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A Role of Environmental Components in Autoimmune Vitiligo of Smyth Line Chickens

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A ROLE OF ENVIRONMENTAL COMPONENTS IN AUTOIMMUNE VITILIGO OF SMYTH LINE CHICKENS

A ROLE OF ENVIRONMENTAL COMPONENTS IN AUTOIMMUNE VITILIGO OF SMYTH LINE CHICKENS

A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy in Cellular and Molecular Biology

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Abstract

The Smyth line (SL) of chickens is a well-established animal model for the study of spontaneous autoimmune vitiligo, displaying all the characteristics of human vitiligo. In humans, phenolic derivative 4-tertiary butyl phenol (4-TBP) has been shown to trigger vitiligo in susceptible individuals. In vitiligo-prone SL-chickens, live turkey herpesvirus (HVT) infection at hatch was identified as the most effective trigger of SL-vitiligo expression. This dissertation examined the role of the environmental factors 4-TBP and HVT in the etiology of SL-vitiligo. Specifically, the generation of reactive-oxygen-species by melanocyte-containing feather tissue (MC-FT) and cultured melanocytes in response to 4-TBP exposure *in vitro* (Study I) was examined. *In vivo* leukocyte infiltration, morphology of melanocytes, and gene-expression post 4-TBP injection into the feather pulp were also monitored (Study II). Results showed that independent of HVT administration at hatch and age of chicken, *in vitro* 4-TBP exposure induced more oxidative stress ($P < 0.05$) in cultured MC-FT and melanocytes from SL- than control-chickens. Alterations in melanocyte morphology and gene-expression of melanogenesis-, apoptosis- and stress-related proteins were observed in *in vivo* 4-TBP-treated SL compared to control feathers. These data support a heightened sensitivity of melanocytes to 4-TBP in SL-chickens. The reduction in MITF and increase in HSP70 and BCL2 observed in control- but not in SL-feathers may be an effective protective response to 4-TBP-induced cellular stress. In Study III, the relationship between HVT and SL-vitiligo expression was further examined. The observed progressive

reduction (90 to 0%) in HVT's ability to trigger SL-vitiligo during the first 10 weeks of age

suggests that HVT exerts its effect on vitiligo expression when adaptive immunity is not

fully developed. The observation of elevated cytokine expression (IL-8, IL-10, IFN-γ and

IL-21) during active SL-vitiligo which was preceded by increased levels of HVT at 1 to 0.5

weeks before SL-vitiligo onset, strengthens the association of HVT-infection and

development of the melanocyte-specific autoimmune response. With the translocation of

HVT to the feather, its presence and resulting immune activity may cause cellular stress in

the inherently defective SL-melanocyte. Stress-associated melanocyte alterations may lead

to immune recognition of SL-melanocytes and development of melanocyte-specific

autoimmune disease.

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INTRODUCTION

Vitiligo is an acquired autoimmune disease in human patients characterized by loss of functioning melanocytes in affected areas of the skin. In order to understand the initial etiological and pathogenic mechanisms of vitiligo, appropriate animal models are crucial to determine the processes involved in the development of this disorder. The value of the Smyth line (SL) chicken as an animal model for human vitiligo is supported by many phenotypical and etiological similarities between human vitiligo and SL vitiligo (SLV) (Literature Review). Like human vitiligo, the spontaneous development of SLV is the result of complicated processes involving the interaction of genetic, immunological, metabolic and environmental factors in susceptible individuals.

Like human vitiligo, there is an inherent susceptibility for vitiligo in SL chickens which is partially manifested by intrinsic melanocyte abnormalities. Melanocytes from SL chickens that never developed vitiligo and from SLV chickens prior to the onset of feather amelanosis appear to be normal when examined by light-microscopy. However, abnormally-shaped melanosomes could be observed in SL melanocytes using transmission electron microscopy. These altered melanosomes were not observed in melanocytes from the parental Brown line (BL) control (Boissy *et al.,* 1983). Cultured embryo-derived melanocytes from SL chickens also exhibited abnormalities such as fewer dendrites and selective autophagocytosis of melanosomes compared to cultured melanocytes from BL

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and Light Brown Leghorn (LBL) controls (Boissy *et al.,* 1986). Together, these observations support the presence of inherent melanocyte defects in SL chickens.

As shown through immunosuppression studies, the inherent melanocyte defect alone is not sufficient for SLV expression without a functioning immune system, but appears to play a role in activating a melanocyte-specific autoimmune response (Smyth *et al*., 1981). Several studies have provided evidence supporting the role of the immune system in the pathology of SLV. A possible role of humoral immunity was demonstrated by the presence of autoantibodies in SLV chickens several weeks before visible onset of SLV that were capable of binding to melanocyte-specific proteins (Austin *et al*., 1992; Austin and Boissy, 1995). Roles of inflammation and cell-mediated immunity in melanocyte loss were suggested by the observation of elevated levels of circulating inflammatory cells just prior to and at visible onset of SLV. Moreover, SLV development was associated with the infiltration of macrophages and lymphocytes into the target tissue, specifically the pulp and melanocyte-containing barb ridge of growing SL feathers. Prior to visible onset of SLV, infiltration consisted primarily of T helper cells (CD4+); whereas, cytotoxic (CD8+) lymphocytes predominated at visible onset of vitiligo and during active melanocyte destruction (Erf *et al*., 2001; Erf *et al.,* 1995; Shresta *et al.,* 1997). The presence of CD8+ cells was related to melanocyte apoptosis in the growing feather from SLV chickens (Wang and Erf, 2003, 2004), supporting a role of cytotoxic T cells in melanocyte loss. Additionally, feather-infiltrating lymphocytes also included a significant proportion of

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IgM+ B cells (Shresta *et al.*, 1997; Shi and Erf, 2012). Recent gene expression analysis of feather tissues collected prior to and near onset of SLV using quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) showed that active SLV was accompanied by high expression of pro-inflammatory cytokines interleukin (IL)-1β, IL-8, IL-12, IL-21 and interferon (IFN)-γ, but low expression levels of IL-4. These findings further support the presence of cellular, Th1-mediated immune activity directed against melanocytes in SLV chickens (Shi and Erf, 2012). Therefore, anti-melanocyte cell-mediated immunity appears to play a crucial role in melanocyte loss in SLV.

In addition to intrinsic melanocyte defects and autoimmune components involved in SLV development, expression of vitiligo in susceptible SL chickens appears to require environmental triggers. Routine administration of live herpesvirus of turkey (HVT) at hatch to protect chicks from infection with Marek's disease virus was revealed as one environmental factor that is highly associated with vitiligo expression in susceptible SL chickens (Erf *et al.,* 2001).

As shown by Manga *et al.* (2006), skin exposure to phenolic derivatives, such as 4-tertiary butyl phenol (4-TBP) can trigger vitiligo in susceptible individuals by inducing oxidative stress that is more readily overcome by melanocytes from normally pigmented individuals than from individuals with vitiligo. The oxidation of 4-TBP by tyrosinase (TYR) to quinones (Thörneby-Andersson *et al.*, 2000), causes futile redox cycling and

oxygen activation forms cytotoxic levels of hydrogen peroxide and glutathione disulfide (GSSG), thereby induces oxidative stress (O'Brien, 1992). In SL vitiligo a disturbed redox state in the target tissue was also reported, including increased levels of reactive oxygen species (ROS) generation in feather samples from SL chickens compared to BL and LBL chickens (Lockhart, 2004). Additionally, feather samples from SLV chickens were found to have decreased anti-oxidative capacity as well as increased levels of lipid peroxidation compared to BL and LBL controls (Erf *et al.*, 2005; Wijesekera, 2004). Oxidative stress has been shown to play a role in triggering various clinical disorders. The exact role of oxidative stress in vitiligo is unclear, but it is assumed to play a part in either the development of the disorder or the progressive loss of melanocytes.

Taken together, the SL autoimmune vitiligo model provides excellent opportunities to study the complex interactions of factors in this multifactorial polygenic disorder which give rise to autoimmune destruction of intrinsically abnormal melanocytes under favorable conditions.

Based on previous studies, we hypothesized that the effect of 4-TBP in SL chickens will be consistent with 4-TBP's effects as a trigger of human vitiligo in susceptible individuals. By using the SL chicken model for human vitiligo, *in vitro* and *in vivo* effects of 4-TBP in melanocytes/melanocyte-containing tissue can be examined. Specifically, examination of 4-TBP's effect on ROS production and gene expression profiles of proteins

related to melanogenesis, apoptosis and stress in SL and controls chickens will provide insight into the mechanisms underlying the phenotypical abnormalities in melanocytes from SL chickens. Additionally, we also hypothesized that determination of cytokine involvement in the initiation of the melanocyte-specific autoimmune response together with assessment of herpesvirus of turkey (HVT) viral load in the target tissue will provide insight into the interplay of genetic susceptibility, immune system activities and environmental factors in the spontaneous expression of SL vitiligo. In summary, this dissertation will provide important new information regarding the complicated nature of this disease and indicate new pathological components in this disorder.

Based on the availability of the chicken genome sequence and limited availability of antibodies to chickens proteins (Burt, 2004), qRT-PCR has become the main approach used to examine gene expression of various targets in this project. The objectives and approaches of the current study were as follows:

Objective 1: To examine the effects of *in vitro* 4-TBP exposure of melanocyte-containing feather tissue and feather- and embryo-derived melanocyte cultures on oxidative stress and stress-, melanocyte-function and apopotosis-related gene expression. For this purpose, pigmented growing feathers were collected from 1-, 4-, 7- and 12-week-old SL, BL and LBL chickens that did or did not receive HVT. The newest three millimeters growth (the melanocyte-containing, active pigmentation site) of the

feathers was cultured with 4-TBP and the production of oxidative radicals was monitored *in vitro*. Additionally, the ROS generation and morphological changes of both feather- and embryo-derived melanocytes from SL and BL chickens were also monitored following exposure to 4-TBP. qRT-PCR was used to determine relative expression of genes for melanogenic proteins including tyrosinase (TYR), tyrosinase-related protein (TRP) 1 and 2, microphthalmia-associated transcription factor (MITF), genes for anti-apoptotic proteins B-cell lymphoma 2 (BCL2) and pro-apoptotic BCL2 homologous antagonist/killer-1 (BAK1), and the gene for heat shock protein (HSP)70.

Objective 2: To examine and monitor effects of 4-TBP on melanocyte morphology and gene-expression *in vivo*. For this study, mononuclear infiltration, morphological changes of melanocytes and gene expression related to melanogenesis and apoptosis, as well as HSP70 was monitored post-4-TBP injection *in vivo*. Specifically, pigmented growing feathers were collected before (0 h) and at 1, 3.5, 6 h, and 3 d after 10 µL of 4-TBP (0.15 mg/mL) were injected into the pulp of growing feathers of 8-week-old SL, BL and LBL chickens. The expression of genes TYR, TRP1, TRP2, MITF, BCL2, BAK1 and HSP70 were examined using qRT-PCR in melanocytes from SL, BL and LBL chickens upon 4-TBP treatment *in vivo*.

Objective 3: To determine the role of HVT in the development of SLV. SL and control chickens were used in this study to determine: 1) the ability of HVT to induce SLV

expression at different ages; 2) primary and recall responses to HVT; and, 3) the association between HVT viral load and cytokine production in the target tissue during SLV development and progression. Specifically, the effect of HVT on the SLV incidence was monitored by HVT vaccination of SL chicks at hatch or at 2-, 4-, 6-, 10-, 14-, and 18-weeks of age. Moreover, the primary and secondary response to HVT was evaluated over a 10 day period based on leukocytes infiltration in the feather pulp in response to HVT injection. To examine HVT viral load and cytokines gene expression throughout SLV development, several approaches were used: HVT load (qPCR) and HVT gp expression (qRT-PCR) was examined in spleen and feathers at various times post-HVT administration; the location of HVT within the feather tissue was examined using laser capture microdissection and qPCR; HVT levels and cytokine expression in feather samples collected prior to and throughout SLV development were examined by qPCR and qRT-PCR, respectively.

Results from this dissertation research provide new insights into the inherent susceptibility of SL melanocytes, the role of environmental factors, and the immune system responses involved in autoimmune loss of SL melanocytes. Furthermore, new insight gained into the complicated etiology of vitiligo in SL chickens will drive the direction of future research in SL and human vitiligo.

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CHAPTER I

LITERATURE REVIEW

LITERATURE REVIEW

Human vitiligo

Vitiligo is an acquired idiopathic dermatological disorder characterized by postnatal loss of epidermal pigment-producing cells (melanocytes) resulting in the formation of white areas of skin. Vitiligo is presumed to affect approximately 1 % of the population in the United States and 0.1 - 2 % worldwide (Moretti *et al.*, 2006), irrespective of age, race, ethnic origin or skin color. Human vitiligo can develop at any age, usually beginning in childhood or young adulthood, with onset of pigmentation loss in the skin typically occurring in individuals between 10 - 30 years of age (Tonsi, 2004). Approximately 20 % of vitiligo patients have at least one first-degree relative with vitiligo and the risk of vitiligo for immediate family members of vitiligo patients increases by 7 to 10 fold (Wolff *et al.*, 2007).

Vitiligo is commonly classified as segmental (asymmetrical distribution of white patches) and nonsegmental (symmetrical distribution of white patches). The appearance of irregularly-shaped white patches is the main symptom of vitiligo. These white patches pose more than aesthetic problems and they have a major impact on the quality of life of the patients. Physiologically, affected skins are more photosensitive than normal skin, resulting in redness and damage from sun exposure. Additionally, vitiligo patients are more prone to developing skin cancer and/or various autoimmune diseases (Nordlund and Lerner,

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1982). Mentally, vitiligo patients tend to experience serious emotional stress, lack of confidence on social contacts, and self-humiliation. Moreover, these physiological and mental disorders interact and enhance each other (Salas-Alanis *et al*., 2011).

Clinical manifestations

Vitiligo is a hypopigmentation disorder where the loss of functioning melanocytes causes the appearance of white patches on the skin. It commonly manifests itself in one to several amelanotic white macules surrounded by a normal or hyperpigmented border (Wolff *et al.,* 2007). Sometimes the patches may have a red inflammatory border.

The lesions usually enlarge at an unpredictable rate and can appear on any part of the body. The most frequent initial sites are hands, forearms, and feet; however, the most commonly affected sites are the face, upper part of the chest, and dorsal aspect of the hands (Tonsi, 2004).

Classification

There are different points of view on the classification of vitiligo. From one point of view, vitiligo is classified as segmental, acrofacial, generalized and universal, based on the pattern of involvement as focal, mixed and mucosal types (Wolff *et al*., 2007). The generalized pattern is the most common one. Another point of view classifies vitiligo based on the extension and distribution of lesions, delineating three types of vitiligo: localized,

generalized and universal vitiligo (Nordlund and Lerner, 1982). Currently, vitiligo is classified into two major subtypes: segmental vitiligo (Type B) and non-segmental vitiligo (Type A) (Le Poole *et al.,* 1993; Koga *et al.*, 1988). Type A is more common and frequently associated with autoimmune disease and the Koebner phenomenon (skin lesions appearing on lines of trauma resulting from either a linear exposure or irritation). Type B is rare and includes focal lesions restricted to a segment of the integument. It usually exhibits a stable course after a rapid onset and evolution (Torello *et al.*, 2008).

Etiology and pathogenesis

Although the etiopathology of vitiligo is still not clearly understood, it has been well established that vitiligo is a multifactorial disorder, involving both genetic and non-genetic factors (Alkhateeb *et al*., 2003).

The genetics of vitiligo cannot be simply explained by Mendelian genetics (Alkhateeb *et al*., 2003) and has been characterized by incomplete penetrance, multiple susceptibility loci, polygenic traits and genetic heterogeneity. Vitiligo susceptible genes play a role in all aspects of vitiligo pathogenesis and respond to environmental inputs hence genetics cannot be considered as a separate phenomenon. Several facts have indicated that genetic factors play an important role in the susceptibility to vitiligo. To begin with, statistics have shown that 15 - 20 % of patients with vitiligo have one or more affected first-degree relatives, which is higher incidence than the sporadic incidence of less than 2 % in the population

(Moretti *et al*., 2006). Similarly, the risk of vitiligo in a patient's sibling among Caucasians is approximately 6.1 % (Alkhateeb *et al*., 2003), which is a 16-fold increase compared to the general Caucasian population where the prevalence of the disease is 0.38 % (Howitz *et al*., 1977). Additionally, the concordance of vitiligo in monozygotic twins is 23 % which is 60-fold greater than that in the general population and 4-fold higher than that for a patient's sibling (Alkhateeb *et al*., 2003).

Currently, several vitiligo susceptibility genes have been identified, including forkhead box D3 (FOXD3), human leukocyte antigen (HLA), PTPN22, NALP1 and cytotoxic T lymphocyte antigen 4 (CTLA4). The majority of these genes are associated with autoimmune susceptibility (Spritz, 2008; Boissy and Spritz, 2009). Specifically, FOXD3 encodes a forkhead transcription factor that is a primary regulator of melanoblast differentiation. Mutations of FOXD3 were co-segregated with vitiligo in the study of a single family (Alkhateeb *et al*., 2005). Additionally, a strong association between HLA-DR4 and vitiligo was illustrated in several populations including American Caucasian, American African and Dutch (Foley *et al*., 1983; Dunston and Halder, 1990; Venneker *et al*., 1993). Recently, allelic variations in the R620W region of the PTPN22 gene that encodes a lymphoid protein tyrosine phosphatase involved in T cell signaling were associated with vitiligo susceptibility (Jin *et al*., 2010). Jin *et al.* (2007) also demonstrated that allelic variants in the NALP1 gene, which encodes a key regulator of innate immunity, increase the risk of vitiligo in Romanian populations. NALP1 protein is a

component of the inflammasome, a cytoplasmic multiprotein complex which mediates the maturation of proinflammatory cytokines such as interleukin (IL)-1 and -18, and stimulates cellular apoptosis. This gene and its activation could be involved in causing melanocyte destruction and thereby vitiligo (Boissy and Nordlund, 2011). Lastly, the variations in the CTLA-4 gene may be associated with secondary disorders such as autoimmune thyroid disease (Spritz, 2010).

Among vitiligo researchers it is generally agreed that there is an inherent susceptibility in certain individuals that together with environmental triggers leads to immune system-mediated loss of melanocytes. In addition to the melanocyte-specific autoimmune hypothesis, different hypotheses have been derived to explain the loss of melanocytes in vitiligo, including the autocytotoxic hypothesis and the neural hypothesis. Each of these hypotheses may play a role independently or in combination with the autoimmune hypothesis in the development and progression of human vitiligo (Alikhan *et al*., 2011).

The autocytotoxic theory postulates that the accumulation of cytotoxic precursors causes cell death. Specifically, by-products of melanin synthesis, such as phenolic radicals or intermediates, may damage the pigment cell, and melanin synthesis in melanocytes (Lerner, 1971). Either overproduction or inability to neutralize these byproducts of melanogenesis contributes to the build-up of cytotoxic intermediates. Melanocytes are genetically equipped with protective mechanisms to eliminate the toxic products

associated with melanogenesis. It has been postulated that these protective mechanisms are deficient in vitiligo patients (Lerner, 1971; Hann and Chun, 2000).

The neural hypothesis of vitiligo is based on observations of segmental vitiligo. It suggests that a neurochemical agent is responsible for the melanocyte death in vitiligo patients. Nerve endings release norepinephrine and other neural chemicals which may be toxic to the melanocytes and their environment. It has been shown that vitiligo-negative patients who experienced thoracoscopic sympathectomy (a surgical procedure where certain portions of the sympathetic nerve trunk are destroyed) developed depigmentation in the areas with disrupted sympathetic innervations (Westphal *et al*., 2009). Recently, it has been shown that Schwann cells are derived from the same multipotent population of neural crest cells as melanocytes. These bilaterally symmetrical characteristics, which relate to innervations pattern, make it possible to hypothesize a new perspective regarding the role of the nervous system in pigmentation and the precursor origin of Schwann cells (Igor and Francois, 2010). This hypothesis may be suited to describe the symmetrical distribution and abnormal sweating with segmental vitiligo (Nordlund and Lerner, 1982). However, it is difficult to provide a complete picture of vitiligo development through the neural hypothesis alone.

Previous studies point to inheritable biological properties from vitiligo patients that might make the melanocyte susceptible to environmental factors or stressors, resulting in

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the death of melanocytes by necrosis or apoptosis. Consequently antigens will be presented, immune tolerance may be lost, and autoimmunity will be ultimately activated directly against melanocytes (Boissy and Spritz, 2009; Mahoney and Rosen, 2005).

Indeed, melanocyte-specific autoimmunity is the most accepted and supported hypothesis to explain melanocyte loss in vitiligo. The coexistence of other autoimmune diseases with vitiligo (Daneshpazhooh *et al.,* 2006) and the higher frequency of autoimmune disorders in first degree relatives of vitiligo patients have always been viewed as strong support for an autoimmune etiology of vitiligo. For example, vitiligo is frequently associated with autoimmune thyroid disease (Boelaert *et al*., 2010), alopecia areata (Ahmed *et al*., 2007), Addison's disease (Zelissen *et al*., 1995), autoimmune polyendocrine syndromes (Neufeld *et al*., 1981), and pernicious anemia. In addition, the majority of vitiligo susceptible genes identified are related to the immune system and autoimmune diseases (Spritz, 2010).

More direct evidence in favor of autoimmune etiology of vitiligo is the presence of autoantibodies and circulating T-cells directed against melanocytes and their components. Moreover, the margins of active generalized vitiligo lesions have been shown to be infiltrated with cytotoxic T-cells which are thought to play a primary role in melanocyte loss (Le Poole and Luiten, 2008; Ongenae *et al.,* 2003). Furthermore, the elevated ratio of $CD8^+$ to $CD4^+$ T-cells found in vitiligo patients further supports a role of cell-mediated

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immunity in vitiligo. Analysis of epidermal cytokines at sites of vitiligo lesions revealed increased levels of soluble IL-2, IL-6 and IL-8, suggesting a role of inflammatory immunity in vitiligo pathogenesis (Mandelcom-Monson *et al*., 2003). Moreover, an imbalance of cytokines can affect melanocyte activity and survival even though the exact roles in vitiligo pathogenesis of these inflammatory cytokines remain to be determined. Along with a role of cell-mediated immunity in vitiligo, humoral immunity was also found to contribute to the loss of melanocytes in this disorder. Antibodies to melanocytes occur at a significantly increased frequency in the sera of vitiligo patients compared with healthy individuals (Cui *et al.*, 1992). The ability of melanocyte-specific autoantibodies to kill melanocytes was eloquently demonstrated *in vivo.* By grafting human skins onto nude mice that were injected with purified IgG obtained from vitiligo patients showed melanocyte loss in this model system. This *in vivo* approach demonstrated the ability of melanocyte-specific autoantibodies to kill melanocytes (Gilhar *et al*., 1995). Vitiligo-associated antibodies were also shown *in vitro* to be able to destroy melanocytes by antibody-dependent cellular cytotoxicity and complement-mediated lysis (Gottumukkala *et al*., 2006). Targets of vitiligo autoantibodies have been reported to include tyrosinase (TYR) (Song *et al*., 1994), tyrosinase-related protein 1 (TRP1) (Li *et al*., 2010), TRP2 (Okamoto *et al*., 1998), tyrosine hydroxylase (Kemp *et al*., 2011), lamin A (Li *et al*., 2010), melanosomal matrix protein pg100 (Pmel17) (Kemp *et al.*, 1998), and melanin-concentrating hormone receptor 1(MCHR1) (Li *et al.,* 2010). In summary,

autoimmunity is the most comprehensive and widely accepted hypothesis for vitiligo with reasonable certainty.

Oxidative stress may also play an important pathogenic role in vitiligo. It is believed to be an initial event causing melanocyte degeneration in vitiligo development. Notably, studies *in vitro* have provided a link connecting cellular oxidative stress with autoimmune diseases (Dell'Anna and Picardo, 2006). Other studies also have indicated that intrinsic damage to melanocytes could be the initiating event in vitiligo development leading to recognition of melanocytes proteins and development of melanocyte specific immunity and progressive immune mediated loss of melanocytes (Van den Boorn *et al.,* 2011; Le Poole and Luiten, 2008). Oxidative stress originally results from either decreased levels of antioxidants such as glutathione, catalase and vitamin E or increased levels or reactive oxygen species, like superoxide anion, hydrogen peroxide and hydroxyl radicals. It is suggested that the imbalance in the oxidant-antioxidant system, which is probably due to melanocyte deficiency rather than oxidative stress, might play such roles in vitiligo (Bagherani *et al*., 2011). Nevertheless, melanocyte cultures from vitiligo patients exhibited intracellular hyperproduction of ROS (Dell'Anna and Picardo*,* 2007). Vitiligo melanocytes were found to be susceptible to external oxidative stress and UV radiation (Jimbow *et al.,* 2001).

In summary, despite years of intensive investigations, the exact pathogenesis of

vitiligo still remain to be defined, and it is anticipated that the discovery of biological pathways leading to development of the disease will provide novel therapeutic targets for future treatment and improve the quality of human life.

Treatment

Vitiligo can cause serious emotional stress and treatment of this aspect could be very important to vitiligo patients. Few studies have paid attention to the effects of treatment interventions on the psychosocial consequence of vitiligo. Currently, repigmentation rate occurs in 1 %-25 % of treated cases (James, 2006). The goal of vitiligo treatment is to halt the progression of depigmentation and try to induce repigmentaion in the white patches. These treatments mainly include prescription medicine, surgical therapy, and skin camouflage techniques.

Medical therapies either use topical steroid or psoralen. Topical steroid therapy is one of the most widely used vitiligo treatments for repigmentation of white patches. Steroids are potent suppressors of immunity and have proven beneficial in treatment of autoimmune diseases. Topical steroid therapy is used in the form of topical creams and ointments during the early stages of vitiligo. Psoralenphoto chemotherapy is also known as psoralen and ultraviolet A. Psoralen is a natural compound found in the seeds of *Psoraleacorylifolia*. After the depigmented area is exposed to ultraliolet A light, psoralen is either taken by mouth or applied to the depigmented area of skin and this sensitizes the skin to

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repigmentation. Despite the photocarcinogenic properties of psoralen, it was used as a

tanning activator in sunscreens until 1996 (Autier *et al*., 1997). Moreover, depigmentation

is an alternative way to achieve medical therapy. Depigmentation involves the use of

melanocytotoxic agents or photothermolysis to bleach the remaining pigmented areas or to

match white areas in patients with vitiligo (Garg *et al.*, 2010).

Surgical therapy is the latest vitiligo treatment which consists of autologous skin

grafts, epidermal blister grafting, and autologous melanocytes transplants, but all these

treatments must be considered only after less invasive medical therapies have failed

(Khunger *et al*., 2009).

