1.1 VITILIGO

Vitiligo, the most common hypopigmentary skin disorder, is an acquired disease characterized by progressive loss of melanocytes. Vitiligo occurs worldwide with an estimated prevalence of 0.5–1% in most populations (Ezzedine *et al*., 2012); 0.5-2.5 % in India (Handa and Kaur, 1999) and the states of Gujarat & Rajasthan have the highest prevalence i.e ~8.8% (Valia and Dutta, 1996). In almost 50% of patients, vitiligo starts before the age of 20 yrs, and almost males and females are equally affected (Taïeb and Picardo, 2009; Ezzedine *et al*., 2012). Vitiligo has been given a Diagnosis Code (l80) by the World Health Organization (WHO) in its 10th revision of International Statistical Classification of Diseases (Ezzedine *et al*., 2015). The cause is yet unknown and there is no commonly used treatment. Although there are no health complications with this hypopigmentation, vitiligo has a profound and permanent impact on patients' quality of life, especially in younger patients (Silverberg and Silverberg, 2014). Most patients exhibit early age of onset (<20 years), and often face stigmatization during their personal, professional, and psychological development and, girls in particular, are subjected to ostracization from the marital point of view (Mehta *et al*., 1973; Parsad *et al*., 2003; Radtke *et al*., 2009; Noh *et al*., 2013; Silverberg *et al*., 2014; Al-Shobaili, 2014). A significant number of patients feel stressed by their condition and experience anxiety and depression leading to low self-esteem and fear of social isolation (Porter *et al*., 1978; Nogueira *et al*., 2009). Certain cultural groups ostracize affected family members who may never be able to work, marry, or participate in normal social interactions. This decline in quality of life with vitiligo is similar to that of patients with atopic dermatitis and psoriasis, which are not considered as cosmetic diseases (Fang and Yang, 2008).

Vitiligo is a multifactorial polygenic disorder with a complex pathogenesis (Agrawal *et al*., 2001; Shajil *et al*., 2006; Kostopoulou *et al*., 2009; Mashayekhi *et al*., 2010; Wang *et al*., 2011). It has been associated with concomitant occurrence of a number of other autoimmune diseases (Alkhateeb *et al*., 2003; Boelaert *et al*., 2010). In vitiligo patients, skin melanocytes are partially or completely lost, and several theories have been put forward to explain the etiology of vitiligo such as oxidative stress, autoimmune, neural and genetic hypotheses (Agrawal *et al*., 2001; Shajil *et al*., 2006; Kemp *et al*., 2001;

Ongenae *et al*., 2003). Melanocyte death may occur due to intrinsic and/or extrinsic factors. Several studies have investigated the key role of oxidative stress in melanocyte death and anti-melanocyte immune responses; however, the interplay between them remains unclear. The onset of vitiligo is manifested by the crosstalk of the following three components: 1) a complex of susceptibility genes that manipulate the autoimmune response; 2) genetically abnormal melanocytes; and 3) one or more physiological or environmental factors that induce oxidative stress and activate the genetic program for melanocyte destruction (Koshoffer and Boissy, 2014). Oxidative stress is considered to be the initial triggering event in the pathogenesis of melanocyte destruction (Schallreuter *et al*., 1999). Vitiligo is accompanied by overproduction and accumulation of H_2O_2 in the epidermis (Browers *et al*., 1994; Knight *et al*., 1995; Yildirim *et al*., 2004). Melanocytes from vitiligo patients are incapable of battling oxidative stress, thus sustaining cellular damage. This significant damage could trigger apoptosis in the melanocytes and create an environment rich with cellular fragments that could become autoimmune targets and turn on an immune response against melanocytes. Histological investigations have demonstrated presence of inflammatory infiltrate of mononuclear cells in the upper dermis and at the dermal-epidermal junction of peri-lesional skin of vitiligo patients (Picardo *et al*., 2010). The initiation mechanism of this micro-inflammatory reaction is still not clear, however local triggers are reported to signal the innate immune system of skin that trigger adaptive immune responses targeting melanocytes (Kroll *et al*., 2005; van den Boorn *et al*., 2011). Alternatively, oxidatively stressed vitiligo melanocytes could produce self-antigens and/or cytokines to activate an autoimmune response (Koshoffer and Boissy, 2014). It has been suggested that oxidative stress may have a role in vitiligo onset, while autoimmunity contributes towards the disease progression (Laddha *et al*., 2013a; Laddha *et al*., 2014).

1.1.1 Historical Aspects

The oldest text in books about a disease similar to vitiligo as it is known today, date back to 1,500 BC is present in Hindu sacred writings “Vedas”, under the name *kilăsa*, also in texts (papyrus) from ancient Egypt (Millington and Levell, 2007). There are a number of references in the Old Testament, particularly in Leviticus XIII, to the term *Zoráat* or

Tzaraat, which in classical Hebrew means "white spots", but there is argument as to whether this disease is really vitiligo. The word "vitiligo" seems to have been derived from the Latin word "vitium" meaning a blemish or "vitulum" meaning a small blemish (Mercurialis, 1572; Nair, 1978). The term *vitiligo* was first used in the first century AD by Celsus in the classic treatise *De Medicina*, with similar meanings, as *vituli* and *vitelius*, comparing the achromic or hypochromic lesions with vitiligo's white patches observed in calves, or *vitium* meaning defect or flaw (Freilich, 1982). In the 19th century, Brocq and Kaposi were among the first to describe both the clinical aspects, as achromia and hyperpigmentation at the borders of patches, as well as its histopathology, in which Kaposi reported the absence of pigment granules in the basal layer cells of the epidermis (Kopera, 1997).

1.2 SKIN

The skin plays an extremely important role, providing a vast physical barrier against chemical, mechanical and microbial factors which may affect the physiological status of the body (Haake and Holbrook, 1999). In addition, skin acts as a matrix for immune network and through its pigments, provides an exclusive defense system against UV radiations (Pathak, 1995). Melanocytes transfer melanosomes through their dendrites to keratinocytes and form the nuclear melanin caps that reduces UV induced DNA damage in human epidermis. The skin's layers are mainly represented by the epidermis and dermis (Figure 1).

Epidermis: The epidermis is an external, stratified epithelium devoid of blood or nerve supplies of ~5–100 μm thickness (which can reach 600 μm on palms and soles) (Tobin, 2006). It is composed of several distinct cell populations; keratinocytes and melanocytes are the main elements, of which the first comprise ~95% of the epidermis and are arranged in four layers: 1) Stratum basale, 2) Stratum spinosum, 3) Stratum granulosum and 4) Stratum corneum (Figure 1A).

Dermis: The dermis is a 2-4 mm-thick layer of connective tissue and fibroblasts that houses the neural, vascular, lymphatic, and secretory apparatus of the skin (Figure 1B).

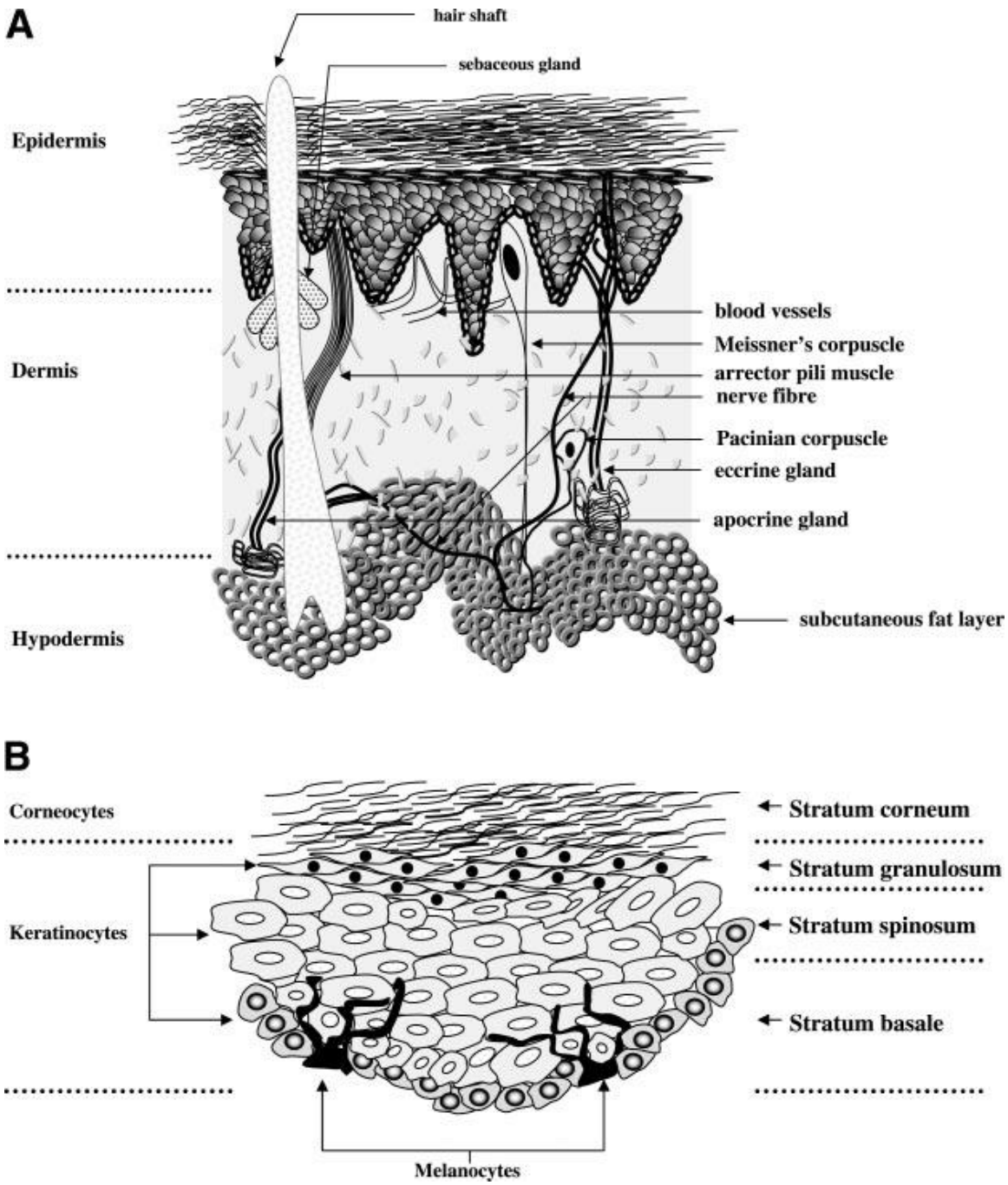

Figure 1. Structure of the skin: A) The different layers and components. **B)** Layers of the epidermis. (Robins, 1991; Costin and Hearing, 2007)

1.2.1 Skin Pigmentation

It is interesting to reveal that one's skin colour is not a 'permanent coat' but the consequence of regular replenishing or 'repainting' according to the turnover of the epidermis. Melanin is produced in the form of pheomelanin (red-yellow shades) and eumelanin (brown-black shades) in melanosomes which are then transferred from melanocyte to associated keratinocytes through its dendrites by phagocytosis. As melanocytes represent only 8–10% of all epidermal cells (Ivanova *et al*., 2006), most of the skin's colour is determined by melanin in the keratinocytes, and only at contained gathering of melanocytes i.e., in melanocytic nevi or 'moles' the melanocytes themselves contribute to the colour. Lighter skin has a higher content of pheomelanin with smaller melanosomes whereas dark skin has a higher proportion of eumelanin, with larger melanosomes. Skin colour is not determined by the number or size of melanocytes. Melanin is considered to filter out UV radiation and scavenge ROS, thereby limiting UVdamage to other cutaneous cells. The supranuclear melanin cap structure presumably minimizes photodamage to the nucleus in keratinocytes (Kollias *et al*., 1991; Kobayashi *et al*., 1998).

1.2.2 Epidermal Melanin Unit

The epidermal melanin unit consists of one melanocyte making contact with ~36 surrounding keratinocytes (Fitzpatrick *et al*., 1963). The ratio of melanocytes to keratinocytes is 1:10 in the epidermal basal layer (Figure 2). This balance is maintained through the human life but the exact mechanism is unknown (Haass and Herlyn, 2005).

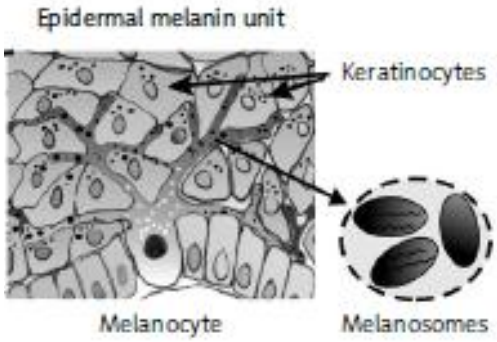

Figure 2. Epidermal melanin unit (Cichorek *et al*., 2013).

About 1200 melanocytes exist per mm^2 of the skin independent of the human race (Miot *et al*., 2009). Adhesion molecules such as E- and P-cadherins participate in building cell-cell contact structures (Haass *et al*., 2005). The contact between the dendritic processes of differentiated melanocyte and keratinocytes is essential for the melanin transport to keratinocytes determining the skin color and is involved in the skin cells' photoprotection. Melanin granules are accumulated above the nucleus of keratinocyte and are removed with the shed of epidermal cells (Plonka *et al*., 1999).

The size of the epidermal melanin unit is similar independent of the human race, but varies between body areas. The racial differences are obvious in an arrangement of the melanocytic dendrites and in the melanogenesis intensity (Miyamura *et al*., 2006). The precise mechanisms that control the organization and number of melanocytes in the epidermis are not known. Melanocytes, keratinocytes and dermal fibroblasts communicate with each other by secreted factors and by cell-cell contacts (Yamaguchi *et al*., 2007). Keratinocytes control melanocyte growth and activity through paracrine growth factors and cell adhesion molecules (Haass and Herlyn, 2005; Lee, 2012). Melanocytes and keratinocytes are also the local source of the various hormones which regulate melanocyte proliferation, melanogenesis and melanocyte dendrite formation (Sulaimon and Kitchell, 2003). The cross-talk of different signaling pathways between keratinocytes and melanocytes is a part of an epidermal complex network implicated in the maintenance of skin homeostasis. In the epidermal melanin unit melanocyte is a very active constituent that secretes several signal molecules targeting not only keratinocytes but also skin immune cells (Lu *et al*., 2002; Tam *et al*., 2007). Thus, melanocytes and keratinocytes form well-organized units in the epidermis. The stable element in each unit is the melanocyte that lives long but keratinocytes die and being shed off. It is still not known how long melanocytes survive in the human skin.

1.3 MELANOCYTES

Melanocytes originate from neural crest cells (NCC) and form a diverse group of cells in the body. Although all of them have ability to produce melanin, their particular functions in all target places are much more than the melanin synthesis only (Plonka *et al*., 1999). In the human body melanocytes' presence does not limit to epidermis, iris and hair where

they give a color to these structures. Melanocytes have also been found in the heart, inner ear and nervous system (Tachibana, 1999; Brito and Kos, 2008). The life cycle of melanocytes consists of different steps including lineage specification from embryonic neural crest cells (melanoblasts), proliferation and migration of melanoblasts, differentiation of melanoblasts into melanocytes, maturation of melanocytes (melanin synthesis in melanosomes, dendritic morphology), transport of mature melanosomes to keratinocytes and eventually cell death. Numerous populations of neural crest cells (cranial, dorsal trunk, ventral trunk) give melanocytes of the skin. Embryonic origin of hair and epidermal melanocytes is the same but development is different (O'Rahilly and Müller, 2007; Ernfors, 2010). The melanocyte stem cells' localization in the adult skin is still a subject of debate. The first such place was a hair bulge (Nishimura, 2011). Experimental data clearly demonstrate that the trunk NCC migrating via a ventral pathway could remain in a myelin sheath of the cutaneous nerves and in particular situations give melanoblasts (Adameyko *et al*., 2009; Cramer, 2009). Melanocytes molecularly are recognizable by identification of melanocyte-specific proteins as tyrosinase (TYR), tyrosinase-related protein 1 and 2 (TYRP1, TYRP2/DCT), microphthalmia transcription factor (MITF), melanosomal matrix proteins (Pmel17, MART-1) (Plonka *et al*., 1999). The microscopic analysis suggests that mature melanocytes are fusiform or oval, dendritic cells, smaller than keratinocytes. There are special membrane-bound organelles present in the cytoplasm, producing melanin, called melanosomes (Seiji and Fitzpatrick, 1961).

Functions of Melanocyte:

- Melanocytes are responsible for skin, hair and eye colour by producing melanin in melanosomes and mediating their transport to neighbouring cells via dendritic processes.
- Melanocytes are responsible for sun tanning, as UV irradiations lead to augmented melanin synthesis and transfer of melanosomes to keratinocytes to protect against UV-induced DNA damage.
- Melanocytes situated in the stria vascularis are necessary for hearing and their loss can result into deafness.

- Melanocyte stem cells exist in the bulge of the hair follicles and loss of the stem cell population leads to hair graying (Goding, 2007).

1.4 MELANOCYTES LOSS IN VITILIGO

When melanocytes die, stop functioning or migrate, skin relapsed to its unpigmented form. This default colour is best exemplified in the single gene disorder albinism, where melanin synthesis is completely absent. An acquired, patchy form of melanocyte failure takes place in vitiligo, with a completely depigmented area having the same colour seen in albinism. Onset is usually gradual; as a number of epidermal turnovers are required before all the remaining pigment in keratinocytes is lost in the process of desquamation and keratinization. Thus, the skin colour disappears in a process of fading, taking quite a few weeks to months. The specific dynamics of this process are difficult to study *in vivo*. Melanocytes in hair follicles appear to be more robust, so that normal hair colour often persists in the white patch (Gilhar *et al*., 2007). Greying and eventual whitening of scalp and body hair in the condition of poliosis is not considered a type of vitiligo, but there might be some shared pathomechanisms; early greying of the hair can be seen in some vitiligo lesions (Anbar *et al*., 2009).

One dogma is that the pigment-forming melanocyte is completely absent in lesional areas (Le Poole *et al*., 1993). However, there is evidence that even in longlasting disease melanocytes still exist but lost their integrity and functionality (Tobin *et al*., 2000). This loss is associated with severe epidermal oxidative stress caused due the accumulation of H_2O_2 up to concentrations in mM range as shown *in vivo* (Schallreuter*et al*., 1999; Schallreuter *et al*., 2001). It is generally accepted that vitiligo results from loss of melanocytes rather than abridged functioning alone. This is supported by histological, ultrastructural and immunohistochemical studies showing that vitiligenous skin is devoid of melanocytes, and by the fact that melanocytes are almost never able to be cultured from lesional skin. Whether melanocytes are eliminated by necrosis or apoptosis in vitiligo has been debated. Histological and ultrastructural investigations support apoptosis by revealing nuclear shrinkage, vacuolization, loss of dendrites and detachment. The known cytotoxins such as phenolic compounds, which can induce leukoderma similar to that seen in vitiligo, leads to melanocyte loss along with plasma membrane blebbing and

DNA fragmentation suggestive of apoptosis; similar mechanisms may occur in vitiligo. The cytokine alterations and immune reactions can initiate apoptosis too, and these are thought to be relevant in melanocyte loss in vitiligo (Huang *et al*., 2002).

1.5 CLINICAL PATTERNS OF VITILIGO

Vitiligo has been classified based on clinical basis into two major forms, namely, segmental vitiligo (SV) and non-segmental vitiligo (NSV), the latter including different variants (acrofacial vitiligo, generalized vitiligo, universal vitiligo) (Taïeb and Picardo, 2007; Ezzedine *et al*., 2012) (Figure 3).

Non-segmental vitiligo (NSV): Clinically, NSV is characterized by depigmented macules that differ in size from a few to several cm in diameter, often involving both sides of the body with tendency toward symmetrical distribution. In NSV, body hairs are usually spared and remain pigmented, although hair depigmentation may occur with disease progression.

