4.1 Introduction

Vitiligo is one of the most common cosmetic disfigurement disorders caused due to the loss of functional melanocytes in epidermis (Poole *et al.,* 1993). The disease can affect individuals of any race or sex and generally manifests before the age of 20 years in approximately half of all cases (Taïeb & Picardo, 2010). Though, the aetiology of vitiligo is complex, several potential precipitating factors such as oxidative stress and autoimmunity in addition to genetic predisposition appear to be involved in vitiligo pathogenesis (Laddha *et al.,* 2013; Mansuri *et al.,* 2014). Various pro-oxidants generated during melanin synthesis and compromised intrinsic antioxidant defence mechanisms make epidermal melanocytes vulnerable to oxidative stress (Denat *et al.,* 2014). Oxidative stress triggers vitiligo onset, while autoimmunity contributes to the disease progression (Laddha *et al.,* 2014). However, despite extensive research, the exact mechanism, which connects the triggering factors with the disease progression, is still obscure. In the skin microenvironment of vitiligo patients, presence of oxidative stress due to altered oxidant-antioxidant balance and inflammatory stress due to the imbalance of pro-and anti-inflammatory cytokines has been reported (Laddha *et al.,* 2013; Singh *et al.,* 2018). Several studies including ours suggest significant increase in homocysteine levels in the blood as well as skin microenvironment of vitiligo patients (Anbar *et al.,* 2016; Jadeja *et al.,* 2018; Tsai *et al.,* 2019). Homocysteine (Hcy), an intermediate of methionine metabolism is known to induce oxidative stress, ER stress and inflammation (Zhang *et al.,* 2001; Nahidi *et al.,* 2014). Elevated Hcy levels are associated with various disorders including cardiovascular diseases, neurodegenerative diseases, diabetes mellitus and autoimmune diseases (Schalinske & Smazal, 2012). The ability of Hcy to induce oxidative stress, endoplasmic reticulum (ER) stress and inflammatory response suggests that it might be a central player in triggering vitiligo and its progression. Earlier, we speculated that the ER stress could be a potential link between oxidative stress and autoimmunity (Mansuri *et al.,* 2014). Dilation of ER is a hallmark of ER stress, which was observed in perilesional skin biopsies as well as melanocytes cultured from vitiligo patients (Boissy *et al.,* 1991; Le Poole *et al.,* 2000). ER stress is induced in cells by both internal and external perturbations (Yoshida, 2007). In the skin, epidermal barrier perturbation, as well as external stress, such as UV irradiation, trauma, and internal perturbation such as oxidative stress may induce ER stress (Mansuri *et al.,* 2014; Harris, 2016). Further, oxidative stress might cause accumulation of misfolded proteins in the ER and lead to ER stress, which activates unfolded protein response (UPR) to resolve the protein folding defects. Under non-stressed condition, the three ER transmembrane signalling molecules ATF6, IRE1, and PERK remain inactive by interaction with BiP/ GRP78. Due to the accumulation of misfolded proteins, GRP78 dissociates from these sensors and results in the activation of protective responses by UPR (Figure 4.1). Activation of IRE1 results in non-canonical splicing of *XBP1* mRNA, which is translated to an active transcription factor *sXBP1*. The dissociation of PERK from GRP78 leads to its phosphorylation and oligomerization that in turn phosphorylates eIF2α to suppress the overall translation while selectively enhancing the translation of mRNAs implicated in UPR such as *ATF4*. ATF4 thus formed initiates the transcription of UPR target genes such as *CHOP* and *GADD34*. Activation of ATF6 results in its translocation to the Golgi apparatus, where it is cleaved by S1P and S2P proteases. The cleaved ATF6 acts as an active transcription factor that enters the nucleus to initiate the transcription of target genes (Walter & Ron, 2011; Hetz & Papa, 2018).