Skin camouflaging is the art of disguising blemishes on the skin by using special

creams without treating its underlying cause. Camouflage therapy varies from

camouflage products, self-tanning dyes and tattooing. The dermatology life quality index

of patients with camouflage improved significantly when compared with that of patients

without camouflage (P < 0.005) (Tanioka *et al*., 2010).

In general, because the pathogenesis of vitiligo is still not clear, there is a plethora of

different treatments. None of these treatments appear to be effective to treat all types of

vitiligo, and hence may either be used alone or in combination depending on clinical type

and stability of vitiligo, age of patient and other factors.

Biology of melanocytes

Melanocytes are dendritic, melanin-producing cells located in the bottom layer of the skin epidermis that are responsible for the pigmentation of skin, hair and eyes through the process of melanogenesis. Each melanocyte in the epidermis has several dendrites that stretch out to connect it with many keratinocytes located in the basal layer. Through these connections, melanosomes, vesicles containing the endogenous photoprotectant melanin, will be transferred from melanocytes to keratinocytes. Together, the cells in the epidermal layer of the skin form a barrier against environmental damage such as pathogens (bacteria, fungi, parasites, and viruses), heat, UV radiation and water loss.

Melanocyte development

Except for pigmented epithelium of the eyes, all other pigment cells in vertebrates are derived from the neural crest that arises from the dorsal part of the neural tube or neural folds early during embryonic development. Depending on the axial level, the neural crest can be a source of bone, cartilage and adipose tissue, endocrine cells, neurons and glial cells and melanocytes. The choice of migratory pathway is determined by both lineage specification and expression of signaling molecules (Erickson and Goins, 1995). Committed precursors for melanocytes are termed melanoblasts.

Several transcriptional factors are crucial to melanoblast lineage commitment and migration behavior. Microphthalmia-associated transcription factor (MITF) is required for development of neural crest-derived melanocytes. Loss of MITF will result in the absence of melanocytes (Mochii *et al.*, 1998). In addition to MITF, stem cell growth factor (SCF) has a crucial role in permitting melanoblast survival and promoting proliferation, both during initial and later migration (Mackenzie *et al.*, 1997). Other factors including endothelin-1 and -3, hepatocyte growth factor and basic fibroblast growth factor are involved in different stages of melanocyte migration from the dermis into the epidermis in humans (Scott *et al.*, 1997).

Melanogenesis

The main function of melanogenesis is to protect skin from UV radiation. Upon the exposure of the skin to UV radiation, melanogenesis is enhanced by the activation of the key enzyme TYR which is a glycoprotein located in the membrane of the melanosome. The structure of TYR includes an inner melanosomal domain that contains the catalytic region (90 % of the protein), a short transmembrane domain and a cytoplasmic domain composed of 30 amino acids (Kwon *et al.*, 1987). It has been shown that histidine residue-binding copper ions located in the inner portion of TYR are required for TYR activity (Hearing and Jimenez, 1987). Two types of melanin are synthesized within melanosomes: a dark brown-black insoluble polymer eumelanin and a light red-yellow

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sulphur-containing soluble polymer pheomelanin (Prota, 1995). TYR catalyses the first

two steps of melanin synthesis: the hydroxylation of L-tyrosine to

L-dihydroxyphenylalanine (L-DOPA) and subsequent oxidation of this L-DOPA to the

corresponding quinone, L-dopaquinone (Ito *et al*., 1984).

Following the formation of L-dopaquinone, the melanin pathway is divided into

synthesis of the black-brown eumelanin and red-yellow pheomelanin. In the eumelanin

pathway, there is spontaneous conversion of L-dopaquinone to dopachrome. Dopachrome

is either spontaneously converted to 5,6-dihydroxyindole or is enzymatically converted to

5,6-dihydroxyindole-2-carboxylic acid by TYR-related protein-2 (TRP2) functioning as

dopachrometautomerase (DCT). TYR-related protein-1 (TRP1) converts the oxidation of

5,6-dihydroxyindole-2-carboxylic acid to a carboxylated indole-quinone at a downstream

point in the melanin biosynthetic pathway (Takeshi, 1994). Finally, the polymerization of

indole and quinines leads to eumelanin formation (Prota, 1988). The pheomelanin pathway

branches from the eumelanin pathway at the L-dopaquinone step and is dependent on the

presence of cysteine which is actively transported through the melanosomal membrane.

Cysteine reacts with L-dopaquinone to form cysteinyl-dopa (Prota, 1988). Then

cysteinyl-dopa is converted to quinoleimine, then to alanine-hydroxyl

dihydrobenzothazine and polymerizes to form pheomelanin.

TRP1 and TRP2, which are structurally related to TYR and share approximately 40 %

amino acid homology, span the melanosomal membrane like TYR. It has been suggested

that TRP1 increases the ratio of eumelanin to pheomelanin (del Marmol *et al.*, 1993).

However, except for the role in melanogenesis, other roles of TRP1 and TRP2 in

melanocyte survival and response to oxidative stress are not yet clarified.

Melanin has an important role in oxidative homoeostasis in skin. Eumelanin has an

ability to scavenge and quench both oxygen- and carbon-derived free radicals

(Rozanowska *et al.*, 1999). Pheomelanin does not have these properties and can even be a

source for free radical production upon UV irradiation (Larsson 1993).

Melanogenic regulation

Melanogenesis is regulated in part by paracrine melanogenic stimulators. These

stimulators include proopiomelanocortin (POMC)-derived peptides: alpha-melanocyte

stimulating hormone (α-MSH), β-MSH and adrenocorticotropic hormone (ACTH) (Thody

and Graham, 1998). Cui *et al.* (2007) showed that p53 activation upon UV radiation

(UVR) exposure stimulates transcription of the POMC promoter in keratinocytes, thus

increasing the release of POMC-derived α-MSH, a key physiological inducer of

melanogenesis. The biological function of keratinocyte-derived α-MSH is then mediated

through its binding to the melanocortin 1 receptor (MC1R), the only melanocortin

receptor expressed in melanocytes. MC1R is a G protein-coupled receptor that activates

adenylate cyclase and cAMP production. The increased intracellular concentration of cAMP mediates the melanogenic response to α-MSH, resulting in increased production of melanin. In general, α-MSH has a key role in the melanogenic response after exposure to UVR. Increased cAMP is followed by activation of protein kinase A (PKA) in melanocytes (Roesler *et al.*, 1998). PKA induces transcription of the MITF gene. In turn, MITF efficiently activates transcription of genes encoding melanogenic enzymes.

During the past decade, scientists found that MITF not only affects melanocyte development, but also pigmentation via its regulatory effects on transcription of TYR, TRP1 and TRP2 (Goding, 2000). MITF also was shown to be a key transcriptional factor for Rab27A which is an important protein for melanosome transport (Chiaverini *et al.*, 2008). Moreover, MITF also regulates expression of Pmel17, Melan-A, and ocular albinism type 1 (OA1) proteins which are involved in the formation of the melanosomal matrix and in melanosome maturation (Berson *et al.*, 2001). Therefore, MITF plays a crucial role in melanin synthesis as well as melanosome biogenesis and transport.

The Smyth line (SL) chicken overview – Avian model for human vitiligo

The Smyth line of chickens, previously known as the DAM (delayed amelanotic) chickens, was developed by Poultry Geneticist Dr. J. Robert Smyth, Jr. at the University of Massachusetts in Amherst in the 1970s. Vitiligo was first observed in 1971 in a hen from the Massachusetts Brown line (BL) population maintained at the University of

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Massachusetts' Poultry Research Center in Amherst, MA (Smyth *et al.*, 1981). This hen

exhibited post-natal, vitiligo-like loss of pigmentation. By back-crossing the vitiliginous

hen with BL males and selecting for vitiligo, the SL line was established. Serotyping of the

SL population identified three major histocompatability complex (MHC) haplotypes: B^{101},

B^{102}, and B^{103}, and sublines of the SL population homozygous to each MHC haplotype

were developed. Three MHC sublines of SL (SL101, SL102, and SL103; $B^{101/101}$, $B^{102/10}$,

and $B^{103/103}$, respectively), three MHC-matched parental BL (BL101, BL102, and BL103)

control lines and one other control line, the Light Brown Leghorn (LBL101) were initially

maintained. With the retirement of Dr. Smyth in 1996 and the closure of the poultry farm at

the University of Massachusetts, SL101 and MHC-matched control lines BL101and

LBL101were relocated to University of Arkansas. All three lines currently are maintained

by Dr. Gisela F. Erf at the Poultry Farm at the University of Arkansas, Division of

Agriculture in Fayetteville, Arkansas. Over the years, this chicken model has made

significant contribution to the understanding of the components and mechanisms involved

in vitiligo and autoimmune diseases in general. SL101, BL101, and LBL101 lines of

chicken are the only remaining genetic lines of chicken animal model for vitiligo.

Characteristics of Smyth line and control chickens

The SL of chickens is a valuable and excellent animal model for human vitiligo. SL

chicks have normal pigmentation at hatch with brown feathers and dark eyes. In general, SL vitiligo (SLV) chickens exhibit a postnatal, spontaneous, vitiligo-like, melanocyte loss in the feather (Erf, 2010), displaying all the characteristics of the human disorder (Wick *et al*., 2006). Under conventional rearing conditions, onset of pigmentation loss in the feathers typically occurs when the chickens are between 6 and 20 weeks of age (young adult) and the incidence of SLV in the SL population ranges between 80% and 95%. The severity varies from a few affected feathers to complete feather depigmentation (Erf, 2010). In addition, SL chickens also exhibit other autoimmune diseases like uveitis, autoimmune thyroiditis and an alopecia-like feathering defect (Smyth *et al*., 1981; Smyth and McNeil, 1999). Interestingly, SL chickens that carry the *Id* sex-lined recessive dermal pigmentation gene (SL-G), resulting in grey-pigmented shanks in *id* females and *id/id* males, very rarely develop SLV (< 5% incidence, Pillai, 1998).

The SL chickens together with the BL parental control (< 1% vitiligo) and the LBL vitiligo resistant control have been established as a very important model for spontaneous vitiligo (Erf, 2010).

Pathogenesis

The exact etiopathology for SLV, like human vitiligo, is not clearly understood,

Similar to human vitiligo, SLV is a multifactorial disorder whereby the expression of SLV involves the interaction between genetic susceptibility, immune system components and environmental factors that trigger the expression of the disorder.

SL chickens exhibit a genetic predisposition to the development of SLV which appears to be manifested in part by an inherent melanocyte defect. As reported by Boissy *et al.* (1984) established SL embryo-derived melanocyte cultures were initially indistinguishable from BL embryo-derived melanocytes. With increased age and passage of cultures, the SL melanocytes became irregularly shaped and developed fewer dendrites than BL control melanocytes. Similar abnormities were also observed in the feather tissue of SL chickens (Boissy *et al.,* 1984). Melanocytes in growing feathers exhibit morphological abnormalities including shrinkage of melanocyte-cell bodies and dendrites and abnormally shaped melanosomes with pigmented membrane extension several weeks before SLV development (Boissy *et al.,* 1983).

However, the inherent melanocyte defect alone is insufficient to lead to SLV without an active immune system (Smyth, 1989). The inherent melanocyte defect does however appear to play a role in provoking a melanocyte-specific autoimmune response. Both cell-mediated and humoral melanocyte-specific immune activities have been demonstrated in chickens with SLV. Cell-mediated immune mechanisms appear to be responsible for the melanocyte loss and to play a key role in the pathogenesis of SLV (Erf *et al*., 1995, Wang

and Erf, 2004). Immunophenotypic analysis of the feather infiltrating leukocytes revealed

the infiltration of more CD8+ cells than CD4+ cells in this target tissue (Erf *et al.*, 1995;

Shresta *et al.*, 1997; Shi and Erf, 2012) whereby CD8+ T cells rather than CD4+ T cells

were located in close proximity association with apoptotic melanocytes in SL feathers

(Wang and Erf, 2004). Recently, it was shown that the elevated leukocyte infiltration in

early and active SLV was accompanied by increased levels of cytokine expression,

especially in IFN-γ, IL-10 and IL-21, suggesting a Th-1 polarized autoimmune disease

(Shi and Erf, 2012).

The presence of melanocyte-specific autoantibodies in serum from SL chickens with

SLV is also well established (Austin *et al.*, 1992, Shi and Erf, 2012). Previous studies have

shown that TYR is a principal autoantigen in human autoimmune vitiligo (Song *et al.*,

1994). Additionally, vitiliginous SL chickens were found to have melanocyte-specific

autoantibodies with demonstrated specificity to tyrosinase related protein (TRP) 1(Austin

and Boissy, 1995; Searle *et al.*, 1993) and heat-shock proteins (HSP) 90 (Erf, 2008).

Moreover, these antibodies also bind to chicken as well as human melanocytes (Austin

and Boissy, 1995). A role of B cells in SLV is also suggested by levels of elevated B cell

infiltration in the pulp of growing feathers from SL chicken with active SLV as compared

to SL chickens that never developed vitiligo (Shi and Erf, 2012).

Herpesvirus of turkey (HVT)

Expression of melanocyte-specific autoimmunity in SLV is greatly augmented in the presence of environmental factors, such as live herpesvirus of turkey (HVT), a routine live virus administration at hatch to protect poultry from lymphoma causing Marek's disease virus (Erf and Wang, 2001).

To date, HVT has been well established as a reliable trigger of the expression of SLV in susceptible SL chickens. HVT is an alpha-herpesvirus commonly used in commercial chicken production as a vaccine to protect chickens from Marek's disease (T cell lymphoma) caused by serotype 1 (oncogenic) Marek's disease viruses (MDV-1). HVT is a non-oncogenic serotype 3 MDV isolated from turkeys. Like all MDV serotypes it exhibits strong tropism for feather follicles, where it infects the feather follicle epithelium (Holland *et al.*, 1998). There were only 10% of non-vaccinated SL chicks raised at biosecurity level 2 that developed vitiligo by 20 weeks of age. This is a dramatic contrast to the greater than 90% incidence of vitiligo observed in SL chickens derived from the same breeder flock and reared in a conventional poultry house. Careful studies revealed that live HVT administration appeared to be an important factor in the expression of SLV (Erf *et al.*, 2001; Erf and Wang, 2001). Moreover, killed HVT had no effect on the expression of SLV, suggesting that an active infection by HVT is needed to trigger SLV development. Other live virus vaccines administered at hatch, such as Newcastle disease virus (NDV) or infectious bronchitis virus (IBV) did not trigger SLV, suggesting that viral infection and associated anti-viral immune activity, as such, are not responsible for triggering the

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expression of SLV (Erf, 2008). Unlike HVT, NDV and IBV do not translocate to the feather; hence, the presence of HVT in the feather where melanocytes are located may be a key to its effect on SLV expression (Holland *et al.*, 1998). In addition, recent studies Marek's disease virus (MDV) serotype 1 (MDV1), MDV2, and MDV3 (HVT) revealed that all three serotypes of MDV are similarly effective in triggering SLV expression in susceptible SL chickens (Erf, personal communication). Lastly, Bateman (2009) showed that there is no difference in viral load in the feathers from HVT-vaccinated SL, BL, and LBL chickens during the 6 weeks sampling period following HVT vaccination.

Oxidative stress overview

Free radicals, such as superoxide anions, hydrogen peroxide, *etc*, are generated during normal metabolic activities. The production of these free radicals sometimes provides advantageous biological effects. The purpose of free radicals during the inflammatory process is to kill or destroy invading microorganisms (e.g., respiratory burst in activated phagocytes) and/or to degrade damaged tissue structures (Fantone and Ward, 1982). Free radicals have been shown to play a role in numerous physiological functions, such as activation of guanylate cyclase (Mittal and Murad, 1977), control of smooth muscle relaxation (Ignarro and Kadowitz, 1985), and activation of transcription factor NF- κB, *etc*. Uncontrolled release of radicals is involved in the pathogenesis of a number of human skin disorders, including vitiligo. Under normal circumstances, the body is able to neutralize

excessive free radicals and prevent them from causing damage. The actual concentration of free radicals is determined by the balance between their rate of production and rate of degradation by antioxidants (Evans and Halliwell, 2001). Antioxidants as a balanced system are used to protect tissues from damage by free radicals that either were produced physiologically or as a response to inflammation, infection, or disease. An imbalance in the radical production-degradation system would result in an increased potential for oxidative stress and bio-molecular damage that is the direct result of free radicals (Halliwell and Whiteman, 2004).

Recent studies also suggest a role of the antioxidant systems and oxidative stress in the pathophysiology of vitiligo (Manga *et al.,* 2006). While the exact role of oxidative stress in vitiligo is unclear, oxidative stress within SL melanocytes has been proposed to be an extensive threat based upon their local environment. The feather barb ridge, where feather melanocytes are located, is poorly vascularized and the limited circulation contributes to the build-up of the free radicals in the barb ridges (Bowers *et al.,* 1999). In humans a heightened sensitivity of vitiligo-melanocytes to oxidative stress was described and found to be due to an imbalance and/or a deficiency in their antioxidant system (Manga, 2006). Similarly, comparisons of SL, BL and LBL embryo-derived melanocyte cultures revealed heightened production of reactive oxygen species (ROS) and lipid peroxidation in SL melanocytes compared to control melanocytes (Erf *et al.,* 2005).

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There are both non-enzymatic and enzymatic reactions of antioxidants which can deactivate free radicals. Non-enzymatic antioxidants include retinoic acid (vitamin A), α-tocopherol (vitamin E), ascorbate (vitamin C), uric acid, and glutathione (GSH). Glutathione, also called L-γ-glutamyl-L-cysteinylglycine, is a ubiquitious intracellular tri-peptide that plays an active role in various cellular functions, including breakdown of ROS (Deneke and Fanburg, 1989). Glutathione is found in two forms, reduced glutathione and oxidized glutathione (GSSG). Reduced GSH neutralizes various ROS, including oxygen free radicals ($O_2{}^-$), hydrogen peroxide (H_2O_2), and hydroxyl radicals (OH^-). These two forms (GSH and GSSG) are recycled between one another through two enzymes: GSH peroxidase and GSH reductase. GSH peroxidase reacts to form GSSG by donating hydrogen to the sulfur groups of two GSH molecules. GSSH can also be converted back to its reduced form via GSH reductase, which reacts with two β–nicotinamide adenine dinucleotide phosphate (NADPH) molecules. In summary, the reduction of GSSG to GSH is critical for cellular function. Increased levels of GSSH can both oxidize cellular proteins and lower the antioxidant capacity (Bottje and Wideman, 1995). Enzymatic antioxidants include catalase, superoxide dismutase and the previously mentioned GSH reductase and peroxidase. Catalase is a homotetramer with ferriheme at its active site, which can reduce hydrogen peroxide by oxidation to produce oxygen and water molecules.

4-tertiary butyl phenol (4TBP) triggers oxidative stress and is toxic to melanocytes

In humans, phenolic derivatives, such as 4-TBP, have been shown to trigger vitiligo in susceptible individuals. Exposure to phenolic compounds, specifically 4-TBP, is widespread in both industrial workers and consumers through contact with synthetic leather, plastic, glues, and a wide variety of germicidal phenolic detergents. Yang *et al*. (1999) reported that occupational vitiligo developed in workers at a 4-TBP manufacturing factory. The occurrence of vitiligo induced by 4-TBP has been shown to be related to the duration and intensity of exposure.

Previous studies have shown that 4-TBP is involved in predisposing melanocytes to the loss of melanin pigment. First of all, 4-TBP is structurally similar to tyrosine, the initial substrate of TYR in melanin synthesis which was shown to be oxidized by TYR. The oxidation of 4-TBP yields a toxic intermediate quinone, which further reacts with cysteine or GSH to produce 4-tert-butyl-6-S-cysteinylcatechol (cys-TBC) and 4-tert-butyl-6-S-glutathionylcatechol (GS-TBC), respectively. Reduced GSH, an antioxidant, helps protect cells from reactive oxygen species (ROS) such as free radicals and peroxides. Thus, the loss of reduced GSH results in a weakened ability to overcome oxidative stress. In general, 4-TBP, a competitive inhibitor of TYR, is metabolized in melanocytes and is able to cause ROS damage to these cells. Moreover, intermediate semiquinones which are generated during 4-TBP's oxidation by TYR facilitate respiratory electron-transport reactions. Approximately 90% of ROS production can be traced back to respiratory chain from normal cell metabolism in the mitochondria. The respiratory

chain is a major site of premature electron leakage to oxygen, thus being a major site of superoxide production and driver of oxidative stress. In summary, 4-TBP induced ROS accumulation may rely on either increase of ROS production during futile respiratory reaction or decrease the ability of anti-oxidative enzymes such as depletion of GSH.

Phenolic compounds are believed to trigger vitiligo by killing the melanocytes in the skin through oxidative stress and causing an overload of hydrogen peroxide in the skin. The expression of most members of the HSP70 family is strongly induced by 4-TBP. HSP70 in turn induces membrane tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) expression in human melanocytes to activate dendritic cell activity towards melanocytes (Kroll *et al.,* 2005). Moreover, Boissy and Manga (2004) showed that human melanocytes were susceptible to apoptosis following exposure to 4-TBP. These observations suggest that apoptosis may be another mechanism by which 4-TBP exerts its toxic effects on melanocytes.

The structure of 4-TBP is similar to tyrosine, the initial substrate of TYR in melanin synthesis, ironically, expression levels of TYR do not correlate with sensitivity to 4-TBP-induced apoptosis, suggesting an alternative enzymatic mediator may be more critical. MITF stimulates melanin synthesis by up-regulating expression of melanogenic enzymes such as TRP1. Elevated expression of TRP1, a key enzyme in melanin production, significantly increased sensitivity to 4-TBP. Melanocytes that express

functional TRP1 were significantly more sensitive to 4-TBP than TRP1-null cells. Thus,

normal melanocytes respond to 4-TBP by reducing expression of MITF and TRP1.

However, the role for TRP1 and MITF in the effects induced by 4-TBP in melanocytes

from SL, BL and LBL chickens is unknown.

In summary, 4-TBP competitively inhibits TYR function and triggers accumulation of

ROS in melanocytes by inhibition of GSH activity. However, it is unclear whether ROS

accumulation during 4-TBP exposure is due to increased production from the respiratory

chain and/or inhibition of anti-oxidative enzymes. Additionally, gene expression profiles

related to melanogenesis and apoptosis with respect to 4-TBP treatment are still unknown.

MITF overview

Various environmental and genetic factors affect the development and progression of

vitiligo. Vitiligo development is initially triggered by environmental factors, and then

genetic/epigenetic alterations occur in the skin. This first trigger alters the conditions of

numerous genes and proteins, and the altered gene expression, and protein stability result

in vitiligo progression. MITF is discussed as the master gene for melanocytic survival and

a key transcription factor regulating the expression of TYR, TRP1 and TRP2.

<u>Introduction</u>

MITF is a basic helix-loop leucine zipper protein which plays a predominant role in both

melanocyte development and regulation of melanogenesis (Hughes *et al.,* 1993). It is not

only expressed in melanocytes, but also in retinal pigmented epithelium, mast-cells,

osteoclasts, and melanoma (Kumasaka *et al.,* 2005; Steingrimsson *et al.,* 2004). MITF

protein forms dimers and binds to specific consensus DNA sequences, such as M-box

promoter elements, in promoter regions of various target genes to regulate proliferation,

melanogenesis and tumorigenesis (Goding, 2000).

Regulation of MITF expression and activity

Several transcription factors directly control MITF gene transcription to regulate

melanocyte development. The first and most important transcriptional factor in

melanogensis is cAMP response element binding protein (CREB). Activation of MC1R

by binding of α-MSH induces cAMP production via activation of adenylate cyclase and

phosphorylates cAMP. Second messager cAMP can activate the protein kinase A, which in

turn response to CREB. Phosphorylated CREB directly binds to the MITF promoter

region and stimulates MITF transcription (Bertolotto *et al*., 1998). Sry-related HMG box

10 (SOX10) is a high-mobility group (HMG) transcription factor whose presence slightly

correlated with melanocyte development and melanogenesis (Khong and Rosenberg,

2002). SOX10 can positively regulate MITF expression by directly binding to MITF

promoter regions in mice and humans (Bondurand *et al*., 2000). There are other

transcriptional factors such as paired box 3 (PAX3), signal transducer and activator of

transcription 3 (STAT3), lymphoid-enhancing factor-1 (LEF-1), immunoglobulin transcription factor-2 (ITF2) and forkhead-box transcription factor D3 (FOXD3), which have been reported to induce and/or reduce MITF gene expression to control melanocyte proliferation (Ping *et al.,* 2011). Furthermore, several factors can modify the MITF protein after translation. P38 stress-activated kinase induces the phosphorylation at Ser 301 of MITF, and glycogen synthase kinase 3β (GSK-3β) induces Ser 298 phosphorylation, resulting in stimulation of MITF transcriptional activity (Takeda *et al*., 2000; Mansky *et al*., 2002).

Target of MITF

MITF is well known as a master regulator of melanin synthesis. Melanin pigment is synthesized from tyrosine via an enzymatic and non-enzymatic process. The enzymatic process is catalyzed by TYR, TRP1 and TRP2, all of which are all regulated by MITF.

Another important gene regulated by MITF is an anti-apoptotic gene B-cell leukemia/lymphoma 2 (BCL2) which is widely expressed in human melanomas (Selzer *et al*., 1998). Oxidative stress from environmental factors, such as UV radiation, causes DNA damage and apoptosis. BCL2 has been identified as an MITF target gene and has been shown to be partially important for cell rescue from oxidative stress-induced apoptosis in MITF-depleted cells (Yajima *et al*., 2011).

In addition to the MITF requirement for development of neural crest-derived melanocytes, MITF also has been shown to be involved in cellular events for the initiation of tumorigenesis. MITF directly binds to the promoter regions of P16$^{\text{Ink4a}}$, P21$^{\text{Cip1}}$, and Cyclin D1 and induces their transcription (Carreira *et al*., 2005; Loercher *et al*., 2005). These genes are well-known to be related to melanoma formation.

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CHAPTER II

A ROLE OF MITF AND TRP1 EXPRESSION IN THE HEIGHTENED 4-TERTIARY BUTYL PHENOL SENSITIVITY OF MELANOCYTE CULTURES FROM AUTOIMMUNE VITILIGO-PRONE SMYTH LINE CHICKENS

A role of MITF and TRP1 expression in the heightened 4-tertiary butyl phenol sensitivity of melanocyte cultures from autoimmune vitiligo-prone Smyth line chickens

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Abbreviations:

4-TBP: 4-tertiary butyl phenol; BL: Brown line; GSH: glutathione; HVT: turkey herpesvirus; LBL: Light Brown Leghorn; MC: melanocyte; MITF: microphthalmia-associated transcription factor; qRT-PCR: quantitative reverse

transcription-polymerase chain reaction; SL: Smyth line; SLV: Smyth line vitiligo; SL-G:

SL chickens with dermal (grey) pigmentation of the shanks; TRP: tyrosinase related

protein; TYR: tyrosinase; ROS: reactive oxygen species; α-MSH:

α-melanocyte-stimulating hormone; BCL2: B-cell lymphoma 2; BAK1: BCL2

homologous antagonist/killer; HSP: heat shock protein;

Abstract

The Smyth line (SL) chicken model for vitiligo exhibits spontaneous, autoimmune loss of

MCs in growing feathers. It is an excellent animal model for human autoimmune vitiligo.

