6.1 INTRODUCTION

As the largest organ in the human body, skin provides important protection against physical and/or chemical trauma from the environment (Lippens *et al*., 2009). The skin comprises of epidermis, the dermis, and a basement membrane separating them. The majority of the basement membrane is composed of melanocytes originating from neural crest cells (Fuchs and Raghavan, 2002; Proksch *et al*., 2008). Owing to their location, melanocytes are vulnerable to be attacked by a large number of exogenous chemicals (Bickers and Athar, 2006). These environmental toxicants or their metabolites are inherent oxidants driving the production of various reactive oxygen species (ROS), including hydrogen peroxide (H_2O_2) (Bogeski *et al*., 2012). ROS induce apoptosis in melanocytes, indicating that apoptosis may be an important mechanism in the destruction of melanocytes (Yang *et al*., 2000; Jian *et al*., 2011; Liu *et al*., 2012). Melanocytes serve as ROS scavengers and play a role in the skin immune system (Kobayashi *et al*., 1998), secreting a wide range of signal molecules and responding to growth factors and cytokines (Choi *et al*., 2013). However, uncontrolled release of ROS drives the induction of oxidative stress that can cause melanocytes to become susceptible to apoptosis and may be critical for the pathogenesis of human skin disorders including vitiligo (Briganti and Picardo, 2003; Rezaei *et al*., 2007; Spritz, 2008; van den Boorn *et al*., 2009; Jin *et al*., 2010; Wu *et al*., 2013; Mosenson *et al*., 2013).

Multiple factors contribute to susceptibility for vitiligo (Le Poole and Boissy, 1997). Several evidences demonstrate a correlation between environmental factors and onset of vitiligo (Manga *et al*., 2006). Vitiligo patients frequently refer to skin trauma as an initiating factor for their disease. Following melanocyte loss, the skin is deprived of pigment protection, leaving it more susceptible to solar damage, and occasionally, compromised immunity may result (Nordlund, 1992). Melanocyte overexposure to ultraviolet rays may cause deregulation of melanization and/or of mitosis, inducing a stress response in the cell (Jean *et al*, 2001). Sites of mechanical stress will express elevated levels of stress proteins (Kippenberger *et al*, 1999). Burns and cuts have been documented as initiation sites for progressive depigmentation, and the Koebner phenomenon is often observed in vitiligo patients (Le Poole and Boissy, 1997). Finally,

in individuals sensitive to bleaching phenols, exposure to phenolic compounds in the workplace can cause what has been coined "occupational vitiligo" (Boissy and Manga, 2004). In contact/occupational vitiligo, the environmental trigger is exposure to specific chemotoxins, which include phenol and catechol derivatives (Gellin and Maibach, 1985; Cummings and Nordlund, 1995; Boissy and Manga, 2004) such as 4-*tert*-butylphenol (4-TBP) (James *et al*., 1977). A number of these chemicals induce cellular oxidative stress. Thus, melanocyte susceptibility to oxidative stress may play a role in the pathogenesis of vitiligo.

Skin trauma leads to oxidative stress, and evidence of oxidative stress has been demonstrated in both skin (Schallreuter *et al.*, 1999) and blood (Beazley *et al*., 1999) of patients with vitiligo. H_2O_2 accumulates in the epidermis of patients, concomitant with reduced levels of catalase (Schallreuter *et al.*, 1999). Epidermal levels of additional antioxidants, including ubiquinol, vitamin E, and reduced glutathione (GSH) were also found to be significantly reduced in active vitiligo (Passi *et al*., 1998). Altered antioxidant levels, including catalase (CAT), GSH, glutathione peroxidase (GPX), glucose 6-phosphate dehydrogenase (G6PD) and superoxide dismutase (SOD), and lipid peroxidation (LPO), a hallmark of ROS-induced damage, was observed in vitiligo patients (Koca *et al*., 2004; Agrawal *et al*., 2004; Shajil and Begum, 2006; Shajil *et al*., 2007; Laddha *et al*., 2013; Laddha *et al*., 2014; Mansuri *et al*., 2016), all suggesting oxidative stress in patients with vitiligo. These conditions may induce expression of stress proteins including heat shock protein 70 (HSP70) and will enhance the activity of anti-oxidant enzymes to protect the cell (Currie and Tanguay, 1991; Calabrese *et al*, 2001; Renis *et al*, 2003).

G6PD plays an essential role in the regulation of oxidative stress by primarily regulating NADPH, the main intracellular reductant (Tian *et al*., 1998 & 1999). Importantly, it has been shown that modest changes in G6PD activity itself have significant effects on cell growth and cell death in a variety of cell types (Tian *et al*., 1998 & 1999). Thus, proper activity of G6PD is required for adequate defense against oxidative stress and prevention of cell damage/death. Inhibition of G6PD by 6-aminonicotinamide (6-ANAD) showed increased cell death in bovine aortic endothelial cells (Zhang *et al*., 2000). Recently,

Tsouko *et al*. (2014) showed inhibition of G6PD by 6-ANAD suppressed prostate cancer cells (LNCaP) proliferation. G6PD per se was not evaluated in melanocytes, nor was the effect of G6PD inhibition reported in melanocytes. Thus, we hypothesize that under the condition of 6-ANAD with impaired G6PD activity, melanocytes would be predisposed to oxidant damage and cell death. G6PD inhibition was also shown to decrease intracellular free calcium ($[Ca^{2+}]_i$) in vascular smooth muscle cells (Ata *et al*., 2011).

The transient receptor potential cation channel, subfamily M, member 1 (TRPM1) is a constitutively active Ca^{2+} channel, which is highly expressed in melanocytes and its activity is critical for melanocyte homeostasis (Devi *et al*., 2009). Schallreuter *et al*., (1996) have shown that cells from lesional skin showed decreased Ca^{2+} uptake compared to non-lesional of vitiligo patients and control skin. Moreover, it has been reported that the *TRPM1* expression is MITF dependent (Levy *et al*., 2010). The microphthalmia-associated transcription factor (MITF) is considered as the "master regulator of the melanocyte," (Widlund and Fisher, 2003), controlling expression of the major melanocyte-specific proteins required for melanin synthesis including tyrosinase (TYR) and tyrosinase-related protein 1 (TYRP1) (Fang and Setaluri, 1999). The expression of *MITF* results in a reduced response to oxidative stress induced by H_2O_2 (Jimenez-Cervantes *et al*., 2001), suggesting a role for this transcription factor in melanocyte survival and response to oxidative stress. However, the mechanisms underlying the aberrant responses induced by ROS-mediated melanocyte loss are not completely understood. Hence, there is a need to understand the process of melanocyte destruction during oxidative stress.

Therefore, the present study is aimed to evaluate melanocyte viability and to investigate the mRNA expression levels of anti-oxidant genes (*CAT, GPX1, G6PD* and *PRDX3*), stress related genes (*HSP60, HSP70, SERP1, SIRT1* and *POLH*) and melanocyte specific genes (*MITF, TYR, TYRP1, TRPM1, EDN1* and *LAMP1*) in NHM, PIG1 and PIG3V cells under H_2O_2 induced oxidative stress and G6PD inhibition.

6.2 MATERIALS AND METHODS

6.2.1 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 participants and written consent was obtained from all subjects before performing the studies.

6.2.2 Culture establishment of primary normal human melanocytes (NHM)

Melanocytes were isolated from human skin biopsy samples and cultured successfully using the standard protocol with slight modifications (Im and Park, 1992; Czajkowski *et al*., 2007). Briefly, the epidermis was separated from the dermis after an overnight incubation of skin biopsies in 0.25% Dispase II protease (Sigma Aldrich, USA) prepared in phosphate buffer saline (PBS) (Himedia, India) at 40˚C. In order to separate epidermal cells, the epidermis was incubated at 37˚C for 10 min, in a solution of 1X trypsin phosphate versene glucose solution (Himedia, India). Cellular suspension was centrifuged at 1,300 rpm for 5 min at room temperature to harvest cells. Melanocytes were selectively cultured in M254 medium with human melanocyte growth supplements (Gibco® life Technologies™, Cascade Biologics™, Portland, Oregon) and 1X antibiotic-antimycotic solution (Himedia, India). The cells were maintained in a humidified incubator with 5% CO_2 at 37˚C and media replenishment was given after every 2-3 days. Melanocyte-keratinocyte mixed population starts appearing around days 4-9. Melanocytes were purified from keratinocytes by differential trypsinisation, which is based on the more sturdy and adherent property of keratinocytes. In addition, melanocytes were given G418 (Geneticin ® Disulfate Salt, MP Biomedicals, France) treatment for gradual removal of fibroblasts to obtain primary human melanocyte and further were splitted at 1:3 ratio, when cell confluency reached 80%. Media was replenished after every 48-72 hrs; melanocytes cultured up to the fifth passage were used for experiments.

Confirmation of melanocytes by DOPA staining:

The pure culture of melanocytes was confirmed by L-DOPA (L-3,4-dihydroxy phenylalanine, Hi-Media, India) staining (Iijima and Watanabe, 1957). NHM were

trypsinized, plated on coverslips and cultured for ~3 days before detection of tyrosinase activity. For DOPA reaction, culture media was removed and cells were rinsed twice with PBS, fixed for 20 min in 4% formaldehyde solution in PBS, washed three times with PBS and then incubated at 37°C for 18 hrs in the dark with 10 mM L-DOPA. After incubation, the cells were rinsed with distilled water, dehydrated, mounted and number of cells positive for tyrosinase activity were observed using light microscopy.

6.2.3 Human Melanocyte Cell lines

Immortalized human melanocyte cell lines PIG1 (derived from healthy individual) and PIG3V (derived from vitiligo patient) (a gift from Dr. I.C. Le Poole, Loyola University, Chicago, Illinois) were cultured as described by Le Poole (Le Poole *et al*., 1997; Le Poole *et al*., 2000).

6.2.4 MTT assay

The number of viable cells was recorded using MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium salts], as described by Mosmann (Mosmann, 1983). NHM/ PIG1/ PIG3V were seeded in 96-well plates at an initial density of 5×10^3 cells per well and after overnight incubation, cells were treated with cumene H_2O_2 (Sigma Aldrich, USA) or 6-amino nicotinamide (6-ANAD) (MP Biomedicals, France) in dose dependent manner (10, 20, 40, 60, 80 & 100 μM H_2O_2 or 0.5, 1, 2, 4 & 5 mM 6-ANAD). After 24 hrs of treatment, MTT (Molecular probes® by Life Technologies™, China) was added to each well and the cells were incubated at 37°C for 4 hrs. The medium was removed and dimethyl sulfoxide (DMSO) (Sisco Research Laboratories, Mumbai) was added to dissolve the formazan crystals. The absorbance was measured at 570 nm using ELISA plate reader (Thermo scientific Multiskan EX, Shanghai, China).