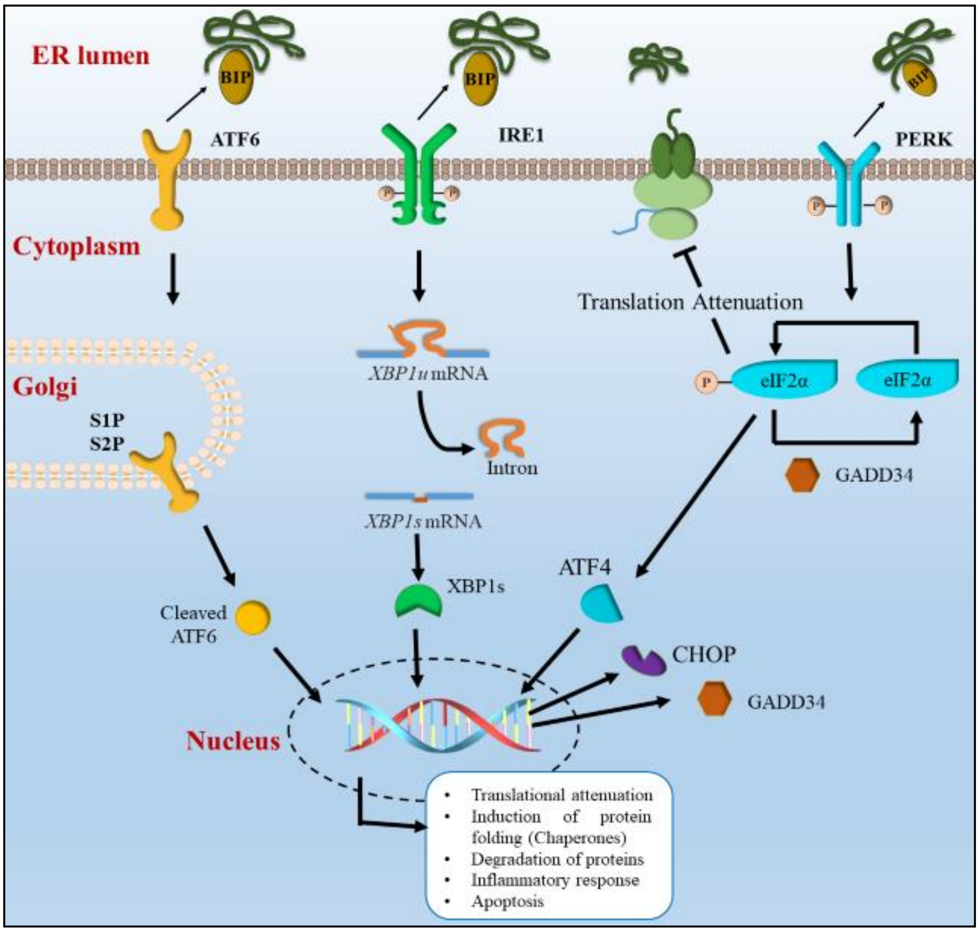

Figure 4.1: The unfolded protein response signalling pathways

When there is chronic or severe ER stress, or the UPR is compromised and not able to restore the protein folding homeostasis, apoptotic signalling pathways are activated. UPR might also induce the production of various pro-inflammatory cytokines such as TNF-α, IL-6, IFN-γ etc. (Garg et al., 2012). Furthermore, UPR can contribute to the activation of autoimmune response by generating altered antigens during the degradation of misfolded proteins, besides release of neo-antigens by apoptotic cells (Lipson et al., 2006; Todd et al., 2008). Based on these evidences, we hypothesized that Hcy induced oxidative stress and/ or ER stress in addition to other intrinsic stressors in the skin microenvironment might induce an inflammatory response and lead to melanocyte destruction in vitiligo. Hence, in the present study, we aimed to explore the effect of Hcy on the cell viability, mode of cell death, ROS generation, UPR activation, expression of cytokines and melanogenesiss in *in vitro* cultured Normal Human Melanocytes (NHM). Further, based on the findings of our population studies, we also monitored the expression of selected candidate genes such as *PSMB8, TAP1, MTHFR* and *HSP70* in Hcy treated NHM.

4.2 Materials and methods

4.2.1 Ethics statement

The study plan was approved by the Institutional Ethics Committee for Human Research (IECHR), Faculty of Science, The Maharaja Sayajirao University of Baroda, Vadodara, Gujarat, India. The importance of the study was explained to all the participants for collecting the skin biopsies for NHM and written consent was obtained.

4.2.2 Culture establishment of primary normal human melanocytes (NHM)

Primary Normal Human Melanocytes (NHM) were isolated from human foreskin samples collected during circumcision surgery and cultured *in-vitro* as per the previous protocol with minor modifications (Im *et al.*, 1993; Czajkowski *et al.*, 2007). To state it briefly, the epidermis and dermis were separated by incubating the pieces of skin biopsies in 0.25% Dispase-II protease (Sigma-Aldrich, USA) overnight at 4°C. The epidermal cell separation was prepared by incubating the epidermis at 37°C for 10 min, in 1X Trypsin Phosphate Versene Glucose (TPVG) Solution (Himedia, India). The epidermal cell suspension was centrifuged at 1,300 rpm for 5 min at room temperature and the cells were cultured in M254 medium with human melanocyte growth supplement HMGS-2 (Gibco™, Thermo Fischer Scientific, USA) and 1X antibiotic-antimycotic solution (Himedia, India). The cells were maintained at 37°C in CO_2 incubator. Melanocyte-keratinocyte mixed population started to appear in about 4-9 days. Melanocytes were then purified from keratinocytes by differential trypsinization. In addition, melanocytes were given G-418 (MP Biomedicals, France) treatment for the gradual removal of fibroblasts to obtain primary human melanocyte and further were split at a ratio of 1:3, when cell confluency reached 80%. Melanocytes were used for experiments in their 5th or 6th passage.

4.2.3 Cell viability assay

The cell viability was monitored using MTT [3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyl tetrazolium salts] (Molecular probes®, Life Technologies™, China) assay. Cells were seeded in 96-well plate at a density of about 5000 cells in each well. Cells were treated with D,L-Homocysteine (Sigma Aldrich, USA) in a dose and time dependent manner as indicated. After completion of the treatment period, MTT assay was performed as per the manufacturer's instructions. In brief, MTT was added to each well and the cells were incubated at 37°C for 4 hrs. The medium was removed and dimethyl sulfoxide (DMSO) (SRL™, India) was added to dissolve the formazan crystals and the absorbance was measured at 570 nm (Thermo Scientific Multiskan EX, Shanghai, China).

4.2.4 Cellular reactive oxygen species (ROS) estimation

Total cellular reactive oxygen species levels were monitored by using a fluorescent dye 2', 7' dichloro-dihydro-fluorescein diacetate (DCFDA). Oxidation of DCFDA by ROS converts the molecule to 2', 7' dichloro-fluorescein (DCF), which is highly fluorescent. Upon stimulation, the resultant production of ROS causes an increase in fluorescence over time. $5x10^4$ cells per well were seeded in 24-well plate. Cells were treated at the indicated time with 2mM Hcy. Post-treatment, cells were incubated with DCFDA for 20 min at 37°C. The cells were then washed with 1X PBS and the signal was captured using a fluorescence microscope (Nikon eclipse Ti2-E, Tokyo, Japan). Total cellular ROS levels was estimated by calculating corrected total cell fluorescence (CTCF) using ImageJ software.

4.2.5 Assessment of mode of cell death by AnnexinV-FITC/PI dual staining

Apoptotic and necrotic cell death can be differentiated by Annexin-V /PI dual staining method. About $5x10^4$ cells per well were seeded in a 24-well plate and the cells were treated with 2mM Hcy for the indicated time. Post treatment, cells were stained with Annexin-V/FITC and PI using Annexin V-FITC/PI apoptosis detection kit (Molecular Probes™, USA) as per the manufacturer's instructions. Fluorescence signals were monitored using a fluorescence microscope (Nikon eclipse Ti2-E, Tokyo, Japan).

4.2.6 Gene expression analysis

About $2x10^5$ cells per well were seeded in 6-well plates and treated with 2mM Hcy for the indicated duration. There after the total RNA was isolated from the cultured NHM using Trizol® reagent (Invitrogen, Carlsbad, CA, USA), and cDNA was prepared using High-Capacity cDNA Reverse Transcription kit (Applied Biosystems™, USA) according to manufacturer's instructions. The transcript levels of different genes in treated and untreated cells were estimated by SYBR green method, using real-time PCR and gene specific primers (Eurofins, Bangalore, India) as shown in Table 4.1. β-Actin (*ACTB)* was considered as a housekeeping gene. Real-time PCR was performed in duplicates in 20 µl volume using LightCycler®480 SYBR Green I Master Mix following the manufacturer's instructions and carried out in the Light Cycler 480 Real-Time PCR (Roche Diagnostics GmbH, Mannheim, Germany).

Table 4.1: Details of primers used for gene expression analysis.