Skin exposure to phenolic derivatives, such as 4-tertiary butyl phenol (4-TBP) can trigger

vitiligo in susceptible individuals by inducing oxidative stress that is more readily

overcome by melanocytes (MCs) from normally pigmented individuals than from

individuals with vitiligo (Manga *et al*., 2006). MC degeneration and/or destruction in

vitiligo may be enhanced or initiated by oxidative stress. The mechanism of 4-TBP on MC

loss in vitiligo is currently unclear. In this study, the generation of reactive oxygen species

(ROS) in response to 4-TBP exposure of MC-containing feather tissue (MC-FT, newest 3

mm growth of growing feathers) and MCs derived from growing feathers (MC-F) or from

the neural crest of 72 h embryos (MC-E) was examined. MC-FT were collected from SL

chickens (80 - 95% vitiligo incidence), SL chickens with dermal (grey) pigmentation of the

shanks (SL-G, < 5% vitiligo), Brown line chickens (BL, parental control, < 1% vitiligo)

and Light Brown Leghorn (LBL, vitiligo resistant control) chickens at 1, 4, 7, and 12 weeks

of age (prior to and after SL vitiligo (SLV) development). To examine the influence of

turkey herpesvirus HVT administration at hatch on the response of MC to 4-TBP, the ROS

generation in MC-FT was measured from chickens with and without HVT vaccination.

Independent of HVT treatment and age, 4-TBP induced more oxidative stress (P < 0.05) in

MC-FT of SL at 1 h co-culture with 4-TBP than SL-G, BL, and LBL chickens. Similar

results were also observed in SL, SL-G, and BL MC-F and MC-E cultures exposed to

4-TBP. As expected, ROS production was not observed in white SLV MC-FT (no MCs)

stimulated with 4-TBP. Elevated ROS production in response to 4-TBP in SL MC-F and

MC-E was accompanied by increased RNA expression levels of tyrosinase related

protein-1 (TRP1) post 1 h culture with 4-TBP, whereas normal MCs from SL-G and BL

chicken responded to 4-TBP by reducing expression of microphthalmia-associated

transcription factor (MITF) and TRP1 to protect MC from further oxidative stress. By

comparison with gene expression in MC-FT, only the relative MITF expression was

reduced in SL-G, BL, and LBL chickens. Taken together, these data suggested a

heightened sensitivity of MCs to 4-TBP in vitiligo-prone SL chickens. The reduced ability

to withstand oxidative stress in SL chickens may due to a disruption in MITF regulation.

Introduction

Vitiligo is an acquired pigmentary disorder characterized by a loss of epidermal

melanocytes (MCs) and depigmentation of the skin (Lerner and Nordlund, 1978). This

disease is found in 0.1 - 2 % of the world's human population (Lerner, 1971). MC death

results in the loss of pigment protection of the skin from solar damage. Although the

etiopathological mechanisms of this disease are not well understood, it is known that many

factors contribute to vitiligo susceptibility, including genetic factors, environmental

components and immune system involvements (Reza, 2011). Exposure to specific

chemotoxins, which include phenol derivatives such as 4-tertiary butyl phenol (4-TBP),

appears to be an environmental trigger for human vitiligo (Boissy and Manga, 2004),

altering MC gene expression and protein stability and activity, and resulting in vitiligo

expression and progression in susceptible individuals (James *et al.,* 2006).

Manga *et al.* (2006) demonstrated that 4-TBP induced oxidative stress in cultured MCs

could be more readily overcome by MCs from normally pigmented individuals than from

individuals with vitiligo. 4-TBP is structurally similar to tyrosine, the initial substrate of

tyrosinase (TYR) in melanin synthesis, and like tyrosine, has been shown to be oxidized by

tyrosinase. The oxidation of 4-TBP yields toxic intermediate semiquinones which facilitate

respiratory electron-transport reaction, a major site of premature electron leakage to

oxygen (Kirsten *et al.,* 2000). Thus, the oxidation of 4-TBP in MCs results in the

generation of reactive oxygen species (ROS). In addition, intermediate quinine can further

react with cysteine or glutathione (GSH) to produce 4-tert-butyl-6-S-cysteinylcatechol and

4-tert-butyl-6-S-glutathionylcatechol, respectively (Kirsten *et al.,* 2000). The loss of GSH,

an antioxidant protecting cells from ROS, results in the inability of MCs to overcome the

oxidative stress induced by exposure to 4-TBP. While the exact role of oxidative stress in

vitiligo is unclear, it has been shown to play a role in the development of the disorder

and/or the progressive loss of MCs.

Previously, it has been shown that MCs cultured in the presence of 4-TBP experience

dose-dependent apoptosis and the percentage of cells that die following exposure to

4-TBP can be significantly reduced by excluding α-melanocyte-stimulating hormone

(α-MSH; Yang *et al*., 1999). α-MSH is a key physiologic inducer of melanogenesis. The

biological function of α-MSH is mediated through its binding to MC1R, the only

melanocortin receptor expressed in MCs (Pavey and Gabrielli, 2002). MC1R is a G

protein-coupled receptor that activates adenylate cyclase. Therefore, the increased

intracellular concentration of cAMP mediates the expression of the MC-specific isoform of

the microphthalmia-associated transcription factor (MITF; Widlund and Fisher, 2003).

MITF in turn activates the expression of many downstream genes, including TYR,

tyrosinase-related protein (TRP) 1 and TRP2, resulting in melanogenesis.

Although MITF is an important factor in MC development, survival, and differentiation,

recent studies have shown that MITF is the substrate of caspases and plays a key role in the apoptosis of melanoma cells (Larribere *et al.*, 2005). B-cell lymphoma 2 (BCL2), an anti-apoptotic factor, has been shown to directly interact with MITF and MITF regulates endogenous levels of BCL2 in MCs (Larribere *et al.*, 2005). BCL2 expression is very critical for MC survival. Further studies also showed that levels of heat shock protein (HSP) 70 were elevated in MCs in response to 4-TBP exposure. Elevated HSP70 subsequently activated dendritic cell (DC) effector functions towards stressed MCs (Kroll *et al.*, 2005). Since MCs are preferentially targeted in vitiligo, we considered MITF and its downstream regulated genes TYR, TRP1 and TRP2, apoptosis related genes BCL2 and BCL2 homologous antagonist/killer (BAK1), and HSP70 as ideal candidates to examine MC stress induced by 4-TBP in the vitiligo prone Smyth line (SL) chickens.

The SL of chickens is a well-established animal model for the study of spontaneous development of autoimmune vitiligo, displaying all the characteristics of the human disorder (Smyth, 1981 and 1989; Wick *et al.*, 2006; Erf, 2010). Like human vitiligo, SL vitiligo (SLV) is a multifactorial disorder involving genetic susceptibility, environmental triggers, and cellular as well as humoral immune system components in the progressive loss of MCs. Under conventional rearing conditions including routine administration of turkey herpesvirus (HVT) at hatch, approximately 80 - 95 % of SL chickens develop SLV between 6 - 20 weeks of age. Without the HVT environmental trigger, the incidence of SLV is reduced to < 20% (Erf and Wang, 2001). Additionally, SL chickens that carry the *Id*

sex-lined recessive dermal pigmentation gene, resulting in grey-pigmented shanks (SL-G), very rarely develop SLV (< 5% incidence, Pillai, 1998). SL and SL-G chickens together with MHC-matched the Brown line (BL) parental control (< 1% vitiligo) and the Light Brown Leghorn (LBL; vitiligo-resistant) control chickens constitute an excellent animal model to study vitiligo. Another excellent attribute of the SL chicken model for vitiligo is the location of MCs in the growing feathers, rather than the skin (Figure 1). Growing feathers are easily and repeatedly accessible for sampling, providing unique opportunity to examine the evolving lesion prior to and throughout the development of SLV in the same individuals. Additionally, MC cultures can be established from growing feathers as well as from the neural crest of 72 h embryos for *in vitro* study of MCs that differentiated in different environments.

In this study, we explore the hypothesis that MCs from vitiligo-prone SL chickens are more sensitive to 4-TBP treatment than MCs from SL-G, BL and LBL chickens. To address this hypothesis, the objectives of the current study were to examine the production of ROS and monitor gene-expression of HSP70 and melanogenesis- and apoptosis-related proteins in response to 4-TBP exposure of MC-containing feather tissue (MC-FT, newest 3 mm growth of growing feathers) and MCs derived from growing feathers (MC-F) or from the neural crest of 72 h embryos (MC-E). Characterization of these pathways will shed more light on the underlying etiology of MC loss in response to 4-TBP.

Materials and Methods

Animals

Three lines of MHC-matched ($B^{101/101}$) chickens were used for this study: 1) the

vitiligo-prone SL chickens which exhibits an 80 - 95 % incidence of SLV by 20 weeks of

age under conventional rearing conditions and SL chickens with grey shanks (SL-G). Since

the establishment of SL breeder populations at the University of Arkansas, none of the

$B^{101/101}$ SL-G chickens (id^+ females and id^+/id^+ males) have been observed to develop SLV

by 20 weeks of age (natural end-point of SLV development in adult chickens), 2) the

parental BL control chickens (< 1% incidence of vitiligo), and 3) the vitiligo-resistant LBL

control line of chickens (no incidence of vitiligo; Erf, 2010). On the day of hatch, chicks

from each pedigreed family were tagged using a color coded numbering system and were

randomly assigned to two groups. Chicks in Group 1were vaccinated for Marek's disease

by s.c. injection with cell-free lyophilized live HVT vaccine (0.2 mL, est. 8000 pfu)

following the manufacturer's instructions (MD-Vac CFL serotype 3, live, Fort Dodge

Animal Health, Fort Dodge, Iowa) and reared in floor pens on wood-shavings litter on the

conventional Arkansas Experiment Station, University of Arkansas Poultry Farm in

Fayetteville, Arkansas. Siblings of Group 1 chicks were not vaccinated with HVT (Group 2)

and reared in floor pens on wood-shavings litter in a HEPA-filtered room at the Arkansas

University of Arkansas, Division of Agriculture, Poultry Health Laboratory. Under these

conditions, the incidence of SLV in HVT-negative chickens has been repeatedly shown to be less than 10% (Erf, personal communication). All chicks were shown the location of food and water and the well-being of the chicks were checked daily. Food and water were available *ad libitum* and standard temperature and lighting protocols were followed. All procedures for this study were approved by the Institutional Animal Care and Use Committee (IACUC) Protocol #08011.

Establishment of MC-F cultures from feathers *in vitro*

MC-F cultures were prepared from each 12-week-old 10 SL, 6 SL-G and 6 BL growing feathers following a modified procedure (Boissy *et al.,* 1987). Briefly, MCs isolated from feathers were incubated at $40.5^{o}C$ in a humidified incubator with 5 % CO_2 in complete Ham's F12 medium supplemented with 10 % BSA, 1% glutamine-penicillin-streptomycin, 1% fungizone and 85 nM TPA. The culture medium was changed every 3 to 4 days. When the cells reached 50 - 70% confluence they were subcultured. Cultures at passage 4 to 5 were used for this study. Twenty-four hours before treatment, all MC-F cultures were cultured in MC-F culture medium without TPA and phenol red (RPMI 1640 medium without phenol red containing 10% FBS, 1% L-glutamine/penicillin/streptomycin, 1% fungizone, and 5% µg/mL insulin). These cultured MC-F were used for ROS generation and gene expression analysis to examine the effect of 4-TBP treatment. Specifically, 100 µL of 0 µM, 400 µM and 2000 µM 4-TBP was added to each well containing 1×10^{4}

MC/well (triplicate cultures; final 4-TBP concentration of 0 µM, 200 µM and/or 1000 µM) in a 96-well flat-bottom tissue culture microtiter plate. Cultures were then incubated for 1 h at 40.5°C humidified incubator with 5% CO_2 before subjected to ROS analysis. For gene expression analysis, 1 x 10^6 MC/well were cultured in triplicates for 1 h with 0 µM or 200 µM 4-TBP in a 24-well flat-bottom tissue culture plate as described above.

Establishment of MC-E cultures from embryos *in vitro*

A modified method based on Boissy and Halaban (1985) was used to establish MC-E cultures from the neural crest of embryos. Briefly, fertilized 10 SL, 6 SL-G and 6 BL eggs from different hens were incubated at 40.5 °C for 72 h. Following careful removal of the embryo, the posterior portion of the embryonic trunk containing the neural crest was dissected and trimmed to obtain the neural crest. The neural crest was incubated at 40.5°C in a humidified incubator with 5% CO_2 in embryo-MC culture medium [10% FBS, 5% NuSerum (Becton Dickson Labware, Bedford, MA, USA), 1% L-glutamine/penicillin/streptomycin, 1% fungizone, 80 nM 12-O-tetradecanoylphorbol-l3-acetate (TPA) and 40 ng/mL cholera toxin in Ham's F10 medium]. After 72 h of incubation, the neural crest was removed and discarded, and the attached MCs remained in the culture plate. The culture medium was changed every 3 to 4 days. When the cells reached 50 - 70% confluence they were subcultured. Cultures at passage 4 to 5 were used for this study. Twenty-four hours before treatment, MC-E cultures

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were cultured in MC-E culture medium without TPA, cholera toxin, and phenol red (10% FBS, 5% NuSerum (Becton Dickson Labware, Bedford, MA, USA), 1% L-glutamine/penicillin/streptomycin, 1% fungizone in 1640 medium). These cultured MC-E were used in ROS generation assays and for gene expression analysis to examine the effect of 4-TBP treatment as described for MC-F above.

Establishment of MC-FT cultured with 4-TBP *in vitro*

Ten SL, 6 SL-G, 10 BL, and 10 LBL HVT-vaccinated chickens (Group 1) and the same number of siblings from Group 1 (not HVT-vaccinated; Group 2) were used in this study. For each chick, 7 growing feathers were collected from each wing at 1 week of age and from each breast track when the chickens were 4-, 7- and 12-weeks of age. The feather tips (Figure 1) from each bird were stored separately in PRMI 1640 medium without phenol red on ice until use. For tissue culture, the bottom 3 mm newest melanocyte-containing growth (MC-FT) (Figure 1) of each feather tip was cut, weighed and cultured in complete-culture medium [RPMI 1640 without phenol red containing 10% FBS, 1% L-glutamine/penicillin/streptomycin, 1% fungizone, and 5% μg/mL insulin] for 1 h at 40.5°C in a humidified incubator with 5% CO_2. These cultured MC-FT were used for ROS generation and gene expression analysis to examine the effect of 4-TBP treatment. Specifically, 7 MC-FT from each bird were used for ROS examination. For this, 1 MC-FT per well was randomly assigned to wells in 96-well flat-bottom tissue culture microtiter

plates as follows: 3 MC-FT were cultured in medium without 4-TBP stimulation (unstimulated control group), 3 MC-FT were cultured in medium with 200 µM 4-TBP (treatment group), and 1 MC-FT was cultured in medium to determine background fluorescence in the ROS assay. For use in gene-expression analysis 6 MC-FT were cultured as described above (3 MC-FT without 4-TBP stimulation and 3 MC-FT with 200 µM 4-TBP treatment).

Assay for ROS generation

The MC-FT, MC-F, and MC-E cultures were incubated with 2',7'-dichlorofluorescin-diacetate (DCF-DA; D-6883, Sigma, St. Louis, MO, USA), and ROS generation was measured using a kinetic read method (every 10 minutes for 90 minutes) on a micro-plate reader with an excitation wavelength of 485 nm and an emission wavelength of 530 nm. The slope of ROS generation for each sample was calculated and expressed as fluorescence units/min (f.u./min). For MC-F and MC-E cultures, the slope (f.u./min) of ROS generation was determined for each 4-TBP treatment (0, 200, and 1000 µM in MC-F cultures; 0 and 200 µM in MC-E cultures). For the analysis of ROS generation in the MC-FT, the slope of ROS generation over time (f.u./min) from untreated samples was subtracted from the slope (f.u./min) of 4-TBP-treated samples before data analysis of 4-TBP induced ROS generation.

RNA extraction, quantification, and cDNA synthesis

Total RNA was isolated from collected MC-E, MC-F, and MC-FT cultures using the

RNeasy® Mini Kit (Qiagen, Valencia, CA) and following the protocol for "Purification of

Total RNA from Animal Tissues" with modifications. MC-FT were homogenized by

Tissue Tearor (BioSpec Products, Bartlesville, OK, Model: 985370-395) provided in the

kit, whereas, MC culture were homogenized in lysis buffer by vortex. Total RNA was

isolated from homogenates with on-column DNA digestion (Qiagen). RNA was eluted in

30 µL RNase-, DNase-free water and stored at -80°C until use. RNA quality and quantity

were determined by Experion^{TM} RNA StandardSens Reagents and Supplies (Bio-Rad

Laboratories, Hercules, CA) following the manufacturer's instructions. RNA (175 ng) was

reverse transcribed into cDNA by High Capacity cDNA Reverse Transcription Kit

(Applied Biosystems, Foster City, CA) in a 10 µL reaction volume according to the

manufacturer's instruction. A Biometra® personal cycler (Biomedizinische Analytik

GmbH, Germany) was programmed as follows: 25ºC for 10 min, 37ºC for 2 h, 85ºC for 5

min and 4ºC holding temperature. Then cDNA was stored at -80ºC until further analysis.

Taqman® quantitative PCR (qPCR)

Primer and probes are shown in Table 1. qPCR was performed in 25 µL reaction volume on

an ABI PRISM 7300 sequence detection system (Applied Biosystems, Foster City, CA)

using the Universal PCR Master Mix (Applied Biosystems, Foster City, CA) and following

the manufacturer's instruction. In each plate, a calibrator sample (a pool of cDNA from

age-matched BL birds from control group), a non-template control, and sample cDNA were included. For each sample 28S ribosomal RNA served as the endogenous control gene. Cycling profiles used were: 94ºC for 15 min, and 50 cycles of denaturation at 94ºC for 60 s, and annealing/extension at 60ºC for 60 s. The relative gene expression was determined by the delta delta Ct *(ΔΔCt)* method (Wong and Medrano, 2005).

Statistical Analysis Statistical Analysis

For *in vitro* MC-FT, two-way analysis of variance (ANOVA; JMP Pro 9, JMP Software Inc., Cary, NC) using the full factorial model procedure and Fisher's LSD multiple means comparisons was carried out to investigate the main effect of type of chicken (SL, SL-G, BL, and LBL), the main effect of HVT (HVT and non-HVT group) and their interaction on the generation ROS and expression of the various target genes examined. In the absence of line of chickens by HVT interaction $(P > 0.1)$, the data were pooled across HVT and the main effect of type of chicken was presented. Two-way and/or one-way ANOVA was also used to analyze MC-F and MC-E ROS generation and gene expression for each line with or without 4-TBP treatment. In the presence of significant main effect(s), as determined by ANOVA, Fisher's LSD multiple means comparison was used to determine differences among groups means. P values ≤ 0.05 were considered significant. Data were reported as means ± SEM.

Results

ROS generation in SL, SL-G, BL and LBL MC-containing feather tissue (MC-FT) cultures in response to 4-TBP treatment

To examine the effect of 4-TBP treatment on ROS generation, the slope of fluorescence (f.u./min) from untreated samples was subtracted from the 4-TBP treated samples. Two-way ANOVA revealed that ROS generation (f.u./min) in response to 4-TPB was not affected by HVT vaccination. Hence, for each line of chickens, data for HVT and no HVT groups were pooled to compare the effect of 4-TBP on MC-FT from the different lines of chickens at 1-, 4-, 7-, and 12-weeks of age. For each time point (1-, 4-, 7- and 12-weeks of age), multiple mean comparisons revealed that culture with 200 µM 4-TBP resulted in higher ($P < 0.05$) ROS generation in SL MC-FT compared to SL-G, BL, and LBL MC-FT cultures. ROS generation in response to 4-TBP of MC-FT from SL-G, BL, and LBL chickens was similar at all time points except at 4 weeks of age when BL MC-FT had higher ROS generation than SL-G and LBL (Figure 2B). Additionally, at 4 weeks, 4-TBP stimulated ROS generation in SL and BL MC-FT was higher than at any of the other time-points examined (Figure 2B). At 12 weeks of age several SL chickens had developed SLV. Data for depigmented (white) SLV MC-FT exposed to 4-TBP were placed in a separate group for the 12-week statistical analysis. Stimulated ROS generation in response to 4-TBP was not observed in these SLV MC-FT cultures (Figure 2D).

ROS generation in SL, SL-G, and BL feather-derived MC (MC-F) and

embryo-derived MC (MC-E) cultures in response to 4-TBP treatment

Two-way ANOVA examination of the effect of line and 4-TBP dose and their interaction revealed a significant ($P < 0.05$) effect of line of chicken, dose of 4-TBP, and no line by dose interactions on the ROS generation of MC-F in response to 4-TPB treatment. Specifically, without 4-TBP, SL and SL-G MC-F cultures had higher ROS generation than BL MC-F (Figure 3). Treatment with 200 µM 4-TBP resulted in increased ROS generation by SL but not SL-G and BL MC-F. Treatment of MC-F cultures with 1000 µM 4-TBP increased ROS generation in both SL and SL-G MC-F compared with no 4-TBP, whereby ROS generation levels where higher in SL MC-F than SL-G MC-F (Figure 3).

Culture of MC-E with 200 µM 4-TBP resulted in higher ($P < 0.05$) ROS generation in SL MC-E compared to SL-G and BL MC-E cultures, whereas, without 4-TBP, there was no difference among MC-E cultures from BL, SL-G, and SL (Figure 4).

Morphological characteristics of MC-F and MC-E cultured with and without 4-TBP

MC dendrite retraction and aggregation at 24 h post 200 µM 4-TBP treatment was observed in MC-F (Figure 5) and MC-E from SL chickens. Mass aggregation was also observed in MC-F from SL chickens 24 h post 1000 µM 4-TBP treatment (Figure 5), whereas, these 4-TBP induced alterations were less prominent in MC-F (Figure 5) and MC-E from BL and SL-G chickens.

Gene expression in SL, SL-G, BL and LBL MC-containing feather tissue (MC-FT) cultures in response to 1 h treatment with 4-TBP

Two-way ANOVA conducted at each age (1-, 4-, 7- and 12-weeks) to examine the effect of line of chicken, HVT, and line by HVT interaction on the gene-expression of various target genes following 1 h exposure of tissues at 0 and 200 µM 4-TPB levels, revealed a significant ($P < 0.05$) effect of line of chicken and no effect of HVT or line by HVT interactions. Hence, for each line of chicken, data for HVT and no HVT groups were pooled for one-way ANOVA to examine the effect of line, age and line by age interactions for MC-FT cultured with 200 µM or without 4-TBP. Analysis revealed no line by age interactions for any of the target genes analyzed. Hence, multiple means comparisons were conducted comparing the line main effect means for each target gene expression (Figures 6-8 and Table 2-3) for each 4-TBP treatment group (0 and 200 µM).

Independent of age of chicken, there were no differences in relative MITF gene expression in MC-FT from SL, SL-G, BL, and LBL cultured without 4-TBP for 1 h. Compared to no 4-TBP controls, treatment with 200 µM 4-TBP for 1 h resulted in lower MITF-expression in SL-G, BL and LBL MC-FT, whereas MITF expression in SL MC-FT was not affected by 4-TBP treatment (Figure 6). The relative expression of TYR, TRP1, TRP2, BCL2, BAK1 and HSP70 expression in MC-FT from the different lines was not affected by 1 h culture with 4-TBP, although TYR expression in LBL MC-FT cultures was higher than those from

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the other line of chickens (Table 2).

Gene expression in SL, SL-G, and BL feather-derived (MC-F) and embryo-derived (MC-E) MC cultures in response to 1 h treatment with 4-TBP

One way ANOVA examining the effect of line on the gene-expression of various targets by MC-F and MC-E cultured with and without 200 µM 4-TBP for 1 h revealed a significant (P < 0.05) effect of line of chicken for both MC-F and MC-E. MC-F from SL-G and BL chickens treated with 200 µM 4-TBP for 1 h had lower relative gene-expression levels of MITF and TRP1 than those without 4-TBP treatment. By contrast, MITF and TRP1 expression in SL MC-F was not affected by 4-TBP treatment for 1 h (Figure 7).

Similarly, BL MC-E treated with 200 µM 4-TBP showed reduced relative expression of MITF and TRP1 compared to no 4-TBP treatment. MITF expression in SL and SL-G MC-E was not significantly affected by 4-TBP treatment (Fig. 8A). However, compared to no 4-TBP cultures, expression of TRP1 was reduced in SL-G MC-E and increased in SL MC-E (Figure 8B). For TYR, TRP2, BCL2, BAK1 and HSP70 there were no statistical differences in gene-expression between SL, SL-G and BL MC-F and MC-E cultures with and without 4-TBP treatment (Table 3-4).

Table 1. Primer[1] and probe[1,2] sequences used to examine the relative expression of target genes

Target	Primer/probe	Sequences (5' to 3')	Accession NO.
28S	Forward	GGCGAAGCCAGAGGAAACT	X59733

	Reverse	GACGACCGATTTGCACGTC	
	Probe	AGGACCGCTACGGACCTCCACCA	
TYR	Forward	ATAATGCCCTTCACATCTACATGAAT	NM_204160.1
	Reverse	GCTCAAAAATGCTGTCAACAAATG	
	Probe	CTCAATGTCCCAAGTACAAGGCTCTGCG	
TRP1	Forward	GGAACCATTTGTAACAGCACTGAAG	NM_205045.1
	Reverse	CCATAGGCCGTGCAACATTT	
	Probe	CGGTCCCATCCGTAGAAATCCTGCTG	
TRP2	Forward	CCTTTCCCGGCATGAGTTT	NM_204935.1
	Reverse	AGCGCATTCCTGAAGCTGAA	
	Probe	CAGTCCTCCGTTTTTCCGCAATTCCA	
MITF	Forward	AAGAACTGGGCACCTTGATACC	NM_205029.1
	Reverse	GATGTAGTCCACTGATGCTTTTAGAATAG	
	Probe	AAATCAAACGACCCGGATATGCGCTG	
HSP70	Forward	GACTGCTCTCATCAAGCGTAACA	FJ217667.1
	Reverse	TCATACACCTGGACGAGGACACT	
	Probe	CACCATTCCCACCAAACAAACACAGACC	
BCL2	Forward	GCGTCAACCGGGAGATGT	NM_205339.1
	Reverse	GGTTCAGGTACTCGGTCATCCA	
	Probe	CCGCTGGTGGACAACATTGCCA	
BAK1	Forward	CCCTGCTGGGTTTCGGTTA	NM_001030920.1
	Reverse	CGCAGGAAGCCCGTGAT	
	Probe	TGCATGGCCATCCACGTCTACCAG	

[1] Primer and probe oligos were synthesized by MWG Biotech, Hight Point, NC.

