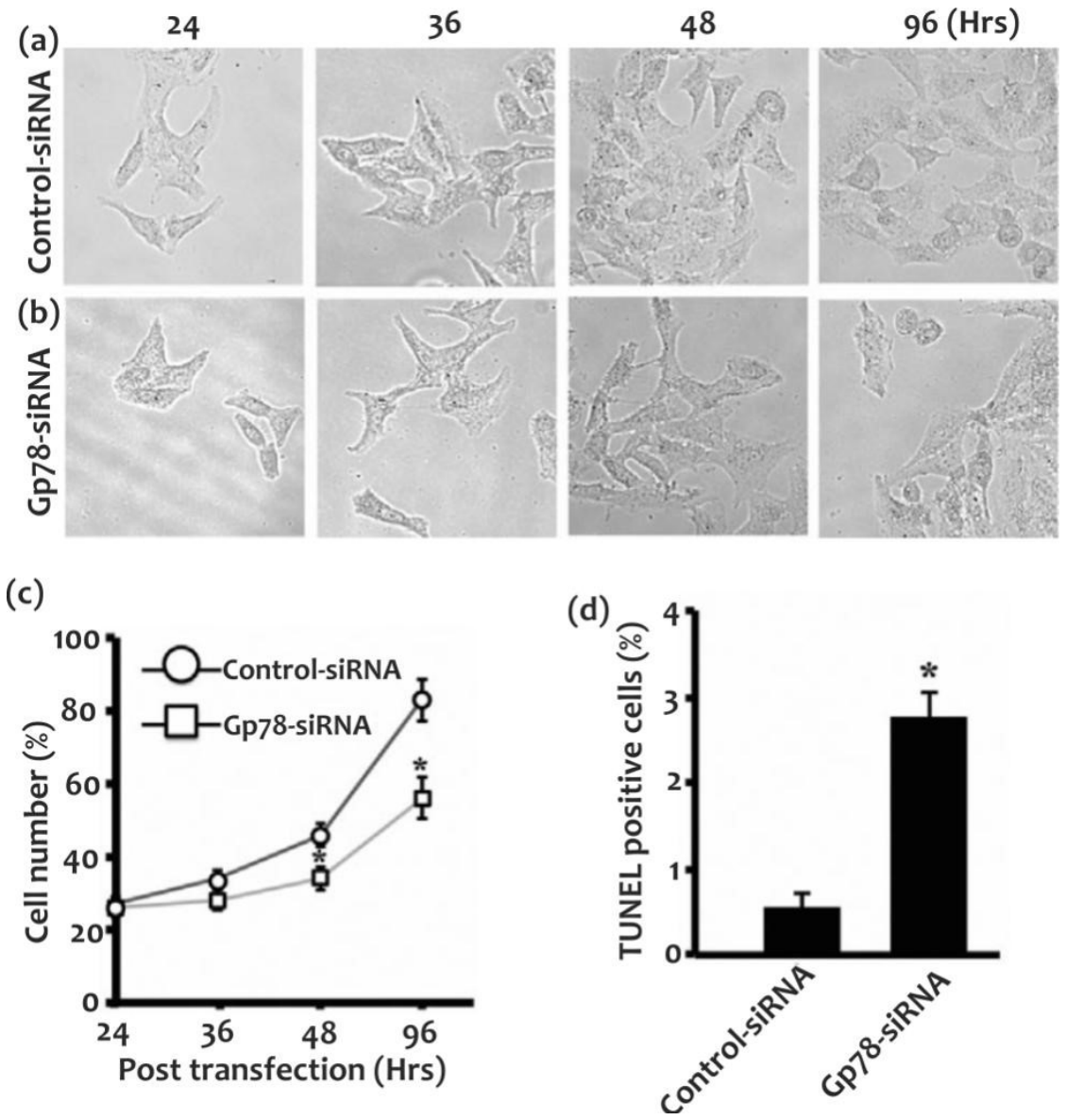

Figure 3.17 : *Gp78 depletion affects cellular proliferation:* (a-b) A549 cells were transfected with control and Gp78-siRNA, and after 24 Hrs of transfection, visualized under bright field microscope at different time intervals up to 96 Hrs. (c) Percentage of post-transfected cell number at a different time interval in control siRNA and Gp78-siRNA transfected cells were represented as a graph. Values presented have means ± S.D.; $n = 3$ and $*p < 0.05$. (c) A549 cells, transfected with siRNAs, as in Figure a, were subjected to TUNEL assay, the percentage of TUNEL positive cells are represented as mean ±S.D. of three independent experiments; $*p < 0.05$ with control.

To further confirm these results, next Gp78 was overexpressed in A549 cells that were observed at different time periods Figure 3.18 (a-b) and noticed that exogenous expression of Gp78 resulted in an increase in the cell proliferation Figure 3.18 c. As shown in Figure 3.18 (d) overexpression and downregulation of Gp78 was further confirmed by using above set of samples for immunoblot analysis, using antibodies of GFP, Gp78, p27, and β-actin.