Primer	Sequence (5' to 3')	Amplicon size(bp)
ACTB FP	ACTCTTCCAGCCTTCCTTCC	104
ACTB RP	CGTACAGGTCTTTGCGGATG	
U-XBP1 FP	TCCGCAGCACTCAGACTAC	Unspliced:186 Spliced:163
S-XBP1 FP	GAGTCCGCAGCAGGTGC	
XBP1 RP	TGGCAGGCTCTGGGGAAG	
GRP78 FP	ACCACCTACTCCTGCGTC	152
GRP78 RP	TTGGAGGTGAGCTGGTTCT	
CHOP FP	CCTGGAAATGAAGAGGAAGAATC	135
CHOP RP	ACTGGAATCTGGAGAGTGAGG	
GADD34 FP	AGCCGCCCAGACACATGGCCC	281
GADD34 RP	AGGGCCTCCACTGTCTTCAGCCTC	
PSMB8 FP	TCCTACATTAGTGCCTTACGGG	135
PSMB8 RP	CAGATAGTACAGCCTGCATTCC	

TAP1 FP	GGACCACTAGTATTTCAGGTATGC	149
TAP1 RP	GAGCAGTACCTCCACAGCC	
HSP70 FP	TGAAGAAGGGTCAAGTGACTGTG	162
HSP70 RP	ACTGAAAACTGAGCTATAGCAGG	
MITF FP	CAAATGATCCAGACATGCGCTGG	180
MITF RP	CTCGAGCCTGCATTTCAAGTTCC	
TYR FP	AGCACCCCACAAATCCTAACTTAC	92
TYR RP	ATGGCTGTTGTACTCCTCCAATC	
MTHFR FP	TGCGGGGAGACCCAATAGG	129
MTHFR RP	TGGGGTAACCTGCCACACAG	
IL6 FP	AAATTCGGTACATCCTCGACGGCA	88
IL6 RP	AGTGCCTCTTTGCTGCTTTCACAC	
TNFA FP	GCCCCCAGAGGGAAGAGTTCCCCA	124
TNFA RP	GCTTGAGGGTTTGCTACAACATGGGC	
IFNG FP	TTGGAAAGAGGAGAGTGACAG	212
IFNG RP	GGACATTCAAGTCAGTTACCGA	
IL10 FP	ACCTGGGTTGCCAAGCCTT	189
IL10 RP	CCACGGCCTTGCTCTTGTT	

4.2.7 Western blot analysis

To detect gene expression at protein levels, 1.8×10^6 cells were seeded in 10 cm plates, and treated with 2 mM Hcy for indicated time. Total cell lysates were obtained by Laemmli buffer extraction. 30 µg of total protein was subjected to electrophoresis in 8-12% SDS gel under reducing conditions and subsequently transferred to polyvinylidene difluoride (PVDF) membranes (Immuno-Blot®, Bio-Rad Laboratories Inc,). The membranes were incubated with the following antibodies: p-PERK, p-eIF2α, eIF2α, CHOP, GRP78 and β-Actin. β-actin was used as a loading control. Protein bands were detected by Clarity™ Western ECL substrate (Bio-Rad Laboratories Inc) and relative densitometric analysis of the protein expression was carried out by ImageJ software.

4.2.8 Assessment of tyrosinase activity by zymography

The tyrosinase enzyme activity was assessed by zymography as previously described (Lin *et al.,* 2011). 1x10^6 cells were seeded in T25 flasks and treated with 2 mM Hcy at the indicated dose and time. The cells were washed 3 times in ice-cold PBS and lysed in cold lysis buffer (20mM sodium phosphate pH 6.8, 1% Triton X-100, and 1mM PMSF) containing a protease inhibitor cocktail. An aliquot of the lysate was used to determine the protein content by Bradford assay using BSA as standard. The proteins (30 µg) were mixed with sampling buffer (no β-mercaptoethanol or heating) and separated by 10% Native-polyacrylamide gel electrophoresis. The gel was rinsed in 200mL of 100mM sodium phosphate buffer (pH 6.8) and equilibrated at room temperature with gentle shaking. After 30min, the rinse buffer was replaced with fresh buffer. The gel was transferred to 200mL of a staining solution containing the rinse buffer supplemented with 5mM L-DOPA, and the mixture was incubated in the dark at 37^{o}C for 1h. Tyrosinase activity was visualized in the gel as a dark melanin-containing band. The signal intensity of each band was quantified densitometrically using ImageJ software.

4.2.9 Statistical analyses

All the experiments were performed independently, at least three times in triplicates on different days using different batches of cells, and data are presented as the mean ± SEM. Results were analysed by applying unpaired t-test for comparison between two groups and one-way ANOVA for comparison among three groups, as applicable using Prism 6 software (Graph Pad Software, USA) to determine the statistical significance of data. $p<0.05$ was considered statistically significant.

4.3 Results

4.3.1 Monitoring the dose and time dependent effect of Hcy on NHM viability

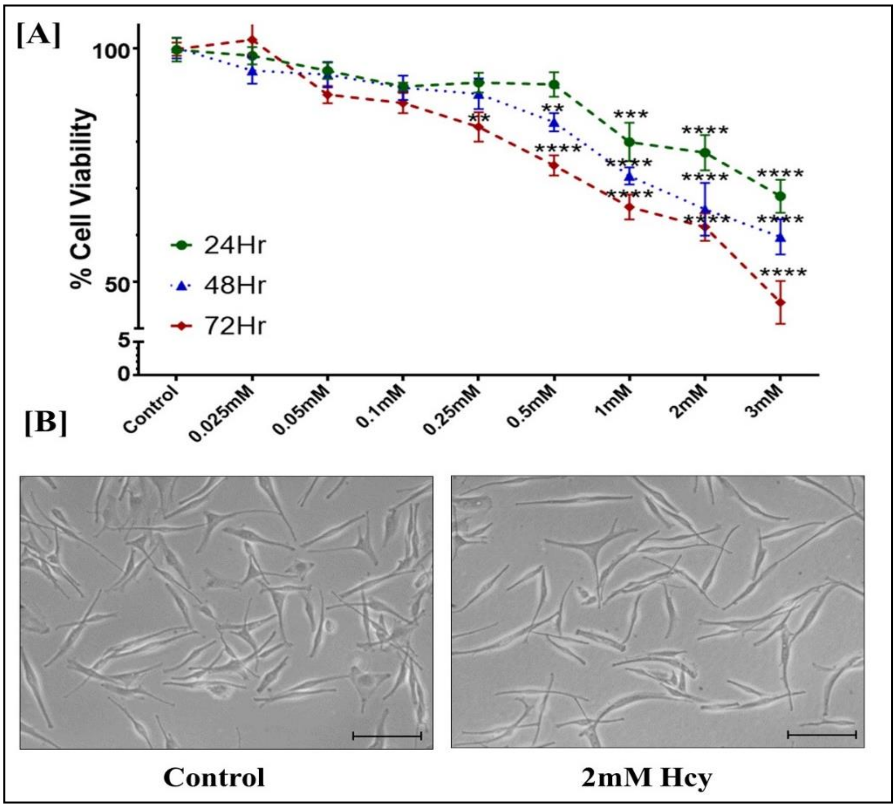

Figure 4.2: Effect of Hcy on NHM viability. [A] Dose and time dependent effect of Hcy on NHM viability. [B] Effect on NHM morphology upon 2 mM Hcy treatment for 24 hrs (Scale bar: 100 µm, Magnification: 20x). [**p<0.01, ***p<0.001, ****p<0.0001; n=3].