[2] Probes had FAM (6-carboxyfluorescein) at 5' end and TAMRA (6-carboxytetramethylrhodamine) at 3' end.

Table 2. Relative expression[1] of target genes from MC-FT not affected by 4-TBP at 1 h co-culture[2]

Gene	SL	SL-G	BL	LBL
TYR	1.08 ± 0.05^a	1.12 ± 0.07^a	1.06 ± 0.05^a	1.30 ± 0.05^b
TRP1	0.89 ± 0.08	0.82 ± 0.18	0.87 ± 0.11	0.98 ± 0.18
TPR2	2.09 ± 0.18	1.78 ± 0.23	1.64 ± 0.68	2.24 ± 0.19
BCL2	1.89 ± 0.28	2.12 ± 0.32	1.98 ± 0.26	2.01 ± 0.26
BAK1	1.15 ± 0.14	1.14 ± 0.15	1.13 ± 0.09	0.96 ± 0.18
HSP70	0.81 ± 0.25	1.41 ± 0.32	1.38 ± 0.18	1.72 ± 0.68

[1] Relative expression of target genes was calculated by the delta delta Ct method of data obtained by qRT-PCR, where 28S served as endogenous control gene and a pool of cDNA from feathers of age-matched parental line BL chickens were used as the calibrator sample. Data were analyzed by fit model of the JMP Pro 9 software and were shown as mean ± SEM.

[2] Due to the absence of interactions between the effects of the line of chickens and 4-TBP treatment, data on gene expression were pooled across treatment. Only the main effects of type of chicken are shown.
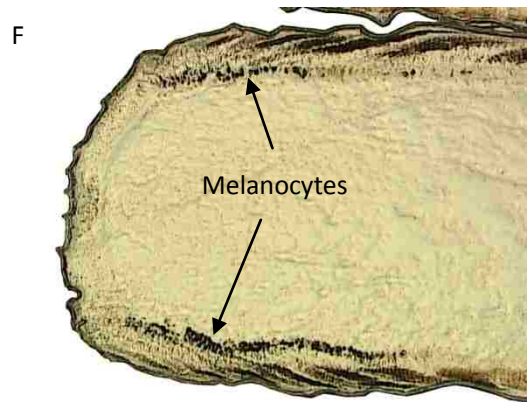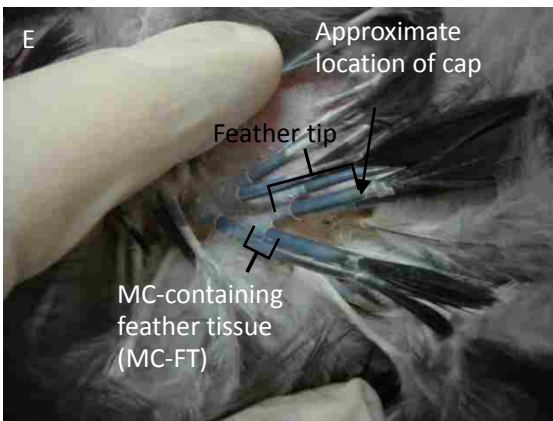
[a,b] For each target gene, means without a common letter are significantly different ($P < 0.05$).

Table 3. Relative expression[1] of target genes in feather-derived MCs that were not affected by 4-TBP treatment for 1 h[2]

Gene	SL	SL-G	BL
TYR	1.87 ± 0.15	2.09 ± 0.21	2.16 ± 0.12
TPR2	1.10 ± 0.22	0.88 ± 0.29	0.91 ± 0.11
BCL2	0.89 ± 0.28	1.10 ± 0.12	1.08 ± 0.31
BAK1	1.02 ± 0.09	1.03 ± 0.11	1.05 ± 0.12
HSP70	0.91 ± 0.24	1.21 ± 0.22	1.07 ± 0.21

[1] Relative expression of target genes was calculated by the delta delta Ct method of data obtained by qRT-PCR, where 28S served as endogenous control gene and a pool of cDNA from BL feather-derived MCs was used as the calibrator sample. Data were analyzed by fit model of the JMP Pro 9 software and were shown as mean ± SEM.

[2] Due to the absence of interactions between the effects of the line of chickens and 4-TBP treatment, data on gene expression were pooled across treatment. Only the main effects of type of chicken are shown.

Table 4. Relative expression[1] of target genes in embryo-derived MCs that were not affected by 4-TBP treatment for 1 h[2]

Gene	SL	SL-G	BL
TYR	3.18 ± 0.21	2.92 ± 0.19	3.29 ± 0.35
TPR2	3.24 ± 0.16	3.54 ± 0.23	3.45 ± 0.45
BCL2	2.98 ± 0.27	2.73 ± 0.26	2.98 ± 0.34
BAK1	2.24 ± 0.34	2.56 ± 0.12	2.64 ± 0.24
HSP70	3.89 ± 0.59	4.56 ± 0.46	4.24 ± 0.34

[1] Relative expression of target genes was calculated by the delta delta Ct method of data obtained by qRT-PCR, where 28S served as endogenous control gene and a pool of cDNA from BL embryo-derived MCs was used as the calibrator sample. Data were analyzed by fit model of the JMP Pro 9 software and were shown as mean ± SEM.

[2] Due to the absence of interactions between the effects of the line of chickens and 4-TBP treatment, data on gene expression were pooled across treatment. Only the main effects of type of chicken are shown.

A

B

C

D

E
Approximate location of cap
Feather tip
MC-containing feather tissue (MC-FT)

F
Melanocytes

Figure 1. Autoimmune chicken model for human vitiligo and morphology of growing feathers from pigmented SL chicken. A) Brown line (BL) parental control (< 1% vitiligo), B) Smyth line (SL) chicken with vitiligo (loss of feather pigmentation due to loss of melanocytes (MCs) in the feather), and C) Light Brown Leghorn (LBL) vitiligo-resistant control. Together, these lines of chicken constitute an excellent animal model to study vitiligo. D) Illustration of dermal (grey) pigmentation in shanks from SL-G chickens (<5% vitiligo); bottom: the grey shanks of a SL-G chicken; top: normal shank color of a SL chicken. E) Normally pigmented growing feathers at one side of breast track from SL chickens. Pigmented growing feathers collected from SL chickens showed the living part (feather tip) of growing feathers (from the newest growth to the epidermal cap) and the bottom 3 mm of the newest growth of growing feathers (MC-FT). F) Micro-structure of the newest growth of a MC-FT with normal pigmentation. Longitudinal, unstained, frozen sections examined at 50 x original magnification under a bright-field microscope.

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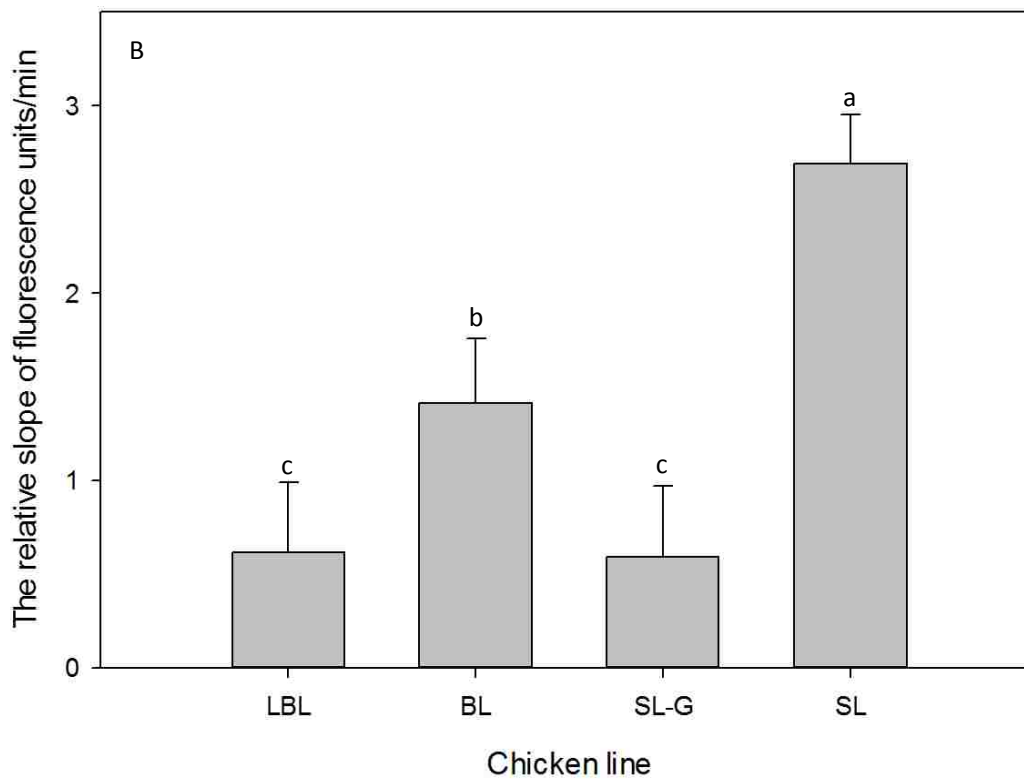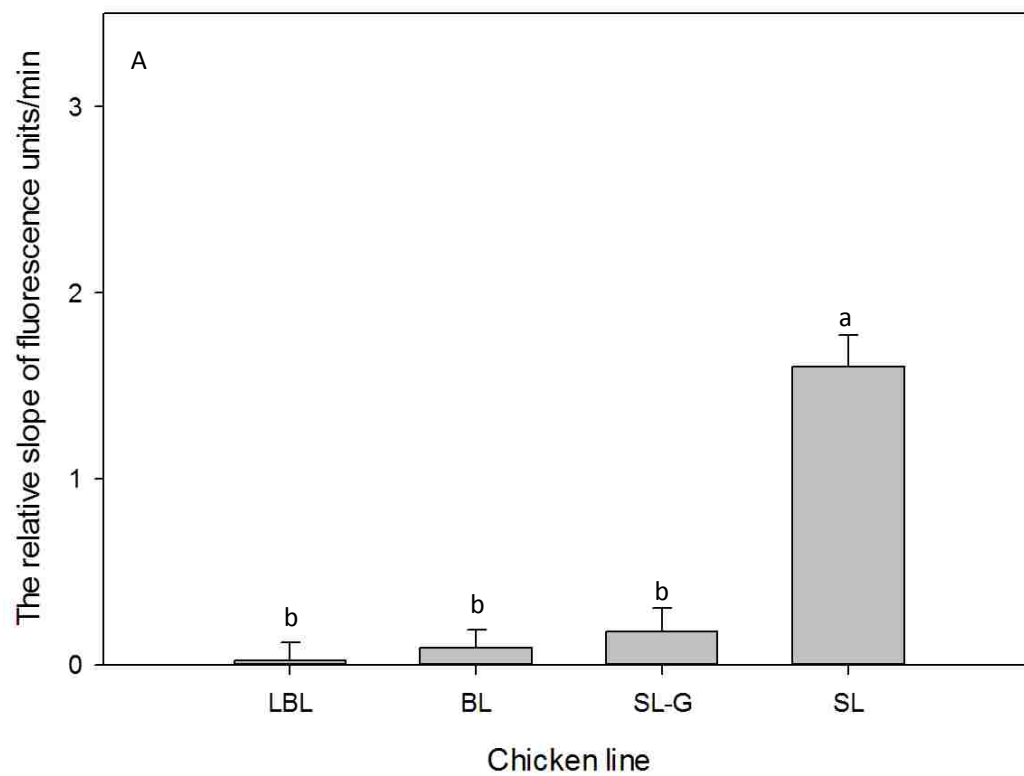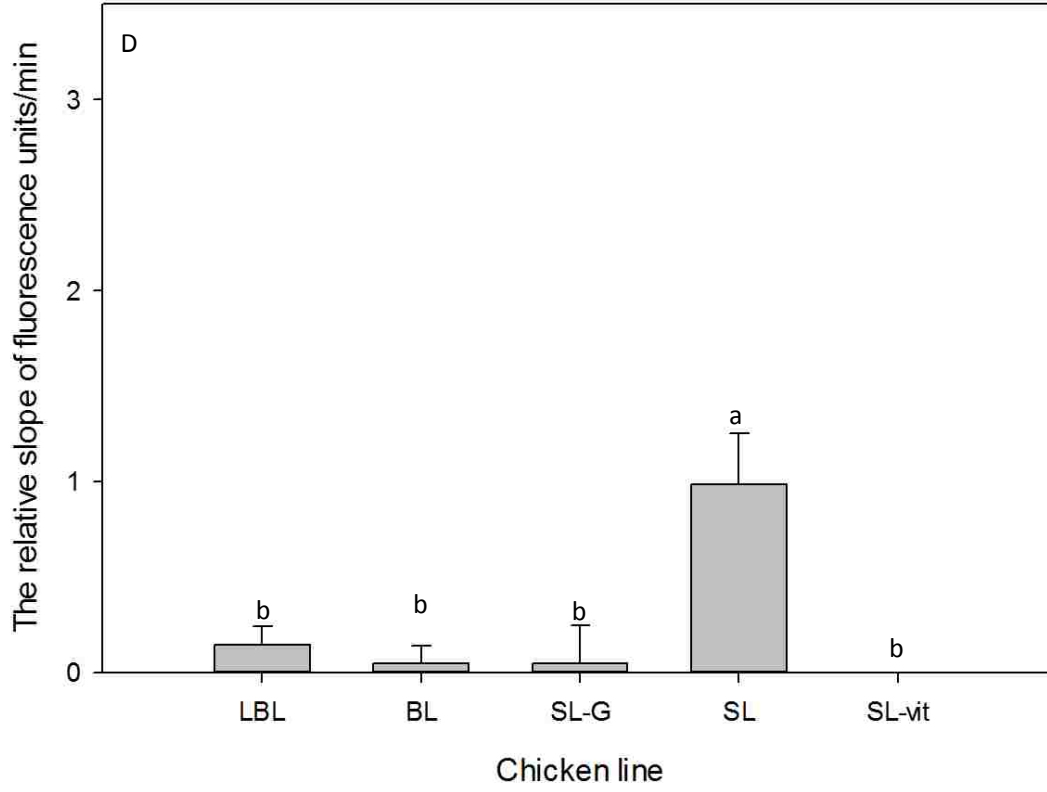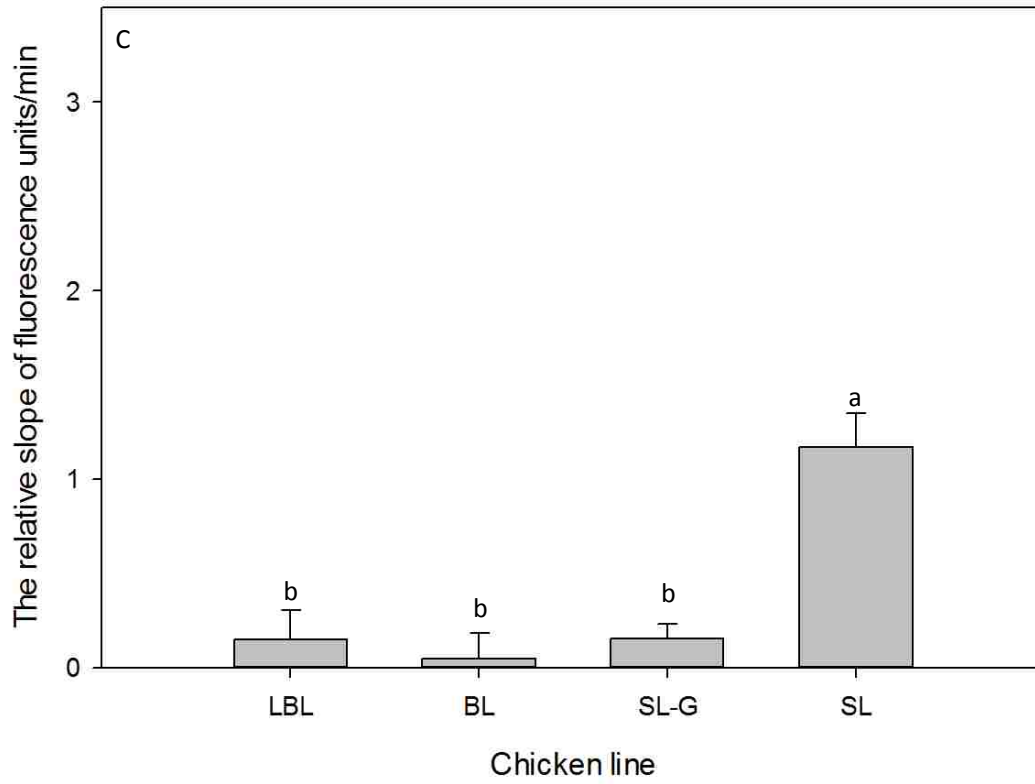
Figure 2. Relative ROS generation in response to 4-TBP treatment of melanocyte-containing feather tissue (MC-FT) cultures. Growing feather were collected from non-HVT-/HVT-injected SL (N = 10), SL-G (N = 6), BL (N = 10), and LBL (N = 10) chickens when the chickens were A) 1-, B) 4-, C) 7- and D) 12-weeks of age. The MC-FT were cultured without or with 200 µM 4-TBP for 1 h. After the incubation, 20 µL of 2',7'-DCF-DA was added and ROS production measured over 90 min period. There was no difference in ROS production by tissues from HVT-positive and HVT-negative chickens and data pooled across HVT treatment are shown. Data shown are the mean ± SEM of the slope of ROS generation (fluorescence units/min; f.u.) due to 4-TPB treatment (ROS treated – ROS untreated). SL = Smyth line, SL-G = SL with grey shanks), BL = Brown line, LBL = Light Brown Leghorn and SL vit = SL with vitiligo chickens. [a,b,c] Means without a common letter are significantly different ($P < 0.05$).
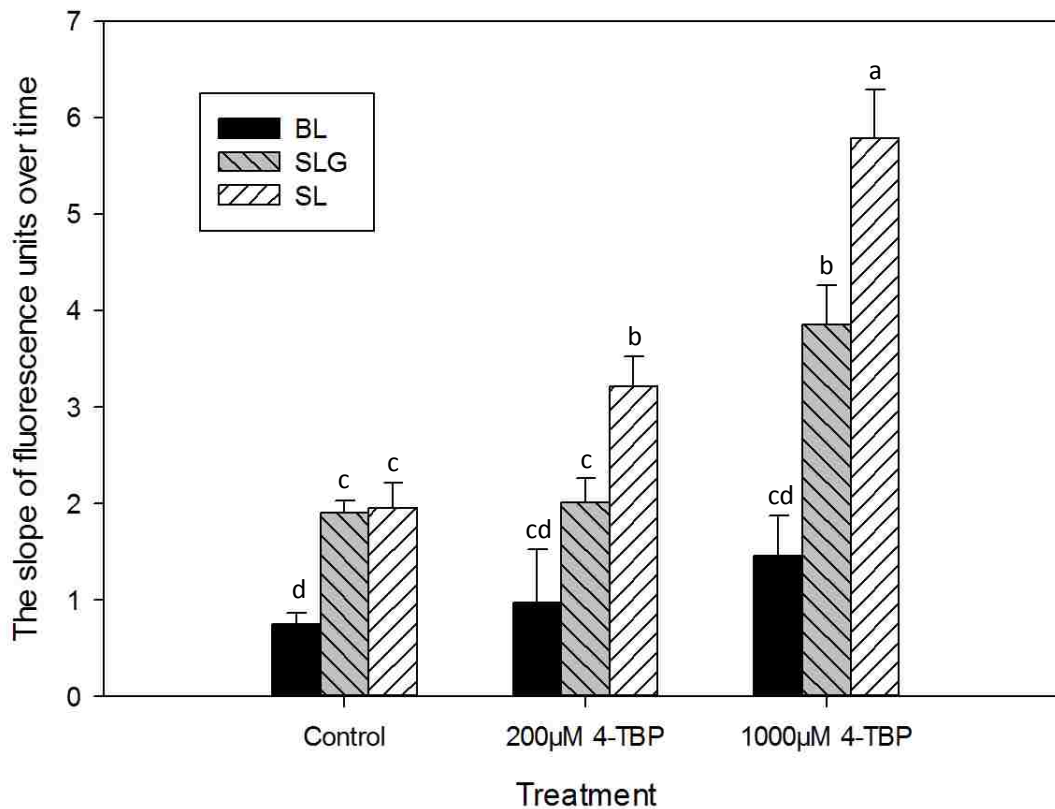
Figure 3. Relative ROS generation in SL, SL-G, and BL feather-derived melanocytes (MC-F) cultured with and without 4-TBP. MC-F cultures were prepared from grwoing feathers of 10 SL, 6 SL-G, and 6 BL 12-week-old chickens following a modified procedure (Boissy *et al*., 1987). MC-F cultures (4th or 5th passage) were deprived of TPA prior to treatment with 0, 200 and 1000 µM 4-TBP. The generation of ROS was determined using 2',7'-dichloro-fluorescein diacetate (2',7'-DCF-DA) after 1 hour incubation of 1×10^4 cells with 4-TBP treatment at 40.5°C humidified incubator with 5% CO_2. ROS generation for each sample is expressed as the slope of fluorescence units over time. Each histogram represents the mean ± SEM ROS generation for each line of chicken. [a,b,c,d] For each line of chicken, means without a common letter are significantly different ($P < 0.05$).
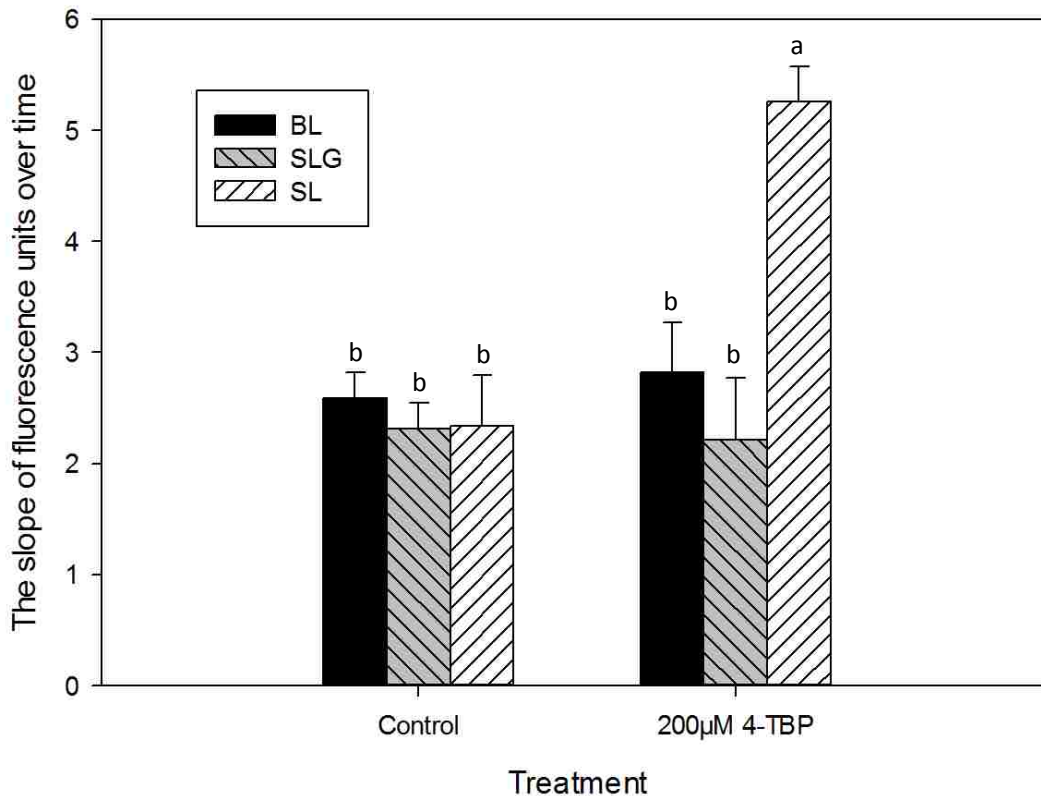
Figure 4. Relative ROS generation by embryo-derived SL, SL-G, and BL MC cultured with and without 4 TBP. MC-E cultures were prepared from 10 SL, 6 SL-G, 6 BL 72 h embryos following a modified method based on Boissy and Halaban (1985). Feather-derived MC cultures (4th or 5th passage) were deprived of TPA and cholera toxin prior to treatment with 4-TBP. The generation of ROS was determined using 2',7'-dichloro-fluorescein diacetate (2',7'-DCF-DA). Fluorescence generated by 1 x 10^{4} cells was determined every 10 min over a 90 min period. ROS generation for each sample was expressed as the slope of fluorescence units/min. Each histogram represents the mean ± SEM ROS generation for each line. [a,b] Means within a type of chicken without a common letter are significantly different (P < 0.05).
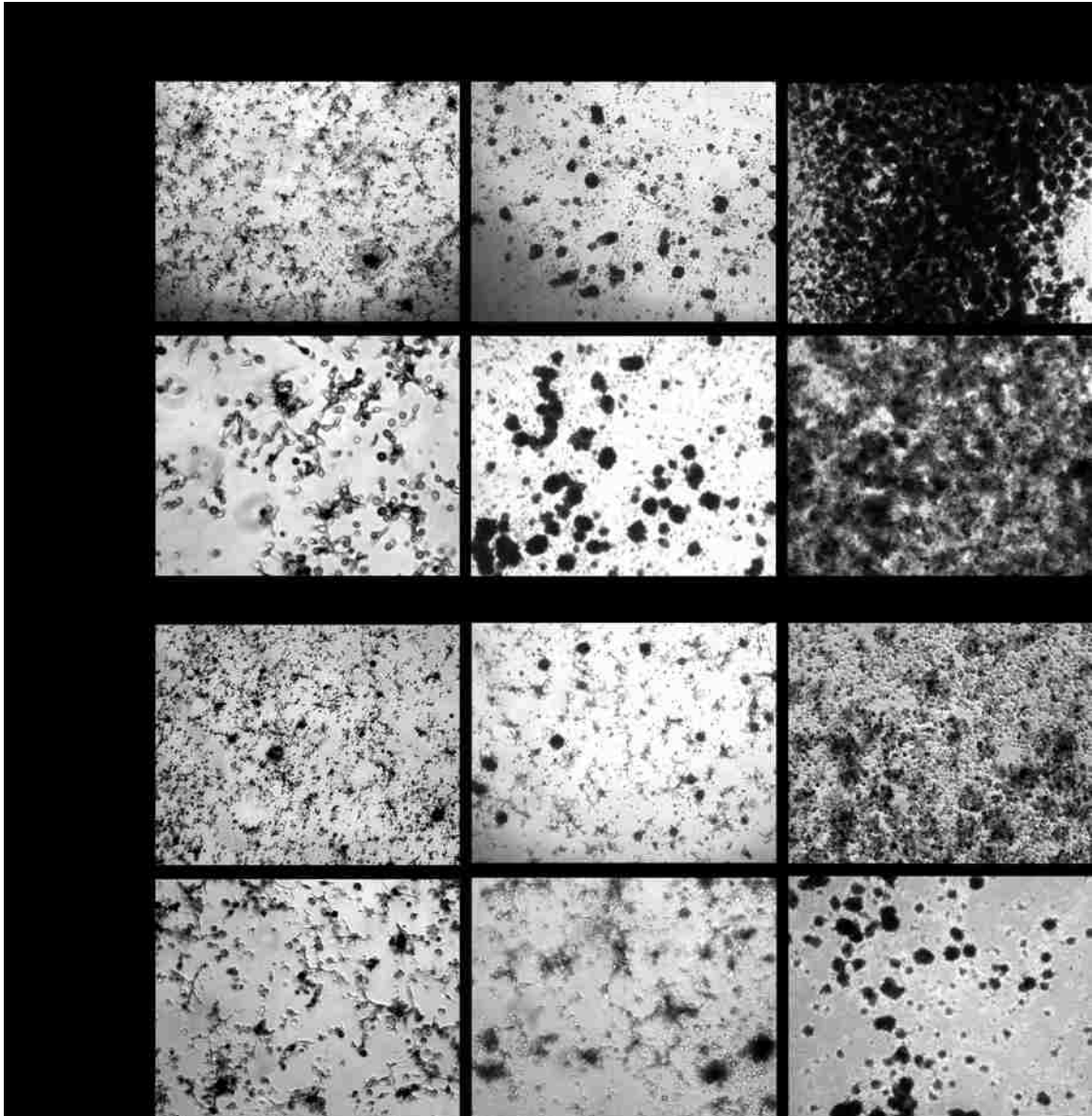
Figure 5. Effects of 4-TBP treatment on the morphology of feather-derived melanocyte (MC-F) cultures. MC-F from vitiligo-prone Smyth line (SL and BL chickens were cultured (1 x 10^4 cells/well; 96 well culture plates) in RPMI 1640 medium w/o phenol red for 24 h. After acclimation to the medium, cells were subjected to 4-TBP treatment (0, 200 or 1000 µM 4-TBP). Morphology of MCs viewed at a) 100x; b) 400x magnification using a bright-field inverted microscope following 3 h of 4-TBP treatment.
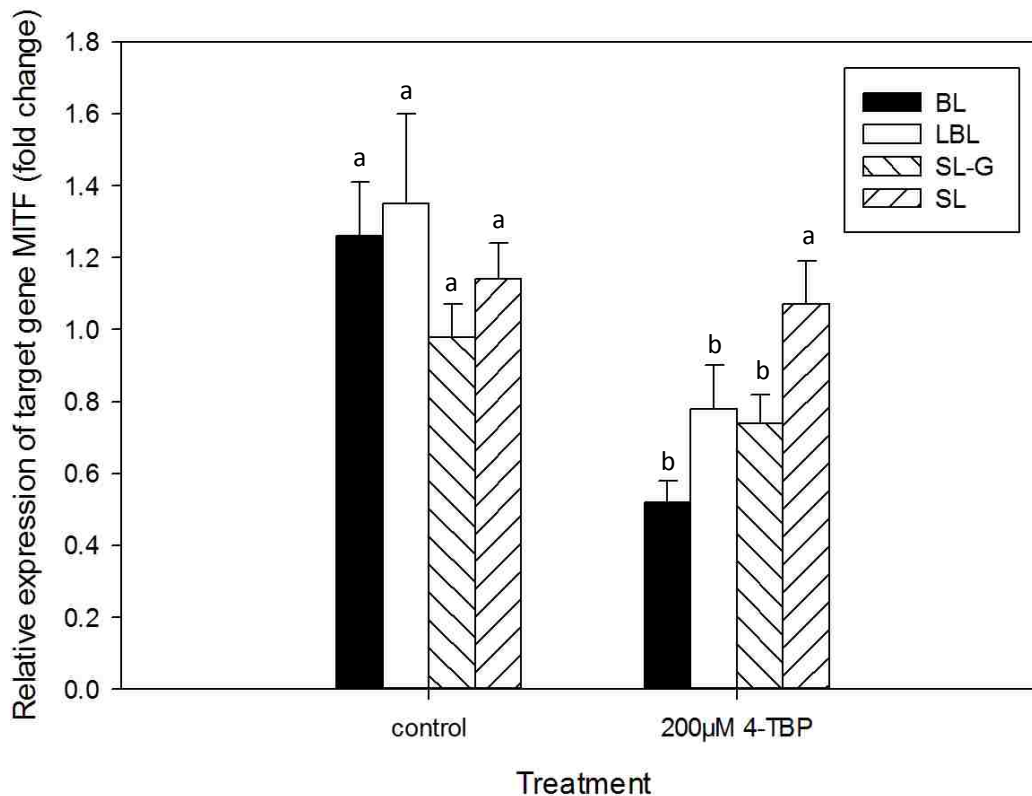
Figure 6. Relative gene expression of MITF in melanocyte containing feather tissue (MC-FT) cultured with and without 4-TBP for 1 h from SL, SL-G, and BL chickens. Growing feather were collected from non-HVT-/HVT-injected SL and SL-G, BL, and LBL control chickens (N = 10/line/HVT group) when the chickens were 1-, 4-, 7-, 12-weeks of age. The MC-FT were cultured without or with 200 μM 4-TBP for 1 h. MITF expression was not affected by HVT status or age of chicken; hence, data were pooled within a type of chicken. Relative expression was determined by the delta delta Ct method in which a pool of cDNA from BL MC-FT (untreated) was used as the calibrator sample and 28S was used as the endogenous control in the qPCR. Data shown are the main effect mean ± SEM for each type of chicken. [a,b] Means without a common letter are significantly different between control and treatment from each chicken line (P < 0.05).
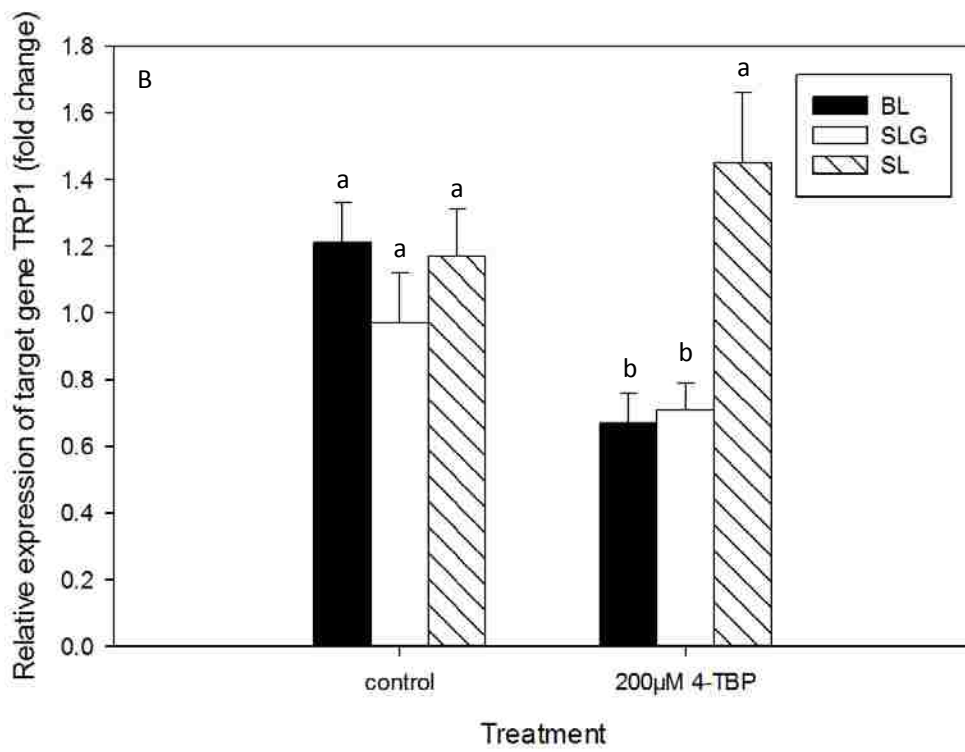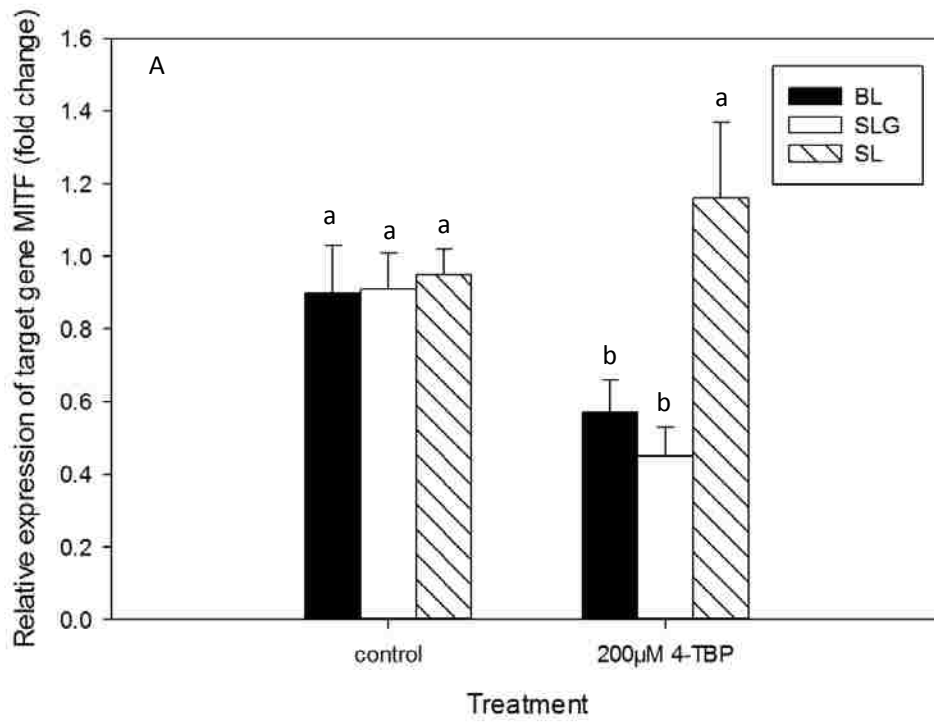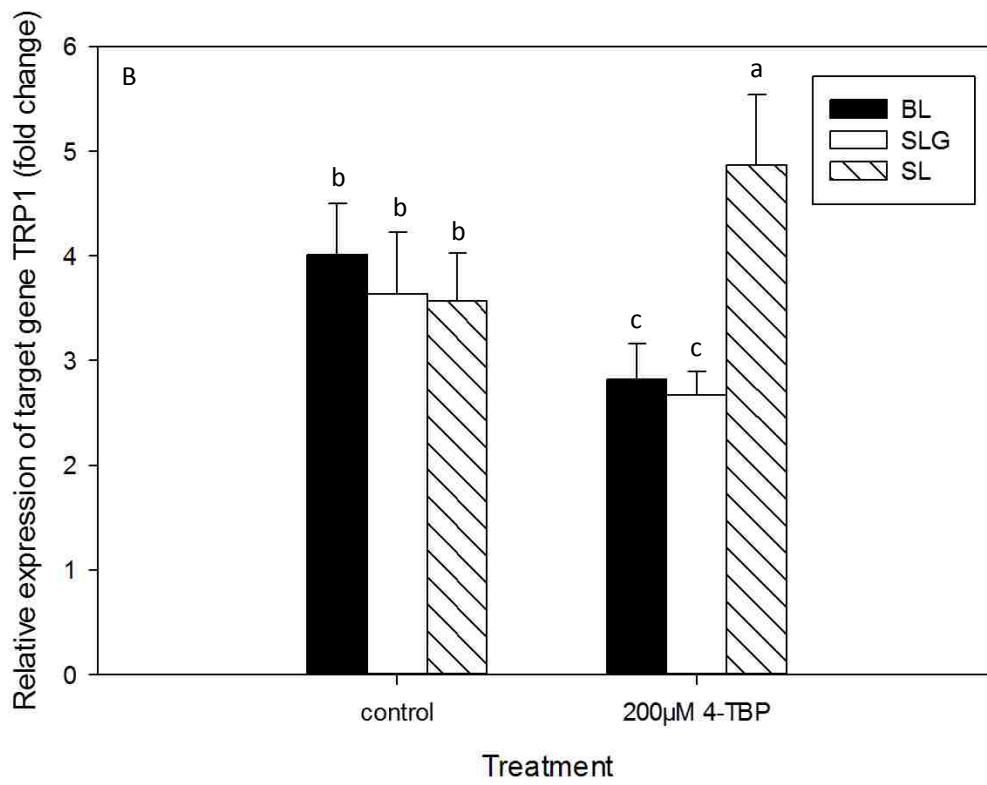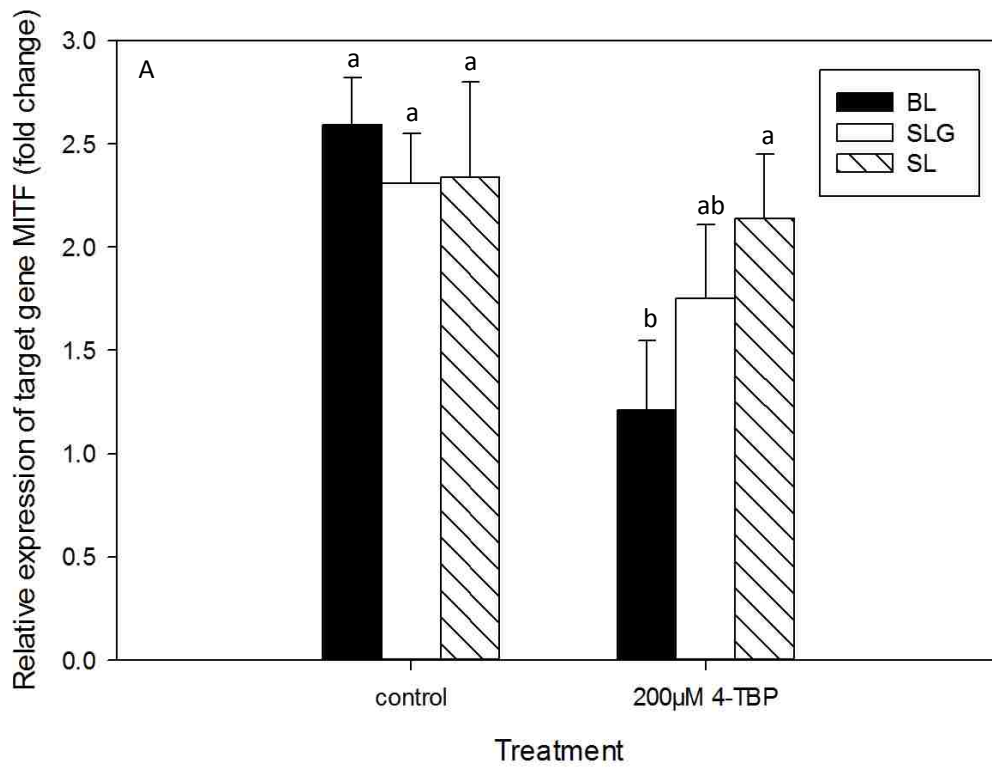
Figure 7. Relative gene expression of A) MITF and B) TRP1 in SL, SL-G, and BL feather derived melanocytes (MC-F) cultured with and without 4-TBP for 1 h. MC-F cultures were prepared from growing feathers of 12-week-old SL (10), SL-G (6), BL(6) chickens following a modified MC culture procedure (Boissy *et al*., 1987). MC-F cultures (4th or 5th passage) were deprived of TPA prior to 4-TBP. Relative target gene expression was determined by the delta delta Ct method in which a pool of cDNA from BL MC-F (untreated) was used as the calibrator sample and 28S was used as the endogenous control in the qPCR. Data shown are the mean ± SEM. [a,b] Means without a common letter are significantly different between control and treatment from each chicken line (P < 0.05).