Focal vitiligo: Focal vitiligo refers to an acquired, small, isolated hypopigmented patch that does not fit a typical segmental distribution, and which has not evolved into NSV after a period of 1-2 years.

Mucosal vitiligo: Mucosal vitiligo classically refers to the involvement of the oral and/or genital mucosae.

Universal vitiligo: Universal vitiligo corresponds to complete or nearly complete depigmentation of the skin. This term is commonly used when NSV progressively leads to complete depigmentation of the skin and body hair, and sometimes oral/genital mucosae.

Segmental vitiligo (SV): Segmental vitiligo typically has a rapidly progressive but limited course, depigmentation spreads within the segment during a period of 6–24 months and then stops; further extension is rare.

Mixed vitiligo: The coexistence of SV and NSV or NSV patient with preexisting segmental lesions is termed as ‘mixed vitiligo’ (Gauthier *et al*., 2003; Mulekar *et al*., 2006; Ezzedine *et al*., 2011).

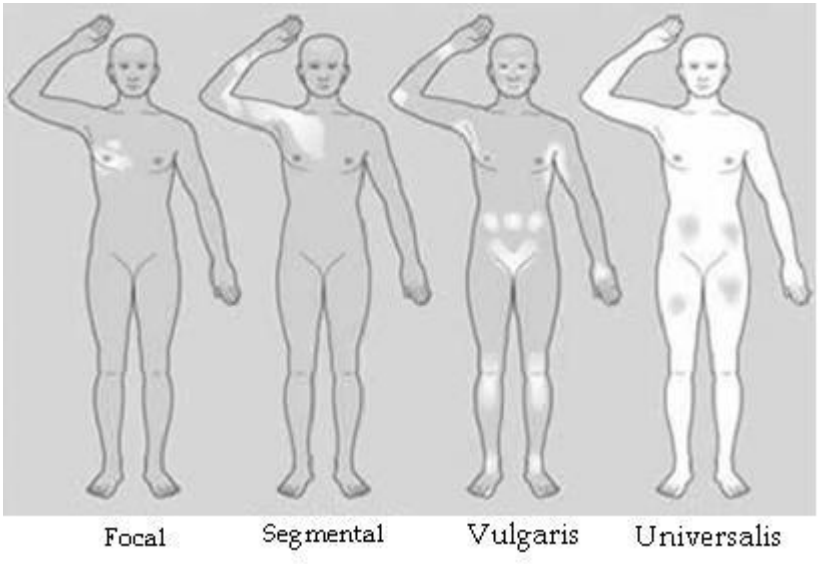

Figure 3: Clinical patterns of vitiligo.

(Source: http://redvitiligo.blogspot.in/2011/08/tipos-de-vitiligo.html)

Occupational/ contact vitiligo: The terms 'contact' or 'occupational vitiligo' (CV) have been used to describe a distinct form of vitiligo induced by exposure to certain chemicals in the workplace or at home, principally aliphatic or aromatic derivatives of catechols and phenols (Boissy, 2010; Boissy and Manga, 2004; Boissy and Nordlund, 1995; Lerner, 1971).

1.6 ETIOPATHOGENESIS OF VITILIGO

The etiopathogenesis of vitiligo has not been fully elucidated and several theories have been proposed (Passerson and Ortonne, 2005; Dell'Anna and Picardo, 2006; Westerhof and d'Ischia, 2007; Schallreuter *et al*., 2008; Boissy and Spritz, 2009), but regardless of tremendous advancement in molecular biology and genetics, there is still no universally accepted hypothesis. It could well be that vitiligo corresponds to a 'syndrome' rather than one disease, with numerous different but not mutually exclusive pathways resulting into melanocyte failure or disappearance. Genetic factors probably determine which particular pathway is predominant in a specific patient. It was first proposed in the 'convergence' theory by Le Poole *et al*. (1993) and later refined by Schallreuter *et al*. (2008). One proposed scheme is as follows: increased concentrations of endogenous or exogenous

phenol/catechol around the melanocyte compete with tyrosine for tyrosinase binding sites in the melanocyte, producing abnormal substrates and curtailing melanin production. These aberrant substrates generate reactive quinones, especially in the presence of reactive oxygen species (ROS) and disturbed redox balance, which impair cell functions and bind covalently to the catalytic centre of tyrosinase, impairing or inactivating the enzyme and further reducing melanogenesis (Passerson and Ortonne, 2005). Analogous detrimental effects occur with other peptides and enzymes from excessive ROS. Another proposal emphasizes the immunogenicity of melanosomal proteins and melanocytes, with aberrant T-cell attack resulting in melanocyte destruction by apoptosis (Kobayashi *et al*., 1998). Within these polar views of vitiligo pathogenesis, there is speculation that ROS and the immune system may somehow interact synergistically, so that both mechanisms might be relevant. Further hypotheses focus on cytokines, calcium imbalance, hormones, neural dysregulation, infections and melanocytorrhagy in the causation of vitiligo (Gauthier *et al*., 2003; Namazi, 2007).

1.6.1 Oxidative Stress Hypothesis of Vitiligo

Oxidative stress is a consequence of imbalance between pro- and anti-oxidant activities in cells. The occurrence of oxidative stress affecting the overall epidermis does appear to be involved. *In vivo*, increased H_2O_2 production, together with a decrease in the expression and activity of catalase has been described in the epidermis of vitiligo patients (Maresca *et al*., 1997; Schallreuter *et al*., 1999). In cultured melanocytes, an altered redox status is associated with an increased susceptibility of the melanocytes to pro-oxidant agents (Boissy and Manga, 2004; Dell'Anna *et al*., 2006). In addition to the involvement of cells of the epidermis, clinical and experimental evidence suggests the occurrence of oxidative stress in non-epidermal cells (Dell'Anna *et al*., 2001; Akyol *et al*., 2002; Dell'Anna *et al*., 2003; Giovannelli *et al*., 2004). Various studies have reported increased ROS production with subsequent alterations in the redox status of cells from vitiligo patients, even in erythrocytes, peripheral blood mononuclear cells (PBMC), serum, plasma or whole blood.

Skin serves as an important environmental interface providing a protective envelope that is critical for homeostasis. Also, it is a major target for toxic insults by a broad spectrum

of chemical (xenobiotic) and physical (UV radiation) agents that are capable of altering its structure and function. Many environmental pollutants act as oxidants or catalyze the production of reactive oxygen species (ROS) directly or indirectly. ROS act largely by driving several important molecular pathways that play important roles in various pathologic conditions including ischemia–reperfusion injury, atherosclerosis, and inflammatory responses. The skin holds a range of defense mechanisms that interact with toxicants to obviate their deleterious effect. These include nonenzymatic and enzymatic molecules that function as potent antioxidants or oxidant-degrading systems.

Unfortunately, these homeostatic defense mechanisms, though highly effective, have limited capacity and can be overwhelmed thereby leading to augmented ROS in the skin that can promote the development of dermatological diseases. One approach to treating or preventing these ROS-mediated diseases is based on the administration of various antioxidants in an effort to restore homeostasis. Even though many antioxidants have revealed considerable efficiency in cell culture systems and in animal models of oxidant damage, definite confirmation of their beneficial effects in human populations remains elusive (Bickers and Athar, 2006).

1.6.1.1 *Reactive oxygen species (ROS)*

Skin provides a major interface between the environment and the body, which is constantly exposed to different physical and chemical environmental pollutants (Athar, 2002). In addition, an immense number of drugs and dietary contaminants can manifest their toxicity in skin (Sander *et al*., 2004). These environmental toxicants and/ their metabolites are inherent oxidants and/or directly or indirectly drive the production of a range of reactive oxygen species (ROS). ROS are short-lived entities that are incessantly produced at low levels during the course of normal aerobic metabolism. ROS include singlet oxygen ($^1O_2$), superoxide anion (O_2^-), H_2O_2, the hydroxyl radical ($OH^\cdot$), etc. $^1O_2$ is formed by the transfer of chemical or physical energy to O_2, which at ambient temperatures behaves as a triplet and is paramagnetic. $^1O_2$ is a very strong oxidant and does not have unpaired electrons. The stepwise sequential univalent reduction of O_2 leads to the formation of O_2^-, H_2O_2, and $OH^\cdot$. Free radical reactions differ from nonradical ones in that new radical species are produced. Free radical-driven reactions are usually

chain reactions. For example, acting as an electron donor, O_2^-can lead to generation of OH° through an O_2^- driven Fenton reaction, and by reacting with NO, can make highly reactive peroxynitrite ($ONOO^-$). Electron acceptors such as molecular oxygen readily interact with free radicals to be converted into free radicals. An additional source of oxygen radicals in skin is infiltrating activated leukocytes that possess abundant systems capable of generating these species, among which are O_2^- and hypochlorite, which are important sources of ROS *in situ*. The fundamental purpose of the discharge of big amounts of ROS during the inflammatory process is to kill or destroy invading microorganisms and/or to degrade injured tissue components. It is the imprecise targeting of ROS that can induce oxidative stress in adjacent normal cells leading to enhancement of pathologic processes (Cerutti *et al*., 1992).

Fe^{2+} is an important catalyst which generates free radical-driven ROS through the Fenton reaction (Golberg *et al*., 1962). Cu^+ is almost as effective as iron as a catalyst in the Fenton reaction nevertheless is a more potent mutagen than iron owing to its potential to directly interact with DNA bases. Along with aerobic metabolism antioxidant defense systems have also co-evolved to counteract the destructive effects of ROS to diminish their potential to cause tissue damage. In spite of these antioxidant defense mechanisms, which are probably programmed genetically, ROS dependent damage of proteins, DNA, and other macromolecules accumulates during the life span of aerobic organisms. It is known that many age-dependent diseases such as osteoarthritis, atherosclerosis, cancer and neuro-degenerative disorders involve ROS during some stage of their progression (Serri *et al*., 1979; Lopez-Torres *et al*., 1994).

1.6.1.1.1 *Melanogenesis and ROS*

Melanin synthesis involves oxidation reactions and superoxide anion (O_2^-) and H_2O_2 generation, which leads to oxidative stress in melanocytes (Koga *et al*., 1992; Simon *et al*., 2009). Detention of melanin synthesis to melanosomes protects other cellular components from oxidative damage. Tyrosinase, the rate-limiting enzyme of melanin synthesis, oxidizes tyrosine to DOPA, and DOPA to dopaquinone, a specific orthoquinone which can react with nucleophilic compounds such as amino groups or thiols. The catalytic activity of tyrosinase also results in the generation of O_2^- (Tomita *et*

al., 1984; Koga *et al*., 1992). Dopaquinone is converted into dopachrome by a redox exchange. Subsequent to spontaneous decarboxylation, dopachrome either produces dihydroxyindole (5,6-DHI), that is oxidized into indole quinone or generates dihydroxyindole carboxylic acid (5,6-DHICA) after tautomerisation by TRP2, and 5,6-DHICA is subsequently converted into corresponding quinone. TRP1 has been shown to be involved not only in melanin synthesis but also in prevention of the premature melanocyte death. It has been suggested that the early cell death of vitiligo melanocytes is linked to their higher sensitivity to oxidative stress, which may occur due to complex processes of anomalous synthesis and processing of TRP1 and its interaction with calnexin (Jimbow *et al*., 2001). In addition, TRP2 protects against oxidative stress by rising glutathione levels and by reducing the DNA damage induced by free radicals and toxicity of quinones (Michard *et al*., 2008). The redox cycling from indoles to quinones generates ROS (Nappi and Vass, 1996) and polymerization of these reactive quinones ultimately leads to the formation of the brown/black eumelanin. The pheomelanin (red-yellow) varies from eumelanin in that it has a higher ratio of sulfur to quinones, and its synthesis involves the generation of cysteinyl-DOPA (instead of DOPA), which is converted into benzothiazine derivatives. These variations account for higher sunlight-induced pro-oxidant property of pheomelanin compared to eumelanin.

In the skin, the equilibrium between the antioxidant and pro-oxidant properties of melanin are mainly determined by the relative pheomelanin and eumelanin contents, the levels of melanin intermediates, and the concentrations of reactive metals in the melanosome microenvironment (Di Donato *et al*., 2002; Liu *et al*., 2005). There are conflicting reports about the role of melanin intermediates or melanin as pro-oxidants or antioxidants. Constitutive pigmentation has been directly correlated with catalase activity in cultured human melanocytes, and with the levels of thioredoxin reductase in human skin (Maresca *et al*., 2008). Production of H_2O_2 in response to UV correlates inversely with constitutive pigmentation, proposing an antioxidant effect of melanin (Song *et al*., 2009). As compared to keratinocytes, the induction of 8-hydroxydeoxyguanosine (8-OHdG), a major form of oxidative DNA damage, and expression of various base excision repair genes are higher in melanocytes (Mouret *et al*., 2012). Paradoxically, cultured human melanocytes with high melanin content were reported to be more susceptible to

UVA-induced, but less susceptible to H_2O_2 -induced oxidative DNA damage compared to their counterparts with redued melanin content (Hoogduijn *et al*., 2004; Wang *et al*., 2010). Stimulation of melanin synthesis in human melanocytes or mouse melanoma cells has been reported to increase UVA induced DNA damage (Wenczl *et al*., 1998; Marrot *et al*., 1999; Kvam and Tyrrell, 1999). In contrast, stimulation of melanogenesis in cultured human melanocytes by α-MSH (a melanocortin) raises the activity and protein levels of catalase, and markedly reduces UV-induced H_2O_2 production (Maresca *et al*., 2008; Song *et al*., 2009). In human melanoma cells, increased pigmentation protects against H_2O_2 or UV-induced mitochondrial DNA damage (Swalwell *et al*., 2011). The controversy about the pro-oxidant versus antioxidant effects of melanin intermediates and melanin is fueled by reports using purified melanin or melanin intermediates exogenously added to naked DNA or cultured cells (Tomita *et al*., 1984; Kipp and Young, 1999; Kovacs *et al*., 2012). Though these reports support the oxidative nature of melanin, the experimental conditions used are unlikely to be physiologically pertinent, as melanin is normally confined in melanosomes.

1.6.1.1.2 *ROS and Vitiligo*

Human skin is constantly exposed to a broad array of chemical, physical and biological agents, many of which are either inherent oxidants or catalyse the generation of ROS. ROS can denature proteins, damage nuclear and mitochondrial DNA, alter apoptotic pathways and mediate release of proinflammatory cytokines (Shigenaga *et al*., 1994; Briganti and Picardo, 2003). ROS are believed to be involved in the pathogenesis of inflammatory skin diseases, carcinogenesis, photoaging and hair greying (Bickers and Athar, 2006; Wood *et al*., 2009). Mitochondria are the most important endogenous source of ROS; however they are too a target of ROS-mediated damage. Thus, ROS can lead to mitochondrial dysfunction, reduced efficiency and more ROS in a vicious cycle of oxidant imbalance (Shigenaga *et al*., 1994). Several convincing evidences have shown the oxidative stress throughout the epidermis of patients with vitiligo, attributed to massive amounts of H_2O_2 in the mM range (Schallreuter *et al*., 1999). Impetus for this research came from the finding of reduced catalase levels in the epidermis of vitiligo patients, first reported in 1991 (Schallreuter *et al*., 1991). Catalase removes H_2O_2 by

converting it into water and oxygen. Generation of H_2O_2 is a physiological reaction in all cells via a number of metabolic pathways. There are also several exogenous direct and indirect sources of epidermal H_2O_2. While low concentrations, of the order of μM, are necessary for cell signalling and transcription, high concentrations can have harmful effects. High levels of epidermal H_2O_2 and methionine sulfoxide have been demonstrated *in vivo* in vitiligo using FT (Fourier Transform) Raman spectroscopy (Schallreuter *et al*., 2008, 1999). This augmented previous findings of increased H_2O_2, which were based on cell culture and skin biopsies (Schallreuter *et al*., 1999). FT Raman spectroscopy also showed oxidation of L-tryptophan in epidermal albumin, and HPLC showed the presence of allantoin in the epidermis, verifying the presence of oxidative stress in vitiligo (Rokos *et al*., 2008; Shalbaf *et al*., 2008).

Lipid peroxidation is one of the hallmarks of oxidative stress. Oxidative destruction of polyunsaturated fatty acids of phospholipids is called as lipid peroxidation (LPO) and Malondialdehyde (MDA) is an end-product of LPO, and elevated serum levels of MDA have been documented in patients with vitiligo (Koca *et al*., 2004; Jain *et al*., 2008; Khan *et al*., 2009). Ultrastructural changes suggestive of LPO have been demonstrated in melanocytes, Langerhans cells and keratinocytes in the skin of vitiligo patients, both in affected and perilesional areas (Moellmann *et al*., 1982; Bhawan and Bhutani, 1983; Boissy *et al*., 1991; Tobin *et al*., 2000). Dell'Anna *et al*. (2003) have shown that the membrane lipid defects are responsible for the generation of ROS in peripheral blood mononuclear cells (PBMCs) from vitiligo patients.

1.6.1.1.3 *Sources of ROS in Epidermis*

To date there is plenty evidence that vitiligo patients accumulate millimolar concentrations of H_2O_2 in their epidermis as well as in their blood. Several enzymes are affected by this H_2O_2 including catalase, glutathione peroxidase and 4a-carbinolamine dehydratase. The 4a-carbinolamine dehydratase enzyme disrupts the recycling of essential cofactor (6*R*)-L-erythro-5,6,7,8-tetrahydrobiopterin ($6BH_4$) for the aromatic amino acid hydroxylases and nitric oxide synthases. Furthermore, decreased dihydropteridine reductase (DHPR) activity, the last enzyme in the $6BH_4$-recycling process in whole blood of vitiligo patients have also been demonstrated, which was

normalized after removal of epidermal H_2O_2 with a topical pseudocatalase (PC-KUS) (Hasse *et al*., 2004).

NADPH oxidase activity in neutrophils and macrophages generates H_2O_2 (Darr and Fridovich, 1994). TNF-α (tumour necrosis factor-α) also contributes in the formation of H_2O_2 indirectly, by inducing manganese superoxide dismutase (Moretti *et al*., 2002). The other cytokines have also been shown to generate H_2O_2: TGF-β, EGF and PDGF (Thannickal *et al*., 2000). Monoamine oxidase A activity in the epidermis generates H_2O_2 (Schallreuter *et al*., 1996). Nitric oxide synthases generate H_2O_2 in an environment deficient in L-arginine (Schallreuter *et al*., 1999). Oxidation of aromatic phenols like 17β-oestradiol to catechols by an NADPH-dependent CYP (cytochrome *P*450) yields superoxide anion, which disproportionates to H_2O_2; this diffuses into the epidermis (Schallreuter *et al*., 2006). Photo-oxidation of epidermal 6-biopterin and sepiapterin yields H_2O_2 (Rokos *et al*., 2002). The enzymes phenylalanine hydroxylase and tyrosine hydroxylase also produce H_2O_2 as by-products. External phenols, para- and ortho-quinols, UVB and UVA, and X-rays also generate H_2O_2 in the mM range (Schallreuter *et al*., 1999). Metabolism of xenobiotics, mainly by CYP enzymes, generates ROS and H_2O_2, through toxic quinone and semiquinone intermediates (Namazi, 2009). Several of these epidermal sources of H_2O_2 are shown to be augmented in vitiligo, providing the presumed source for the high levels, which have been documented, both in the lesional and non- lesional skin of vitiligo patients (Table 1 & 2; Figure 4). Elavated epidermal and serum TNF-α levels have been shown (Moretti *et al*., 2002; Laddha *et al*., 2012). Increased photo-oxidation of epidermal 6-biopterin and sepiapterin has been demonstrated (Rokos *et al*., 2002). Impaired recycling of the essential cofactor $6BH_4$ by elevated H_2O_2 causes accumulation of H_2O_2 in the epidermis and influences all cofactor-dependent mechanisms. $6BH_4$ is an essential electron donor in the hydroxylation of the aromatic amino acids L-tyrosine, L-tryptophan and L-phenylalanine. These amino acids are substrates for melanogenesis, and thus, $6BH_4$ is an essential component of the pigmentary system (Schallreuter *et al*., 2001). Inducible nitric oxide synthase levels are elevated in epidermis of patients with vitiligo, producing both H_2O_2 and peroxynitrite (Salem *et al*., 2009).