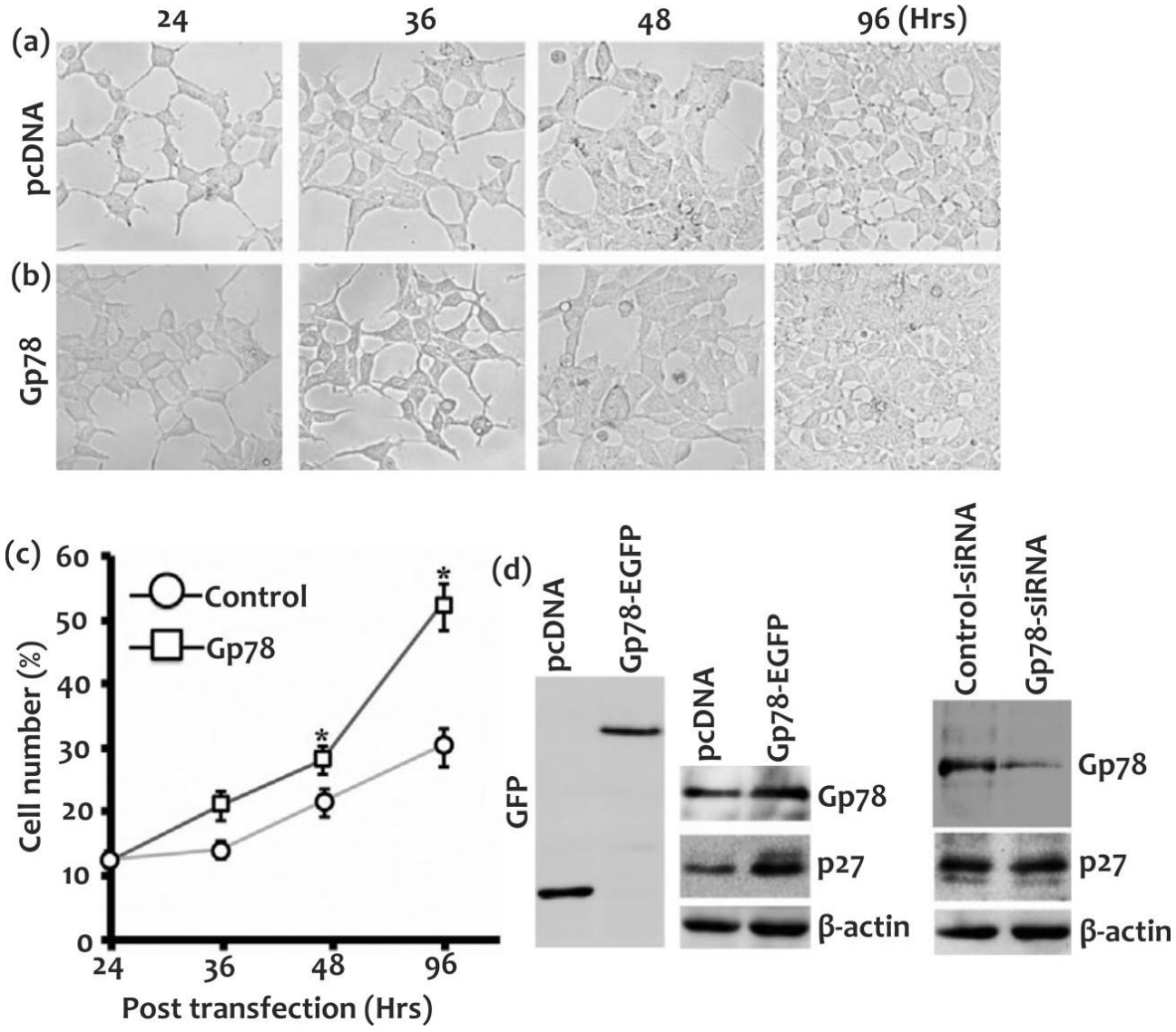

Figure 3.18 : *Effect of Gp78 overexpression on cell proliferation:* (a-b) pcDNA and Gp78 were transfected in A549 cells for different time intervals and images were taken by bright field microscope. (c) Cell number of above-transfected cells were counted and represented by graph with respect to transfection time intervals. Values are mean ± S.D obtained from three different experiments. *$p < 0.05$ with control. (d) As described in previous sections, cells were used for immunoblotting by using GFP, Gp78, p27, and β-actin antibodies.

As shown in Figure 3.19, knockdown of p27 changes cell proliferation, this effect can be marginally suppressed by overexpression of Gp78 in cells. Earlier study has also found that Gp78 overexpression stimulates cellular transformation in fibroblast cells [Onishi *et al.*, 2003]. Taken together, present findings, which are also supported by earlier studies, suggest that expression of Gp78 in cells could affect cellular proliferation that might be influenced by deregulated levels of p27 in cells.

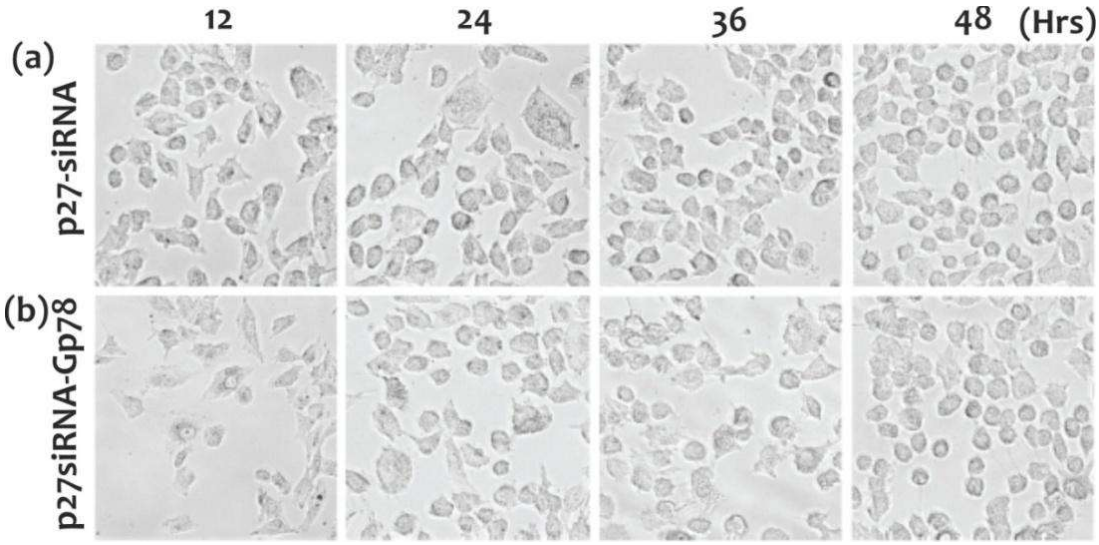

Figure 3.19 : *Effect of p27-siRNA on cell proliferation:* (a-b) p27-siRNA and p27 siRNA-Gp78 were transfected in A549 cells for different time intervals and images were taken with bright field microscope.