Figure 8. Relative gene expression of A) MITF and B) TRP1 in SL, SL-G, and BL embryo-derived melanocytes (MC-E) cultured with and without 4-TBP for 1 h. MC-E cultures were prepared from 10 SL, 6 SL-G, 6 BL 72 h embryos following a modified method based on Boissy and Halaban (1985). Feather-derived MC cultures (4th or 5th passage) were deprived of TPA and cholera toxin prior to treatment with 4-TBP. Relative target gene expression was determined by the delta delta Ct method in which a pool of cDNA from BL MC-E (untreated) was used as the calibrator sample and 28S was used as the endogenous control in the qPCR. Data shown are the mean ± SEM. [a,b,c] Means without a common letter are significantly different between control and treatment from each chicken line ($P < 0.05$).

Discussion

Phenol derivative compounds, like 4-TBP, have been shown to induce vitiligo in susceptible individuals. *In vitro* studies on the effect of 4-TBP on MC from healthy donors and vitiligo patients showed that incubation with 4-TBP altered antioxidant levels, including catalase, GSH and superoxide dismutase, and resulted in lipid peroxidation (Koca *et al*., 2004). Manga *et al.* (2006) demonstrated that 4-TBP induces oxidative stress that is more readily overcome by MCs from normally pigmented individuals than from individuals with vitiligo. 4-TBP is oxidized by tyrosinase to quinones (Thorneby-Andersson *et al*., 2000), which cause futile redox cycling and cytotoxic levels of hydrogen peroxide and glutathione disulfide (GSSG), and hence oxidative stress (O'Brien, 1992). Based on these reports, the ability of MC to respond to oxidative stress may play a role in the etiology of vitiligo.

SL chickens exhibits a genetic predisposition to the development of vitiligo (SLV). Comparison of the response of SL MC to 4-TBP in the current study, revealed that 4-TBP induced higher levels of ROS generation, and hence oxidative stress, in MC-FT from susceptible SL chickens compared to SL-G, BL and LBL MC-FT (Figure 1 and 2). Similar results were also observed in MC-F and MC-E cultures stimulated with 4-TBP (Figure 3 and 4). These observations support our hypothesis that, like vitiligo-susceptible humans (Manga *et al*., 2006), the vitiligo-susceptible SL chickens are more sensitive to 4-TBP and,

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like in humans, this heightened sensitivity to 4-TBP induced oxidative stress may be due to depletion of the GSH antioxidant system (Thorneby-Andersson *et al*., 2000; O'Brien, 1992).

In the current study, an important confirmation of MC-specific effects of 4-TBP in chickens was the lack of 4-TBP stimulation of ROS generation in white (no MC) feather tips from vitiliginous SL chickens (Figure 2D). Additionally, the observation that 4-TBP treatment of MC-FT from SL chickens with grey shanks (SL-G), which do not develop vitiligo, resulted in similarly low ROS production as that observed in BL and LBL controls strongly supports association between SL vitiligo-susceptibility and heightened 4-TBP sensitivity (Figure 2, 3 and 4). The grey shanks in SL-G chickens are due to the *Id* dermal pigmentation gene. This is a sex-linked recessive gene where Id^+ females, Id^+Id^+ males and Id^+id^+ males will have yellow shanks, and id^+ females and id^+id^+ males will have grey shanks. SLV is rarely observed in SL-G, even if SLV develops aggressively in their Id^+ SL siblings. How id^+/id^+id^+ genotype exerts its protective effects regarding SLV expression needs to be further examined. Based on the ROS and gene-expression studies it appears that MC in SL-G chickens are more robust and better able to cope of the effects of 4-TBP exposure. Another important finding of the current study was that HVT infection, a known environmental trigger of SLV expression in susceptible SL chickens, did not influence the effects of 4-TBP on MCs in MC-FT collected from HVT vaccinated chickens. This may due to the direct effect of 4-TBP on MCs compared to a likely bystander effect of HVT in

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the feather.

In addition to altered ability of SL MC to cope with oxidative stress, results from the current study on the effects of 4-TBP on SL MC also revealed morphological changes in MC-F and MC-E in the presence of 4-TBP and ROS. These changes included morphological abnormalities such as dendrite retraction and mass aggregation in SL MC, which were less prominent in BL MC cultures (Figure 5). SLV susceptibility of SL chickens was previously shown to be manifested in part by an inherent MC defect. Abnormally-shaped melanosomes could be observed in SL MCs before SLV using transmission electron microscopy. These altered melanosomes were not observed in MCs from the parental Brown line (BL) control (Boissy *et al.,* 1983). MC-E from SL chickens also exhibited abnormalities such as fewer dendrites and selective autophagocytosis of melanosomes as compared to cultured MC-E from BL and LBL controls (Boissy *et al.,* 1986). In the current study, the observed higher level of ROS accumulation in SL MC-E cultures compared to those derived from SL-G and BL embryos further emphasizes that these altered responses of SL MC are inherent and not induced by differentiation in the feather environment of SL chickens (Figure 4).

Studies on human melanocytes have shown that melanogenic proteins are involved in the response to 4-TBP treatment. 4-TBP, as a substance, is metabolized by TYR in MCs (Yang and Boissy, 1999). MITF, which is master regulator for both MC proliferation and melanin

synthesis, was shown to be down regulated in response to oxidative stress (Jimenez-Cervantes *et al*., 2001). 4-TBP-induced cytotoxicity in human vitiligo MC was associated with increased expression levels of MITF and TRP1 (Manga *et al*., 2006). In our study, MITF-expression was down-regulated in MC-FT, MC-F, and MC-E cultures from control SL-G and BL chickens within 1 h exposure to 4-TBP. This drop in MITF was accompanied by a drop in TRP1 expression in MC-F and MC-E cultures. In SL MC-FT, MC-F, and MC-E cultures these alterations in MITF were not observed and TRP1 expression was increased in MC-F and MC-E cultures (Figure 7 and 8). The divergent response in control and SL MC regarding MITF- and TRP1-expression are in line with those in humans, suggesting that lower expression of these proteins may play a similarly protective role in chicken MCs during 4-TBP exposure. How MITF and its downstream product TRP1 influence the sensitivity of SL MC to 4-TBP is not well understood. MITF also regulates the anti-apoptotic factor BCL2 (McGill *et al*., 2002) to induce apoptosis in response to 4-TBP (Hariharan *et al*., 2010). BCL2 has been identified as an MITF target gene and has been shown to be partially important for cell rescue from oxidative stress-induced apoptosis in MITF-depleted cells (Yajima *et al*., 2011). Previous study also indicated human pro-apoptosis gene BAK1 and cellular stress related protein HSP70 have potential effects in the response to 4-TBP (Kroll *et al*., 2005). However, in the current study there were no statistical differences in BCL2, BAK1 and HSP70 expression in MC-FT, MC-F, and MC-E after 1 h of 4-TBP treatment (Table 2-4). A longer exposure time of SL,

BL and LBL MC to 4-TBP would have provided more insight into protective response mechanisms initiated in MC during 4-TBP exposure.

The current study clearly showed that MCs from vitiligo-prone SL chickens, like those form vitiligo patients, are inherently more sensitive to 4-TBP treatment than MCs from SL-G, BL and LBL chickens. Exposure to 4-TBP induced oxidative stress in MCs, which in SL-G, BL, and LBL control MC triggered down-regulation of MITF and TRP1 expression and possibly other genes that may allow MC to cope and control the oxidative stress. Whereas, these protective responses to 4-TBP in the inherently abnormal MCs from SL chickens were not effectively initiated. In addition to altered antioxidant capacity in SL chickens (Wijesekera, 2004), the reduced ability of SL-MC to withstand oxidative stress may be due in part to a disruption in the regulation of MITF and TRP1 expression.

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CHAPTER III

ALTERATIONS IN MELANOCYTE MORPHOLOGY, GENE-EXPRESSION AND ROS GENERATION IN GROWING FEATHERS FROM AUTOIMMUNE VITILIGO-PRONE SMYTH LINE CHICKENS TREATED WITH 4-TERTIARY BUTYL PHENOL *IN VIVO*

Alterations in melanocyte morphology and gene-expression in growing feathers from autoimmune vitiligo-prone Smyth line chickens treated with 4-tertiary butyl phenol *in vivo*

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Abbreviations:

4-TBP: 4-tertiary butyl phenol; α-MSH: α-melanocyte-stimulating hormone; BAK1: BCL2-antagonist/killer 1; BCL2: B-cell lymphoma 2; BL: Brown line; GSH: glutathione; HVT: turkey herpesvirus; IL: interleukin; LBL: Light Brown Leghorn; MC: melanocyte;

MCHR1: melanin-concentrating hormone receptor 1; MITF: microphthalmia-associated transcription factor; ROS: reactive oxygen species; SL: Smyth line; SLV: Smyth line vitiligo; qRT-PCR: quantitative reverse transcription-polymerase chain reaction; TRP1: tyrosinase-related protein 1; TRP2: tyrosinase-related protein 2; TYR: tyrosinase.

Abstract

Smyth line (SL) chicken is an animal model for human autoimmune vitiligo. SL vitiligo

(SLV) is characterized by spontaneous, post-hatch autoimmune loss of melanocyte (MC)

resulting in feather depigmentation. In both chickens and humans, multiple factors

contribute to the loss of MCs and development of vitiligo, including genetic susceptibility,

immune system involvement and environmental triggers. In humans, phenolic derivatives,

such as 4-tertiary butyl phenol (4-TBP), have been shown to trigger vitiligo in susceptible

individuals. Recent studies also have shown heightened sensitivity of SL MCs to 4-TBP *in*

vitro (Chapter II). In order to examine the effects of 4-TBP on SL MC *in vivo*, leukocyte

infiltration, morphology of MCs, and gene expression were monitored in pigmented

growing feathers injected with10 µL 4-TBP (0.15 mg/mL) into the pulp. Feathers were

collected before (0 h) and at 1-, 3.5-, 6-, and 72-h post-4-TBP injection from

eight-week-old SL, Brown line (BL, parental control, < 1% vitiligo) and Light Brown

Leghorn (LBL, vitiligo resistant) chickens. Leukocyte infiltration post-4-TBP injection

was elevated in SL compared to BL and LBL feathers. 4-TBP injection into SL growing

feathers resulted in MC detachment and dendrite retraction at 1 h and 3.5 h post-injection.

By 6 h these alterations were less prominent and normal MC morphology was restored 3 d

after 4-TBP injection. For BL and LBL chickens, MC morphology was not affected by

4-TBP injection into growing feathers. Gene expression analysis revealed a drop in the

expression of microphthalmia-associated transcription factor (MITF) following 4-TBP

injection. In SL feathers this decrease in MITF expression was much less prominent than in BL and LBL feathers and returned to pre-injection level by 6 h. However, at 3 d post injection, MITF levels where similarly low in injected feathers from all chickens. In SL feathers, tyrosinase related protein (TRP) 1 and TRP2 expression was elevated at 6 h compared to pre-injection levels and to BL and LBL samples. The expression of anti-apoptotic B-cell lymphoma 2 (BCL2) was lower in SL feathers by 6 h and 3 d compared to pre-injection levels , whereas BCL2 in samples from BL and LBL controls were elevated at 3 d compared to pre-injection levels and were higher than in SL samples at all times post-4TBP injection. At 1 h post-4-TBP injection, heat shock protein (HSP) 70 expression levels were higher in BL and LBL compared to SL feathers, reached peak levels at 6 h and remained elevated at 3 d. HSP70 levels in SL were not affected by 4-TBP. These different responses of vitiligo-prone SL and controls to *in vivo* administration of 4-TBP into melanocyte-containing feather tissue, further emphasize the heightened sensitivity of SL MC to 4-TBP which may be related to heightened TRP 1 expression. The reduction in MITF and increase in HSP70 and BCL2 observed in control samples may be a protective response to 4-TBP induced cellular stress.