6.2.5 RNA isolation and cDNA synthesis

Total RNA from NHM/ PIG1/ PIG3V cells was extracted using RNA isolation kit (Ambion®, Carlsbad, CA, USA) following the manufacturer's instructions. cDNA synthesis was performed using Verso cDNA Kit (Thermo Fisher Scientific Inc., USA) according to the manufacturer's instructions in the Mastercycler Gradient PCR (Eppendorf, Germany). Details are described in 'Chapter II'.

6.2.6 mRNA Expression

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 1. Expression of *GAPDH* gene was used as a reference. Real-time PCR was performed in duplicates in 20 µl volume using LightCycler®480 SYBR Green I Master following the manufacturer's instructions and carried out in the Light Cycler 480 Real-Time PCR (Roche Diagnostics GmbH, Mannheim, Germany). Details are described in 'Chapter II'.

6.2.7 Data analysis

All the experiments were performed at least three times in triplicates independently on different days using different batches of cells, and data are presented as the mean ± SEM. To evaluate the MTT or gene expression results, the absorbance or ΔCt values respectively, were compared between different groups and analysis was plotted and analyzed by nonparametric unpaired t-test using Prism 4 software (Graph Pad Software, USA, 2003) to determine the statistical significance of data. Statistical power of detection of association with the disease at 0.05 level of significance was determined by using G* Power software (Faul *et al*., 2007). *p*-values less than 0.05 were considered as statistically significant. Fold change in mRNA was calculated according to $2^{-\Delta\Delta Ct}$ method.

Table 1. Details of primers used for mRNA expression of the candidate genes.

Gene	Forward/ reverse primer	Primer Sequence (5' to 3')	Annealing Temperature (°C)	Amplicon size (bp)
EDN1	FP	ACTTCTGCCACCTGGACATCA	63	91
	RP	TCCAAGGCTCTCTTGGACCTAG		
EZR	FP	TCCCTC AAAGAG TGATGG ACCAG	65	100
	RP	TTA TCT TTG AGC ATC CCA CGG TG		
LAMP1	FP	GCGAGCTCCAAAGAAATCAA	63	95
	RP	TGGACCTGGGTGCCACTAA		
POLH	FP	ATCATGGAAGGGTGGTGGAAT	63	167
	RP	TGGCTTCCCGGTACTTGG		
SIRT1	FP	ACG CTG GAA CAG GTT GCG G	64	168
	RP	AAG CGG TTC ATC AGC TGG GC		
SERP1	FP	TCGCCAAGACCTCGAGAAATG	62	101
	RP	CTGGAAAATTGCAGAACCACAGAC		
TYR	FP	AGCACCCCACAAATCCTAACTTAC	63	92
	RP	ATGGCTGTTGTACTCCTCCAATC		
TRPM1	FP	ACTCTAACAGGTGTTGCTGTGG	62	153
	RP	CTGTTGGGTAGCTCTGGGTG		
HSP60	FP	CTGGTGGTGCAGTGTTTGG	62	269
	RP	TGTCCCACCAACCTTCAGC		
HSP70	FP	TGAAGAAGGGTCAAGTGACTGTG	62	162
	RP	ACTGAAAACTGAGCTATAGCAGG		
PRDX3	FP	TTCAGCACCAGTTCCTCATG	60	168
	RP	AGGACACACAAAGGTGAAATCC		
TYRP1	FP	TTT GTA ACA GCA CCG AGG ATG	62	192
	RP	TGG GGT CAC TGT AAC CTT CCA C		
G6PD	FP	TGAGCCAGATAGGCTGGAA	63	225
	RP	TAACGCAGGCGATGTTGTC		
MITF	FP	CAAATGATCCAGACATGCGCTGG	61	180
	RP	CTCGAGCCTGCATTTCAAGTTCC		
CAT	FP	TAAGACTGACCAGGGCATC	63	201
	RP	CAAACCTTGGTGAGATCGAA		
GPX1	FP	GTTTGGGCATCAGGAGAACGCC	64	147
	RP	AGGAAGGCGAAGAGAGGGTGC		
GAPDH	FP	ATCCCATCACCATCTTCCAGGA	65	122
	RP	CAAATGAGCCCCAGCCTTCT		

'FP': forward primer; 'RP': reverse primer; 'bp': base pair.

6.3 RESULTS

6.3.1 Dose dependent effect of H_2O_2 on Melanocyte viability

NHM/ PIG1/ PIG3V cells were treated with different doses of H_2O_2 (10, 20, 40, 60, 80 & 100 μM) and observed after 24 hrs for viability (n=3). The NHM showed significant decrease in viability (mean ± SEM) upon: 90.94 ± 1.50 % at 10 μM (p=0.049), 79.08 ± 4.37 % at 20 μM (p=0.016), 76.40 ± 5.81 % at 40 μM (p=0.022), 66.10 ± 2.48 % at 60 μM (p=0.0009), 62.96 ± 6.95 % at 80 μM (p=0.008) and 53.83 ± 7.09 % at 100 μM (p=0.004) H_2O_2 treatment for 24 hrs as compared to untreated NHM (Figure 1A). The PIG1 cells showed significant decrease in viability (mean ± SEM) upon: 85.77 ± 1.60 % at 10 μM (p= 0.036), 76.78 ± 3.43 % at 20 μM (p=0.014), 72.17 ± 1.90 % at 40 μM (p=0.006), 57.71 ± 1.03 % at 60 μM (p=0.002), 52.74 ± 6.93 % at 80 μM (p=0.005) and 53.60 ± 8.23 % at 100 μM (p=0.008) H_2O_2 treatment for 24 hrs as compared to untreated cells (Figure 1B). The PIG3V cells showed significant decrease in viability (mean ± SEM) upon: 78.16 ± 3.27 % at 10 μM (p= 0.008), 56.63 ± 2.32 % at 20 μM (p= 0.0003), 55.44 ± 3.57 % at 40 μM (p= 0.0006), 57.23 ± 2.54 % at 60 μM (p= 0.0004), 53.80 ± 3.35 % at 80 μM (p= 0.0005) and 47.71 ± 3.54 % at 100 μM (p= 0.0002) H_2O_2 treatment for 24 hrs as compared to untreated cells (Figure 1C).

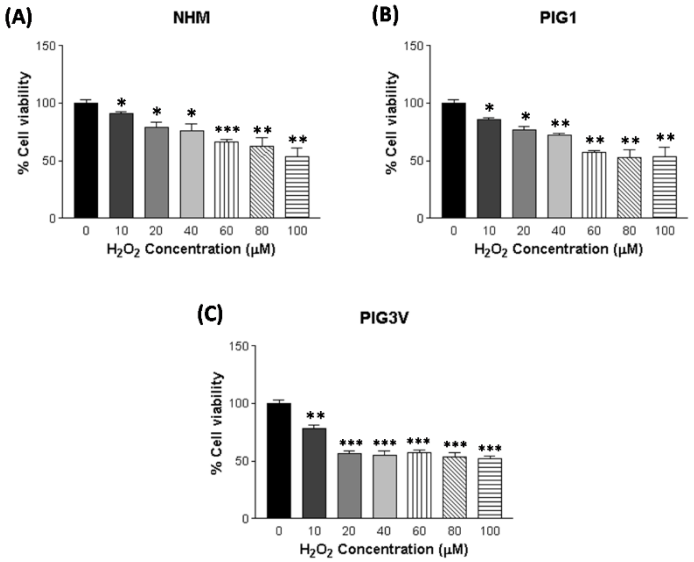

Figure 1. Dose dependent effect of H_2O_2 on melanocyte viability: (A) NHM, (B) PIG1 and (C) PIG3V cells showed significantly decreased viability upon 10, 20, 40, 60, 80 and 100 μM H_2O_2 treatments for 24 hrs as compared to untreated cells (n=3).

6.3.2 Dose dependent effect of 6-ANAD on Melanocyte viability

NHM/ PIG1/ PIG3V cells were treated with different doses of 6-ANAD (0.5, 1, 2, 4 & 5 mM) and observed after 24 hrs for viability (n=3). The NHM showed significant decrease in viability (mean ± SEM) upon: 70.55 ± 1.03 % at 0.5 mM (p=0.0007), 68.52 ± 0.82 % at 1 mM (p=0.0005), 67.75 ± 0.44 % at 2 mM (p=0.0004), 64.98 ± 3.24 % at 4 mM (p=0.001) and 60.41 ± 1.12 % at 5 mM (p=0.0002) 6-ANAD treatment for 24 hrs as compared to untreated NHM (Figure 2A).

The PIG1 cells showed significant decrease in viability (mean ± SEM) upon: 69.40 ± 6.15 % at 0.5 mM (p= 0.014), 66.91 ± 4.93 % at 1 mM (p= 0.008), 70.21 ± 1.52 % at 2 mM (p= 0.005), 66.64 ± 3.69 % at 4 mM (p= 0.006) and 67.03 ± 2.49 % at 5 mM (p= 0.004) 6-ANAD treatment for 24 hrs as compared to untreated cells (Figure 2B).

The PIG3V cells showed significant decrease in viability (mean ± SEM) upon: 58.93 ± 3.95 % at 0.5 mM (p= 0.0006), 59.84 ± 3.77 % at 1 mM (p= 0.0005), 54.73 ± 3.17 % at 2 mM (p= 0.0002), 55.46 ± 4.50 % at 4 mM (p= 0.0006) and 54.36 ± 2.950 % at 5 mM (p= 0.0001) 6-ANAD treatment for 24 hrs as compared to untreated cells (Figure 2C).