3.1.6 Gp78-Mediated p27 Elevation Might be Involved in the Upregulation of CDK4, CyclinD1, and Cyclin E

Few crucial reports indicated enhanced levels of p27 and other cell cycle regulatory proteins such as CyclinD1 and CDK4 in cancer cells [Fredersdorf *et al.*, 1997; Larrea *et al.*, 2008; Reed *et al.*, 1999; Sgambato *et al.*, 1997]. Prior works also indicated that high expression of p27 in various cancers is linked with the elevated level of Cyclin E [Bales *et al.*, 1999; Iida *et al.*, 1997; Zhou *et al.*, 2004]. As current observation suggests that overexpression of Gp78 leads to an increase in the p27 levels in cells, further it is important to check whether the Gp78 mediated p27 elevation can also elevate the levels of cell cycle regulatory proteins CDK4, CyclinD1, and Cyclin E. The pcDNA-EGFP (control) and Gp78-EGFP plasmids were used for transient transfection in cells; and after 48 Hrs of transfection, cells were processed for immunofluorescence staining with relevant antibodies of above-described cell cycle regulatory proteins. As shown in Figure 3.20, 3.21, 3.22 overexpression of Gp78 resulted in the increased cellular levels of CDK4, Cyclin D1, and Cyclin E, in comparison to their respective controls.

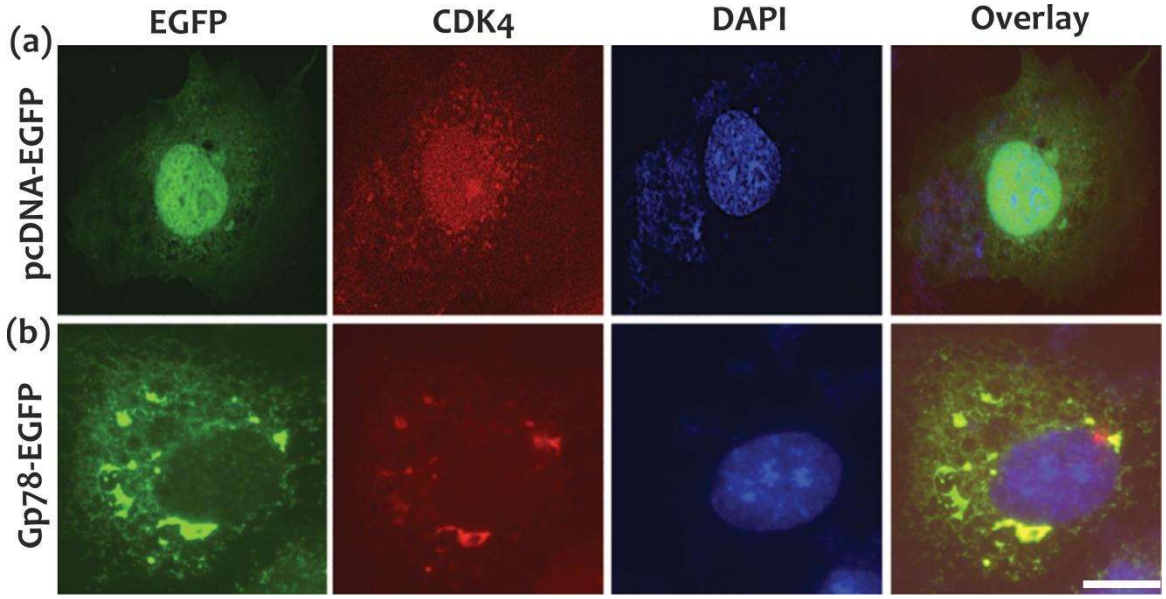

Figure 3.20 : *Effect of Gp78 overexpression on the levels of CDK4:* (a-b) Transfected Cos-7 cells were fixed and processed for immunofluorescence staining using CDK4 antibody (red). Scale bar 20 µm.

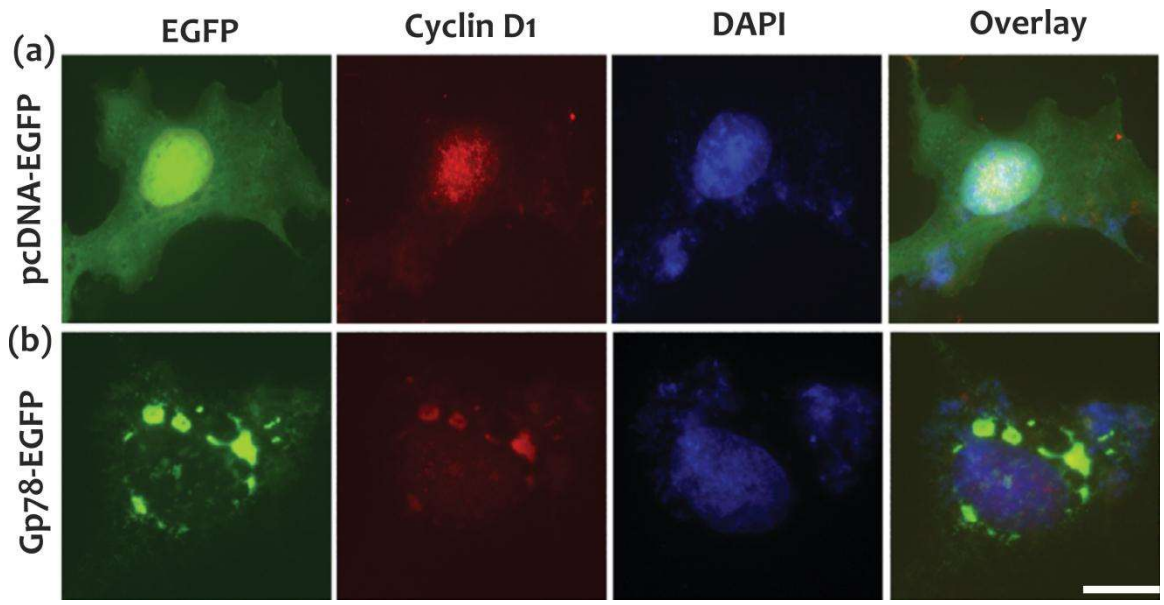

Figure 3.21 : *Analysis of Cyclin D1 in Gp78 overexpressed cells:* Cos-7 cells were transfected as described above in Figure 3.19 and immunofluorescence staining was performed for CyclinD1 (red). Scale bar 20 μm.