Table 1. Sources of ROS in vitiligo. (Glassman, 2011)

Source	Level	Reference(s)
Catalase	Decreased	Schallreuter *et al.*, 1991; Maresca *et al.*, 1997; Dell'Anna *et al.*, 2001; Agrawal *et al*., 2004; Shajil and Begum, 2006; Dammak, *et al.*, 2009; Shajil *et al*., 2007
GPX/ GSH	Decreased	Beazley *et al.*, 1999; Dell'Anna *et al.*, 2001; Agrawal *et al*., 2004; Shajil and Begum, 2006; Shajil *et al*., 2007
H_2O_2	Increased	Schallreuter *et al.*, 1999
Peroxynitrite	Increased	Salem *et al.*, 2009
NADPH oxidase	Increased	Darr *et al.*,1994
TNF-α	Increased	Moretti *et al.*, 2002; Laddha *et al*., 2012
Oxidized pterins	Increased	Rokos *et al.*, 2002
$6BH_4$ recycling	Decreased	Schallreuter *et al.*, 1994b; Kaufman, 1997; Schallreuter *et al.*, 2001; Rokos *et al.*, 2002
iNOS	Increased	Salem *et al.*, 2009
Hcy	Increased	Shaker *et al.*, 2008
Monoamine oxidase A	Increased	Schallreuter *et al.*, 1996
SOD	Increased	Koca *et al.*, 2004; Picardo *et al.*, 1994; Agrawal *et al*., 2004; Yildirim *et al.*, 2004; Hazneci *et al.*, 2005; Shajil and Begum, 2006; Khan *et al.*, 2009; Dammak, *et al.* ,2009; Laddha *et al*., 2013
Thioredoxin reductase	Decreased	Gibbons *et al.*, 2006
Xanthine oxidase	Increased	Shalbaf *et al.*, 2008
Catecholamines	Increased	Westerhof *et al.*, 2007
GTP-cyclohydrolase I	Increased	Schallreuter *et al.*, 1994; Chavan *et al.*, 2009
Vitamin E	Decreased	Agrawal *et al*., 2004; Jain *et al.*, 2008; Khan *et al.*, 2009
Monoamine oxidase A	Increased	Schallreuter *et al.*, 1996
NADPH oxidase	Increased	Schallreuter *et al.*, 1999
Photooxidation of pterins	Increased	Rokos *et al.*, 2002
Nitric oxide synthases	Increased	Gibson and Lilley, 1997
TYRP1	Decreased	Jimbow *et al.*, 2001

Hcy oxidation also generates ROS, and elevated serum Hcy levels have been reported in vitiligo patients (Shaker *et al*., 2008). Furthermore, MTHFR which is involved in Hcy metabolism, affects ROS generation and the apoptosis process via downregulation of antioxidant enzymes such as GPX1 (Weiss *et al*., 2001; wang *et al*., 2012;). Chen *et al*. (2014) have suggested that an increased risk of vitiligo was associated with higher levels of total Hcy indicating the possible involvement of MTHFR and Hcy in pathogenesis of vitiligo. Increased monoamine oxidase A activity has been found in the epidermis of patients with vitiligo, elaborating H_2O_2 (Schallreuter *et al*., 1996). SOD is a group of metallo-enzymes that scavenge and dismutate superoxide (O_2^-) anion in its detoxification process resulting in the formation of O_2 and H_2O_2 (Koca *et al*., 2004). Increased SOD levels have been reported in lesional and non- lesional skin from vitiligo patients (Sravani *et al*. 2009). Our earlier lab studies also showed significantly higher activities and/ or transcript levels of SODs in vitiligo patients (Agrawal *et al*., 2004; Laddha *et al*., 2013). Other reports have also shown elevated serum/ PBMCs and tissue SOD activity in vitiligo patients (Picardo *et al*., 1994; Dell'Anna *et al*., 2003; Koca *et al*., 2004; Yildirim *et al*., 2004; Hazneci *et al*., 2005; Dammak *et al*., 2009). Elevated SOD activity would seem a likely source of H_2O_2 in vitiligo, but results have been contradictory. Various studies indicate the importance of FOXO3a as a transcription activator of *SOD2*, catalase (*CAT),* and peroxiredoxin 3 genes (Kops 2002; Nemoto 2002; Chiribau 2008). FOXO3a is a member of the forkhead class O (FOXO) transcription factors, which plays an important role in cell cycle regulation, apoptosis, oxidative stress, and DNA repair (Brunet 1999; Furukawa-Hibi 2005). Olmos *et al*. (2009) reported that FOXO3a can protect cells from oxidative stress by regulating *CAT* and *SOD2*. Also, FOXO3a-deficient hematopoietic stem cells have shown reduced expression of ROS detoxification genes leading to elevated levels of ROS (Yalcin 2008). Ozel Turkcu *et al*. (2013) have shown decreased FOXO3a levels in vitiligo patients and its association with oxidative stress and active vitiligo suggestive of its significant role in oxidative stress mediated pathogenesis of vitiligo. XO (xanthine oxidase) catalyses the conversion of purine bases into uric acid producing H_2O_2 as a by-product (Shalbaf *et al*., 2008). XO contains two non-haem Fe atoms in its structure, which can react with H_2O_2 to produce $OH^{-}\cdot$, the most potent ROS with respect to DNA damage. Because H_2O_2 also oxidizes uric acid to allantoin, this

metabolite is a useful marker of oxidative stress. XO activity has been shown in skin, and high plasma levels have been observed in vitiligo patients. XO activity in melanocytes and keratinocytes was demonstrated by Shalbaf *et al*. (2008), with H_2O_2 regulating enzyme activity in a concentration dependent manner: low levels (μM) up-regulated activity, whereas elevated levels were suppressive. H_2O_2 mediated oxidation of tryptophan and methionine residues in XO are thought to be the mechanism for this effect. Allantoin was also detected in epidermis of acute vitiligo, further supporting a role for ROS in vitiligo pathogenesis (Shalbaf *et al*., 2008).

Table 2. Downstream effects of ROS in vitiligo. (Glassman, 2011)

Effect	Level	Reference(s)
Tyrosinase	Decreased	Wood *et al.,* 2004
TYRP1	Decreased	Jimbow *et al.,* 2001
MSR	Decreased	Zhou *et al.,* 2009 Schallreuter *et al.,* 2008
Catalase	Decreased	Schallreuter *et al.,* 1991; Dammak, *et al.,* 2009; Gibbons *et al.* ,2006
Thioredoxin reductase	Decreased	Gibbons *et al.,* 2006
Tyrosine hydroxylase	Decreased	Schallreuter *et al.,* 2001, Rokos *et al.,* 2002
POMC peptides	Decreased	Graham *et al.,* 1999; Spencer *et al.,* 2005; Spencer *et al.,* 2007
L-Phenylalanine	Increased	Schallreuter *et al.,* 1998
Acetylcholine	Increased	Gibbons *et al.*,2006; Schallreuter *et al.,* 2004
$6BH_4$ recycling	Decreased	Schallreuter *et al.,* 2001, Hasse *et al.,* 2004
Calmodulin, furin	Decreased	Schallreuter *et al.,* 1998; Schallreuter *et al.,* 2007; Spencer *et al.,* 2007
Albumin (epidermal)	Decreased	Rokos *et al.,* 2002
Malondialdehyde	Increased	Koca *et al.,* 2004; Jain *et al.,* 2008; Khan *et al.,* 2009
Methionine sulfoxide	Increased	Schallreuter *et al.,* 2008; Zhou *et al.,* 2009, Schallreuter *et al.,* 2008
Allantoin	Increased	Shalbaf *et al.,* 2008

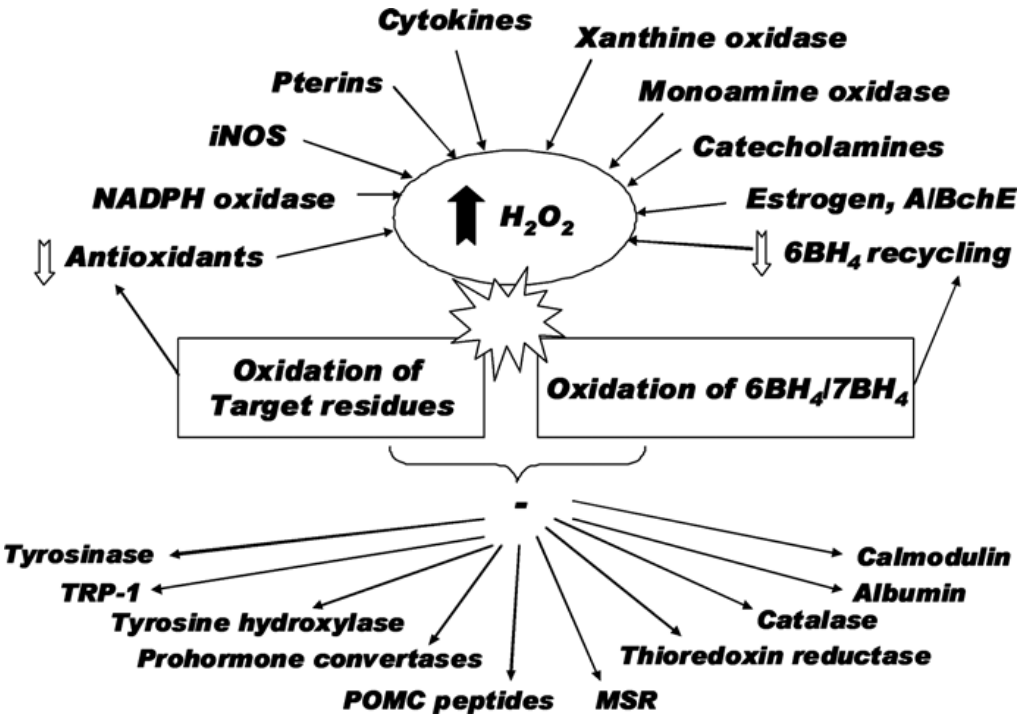

Figure 4. Sources, effects and interactions of ROS/ H_2O_2 in vitiligo. (Glassman, 2011)

1.6.1.2 ANTIOXIDANTS

Antioxidant molecules interact with ROS or their by-products to either remove them or to diminish their deleterious effects (Figure 5). The antioxidant system comprises of different types of functional components such as enzymatic and nonenzymatic antioxidants. The enzymatic antioxidants comprise of superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase and glutathione S-transferase. The non-enzymatic antioxidants include reduced glutathione, vitamin C, vitamin E (α - tocopherol), uric acid, carotenoids, flavanoids, ubiquinol etc.

1.6.1.2 .1 Enzymatic antioxidants

Superoxide dismutase:

Superoxide dismutases (SODs) are metalloenzymes found widely distributed in prokaryotic and eukaryotic cells (Fridovich, 1995). They constitute an enzyme family that catalyzes the conversion of supeoxide anion to H_2O_2.

$$2O^{2-} + 2H^{+} \rightarrow O_2 + H_2O_2$$

Several common forms of SOD exist; however, there are three major families of superoxide dismutase, depending on the protein fold and the metal cofactor: the Cu/Zn type, Fe and Mn types, and the Ni type. These SODs form the major superoxide scavenging system in the mitochondria, nucleus, cytoplasm and extracellular spaces. These SODs are the products of different genes and are designated by their primary location as follows. SOD1 (Cu-Zn SOD, cytoplasmic), SOD2 (Mn SOD, mitochondrial), SOD3 (Cu-Zn SOD, extracellular) (Johnson and Giulivi, 2005).

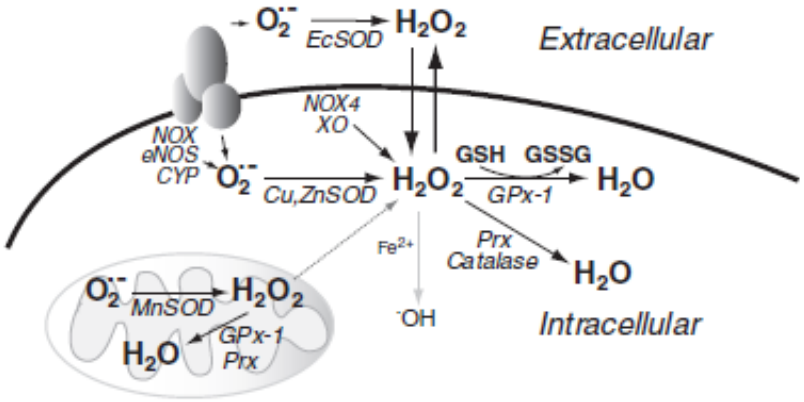

Figure 5. Modulation of cellular ROS. Superoxide originates from normal mitochondrial respiration or from enzymatic sources, such as uncoupled endothelial nitric oxide synthase (eNOS), NADPH oxidases (NOX), or P450 (CYP) isoforms. Superoxide is dismutated enzymatically or spontaneously to H_2O_2. Mitochondrially localized MnSOD, intracellular Cu, ZnSOD or extracellular SOD are enzymatic sources of this conversion. H_2O_2 can also be produced directly by xanthine oxidase and NADPH oxidase subtype 4. Under certain oxidative stress conditions, H_2O_2 can react with free iron to promote the formation of OH°. Under normal cellular conditions the amount of free iron in the cell is low limiting the flux via this pathway (shown by light gray). H_2O_2 is subsequently enzymatically reduced by GPX, including GPX1, as well as catalase and Prx. Catalase is primarily in the peroxisomes, while various Prxs localize to the mitochondria (e.g., Prx 3,5) or cytosol (such as Prx 1,2). Most Prxs use thioredoxin as a source of reducing equivalents, although Prx 6 appears to function as a GSH-dependent peroxidase. Importantly, GPX1 can be found in the cytosol, in mitochondria, and also in peroxisomes. GPX1 utilizes GSH as a cofactor to reduce H_2O_2, resulting in the formation of GSSG. (Lubos *et al*., 2011)

Catalase:

Catalase is present in the peroxisomes of nearly all aerobic cells and protects the cell from the toxic effects of H_2O_2 by catalyzing its decomposition into molecular oxygen and water without the production of free radicals and the overall reaction is as follows:

$$2\ H_2O_2 \longrightarrow 2\ H_2O + O_2$$

This reaction is a first order reaction and depends entirely on the concentration of H_2O_2. At high substrate concentrations the rate of reaction is usually rapid. However, millimolar levels of H_2O_2 ($\geq 10^{-3}$ M) are reported to result in the inactivation of catalase (Schallreuter *et al.*, 1991).

Glutathione peroxidase (GPX):

Glutathione peroxidase catalyzes the reduction of various organic hydroperoxides, as well as H_2O_2, with glutathione as hydrogen donor. There are two locations of GPX in the cells, mitochondria and cytosol. The cytosolic from of GPX (GPX1) is the first and best characterized selenoprotein (Arthur, 2000). GPX1 is the most abundant version, found in the cytoplasm of nearly all mammalian tissues, whose preferred substrate is H_2O_2. The reaction catalyzed by GPX is given below.

$$2GSH + H_2O_2 \xrightarrow{GPX} GSSG + 2H_2O$$

Glutathione reductase then reduces the oxidized glutathione to GSH

$$GSSG + NADPH + H^+ \xrightarrow{GR} 2\ GSH + NADP^+$$

Glutathione reductase:

Glutathione reductase (GR) is a ubiquitous enzyme associated with the hexose monophosphate shunt of glucose metabolism. It catalyses reduction of oxidized glutathione (GSSG) to reduced GSH, with the concomitant conversion of NADPH to $NADP^+$ (Beutler and Yeh, 1963).

Glutathione S transferase:

Glutathione S-transferases (GSTs) are a super family of enzymes. It plays an important role in the detoxification and elimination of xenobiotics. This process involves conjugation of glutathione with electrophilic metabolites and extrusion of the conjugate out of the cell for further metabolism. GSTs also function as glutathione peroxidases by

reducing organic hydroperoxides to the corresponding alcohols, of importance for protection against oxidative stress and consequent lipid peroxidation. The reaction catalyzed by GST is given below:

$$RX + \text{glutathione} \xrightarrow{GST} HX + \text{R-S-glutathione}$$

Glucose -6- phosphate dehydrogenase (G6PD):

The intracellular redox potential is determined by the concentrations of oxidants and reductants. A critical modulator of the redox potential is NADPH, the principal intracellular reductant in all cell types. Glucose -6-phosphate dehydrogenase (G6PDH), the rate-limiting enzyme of the pentose phosphate pathway (PPP) determines the amount of NADPH by controlling the metabolism of glucose via PPP (Kletzien *et al.*, 1994). The amount of NADPH maintains an adequate level of reduced glutathione (GSH). G6PDH is present in all human cells but is particularly important to red blood cells. NADPH protects the sulfhydryl groups (-SH) of hemoglobin and the red cell membrane from oxidation by the reactive oxygen species. The reaction catalyzed by G6PD is given below:

$$\text{Glucose -6-phosphate} + NADP^{+} \longrightarrow \text{Glucono-1, 5-lactone 6-phosphate} + NADPH + H^{+}$$

1.6.1.2 .2 Non-Enzymatic antioxidants

Reduced glutathione :

Glutathione (γ-glutamylcysteinylglycine, GSH) is a sulfhydryl (-SH) antioxidant, antitoxin, and enzyme cofactor. Glutathione is ubiquitous in animals, plants, and microorganisms, and being water soluble is found mainly in the cell cytosol. GSH is present in millimolar concentrations in virtually all healthy cells. Glutathione exists in two forms: the antioxidant "reduced glutathione" tripeptide is conventionally called glutathione (GSH) and the oxidized form is a sulfur-sulfur linked compound, known as glutathione disulfide (GSSG). The GSSG/GSH ratio may be a sensitive indicator of oxidative stress (Rahman *et al.*, 2005). Glutathione status is homeostatically controlled both inside and outside the cell, being continually self-adjusting with respect to the

balance between GSH synthesis (by GSH synthetase enzymes), its recycling from GSSG (by GSH reductase), and its utilization (by peroxidases, transferases, transhydrogenases, and transpeptidases).

The GSH can act as a free radical scavenger and as an antioxidant enzyme cofactor. Glutathione is most concentrated in the liver (10 mM), where the "P450 Phase II" enzymes require it to convert fat soluble substances into water soluble GSH conjugates, in order to facilitate their excretion. GSH depletion leads to cell death, and has been documented in many degenerative conditions. Mitochondrial GSH depletion may be the ultimate factor determining vulnerability to oxidant attack. Rare mutations in genes encoding glutathione synthase, glutamate-cysteine ligase and g-glutamyl transferase enzymes in the g-glutamyl cycle can reduce tissue and blood GSH levels (Schulman *et al*., 1975; Oshima *et al*., 1976; Dalton *et al*., 2004). The depletion of this tripeptide augments oxidant injury, while administration of agents that increase tissue GSH levels such as 4-oxothiazoladine carboxylate or N-acetyl cysteine affords protection against the noxious effects of ROS-generating agents (Wu *et al*., 2004).

Vitamin E:

Vitamin E refers to a group of antioxidants, which consists of tocopherols and tocotrienols, in which α-tocopherol has the highest biological activity. Vitamin E is membrane-bound. Alpha tocopherol is the major lipid soluble, chain breaking antioxidant, which protects mammalian membranes and lipoproteins from damage. Vitamin E is mainly found on membranes where they either interrupt the propagation step of lipid peroxidation by destroying peroxyl radicals ($ROO^{\cdot}$) or block the formation of hydroperoxides from singlet oxygen (Halliwel and Chirico, 1993).