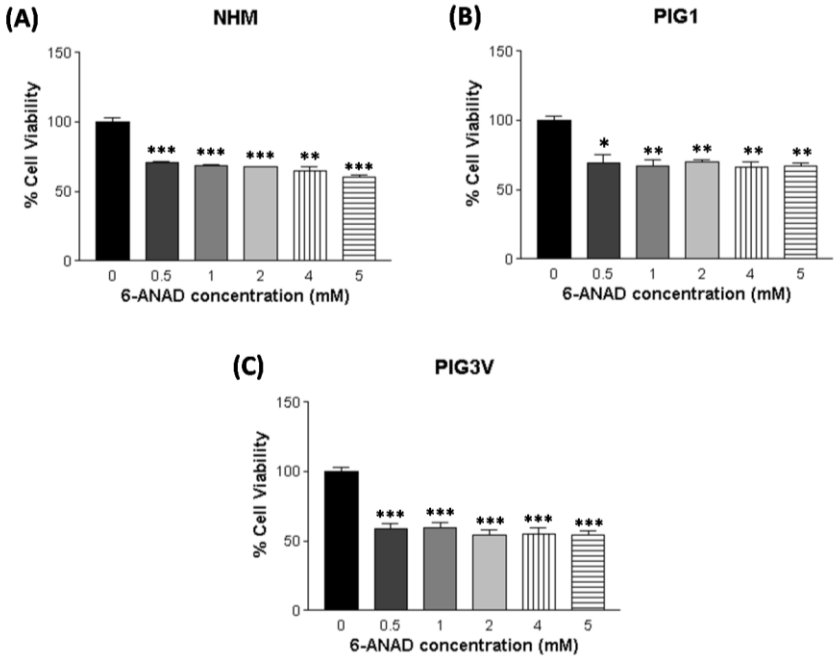

Figure 2. Dose dependent effect of 6-ANAD on melanocyte viability: (A) NHM, (B) PIG1 and (C) PIG3V cells showed significantly decreased viability upon 0.5, 1, 2, 4 and 5 mM 6-ANAD treatment for 24 hrs as compared to untreated cells (n=3).

6.3.3 Gene expression profile of Melanocytes under normal condition

The mRNA expression levels of anti-oxidant genes (*CAT, GPX1, G6PD* and *PRDX3*), stress related genes (*HSP60, HSP70, SERP1, SIRT1* and *POLH*) and melanocyte specific genes (*MITF, TYR, TYRP1, TRPM1, EDN1* and *LAMP1*) in NHM, PIG1 and PIG3V cells were analyzed and compared (Figure 3). However, there was no difference in the expression of *CAT, GPX1, G6PD* and *PRDX3* between NHM and PIG1 cells (p= 0.976, p=0.7458, p=0.128, p=0.983 respectively; Figure 3A). Further, no difference was observed in the expression of *CAT, GPX1, G6PD* and *PRDX3* between NHM and PIG3V cells (p=0.318, p=0.867, p=0.714 and p=0.664 respectively). Also, there was no difference in the expression of *CAT, GPX1, G6PD* and *PRDX3* between PIG1 and PIG3V cells (p=0.217, p=0.883, p=0.070 and p=0.666 respectively).

PIG3V cells showed significantly higher expression of *HSP70* and *SERP1* as compared to PIG1 cells (p=0.015 and p=0.049 respectively; Figure 3B). However, there was no difference observed for *HSP70* and *SERP1* expression between NHM and PIG3V cells (p= 0.095 and p=0.447 respectively). Also, there was no difference observed in the expression of *HSP60, SIRT1* and *POLH* between NHM and PIG3V cells (p=0.914, p=0.312 and p=0.287 respectively). Further, no difference was observed in the expression of *HSP60, HSP70, SERP1, SIRT1* and *POLH* between PIG1 and NHM (p=0.4, p=0.091, p=0.054, p=0.875 and p=0.217). Also, there was no difference observed in the expression of *HSP60, SIRT1* and *POLH* between PIG1 and PIG3V cells (p=0.355, p=0.355, p=0.951).

The PIG3V cells showed significantly lower expression of *TYR, TRPM1* and *EZR* as compared to NHM (p=0.031, p=0.012 and p=0.022 respectively). However, there was no difference in the expression of *MITF, TYRP1, EDN1* and *LAMP1* between PIG3V and NHM (p=0.155, p=0.134, p=0.647 and p=0.151 respectively). Further, no difference was observed in the expression of *MITF, TYRP1, TRPM1, EDN1, EZR* and *LAMP1* between PIG1 and NHM (p=0.197, p=0.405, p=0.986, p=0.968, p=0.331, p=0.692 and p=0.685 respectively). Also, there was no difference observed in the expression of *MITF, TYRP1, TRPM1, EDN1, EZR* and *LAMP1* between PIG3V and PIG1 cells (p=0.725, p=0.179, p=0.216, p=0.022, p=0.564, p=0.125 and p=0.273 respectively).

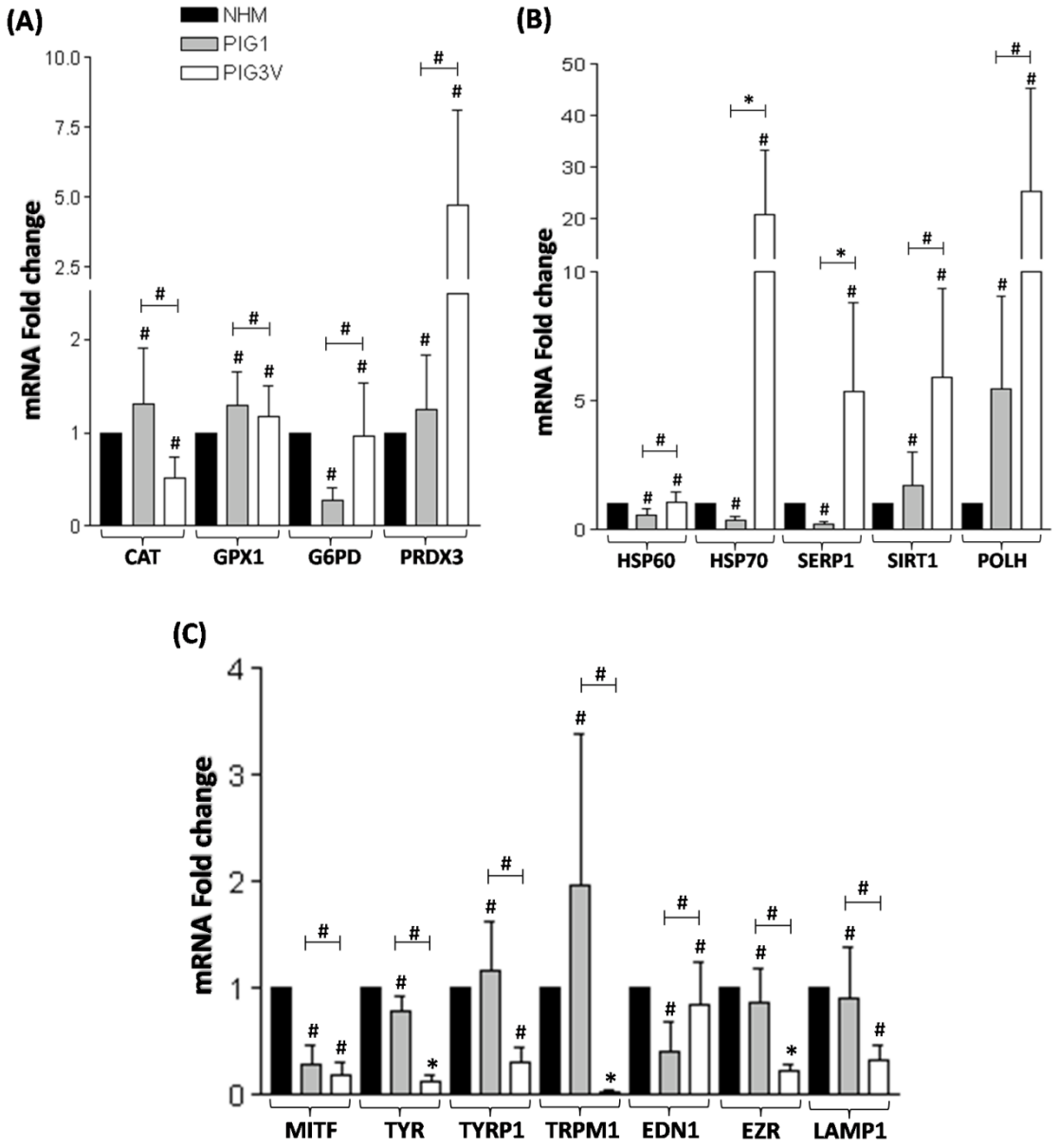

Figure 3. Gene expression profiles of primary NHM, PIG1 and PIG3V cells under normal condition. (A) Anti-oxidants: There was no difference in the expression of *CAT*, *GPX1*, *G6PD* and *PRDX3* among NHM, PIG1 and PIG3V cells. **(B) Stress related genes:** PIG3V cells showed significantly higher expression of *HSP70* and *SERP1* as compared to PIG1 cells. There was no difference in the expression of *HSP60*, *SIRT1* and *POLH* among NHM, PIG1 and PIG3V cells. **(C) Melanocyte specific genes:** PIG3V cells showed significantly lower expression of *TYR*, *TRPM1* and *EZR* as compared to NHM. There was no difference in the expression of *MITF*, *TYRP1*, *EDN1* and *LAMP1* among NHM, PIG1 and PIG3V cells. [$^{*}p<0.05$; $^{\#}p>0.05$ or non-significant]