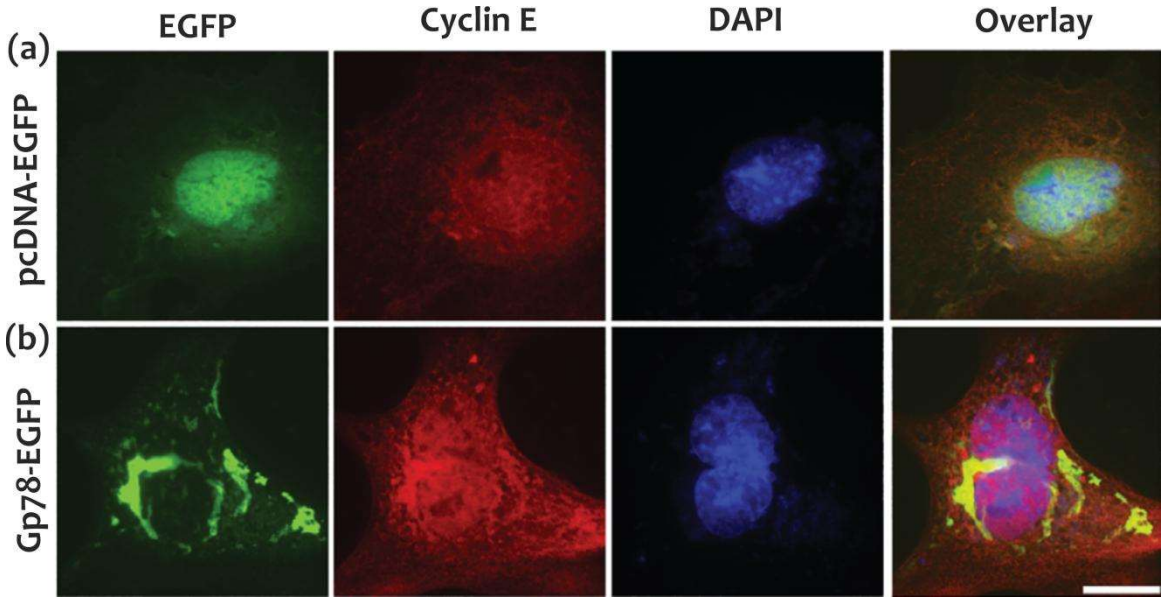

Figure 3.22 : *Analysis of Cyclin E level in Gp78 overexpressed cells:* Cos-7 cells were transfected as described above in Figure 3.20 and after 48 Hrs of transfection cells were processed for immunofluorescence analysis using Cyclin E antibody (red). Nuclei were stained with DAPI. Scale bar 20 μm.

Similar sets of samples were also used for immunoblotting with their respective antibodies, as shown in Figure 3.23. β-actin was used as loading control. To further ascertain results, next the overexpression of exogenously expressed Gp78 protein was confirmed through immunoblot analysis, using GFP and β-actin antibodies. On the basis of previous findings and present observations, it is most likely that Gp78 affects p27 endogenous levels, which could be linked with the elevation of other cell cycle regulatory proteins.

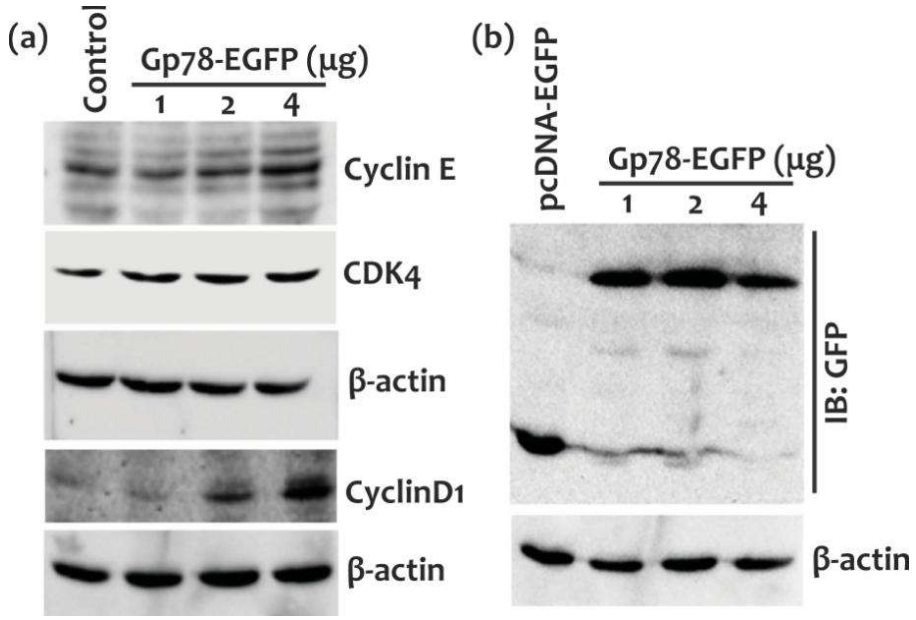

Figure 3.23 : *Immunoblot analysis of various cell cycle regulatory proteins:* (a) After 48 Hrs of concentration dependent transfection of Cos-7 cells with Gp78-EGFP expression constructs, lysates were collected and subjected to immunoblots analysis with CDK4, Cyclin D1, Cyclin E, and β-actin antibodies. (b) Expression of constructs was confirmed in above-transfected cells by immunoblotting, using anti-GFP antibody.