Initially, we assessed a dose (0.025 mM–3mM) and time dependent (24 hrs, 48 hrs and 72 hrs) effect of Hcy on NHM viability. The results suggested a significant decrease in viability of NHM as compared to untreated cells in a dose and time dependent manner upon Hcy treatment (Figure 4.2 A). The Hcy treatment with 0.25mM Hcy resulted in about a 16.6% decrease in cell viability (p=0.0034) after 72 hrs of treatment, while about 15.9% decrease (p=0.0026) was observed with 0.5mM after 48 hrs. Treatment with 1mM Hcy resulted in about a 19.7% decrease in NHM viability (p=0.0001) after 24 hrs, while 2mM Hcy resulted in about 22% (p<0.0001), 34.5% (p<0.0001) and 38% (p<0.0001) decrease in viability after 24, 48 and 72 hrs post treatment, respectively. Further, treatment of NHM with 3mM Hcy resulted in about 31.3% (p<0.0001) decrease in viability after 24hrs, 40.4% (p<0.0001) after 48 hrs and 54.2% (p<0.0001) after 72 hrs post treatment as compared to untreated cells. The minimum effective dose of 2mM Hcy was selected for further experiments. Microscopic observation revealed adverse effect of 2mM Hcy on melanocyte morphology after 24 hours treatment (Figure 4.2 B).

4.3.2 Determination of the mode of cell death and total cellular ROS levels upon Hcy treatment in NHM

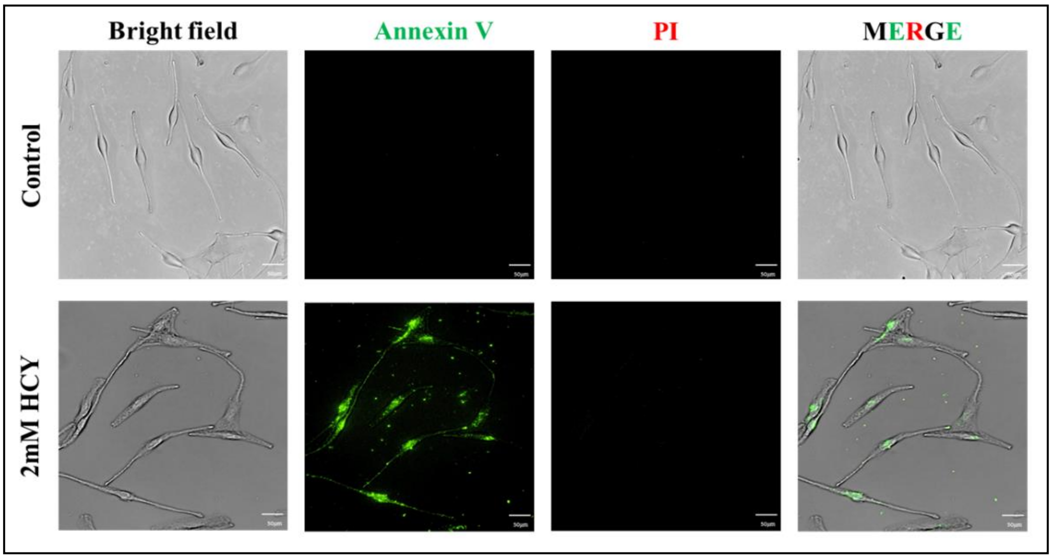

Figure 4.3: Determination of mode of cell death by Annexin-V/PI dual staining method (Scale bar: 50µm Magnification: 40X; n=3).

Annexin-V positive cells were detected upon 2mM Hcy treatment suggesting the externalization of Phosphatidyl Serine (PS). However, PI positive cells were not detected (Figure 4.3). Hence, the results suggest that Hcy induced apoptotic cell death in NHM.

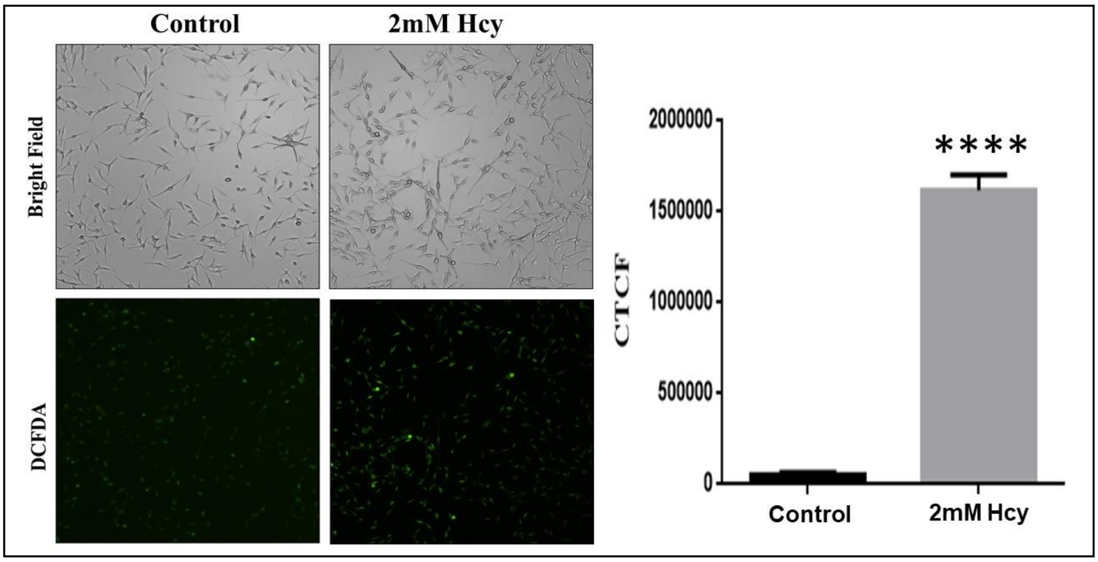

Figure 4.4: Analysis of ROS levels by DCFDA method (Magnification: 10X; CTCF: corrected total cell fluorescence). [****$p<0.0001$; n=3].

Total cellular ROS levels in NHM treated with 2mM Hcy were monitored using DCFDA method (Figure 4.4). A significant increase in cellular ROS levels was observed in NHM upon 2mM Hcy treatment for 3 hrs as compared to untreated cells (p<0.0001).