Introduction

Vitiligo is an acquired pigmentary disorder characterized by loss of epidermal melanocytes

(Lerner, 1978) which results in patches of depigmentation, and in some patients, complete

depigmentation of the skin and its derivatives (Ortonne, 1993). This disorder is found in

0.1 - 2 % of the world's human population. Although the etiopathological mechanisms of

vitiligo are not well understood, it is believed that many factors contribute to vitiligo

development including both genetic and non-genetic factors (Reza *et al.*, 2011). Oxidative

stress has been shown to play a role in various clinical disorders. The exact role of

oxidative stress in vitiligo is unclear, but it is assumed to play a part in either the

development of the disorder or the progressive loss of MCs (Jian *et al.*, 2011). Phenolic

derivatives, such as 4-tertiary butyl phenol (4-TBP), have been shown to trigger vitiligo in

susceptible individuals. Manga *et al.* (2006) demonstrated that 4-TBP induces oxidative

stress that was more readily overcome by MCs from normally pigmented individuals than

from individuals with vitiligo. 4-TBP is structurally similar to tyrosine, the initial substrate

in melanogenesis that is oxidized by tyrosinase (Yang and Boissy, 1999). The oxidation of

4-TBP yields a toxic intermediate called quinone that further reacts with cysteine or

glutathione (GSH) to produce 4-tert-butyl-6-S-cysteinylcatechol and

4-tert-butyl-6-S-glutathionylcatechol, respectively (Kirsten *et al.,* 2000). The oxidation of

4-TBP in MCs results in the generation of reactive oxygen species (ROS). In addition,

GSH, an antioxidant, helps to protect cells from ROS, such as free radicals and peroxides.

The loss of GSH reduces the ability of cells to overcome oxidative stress. However, the exact role of oxidative stress induced by 4-TBP in vitiligo is currently unclear.

Previously, it has been shown that human MCs cultured in the presence of 4-TBP experience dose-dependent apoptosis (Manga, 2006) and the percentage of cells that die following exposure to 4-TBP can be significantly reduced by excluding α-melanocyte-stimulating hormone (α-MSH; Yang *et al*., 1999). α-MSH is a key physiologic inducer of melanogenesis. The biological function of α-MSH is mediated through its binding to MC1R, the only melanocortin receptor expressed in MCs (Pavey and Gabrielli, 2002). MC1R is a G protein-coupled receptor that activates adenylate cyclase. The increased intracellular concentration of cAMP mediates the expression of the MC-specific isoform of the microphthalmia-associated transcription factor (MITF; Widlund and Fisher, 2003). MITF in turn activates the expression of many downstream genes, including tyrosinase (TYR), tyrosinase-related protein 1 (TRP1) and TRP2, needed for melanogenesis.

Although MITF is an important factor in MC development, survival, and differentiation, recent studies also have shown that MITF is a substrate of caspases and plays a key role in the apoptosis of melanoma cells (Larribere *et al*., 2005). Over-expression of BCL2 has been shown to overcome the melanocyte apoptosis triggered by disruption of MITF (McGill *et al*., 2002). In addition, MITF regulates endogenous levels of BCL2 in MCs.

Thus, BCL2 expression is very critical for MC survival and may prevent apoptosis during cellular stress, such as 4-TBP related oxidative stress. Furthermore, exposure of the human MC immortalized vitiligo cell line (PIG3V) to 4-TBP resulted in elevated extracellular levels of heat shock protein (HSP) 70 and activation of dendritic cell (DC) effector functions focused on the stressed MCs (Kroll *et al*., 2005).

The Smyth line (SL) of chickens is a well established animal model for the study of spontaneous development of autoimmune vitiligo, displaying all the characteristics of the human disorder (Smyth, 1981 and 1989; Wick *et al.*, 2006; Erf, 2010). Like human vitiligo, SL vitiligo (SLV) is a multifactorial disorder involving genetic susceptibility, environmental triggers, and cellular as well as humoral immune system components in the progressive loss of MCs. Under conventional rearing conditions, including routine administration of turkey herpesvirus (HVT) at hatch, approximately 80 - 95 % of SL chickens develop SLV between 6 - 20 weeks of age. Without the HVT environmental trigger, the incidence of SLV is reduced to < 20% (Erf *et al*., 2001). SL chickens together with the MHC-matched Brown line (BL) parental control (< 1% vitiligo) and the Light Brown Leghorn (LBL) vitiligo-resistant control chickens constitute an excellent animal model to study vitiligo. Another excellent attribute of the SL chicken model for vitiligo study is the location of MCs in the growing feathers, rather than the skin (Figure 1). Growing feathers are easily and repeatedly accessible for sampling, providing unique opportunity to examine the evolving lesion prior to and throughout the development of

SLV in the same individuals.

Recent *in vitro* studies have shown a heightened sensitivity of SL MC to 4-TBP (Chapter II). Additionally, based on the divergent ROS and MITF- and TRP1-expression response to a 1 h 4-TBP exposure in SL MC versus BL- and LBL-MC, it appears that aberrant regulation of these melanogenesis-related proteins may be related to the reduced ability of SL MC to withstand oxidative stress. Specifically, the culture of MC-containing feather tissue, feather-derived MC or embryo-derived MC with 4-TBP resulted in higher ROS generation in SL compared to BL and LBL MCs. The elevated ROS accumulation in SL MC was accompanied by increased RNA expression levels of TRP1 in feather- and embryo-derived MC. On the other hand, the low levels of 4-TBP induced ROS generation in BL and LBL MC cultures were associated with a decrease in MITF and TRP1 expression levels. The expression of TYR, TRP2, BCL2, BAK1 and HSP70 was however not affected by *in vitro* 4-TBP treatment of MC for 1 h in SL and controls. In the current study, we will explore the effects of 4-TPB on SL and control MC in situ by *in vivo* administration of 4-TBP into the pulp of melanocyte-containing growing feathers. By injecting multiple growing feathers in the same individual, injected feathers can be collected for *ex vivo* analysis at various times post-4-TBP injection to monitor the local *in vivo* effects of 4-TPB. To assess the *in vivo* effects of 4-TBP in the feather, MC morphology, leukocyte infiltration, and expression of HSP70 as well as melanogenesis- and apoptosis-related genes will be examined before and at various times post-4-TBP injection (0, 1, 3.5, 6, and 72 h). We

hypothesize, that similar to *in vitro* observations, SL MC will have heightened sensitivity

to 4-TBP *in vivo* compared to BL and LBL melanocytes. Characterization of these

activities in the vitiligo-prone SL, parental BL and vitiligo-resistant LBL chickens will

provide important insight into the underlying MC defect and etiology of vitiligo.

Materials and Methods

Experimental Animal

Three lines of MHC-matched ($B^{101/101}$) chickens were used for this study: 1) the vitiligo-prone SL chickens which exhibit an 80 - 95% incidence of SLV by 20 weeks of age under conventional rearing conditions, 2) the parental BL (< 1% incidence of vitiligo) control chickens, and 3) the vitiligo-resistant LBL (no incidence of vitiligo) control line of chickens (Erf, 2010). On the day of hatch, chicks from each pedigreed family were tagged using a color coded numbering system. Chicks were not vaccinated with live herpesvirus of turkey, a known environmental trigger of SLV expression in susceptible individuals (Erf *et al*, 2001). All chicks were reared in floor pens on wood-shavings litter in a HEPA-filtered room in the University of Arkansas Poultry Health Laboratory. Using these rearing conditions the incidence of SLV has repeatedly been shown to be < 10% (Erf, personal communication). Food and water were available *ad libitum.* Standard diet, temperature and lighting protocols were followed and the well-being of chicks was checked daily. All procedures for this study were approved by the Institutional Animal Care and Use Committee (IACUC) Protocol #08011.

Feather Injection Study

At eight weeks of age, melanocyte-containing growing feathers of 10 SL, 5 BL, and 5 LBL

chickens were injected with 4-TBP. Specifically, 10 µL of 0.15 mg/mL (1mM) 4-TBP

(Sigma-Aldrich Inc., St. Louis, MO, USA) were injected into the pulp of 8 growing

feathers on each breast track from each bird using insulin syringes with the BD

Ultra-Fine^{TM} needle (Capacity: 3/10cc,30 unit; Length: 8 mm; Gauge: 31 G; NDC/HRI#

08290-8438-01, Becton, Dickinson and Company). Prior to injection, emerging barbes of

the growing feathers were cut, leaving the epidermal cap surrounding the pulp (dermis)

intact. This approach made it easier to inject into the center of the pulp and allowed for

identification of injected feathers at collection time. Three samples of feathers (feather tips)

were collected before (0 h) and at 1-, 3.5-, 6- and 72-h post-4-TBP injection. At each time

point, one feather tip was placed into 10% buffered formalin for conventional histology

and two feather tips were snap frozen in liquid N_2 and stored at -84°C for later RNA

analysis. Based on previous studies, feather pulp injection with vehicle alone does not

cause alteration in this tissue; hence, this control was not included in this study (Erf *et al*.,

2007).

Assessment of leukocyte infiltration and MC morphology

Formalin fixed, processed, and paraffin embedded feather samples were cut into

longitudinal sections (6 µm thick) and stained with Hematoxylin/Eosin stain. Using a

bright-field microscope at 50x magnification and a 10 x 10 mm ocular inserted (1 square),

the extent of leukocyte infiltration was determined based on the percentage of affected pulp

area and on the cell density of infiltration. Area scores ranging from one to five were assigned depending on the percentage area affected by leukocyte infiltration: <20% (score 1); >20%,<40% (score 2); >40%,<60% (score 3); >60%,<80% (score 4); and >80%,<100% (score 5). Density scores ranging from zero to three were given depending on the number of leukocytes/square with 0-20 cells, 20-100 cells, 100-150 cells, and >150 cells receiving score 0 to 3, respectively. The morphological assessments of melanocyte and the nature of the leukocyte infiltrate [granulocytes and mononuclear cells (lymphocytes and monocytes/macrophages)] were carried out under a bright field microscope (100x, 400x and 1000x magnification).

RNA extraction, quantification, and cDNA synthesis

Total RNA was isolated from the 3 mm newest growth of the snap frozen feather tips which contains the epidermal tissue where MCs are located. RNA was isolated using the RNeasy® Mini Kit (Qiagen, Valencia, CA) and following the protocol for "Purification of Total RNA from Animal Tissues" with modifications. MC-F were homogenized by Tissue Tearor (BioSpec Products, Bartlesville, OK, Model: 985370-395) provided in the kit. Total RNA was isolated from homogenates with on-column DNA digestion (Qiagen). RNA was eluted in 30 µL RNase-, DNase-free water and stored at -80°C until use. RNA quality and quantity were determined by Experion^{TM} RNA StandardSens Reagents and Supplies (Bio-Rad Laboratories, Hercules, CA) following manufacturer's instructions (Figure 5).

RNA (175 ng) was reverse transcribed into cDNA by High Capacity cDNA Reverse

Transcription Kit (Applied Biosystems, Foster City, CA) in a 10 µL reaction volume

according to manufacturer's instruction. A Biometra® personal cycler (Biomedizinische

Analytik GmbH, Germany) was programmed as follows: 25ºC for 10 min, 37ºC for 2 h,

85ºC for 5 min and 4ºC holding temperature. Then cDNA was stored at -80ºC until

further analysis.

Taqman® quantitative PCR (qPCR)

qPCR was performed on the cDNA samples (Primer and probes are shown in Table 2) in 25

µL reaction volume on a ABI PRISM 7300 sequence detection system (Applied

Biosystems, Foster City, CA) using the Universal PCR Master Mix (Applied Biosystems,

Foster City, CA) and following manufacturer's instruction. In each plate, a calibrator

sample (a pool of cDNA from feather tips of age-matched BL birds), a non-template

control, and sample cDNA were included. 28S ribosomal RNA was the endogenous control

gene. Cycling profiles used were 94ºC for 15 min, and 50 cycles of denaturation at 94ºC for

60 s, and annealing/extension at 60ºC for 60 s. The relative gene expression was

determined by the delta delta Ct *(ΔΔCt)* method (Wong and Medrano, 2005).

Statistical Analysis

Statistical analysis was carried out using JMP Pro 9 (JMP Software Inc., Cary, NC).

Two-way ANOVA was used to examine the effects of line of chicken (SL, BL, and LBL), time (0-, 1-, 3.5-, 6- and 72-h), and line by time interactions, on the expression of each target gene and area and density of leukocyte infiltration. In the presence of significant main effects for line and/or time, and the absence of line by time interactions, Fisher's LSD multiple means comparison was used to determine differences among group means. P values ≤ 0.05 were considered significant. Data were reported as means $\pm$ SEM.

Results

Mononuclear cell infiltration into the pulp of 4-TBP injected growing feathers

4-TPB injection resulted in mononuclear cell (lymphocytes and macrophages) infiltration into the feather pulp (Figure 2). In terms of area score, 2.5 fold increase ($P < 0.05$) in mononuclear cell infiltration could be observed in SL feathers at 1, 3.5, and 6 h post-4-TBP injection compared to pre-injection levels. By 3d post-4-TBP injection leukocyte infiltration was near pre-injection levels. Compared to pre-4-TBP treatment levels, mononuclear cell infiltration in BL feathers increased 2 fold ($P < 0.05$) at 1, 3.5 and 6 h and returned to pre-injection levels at 3 d. The extent of mononuclear cell infiltration in LBL feathers was nearly identical to that observed in BL feathers except that pre-injection levels were already restored at 6 h post-4-TBP injection. Generally, mononuclear cell infiltration area scores tended to be higher in SL than in BL and LBL feathers, with higher ($P < 0.05$) levels observed at 3.5 h and 6 h post-4-TBP treatment compared to BL and LBL feathers, respectively (Figure 3A).

Infiltration density scores were not different in 4-TBP injected SL, BL and LBL feathers. Compared to pre-4-TBP injection levels, the density scores for SL, BL and LBL chickens were elevated ($P < 0.05$) at 1 h, remained at this level at 3.5 and 6 h and returned to pre-4-TBP injection levels by 3 d post-4-TBP treatment (Figure 3B).

Melanocyte morphology in 4-TPB injected growing feathers

In SL chickens, injection of 4-TBP into the pulp of growing feathers resulted in MC detachment and dendrite retraction at 1 and 3.5 h post-injection. By 6 h these alterations were less prominent and normal MC morphology was restored 3 d after 4-TBP injection. These 4-TBP induced morphological alterations in SL MC were not observed in BL and LBL injected feathers (Figure 4).

Expression of target genes in 4-TBP injected growing feathers

Compared to pre-injection levels, the relative gene expression of MITF in SL feathers dropped slightly at 1 and 3.5 h (P < 0.05), increased significantly at 6 h and dropped (P < 0.05) below pre-injection levels at 3 d after 4-TBP treatment. By comparison, in BL and LBL feathers, MITF expression levels dropped substantially (P < 0.05) below pre-injection levels within 1 h and remained at this low level throughout the 3 d sampling period (Figure 6A). Compared to BL and LBL controls, MITF gene expression levels were higher in SL feathers at 1, 3.5, and 6 h, but lower at 3 d (Figure 6A).

Compared to pre-injection levels, the relative TRP1 expression in SL feathers increased significantly (P < 0.05) at 6 h and returned to pre-injection level at 3 d post-4-TBP treatment. In comparison, the level of TRP1 expression in BL and LBL dropped (P < 0.05) below pre-injection levels at 1 h, stayed at this level for the 3.5 h and 6 h time points, and

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returned to pre-injection levels at 3 d (Figure 6B). TRP1 gene expression levels were

higher in SL feathers compared to BL and LBL controls at all time-points post-4-TBP

injection (Figure 6B).

The relative expression of TRP2 in SL feathers increased ($P < 0.05$) starting at 3.5 h

post-4-TBP injection, reached peak levels at 6 h, and remained elevated at 3 d, whereas

TRP2 expression levels in BL and LBL feathers remained at the pre-injection level

throughout the 3 d sampling period (Figure 6C). TRP2 gene expression levels were higher

($P < 0.05$) in SL compared to BL and LBL feathers at 3.5 h, 6 h and 3 d post-4-TBP

injection (Figure 6C).

Compared to pre-injection levels, the relative expression of anti-apoptotic BCL2 decreased

($P < 0.05$) in SL chickens starting at 6 h post-4-TBP treatment and remained at low levels

throughout the sampling period. In BL and LBL controls the level of BCL2 expression was

elevated ($P < 0.05$) at 3 d post-4-TBP treatment (Figure 6D). Compared to BL and LBL

controls, BCL2 gene expression levels in SL feathers were higher at 1 h and lower ($P < 0.05$)

at 6 h and 3 d (Figure 6D).

The relative HSP70 expression in SL feathers was not affected by 4-TBP treatment during

the 3 day sampling period, whereas the expression of HSP70 in BL and LBL feathers

increased ($P < 0.05$) starting at 1 h, reached peak levels at 6 h and remained at high levels in

feather samples collected 3 d post-4-TBP injection (Figure 6E). HSP70 gene expression

levels were lower (P < 0.05) in SL feathers at 1 h, 3.5 h, 6 h and 3 d (Figure 6E) compared to levels observed in BL and LBL feathers.

The relative expression levels of TYR and the pro-apoptotic BAK1 protein in feather tips from the different lines of chickens were not affected by 4-TBP treatment during the 3 d observation period. However, the main effect mean for TYR expression over the course of this study was higher in LBL compared to BL and SL feathers (Table 3).

Table 1. Mononuclear cell infiltration into 4-TBP injected growing feathers from vitiligo prone Smyth line (SL) and normally pigmented Brown line (BL), and Light Brown Leghorn (LBL) chickens.

Mononuclear cell infiltration	SL (N=10)	BL (N=5)	LBL(N=5)
Area[1] at 0h	1.100 ± 0.076	1.000 ± 0.109	1.000 ± 0.109
Area[1] at 1h	2.400 ± 0.148	2.167 ± 0.214	2.000 ± 0.214
Area[1] at 3.5h	2.500 ± 0.148^a	2.000 ± 0.214^b	1.833 ± 0.214^b
Area[1] at 6h	2.300 ± 0.218^a	2.000 ± 0.315^a	1.167 ± 0.315^b
Area[1] at 3d	1.300 ± 0.120	1.000 ± 0.173	1.000 ± 0.173
Density[1] at 0h	0.400 ± 0.148	0.000 ± 0.214	0.167 ± 0.214
Density[1] at 1h	1.900 ± 0.182	1.833 ± 0.263	1.583 ± 0.263
Density[1] at 3.5h	1.500 ± 0.165	1.583 ± 0.239	1.667 ± 0.238
Density[1]at 6h	1.450 ± 0.138	1.167 ± 0.199	1.000 ± 0.199
Density[1] at 3d	0.700 ± 0.263	0.417 ± 0.379	0.250 ± 0.379

[1] Mononuclear cell infiltration was evaluated based on area and density of infiltration. Areas scores ranging from one to five were given to each feather after visual examination. Score one to five were given according to the following infiltration: <20% (score 1); >20%,<40% (score 2); >40%, <60% (score 3); >60%, <80% (score 4); and >80%, <100% (score 5). Density scores ranging from zero to three were given depending on the number of leukocytes in 10 squares of a 10 mm by 10 mm ocular insert (1 square) viewed at 50 x magnification under a microscope. The density scores were assigned according to the following criteria: 0-20 cells (score 0), 20-100 cells (score 1), 100-150 cells (score 2), and >150 cells (score 3) per square.

N = number of birds sampled at each time point

[a,b] For each time point, means without a common letter are significantly different (P < 0.05).

Table 2. Primer[1] and probe[1,2] sequences for target genes examined in the relative gene expression analysis

Target	Primer/probe	Sequences (5' to 3')	Accession NO.
28S	Forward	GGCGAAGCCAGAGGAAACT	X59733
	Reverse	GACGACCGATTTGCACGTC	
	Probe	AGGACCGCTACGGACCTCCACCA	
TYR	Forward	ATAATGCCCTTCACATCTACATGAAT	NM_204160.1
	Reverse	GCTCAAAAATGCTGTCAACAAATG	
	Probe	CTCAATGTCCCAAGTACAAGGCTCTGCG	
TRP1	Forward	GGAACCATTTGTAACAGCACTGAAG	NM_205045.1
	Reverse	CCATAGGCCGTGCAACATTT	
	Probe	CGGTCCCATCCGTAGAAATCCTGCTG	
TRP2	Forward	CCTTTCCCGGCATGAGTTT	NM_204935.1
	Reverse	AGCGCATTCCTGAAGCTGAA	
	Probe	CAGTCCTCCGTTTTTCCGCAATTCCA	
MITF	Forward	AAGAACTGGGCACCTTGATACC	NM_205029.1
	Reverse	GATGTAGTCCACTGATGCTTTTAGAATAG	
	Probe	AAATCAAACGACCCGGATATGCGCTG	
HSP70	Forward	GACTGCTCTCATCAAGCGTAACA	FJ217667.1
	Reverse	TCATACACCTGGACGAGGACACT	
	Probe	CACCATTCCCACCAAACAAACACAGACC	
BCL2	Forward	GCGTCAACCGGGAGATGT	NM_205339.1
	Reverse	GGTTCAGGTACTCGGTCATCCA	
	Probe	CCGCTGGTGGACAACATTGCCA	
BAK1	Forward	CCCTGCTGGGTTTCGGTTA	NM_001030920.1
	Reverse	CGCAGGAAGCCCGTGAT	
	Probe	TGCATGGCCATCCACGTCTACCAG	

[1] Primer and probe oligos were synthesized by MWG Biotech, Hight Point, NC.

[2] Probes had FAM (6-carboxyfluorescein) at 5' end and TAMRA (6-carboxytetramethylrhodamine) at 3' end.

Table 3. Relative expression[1] of target genes not affected by 4-TBP during the course of the 3 d study[2]

Gene	Smyth line	Brown line	Light Brown Leghorn
TYR	1.069 ± 0.062^{b}	1.081 ± 0.061^{b}	1.241 ± 0.061^{a}
BAK1	1.084 ± 0.059	1.167 ± 0.059	1.170 ± 0.059

[1] Relative expression of target genes was calculated by delta delta Ct method of data obtained by qRT-PCR, where 28S served as endogenous control gene and a pool of cDNA from feathers of age-matched parental line BL was use as the calibrator sample. Data were analyzed by fit model of the JMP Pro 9 software and were shown as mean $\pm$ SEM.

[2] Due to the absence of interactions between the effects of the line of chickens and time points examined (0, 1, 3.5, 6 h and 3 d), the main effect means for each line of chicken are shown.