Alpha tocopherols are efficient scavengers of peroxyl radicals in phospholipid bilayers. It scavenges lipid peroxyl radicals ($LOO^{\cdot}$) through hydrogen atom transfer. The α-tocopherol radical might also react with a further peroxyl radical to give a non radical product i.e. one molecule of α-tocopherol is capable of terminating two peroxidation chains (Chaudiere and Ferrari-Iliou, 1999).

$$\alpha\text{-TOH} + LOO^{\cdot} \longrightarrow \alpha\text{-TO} + LOOH$$

$$LOO^{\cdot} + \alpha\text{-}TO^{\cdot} \longrightarrow \alpha\text{-TocOOL}$$

1.6.1.3 ANTIOXIDANT STATUS IN VITILIGO

SOD activity has been shown to increase in both lesional and non-lesional epidermis (Schallreuter *et al*., 1991; Sravani *et al*., 2009) as well as in melanocytes (Maresca *et al*., 1997). Also, there are several reports including ours suggesting increased systemic activities of SOD1, SOD2 and/ SOD3 in vitiligo patients compared to controls (Hazneci *et al* 2005, Yildirim *et al* 2003, 2004; Dell'Anna *et al* 2001; Chakraborty *et al* 1996; Ines *et al* 2006; Laddha *et al*., 2013). Our previous lab study has shown that the association of Thr58Ile has a profound effect on SOD2 activity in vitiligo patients and the increased activity of SOD2 may in part be contributed by the polymorphism as well as increased SOD2 mRNA levels. Also, the SOD3 Arg213- Gly polymorphism and increased levels of SOD3 transcripts in combination may be responsible for increased activity of SOD3 in extracellular fluids, resulting in increased H2O2 production (Laddha *et al*., 2013).

Catalase is deactivated by H_2O_2 by oxidation its porphyrin ring and tryptophan and methionine residues in the structure of the enzyme-active site and the cofactor NADPH binding site. This explains why catalase levels are low in vitiligo (Gibbons *et al*., 2006). Low tissue levels of catalase in vitiligo patients were confirmed, with lower levels in active rather than stable disease (Dammak *et al*., 2009; Sravani *et al*., 2009). Numerous heterogeneous SNPs in the catalase gene, observed in vitiligo patients, might render these catalase variants particularly vulnerable to deactivation by H_2O_2, thereby predisposing to vitiligo (Casp *et al*., 2002; Wood *et al*., 2008). Further proof of the role of H_2O_2 and ROS requires evidence of improvement in lesions with antioxidant therapies. Topical pseudocatalase (PC-KUS) is a narrowband UVB-activated bis-MnIII-$(EDTA)_2$-$(HCO_3^-)_2$ complex that is applied as a lotion to the entire skin, followed by low-dose UVB exposure. It functions as a synthetic catalyst to oxidize H_2O_2 into O_2 and H_2O, thus mimicking natural catalase. This regimen causes rapid repigmentation of even long-standing vitiligo (Schallreuter *et al*., 2002; Schallreuter *et al*., 2008). Consequent with this is a marked reduction in epidermal H_2O_2 as measured *in vivo* with FT Raman spectroscopy. Bathing in Dead Sea water also had a pseudocatalase effect, though milder, perhaps from its high concentration of transition metals like Mn (Schallreuter *et al*., 2002). A randomized controlled trial contrasting narrow-band UVB with or without a

pseudocatalase cream did not confirm additional benefit from the cream, although the methodology was different, and the pseudocatalase was not the same as PC-KUS (Bakis-Petsoglou *et al*., 2009). A catalase of vegetable origin, combined with SOD in a microsphere formulation, showed equivalent efficiency to topical betamethasone, in combination with sunlight exposure in the treatment of vitiligo (Sanclemente *et al*., 2008). Oral vitamin E appeared to confer some additional benefit to narrow-band UVB therapy of vitiligo, with a corresponding decrease in plasma MDA levels in vitamin E group (Elgoweini *et al*., 2009).

Glutathione peroxidase (GPX) and selenium levels have been found be variable in tissue and blood of patients with active vitiligo (Passi *et al*., 1998; Boisseau-Garsaud *et al*., 2002; Yildirim *et al*., 2004; Khan *et al*., 2006). Various studies reported decreased GPX activity in plasma and skin biopsy samples of vitiligo patients (Dammak *et al*., 2004; Beazley *et al*., 1999). Hazneci *et al*. (2005) have shown lower levels of GPX in lesional and non-lesional skin from vitiligo patients, whereas, Maresca *et al*. (1997) have reported higher levels of GPX in vitiligo patients. Our previous lab studies have shown decreased erythrocyte GPX activity in patients with vitiligo (Agrawal *et al*., 2004; Shajil *et al*., 2007). Total blood antioxidant status was also shown to be increased in vitiligo patients (Boisseau-Garsaud *et al*., 2002).

Glucose 6-phosphate dehydrogenase (G6PD) levels were reported to be decreased in vitiligo patients by various studies including ours (Agrawal *et al*., 2004; Shajil and Begum, 2006; Arican and Kurutas, 2008; Farahi-Jahromy *et al*., 2014). However, there is no information about the molecular basis of G6PD deficiency in vitiligo. Variations in *G6PD* gene lead to G6PD deficiency; most of which are single amino acid substitutions and the resulting G6PD variants have differential enzyme activity and were found to be associated with a variety of clinical phenotypes, resulting from an increased susceptibility of individuals to oxidative stress (Tishkoff *et al*., 2001; Cappellini and Fiorelli, 2008).

Glutathione transferase (GST) is a superfamily of widely expressed isoenzymes involved in defence against oxidative stress. Polymorphisms in the *GST* gene have been reported, which confer risk of vitiligo (Liu *et al*., 2009).

Thioredoxin reductase, another antioxidant enzyme system, is impaired by high H_2O_2 levels through oxidation of the NADPH cofactor-binding site and the enzyme active site. Low activities of thioredoxin reductase have been shown in vitiligo (Chavan *et al*., 2009).

Methionine sulfoxide reductase (MSR) has been shown to be low in vitiligo patients. Oxidation of methionine sulfoxide reductase A (MSRA) and MSRB by H_2O_2 leads to reduced functioning of this significant protein-repair mechanism. In addition, *in vitro* down-regulation of MSRA in normal cultured melanocytes rendered them very susceptible to oxidative stress (Ogawa *et al*., 2006; Schallreuter *et al*., 2008).

Melatonin also has antioxidant effect, and an abnormality of melatonin receptors was proposed as a mechanism for the development of vitiligo (Slominski *et al*., 1989). Melatonin is synthesized in the skin yielding potent free radical scavengers like *N*1-acetyl-*N*2- formyl-5-methoxykynuramine, which also stabilizes the mitochondrial electron transport chain (Reiter *et al*., 2007).

Antioxidants and Apoptosis

Antioxidants react in tandem with proteins like Bcl-2 and caspases to regulate apoptosis, which can result from oxidative challenge (Simon *et al*., 2000). For example, Bcl-2 expression prevents cell death from H_2O_2 and menadione (Lee *et al*., 2001). Bcl- 2 is expressed by melanocytes and keratinocytes and is crucial for their survival. Melanocytes from patients with vitiligo have lower levels of Bcl-2 than normal, presumably contributing to melanocyte fragility (Boissy and Manga, 2004). Elevated levels of Bcl-2 have also been reported in vitiligo (Salem *et al*., 2009), as well as normal levels (Van den Wijngaard *et al*., 2000), and the role of melanocyte apoptosis in vitiligo is still unclear.

1.6.2 AUTOIMMUNE HYPOTHESIS OF VITILIGO

Melanocytes also contribute to the skin immune system, secreting a wide range of signal molecules and responding to cytokines and growth factors. Melanocytes can phagocytize and eliminate exogenous antigens, which have penetrated the skin barrier (Le Poole *et al*., 1993), and they can process and present antigen in the form of peptides with HLA (human leucocyte antigen) class II molecules to T-cells, triggering an adaptive immune

response. Melanosomal proteins are involved in this antigen processing (Le Poole and Luiten, 2008). Activation of T-cells by melanocytes has shown by the secretion of co-stimulatory molecules like ICAM (intercellular adhesion molecule)-1 and LFA (leucocyte fusion-associated molecule)-3 (Le Poole *et al*., 1993; Das *et al*., 2001). Case reports on inflammatory vitiligo furnished the involvement of T cells in the pathogenesis of vitiligo (Garb and Wise, 1948; Buckley, 1953; Michaelsson, 1968). Immune-mediated responses are consistently observed in progressive vitiligo at the periphery of depigmenting patches. Histopathological investigations of the peri-lesional skin suggest lymphocyte involvement in the depigmentation process. Immunohistochemical studies have also confirmed the presence of infiltrating T cells and their frequent opposition to peri-lesional melanocytes in skin biopsies from vitiligo patients (Le Poole *et al*, 1996; Yagi *et al*, 1997). Notably, similar *in situ* T cell infiltrates, primarily $CD8^+$ T cells, have also been detected in generalized vitiligo (Gross *et al*, 1987; Badri *et al*, 1993; Abdel-Naser *et al*, 1994; van den Wijngaard *et al*, 2000; Sanchez-Sosa *et al*., 2013; Bertolotti *et al*., 2014). T cells are more prevalent in vitiligo peri-lesional skin than in surrounding non-lesional skin. The lymphocyte infilterate consists essentially of $CD8^+$ T cells with occasional $CD4^+$ T cells (Le Poole *et al.*, 1996). The prevalence of cytotoxic T cells and their co–localization with surviving melanocytes suggest T cell mediated cytotoxicity towards the melanocytes (Wankowicz-Kalinska *et al.*, 2003). Various other studies including ours show decrease in systemic $CD4^+$ T-cells and an increase in $CD8^+$ T-cells with consequent decrease in $CD4^+/CD8^+$ ratio in vitiligo patients, suggesting role of $CD8^+$ cells in melanocyte death (Grimes *et al*., 1986; Halder *et al*., 1986; Nigam *et al.*, 2011; Dwivedi *et al.*, 2013). Several studies have shown a defective functionality and decreased frequency of regulatory T cells (Tregs) in vitiligo patients suggestive of the unchecked activation of $CD8^+$cells (Dwivedi *et al*., 2013a; Lili *et al*., 2012). Furthermore, Bertolotti *et al*. (2014) have also reported the presence of IFNα secreting plasmacytoid dendritic cells (pDC) in the infilterate of progressive vitiligo. IFNα induces the expression of *MxA*, which encodes a guanosine triphosphate (GTP)-metabolizing protein. Association of MxA with the expression of chemokine (C-X-C motif) ligand 9 (*CXCL9*) correlates well with the recruitment of chemokine (C-X-C motif) receptor 3^+ ($CXCR3^+$) immune cells. Further, they also showed increased expression of *MxA* in peri-

lesional skin in close opposition to surviving melanocytes within the T-cell infiltrate. In contrast, MxA was not evident in lesional skin, suggesting that IFNα production is an early event in the progression of the disease. Autoimmune aspect of vitiligo pathogenesis is strongly supported by the presence of autoreactive T-cells (Ongenae *et al*., 2003; Das *et al*., 2001; Le Poole *et al*., 1996). They target melanocyte-specific antigens, such as melan-A/MART1, Gp100/Pmel 17 (a melanosomal matrix glycoprotein), tyrosinase (Fishman *et al*., 1993; Song *et al*., 1994; Kemp *et al*., 1997), TRP1 and TRP2 (Wankowicz-Kalinska *et al*., 2003; Palermo *et al*., 2001) that are localized primarily on melanosomes (Kemp *et al*., 1998a; Kemp *et al*., 1998b; Hearing *et al*., 1999). In fact auto-antibodies against melanocyte antigens have been detected in the sera of vitiligo patients (Alkhateeb *et al*., 2003, Pradhan *et al*., 2013; Laddha *et al*., 2014). The transcription factors SOX9 and SOX10 have also been identified as melanocyte auto antigens (Hedstrand *et al*., 2001). Even auto antibodies against HLA Class I molecules have been detected in vitiligo (Ongenae *et al*., 2003). A positive correlation has been seen between the level of melanocyte antibodies and disease progression in vitiligo (Harning *et al*., 1991). This is further supported by the concentration of these antibodies in propotion to extent of skin lesion (Naughton *et al*., 1986). Overall all these point to a new innate immune pathway leading to the progression of vitiligo. Genes within class II region of the major histocompatibility complex (MHC) are associated with several autoimmune diseases (Pamer and Cresswell, 1998). This highly polymorphic region includes several genes involved in the processing and presentation of antigens to the immune system including low molecular weight polypeptide 2 and 7 (LMP2 and LMP7) and transporter associated with antigen processing 1and 2 (TAP1 and TAP2). Though *LMP/TAP* gene cluster is located on MHC class II region of chromosome 6, it is involved in antigen presenting function of MHC class I molecule. Different researchers have reported association of *LMP7* and *TAP1* with susceptibility to vitiligo (Casp *et al.*, 2003). LMP2 and LMP7 are also involved in the degradation of ubiquitin tagged cytoplasmic proteins to peptides while, TAP1 and TAP2 are involved in transportation of peptides into the endoplasmic reticulum for exposure to nascent MHC class I molecules (Pamer and Cresswell, 1998). MHC-I molecules are critical in the regulation of cytotoxic effector functions of natural killer (NK) cells and T cells. MHC-I molecules present antigens to

cytotoxic T cells and are part of the recognition signals that regulate activation of NK cells (Ulianich *et al*., 2011). Usually, antigenic peptides are generated by proteasomal degradation of cytosolic proteins and consequently translocated to ER by TAP. In the ER, assembly of MHC class I α chain, β2-microglobulin and peptides is guided by chaperones (Pamer *et al*., 1998). On the other hand antigenic peptides are associated with the chaperones GRP94, GP96, PDI and calreticulin (Nieland *et al*., 1996; Lammert *et al*., 1997; Spee and Neefjes, 1997; Nicchitta, 1998). Functional class I-peptide complexes are then transported to the cell surface. A necessary condition for the successful completion of this complex process is glycosylation and correct folding of MHC class 1 heavy chains in the ER. A failure in the above results in their slow or inefficient transport to the cell surface (Carreno *et al*., 1995; Degen *et al*., 1992; Noessner *et al*., 1995; Rajagopalan and Brenner, 1994; Parham, 1996). ER stress may result in reduced expression of MHC class I on the cell surface, thereby preventing the recognition of cells by the adaptive and innate immune system (Gleimer *et al*., 2003; Hickman-Miller *et al*., 2004). It has been reported that defects in the expression of different elements of the MHC class I antigen processing machinery, such as the proteasomal subunits LMP2 and LMP7 and the peptide transporters TAP1 and TAP2, account for impaired MHC class I surface expression (Casp *et al.*, 2003). In addition, an inappropriate functioning or expression of LMP7 might inhibit antigen processing and presentation, leading to a loss of peripheral tolerance to self-antigens and occurrence of several autoimmune diseases (Casp *et al.*, 2003). In this context, Ulianich *et al*. (2011) have shown ER stress induced decrease in surface expression of MHC class I in thyroid cells. This effect was accompanied by activation of NKcells and their cytotoxicity to thyroid cells by increased IFNγ production. Together, these data indicate ER stress induced reduction in MHC class I expression and reduced NK-cells self-tolerance. It has been shown that IFNγ induces LMP and TAP subunits (Casp *et al.*, 2003). Taken together these results suggest IFNγ induced expression of MHC-I, MHC-II and TAP on melanocytes. Recently it has been shown that IFNγ induces senescence in melanocytes (Wang *et al*., 2014). IFNγ signaling impedes maturation of melanosomes by intensive downregulation of a few pigmentation genes that leads to IFNγ–mediated hypo- pigmentation of melanocytes (Natrajan *et al*., 2014). IFNγ and TNFα induce the expression of ICAM1 on melanocytes (Yohn and

others 1990). Our previous lab studies also showed increased levels of IFNγ, TNFα, TNFβ and ICAM1 in vitiligo patients (Dwivedi *et al*., 2013b; Laddha *et al*., 2012; Laddha *et al*., 2013). Further, levels of ICAM1 were found to be upregulated in melanocytes of peri-lesional skin of vitiligo patients and melanocyte- T cell binding was enhanced by elavated expression of ICAM1 on melanocytes (Al Badri 1993). A melanocyte is in close association with ~32 keratinocytes in the epidermal melanin unit. Keratinocytes synthesize cytokines, such as TNFα, IL1α, IL6, and transforming growth factor β (TGFβ), which are paracrine inhibitors of melanocyte proliferation and melanogenesis. In numerous cell types, TNFα plays an important role in apoptosis through activation of the receptor-mediated apoptotic pathway (Gupta and Gollapudi, 2006). Moreover, TNFα can also inhibit melanocyte stem cell differentiation (Alghamdi *et al*., 2012). NACHT leucine-rich repeat protein 1 (NLRP1), known to be involved in inflammation and apoptosis (Martinon *et al*., 2007; Tschopp *et al*., 2003), modulates the response of cells towards proinflammatory cytokines such as IL1β, IFNγ and TNFα. Previously, the increased expression of *NLRP1* in vitiligo patients have been reported from Gujarat (Dwivedi *et al*., 2013). Bassiouny *et al*. (2011) have reported higher level of IL17 and its positive correlation with disease pregression in both the lesional skin and sera of vitiligo patients. Zhao *et al* (2006) found decreased levels of anti-inflammatory cytokine IL10 in vitiligo patients. All the above indicate the significant role of immune mechanisms in the progression of vitiligo which is futher corroborated by the use of immunosuppressive treatments for vitiligo (Lepe *et al*., 2003).

1.6.3 Cross Talk between Oxidative Stress and Immune System in Vitiligo

In vitiligo, augmented reactive oxygen species (ROS) is believed to be involved in disease onset, while melanocyte-specific cytotoxic $CD8^+$ T cells infiltrating the peri-lesional border directly contribute to melanocyte loss (van den Boorn *et al*., 2009). A variety of evidences suggest oxidative stress–autoimmunity-mediated melanocyte destruction as a key convergent pathway (Toosi *et al*., 2012; Zhang *et al*., 2014). However, the exact mechanism linking oxidative stress to autoimmunity is largely unknown. Numerous studies indicate possible cross talk between ER and oxidative stresses; however the mechanistic link is not fully understood (Malhotra *et al*., 2007).

Oxygen-utilizing metabolic processes such as oxidative phosphorylation in mitochondria generate ROS.

The presence of anti-melanocyte antibodies can serve as a marker for the initiation and progression of autoimmunity in vitiligo (Laddha *et al*., 2014; Alkhateeb *et al*., 2003; Pradhan *et al*., 2013). Cytokines have been evidenced to activate the expression of inducible nitric oxide synthase (iNOS), resulting into excessive nitric oxide (NO) production. Interestingly, cytokines are reported to induce ER stress via iNOS and JNK pathways. NO has also been suggested to be an important mediator of cell death. Inflammatory cytokines including IFNγ, TNFα and IL1β can induce iNOS expression which then produces copious amount of NO (Del´epine *et al.,* 2000). JNK pathway is activated by IL1β (Wang *et al.,* 2009). Activation of ER stress pathway is stimulated by IL1β and IFNγ leading to apoptosis via NO synthesis (Cardozo *et al.*, 2005). Previous studies have shown increased expression levels of TNFA (Laddha *et al*., 2012), IFNG (Dwivedi *et al*., 2013) and IL1B (Laddha *et al*., 2014) in patients with vitiligo. Increased level of NO induces DNA damage and results in apoptosis via p53 pathway or necrosis via poly (ADP-ribose) polymerase (PARP) pathway (Oyadomari *et al.,* 2004). In addition, NO depletes ER Ca^{2+} storage by means of activating Ca^{2+} channels or inhibiting Ca2+ pumps (Messmer *et al.,* 1996; Viner *et al.,* 1999). Depleted ER Ca^{2+} levels leads to ER stress and apoptosis through induction of CHOP signaling (Oyadomari *et al.,* 2001). Imbalance of different pro- and anti-inflammatory cytokines has been reported in the microenvironment of vitiliginious skin. IL17 works to activate the production of other cytokines, including IL1 and IL6, and can potentiate other local inflammatory mediators like TNFα (Kolls & Linders, 2004, as cited in Bassiouny & Shaker, 2011). Keratinocyte dysfunction along with higher IL6 and TNFα production has been reported in vitiligo patients (Moretti *et al*., 2009). H_2O_2 is known to induce NFκB in nuclear extracts and amplify phosphorylated p38 MAPK levels in cells. NFκB is present as an inactive NFκB complex in the cytoplasm. In response to different stimuli, the latent cytoplasmic NFκB/IkB a complex dissociates and activated NFκB translocates to the nucleus and induces the expression of relevant genes, including various cytokines genes (Kumar *et al*., 2004). The build-up of ROS causes protein misfolding in the ER which, may result in

possible immune system defects precipitating inappropriate autoimmune response against melanocytes (Figure 6).