4.3.3 Exploring the ER stress induced UPR activation in Hcy treated NHM.

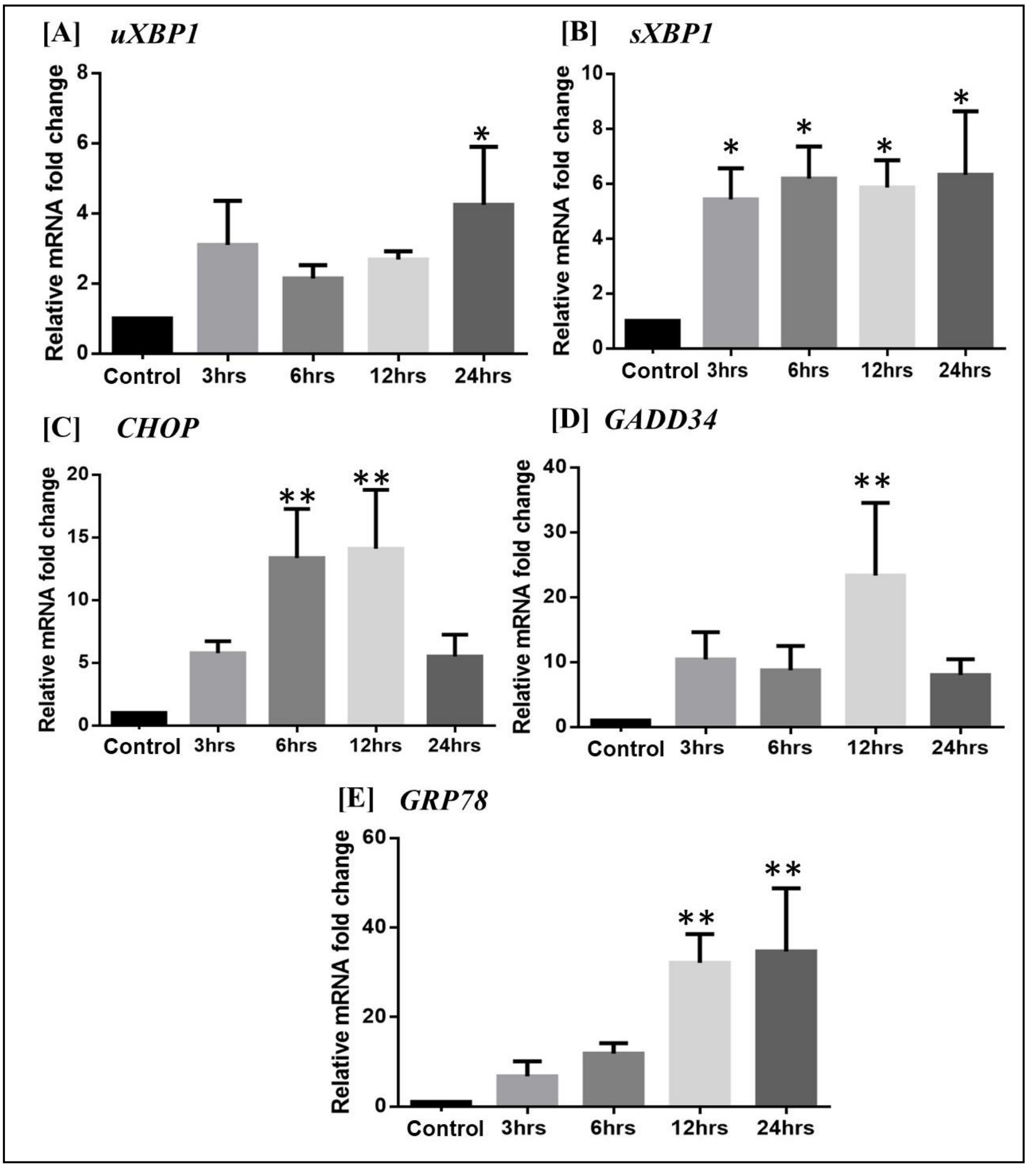

Figure 4.5: Gene expression analysis of UPR target genes. Relative gene expression analysis of [A] unspliced *XBP1*, [B] spliced *XBP1*, [C] *CHOP*, [D] *GADD34* and [E] *GRP78* was carried out after normalization with *ACTB* gene in NHM upon 2mM Hcy treatment [*p<0.05, **p<0.01; n=3].

To get an insight of Hcy induced ER stress in NHM we analysed gene expression of UPR target genes upon treatment with 2mM Hcy for 0-24 hrs (Figure 4.5). Activation of IRE1 signalling was monitored by estimating the transcript levels of spliced and unspliced *XBP1* mRNA (Figure 4.5 A & B). The results suggest significant increase in spliced *XBP1* transcript levels at 3 hrs (p=0.032), 6 hrs (p=0.014), 12hrs (p=0.024) and 24 hrs (p=0.027) whereas unspliced *XBP1* transcript levels were significantly higher at 24 hrs (p=0.032) upon Hcy treatment. Activation of PERK signalling upon Hcy treatment was assessed by monitoring the transcript levels of its downstream target genes such as *CHOP* and *GADD34* (Figure 4.5 C & D). Transcript level analysis suggest a significant increase in expression of *CHOP* at 6 hrs (p=0.002) and 12 hrs (p=0.001) post treatment. Moreover, transcript levels of *GADD34* were significantly increased at 12 hrs (p=0.007) upon Hcy exposure. Further, activation of ATF6 was monitored by estimating the transcript levels of direct transcriptional target gene *GRP78* (Figure 4.5 E). The expression of *GRP78* was significantly elevated at 12 hrs (p=0.006) and 24hrs (p=0.003) of Hcy treatment.

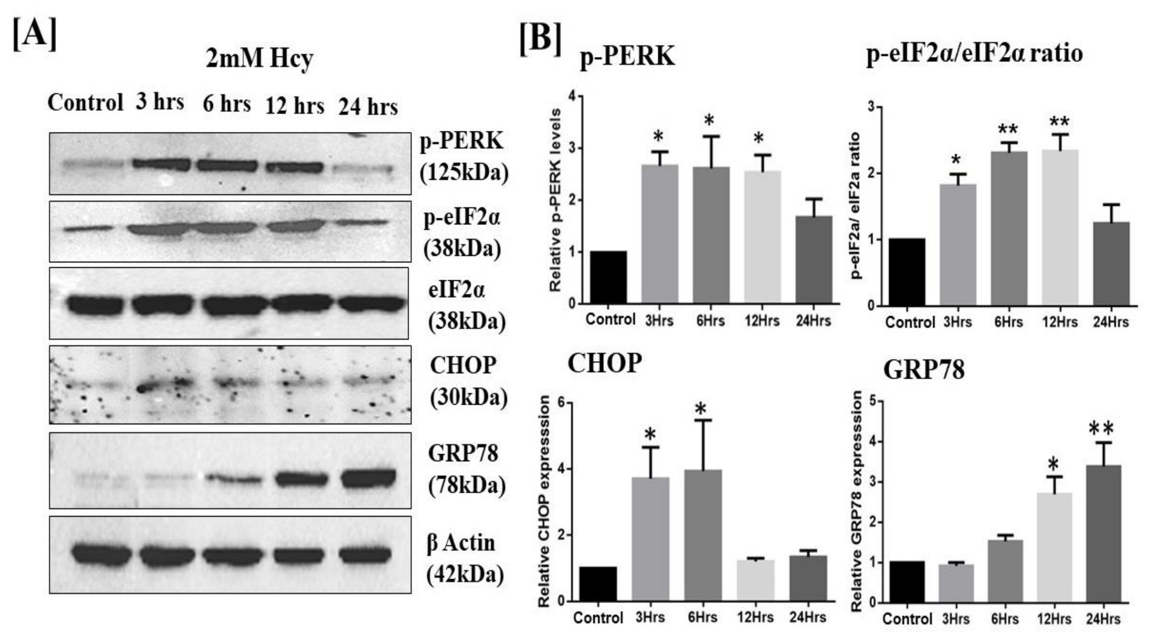

Figure 4.6: Activation of UPR signalling at protein levels by western blot analysis. [A] Representative western blot band images [B] Densitometric analysis of target proteins [*p<0.05, **p<0.01; n=3].