[a,b] For each target gene, means without a common letter are significantly different ($P < 0.05$).
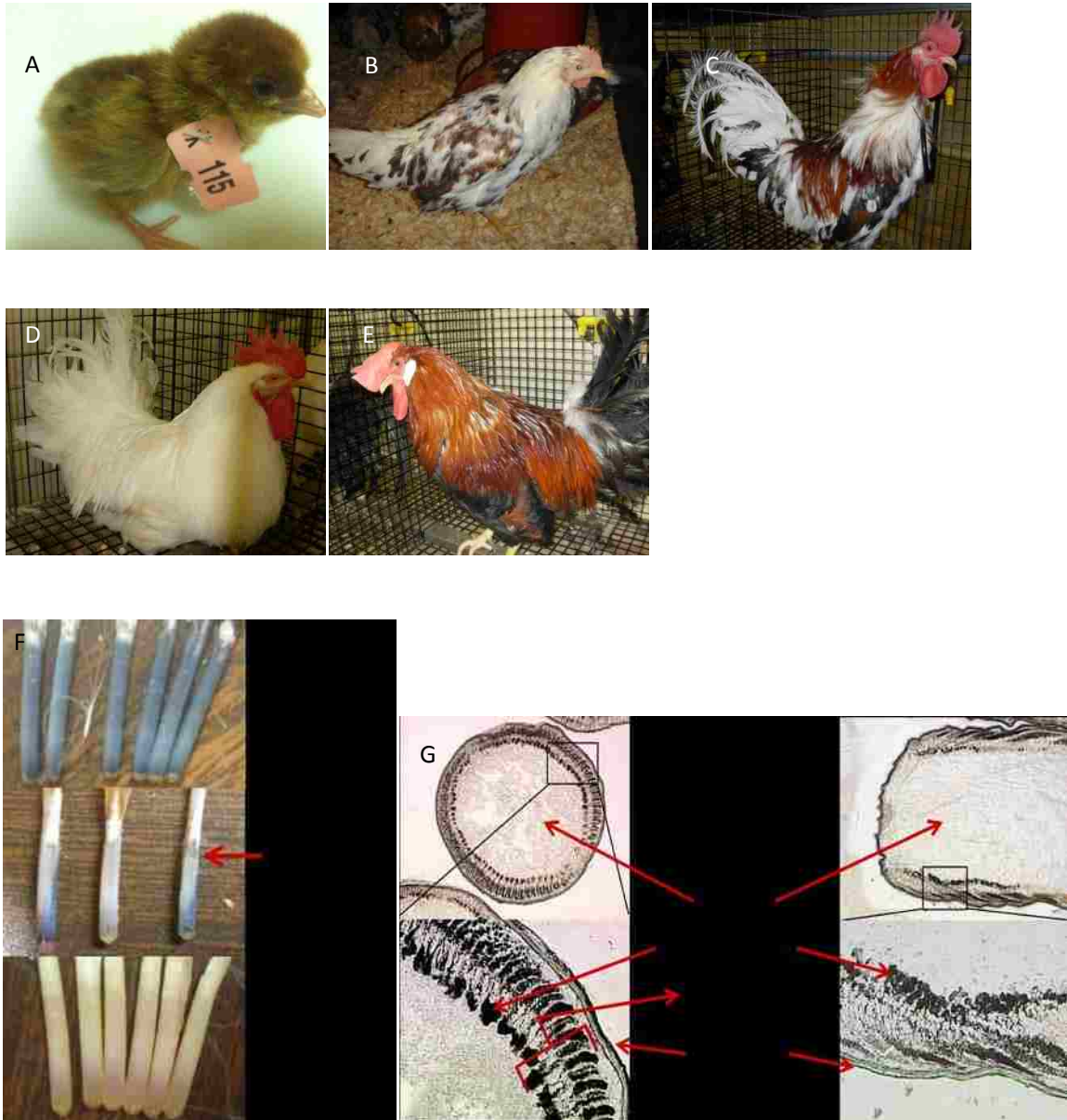
Figure 1. Phenotypic expression of vitiligo in the Smyth line (SL) chicken and structural features of growing feathers. A) pigmented SL chick at hatch; B) 14-week-old SL female with vitiligo; C) 20-week-old vitiliginous SL rooster; D) 20-week-old SL rooster with complete vitiligo; and E) 20-week-old Light Brown Leghorn (LBL) rooster without vitiligo (normal pigmentation); F) growing feathers collected from SL chickens: non-vitiligo (top), active vitiligo (middle) and complete vitiligo (bottom); G) Cross- and longitudinal-sections of a growing chicken feather.
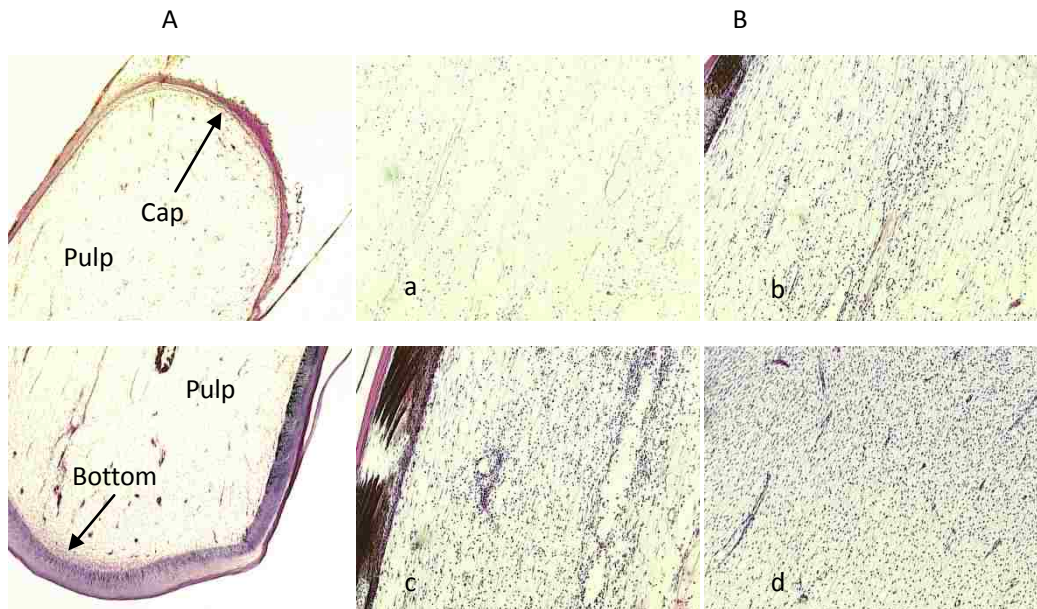
Figure 2. Scoring of leukocyte infiltration into the pulp area injected with 4-TBP. A) The ensheathed unit of living tissue in the newest growing feather. The ensheathed unit of living tissue is approximately 8 - 10 mm from bottom to cap with a 3-4 mm diameter. Most of the tissue consists of the inner pulp (sections were stained with Hematoxylin/Eosin stain and viewed at 50x magnification using a bright-field microscope). Leukocyte infiltration was scored based on area and density. Area scores ranging from one to five were given to each feather after visual examination. Score one to five were given according to the extent of infiltrated pulp area: <20% (score 1); >20%,<40% (score 2); >40%, <60% (score 3); >60%,<80% (score 4); and >80%,<100% (score 5). B) Density scores ranging from zero to three were given according to the number of mononuclear cells in 10 squares of a 10 mm by 10 mm ocular insert (1 squares) when viewed on a bright-field microscope at 100 x magnification a) 0-20 cells (score 0), b) 20-100 cells (score 1), c) 100-150 cells (score 2), and d) >150 cells (score 3) per square.
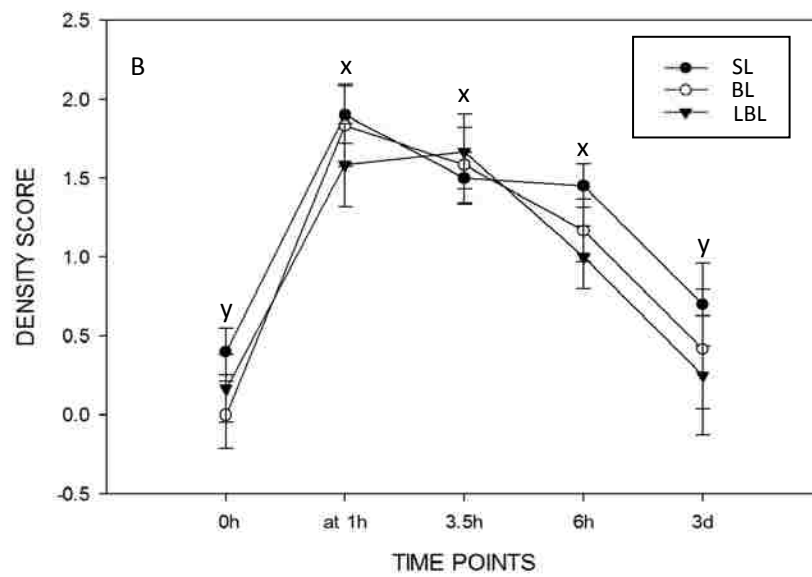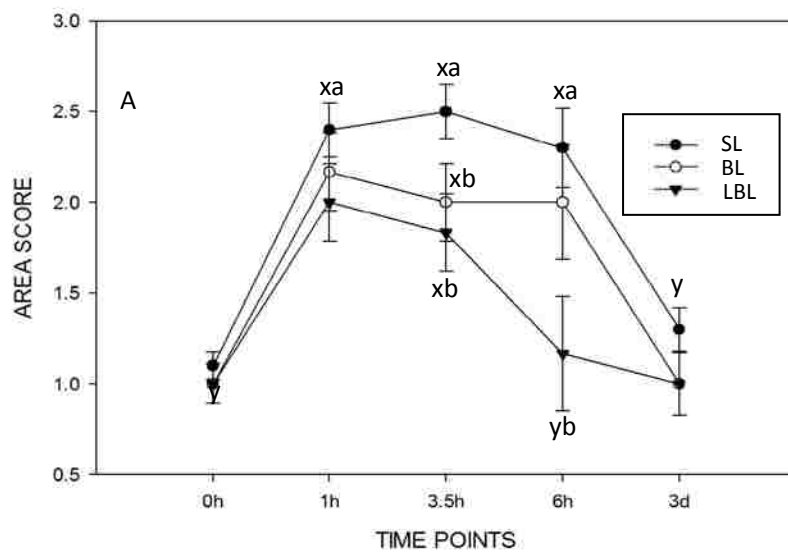
Figure 3. Extent (Area score), density (Density score), and time course of mononuclear cell infiltration into the pulp of 4-TBP injected feathers. Growing feathers of Smyth line (SL), Brown line (BL), and Light Brown Leghorn (LBL) chickens were injected with 10 μL 4-TBP (0.15mg/mL; 1mM) into the feather pulp. Injected feather were collected at 0h, 1h, 3.5h, 6h and 3d after 4-TBP treatment and preserved in 10% buffered formalin for conventional histology. Sections were stained with Hematoxylin/Eosin stain. Areas score ranging from one to five were given to each feather after visual examination. Score one to five were given according to the following infiltration: <20% (score 1); >20%,<40% (score 2); >40%, <60% (score 3); >60%; <80% (score 4); and >80%; <100% (score 5). Density scores ranging from zero to three were given depending on the number of leukocytes in 10 squares of a 10 mm by 10 mm ocular insert (1 square) viewed at 50 x magnification under a microscope. The density scores were assigned according to the following criteria: 0-20 cells (score 0), 20-100 cells (score 1), 100-150 cells (score 2), and >150 cells (score 3) per square. Data were expressed as mean $\pm$ SEM. [x,y] For each line of chickens, means without a common letter are different from the 0 h mean; [a,b] For each time point, means from each line of chickens without a common letter are different ($P < 0.05$).
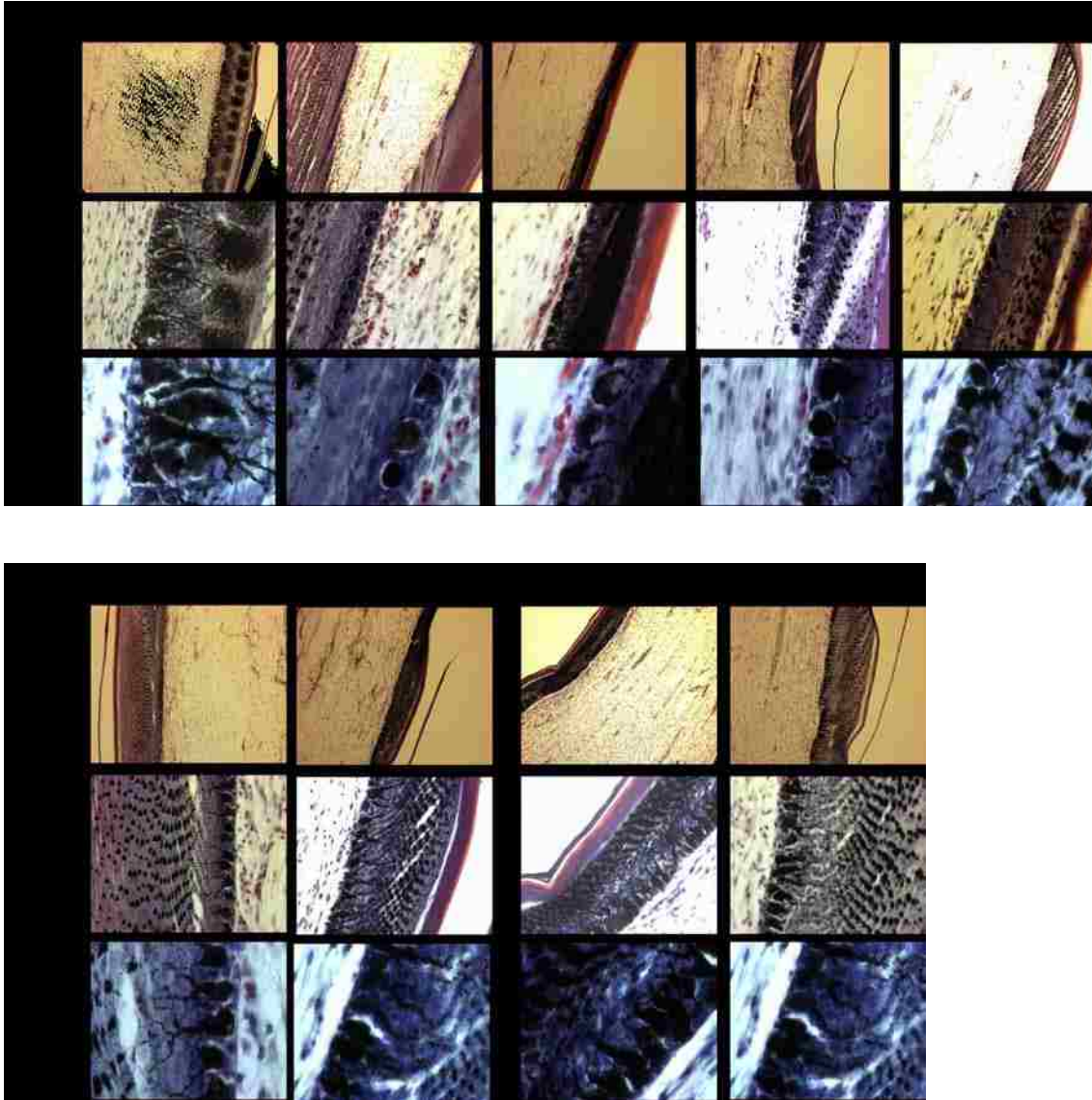
Figure 4. Melanocyte morphology in pigmented growing feathers of 8-week-old Smyth line (SL), Brown line (BL) and Light Brown Leghorn (LBL) chickens before (0 h) and at various times after injection with 10 μL 4-TBP (0.15 mg/mL; 1mM). Injected feathers were collected at 0h, 1h, 3.5h, 6h and 3d after 4-TBP treatment and preserved in 10% buffered formalin for conventional histology. Sections were stained with Hematoxylin/Eosin stain and viewed at a) 100x, b) 400x or c) 1000x magnification using a bright-field microscope.
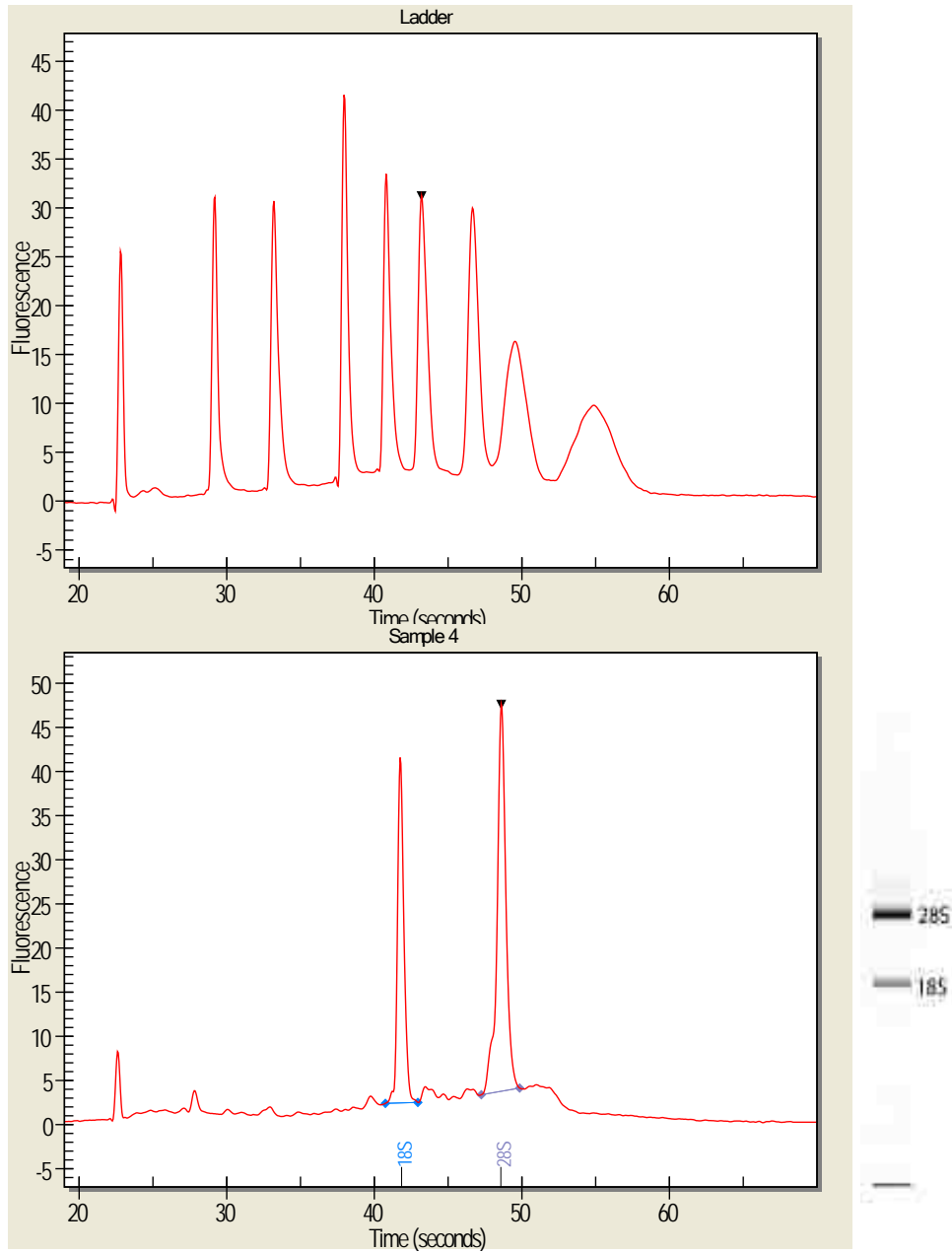
Figure 5. Total RNA quality assessment on the basis of 18S and 28S ribosomal RNA (rRNA). Actual image of total RNA from isolated feather tissue after electrophoresis using Experion StandardSen kit. Top: RNA ladder. Bottom: the intact total RNA with clearly defined 18S and 28S rRNA bands.

A

B
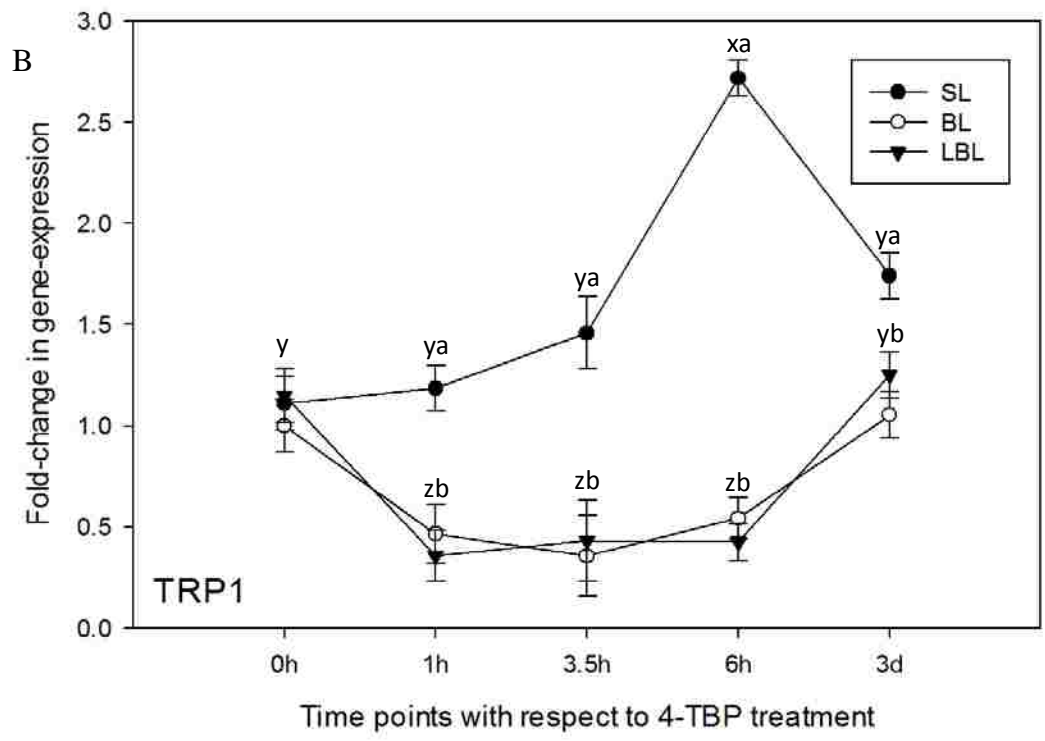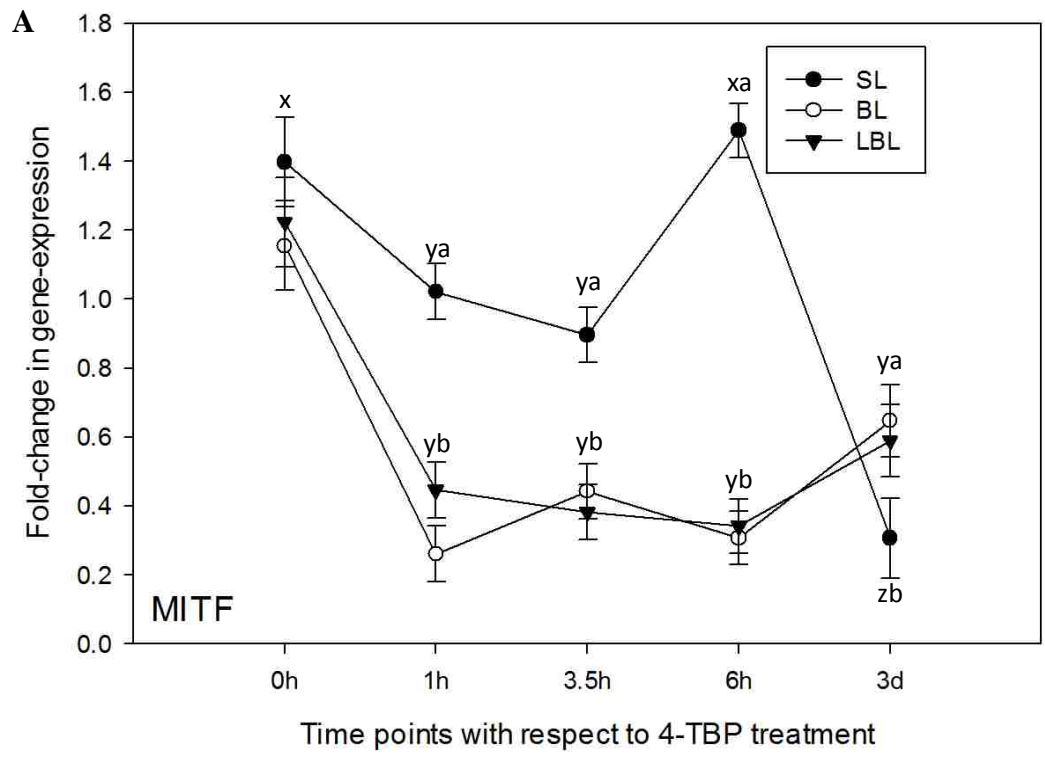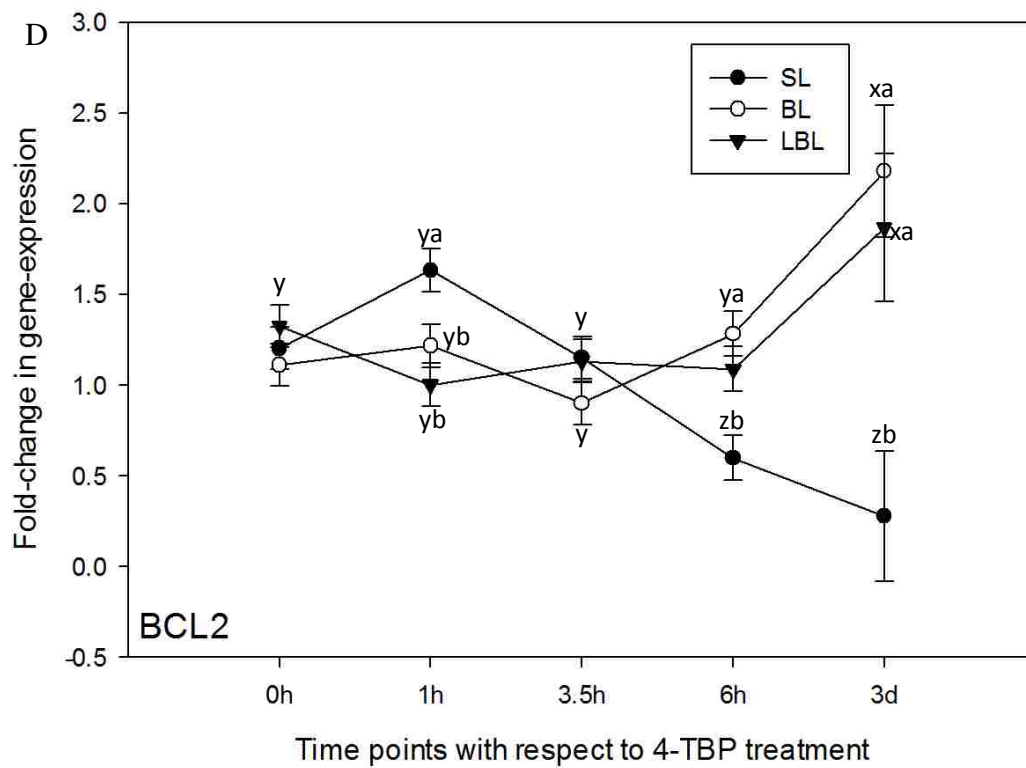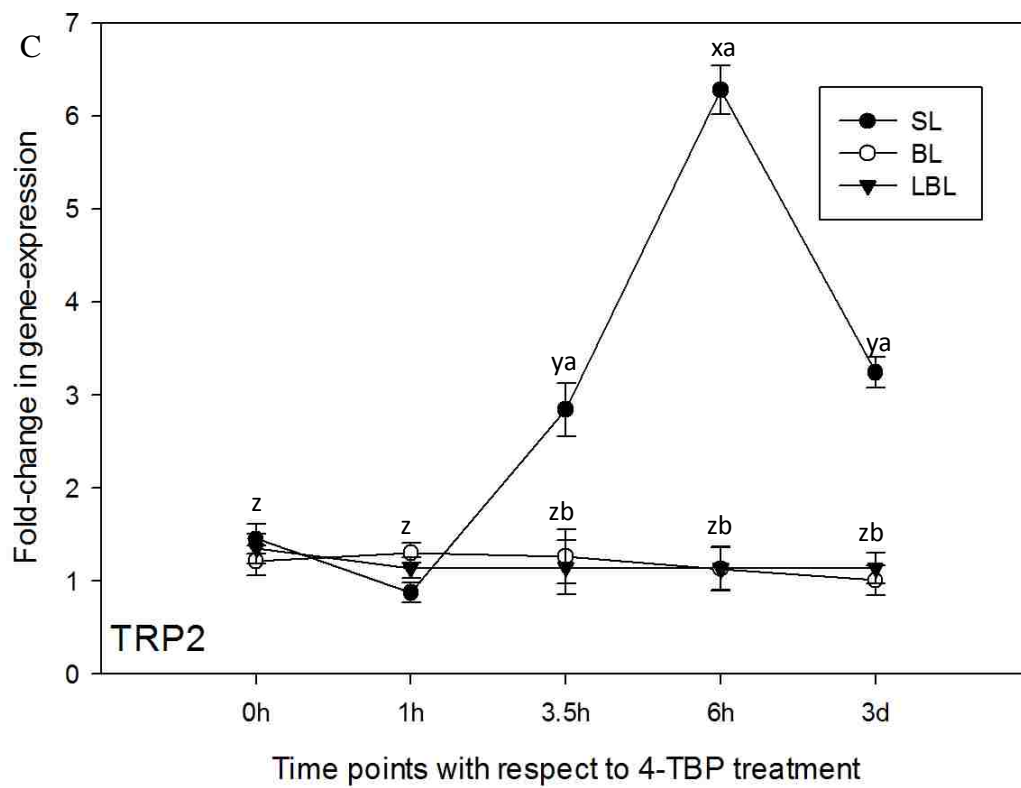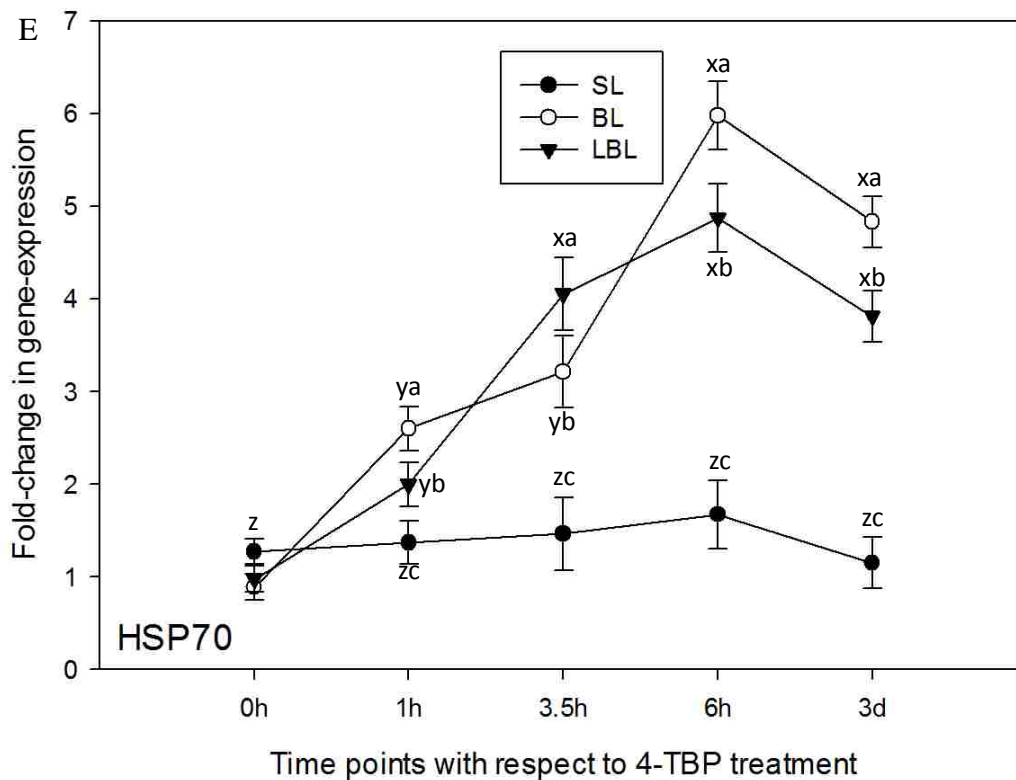
Figure 6. Time course of A) MITF, B) TRP1, C) TRP2, D) BCL2, and E) HSP70 gene expression in Smyth line (SL), Brown line (BL) and Light Brown Leghorn (LBL) feathers injected with 4-TBP. Growing feathers of SL chickens were injected with 10 µL 4-TBP (0.15 mg/mL; 1mM) into the feather pulp. Injected feathers were collected at 0 h (before), and 1 h, 3.5 h, 6 h and 3 d after 4-TBP treatment. The relative gene expression of MITF, TRP1, TRP2, BCL2 and HSP70 was determined by quantitative real-time RT-PCR. The relative change in expression was computed by the *delta delta* Ct method in which a pool of cDNA from BL chickens was used as the calibrator samples and 28S was used as the endogenous control in the qPCR. Data were expressed as mean ± SEM. [x,y,z] For each line of chickens, means without a common letter are different from the 0 h mean. [a,b,c] For each time point, means from each line of chicken without a common letter are different ($P < 0.05$).