3.1.7 Knockdown of Gp78 Does Not Affect the Endogenous Levels of p27 in Cells

The elevated levels of p27 in the Gp78 overexpressed cells has prompted to analyze the impact of Gp78 knockdown on endogenous levels of p27 in cells. First, the cells were transfected with control and Gp78-siRNA in A549 cells and after 48 Hrs, cells were used for immunofluorescence staining using anti-Gp78 antibody to detect the efficiency of knockdown. As shown in Figure 3.24, transient expression of Gp78-siRNA reduces the endogenous levels of Gp78 in cells.

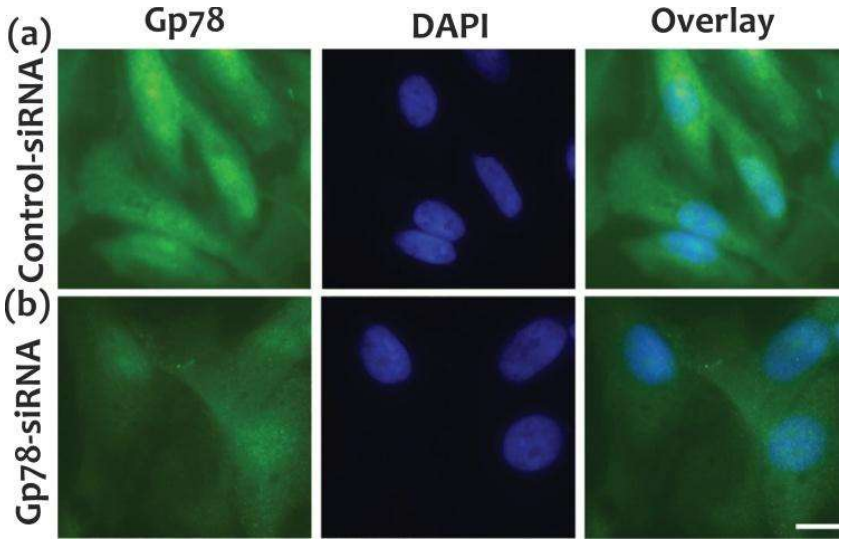

Figure 3.24 : *Immunocytochemistry analysis of Gp78 siRNA:* (a) After 48 Hrs of transient transfection of control and Gp78-siRNA, A549 cells were processed for immunofluorescence staining using the Gp78 antibody. FITC conjugated secondary antibody was used to label Gp78 and stained with DAPI to visualize nuclei under a fluorescence microscope. Scale bar 20 µm.

Few sets of cells were also used for immunofluorescence by anti-Gp78 and anti-p27 antibodies, and nuclear staining was performed by DAPI. These observations clearly

demonstrated that knockdown of Gp78 does not affect the endogenous levels of p27 in cells, Figure 3.25. The RNAi-mediated knockdown of Gp78 gene was also validated by RT-PCR analysis. Depletion of Gp78 does not affect the mRNA levels of p27 as shown in Figures 3.26.

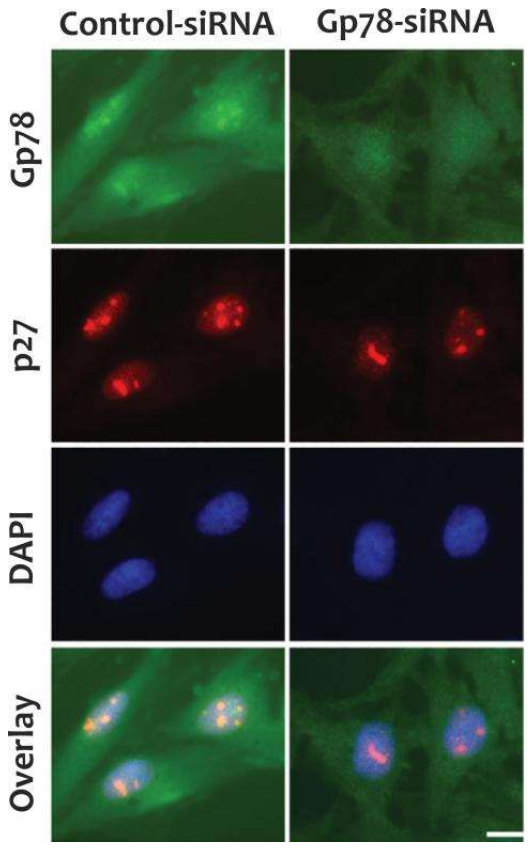

Figure 3.25 : *Effect of Gp78 siRNA on p27:* (a) A549 cells, transfected with control and Gp78-siRNA, were subjected to immunocytochemistry analysis with Gp78 (green) and p27 (red) antibodies. Scale bar 20 µm.

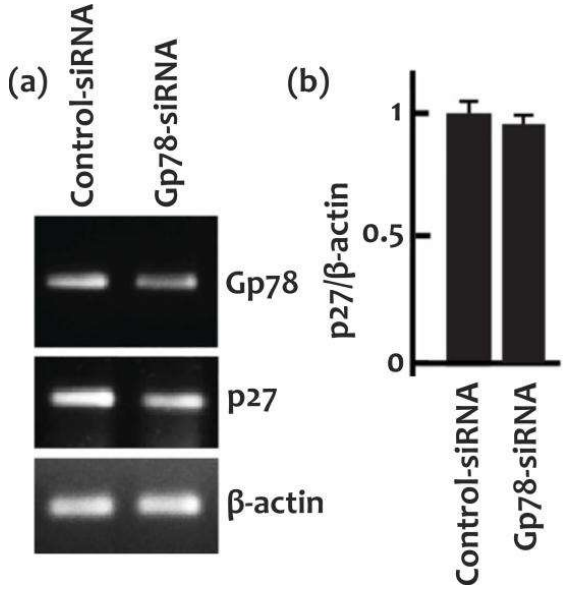

Figure 3.26 : *RT-PCR analysis of Gp78 siRNA:* (a) Few sets of transfected cells were processed for total RNA isolation and subjected to RT-PCR analysis of Gp78, p27, and β-actin. (b) Micrographs of RT-PCR analysis of Figure (a) were used for quantitation of p27/β-actin band intensities, values are mean ± S.D obtained from three independent experiments.