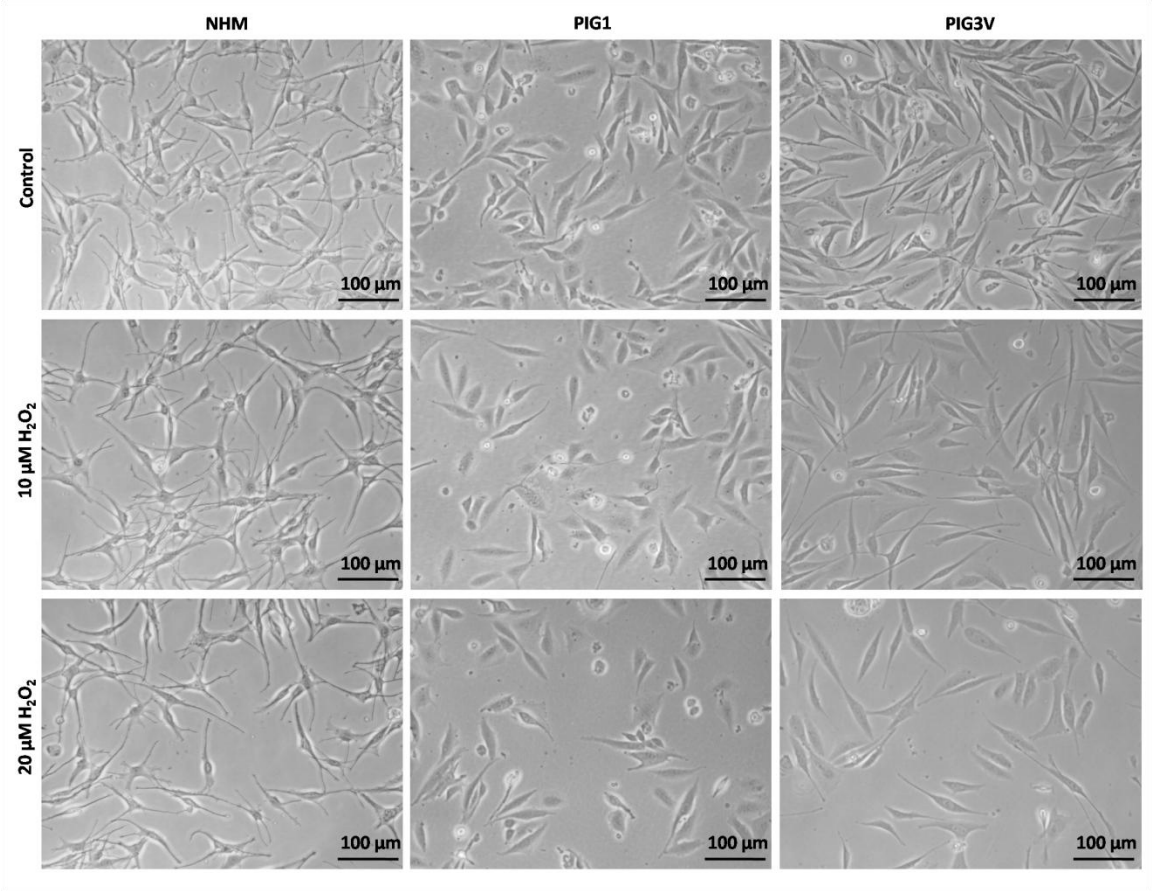

Figure 4. Effect of H_2O_2 on melanocytes: NHM, PIG1 and PIG3V cells showed significant decrease in viability upon 10 and 20 µM H_2O_2 treatments for 24 hrs as compared to untreated cells.

6.3.4 Gene expression profile of melanocytes under H_2O_2 induced oxidative stress

NHM, PIG1 and PIG3V cells were treated with H_2O_2 (10 µM & 20 µM) (Figure 4) and mRNA expression levels of anti-oxidant genes, stress related genes and melanocyte specific genes were analyzed after 24 hrs.

mRNA expression levels at 10 µM H_2O_2

PIG3V cells showed significantly decreased expression of *CAT* as compared to NHM (p=0.012) and significantly increased expression of *PRDX3* as compared to PIG1 cells (p=0.039). However, there was no difference in the expression of *CAT, GPX1, G6PD* and *PRDX3* between NHM and PIG1 cells (p=0.071, p=0.244, p=0.062 and p=0.808 respectively; Figure 5A). Further, no difference was observed in the expression of *GPX1, G6PD* and *PRDX3* between PIG3V and NHM (p=0.703, p=0.923 and p=0.427

respectively). Also, there was no difference in the expression of *CAT, GPX1* and *G6PD* between PIG1 and PIG3V cells (p=0.654, p=0.750 and p=0.128).

PIG3V cells showed significantly increased expression of *HSP70* as compared to NHM (p=0.023), and significantly increased expression of *HSP70, SERP1* and *POLH* as compared to PIG1 cells (p=0.008, p=0.043 and p=0.043 respectively; Figure 5B). However, there was no difference observed in the expression of *HSP60, HSP70, SERP1, SIRT1* and *POLH* between PIG1 and NHM (p=0.579, p=0.636, p=0.124, p=0.534 and p=0.114 respectively). Also, there was no difference observed in the expression of *HSP60, SERP1, SIRT1* and *POLH* between PIG3V and NHM (p=0.969, p=0.476, p=0.352 and p=0.356 respectively). Further, no difference was observed in the expression of *HSP60* and *SIRT1* between PIG1 and PIG3V cells (p=0.361, and p=0.888).

The PIG3V cells showed significantly decreased expression of *MITF, TYR, TRPM1, EDN1* and *EZR* as compared to NHM (p=0.017, p=0.015, p=0.0003, p=0.014 and p=0.037 respectively; Figure 5C). Also, PIG3V cells showed significantly decreased expression of *MITF, TYR, TRPM1, EZR* and *LAMP1* as compared to PIG1 cells (p=0.039, p=0.007, p<0.0001, p=0.009 and p=0.008 respectively). However, there was no difference in the expression of *MITF, TYR, TYRP1, TRPM1, EDN1* and *LAMP1* between PIG1 and NHM (p=0.638, p=0.771, p=0.670, p=0.139, p=0.433, p=0.524 and p=0.661 respectively). Further, no difference was observed in the expression of *TYRP1* and *LAMP1* between PIG3V and NHM (p=0.772 and p=0.145 respectively). Also, there was no difference observed in the expression of *TYRP1* and *EDN1* between PIG3V and PIG1 cells (p=0.589 and p=0.915 respectively).

mRNA expression levels at 20 µM H_2O_2

PIG3V and PIG1 cells showed significantly decreased expression of *CAT* as compared to NHM (p= 0.019 and p= 0.033 respectively; Figure 6A). Further, PIG3V cells showed significantly increased expression of *PRDX3* as compared to NHM and PIG1 cells (p=0.005 and 0.003 respectively). However, there was no difference in the expression of *GPX1, G6PD* and *PRDX3* between NHM and PIG1 cells (p=0.088, p=0.078 and p=0.683 respectively). Further, no difference was observed in the expression of *GPX1* and *G6PD* between PIG3V and NHM (p=0.127 and p=0.746 respectively).

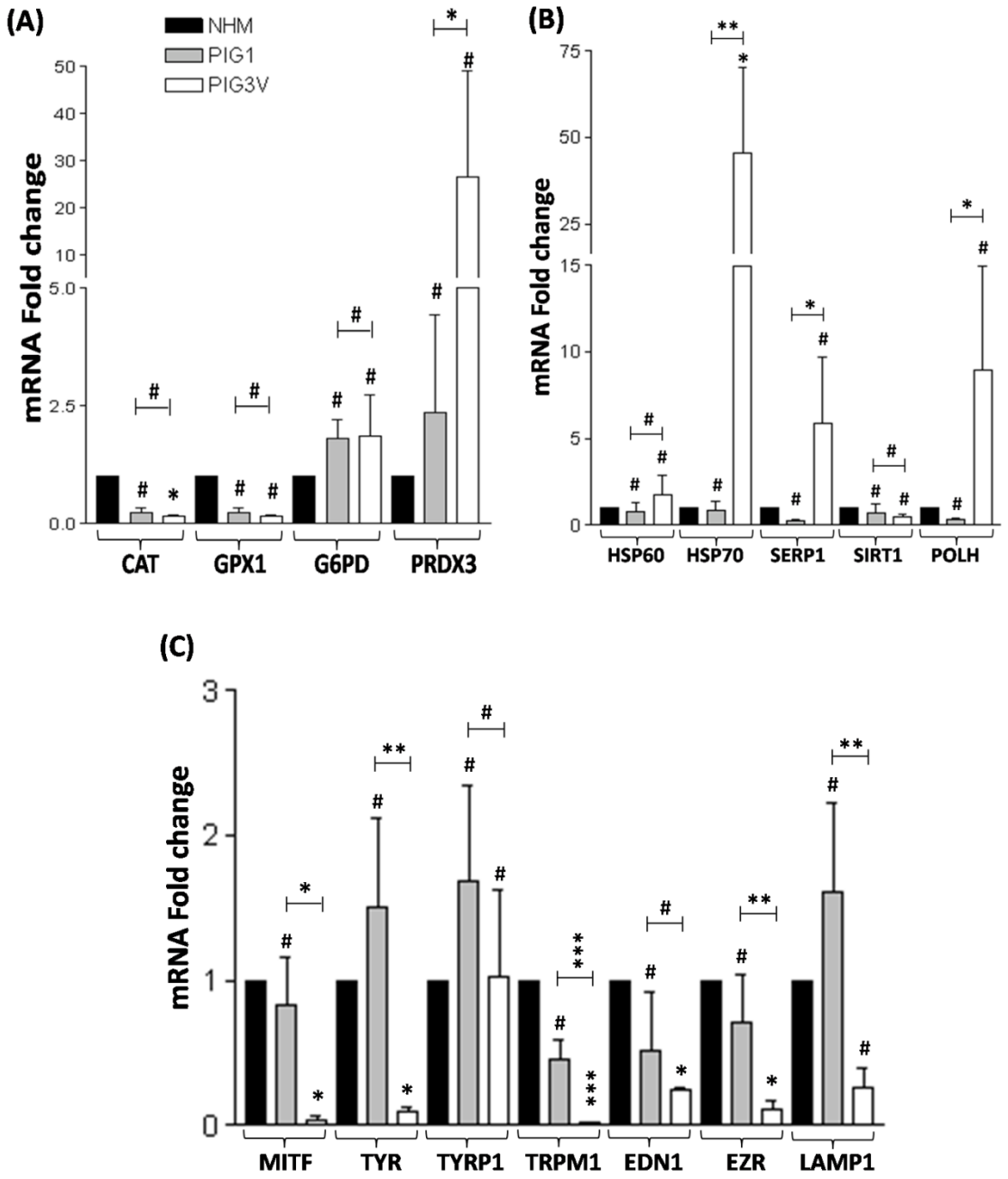

Figure 5. Gene expression profiles of primary NHM, PIG1 and PIG3V cells after 24 hrs of 10µM H_2O_2 treatment. (A) Anti-oxidants: PIG3V cells showed significantly decreased expression of *CAT* as compared to NHM, and significantly increased expression of *PRDX3* as compared to PIG1 cells. There was no difference observed in the expression of *GPX1* and *G6PD* among NHM, PIG1 and PIG3V cells. **(B) Stress related genes:** PIG3V cells showed significantly increased expression of *HSP70* as compared to NHM, and significantly increased expression of *HSP70, SERP1* and *POLH* as compared

to PIG1 cells. There was no difference observed in the expression of *HSP60* and *SIRT1* among NHM, PIG1 and PIG3V cells. **(C) Melanocyte specific genes:** PIG3V cells showed significantly decreased expression of *MITF, TYR, TRPM1, EDN1* and *EZR* as compared to NHM. Also, PIG3V cells showed significantly decreased expression of *MITF, TYR, TRPM1, EZR* and *LAMP1* as compared to PIG1 cells. There was no difference observed in the expression of *TYRP1* among NHM, PIG1 and PIG3V cells. [$^{*}p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$; $^{\#}p>0.05$ or non-significant]

Also, there was no difference in the expression of *CAT, GPX1* and *G6PD* between PIG1 and PIG3V cells ($p=0.159$, $p=0.871$ and $p=0.488$ respectively).