Activation of UPR signalling at protein levels was monitored by detecting the levels of phosphorylated-PERK, phospho-eIF2α, eIF2α, CHOP and GRP78 using β Actin as an internal control by Western blot analysis (Figure 4.6). Protein expression results showed PERK phosphorylation at 3 hrs (p=0.021), 6 hrs (p=0.017) and 12 hrs (p=0.022) after 2mM Hcy treatment as compared to untreated cells. Simultaneously, significant increase in eIF2α phosphorylation was also observed at 3 hrs (p=0.033), 6 hrs (p=0.001) and 12 hrs (p=0.001) after normalizing with total eIF2α. CHOP protein levels were significantly higher at 3 hrs (p=0.040) and 6 hrs (p=0.028) after which there was a decline. A gradual increase in GRP78 protein levels was observed after 2mM Hcy treatment leading to a significant increase at 12 hrs (p=0.043) and 24 hrs (p=0.004) as compared to untreated cells.

4.3.4 Gene expression analysis of cytokines *(TNFA, IL6, IFNG and IL10)* in NHM upon Hcy treatment

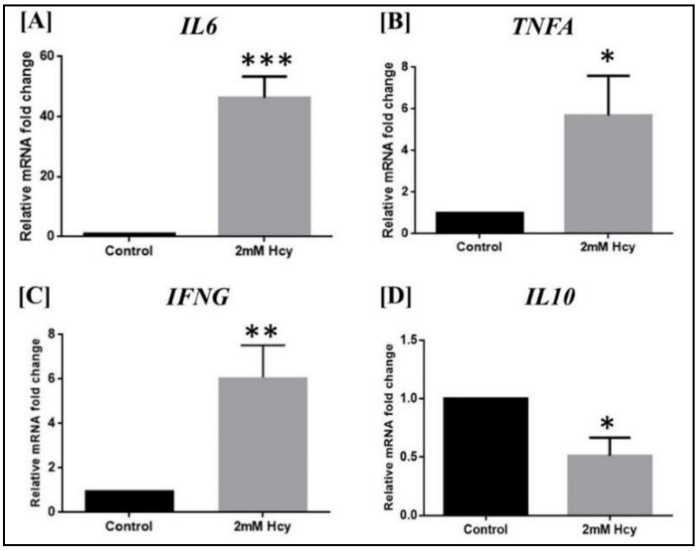

Figure 4.7: Relative gene expression analysis of [A] *IL6*, [B] *TNFA*, [C] *IFNG*, and [D] *IL10* after normalization with *ACTB* gene in NHM treated with 2mM Hcy for 24hrs [*p<0.05, **p<0.01, ***p<0.001; n=3].

To monitor the effect of Hcy on expression of cytokines, transcript levels of *IL6*, *TNFA*, *IFNG* and *IL10* were monitored after treatment of NHM with 2mM Hcy for 24 hrs (Figure 4.7). Transcript level analysis revealed significant increase in the expression of *IL6* (p=0.0006), *TNFA* (p=0.032) and *IFNG* (p=0.008) whereas expression of *IL10* was significantly decreased (p=0.014).

4.3.5 Monitoring the effect of Hcy on melanogenesis in NHM

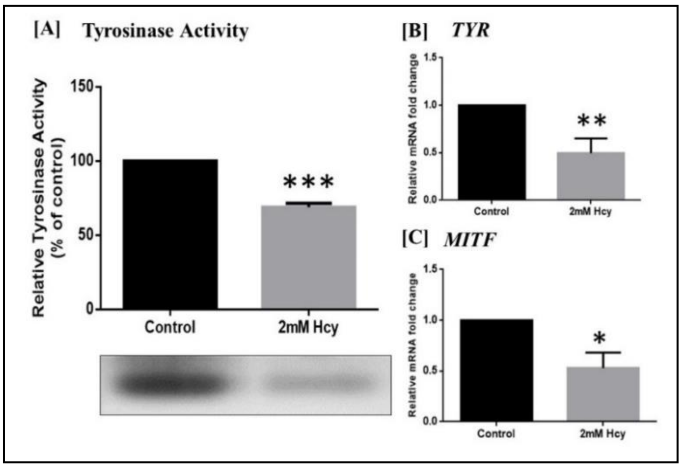

Figure 4.8: Monitoring the effect of Hcy on melanogenesis in NHM. Analysis of tyrosinase activity [A], transcript analysis of *TYR* [B] and *MITF* [C] in NHM treated with 2mM Hcy [*p<0.05, **p<0.01, ***p<0.001; n=3].

The effect of Hcy on melanogenesis was assessed by monitoring tyrosinase activity using zymography and transcript level analysis of key genes involved in melanogenesis i.e. *TYR* and *MITF*. A significant decrease (p=0.0003) of about 31% in tyrosinase activity was observed in 2mM Hcy treated NHM after 24hrs (Figure 4.8A). Interestingly, a significant decrease in *TYR* (p=0.004) and *MITF* (p=0.020) transcript levels was also observed in 2mM Hcy treated NHM after 24hrs (Figure 4.8 B & C).