Immunoblot analysis was also performed using cell lysates, which were prepared from similar sets of transiently transfected cells. Blots were observed by using Gp78, p27, and β-actin antibodies, Figure 3.27, and p27 levels were quantified through NIH image analysis software using β-actin levels for normalization.

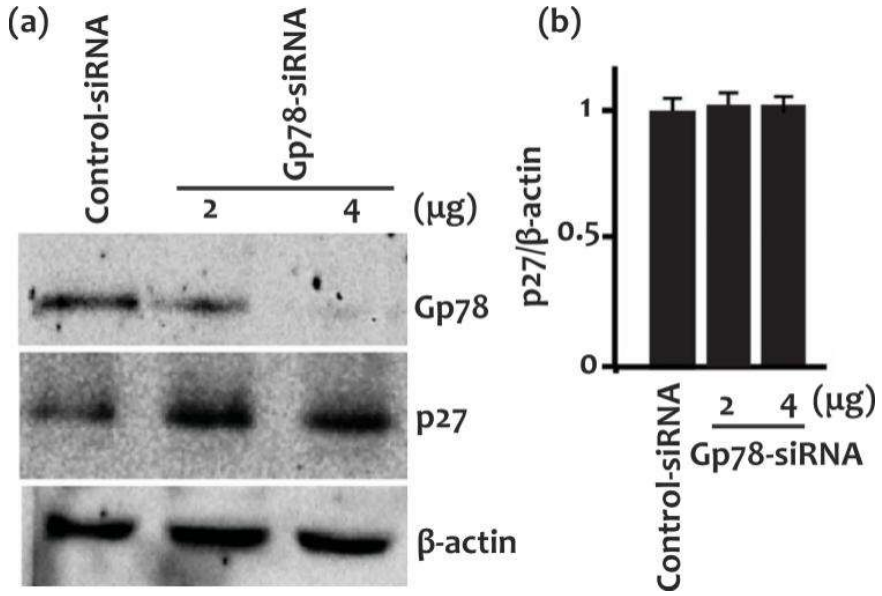

Figure 3.27 : *Immunoblot analysis of Gp78 siRNA transfection:* (a) Control-siRNA and Gp78-siRNA at different concentrations were used for transient transfection of A549 cells; and after 48 Hrs of transfection, cell lysates were collected to perform immunoblotting and Gp78, p27, and β-actin antibodies and were used to probe these proteins. (b) Quantitation of p27/β-actin band intensities of Figure (a) having values means ± S.D obtained from three different experiments.

3.1.8 Cellular Stress Enhances the Endogenous Levels of Gp78 and p27 in Cells

In the current finding, it has been noticed that overexpression of Gp78 influences the endogenous levels of cell cycle regulatory proteins. Previous reports indicate that E3 ubiquitin ligase Gp78 targets various metastasis suppressor and neurodegenerative disease associated proteins for their degradation [Chen *et al.*, 2012]. It has been observed that tunicamycin treatment induces p27 levels, which suppresses cell cycle progression in melanoma cells [Han *et al.*, 2013]. As it was also observed that under endoplasmic reticulum stress condition, Gp78 gets stabilized [Shen *et al.*, 2007]. Therefore, in our conclusive experiments, it was important to check how Gp78 acts and regulates endogenous levels of p27 in cells under stress condition. To find out the answer of this question, first cells were treated in concentration-dependent Figure 3.28 (a) and time-dependent Figure 3.28 (b) manner, with tunicamycin for ER dysfunction; and after tunicamycin exposure, cells were visualized using bright field microscope for analysis.