PIG3V cells showed significantly increased expression of *HSP70* as compared to NHM ($p=0.047$) and significantly increased expression of *HSP70, SERP1* and *POLH* as compared to PIG1 cells ($p=0.001$, $p=0.044$ and $p=0.043$; Figure 6B). PIG1 cells also showed significantly increased expression of *POLH* as compared to NHM ($p= 0.039$). However, there was no difference observed in the expression of *HSP60, HSP70, SERP1* and *SIRT1* between PIG1 and NHM ($p=0.129$, $p=0.217$, $p=0.067$ and $p=0.905$ respectively). Also, there was no difference observed in the expression of *HSP60* and *SIRT1* between PIG3V and NHM ($p= 0.3948$ and $p=0.1537$ respectively). Further, no difference was observed in the expression of *HSP60* and *SIRT1* between PIG1 and PIG3V cells ($p= 0.085$, and $p= 0.505$ respectively).

The PIG3V cells showed significantly decreased expression of *MITF, TYR, TRPM1* and *EZR* as compared to NHM ($p=0.021$, $p=0.0004$, $p=0.032$ and $p=0.009$ respectively; Figure 6C). Also, PIG3V cells showed significantly decreased expression of *TYR, TYRP1, TRPM1* and *EZR* as compared to PIG1 cells ($p=0.012$, $p=0.044$, $p=0.027$ and $p=0.021$ respectively). However, there was no difference in the expression of *MITF, TYR, TYRP1, TRPM1, EDN1* and *LAMP1* between PIG1 and NHM ($p=0.263$, $p=0.081$, $p=0.815$, $p=0.056$, $p=0.916$ and $p=0.613$ respectively). Further, no difference was observed in the expression of *TYRP1, EDN1* and *LAMP1* between PIG3V and NHM ($p=0.176$, $p=0.210$ and $p=0.139$ respectively). Also, there was no difference observed in the expression of *MITF, EDN1* and *LAMP1* between PIG3V and PIG1 cells ($p=0.158$, $p=0.482$ and $p=0.325$ respectively).

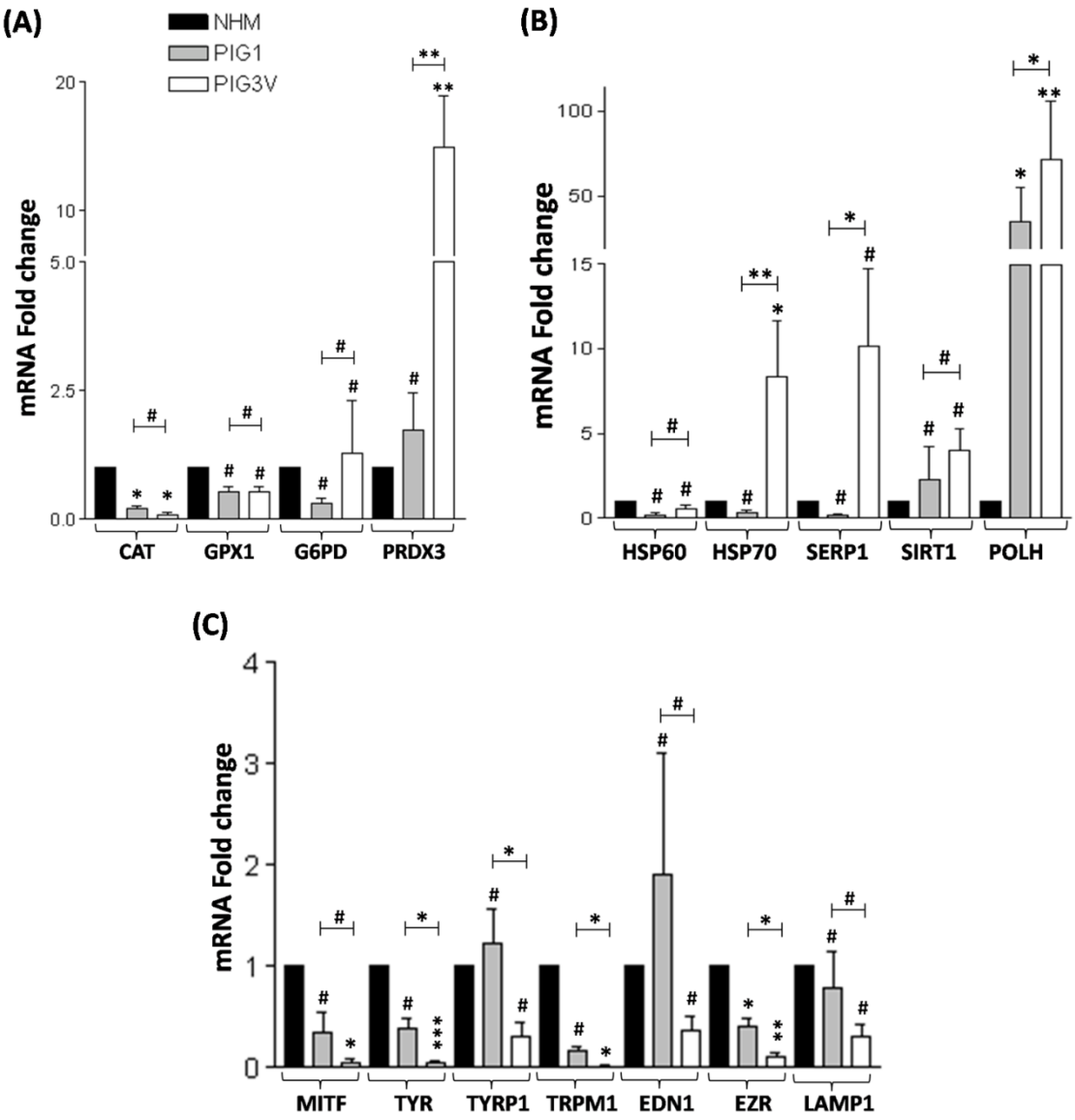

Figure 6. Gene expression profiles of primary NHM, PIG1 and PIG3V cells after 24 hrs of 20µM H_2O_2 treatment. (A) Anti-oxidants: PIG1 and PIG3V cells showed significantly decreased expression of *CAT* as compared to NHM. PIG3V cells showed significantly increased expression of *PRDX3* as compared to NHM and PIG1 cells. There was no difference observed in the expression of *GPX1* and *G6PD* among NHM, PIG1 and PIG3V cells. **(B) Stress related genes:** PIG3V cells showed significantly increased expression of *HSP70* as compared to NHM and significantly increased expression of *HSP70*, *SERP1* and *POLH* as compared to PIG1 cells. PIG1 cells also showed significantly increased expression of *POLH* as compared to NHM. There was no difference observed in the expression of *HSP60* and *SIRT1* among NHM, PIG1 and

PIG3V cells. **(C) Melanocyte specific genes:** PIG3V cells showed significantly decreased expression of *MITF, TYR, TRPM1* and *EZR* as compared to NHM. Also, PIG3V cells showed significantly decreased expression of *TYR, TYRP1, TRPM1* and *EZR* as compared to PIG1 cells. There was no difference observed in the expression of *EDN1* and *LAMP1* among NHM, PIG1 and PIG3V cells. [$^{*}p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$; $^{\#}p>0.05$ or non-significant]

6.3.5 Gene expression profile of Melanocytes under G6PD inhibition

NHM, PIG1 and PIG3V cells were treated with 6-ANAD (0.5 mM) (Figure 7) and mRNA expression levels of anti-oxidant genes, stress related genes and melanocyte specific genes were analyzed after 24 hrs. PIG3V cells showed significantly increased expression of *PRDX3* as compared to NHM ($p= 0.017$) and PIG1 cells ($p= 0.027$; Figure 8A).However, there was no difference in the expression of *CAT, GPX1, G6PD* and *PRDX3* between NHM and PIG1 cells ($p=0.675$, $p=0.718$, $p=0.092$ and $p=0.483$ respectively). Further, no difference was observed in the expression of *CAT, GPX1* and *G6PD* between PIG3V and NHM ($p=0.666$, $p=0.698$ and $p=0.744$ respectively). Also, there was no difference in the expression of *CAT, GPX1* and *G6PD* between PIG1 and PIG3V cells ($p=0.945$, $p=0.519$ and $p=0.145$ respectively).

PIG3V cells showed significantly increased expression of *HSP70, SERP1* and *POLH* as compared to NHM ($p=0.004$, $p=0.018$ and $p=0.012$ respectively; Figure 8B) and PIG1 cells ($p=0.002$, $p=0.034$ and $p=0.039$ respectively). However, there was no difference observed in the expression of *HSP60, HSP70, SERP1, SIRT1* and *POLH* between PIG1 and NHM ($p=0.926$, $p=0.221$, $p=0.630$, $p=0.156$ and $p=0.745$ respectively). Also, there was no difference observed in the expression of *HSP60* and *SIRT1* between PIG3V and NHM ($p=0.962$ and $p=0.895$ respectively). Further, no difference was observed in the expression of *HSP60* and *SIRT1* between PIG1 and PIG3V cells ($p=0.81$, and $p=0.169$ respectively).