4.3.6 Estimation of transcript levels of *PSMB8, TAP1, HSP70* and *MTHFR*

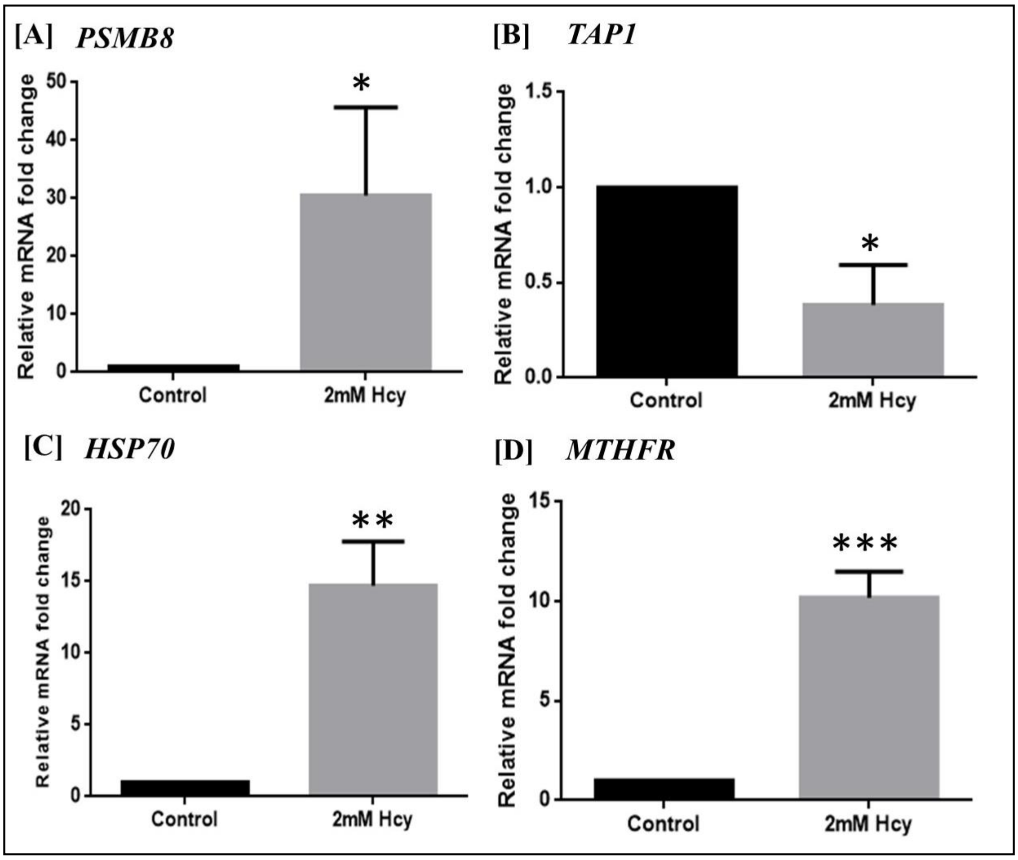

Figure 4.9: Relative gene expression analysis of [A] *PSMB8*, [B] *TAP1*, [C] *HSP70*, [D] *MTHFR* after normalization with *ACTB* gene in NHM treated with 2mM Hcy [*p<0.05, **p<0.01, ***p<0.001; n=3].

We further monitored the effect of Hcy on the expression of selected candidate genes viz., *PSMB8*, *TAP1, HSP70* and *MTHFR* upon 2mM Hcy exposure to NHM after 24 hrs (Figure 4.9). Gene expression analysis revealed a significant increase in the expression of *PSMB8* (p=0.038), *HSP70* (p=0.001) and *MTHFR* (p=0.0004), whereas the expression of *TAP1* (p=0.026) was significantly decreased upon 2mM Hcy treatment to NHM.