Calreticulin (CRT) is a ubiquitous protein localized predominantly in the ER and plays a major role in intracellular Ca^{2+} homeostasis (Krause and Michalak, 1997; Guermonprez *et al*., 2003; Rutkevich and Williams, 2011; Frasconi *et al*., 2012). Cell surface localization of CRT has been reported on T cells, neutrophils, monocytes, macrophages and dendritic cells (Cho *et al*., 2001; Gardai *et al*., 2005; Porcellini *et al*., 2006; Wang *et al*., 2012). Localization of cell surface CRT reportedly affects complement activation and antigen presentation (Donnelly *et al*., 2006; Fogal *et al*., 2008; Del Cid *et al*., 2010), and clearance of apoptotic cells (Gardai *et al*., 2005). Recently, Zhang *et al*. (2014) have reported increased CRT expression in response to H_2O_2 in human melanocytes leading to apoptosis. In addition, CRT-treated peripheral blood mononuclear cells or stressed melanocytes show higher IL6 and TNFα levels. Higher CRT expression in vitiligo patients is positively correlated with lesion area and duration of disease. The exposure of CRT on the plasma membrane can precede anthracycline-induced apoptosis and is needed for cell death to be perceived as immunogenic (Panaretakis *et al*., 2005). Several studies have found translocation of intracellular CRT to the cell surface in response to anthracycline and high doses of irradiation in a range of human and rodent cancer cells, including melanoma (Obeid *et al*., 2007c; Perez *et al*., 2009; Mosca and Robertson, 2011). Surface CRT then initiates an apoptotic signal (Chao *et al*., 2010) which is critical for the recognition and engulfment by DCs. In contrast, apoptotic cells have been suggested to be phagocytized because of their lack of CD47 expression and the coordinated upregulation of cell surface CRT in fibroblasts, neutrophils, and Jurkat T cells (Gardai *et al*., 2005). Interestingly, Zhang *et al*. (2014) have shown increased surface levels of CRT in response to H_2O_2 and this correlates with decreased CD47 levels, suggesting increased susceptibility to H_2O_2 mediated oxidative stress induced apoptosis (Ihara *et al*., 2006). These finding demonstrate translocation of CRT to the cell surface, via H_2O_2 induced oxidative stress to play an essential role in melanocyte apoptosis suggesting a relationship between apoptosis and immune responses during melanocyte destruction. Nevertheless, the relationship between CRT and ROS-induced apoptosis in melanocytes is still not fully explored. However, the defective Ca^{2+}

homeostasis in vitiligo patients (Schallreuter *et al*., 1988; 1996) certainly proposes a crucial role of CRT and thus the ER stress in vitiligo pathogenesis.

Cells under stress halt typical protein synthesis in favor of heat-shock protein (HSP) and/ GRP synthesis (Welch 1993; Jansen *et al*., 2012). In the ER, this can induce the UPR which upregulates HSps (Gardner *et al*., 2013). Among larger HSPs, inducible HSP70 (HSP70i) is unique for its secretion from cells as a chaperokine (Vega *et al*., 2008). The unique secretory property of HSP70i may be attributed at least in part to its cellular location, associated with melanosomes (Chi *et al*., 2006).

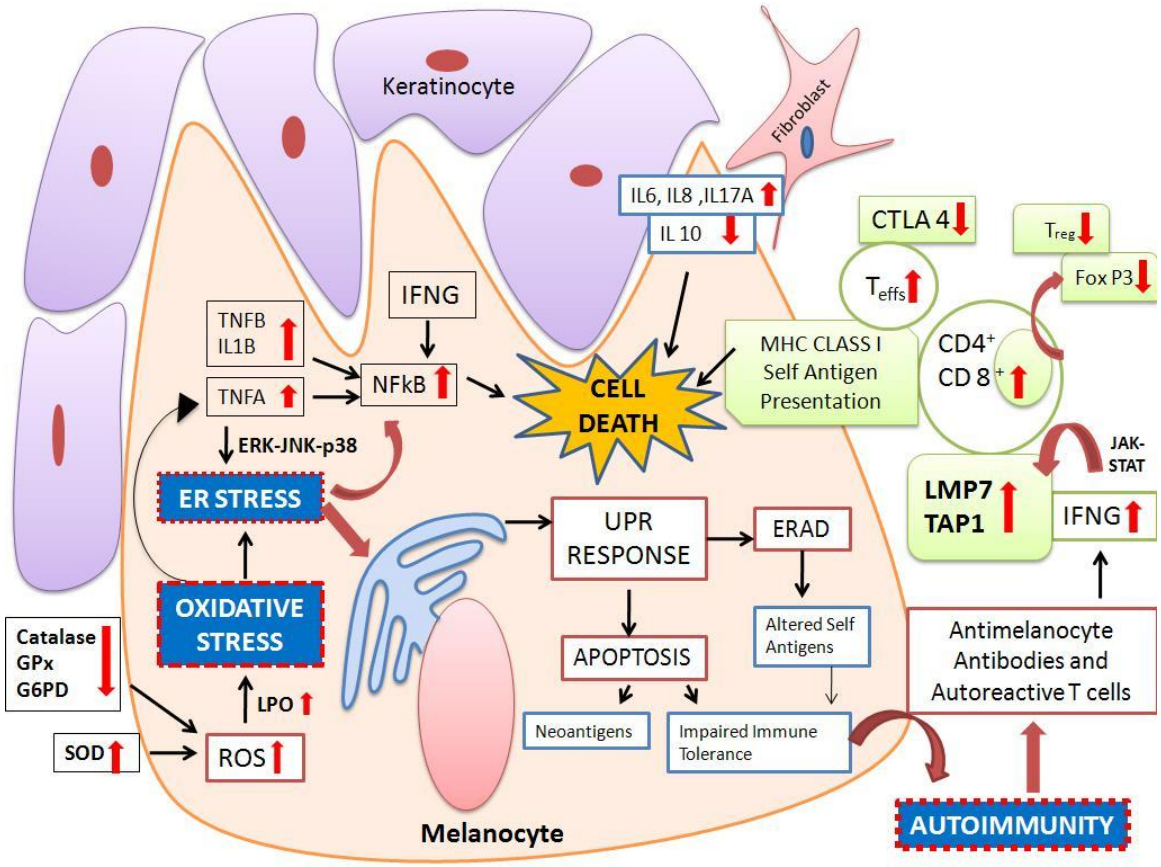

Figure 6. Cross talk between oxidative stress, ER stress and immune System in melanocyte death: Decrease in antioxidant enzymes leads to ROS formation, ultimately leading to oxidative stress. Oxidative stress induces ER stress leading to activation of Unfolded Protein Response (UPR) which in turn activates autoimmune response (Mansuri *et al*., 2014).

HSP70i is exported by cells through the endo-lysosomal pathway (Mambula *et al*., 2006). An increase rise in intracellular Ca^{2+} serves as a signal for exocytosis (Johnson *et al*., 2006). HSP70i can stimulate proliferation and cytotoxicity of NK cells (Nicchitta, 2003), and enhance leukotriene secretion by mast cells (Multhoff 2009). Moreover, HSP70i has shown to induce maturation and type-1 polarizing cytokine production by DCs and stimulate cross-priming of T cells (Kammerer *et al*., 2002), breaking the tolerance and inducing autoimmune mediated tissue destruction in mice (Millar *et al*., 2003). Mosenson *et al*. (2012) reported that modified HSP70i prevents T cell–mediated depigmentation. In addition, they have also shown HSP70i induces an inflammatory DC phenotype in both vitiligo mouse models as well as in vitiligo patients, which is necessary for depigmentation (Mosenson *et al*., 2012; 2013). Interestingly, mutant $HSP70i_{Q435A}$ has been shown to prevent and reverse the depigmentation in different mouse models prone to vitiligo. These findings indicate a vital role of HSP70 in precipitation of vitiligo and targeting HSP70i might be a promising approach towards the treatment of vitiligo (Mosenson *et al*., 2012, 2013).

1.6.4 NEURAL HYPOTHESIS OF VITILIGO

Melanocytes are neural crest derived cells with an embryological connection to the nervous system (Reedy *et al*., 1998). Neural hypothesis suggests that different neurochemical mediators including acetylcholine secreted by the nerve endings to be toxic to melanocytes leading to their destruction. Decreased acetylcholine esterase (AChE) activity has been shown in vitiliginous skin during depigmentation (Iyengar, 1989), suggesting that acetylcholine may aggravate the progression of vitiligo. In addition, decreased sweating in the depigmented epidermis of patients also suggests possible cholinergic involvement in vitiligo (Elwary *et al*., 1997). Moreover, Schallreuter *et al*. (2004) have shown H_2O_2 mediated oxidation of AChE further emphasizing the role of oxidative stress in the precipitation of vitiligo. The inactivation of AChE has been proposed to be due to oxidation of Trp^{432}, Trp^{435} and Met^{436} residues by H_2O_2 (Schallreuter *et al*., 2004). Acetylcholine has an inhibitory effect on melanocyte DOPA oxidase activity affecting melanin production (Iyengar, 1989). Our previous studies has in fact shown decrease in blood AChE activity (Shajil *et al*., 2006) and significant increase in lipid peroxidation levels in vitiligo patients (Agrawal *et al*., 2004; Laddha *et*

al., 2014; Laddha *et al*., 2013b; Shajil and Begum, 2006; Shajil *et al*., 2007). These reports provide evidence for inhibition of AChE in vitiligo patients to be due to high oxidative stress. Thus, accumulated acetylcholine may lead to the destruction of melanocytes resulting in the precipitation of vitiligo.

1.6.5 GENETIC HYPOTHESIS OF VITILIGO

Most human diseases result from an interaction between genetic variants and environmental factors, and to establish the actual involvement of genetic factors is the first step of genetic studies that evaluate complex diseases. In general terms, the search of complex diseases genetic components begins through observational studies, such as: analysis of pools of familial cases, comparative studies of concordance rates of disease occurrence among monozygotic and dizygotic twins and complex segregation analysis (CSA). Though, these studies do not provide information on the exact nature of the genetic component in question, as the location and identity of the involved genes. In order to advance it is essential to perform different studies involving molecular genetic markers, as it is done in association and linkage analysis.

Genetic epidemiological studies have demonstrated that vitiligo can be considered a complex genetic disease because: (i) the disease differs in symptom severity and age of onset, which hinders the definition of the suitable phenotype and the selection of the optimum study population; early age of onset was associated with familial occurrence of generalized vitiligo (Alkhateeb *et al*., 2003; Laberge *et al*., 2005). In addition, early onset vitiligo is associated with more severe disease; (ii) the etiological mechanisms of the disease can vary; etiopathogenesis of vitiligo has not yet been fully elucidated, and several theories have been proposed; (iii) complex genetic diseases are often polygenic and each gene contributes to a fraction of the overall relative risk; linkage analysis performed in vitiligo patients identified susceptible *loci* located on chromosomes 1, 4, 6, 7 , 8, 17 e 22 co-segregating with the disease (Rezaei *et al*., 2007; Castanet and Ortonne, 1997; Morelli and Norris, 1993; Gauthier *et al*., 2003; Dell'anna and Picardo, 2006; Huang *et al*., 2002; Spritz *et al*., 2004; Chen *et al*., 2005). The involvement of genetic factors in vitiligo susceptibility became evident in familial studies, which demonstrated

that vitiligo segregates with a complex standard of polygenic and multifactorial inheritance (Majumder *et al*., 1993). The familial aggregation of cases with 20% of the affected individuals presenting at least one first-degree relative with vitiligo was confirmed in Caucasians (Majumder *et al*., 1993). The relative risk of vitiligo in Denmark and Indian populations was 7 for parents, 36 for off-spring, and 12 for siblings (Majumder *et al*., 1993). The relative risk of vitiligo in first-degree relatives was estimated between 7-10 times higher than in general population (Nath *et al*., 1994). Previous study from Gujarat has shown that ~22% patients had a positive family history of vitiligo, where 13.68% were first-degree relatives affected, 5.66% second-degree relatives and 2.59% third degree relatives (Shajil *et al*., 2006). A study about vitiligo conducted in twins showed 23% agreement rate of generalized vitiligo for 22 pairs of monozygotic twins compared with 0% in 24 pairs of dizygotic twins (Alkhateeb *et al*., 2003). However, the lack of complete agreement for vitiligo between monozygotic twins suggests the importance of non-genetic factors.

1.6.5.1 LINKAGE STUDIES IN VITILIGO

Linkage analysis is a tool which allows the screening of the entire human genome, using multigenerational families in order to recognize genomic regions harboring *loci* responsible for the observed phenotype, even without prior assumptions about the pathogenesis of disease. The principle of linkage analysis is based on the premise that if a polymorphic marker is close to the *locus* which harbors the disease predisposing gene, they will co-segregate in families over generations, more severely than expected under the hypothesis of aleatory transmission expected in an independent heritage of unconnected *loci* (Lander and Schork, 1994). The first evidence of linkage was between vitiligo associated with systemic lupus erythematosus (SLE) and chromosome 17p13 markers (*SLEV1*), in Euro-American families (Nath *et al*., 2001). This result suggests the existence of a probable common genetic autoimmunity determinant of SLE and vitiligo in these families. A subsequent independent study detected linkage between 1p31 *locus*, AIS1 (autoimmunity susceptibility locus 1) and vitiligo in a large multigenerational family presenting multiple cases of vitiligo and Hashimoto's thyroiditis (Alkhateeb *et al*., 2002; Fain *et al*., 2003). Subsequently, a complementary study in which evidence of the

connection with vitiligo was also detected on chromosomes 7 and 8. Furthermore, the evidence of linkage to 17p13 *locus* previously described was confirmed (Chen *et al*., 2005). Thus, the suggestive linkage signal to four *loci* on chromosomes 9, 13, 19 and 22 was detected (Spritz *et al*., 2004). It has been suggested that linkage signals detected for chromosomes 7q and 17p appear to derive primarily from families segregating vitiligo and epidemiologically related autoimmune diseases. In contrast, the linkage signal detected for chromosome 8p derived from families that segregate only vitiligo (Spritz *et al*., 2004). A genomic scan performed in Chinese multiplex families affected by generalized vitiligo (GV) identified the association of markers of region 4q13-q21, 22q12 and 6p21-p22 with the disease (Chen *et al*., 2005; Liang *et al*., 2007). Linkage analyzes carried out in populations with different ethnic backgrounds, using GV as phenotype, demonstrated that the main susceptibility *locus* co-segregating with the disease is not the same in each population, except for 22q11. This proposes that diverse genes may be involved in the pathogenesis of vitiligo in different populations around the world, characterizing a polygenic disease (Spritz *et al*., 2004; Chen *et al*., 2005; Liang *et al*., 2007). The precise definition of the gene/s involved in controlling the phenotype in question depends on further association studies, involving target genes located in genomic regions identified in the linkage analysis.

1.6.5.2 ASSOCIATION STUDIES IN VITILIGO

The association studies are of two main types: population-based and family-based. Population-based studies (case-control) mainly compare the allele frequencies of a genetic marker between affected and unaffected individuals. A particular allele is considered to be associated with the phenotype when it occurs with different frequencies amongst the groups. The immense challenge in this type of study is the proper selection of the population fragment and the sample size, in order to have adequate power to detect the genetic effect, if it really exists. Family-based association studies use the basic trio design which consists of two parents and an affected offspring. The study assesses the frequency with that a particular allele is transmitted from a heterozygous parent to the affected child: a variation in the expected aleatory transmission, according to the first law of Mendel, suggests an association. It is essential to emphasize that, the association

studies have greater power to detect moderate to weak genetic effects as compared to linkage studies, which makes them idyllic for fine mapping of previously detected chromosomal regions connected with the disease (Cordell and Clayton, 2011).

Currently, over 50 candidate genes have already been investigated in association studies for vitiligo susceptibility. Though, few genes, including *DDR1*, *NLRP1*, *XBP1*, *PTPN22* and *COMT*, were constantly associated with vitiligo, either by being situated in a region or by having been replicated in populations of diverse geographic regions, as described in Table 3. In addition to the genes listed above, an evidence of association with vitiligo was found for markers of *ACE, AIRE, CAT, CD4, CLEC11A, COMT, COX2, CTLA4, C12orf10, DDR1, EDN1, ESR1, FAS, FBXO11, FOXD3, FOXP3, GSTM1, GSTT1, IL1RN, IL10, KITLG, MBL2, MC1R, MYG1, Nrf2, NFE2L2, PTGS2, PDGFRA, PRO2268, SCF, SCGF, STAT4, TAP1-PSMB8, TGFBR2, TNF, TSLP, TXNDC5, UVRAG, VDR* and *XBP1* genes, however in independent populations these associations were not replicated (Birlea *et al*., 2010). Most vitiligo susceptibility loci encode melanocyte components, and antioxidant and immune-regulatory proteins (Laddha *et al*., 2013; Jin *et al*., 2012). Previous studies from Gujarat, on genetic association between polymorphisms in catalase (CAT) (Shajil *et al*., 2007), glutathione peroxidase (GPX) (Shajil *et al*., 2007), major histocompatibility complex *(MHC*) (Singh *et al*., 2012; Birlea *et al*., 2013), NLR family pyrin domain containing 1 *(NLRP1)* (Dwivedi *et al*., 2013a), tumor necrosis factor A (TNFA) (Laddha *et al*., 2012), interferon-γ (IFNG) (Dwivedi *et al*., 2013b), intercellular adhesion molecule-1 (ICAM1) (Dwivedi *et al*., 2013b; Laddha *et al*., 2013), tumor necrosis factor B (TNFB) (Laddha *et al*., 2013a), superoxide dismutase (SOD) (Laddha *et al*., 2013b), cytotoxic T lymphocyte associated antigen 4 (*CTLA4*) (Dwivedi *et al*., 2011), interleukin 4 (IL4) (Imran *et al*., 2012), melanocyte proliferating gene (MYG1) (Dwivedi *et al*., 2013c) vitiligo pathogenesis have been established. Nevertheless, several genetic studies for *CAT* and *GPX* genes in populations of different ethnic origins, have produced conflicting results (Casp *et al*., 2002; Liu *et al*., 2010; Shajil *et al*., 2007; Birlea *et al*., 2011; Bulut *et al*., 2011). Generally, the associations of *HLA* variants with vitiligo susceptibility, considering different ethnic groups, have not been consistent; but, there are remarkable exceptions, such as the

association signs observed for *HLA-A2*, *HLA-DR4* and *HLA -DR7* alleles (Tastan *et al*., 2004; Buc *et al*., 1998; Wang *et al*., 2007; Singh *et al*., 2012; Hu *et al*., 2011).

Table 3. Candidate genes for vitiligo susceptibility.