The PIG3V cells showed significantly decreased expression of *MITF, TYR* and *TRPM1* as compared to NHM ($p=0.048$, $p=0.046$ and $p=0.013$ respectively; Figure 8C). Also, PIG3V cells showed significantly decreased expression of *TYR, TRPM1* and *EZR* as compared to PIG1 cells ($p=0.004$, $p=0.028$ and $p=0.009$ respectively). However, there

was no difference in the expression of *MITF*, *TYR*, *TYRP1*, *TRPM1*, *EDN1*, *EZR* and *LAMP1* between PIG1 and NHM (p=0.791, p=0.605, p=0.829, p=0.616, p=0.622, p=0.601 and p=0.914 respectively). Further, no difference was observed in the expression of *TYRP1*, *EDN1*, *EZR* and *LAMP1* between PIG3V and NHM (p=0.516, p=0.913, p=0.089 and p=0.248 respectively). Also, there was no difference observed in the expression of *MITF*, *TYRP1*, *EDN1* and *LAMP1* between PIG3V and PIG1 cells (p=0.135, p=0.122, p=0.171 and p=0.172 respectively).

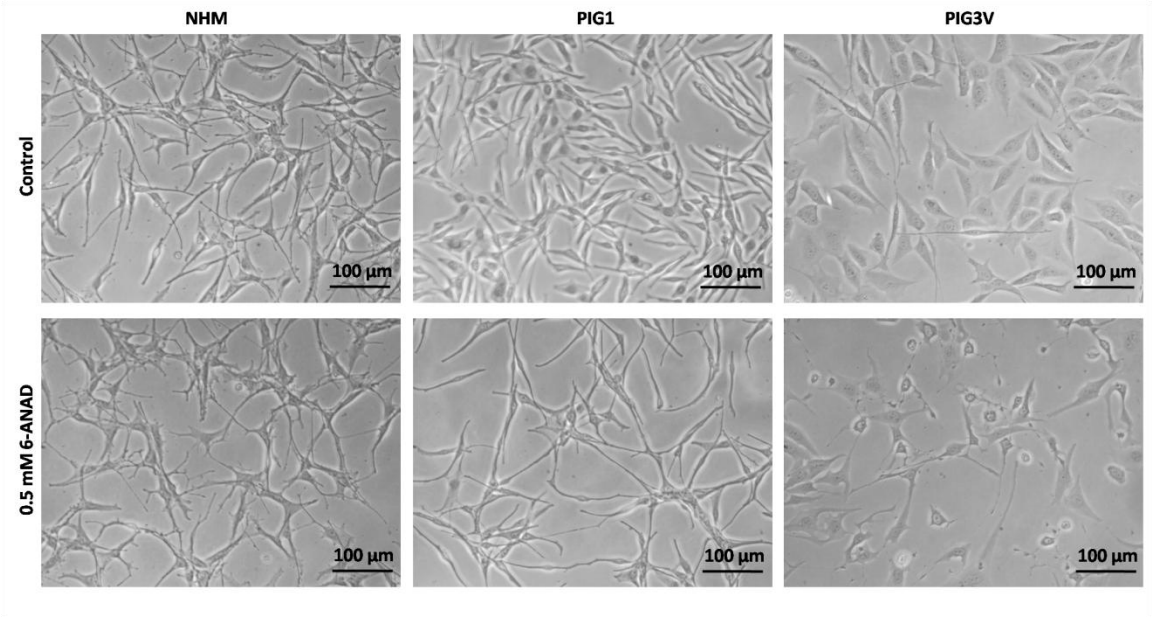

Figure 7. Effect of 6-ANAD on melanocytes: NHM, PIG1 and PIG3V cells showed significant decrease in viability upon 0.5 mM 6-ANAD treatment for 24 hrs as compared to untreated cells.

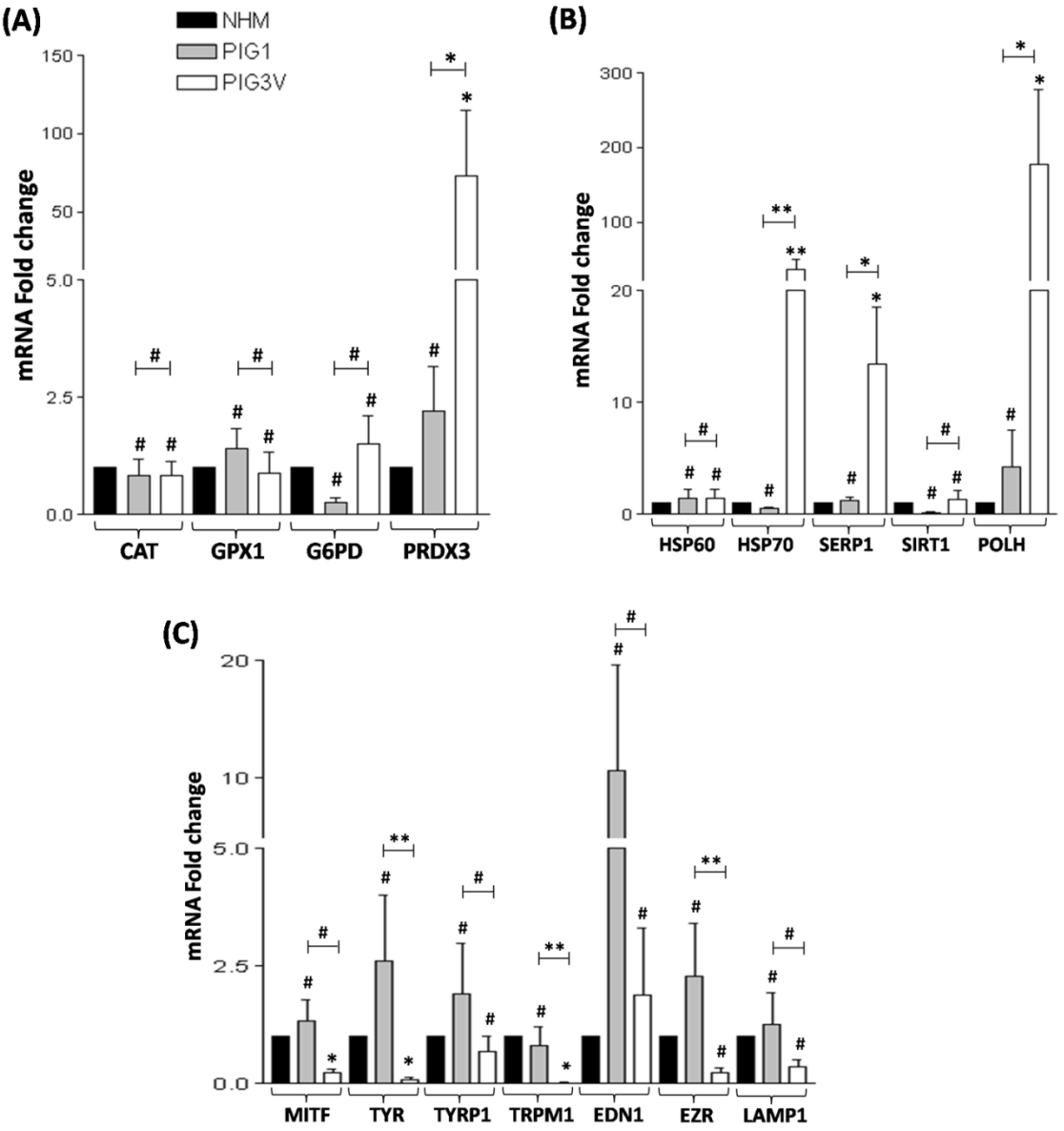

Figure 8. Gene expression profiles of primary NHM, PIG1 and PIG3V cells after 24 hrs of 0.5 mM 6-ANAD treatment. (A) Anti-oxidants: PIG3V cells showed significantly increased expression of *PRDX3* as compared to NHM and PIG1 cells. There was no difference observed in the expression of *CAT, GPX1* and *G6PD* among NHM, PIG1 and PIG3V cells. **(B) Stress related genes:** PIG3V cells showed significantly increased expression of *HSP70, SERP1* and *POLH* as compared to NHM and PIG1 cells. There was no difference observed in the expression of *HSP60* and *SIRT1* among NHM, PIG1 and PIG3V cells. **(C) Melanocyte specific genes:** PIG3V cells showed significantly decreased expression of *MITF, TYR* and *TRPM1* as compared to NHM.

Also, PIG3V cells showed significantly decreased expression of *TYR, TRPM1* and *EZR* as compared to PIG1 cells. There was no difference observed in the expression of *TYRP1*, *EDN1* and *LAMP1* among NHM, PIG1 and PIG3V cells. [$^{*}p<0.05$; $^{**}p<0.01$; $^{\#}p>0.05$ or non-significant]

6.4 DISCUSSION

Evidence of oxidative stress has been demonstrated in both the skin and blood of patients with vitiligo. Perturbed (6R)-L-erythro 5,6,7,8 tetrahydrobiopterin ($6BH_4$) *de novo* synthesis/recycling/regulation; impaired catecholamine synthesis with increased monoamine oxidase A (MOA) activities; low GPX activities; and "oxygen burst" via NADPH oxidase from a cellular infiltrate are the potential sources for epidermal H_2O_2 generation in vitiligo (Schallreuter *et al*, 1999). As a consequence, local and systemic high levels of H_2O_2 are able to alter calcium homeostasis, consequently perturbing the uptake of L-phenylalanine, the amino-acid precursor of tyrosine in melanocytes (Schallreuter *et al*., 2007). H_2O_2 accumulation is associated with low epidermal catalase levels. H_2O_2 can directly oxidize $6BH_4$ to 6-biopterin, which is cytotoxic to melanocytes *in vitro*. H_2O_2 overproduction can cause inactivation of catalase as well as vacuolation in epidermal melanocytes and keratinocytes. Vacuolation was also observed *in vitro* in melanocytes established from lesional and non-lesional epidermis of patients which was reversible upon addition of catalase.

The consequences of increased oxidative stress include oxidation of lipids, proteins, carbohydrates, and nucleic acids that lead to defects in metabolism, cell growth, and other cellular physiologic processes (Fisher 1988; Fridovich 1988). Anti-oxidants involved in redox regulation are GSH, GPX, catalase and SOD; all of these require a reductant for their operation. The main intracellular reductant is NADPH, which is principally produced by G6PD (Simonian *et al*., 1996; Pandolfi *et al*., 1995; Horecker, 1965; Kletzien *et al*., 1994). In the cell, G6PD is an important, but not an exclusive source for NADPH, which can also be produced by 6-phosphogluconate dehydrogenase (PGD), malate dehydrogenase (MDH), and isocitrate dehydrogenase (IDH) (Stanton, 2012).