4.4 Discussion

Vitiligo is a multifactorial disorder related to both genetic and non-genetic factors. Vitiligo can be triggered by stress to the melanocytes, which are the pigment-producing cells of the skin. Stress can be a major reason for melanocyte loss (Yaghoobi *et al.,* 2011). Various exogenous and endogenous stressors are involved in the etiopathogenesis of vitiligo. Triggers/ stressors, which may range from sunburn to chemical exposures lead to autoimmune response that can target melanocytes. In vitiligo pathogenesis and etiology ER stress and oxidative stress both play significant role (Manga *et al.*, 2016). Cellular metabolism produces ROS (reactive oxygen species) including superoxide anions, hydrogen peroxide (H_2O_2) and hydroxyl radicals endogenously. Exogenous stressors like radiation, microorganisms, xenobiotics/drugs and phenolic chemicals are responsible for generation of these free radicals (Trouba *et al.*, 2002; Al-shobaili & Rasheed, 2015). There are numerous sources of ROS in the epidermis. H_2O_2 is one of the major ROS found in high amounts in the epidermis of patients with vitiligo. H_2O_2 is a by-product of many metabolic pathways like methionine metabolism, melanogenesis, mitochondrial processes (Schallreuter *et al.,* 1999). Homocysteine, a by-product of methionine metabolism may also generate oxidative stress via producing hydrogen peroxide, nitric oxide, superoxide anion, etc. (Loscalzo, 1996; Ramakrishnan *et al.*, 2006; Esse *et al.,* 2019). Several studies including ours have reported elevated Hcy levels in the blood as well as skin of active vitiligo patients (Anbar *et al.*, 2016; Jadeja *et al.,* 2018; Tsai *et al.,* 2019). Homocysteine may potentiate autoxidation through its highly reactive free thiol group, which participates in redox reactions at physiological pH, and synthesizes a disulphide bond between two homocysteine molecules (homocystine) or a disulphide bond with the cysteine residue of proteins (Jakubowski *et al.*, 2018). Hence, in the present study we aimed to investigate the effect of Hcy on *in-vitro* cultured primary normal human melanocytes (NHM). Our results suggested a significant decrease in melanocyte viability in a dose and time dependent manner. Moreover, we observed that Hcy exposure leads to a significant increase in total cellular ROS levels in NHM. We have selected an effective dose of 2mM Hcy for further experiments. Liu et al., have reported that under *in-vitro* conditions, a concentration of 1–10 mM Hcy is required to generate ROS and cause subsequent cell damage (Liu *et al.,* 2009). Intracellular levels of Hcy are increased transiently in human umbilical cord vein endothelial cells (HUVECs) exposed to exogenous Hcy (Outinen *et al.,* 1998). Moreover, it is reported that for a 2-6 fold transient increase in intracellular Hcy, an extracellular Hcy concentration of 1 to 5 mM would be needed in HepG2 cells (Werstuck *et al.,* 2001). Numerous reports have suggested toxic effect of Hcy on various cell types such as vascular endothelial cells, retinal ganglion cells, neuronal cells, osteoblasts etc. leading to cell death (Ramakrishnan *et al.*, 2006; Ganapathy *et al.*, 2011; Park *et al.*, 2012; Esse *et al.*, 2019). Oxidative stress causes cellular disturbances including interruption of protein maturation in the ER leading to the activation of the UPR signalling. Analysis of ER stress markers in the present study suggested an activation of all three signalling i.e. IRE1, PERK and ATF6 upon Hcy exposure to NHM. A significant increase in *XBP1* mRNA splicing suggested activation of IRE1 signalling. The active transcription factor, spliced XBP1 (sXBP1) protein directs the transcription of a large range of targets including the expression of chaperones (GRP78) as components of the ERAD pathway, to mitigate ER stress and restore homeostasis (Almanza *et al.*, 2019). GRP78 is robustly increased upon activation of UPR signalling, and it is a direct transcriptional target of cleaved ATF6 (Haze *et al.*, 1999). We observed a significant increase in the expression of GRP78 upon Hcy expression, suggesting activation of ATF6 signalling. Corroborating our results, significantly increased *XBP1* transcript levels were reported in the skin of vitiligo patients (Ren *et al.*, 2009). Furthermore, the present study showed increased phosphorylation of PERK and eIF2α in addition to elevated levels of CHOP and GADD34 suggesting activation of PERK signalling. CHOP is an active transcription factor that activates the transcription of several genes that induce apoptosis as a consequence of UPR (Zhang & Kaufman, 2008). Substantiating our study, Chen *et al.*, (2020) recently reported that Hcy may induce melanocyte apoptosis through activation of PERK signalling. Moreover, we reported earlier that CHOP expression was significantly increased in the skin of vitiligo patients (Mansuri *et al.*, 2016). Activation of CHOP, in early ER stress results in the induction of pro-survival pathways and activation of a number of corrective genes like *XBP1* and chaperones. This subsequently leads to an antioxidant response, increased ERAD, amino-acid biosynthesis, promotes the protein folding and maintains the redox homeostasis to sustain cell survival (Rutkowski & Kaufman 2003). Notably, GADD34 mediated dephosphorylation of eIF2α allows the restoration of protein synthesis upon stress recovery (Novoa *et al.*, 2001). If the stress persists for an extended period it will lead to increased expression of CHOP resulting in cell cycle arrest and further to cell death by initiating the pro-apoptotic pathway (Oyadomari & Mori, 2004). The UPR constitutes a key mechanism safeguarding the fidelity of the cellular proteome (Ron & Walter 2007; Hetz & Papa 2018). The UPR has emerged as a hallmark of several diseases, including inflammatory bowel syndrome, arthritis, neurodegenerative diseases, diabetes mellitus, stroke and cancer (Oakes & Papa 2015; Bettigole & Glimcher 2015; Grootjans *et al.*, 2016). Three major arms of UPR play a vital role in inflammation, mainly regulated by nuclear factor-kappaB (NF-κB) (Hotamisligil & Erbay 2008; Walter & Ron 2011; Verfaillie *et al.,* 2013). NF-κB plays a vital role as a master transcriptional regulator in all pro-inflammatory pathways and involved in encoding crucial pro-inflammatory cytokines and immunomodulatory genes. It has also been suggested that melanocytes from vitiligo patients have intrinsic defects that reduce the capacity to manage cellular stress, resulting in increased ROS production and UPR induction, which in turn activate the innate inflammation (Park *et al.,* 2019). Elsherbiny *et al.,* (2020) have demonstrated that hyperhomocysteinemia induces expression of pro-inflammatory cytokines such as IL-1β, IFN-γ, TNF-α, IL-6 in mouse brain and retina in addition to cultured retinal and microglial cells. In the present study, we observed a significantly increased expression of pro-inflammatory cytokines *IL6, TNFA* and *IFNG* and a decrease in expression of anti-inflammatory cytokine *IL10* on exposure of NHM to 2mM Hcy. Incidentally, IFN-γ, IL-6 and TNFα are the pro-inflammatory cytokines implicated in melanocyte destruction in vitiligo. Elevated levels of these cytokines and decreased anti-inflammatory cytokine IL-10 have been reported in the skin as well as blood of vitiligo patients (Yu *et al.,* 1997; Tu *et al.,* 2003; Laddha *et al.,* 2012; Singh *et al.,* 2018). Toosi et al., (2012) have reported a significant increase in the expression of IL6 upon *XBP1* activation in 4-TBP treated melanocytes. We further monitored the effect of Hcy on melanogenesis. A significant decrease in tyrosinase activity and expression of key genes involved in melanogenesis (*TYR* and *MITF*) was observed upon Hcy treatment of NHM. These results suggest a possible inhibition of melanogenesis in NHM by Hcy. An earlier study shows that tyrosinase activity is inhibited by elevated levels of homocysteine (Reish *et al.,* 1995). Recent findings of Chen et al., also suggest that Hcy interferes with the melanogenesis pathway leading to decreased melanin content in NHM (Chen *et al.,* 2020). Reish *et al.,* (1995) speculated that decreased tyrosinase activity might be due to the interaction of Hcy with copper at the active site of the TYR enzyme inhibiting its activity. Proteasome subunit beta 8 (PSMB8) and transporter associated with antigen processing 1 (TAP1), involved in antigen processing and presentation were found to be associated with several autoimmune diseases including vitiligo (Djilali-Saiah *et al.,* 1994; Casp *et al.,* 2003; Krämer *et al.,* 2007; Jadeja *et al.,* 2017). In the present study, we found significantly increased expression of *PSMB8.* However, *TAP1* expression was significantly decreased in NHM upon Hcy exposure. Lundh *et al.,* (2017) have reported that expression of immunoproteasome subunits including *PSMB8* is induced upon exposure of cytokines leading to apoptosis in human islets. Bartoszewski *et al.,* (2011) have reported that *XBP1* induced miRNA targets *TAP1* gene leading to its downregulation under ER stress conditions which might in turn result in decreased expression of MHC-I molecules. The appropriate MHC class I expression is necessary for self-tolerance and abnormalities in such expression may lead to autoimmunity (Fu *et al.*, 1993). Significantly increased expression of *HSP70* has been reported in the skin of vitiligo patients (Thörneby-Andersson *et al.,* 2000; Mosenson *et al.,* 2012; Mansuri *et al.,* 2016). We observed a significant and concomitant increase in HSP70 upon Hcy treatment. It has been reported that Hcy induces the expression of stress response genes and chaperones including HSP70 in neuronal cells (Althausen & Paschen 2000). Kroll et al., have reported enhanced expression of HSP70 in melanocytes upon exposure to 4-tertiary butyl phenol (4-TBP) (Kroll *et al.,* 2005). Further, Asea et al., (2000) reported that HSP70 induced monocytes/macrophages showed secretion of cytokines such as IL-1, IL-6, and TNF-α. Methylene tetrahydrofolate reductase (MTHFR) is an important regulatory enzyme involved in the conversion of homocysteine to methionine. It catalyses the reduction of 5, 10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, thereby decreasing the Hcy levels (Goyette *et al.,* 1994). We observed a significant increase in *MTHFR* expression upon Hcy treatment to NHM. Interestingly, it was found that ER stress led to increased MTHFR expression via the IRE1 transducer (Leclerc & Rozen, 2008). The elevated *MTHFR* expression upon Hcy treatment might be to reduce Hcy-induced stress. The up-regulation of *MTHFR* through UPR might contribute to amino acid sufficiency by supplying methionine for protein synthesis, allowing the cells to combat the metabolic consequences of the ER stress (Lenz *et al.,* 2006).

Although, Vitiligo pathogenesis is well addressed, much of it is still a conundrum. The present study suggests that Hcy can hamper the melanocyte homeostasis through oxidative stress and ER stress that further affects the expression of cytokines and genes related to melanogenesis in NHM. Overall, we speculate that Hcy along with other intrinsic stressors and inherent defects in melanocytes of vitiligo patients might be playing a vital role in melanocyte destruction.

4.5 References

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