Gene	SNP	Study Population	Reference/s
ACE	rs1799752	Korean, Gujarati Indian	Jin *et al.*, 2004; Dwivedi *et al*., 2008
ASIP	rs6058017	Korean	Na *et al.*, 2003
CAT	rs769217 rs7943316	Caucasian, Korean, Gujarati Indian	Casp *et al.* 2002; Gavalas *et al.* 2006; Park *et al.* 2006, Shajil *et al*., 2009.
CD4	5 nucleotide repeat	Iranian,	Zamani *et al.*,2010
CLEC11A	rs7246355	Taiwanese	Lan *et al.*,2009
COMT	rs4680	Turkish	Tursen *et al.*,2002
CTLA4	rs231775	Caucasian, Gujarati Indian	Kemp *et al.*, 1999; Dwivedi *et al*., 2011
MYG1	rs7975232	Caucasian; Gujarati Indian	Philips *et al.*,2010; Dwivedi *et al*., 2012
DDR1	rs2267641	Brazilian	de Castro *et al.*,2010
EDN1	rs2071942, rs5370	Korean	Kim *et al.*,2007
ESR1	rs2234693	Korean	Jin *et al.*, 2004b
FAS	rs2234767	Chinese	Li *et al.*,2008
FOXD3	rs41285370	Caucasian	Alkhateeb *et al*., 2005
FOXP3	rs11798415	Chinese Han	Song P *et al*., 2013
GSTM1	rs2071487,	Chinese, Korean	Liu *et al.*,2009; Uhm *et al.*,2007
GSTT1	rs2234953	Korean	Uhm *et al.*,2007
IFNG	rs2430561 rs3138557	Gujarati Indian	Dwivedi *et al*., 2013
IL1RN	Intron 2 VNTR	Turkish	Pehlivan S *et al.*,2009
IL1B	rs16944	Gujarati Indian	Laddha *et al*., 2014
IL4	Intron 3 VNTR, rs2243250	Gujarati Indian	Imran M *et al*., 2012

IL10	rs1800872; rs1800871	Saudi	Abanmi *et al*., 2008
KITLG	rs689466	Taiwanese	Lan *et al.*,2009
LMP7	2071627	Caucasian	Casp *et al.*,2003
MC1R	rs2228479	Korean	Na *et al.*, 2003
MBL2	rs1800450	Turkey, Gujarati Indian	Onay *et al.*,2007; Dwivedi *et al*., 2009
NFE2L2	rs6721961	Chinese Han	Song *et al*., 2016
NALP1	rs6502867	Caucasian, Gujarati Indian	Jin *et al.*, 2007; Dwivedi *et al*., 2013
NPY	rs16139 rs16147	Gujarati Indian	Laddha *et al*., 2014
PDGFRA- KIT	rs36901	Chinese	Xu *et al.*,2010
PTPN22	rs2476601	Caucasian, Gujarati Indian	LaBerge *et al*., 2008, Laddha *et al*., 2008
PTGS2	rs689466	Chinese	Li K *et al.*,2009
STAT4	rs7574865	Chinese	Hu *et al.*,2010
SOD	rs1804450, rs11556619, rs11556620	Gujarati Indian	Laddha *et al*., 2013
TAP1	rs1135216	Caucasian	Casp *et al.*,2003
TGFBR2	rs2005061	Korean	Yun *et al.*,2010
TNF	rs1800629	Iranian, Gujarati Indian	Namian *et al*., 2009; Laddha *et al*., 2012
TSLP	rs3806933	Korean	Cheong *et al.*,2009
TXNDC5	rs1043784	Korean	Jeong *et al.*,2010a
UVRAG	rs1458836, rs7933235	Korean	Jeong *et al.*,2010b
VDR	rs7975232	Romanian	Birlea *et al.*,2006
XBP	rs2269577	Chinese	Ren *et al*., 2009

1.6.5.3 GENOME WIDE ASSOCIATION STUDIES (GWAS)

GWAS involve hundreds of thousands of genetic markers, adequate for complete genome coverage, which are tested in combination to establish their association with a particular disease. This method is free of positional or functional hypotheses, as the only aim in selecting the set of markers to be tested is to attain the most considerable coverage achievable, including all the genes in the genome that are investigated in a single experiment. Thus, GWAS allow the detection of genes earlier unsuspected of participating in the pathogenesis control of the studied disease.

The first GWAS in vitiligo was carried out in a genetically isolated population sample under strong founder effect (thus presenting lesser genetic variability) in north-western Romania, with a high prevalence of generalized vitiligo (GV), the authors found an association between vitiligo and *SMOC2* rs13208776; so far, this finding was not replicated (Birlea *et al*., 2010; Spritz, 2012). Subsequently, two independent GWAS for GV, carried out in Caucasian and Chinese populations, observed many signs of associationof vitiligo with polymorphisms of several *loci*, including the MHC (Jin *et al*., 2010; Quan *et al*., 2010). Only 2 of these signals for LPP and IKZF4 genes were replicated in population samples different from the original ones (Jin *et al*., 2007, 2010; Quan *et al*., 2010; Tang *et al*., 2013).

1.7 CANDIDATE GENES ADDRESSESD IN THE PRESENT STUDY

1.7.1 Catalase (*CAT*)

This gene encodes catalase, a key antioxidant enzyme in the body's defense against oxidative stress. Catalase (EC 1.11.1.6) is a heme enzyme that is present in the peroxisome of nearly all aerobic cells. It is a tetrameric enzyme consists of four identical, tetrahedrally arranged subunits of 60 kDa, each containing a heme group and NADPH in its active center. Catalase has two enzymatic activities depending on the concentration of H_2O_2. If the concentration of H_2O_2 is elevated, catalase acts catalytically, i.e. removes H_2O_2 by forming H_2O and O_2 (catalatic reaction). But, at a low concentration of H_2O_2 with the presence of a suitable hydrogen donor, e.g. ethanol, methanol, and phenol,

catalase acts peroxidically, eliminating H_2O_2, but oxidizing its substrate (peroxidatic reaction) (Scibior and Czeczot, 2006).

The polymorphisms in catalase gene have been associated with decreases in its activity. A total, 245 catalase SNPs were detected and described according to the NCBI database in different regions of the *CAT* (Crawford *et al*., 2012a). Significant correlations of the studied polymorphisms and various diseases are shown in Table 4 & Figure 7. It has been shown that genetic variations in the *CAT* gene and its promoter may play a role in glucose disorders and may be risk factors for metabolic diseases such as impaired glucose tolerance, insulin resistance, diabetes mellitus, dyslipidaemia, hypertension and osteoporosis and their comorbidities (Hebert-Schuster *et al*., 2012). There are few reports on association of *CAT* polymorphisms with vitiligo: Casp *et al*. (2002) reported genotypic but not allelic association of the *CAT* SNP rs769217 in a US non-Hispanic white cohort. Further, Gavalas *et al*. (2006) also reported association of rs769217 in a UK non-Hispanic white cohort. However, studies from Korea (Park *et al*., 2006) and China (Liu *et al*., 2010) found no association of rs769217. In our previous study from Gujarat (Shajil *et al*., 2007); we observed no association with rs769217. The meta-analysis of SNP rs769217 established a constant high-risk allele across different studies, but failed to achieve the study significance threshold (Birlea *et al*., 2011). Liu *et al*. (2010) reported an association with 5′-flanking SNP rs7943316.

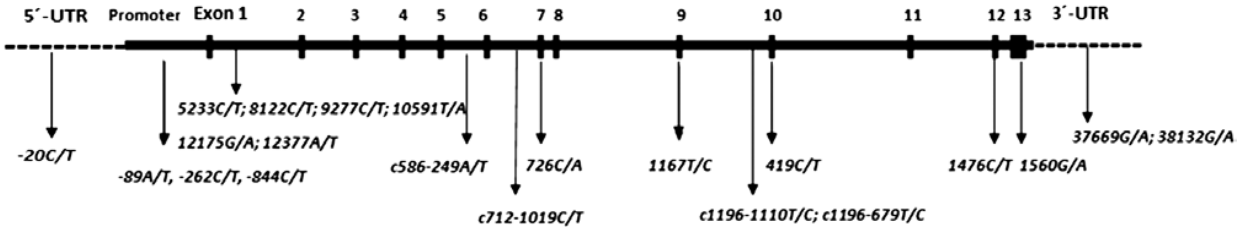

Figure 7. *CAT* gene and relevant single-nucleotide polymorphisms. (Liu *et al*., 2010; Hebert-Schuster *et al*., 2012; Kodydkov á *et al*., 2014)

Table 4. *CAT* SNPs and their associated diseases.

Polymorphism (SNP ID)	Genomic context	Disease	Reference/s
-844C/T or -844G/A (rs769214)	Promoter	Hypertension Malnutrition Asthma	Jiang *et al*., 2001; Watanabe *et al*., 2010; Fabre *et al*., 2008 Hebert-Schuster *et al*., 2011 Podgoreanu *et al*., 2006
-262C/T or 330C/T (rs1001179)	Promoter	Type 1 diabetes Breast cancer Hepatocelullar carcinoma (HCC) Rheumatoid arthritis Asthma Ulcerative colitis Asbestosis Hyperkeratosis Various	Chistiakov *et al*., 2004; Chistiakov *et al*., 2006 Ahn *et al*., 2005;Quick *et al*., 2008 Ahn *et al*., 2006b; Ezzikouri *et al*., 2010 Grabar *et al*., 2009 Mak *et al*., 2006; Islam *et al*., 2008 Khodayari *et al*., 2013 Franko *et al*., 2008 Ahsan *et al*., 2003 Christiansen *et al*., 2004
-89A/T or -21A/T (rs7943316)	Promoter	Asthma Osteonecrosis Vitiligo	Polonikov *et al*., 2009 Kim *et al*., 2008 Liu *et al*., 2010
-20C/T (rs1049982)	5'UTR	Hypertension Ostenecrosis	Mansego *et al*., 2011 Kim *et al*., 2008
1167C/T; 389C/T, 22348 C/T; 111C/T; 389Asp/Asp (rs769217 or rs17880449)	Exon 9 Codon 389	Type 1 diabetes Type 2 diabetes Vitiligo Osteonecrosis Bone mineral density	Tarnai *et al*., 2007; Chistiakov *et al*., 2004 Chistiakov *et al*., 2000 Gavalas *et al*., 2006 Kim *et al*., 2008 Oh *et al*., 2007
5233C/T, c66+78C/T; +144C/T (rs10836235/ rs17886119)	Intron 1	Various	Rajic *et al*., 2009
8122T/C, 3033C/T; c66+2967T/C	Intron 1	Osteonecrosis	Kim *et al*., 2008
19628A/T, 14539A/T; c586-249A/T	Intron 5	Osteonecrosis	Kim *et al*., 2008
29502T/C, 24413T/C; c1196-679T/C (rs2284365)	Intron 9	Osteonecrosis	Kim *et al*., 2008

1.7.2 Glutathione peroxidase 1 (GPX1)

GPX1 represents the first identified mammalian selenoprotein. GPX1 is an intracellular antioxidant enzyme that enzymatically reduces H_2O_2 to water to limit its damaging effects. Certain ROS, such as H_2O_2, are also essential for mitochondrial function, growth factor-mediated signal transduction and maintenance of normal thiol redox-balance (Lubos *et al*., 2011). Thus, by limiting H_2O_2 accumulation, GPX1 also modulates these processes (Figure 8 & 9). Selenocysteine insertion sequence-associating factors, Abl and Arg tyrosine kinases, and adenosine are potent, Se-independent regulators of GPX1 gene, protein, and activity. As a selenocysteine-containing enzyme, GPX1 expression is subject to unique forms of regulation involving the trace mineral selenium (Se) and selenocysteine incorporation during translation. Tempting evidences have been generated the protective role of GPX1 in coping with oxidative injury and death mediated by reactive oxygen species. Intracellular and tissue levels of GPX1 affect apoptotic signaling pathway, protein kinase phosphorylation, and oxidant-mediated activation of NFκB (Lei *et al*., 2007). In addition, GPX1 has been implicated in the development and prevention of many common and complex diseases.

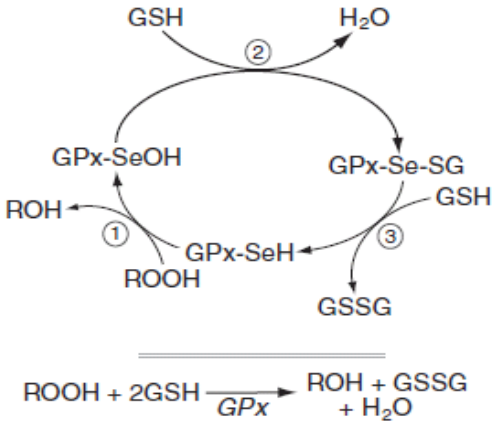

Figure 8. Reduction of H_2O_2 by GPX1. The enzymatic inactivation of peroxides by GPX1 involves the formation of several stable intermediary modifications to the active-site selenocysteine (Sec). The selenol of GPX-SeH (with -SeH representing the Sec active site) forms a selenenic acid (Se-OH) after reacting with peroxides (1). One molecule of GSH reduces selenenic acid leading to the Se-SG intermediate (2), which is reduced by the second GSH, resulting in the formation of GSSG (3). The net reaction is shown in the lower part of the figure. (Lubos *et al*., 2011)

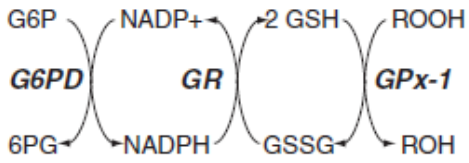

Figure 9. Redox pathways involved in maintaining the cofactors necessary for the activity of GPX1. (Lubos *et al*., 2011)

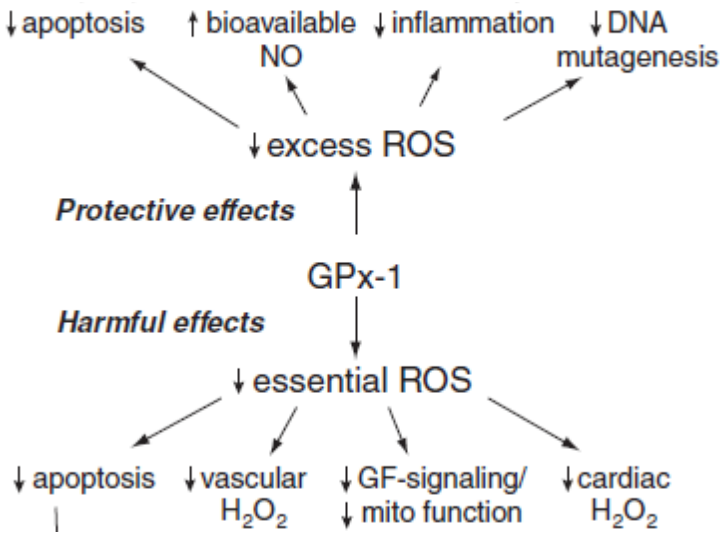

Figure 10. Protective and harmful effects of GPX1. Some of the protective effects and harmful actions of GPX1 that are mediated by decreasing cellular ROS. Thus, elimination of excess ROS can protect cells against apoptotic loss or injury; toxicity to drugs; and susceptible cells against high ROS. Removal of excess ROS can preserve bioavailable NO, preserving vascular function. GPX1 has also been shown to protect against inflammatory stimuli. GPX1 prevents DNA mutagenesis, which also decreases carcinogenic potential. Many of the harmful effects of GPX1 occur under conditions of excess GPX1, which may be induced by physiological conditions such as mechanical stress in the vasculature or under pathological conditions. Removal of essential oxidants can create a reductive stress characterized by loss of normal physiological responses, including reduced vascular responses to H_2O_2 and arachidonic acid; decreased mitochondrial function and growth factor (GF) signaling that can cause insulin insensitivity and decreased cell proliferation; and development of cells by mechanisms not completely understood but presumed to involve a reduction in essential levels of H_2O_2. (Lubos *et al*., 2011)

Although, overall GPX1 can be considered protective against oxidative stress, it is important to consider that changes in redox balance in either direction, oxidative or reductive, may also influence the protective or harmful roles of GPX1. Under some circumstances, enhanced expression of GPX1 may promote reductive stress by removing essential oxidants resulting in detrimental physiological effects, such as diminished growth factor-mediated signaling (which may contribute to insulin resistance), decreased cellular proliferation, and, in some cases, enhanced apoptosis (Figure 10)

The human *GPX1* gene localizes to human chromosome 3p21, a region which has been associated with loss of heterozygosity (LOH), a process that involves deletion of a chromosomal region from one of two autosomes leading to the detection of only one allelic variant in the subjects who are heterozygous in all other tissues. Involvement of the GPX1 gene locus in LOH events has been shown for cancers of the lung, breast, head and neck, and colon (Hardie *et al*., 2000; Hu *et al*., 2003 & 2005; Moscow *et al*., 1994). Overall, 38 SNPs have been reported for the *GPX1*; most of these SNPs are in the 5'- and 3'- flanking region (Foster *et al*., 2006). Of the polymorphisms that alter the protein coding region, two major polymorphic sites of the *GPX1* gene are commonly studied due to their frequency and resulting alterations in the amino acid sequence of *GPX1* (Table 5). One of the polymorphisms involves a variable number of ''GCG'' tri-nucleotide repeats in the first exon of the human *GPX1* gene that results in five, six, or seven alanines near the N-terminus. The other common polymorphism involves a Leu substitution in place of the more common Pro at amino acid 198 due to a substitution of T to C. While one study reported an effect for the Ala repeats in modulating *GPX1* expression in response to Se (Zhuo *et al*., 2004), most of the studies have analyzed the consequences of the Leu/Pro polymorphism (rs1050450, Pro198Leu) on expression and activity. The Leu/Pro polymorphism shows an interesting gene–environment effect in that these allelic forms differ in their expression in response to selenium. However, in previous study on association of Pro198Leu polymorphism with vitiligo patients was uninformative from Gujarat (Shajil *et al*., 2007).

Table 5. *GPX1* SNPs and their associated diseases.

Polymorphism (SNP ID)	Gnomic context	Disease	Reference/s
rs1800668	5'UTR	Glioma, Prostate cancer	Zhao *et al*., 2012; Geybels *et al*., 2014
GCG repeat	Exon 1	Prostate cancer	Kote-Jarai *et al*., 2002
rs1987628	Intron	Noise-induced hearing loss	Wen *et al*., 2014
Pro198Leu (rs1050450)	Exon 2	Vitiligo	Seçkin *et al*., 2016
		Skin cancer	He*et al*., 2010
		Skin aging	Naval1 *et al*., 2014
		Alopecia areata	Kalkan *et al*., 2015
		Diabetic nephropathy	Paulescu*et al*., 2011
		Coronary heart disease	Souiden *et al*., 2016
		Thalassemia major	Bhargava *et al*., 2013
		Coronary artery disease	Zheikova *et al*., 2012
		Alzheimer's disease	Cardoso *et al*., 2012
		Breast cancer	Ravn-Haren *et al*., 2006; Méplan *et al*., 2013
		Brain tumors	Bhatti*et al*., 2009
		Prostate cancer	Arsova-Sarafinovska *et al*., 2009
		Kashin-Beck disease	Xiong *et al*., 2010
rs3448	3'UTR	Noise-induced hearing loss	Wen *et al*., 2014
		Prostate cancer	Geybels *et al*., 2013
		Breast Cancer	Mavaddat *et al*., 2009

1.7.3 Glucose-6-phosphate dehydrogenase (G6PD)

This X-linked gene encodes for glucose-6-phosphate dehydrogenase, which is a cytosolic enzyme whose main function is to produce NADPH, a key electron donor in the defense against oxidizing agents and in reductive biosynthetic reactions. G6PD catalyses the conversion of glucose-6-phosphate (G6P) into 6-phosphoglucono-δ-lactone with the concomitant reduction of NADP+, which is the first and rate-limiting step in the pentose phosphate pathway. The generated NADPH has been shown to be crucial for the protection of cells against oxidative damage (Thomas *et al*., 1991; Pandolfi *et al*., 1995). Several reports have suggested that G6PD exerts its protective effect by supplying NADPH to maintain the intracellular GSH level (Salvemini *et al*., 1999; Preville *et al*., 1999). NADPH, generated by increased G6PD activity, is necessary for regulating the

optimum pH required for the stimulation of cell growth by EGF or (PDGF (Stanton *et al*., 1991; Tian *et al*., 1998).