However, the activity of PGD is completely dependent on G6PD as G6PD is the sole source of substrate for this enzyme (6- phosphogluconate). In most cells this enzyme cannot replace G6PD as the principal source of NADPH. However, in liver, adipose tissue, pancreatic β-cells, and macrophages NADP1-dependent malate dehydrogenase may play a significant role in NADPH production. Thus in most cells, proper activity of G6PD is required for the cell to defend against oxidative stress.

G6PD maintains the levels of NAPDH and GSH; also it may support the survival of cells through maintenance of both extracellular pH and redox potential (Maly *et al*., 1990; Kobayashi *et al*., 2005). There is compelling evidence that G6PD is the principal source of NADPH utilized in redox regulation. Under oxidative stress conditions many studies have shown that G6PD and the pentose phosphate pathway are elevated (Cramer *et al*., 1995; Slekar *et al*., 1996; Ursini *et al*., 1997). Pandolfi *et al*. (1995) produced totally G6PD deficient mouse embryonic stem cells which were exquisitely sensitive to oxidative stress. In addition, the null cells showed decreased growth rates and reduced cloning efficiencies, suggesting that G6PD was critical for NADPH production and was the principal source of NADPH. Another G6PD-deficient Chinese hamster ovary cell line was also found to be more susceptible to oxidative stress (Rosenstraus and Chaisin, 1975). G6PD inhibition was shown to decrease the intracellular free calcium ($[Ca^{2+}]_i$) in vascular smooth muscle cells (Ata *et al*., 2011). Salvemini *et al* (1999) overexpressed G6PD in HL-60 (Human promyelocytic leukemia) cells and showed that the GSH levels in the cell were elevated and the cells were more resistant to oxidative stress. In most circumstances, increased G6PD activity has been reported to protect against oxidative stress-induced cell death (Fico *et al*., 2004). Bhardwaj *et al*. (2011) have shown that a combination of 2-deoxy-D-glucose and 6-ANAD induces oxidative stress mediated selective radiosensitization of malignant cells via mitochondrial dysfunction. Taken together, these results show that G6PD activity is of central importance to cellular redox regulation.

6-ANAD is taken up by cells and transformed into 6-amino-$NADP^+$ by NAD-glycohydrolase, which acts as an analogue of $NADP^+$. 6-ANAD acts as a competitive inhibitor of G6PD and PGD, which also requires $NADP^+$. Importantly, 6-ANAD inhibits

PGD with an inhibitor constant (K_i) of 0.13×10^{-6} M, approximately 400- fold lower than the K_i for G6PD (Kohler *et al*., 1970; Hothersall *et al*., 1998). Thus, we can consider that 6-ANAD inhibits the PPP pathway by acting in both PGD and G6PD (Lei *et al*., 2014).

On the other hand, ROS are able to oxidize and inhibit the activity of proopiomelanocortin derived bioactive peptides ACTH and a-MSH that have crucial role in maintaining efficient melanogenesis, since their release activates a cascade of intracellular signals leading to an upregulation of tyrosinase, TYRP1 and TYRP2 (or dopachrome tautomerase, DCT) (Tachibana, 2000). Yang *et al*. (2000) have shown that normal melanocytes cultured in the presence of 4-TBP undergo dose-dependent apoptosis. The percentage of cells that die following exposure to 4-TBP are unaffected by 12-*O*-tetradecanoylphorbol-13-acetate but can be reduced significantly by excluding α-melanocyte-stimulating hormone (MSH) from the culture medium (Yang *et al*., 1999). MSH, a potent stimulator of melanogenesis, is a ligand for the melanocortin 1 receptor (MC1R). Binding of MSH to MC1R activates adenylate cyclase (Suzuki *et al*., 1996). The subsequent increase in cyclic AMP leads to the expression of melanocyte-specific isoform of MITF (Widlund and Fisher, 2003), which in turn *trans*-activates the expression of downstream genes, including tyrosinase and TYRP1 (Fang and Setaluri, 1999), ultimately resulting in increased melanin production. MITF is most active in its phosphorylated state but is rapidly degraded following ubiquitination (Xu *et al*., 2000). Although MITF promotes survival of melanocytes and melanoma cells in some instances, it can also act as a proapoptotic signal. MITF has been shown to be a target of caspases, and in melanoma cells, the products of caspase-mediated MITF digestion induce apoptosis (Larribere *et al*., 2005). Furthermore, expression of MITF results in a reduced response to oxidative stress induced by H_2O_2 (Jimenez-Cervantes *et al*., 2001), suggesting a role for this transcription factor in melanocyte survival and response to oxidative stress. Oxidative stress induced by H_2O_2 causes down-regulation of MITF in melanoma cells (Jimenez-Cervantes *et al*., 2001).

Further, Manga *et al*. (2006) have reported that melanocytes exhibit reduced expression of MITF and TYRP1 in response to oxidative stress induced by 4-TBP. 4-TBP is

converted to quinines (Thorneby-Andersson *et al*., 2000), which undergo redox cycling within the cell, thereby generating ROS (O'Brien, 1991) and inducing oxidative stress. To clarify the biological role of human TYRP1 in melanocyte survival, cultured melanocyte from an active advancing border of vitiligo were established and studied by Jimbow *et al*. (2001). The established 'vitiligo melanocytes' showed early cell death when exposed to oxidative stress (ultraviolet B) and decreased expression of TRYP1 mRNA as well as increased and abnormal immune-staining and immune-precipitation by antibodies against human and mouse TYRP1, indicating an altered synthesis and processing of TYRP1. Further, vitiligo melanocytes revealed abnormal protein-protein interactions with calnexin, a melanogenesis-associated chaperone, suggesting altered folding and maturation of nascent TYRP1 polypeptides. This study suggests that the early cell death of vitiligo melanocytes is related to their increased sensitivity to oxidative stress, which may arise from complex processes of abnormal synthesis and processing of TYRP1 and its interaction with calnexin. Overall, the above literature advocates the reduced mRNA expression of TYRP1 in vitiligo melanocytes with abnormal processing of TYRP1 polypeptides, which may results in increased and abnormal antigen presentation of TYRP1 peptides on melanocyte membrane leading to autoimmune response via anti-TYRP1 antibodies and/ or T cell attack on melanocytes in patients with vitiligo. Earlier, we have shown the down-regulation of TYR and TYRP1 in lesional as well as non-lesional skin of patients (Chapter V-B). In the present study, we found reduced expression of MITF, TYR and TYRP1 in vitiligo patient derived PIG3V melanocytes under H_2O_2 induced oxidative stress and/ G6PD inhibition (Figure 5, 6 & 8). Therefore, our results are in agreement with previous studies (Jimenez-Cervantes *et al*., 2001; Jimbow *et al*., 2001; Manga *et al*., 2006), supporting oxidative stress mediated pathogenesis of vitiligo.

Stressed cells are characterized by elevated expression of stress proteins. Stress proteins include the HSP family upregulated in response to elevated environmental temperatures and other forms of stress. Stress proteins are evolutionarily very well conserved, and they function as chaperone molecules protecting cellular proteins from premature degradation by supporting proper protein folding (Houry, 2001). Cells with elevated levels of stress proteins are protected from the consequences of subsequent stress episodes (Mestril and

Dillmann, 1995). Exposure to 4-TBP enhanced the expression of HSP70 by melanocytes (Kroll *et al*., 2005). PIG3V vitiligo melanocytes showed a tendency to be more sensitive to 4-TBP as compared to control PIG1 melanocytes (Kroll *et al*., 2005). The putative cytoprotective effect of stress proteins was inadequate to prevent cell death in either cell line as adenoviral overexpression of either HSP27 or HSP70 failed to protect PIG1 or PIG3V melanocytes from 4-TBP-induced cell death. Such a lack of protection can possibly be explained by inadequate activation of antioxidant enzymes, as supported by the therapeutic potential of pseudocatalase in vitiligo (Schallreuter *et al*, 1999). Both the PIG1 and PIG3V cell lines upregulated HSP70 expression in response to 4-TBP (Kroll *et al*., 2005). Release of HSP70 from viable cells was reported for the constitutive as well as the inducible form of HSP70 (Barreto *et al*, 2003; Broquet *et al*, 2003). The mechanism reportedly involves the release of membrane-bound HSP70 from lipid rafts (Broquet *et al*, 2003). For the constitutive form of HSP70, it was demonstrated that release was inducible by a variety of cytokines, most notably IFN-γ (Barreto *et al*, 2003). The IFN-γ is generated by infiltrating T cells in perilesional vitiligo skin (Le Poole *et al*, 2003; Wankowicz-Kalinska *et al*, 2003).

Stress proteins occasionally secreted by viable cells have been coined "chaperokines", reflecting the intercellular effects commonly assigned to cytokines while chaperoning peptides specific to the cell type from which they were derived (Asea *et al*, 2000; Asea, 2003). This may reflect an early phase of an immune response, as HSP70 was shown to induce secretion of primary cytokines IL-1, IL-6, and TNF-α by monocytes/macrophages in a C14-dependent fashion (Asea *et al*, 2000). It was previously shown that melanocytes can generate primary cytokines as well (Kruger- Krasagakes *et al*, 1995). Under oxidative stress, anti-apoptotic HSP60 and HSP70 have also been reported to favor the apoptosis by reducing their expression (Xu *et al.,* 2007), which inhibit the mitochondrial death pathway (Vecchione *et al.,* 2010). Differential expression of HSP60 and HSP70 has been reported in skin of vitiligo patients (Mosenson *et al.,* 2012; Chapter V-B). Previous studies including ours have shown decreased G6PD activity and expression in vitiligo patients (Agrawal *et al.,* 2004 and Arican *et al.,* 2008; Chapter IV). As the gene expression data shown in Figure 5, 6 & 8 support upregulated expression of HSP70 by PIG3V melanocytes in response to H_2O_2 induced oxidative stress and G6PD inhibition.

Peroxiredoxins (PRDXs) are involved in the degradation of H_2O_2, organic hydroperoxides and peroxynitrites (Rhee *et al.*, 2005). PRDX3 encodes an antioxidant mitochondrial protein (Wood *et al.*, 2003). In addition to its peroxidase activity, PRDX3 also acts as free radical scavenger (Gourlay *et al.*, 2003) and participates in redox-related signaling transduction pathways (Rhee *et al.*, 2005). PRDX3 was found to be up-regulated in lesional and non-lesional skin as well as circulation of patients especially with GV and AV (Chapter V-B), suggesting the protective role of PRDX3 against oxidative stress in vitiligo. Interestingly, in the present study we observed upregulation of PRDX3 in response to both H_2O_2 induced oxidative stress and G6PD inhibition (Figure 5, 6 & 8).