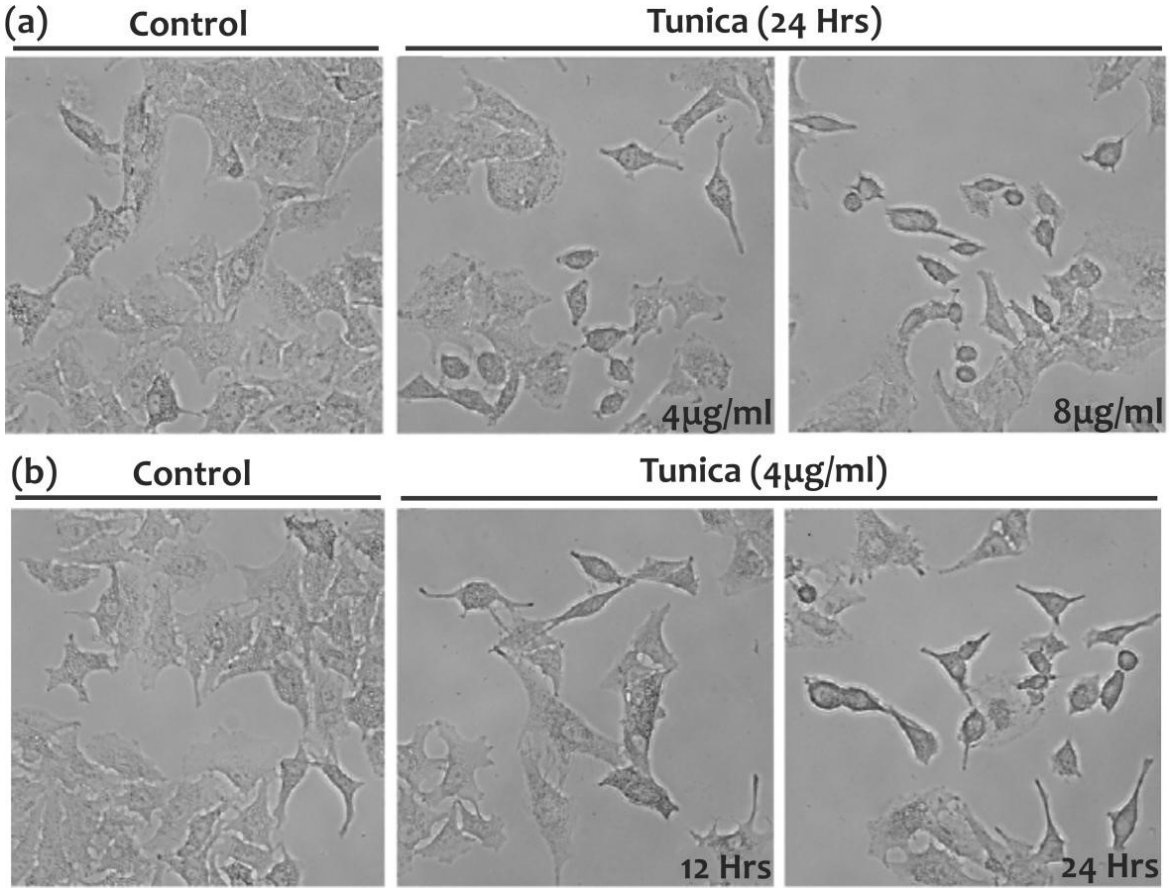

Figure 3.28 : *Effect of tunicamycin treatment on cells:* Tunicamycin (Tunica) was given to A549 cells in concentration (a) and time (b) dependent manner and bright field micrographs were analyzed compared to control cells.

Using immunoblot analysis, next the effects of above treatments were examined on the protein levels of p27. As shown in Figure 3.29 and Figure 3.30, level of p27 was elevated after tunicamycin treatment in cells. To further determine the effects of tunicamycin-mediated ER dysfunction on p27 protein levels, immunoblot analysis was performed by p27 antibody.

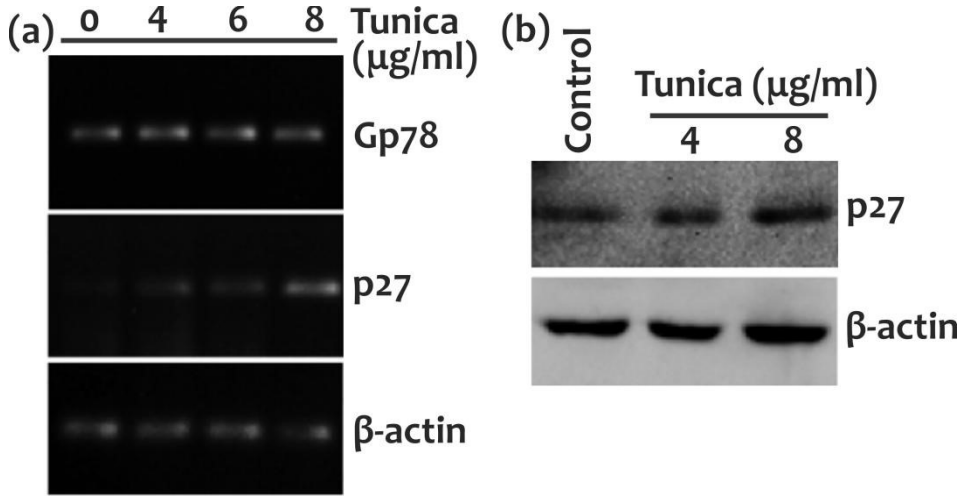

Figure 3.29 : *PCR analysis after tunicamycin concentration dependent treatment:* (a) Total RNA sample of cells were collected and PCR analysis was performed for Gp78, p27, and β-actin. (b) Similar set of cells were also used for western blot analysis using p27 and β-actin antibodies.

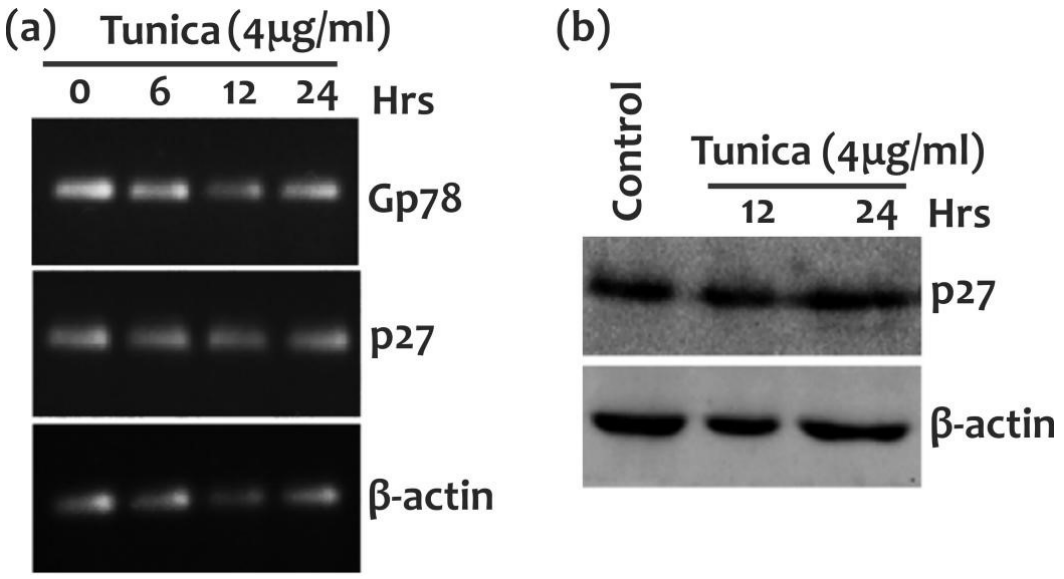

Figure 3.30 : *Expression analysis of Gp78 and p27 after tunicamycin treatment:* (a) A549 cells, treated in time dependent manner were exposed to tunicamycin (4 µg/ml) for different time intervals and RNA sample were collected in similar sets of cells for PCR analysis of Gp78, p27 and β-actin. (b) Cell lysates were collected after 0, 12, and 24 Hrs of tunicamycin treatment and western blot analysis were performed by p27 and β-actin antibodies. Published as Joshi and Mishra et al., 2018.