G6PD is remarkable for its genetic diversity. Variations in *G6PD* gene lead to G6PD deficiency; most of which are single amino acid substitutions and the resulting G6PD variants have differential enzyme activity and were found to be associated with a variety of clinical phenotypes (Table 6), resulting from an increased susceptibility of individuals to oxidative stress (Tishkoff *et al*., 2001; Cappellini and Fiorelli, 2008). G6PD deficiency may cause acute hemolysis, neonatal jaundice, or severe chronic non-spherocytic hemolytic anemia. Various studies including reports from Gujarat have reported decreased G6PD levels in vitiligo patients (Agrawal *et al*., 2004; Shajil and Begum, 2006; Arican and Kurutas, 2008; Farahi-Jahromy *et al*., 2014). However, there is no information about the molecular basis of G6PD deficiency in vitiligo.

Table 6.*G6PD* SNPs and their associated diseases.

Polymorphism	Gnomic context	Disease/ Condition	Reference/s
C770+175 T (rs2515905)	Exon 1	G6PD deficiency	Al-Sweedan SA *et al*.,2012
rs1050828 (G202A)	Exon 4	G6PD deficiency, Malaria	Beutler *et al*., 1991; Eid *et al*., 2010
rs1050829 (A376G)	Exon 5	Asymptomatic malaria	Ouattara *et al*., 2014; Eid *et al*., 2010
rs5030872 (A542T)	Exon 5	Diabetes, Malaria, G6PD deficiency	Clark *et al*., 2009; Beutler *et al*., 2002
C563 T (rs5030868)	Exon 6	G6PD deficiency	Vulliamy TJ *et al*.,1998
G680T	Exon 7	Asymptomatic malaria, G6PD deficiency	Ouattara *et al*., 2014; Hirono A *et al*., 1988
T968C+	Exon 9	Asymptomatic malaria, G6PD deficiency	Ouattara *et al*., 2014; Carter N *et al*., 2011
G1003 A (rs5030869)	Exon 9	G6PD deficiency	Al-Sweedan SA *et al*.,2012
C1024T	Exon 9	G6PD deficiency	Chiu DT *et al*., 1991a
C486-60 G (rs2515904)		G6PD deficiency	Al-Sweedan SA *et al*.,2012
C1311 T (rs2230037)	Exon 11	G6PD deficiency	Gabriella De Vita *et al*., 1989

1.8 MicroRNAs (miRNAs)

MicroRNAs (miRNAs) are approximately 22-nucleotide-long non-coding RNAs that are important regulators of gene expression in eukaryotes. miRNAs are small, hitherto they are influential due to their intrinsic involvement in regulation of protein translation and mRNA (messenger RNA) stability. Besides, as they do not require perfect complementarity to the target mRNAs, in some cases only requiring 6-7 nucleotides to bind, each miRNA has the predicted ability to bind and 'fine-tune' thousands of genes. miRNAs are governed by the same mechanisms that apply to all the other RNAs in a cell. miRNAs are first transcribed as long primary transcripts, which then undergo a series of processing steps to produce the single-stranded mature miRNAs. There are two well established principles that explain many findings regarding miRNA biogenesis. 1) The first principle is that most RNAs undergo processing before maturation. For example, rRNAs (ribosomal RNAs) are produced by cropping of their primary transcripts and contain numerous nucleotide modifications, and eukaryotic mRNAs undergo 5' and 3' modifications as well as splicing. miRNA biogenesis also involves processing, which is achieved by proteins and enzymes with specialized activities. 2) The second principle is that cellular RNAs associate continuously with proteins that can regulate RNA transcription, processing, localization, function and/or degradation. There are hundreds of human proteins with specialized RNA-binding motifs, and the actual figure of RNA-binding proteins *in vivo* is probably much higher. Also, additional proteins may be employed to RNAs through interaction with RNA-binding proteins. It is difficult to ascertain the exact mechanism and function of such proteins in the miRNA pathway *in vivo,* because these proteins are quite often promiscuous.

1.8.1 BIOGENESIS OF miRNA

Complex genomes encode hundreds of miRNA genes; e.g., the human genome has over 1000 miRNA genes as categorized by miRBase (Kozomara and Griffiths-Jones, 2011). miRNA genes are transcribed by RNA polymerase II or occasionally by RNA polymerase III, to produce the primary miRNA transcripts (primiRNAs) (Cullen, 2004). As a result, pri-miRNAs are long and may contain 5' and 3' modifications identical to those present in mRNAs or pre-mRNAs. In fact, many miRNA-coding sequences are

located inside or overlap with annotated genes for mRNAs or other RNAs, which are usually referred as the host genes for the miRNAs. Though, miRNA genes are not well defined experimentally and pri-miRNAs are not as widely characterized as other transcripts such as mRNAs. When DNA sequence coding for a mature miRNA is located within or near a known gene, it is often assumed that transcription of the host gene produces a transcript that eventually gives rise to the miRNA. However, it is possible that the miRNA has its independent promoter which has nothing to do with the "host" gene, or that the known host transcript/s is just one of two or more likely pri-miRNAs that produce the same miRNA. There are also examples whereby miRNAs emerge to form their own transcription units, while it remains possible that they share transcripts with other genes too.

Mammalian miRNAs can be divided into two wide classes, canonical and non-canonical, based on how the pri-miRNAs are processed resulting into the production of mature miRNAs. In the canonical pathway (Figure 11), a pri-miRNA is cleaved by Drosha bound through its regulatory subunit DGCR8, to release a hairpin structured precursor, or pre-miRNA, of ~60–70 nucleotides (nt) in the nucleus (Lee *et al*., 2003; Denli *et al*., 2004; Gregory *et al*., 2004; Han *et al*., 2004; Landthaler *et al*., 2004). The pre-miRNA often contains a 2 nt 3' overhang, as a result of Drosha's RNase III activity, and is transported to the cytoplasm by Exportin5 (Exp5) associated with its Ran co-factor coupled to GTP (Yi *et al*., 2003; Bohnsack *et al*., 2004; Lund *et al*., 2004). GTP is replaced by GDP in the cytoplasm, inducing Exp5 to liberate its pre-miRNA cargo. The pre-miRNA is then cleaved by an other RNase, Dicer, to generate a miRNA duplex intermediate of ~22 base pairs (Billy *et al*., 2001; Grishok *et al*., 2001; Hutva´gner *et al*., 2001; Ketting *et al*., 2001; Provost *et al*., 2002; Zhang *et al*., 2002). An Argonaute (Ago) protein binds to the duplex and incorporates the mature, single- stranded miRNA into the Ago:RNA complex, whereas the other strand in the original duplex is discarded (Hammond *et al*., 2001; Hutva´gner *et al*., 2002; Mourelatos *et al*., 2002). It depends on the relative thermodynamic stability of the two ends of the duplex intermediate that which strand is retained (Khvorova *et al*., 2003; Schwarz *et al*., 2003). Other RNA- and Dicer-binding proteins, such as TRBP and PACT, may facilitate the formation of miRNA duplexes and/or the transport of mature miRNAs to Ago proteins (Chendrimada *et al*.,

2005; Fo¨rstemann *et al*., 2005; Gregory *et al*., 2005; Haase *et al*., 2005; Jiang *et al*., 2005; Saito *et al*., 2005; Lee *et al*., 2006).

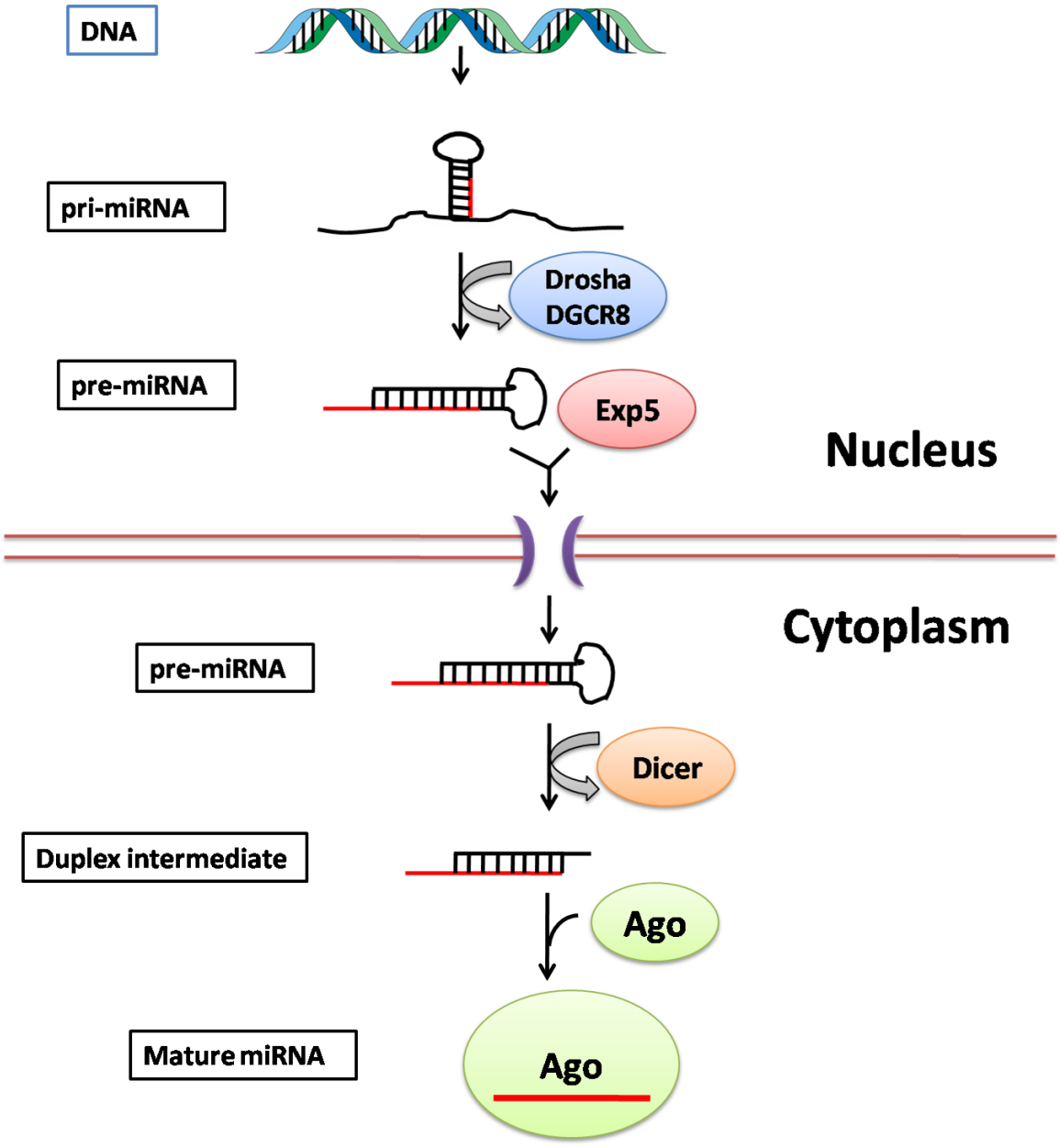

Figure 11. The canonical miRNA biogenesis pathway in animal cells. (Graves and Zeng, 2012)

For non-canonical miRNAs, their processing does not need all the above mentioned protein factors. For example, pre-miRNAs of mirtron genes are produced by splicing, not by Drosha cleavage (Berezikov *et al*., 2007; Okamura *et al*., 2007; Ruby *et al*., 2007; Bogerd *et al*., 2010). Pre-miR-451 is cleaved by Ago2, bypassing Dicer (Cheloufi *et al*., 2010; Cifuentes *et al*., 2010). Certain pri-miRNAs are small hairpin RNAs which perhaps serve as premiRNAs and Dicer substrates directly (Babiarz *et al*., 2008). Such RNAs may also be defined as the endogenous small interfering RNAs (siRNAs). Canonical and non-canonical miRNAs can be differentiated by changes in their expression when a certain miRNA processing factor is knocked down or knocked out. Loss of Dicer, Drosha or DGCR8 should immensely reduce the expression of canonical miRNAs, whereas the non-canonical miRNAs would have different responses depending on individual proteins (Cheloufi *et al*., 2010; Babiarz *et al*., 2008). The majority of ~22-nt long RNA species in mammals are canonical miRNAs (Babiarz *et al*., 2008). It is unclear whether canonical miRNA genes out figure non-canonical miRNA genes, as increasingly miRNAs with low abundance are being recognized through deep-sequencing and deposited to miRBase, while how these RNAs are processed has rarely been scrutinized. RNA editing can introduce additional variation in miRNAs. RNA adenosine deaminases convert adenosine into inosine residues that can form basepairs with cytosines or uracils (Luciano *et al*., 2004; Blow *et al*., 2006; Chiang *et al*., 2010). Pri-miRNA, pre-miRNA and mature miRNA sequences may be modified through deaminases, which potentially impact miRNA processing and target recognition.

What decides how fast and when a mammalian miRNA is degraded is inadequately understood. The MCPIP1, a mammalian RNase, digests pre-miRNAs to block the Dicer processing step (Suzuki *et al*., 2011). Once made, most miRNAs are thought to be stable, though the half-lives of miRNAs differ as a result of intrinsic stability or treatments (Bail *et al*., 2010; Gantier *et al*., 2011; Rissland *et al*., 2011). It is uncertain whether a mature miRNA can be stripped away from its Ago partner for degradation, or whether the decomposition of a miRNA must be tied to that of the Ago protein. miRNAs also undergo tailing and trimming at their 3' end (Ameres *et al*., 2010; Liu *et al*., 2011). Such modifications are presumably coupled to miRNA function and degradation, although their significance in mammalian systems remains to be determined.

1.8.2 MECHANISMS OF ACTION OF miRNAs

miRNAs are principally negative regulators of mRNA translation and exert their effects through various mechanisms (Figure 12 & 13). As a first step, the pre-miRNAs are assembled in large protein complexes known as miRNA RNA-inducing silencing complex (miRISC or RISC). Some of the recognized proteins which form RISC include the RNA-specific endonuclease Dicer, that is involved in the processing of pre-miRNA into the mature form (Hutvagner *et al*., 2002); the Ago protein, which has four isoforms, of which only Ago2, also known as "slicer," has the ability to cleave the target mRNA (Meister *et al*., 2004; O'Carroll *et al*., 2007); PW182 a P-body protein (Liu *et al*., 2005); the human immunodeficiency virus (HIV) transactivating response RNA-binding protein (TRBP), which employs Ago2 to the complex (Chendrimada *et al*., 2005); and fragile X mental retardation protein (FMRP1) (Jin *et al*., 2004), which is known to be associated with polyribosomes (Feng *et al*., 1997); amongst other proteins whose functions within the RISC have not been fully studied yet (Hock *et al*., 2007). Guided by partial sequence complementarity between the miRNA and its target mRNA, the complex binds to miRNAs target sites, mainly in the 3'-UTR. It was first reported in *C. elegans* that miRNAs have the ability to inhibit translation of its target mRNA at a post-initiation step, as lin-4 inhibits the translation of lin-14 without dropping mRNA abundance, polyadenylation, or polyribosomal content (Olsen *et al*., 1991). Similar results were observed with inhibition of lin-41 by let-7 in a HeLa cell background, where the suppressed mRNA was associated with active polyribosomes comparable to its unsuppressed counterpart (Nottrott *et al*., 2006). In favor of this mechanism, cap-independent translation of mRNAs has also been successfully inhibited by recombinant miRNA (Petersen *et al*., 2006). It was suggested that this result could be a consequence of premature dissociation of ribosomes during translation elongation.

A second mode of function utilized by miRNAs is inhibiting translation initiation, which requires both the cap (Humphreys *et al*., 2005; Pillai *et al*., 2005; Thermann *et al*., 2007) and poly(A) tail structures (Humphreys *et al*., 2005). It has been shown that both of these structures act in concert to assist translation. It was demonstrated by a model in which eukaryotic initiation factors 4G (eIF4G), a subunit of the 5'cap-binding initiation factor,

binds the poly(A)- binding protein (PABP) and promotes circularization of the mRNA, a structure that is translationally superior to the linear form (Sachs, 2000). A cell-free translation system demonstrated that miRNA inhibits translation via inhibiting the assembly of the 80S ribosomal complex in a cap (m7GpppG)-dependent manner (Mathonnet *et al*., 2007). This effect could be partly accredited to the employment of the anti-association factor eIF6 by RISC, which averts the assembly of the 80S complex (Chendrimada *et al*., 2007). In addition, the Ago2 protein within the RISC complex has a cap-binding domain that competes with eIF4E and restrains binding of the initiation complex to the m7GpppG cap (Kiriakidou *et al*., 2007). Ago2 also has the ability to compete with eIF4E for its interaction with eIF4G and further disrupts assembly of the initiation complex (Iwasaki *et al*., 2009).

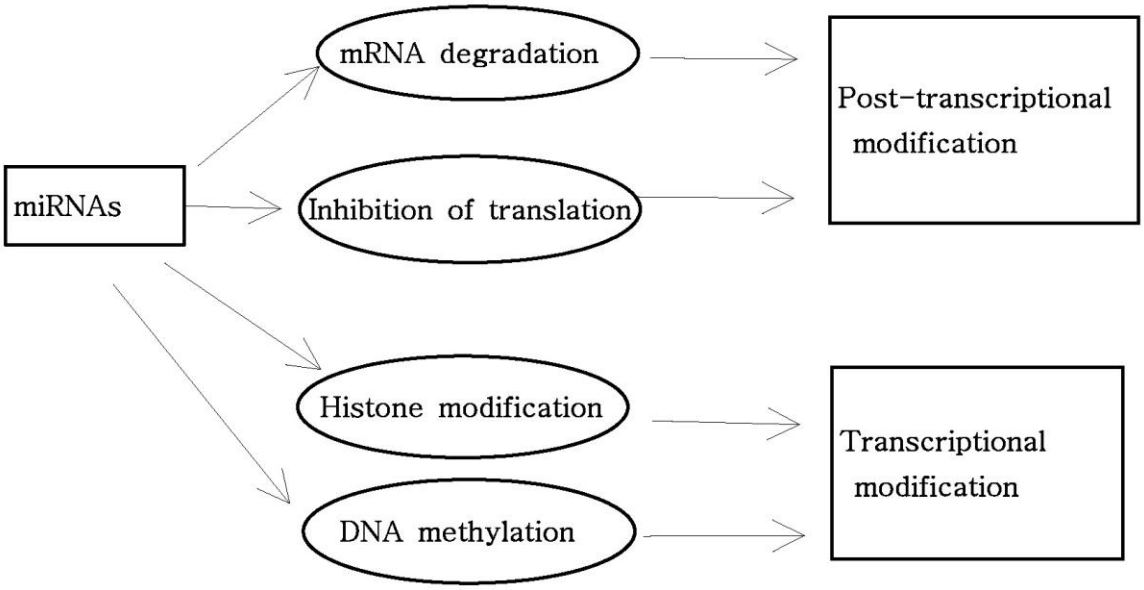

Figure 12. Modes of gene regulation by miRNAs. Diverse roles of miRNAs, such as mRNA degradation, inhibition of translation, histone modification, and DNA methylation. miRNAs not only regulate gene expression at the post-transcriptional level, but they are also capable of modifying transcription. (Ha, 2011)

A more intricate mechanism became apparent when it was discovered that miRNAs also induce deadenylation of mRNAs (Behm-Ansmant *et al*., 2006; Wu *et al*., 2006; Wakiyama *et al*, 2007), which accomplishes translational inhibition but is independent of it (Fabian *et al*., 2009). This function is mediated by the interaction of Ago protein with chromatin assembly factor 1 (CAF1), which employs the CCR4-NOT protein complex, harboring both chemokine (C-C motif) receptor (CCR4) and CAF1 deadenylase enzymes (Fabian *et al*., 2009). In addition to GW182's interaction with PABP, this induces mRNA deadenylation, which may be associated with an extra step involving the PABP-dependent poly(A) nuclease 2 (Pan2)-Pan3 deadenylase complex (Chen *et al*., 2009). Though, the results of the latter study disagree with direct physical association between miRISC and any of the deadenylase enzymes and suggests an another model in which miRNAs render mRNAs more liable to deadenylation via the degradation machinery, plausibly, by disrupting PABP's association with the poly(A) tail (Chen *et al*., 2009). Generally, deadenylation leads to destabilization of mRNA. Although mammalian miRNAs are commonly documented for inhibiting translation versus inducing mRNA degradation, there is considerable evidence that supports the latter (Bagga *et al*., 2005). In fact, Farh *et al*. (2005) reported that the levels of tissue-enriched miRNAs conversely correlated with levels of some of their mRNA targets compared with their concentrations in other tissues. The question of whether a miRNA induces translation inhibition or mRNA degradation is best addressed at the level of individual miRNAs, specific targets, and cell milieu.