Several studies have indicated that SIRT1 protects against stress-related diseases by deacetylating targets such as p53, FOXO, NFκB and PGC1α which regulate diverse cellular processes, including stress response, replicative senescence, inflammation and metabolism (Saunders *et al.,* 2007). Han *et al*. (2008) have reported high SIRT1 levels in mouse to protect embryonic stem cells from oxidative stress. SIRT1 is critical for cellular development, loss of SIRT1 in mice leads to postnatal lethality (Cheng *et al.,* 2003). Recently, Becatti *et al*. (2014) have shown that SIRT1 regulates MAPK pathway via Akt-apoptosis signal-regulating kinase-1 in peri-lesional vitiligo keratinocytes. Two key mediators of skin damage, UV radiation and H_2O_2, down-regulate SIRT1 in keratinocytes (Cao *et al.,* 2009). Previously, we showed increased expression of SIRT1 in lesional skin and in circulation of patients (Chapter V-B). However, in the present study we did not find any significant difference in the expression of SIRT1 in response to H_2O_2 induced oxidative stress and G6PD inhibition (Figure 5, 6 & 8) in melanocytes.

Endothelin 1 (EDN1) is a paracrine growth factor synthesized by numerous cell types including keratinocytes, which interacts synergistically with α-MSH and basic fibroblast growth factor that together affect melanocyte proliferation, migration, tyrosinase activity, melanogenesis, and dendrite formation (Hara *et al.,* 1995 and Tada *et al.,* 1998). EDN1 also increases the expression and phosphorylation of MITF (Kadekaro *et al*., 2005). Manga *et al*. (2006) have shown that EDN1 caused an increase in melanocyte sensitivity to 4-TBP-induced cytotoxicity and that MITF expression is sensitive to the redox state of cells (Jimenez-Cervantes, *et al*., 2001). Previously, we showed that EDN1 was down-

regulated in lesional skin and, blood of vitiligo patients (Chapter V-B). The present study, we found significantly decreased expression of EDN1 in patients derived PIG3V melanocytes as compared to NHM in response to 10 µM H_2O_2 (Figure 5), suggesting an important role for EDN1 in the regulation of human melanocytes.

The first mouse model for vitiligo was a MITF mutant (Lamoreux *et al*., 1992); however, mutations at the MITF locus do not result in human vitiligo (Tripathi *et al*., 1999). Pathways involved in MITF regulation have been shown to be defective in vitiligo (Kitamura *et al*., 2004) and may contribute to the increased risk of melanocyte death. Reduced expression of MITF would decrease expression of TYRP1 and additional genes that promote apoptosis during oxidative stress. Although its precise function is not known, TYRP1 has been shown to have several catalytic activities, including tyrosine hydroxylase activity (Jimenez-Cervantes *et al*., 1993; Zhao *et al*., 1994). Therefore, reduction in expression of MITF and subsequently TYRP1 will protect cells against further oxidative stress. TYR and TYRP1 are expressed in melanocytes and mainly localized in melanosomes where they play key roles in promoting melanogenesis. While TYR catalyses the key initial step in melanin production, TYRP1 and TYRP2 act at subsequent steps, influencing quality and quantity of melanins (Sturm *et al.,* 2012). LAMP1 is the melanocyte vesicular membrane glycoprotein (Zhou *et al.*, 1993) and TYR, TYRP1 as well as LAMP1 are expressed as a multi-protein complex and function together by stabilizing the enzyme-protein complex within the melanosome and prevent the premature death of melanocytes due to tyrosinase-mediated cytotoxicity (Ghanem *et al.*, 2011). Jimbow *et al*. (2001) have shown that the early cell death of vitiligo melanocytes is related to their increased sensitivity to oxidative stress, which may arise from abnormal synthesis and processing of TYRP1 and its interaction with calnexin, a melanogenesis-associated chaperone, suggesting altered folding and maturation of nascent TYRP1 polypeptides. Also decreased expression of TYRP1 mRNA in vitiligo melanocytes was demonstrated by Northern blot (Jimbow *et al.*, 2001). TYR and TYRP1 promoter contains a motif termed M-box that can bind to MITF, which is a master regulator in melanocyte proliferation, development and survival. MITF stimulates melanin synthesis by regulating expression of melanogenic enzymes (TYR and TYRP1) (Manga *et al.*, 2006 and Luo *et al.*, 1994) and reduced expression of MITF and TYRP1 in

melanocytes was observed under oxidative stress (Manga *et al.,* 2006). Overall, the above studies advocate the reduced mRNA expression of TYRP1 in vitiligo melanocytes with abnormal processing of TYRP1 polypeptide, which may results in increased and abnormal antigen presentation of TYRP1 peptides on melanocyte membrane leading to autoimmune response via anti-TYRP1 antibodies and/ or T cell attack on melanocytes in patients with vitiligo. Earlier, we have shown decreased expression of TYR, TYRP1 and LAMP1 in lesional and non-lesional skin (Chapter V-B). In the present study, we found the down-regulation of MITF, TYR, TYRP1 and LAMP1 in PIG3V melanocytes as compared to NHM and / PIG1 melanocytes in response to H_2O_2 induced oxidative stress and G6PD inhibition (Figure 5, 6 & 8). Therefore, our results are in agreement to previous studies (Wu *et al.,* 2008; Jimbow *et al.,* 2001 and Manga *et al.,* 2006), supporting oxidative stress mediated pathogenesis of vitiligo.

SERP1 stabilizes membrane proteins during stress and facilitates subsequent glycosylation (Yamaguchi *et al.,* 1999), which protects unfolded target proteins against degradation during ER stress. Whereas, EZR acts as a linker between plasma membrane and cytoskeleton; and interacts with intercellular adhesion molecules 1 and 2 (Vaheri *et al.,* 1997). Epithelial cells expressing mutant EZR (Y145F) showed defect in cell proliferation and adhesion mediated events (Srivastava *et al.,* 2005). Also, EZR expression has been correlated with tumor thickness and level of invasion in primary cutaneous melanoma (Ilmonen *et al.,* 2005). Phosphorylation of ezrin/radixin/moesin (ERM) proteins mediates melanocyte proliferation and migration (Jeon *et al.,* 2009). SERP1 and EZR were found to be down-regulated in lesional and non-lesional skin and in blood of patients (Chapter V-B). In contrast, patients derived PIG3V melanocytes exhibit higher levels of SERP1 and lower levels of EZR as compared to PIG1 and NHM respectively (Figure 3), indicating accumulation of unfolded proteins in vitiligo melanocytes. In addition, PIG3V melanocytes showed significantly increased expression of SERP1 in response to H_2O_2 induced oxidative stress and G6PD inhibition as compared to NHM and/ PIG1 cells(Figure 5, 6 & 8). However, EZR was down-regulated in PIG3V melanocytes in response to H_2O_2 induced oxidative stress and G6PD inhibition as compared to NHM and/ PIG1 cells (Figure 5, 6 & 8). Previously, we have proposed that ER stress could be a major link between oxidative stress and autoimmunity in vitiligo

(Mansuri *et al.,* 2014b). This is further supported by decreased expression of EZR and increased expression of SERP1 in vitiligo melanocytes.

TRPM1, whose expression is restricted to the melanocyte lineage (Hunter *et al.,* 1998 and Gaur *et al.,* 2007), is a constitutively active Ca^{2+} channel, which is highly expressed in melanocytes and its activity is critical for melanocyte homeostasis (Devi *et al.,* 2009). Schallreuter *et al*. (2009) have shown that cells from lesional skin showed decreased Ca^{2+} uptake compared to non-lesional of vitiligo patients and control skin. Moreover, it has been reported that TRPM1 expression is MITF dependent (Levy *et al.,* 2010). In addition, the present study suggests that TRPM1 is down-regulated in vitiligo melanocytes (Figure 3). In addition, PIG3V melanocytes showed significantly decreased expression of TRPM1 in response to H_2O_2 induced oxidative stress and G6PD inhibition as compared to NHM and/ PIG1 cells (Figure 5, 6 & 8), indicating ITS essential role in vitiligo pathogenesis.

POLH is a member of nucleotide excision repair family genes (Yu *et al.,* 2012) which encodes Pol, a specialized polymerase that is able to bypass UV lesions. When POLH is defective, UV-induced DNA lesions are replicated by a more error-prone polymerase that produces more mutations (Flanagan *et al.,* 2007). Melanins, produced by melanocytes, play an important role in protecting the skin against UV radiation. UV-induced DNA damage in melanocytes is more effectively prevented in darker skin (Lee *et al.,* 2013). It has been reported that stimulation of melanogenesis in human melanocytes increase UVA-induced DNA damage (Denat *et al.,* 2014). Upon UVA exposure, oxidative lesions contribute to a larger extent to DNA damage in melanocytes (Mouret *et al.,* 2012). While UVB induces changes in the skin; causing a carcinogenic DNA damage and various epigenetic effects (Canguilhem *et al.,* 2005) and higher doses of NB-UVB induces DNA damage leading to cell-cycle arrest, which gives cells time to repair DNA mutations (Reich *et al.,* 2013). In conditions of vitiligo, melanocytes are under oxidative stress UV-induced DNA damage and activation of POLH is obvious. Our previous study has shown the upregulated expression of POLH in lesional and non-lesional skin as well as in blood of patients especially with SV (Chapter V-B), indicating its protective role against UV-

induced DNA damage in vitiligo. In addition, PIG3V melanocytes showed significantly increased expression of POLH in response to H_2O_2 induced oxidative stress and G6PD inhibition as compared to NHM and/ PIG1 cells (Figure 5, 6 & 8).

Understanding the mechanism(s) by which H_2O_2 causes melanocyte death may elucidate the pathology underlying vitiligo in general. Thus, Melanocyte susceptibility to oxidative stress may play a role in the pathogenesis of vitiligo. In this study, we show that H_2O_2 and 6-ANAD induce oxidative stress in melanocytes and that melanocytes from individuals with vitiligo are more sensitive to H_2O_2 induced oxidative stress and G6PD inhibition compared to normal melanocytes. Therefore, vitiligo may result from an insufficient response to oxidative stress induced by H_2O_2 and 6-ANAD inhibition.

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