3.2 DISCUSSION

In ubiquitin proteasome system, E3 ubiquitin ligases play an essential function of the specific recognition of cellular proteins to coordinate the processes of protein degradation in cells [Chhangani *et al.*, 2012]. Interruption of the UPS plays a critical role in numerous neurodegenerative diseases, aging, and cancer [Chhangani and Mishra, 2013; Paul, 2008]. In the present study, it has been found that cell cycle regulatory protein p27 is subjected to stabilization and its endogenous level is also elevated in cells having exogenous expression of E3 ubiquitin ligase Gp78. The upregulation of p27 at mRNA and protein levels were observed in cells, exogenously overexpressing Gp78. Overexpression of Gp78 also demonstrates massive accumulation of p27-positive inclusion-like structures in cells. E3 ubiquitin ligase Gp78 overexpression most likely induces the stabilization of both phosphorylated and unphosphorylated forms of p27 in cells. Functional interactions of E3 ubiquitin ligases are widely viewed as a pivotal event in cell cycle transition, and the disruption of the critical regulators of this mechanism are chiefly observed in different types of cancers [Bernassola *et al.*, 2008; Nakayama and Nakayama, 2006]. Therefore, it is important to understand the basic mechanisms involved in the control of cell cycle regulatory proteins that is mediated by E3 ubiquitin ligases and other basic components of protein quality control system. In addition to the multiple E3 ubiquitin ligases that regulate cell cycle regulatory protein p27, previous studies have also shown that in various cancers, endogenous levels of p27 gets disturbed and its level is found to be elevated in breast cancer cell lines [Cao *et al.*, 2011; Hattori *et al.*, 2007; Mishra *et al.*, 2009; Sgambato *et al.*, 1997]. Another report indicates inverse correlation between the expression of p27 and degree of malignancy in some human breast cancer tissues [Fredersdorf *et al.*, 1997]. Few reports also strongly observed the overexpression of AMFR in various types of human cancers [Kawanishi *et al.*, 2000; Kaynak *et al.*, 2005; Timar *et al.*, 2002].

Previous findings revealed that Gp78 and its ligand AMF mediated signaling in cells results in increased rate of cell proliferation [Silletti *et al.*, 1993; Yanagawa *et al.*, 2004]. It was also observed that overexpression of Gp78 induces a proportional increase in proliferation of

transfected cells [Fairbank *et al.*, 2009; Onishi *et al.*, 2003]. Recently, a study in THP-1 cells reported that Gp78 siRNA treated cells showed a significant increase in the overall rate of apoptosis [Wang *et al.*, 2015]. In consistent with above findings, it has also been observed that knockdown of Gp78 reduces cellular proliferation and induces apoptosis in cells; whereas overexpression of Gp78 enhances overall cellular proliferation. Earlier, several human cancer types have also been reported for the high rates of cell proliferation with increased Gp78 expression [Chiu *et al.*, 2008]. Disruption of cell cycle regulation leads to improper cellular growth. Results from other studies propose that induced levels of p27 in different types of cancers are also associated with increased levels of Cyclin E [Bales *et al.*, 1999; Iida *et al.*, 1997; Zhou *et al.*, 2004]. As p27 plays a central role in cell cycle regulation, next effects of Gp78 overexpression was checked on it and on other cell cycle-associated proteins.

Several findings have established the relationship between various metabolic stresses and altered cancer cell metabolism [Cairns *et al.*, 2011; DeBerardinis *et al.*, 2008]. Previous studies indicate that cancer cells have distinguished metabolic demands, as compared to normal cells; therefore, modulation of these mechanisms generate cellular stress responses, such as, proteasomal deregulation, autophagy dysfunction and unfolded protein response (UPR) [Dang, 2012; Jin and White, 2007]. Interestingly, few findings indicate that cell cycle regulation, protein synthesis, and variations in cellular stress responses in cancer cells can act as possible key alternative anti-proliferative strategies, despite their therapeutic intervention is not well understood [Griffin and Shockcor, 2004; Hsu and Sabatini, 2008]. It has been observed that tunicamycin mediated activation of UPR leads to accumulation of p27 and induces the cell cycle arrest in melanoma cells and levels of Skp2, an E3 ligase for p27 was reduced after tunicamycin treatment [Han *et al.*, 2013]. Therefore, it was important to monitor the effects of ER stress, mediated by tunicamycin, on the endogenous levels of p27 in cells. A significant enhancement of p27 was observed in cells after exposure to ER stress, as compared to Gp78 levels.

There are multiple lines of emerging evidence that suggest different cellular stress conditions impair normal cellular metabolic processes, including cell cycle regulation [Bakkenist and Kastan, 2004; Klein and Ackerman, 2003]. To cope up with such conditions, PQC mechanism and its critical components (UPS and autophagy) do their functional intersections in various ways at different levels, which is important for the regulation of cellular proliferation and other cellular physiological functions [Koepp *et al.*, 1999; Pagano, 1997]. Currently, the role of Gp78 in PQC mechanisms and cellular proliferation is not completely understood. In near future, these findings may suggest a potential strategy that can generate promising possibilities of therapeutic interventions linked with anti-proliferation mechanism.

3.3 CONCLUDING REMARKS

In this work, Gp78 overexpressing cells were used for immunofluorescence staining using p27 antibody, which confirmed the substantial accumulation of intra cellular p27-positive inclusion-like structures, mainly in the nucleus, as compared to control (pcDNA-EGFP) cells. It has been demonstrated that overexpression of Gp78 leads to increase in steady-state levels of p27 in cells. Collectively, results of cycloheximide chase experiment analysis and immunofluorescence staining with overexpression of Gp78 suggests that induced level of Gp78 enhances the endogenous steady state levels of both unphosphorylated or hypo-phosphorylated and phosphorylated p27 forms in cells. The current findings are in line with earlier studies and proposes a possible mechanism that overexpression of Gp78 most likely disturbs the functional competency of p27 protein in cells. Current study overall suggests a possibility that cellular quality control E3 ubiquitin ligase Gp78 may play multilevel regulatory roles linked with PQC mechanism and cellular proliferation.