Surprisingly, miRNAs have also been monitored to enhance translation in quiescent cells (Vasudevan *et al*., 2007). Particularly, miR-369-3p increases the translation of TNFα in serum-deprived, cell cycle-arrested, 293HEK cells. Fascinatingly, this effect switches to an inhibitory one, upon re-entry of the cells into the cell cycle. An analogous effect was observed with the regulation of high mobility group A2 (HMGA2) protein and its targeting miRNA, Let-7, proposing the ubiquity of this mechanism, but not with siRNA. During miRNA-enhanced translation, it was illustrated that the FMRP1 protein is employed to RISC; but, the complete mechanism of this functional aspect of miRNAs awaits explanation.

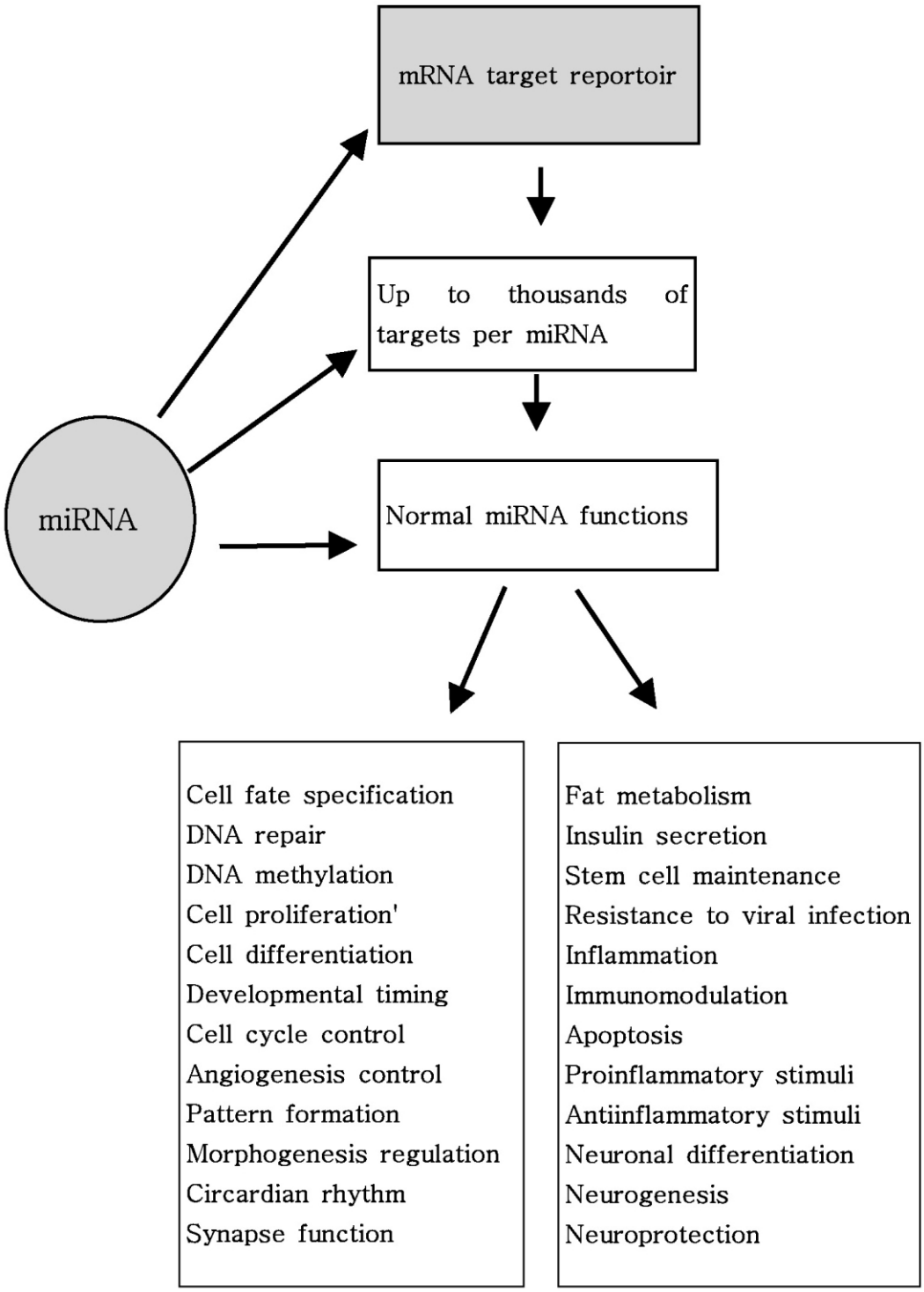

Figure 13. Biological and cellular functions of miRNAs. miRNAs exhibit multiple fundamental biological roles. Various miRNAs may interact with thousands of different mRNA targets. Post-transcriptional RNA editing may change mRNA target specificity. (Ha, 2011)

1.8.3 miRNAs IN HUMAN DISEASES

The immense discovery of miRNAs has transformed current cell biology and medical science. New members of the miRNA family are being exposed on a daily basis and emerging facts has demonstrated that miRNAs play a key role in a broad range of developmental process including cell proliferation, cell differentiation, cell cycle, developmental timing, apoptosis, neuronal gene expression, neuronal cell fate, brain morphogenesis, muscle differentiation, stem cell division and metabolism. In addition, several studies have reported links between alterations of miRNA homeostasis and pathological conditions such as cardiovascular disease, neurological diseases, cancer and autoimmune diseases. Interestingly, miRNA deficiencies or excesses have been correlated with numerous clinically important diseases ranging from myocardial infarction to cancer. miRNAs can suppress the mRNA translation of hundreds of target genes and are therefore well-positioned to target a multitude of cellular mechanisms. As a result of extensive participation in normal functions, it is quite reasonable to ask the question if abnormalities in miRNAs ought to have importance in human diseases. Great discoveries and rapid advancement in the past few years on miRNAs give the hope that miRNAs will in the near future have a great potential in the diagnosis and treatment of many diseases (Ha, 2011).

Significantly, it has been more and more reported that miRNAs are associated with diseases. However, the pattern amongst the miRNA-disease association remains largely imprecise. In order to dissect the patterns of miRNA-disease associations, Lu *et al*. (2008) carried out a comprehensive analysis to the human miRNA disease association data, which was manually collected from publications and a human miRNA association disease network was made. Interestingly, miRNAs show similar or different dysfunctional evidence for the similar or different disease groups, respectively (Lu *et al*., 2008). Additionally, they also found that there is a negative correlation between the tissue-specificity of a miRNA and the number of diseases it is associated and that there is an association between miRNA conservation and disease (Lu *et al*., 2008). Moreover, miRNAs associated with the same disease tend to appear as predefined miRNA groups. These findings can not only provide help in understanding the association between

miRNAs and human diseases, but also recommend a new approach to identify novel disease-associated miRNAs (Lu *et al*., 2008).

miRNA expression can be induced or supressed by a variety of stimuli and mechanisms. These stimuli comprise direct transcriptional activation or repression from transcriptional enhancers, epigenetic modifications of the genome, genomic amplification or deletion, cellular stress and inflammatory stimuli (O'Neill *et al*., 2011; Guo *et al*., 2010; Tomankova *et al*., 2010). The effect of inducing or supressing miRNA expression can persuade most biological processes, including cell proliferation, cell fate specification, DNA methylation, DNA repair and apoptosis and provide pro- or anti-inflammatory stimuli (Belver *et al*., 2010; Pallante *et al*., 2010). The biological outcome of a specific miRNA will depend on the cellular milieu in which it is expressed, its turnover rate and the target sequence to that the miRNA can bind. Since a single miRNA can bind to its target sequence with imperfect complementarity, a specific miRNA can have many potential targets (O'Neill *et al*., 2011; Guo *et al*., 2010). This allows a single type of miRNA to concurrently regulate the translation of various genes in multiple pathways. In this way, relatively small changes in miRNA expression can lead to unpretentious changes in the levels of multiple proteins and together these can add up to large alterations in biology (Selbach *et al*., 2008; Baek *et al*., 2008; Germain *et al*., 2011). Selbach *et al*. (2008) have revealed that, in addition to down-regulating mRNA levels, miRNAs also directly suppress translation of hundreds of genes and that a miRNA can, by direct or indirect means, tune protein synthesis from thousands of genes (Selbach *et al*., 2008). miRNAs also have a necessary role in both the innate and adaptive immune system. Appropriate miRNA expression is requisite for proper differentiation of immune cells (Lu *et al*., 2009). Immune responses are symphonies of cellular and molecular interactions, with each participant doing its part to generate the composite behavior we see as efficient host defense, or when dis-coordinated, as immunodeficiency or immunopatholgy (Germain *et al*., 2011). Therefore it is not surprising that miRNAs have been implicated in a range of human diseases like cancer (O'Connell *et al*., 2010; Pallante *et al*., 2010; Liston *et al*., 2010; Schetter *et al*., 2010; Yu *et al*., 2010; Liu *et al*., 2011; Navarro *et al*., 2008), cardiovascular diseases (Wang *et al*., 2010.; Contu *et al*., 2010; Small *et al*., 2011; Qian *et al*., 2011), neurological diseases (Miller *et al*., 2010; Haramati

et al., 2010), psychiatric diseases (Beveridge *et al*., 2008; Wang *et al*., 2008), asthma (Tan *et al*., 2007; Polikepahad *et al*., 2010), microbial infection (O'Neill *et al*., 2001; Li *et al*., 2010; Witwer *et al*., 2010; Belair *et al*., 2009; Liu *et al*., 2010; Zhang *et al*., 2010), autoimmune diseases (Tili *et al*., 2008), multiple sclerosis (De Santis *et al*., 2010), diabetes (Pandey *et al*., 2009; Leeper *et al*., 2011), rheumatoid arthritis (Fulci *et al*., 2010) and skin diseases (Buckner *et al*., 2010; Sonkoly *et al*., 2009; Yi *et al*., 2010) including psoriatic arthritis (Chatzikyriakidou *et al*., 2010). An understanding of how miRNAs influence the body's response to certain drugs and how these influence the expression of miRNAs, will be of key importance in developing drugs with greater efficacy and safety (Garofalo *et al*., 2008).

1.8.4 miRNAs IN MELANOCYTE BIOLOGY

Besides the approximately hundred genes are known to influence melanocyte development, there are numerous miRNAs, which target genes or pathways implicated in melanocyte biology. Several of these have been recognized through studies in melanoma cells or tissue samples, and for the immense majority, their role/s, if any, in melanocyte biology or development is still unidentified. The first evidence of an involvement of miRNAs in melanocyte biology came from the scrutiny that Dicer is required for survival of differentiating neural crest cells (Zehir *et al*., 2010). However, until today, only a few miRNAs regulating neural crest differentiation, specification and migration have been reported. Eberhart *et al*. (2008) showed that miR-140 influences cranial neural crest cell dispersion. Further studies have identified numerous miRNAs playing diverse roles in neural crest development (Amaral and Mattick, 2008; Cordes and Srivastava, 2009; Ivey and Srivastava, 2010; Subramanyam and Blelloch, 2011; Xin *et al*., 2009). MiR-143 and miR-145 have been reported to directly exit from pluripotency in embryonic stem cells (ESCs) by targeting Sox2, Oct4 (octamer binding transcription factor 4) and Klf4 (Kruppel like factor 4) (Xu *et al*., 2009), thus allowing differentiation toward diverse lineages, including neural crest fate. Conditional deletion of Dicer from neural crest lineage does not stop induction and migration of neural crest, but impairs patterning and migration of cardiac precursors, leading to a variety of cardiovascular abnormalities (Huang *et al*., 2010b; Nie *et al*., 2011; Zehir *et al*., 2010). Dicer-induced phenotypes are,

at least partially, mediated by miR-21 and miR-181a, which sequentially affects the MEK/ ERK signaling pathway (Huang *et al*., 2010a,b).

Dynoodt *et al*. (2013) identified 16 miRNAs differentially expressed in melan-a treated cells using miRNA profiling on mouse melanocytes after successive treatments involving forskolin and solar-simulated UV (ssUV) irradiation. Down-regulation/ over-expression of miR-145 in melan-a cells revealed increased or reduced expression of MITF, Tyr, Trp1, Sox9, Myo5a, Fscn1, and Rab27a, respectively, placing miR-145 in a central role in regulation of melanogenesis in response to external stimuli. Recently, miR-125b was recognized as a strong regulator of steady state melanogenesis. Kim *et al*. (2014) found that the expression of miR-125b is conversely related to pigment levels. MiR-125b targets TYR and DCT, two key enzymes of melanogenesis. The miR-125b promoter was hypermethylated in darkly pigmented cells. While a down-regulation of miR-125b has been reported in melanoma, no association to pigmentation was observed in melanoma cell lines or samples.

A wide network of miRNAs centered on MITF in melanocyte differentiation is emerging through the efforts of different researchers. Levy *et al*. (2010) reported MITF regulated DICER transcription in melanocytes. MITF binds and activates a conserved regulatory element upstream of Dicer's transcriptional start site leading to melanocyte differentiation, therefore allowing maturation of specific miRNAs implicated in melanocyte survival. Targeted knock out (KO) of Dicer is lethal to melanocytes, similar to MITF deficiency (Goding, 2000) partly through Dicer-dependent processing of the pre-miRNA-17-92 cluster targeting BIM, a well-known pro-apoptotic regulator of melanocyte survival (Levy *et al*., 2010). Dicer is robustly upregulated during melanocyte differentiation. Consecutively, MITF was suggested to be targeted by miR-25 in melanocytes. Microarray screening of miRNAs identified differentially expressed miR-25 in the skin of alpacas with pigmented versus white coat color, conserved binding sites of miR-25 are found in the 3' UTR of MITF from human, mouse, rat, and dog. Up-regulation of miR-25 in cultured melanocytes, in fact, decreased MITF mRNA and protein levels as well as the expression of the MITF-regulated enzymes tyrosinase and TYRP (Zhu *et al*., 2010). In 2012, Dong *et al*. (2012) demonstrated miR-137 mediated

down-regulation of MITF in a transgenic mouse model expressing multiple copies of pre-miR-137 under a CMV (cytomegalovirus) promoter. Transgenic mice developed coat color changes related to the levels of miR-137 expression. Molecular analyses of the transgenic mice with three copies of pre-miR-137 under CMV promoter, showed decreased expression of MITF only at the protein level and of its downstream genes, including TYR, TYRP1 and TYRP2. As for miR-145, melanogenesis altered by miR-137 was different from that affected by UV radiation. A response to UV, however, is also present in transgenic mice and is mediated by α-MSH. Alpha-MSH supresses the expression of endogenous miR-137 (Bemis *et al*., 2008), activates MITF, and generates more melanin in skin to partly compensate for the melanin drop induced by exogenous miR-137. Therefore, a complex regulatory loop is established that compensates for alteration in miR-137 expression. The association of miR-340 with MITF regulation requires further investigation; though, miR-340 was reported to regulate UVB-induced dendrite formation via the regulation of RhoA expression in melanocytes (Jian *et al*., 2014). In response to skin penetration by UV rays and subsequent DNA damage, melanocytes form dendrites that transport melanosomes to their tips for transport to the surrounding keratinocytes and protect them from UV irradiation (Eller *et al*., 1997). UV-induced DNA damage produces thymidine dinucleotide fragments, which induce melanin synthesis and lead to melanosomes fomation (Yamaguchi *et al*., 2007). Jian *et al*. (2014) showed that miR-340 was up-regulated in pigmented cells treated with UVB irradiation. Over-expression of miR-340 considerably supressed RhoA protein expression, amplified the total length and number of dendrites per cell, and encouraged melanosome aggregation in dendritic tips of melanocytes.

miRNAs have been shown to direct skin pigmentation in fish (Yan *et al*., 2013). Thirteen miRNAs were found to be differentially expressed between red and white skin carps (*Cyprinus carpio*), and 10 of them showed the similar behavior in red/white tilapia. The analysis of miRNA spatial and temporal expression patterns indicates that miR-429 may be a potential regulator of skin pigmentation in carps. MiR-429 silencing in red-skin carp causes depigmentation. Bioinformatics analysis and a luciferase reporter assay demonstrated that miR-429 directly regulates expression of FOXD3 (forkhead box D3) by targeting its 3'UTR. miR-429 silencing leads to a considerable increase in the

expression of FOXD3 *in vivo*, thus repressing the transcription of MITF and its downstream genes, such as TYRP1, TYRP2 or TYR. FOXD3 has been shown to have a role in the specification of different downstream neural crest derivatives. miR-429-mediated pigmentation is different from α-MSH-mediated pigmentation upon UV radiation. However, it was found that UV radiation significantly upregulated the level of miR-429 in the melanocyte, supporting convergence of pro-pigmentation signals on miR-429. The alteration in miR-429 expression was detected as early as 1 h after UV treatment, thus showing that miRNA regulation takes place earlier than most gene transcription responses, resulting into reduced in FOXD3 expression and increased MITF expression at mRNA and protein levels. MiR-429 is a member of the miR-8 family. In Drosophila, miR-8 is essential for proper spatial patterning of pigment on adult female abdomens. Loss of miR-8 in the developing cuticle leads to cell-autonomous loss of pigmentation (Kennell *et al*., 2012). FOXD3 is one of the initial molecular markers of the neural crest lineage; FOXD3 can also regulate the lineage choice between glial or neural and pigment cells by repressing MITF during the early stage of neural crest migration. miR-429 expression was shown to start at the gastrula stage, which leads to a gradual decrease in FOXD3expression. The progressive decrease of MITF supression by FOXD3 could contribute to the formation of pigment cells.

On the other hand, in the retinal pigmented epithelium (RPE), MITF promoted differentiation by regulating miR-204/211 expression. miR-204/211 transfection in de-differentiated RPE cells was adequate to induce epithelial differentiation (Adijanto *et al*., 2012). Numerous miRNAs were deregulated in MITF knocked down melanocytes (Wang *et al*., 2012). The changes in miRNA profile were correlated with the down-regulation of TYRP1, TYRP2 and TYR mRNA expressions, but miRNA target sites are poorly conserved in these potential targets, which might be regulated directly by MITF (Wang *et al*., 2012). Various groups reported miRNA profiling results in normal epidermal melanocytes. Such as, microarray analysis demonstrated that miR-192 and miR-194 are being expressed at high levels in the normal epidermal melanocytes (Caramuta *et al*., 2010). Deep sequencing of epidermal melanocyte libraries shown relatively high levels of expression for some miRNAs, including the members of the let-7 (let-7a-i), miR-320a, miR-219a, and miR-378 families (Stark *et al*., 2010). Still, the role of these miRNAs and

their targets in pathogenesis of vitiligo requires further investigations. As DICER, a key component of the miRNA processing machinery, is a target of the melanocyte-specific master regulator MITF, this suggests that miRNAs are important components of cell type-specific transcriptional programs. In turn, MITF is itself a target of individual miRNAs (mir-137, miR-125b and miR-25, and possibly miR-340), establishing a negative feedback mechanisms that controls MITF expression in differentiating cells (Botchkareva, 2012). These results indicate the existence of a crosstalk between the miRNAs and pigment-specific master regulators, which is imperative for a proper balance of gene expression in melanocytes. There is still inadequate information available about the functions of further individual miRNAs in melanocyte biology.

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