Discussion

Various environmental and genetic factors affect the development and progression of vitiligo. In susceptible individuals, vitiligo development is initially triggered by environmental factors including 4-TBP. Yang *et al.* (1999) reported that exposure to 4-TBP of industry workers and consumers is widespread through phenolic detergents, synthetic leather, glues, *etc*. 4-TBP is oxidized by tyrosinase to quinones (Thörneby-Andersson *et al.*, 2000), which cause futile redox cycling and cytotoxic levels of hydrogen peroxide and glutathione disulfide (GSSG), thereby inducing and oxidative stress (O'Brien, 1992). After 4-TBP exposure, genetic/epigenetic alterations may occur in affected melanocytes resulting in vitiligo onset and progression in human.

In Chapter II of this dissertation, morphological abnormalities such as dendrite retraction and mass aggregation were observed in SL MC cultures exposed to 4-TBP treatment, which were less prominent in BL MC cultures. More importantly, MC-E cultures established from SL chickens expressed a higher level of ROS accumulation in comparison to those cultures derived from SL-G and BL embryos, emphasizing that these deficiencies are inherent and not induced by differentiation in the feather environment of SL chickens. In the current study, these morphological abnormalities were also observed in 4-TBP injected melanocyte-containing feather tissue from SL chickens, including MC detachment and dendrite retraction at 1 and 3.5 h post-injection. These MC alterations in response to

4-TBP exposure *in vivo* were much less prominent in BL and LBL feather tissue (Figure 4).

In the previous study (Chapter II), the ROS generation response of melanocytes to 4-TBP treatment *in vitro* were examined in SL and control chickens. Exposure of melanocytes to 4-TBP resulted in greatly heightened ROS generation in SL compared to control melanocytes. This heightened sensitivity of SL melanocytes was associated with altered MITF and TRP1 expression levels compared to controls 1 h after 4-TBP (the only time-point examined), suggesting that the reduced ability to cope with oxidative stress in SL chickens may be related to MITF regulation. In the current study, divergent responses *in vivo* to 4-TBP injection of MC containing feathers were observed in SL compared to controls regarding MITF, TRP1, TRP2, BCL2, and HSP70 expression.

MITF is considered as the master gene for melanocytic survival and a key transcription factor for melanogenesis which can positively regulate genes of the melanogenic enzymes TYR, TRP1, and TRP2. In the current study, it was revealed that the relative gene expression of MITF in 4-TBP injected SL feathers dropped slightly at 1 and 3.5 h, increased significantly at 6 h and dropped ($P < 0.05$) below pre-injection levels at 3 d after 4-TBP treatment. This increase from 3.5 h to 6 h was accompanied by elevated TRP1 and TRP2 expression compared to pre-injection levels (Figure 6A-C). This is in accordance with the regulation of TRP1 and TRP2 by MITF. In human vitiligo studies, 4-TBP sensitivity of MC was directly related to TRP1 levels (Manga *et al*., 2006). Thus,

up-regulation of TRP1 in SL chickens in response to 4-TBP may be similarly responsible

for the heightened 4-TBP sensitivity of SL MC (e.g. ROS production; Chapter II). The

increase in MITF, TRP1, and TRP2 observed between 3.5 and 6 h post-4-TBP injection is

contrary to the apparently protective response of decreased MITF and TRP1 expression in

BL and LBL feathers (Figure 6). The elevated levels of TRP2 in SL may however play a

protective role. In addition to the role in eumelanogenesis, where TRP2 catalyzes the

isomerization of DHICA from dopachrome, TRP2 may also plays a role in MC protection

during oxidative stress. Over-expression of TRP2 in the amelanotic WM35 melanoma cell

line was shown to reduce sensitivity to oxidative stress. However, these properties of TRP2

appeared to depend on a particular cell environment (Michard *et al*., 2008). Overall, the

divergent response to 4-TBP regarding MITF expression observed in feather samples from

vitiligo-susceptible SL compared to controls are in agreement with those reported for

human MC by Manga *et al*. (2006).

Yang *et al*. (2000) have reported that the destruction of MC by 4-TBP is through apoptosis.

Examination of BCL2 (anti-apoptotic protein) expression following 4-TBP exposure *in*

vivo in the present study revealed a gradual decrease of BCL2 in SL feathers, whereas

BCL2 expression in BL and LBL feathers was found to steadily increase (Figure 6D). This

divergent response in BCL2 expression in SL compared to controls, further supports a lack

of a protective response to 4-TBP exposure in vitiligo-susceptible individuals. While MITF

has been shown to control MC survival via BCL2 (McGill *et al*., 2002; Nishimura *et al*.,

2005), the MITF expression pattern observed in controls (decrease) and SL (little change) during the first 6 h following 4-TBP exposure does not suggest a regulatory role of MITF in BCL2 expression in this situation.

In the current study, expression levels of the pro-apoptotic BCL2 homologs BAK1 did not change following 4-TBP treatment in SL, BL and LBL chickens. It has been suggested (Willis *et al*., 2007; Huang *et al*., 2012) that an increase in the BCL2:BAK1 ratio enables greater engagement of anti-apoptotic BCL2 by the pro-apoptotic BH3 ligand, therefore prompting survival of the cell. With reduction of BCL expression and no change in BAK1 expression in SL feathers following 4-TBP exposure, the proportions between these proteins further support initiation of activities favoring apoptosis in SL MC.

ROS has the potential role to regulate the expression of HSP70. It appears that these proteins in turn have the ability to protect cells and tissues from the deleterious effects of inflammation. The mechanisms by which such protection occurs include prevention of ROS-induced DNA strand damages, lipid peroxidation as well as protection of mitochondrial function (Jacquier-Sarlin MR *et al*., 1994). Exposure of melanocytes to 4-TBP *in vitro* was found to result in HSP70 generation in cells from both the immortalized human vitiligo MC cell line PIG3V and the normal human MC cell line PIG1. In 4-TBP stimulated PIG3V MC, HSP70 was found to be released into the medium, a phenomenon not observed in PIG1 MC. Moreover, high extracellular levels of HSP70 resulted in

activation of dendritic cell (DC) effector functions focused on the stressed MCs. (Kroll *et al*., 2005). Our study showed early and sustained elevation of HSP70 expression in BL and LBL upon 4-TBP exposure, and no change in HSP70 expression levels in SL feathers (Figure 6E). The complete lack of a HSP70 response to 4-TBP in SL-MC, which clearly exhibit signs of cellular stress due to treatment, may reflect a serious alterations in their ability to generate protective mechanisms during cellular stress.

Based on the *in vivo* effects of 4-TBP revealed in this study, it appears that reduced MITF and TRP1 expression and increased HSP70 and BCL2 expression following MC exposure to 4-TBP are protective responses that failed to be initiated in SL MC. A decrease in the BCL2:BAK1 ratio in response to 4-TBP observed in SL MC may suggests that SL MC are on a path towards apoptosis. Apoptotic MC may subsequently lead to immune recognition of MC and initiation of a melanocyte-specific immune response. The mechanisms underlying altered gene expression in SL MC upon exposure to 4-TBP need to be further examined to gain insight into the etiology of the vitiligo. Specifically, functional studies need to further examine the role the MITF, TRP1, and TRP2 in the response of MC to oxidative stress.

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CHAPTER IV

ASSOCIATION OF HERPESVIRUS INFECTION AND IMMUNE ACTIVITIES IN EVOLVING AUTOIMMUNE VITILIGO LESIONS IN SMYTH LINE OF CHICKENS

Association of herpesvirus infection and immune activities in evolving autoimmune vitiligo lesions in Smyth line of chickens

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Abbreviations:

BL: Brown line; HVT: turkey herpesvirus; IL: interleukin; LBL: light brown leghorn; MC: melanocyte; qRT-PCR: quantitative reverse transcription-polymerase chain reaction; SL: Smyth line; SLV: Smyth line vitiligo; MDV: Marek's disease viruses; IL: interleukin; IFN-gamma: interferon-gamma.

Abstract

The Smyth line (SL) of chicken is an animal model for human autoimmune vitiligo.

Multiple factors contribute to the death of melanocytes (MCs), including genetic

susceptibility to vitiligo (manifested in part as an inherent MC defect), as well as,

environmental factors that trigger autoimmune loss of SL MCs. Live turkey herpesvirus

(HVT, serotype 3 Marek's disease virus; MDV) was identified as the most effective trigger

of SL vitiligo (SLV) expression in susceptible individuals. With the administration of live

HVT at hatch, onset of SLV can be observed between 6 and 20 wks of age and affects 80 -

95 % of SL chickens. However, little is known regarding the role and activities of HVT in

SLV development. Using SL and control chickens this study was conducted to determine

the ability of HVT to induce SLV expression at different ages; examine primary and recall

responses to HVT; and determine the association between HVT viral load and cytokine

production in the target tissue during SLV development and progression. The

SLV-triggering effect of HVT dropped substantially within the first 6 weeks of age and was

no longer observed at 10 weeks and onward. The primary and recall responses were similar

in SL chickens and BL- and LBL-controls in terms of leukocyte infiltration to the site of

HVT injection. Between 4.5 and 7 weeks post-HVT administration at hatch, the relative

HVT viral load (DNA) in SLV chickens was higher than in non-vitiliginous SL and BL

controls and reached peak-levels between 1 to 0.5 weeks before visible onset of SLV.

Moreover, there was a strong direct association between HVT levels, cytokine expression

and SLV onset. Specifically, the increased levels of HVT at 1 to 0.5 weeks before SLV

onset was accompanied by elevated cytokine expression of IL-8, IL-10, IFN-γ, and most

remarkably IL-21, supporting a Th1 phenotype of the autoimmune response. Low relative

expression of IL-4 did not suggest an important role of Th2 cells in SLV pathogenesis.

Taken together this study further supports a role of HVT in the expression of SLV in

susceptible individuals. HVT appears to exert its effect early in the life of a chick when

adaptive immunity is not fully developed. With the translocation of HVT to the feather and

the higher viral load in SL feathers just before SLV onset, the resulting immune activity

may cause cellular stress in the inherently fragile SL MC. Stress-associated MC alterations

may lead to immune recognition of MC and development of MC-specific autoimmune

disease.

Introduction

Vitiligo is an acquired pigmentary disorder characterized by loss of epidermal melanocytes (MCs) (Lerner, 1978) which results in patches of depigmentation, and in some patients, complete depigmentation of the skin and its derivatives. This disorder is found in 0.1 - 2 % of the world's human population. Although the etiopathological mechanisms of this disease are not well understood, it is generally believed that many factors contribute to vitiligo development, including genetic susceptibility, environmental triggers, and immune system involvement (Reza, 2011).

The Smyth line (SL) of chicken, exhibits many phenotypic and etiopathological similarities with human vitiligo including the multifactorial nature of the disorder, high incidence of vitiligo in the genetically susceptible group, and spontaneous onset, *etc*. (Smyth, 1989; Erf, 2010). Together with the Brown line (BL) parental control (< 1 % vitiligo) and Light Brown Leghorn (LBL) vitiligo resistant control the SL chicken model has been established as a very important model for spontaneous autoimmune vitiligo (Erf, 2010; Wick *et al*., 2006). Live HVT is a reliable environmental trigger for vitiligo expression in susceptible SL individuals. Onset of SL vitiligo (SLV) can be observed between 6 to 20 weeks of age and affects 80 - 95 % of SL chickens receiving HVT at hatch. Without HVT administration at hatch, the incidence of SLV drops to < 20 % and under certain housing conditions to < 10%. HVT is an α-herpesvirus that belongs to the group of

Marek's disease viruses (MDV) (Calnek and Witter, 1991). MDVs include three serotypes: MDV serotype-1 (MDV1) consists of oncogenic viruses and their attenuated derivatives; MDV2 consists of related non-oncogenic viruses isolated from chickens; and HVT (MDV3) consists of non-oncogenic viruses isolated from turkey (Erf *et al*., 2001). HVT is used as a vaccine in chickens to protect them from MDV1 infection which causes T-cell lymphomas and numerous mononuclear cell infiltration-associated lesions. Interestingly, all MDV serotypes exhibit special tropism for feather tissue where MCs are located (Holland *et al*., 1998) and all have similar effects on triggering vitiligo expression in susceptible individuals (Erf, personal communication). Target MCs in chickens are located in growing feathers which can be easily removed. Hence, HVT infection and the evolving autoimmune lesion can be monitored prior to and throughout SLV development in the same individual.

Similar to human autoimmune vitiligo, both humoral and cellular immunity play an important role in SLV, with a more prominent role attributed to cellular immunity in MC loss based on phenotypical analysis of leukocytes infiltrating the target tissue (Wang and Erf, 2004; Shresta *et al*., 1997; Erf *et al*., 1995). Moreover, the presence of MC-specific cell-mediated immunity was demonstrated *in vivo* based on the delayed wattle-swelling response to injection of syngeneic MC lysates in chickens with SLV (Wang and Erf, 2003). Immune functional gene expression activities during SLV development were recently shown to be associated with IFN-γ, IL-21 and IL-10 co-expression in evolving

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autoimmune vitiligo lesions of SL chickens, indicating a Th1-polarized immune response (Shi and Erf, 2012). Immune functional activities associated with the environmental trigger, HVT, in SLV development have not been thoroughly examined. Preliminary data showed elevated levels of CD4+ and CD8+ lymphocytes in skin and feather pulp from SL chickens compared to BL chickens during a 1 to 6 week observation period post-HVT vaccination at hatch. Likewise, HVT-vaccinated SL and BL chickens showed higher CD4+ and CD8+ cell infiltration than non-HVT- vaccinated SL and BL chickens in skin and feather pulp, indicating a role of HVT infection in leukocyte infiltration in the feather pulp (Erf, personal communication).

Using the SL vitiligo chicken model, the objectives of this study were to determine the ability of HVT to induce SLV expression at different ages; examine the primary and recall response activities to HVT in SL and control lines; and determine the association between HVT viral load and immune function activities (i.e. cytokine production) in the evolving autoimmune lesion. Specifically, the effect of HVT on SLV incidence was monitored by giving HVT vaccination at hatch or when SL chickens were 2-, 4-, 6-, and 10-weeks of age. Immune responses to HVT in SL, BL and LBL chickens were examined by monitoring leukocytes infiltration in response to a primary and a secondary injection of HVT into growing feathers over a 10 day period. To determine HVT viral load and cytokines gene expression throughout SLV development, growing feather were collected twice a week from the same individuals starting when the chicks were 2.5 weeks of age until SLV was

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fully established. Collected SL and control feather samples were then used for *ex vivo*

analysis. Using quantitative PCR (q-PCR), the presence of relative HVT viral load in

whole cross-section of feathers and in different areas of the cross-sections (MC area,

keratinocyte area, and pulp area) was monitored. Gene expression profiles in collected

feather tips were also established for cytokines of innate immunity (IL-1, IL-8, IL-10),

signature cytokines of T helper (Th)1 and Th2 (IFN-γ and IL-4, respectively) lymphocytes,

a pleiotropic cytokine IL-21, and for HVT-glycoprotein by qRT-PCR. Knowledge gained

from this study will broaden and deepen our understanding of pathogenic mechanisms

involved in the autoimmune loss of MCs in vitiligo development.

Materials and Methods

Animals:

Three lines of MHC-matched ($B^{101/101}$) chickens were used for these studies: 1) the vitiligo-prone SL chickens which exhibits an 80 - 95% incidence of SLV by 20 weeks of age under conventional rearing conditions, 2) the parental BL ($< 1\%$ incidence of vitiligo) control chickens, and 3) the vitiligo-resistant LBL (no incidence of vitiligo) control line of chickens (Erf, 2010). For all studies, each bird was tagged using a color-coded numbering system to identify individual birds and birds from different lines. The chicks were raised in floor pens on wood shavings litter. Food and water were available *ad libitum.* Chicks were reared under standard diet, lighting and temperature protocols and their well-being was checked daily. Vitiligo development was monitored by visual evaluation of pigmentation in growing feathers and assigning a score ranging from 1 to 5 depending on the percentage of growing feathers exhibiting pigmentation loss. [0 % (score 1), < 20 % (score 2), 20-60 % (score 3), 60-99 % (score 4) and 99-100 % (score 5)] All procedures involving animals were approved by the Institutional Animal Care and Use Committee (IACUC) Protocol #08011.

Study 1. SLV incidence post HVT injection at different age of chickens

Experimental design:

Forty newly hatched SL chicks from 35 families were assigned to Group 1 for HVT
administration on the day of hatch. For HVT administration, chicks were injected s.c. with
cell-free lyophilized live HVT vaccine (0.2 mL, est. 8000 pfu) following the
manufacturer's instructions (MD-Vac CFL serotype 3, live, Fort Dodge Animal Health,
Fort Dodge, Iowa). Chicks in Group 1 where then raised under conventional farm
conditions at the Arkansas Experiment Station Poultry Farm in Fayetteville, AR.
One-hundred and twenty HVT-negative siblings of chicks from Group 1 were not HVT
vaccinated and reared in an isolation room equipped with a high efficiency particulate air
(HEPA) filter at the University of Arkansas, Division of Agriculture's Poultry Health
Laboratory in Fayetteville, AR. Under these housing conditions, the incidence of SLV in
HVT-negative chickens has been repeatedly shown to be less than 10 % (Erf, personal
communication). Siblings from Group 1 (40/age group) were injected with HVT at 2-, 4-,
6-, or 10-weeks of age, and then moved to the Arkansas Experiment Station Poultry Farm
in Fayetteville, AR and reared under conventional farm conditions. SLV incidence was
monitored once per week until the chickens were 20 weeks of age.

Study 2. Leukocyte infiltration in response to a primary and second administration of HVT at hatch

Experimental design:

Two groups of SL, BL, and LBL chickens (N = 5 per line and group) were used for this study. The first group (Group 1) was injected with live HVT vaccine (MD-Vac CFL serotype 3, live, Fort Dodge Animal Health, Fort Dodge, Iowa) at hatch and reared on Arkansas Experiment Station Poultry Farm in Fayetteville, AR; The second group (Group 2) was not injected with HVT at hatch (HVT-negative) and reared in an isolation room at the University of Arkansas, Division of Agriculture's, Poultry Health Laboratory in Fayetteville, AR, as described above. When the birds were 18 weeks of age, 10 µL of HVT (16000 pfu) were injected into 8 growing feathers on each breast track using insulin syringes with the BD Ultra-Fine™ needle (Capacity: 3/10cc,30 unit; Length: 8 mm; Gauge: 31 G; NDC/HRI# 08290-8438-01, Becton, Dickinson and Company). Growing feathers were collected before (day 0) and at 1-, 2-, 3-, 5-, 7-, and 10 days after HVT injection and placed into 10% buffered formalin for conventional histology. SL chickens in Group 1 had complete vitiligo with 100% of amelanotic feathers, whereas all of the SL chickens in Group 2 exhibited no sign of SLV at the time of treatment.

Assessment of leukocyte infiltration into HVT injected feathers

Formalin fixed, processed and paraffin-embedded feather samples were cut into longitudinal sections (6 μm thick) and stained with Hematoxylin/Eosin (University of Arkansas, Division of Agriculture, Histology Laboratory). Sections were examined for leukocyte presence and type of leukocyte (granulocyte or mononuclear cells using a bright field microscope (400 x magnification). The intensity of leukocyte infiltration into the ensheathed unit of living feather tissue in response to HVT was assessed based on the proportion of infiltrated to total tissue area (% area) and density of infiltration. Area scores ranging from one to five were given to each feather after visually examination under a bright field microscope (50 x magnification). Area scores one to five were given according to the following infiltration levels: <20 % (score 1); >20 %,<40 % (score 2); >40 %,<60 % (score 3); >60 %,<80 % (score 4); and >80 %,<100 % (score 5). Density scores ranging from zero to three were given depending on the number of leukocytes in 10 squares of a 10 mm by 10 mm ocular insert (1 square) viewed at 50 x magnification under a microscope. The density scores were assigned according to the following criteria: 0-20 cells/square (score 0), 20-100 cells/square (score 1), 100-150 cells/square (score 2), and >150 cells/square (score 3).

Study 3. Examination of relative HVT-glycoprotein (gp) expression in spleen and growing feathers

Experimental design:

Feather tips and spleen from a previous study (Bateman, 2008) were used to examine   the expression of HVT-gp in growing feathers and spleen from SL, BL and LBL chicks that were HVT-vaccinated at hatch. Spleen and growing feathers were collected from 5 to 10 chicks/line at day 5, 8, 12, 15, 21, 28, 35 and 42 post-hatch. Portions of each tissue sample were placed in RNA stabilizing buffer (RNAlater, Quiagen, Valincia, CA) for RNA isolation. These tissues were then placed in the -80°C ultra low freezer for storage until use for RNA extraction, cDNA prearation and qPCR (see Study 4).

Study 4. Assessment of the relative HVT viral load and cytokines profiles in growing feathers throughout vitiligo development

Experimental design:

To study the relative HVT viral load and expression of immune-response related cytokines throughout SLV development and progression from each individual chicken, 18 SL and 6 BL chickens were randomly selected from different families on the day of hatch after HVT vaccination. Two feathers were snap frozen in liquid nitrogen for RNA analysis and four feathers were collected from SL and BL chickens every half week starting when the chicks

were 2.5 weeks of age until stable SLV development in SL chickens. At each collection time, and once per week thereafter until all SL chicks were 20 weeks of age, SLV development was monitored and the extent of depigmentation scored as described above. [20 weeks of age is the natural end-point of SLV development as feather growth, and hence pigmentation, is complete in adult chickens]. Two of the 4 feathers were snap frozen in liquid nitrogen for RNA analysis, the other 2 were placed in embedding medium (Optimal Cutting Temperature, O.C.T, VWR International, Bridgeport, NJ), and snap frozen in liquid nitrogen. All samples were stored at -80°C until use. Based on SLV development in SL chickens by 20 weeks of age, SL samples were placed into two SL groups: SLV and SL no vitiligo (SLnoV).

RNA extraction, quantification, and cDNA synthesis:

Total RNA was isolated from spleen (Study 3) and the 3 mm newest growth of the snap frozen feather tips which contains the epidermal tissue where MCs are located. Tissues were homogenized by Tissue Tearor (BioSpec Products, Bartlesville, OK, Model: 985370-395) RNA was isolated using the RNeasy® Mini Kit (Qiagen, Valencia, CA) and following the protocol for "Purification of Total RNA from Animal Tissues" with modifications.) Following RNA isolation from homogenates with on-column DNA digestion (Qiagen), RNA was eluted in 30 µL RNase-, DNase-free water and stored at -80°C until use. RNA quality and quantity were determined by Experion^{TM} RNA

StardardSens Reagents and Supplies (Bio-Rad Laboratories, Hercules, CA) following

manufacturer's instructions. RNA (175 ng) was reverse transcribed into cDNA by High

Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) in a 10

µL reaction volume according to manufacturer's instruction. A Biometra® personal cycler

(Biomedizinische Analytick GmbH, Germany) was programmed as follows: 25ºC for 10

min, 37ºC for 2 h, 85ºC for 5 min and 4ºC holding temperature. Then cDNA was stored at

-80ºC until further analysis.

Feather Section Collection Using Laser Capture Microdissection (LCM):

Frosted glass slides were first wiped with 70% alcohol (70 volume absolute alcohol and 30

volume RNase-, DNase- free water; MP Biomedials, LLC, Solon, Ohio). After being

air-dried at room temperature, the slide was wiped with RNase *AWAY®* (Molecular

Bioproducts, Inc., San Diego, CA) in a hood to remove RNase and DNA contamination.

Frozen O.C.T blocks containing feather tips were trimmed and placed onto a tissue holder

in the proper cutting orientation to obtain cross-sections starting at the bottom of the

feather tip (newest growth). Frozen sections were cut at -20ºC (12 µm thick) in the cryostat

and were closely checked for melanin deposition under a bright-field microscope. Actual

section collection onto pre-treated and pre-cooled slides started shortly after the first

sections with a clearly pigmented barb ridge was identified. Four sections were placed onto

each slide. Slides were then exposed to the following dehydration procedure: 30 s in 75%

alcohol, 30 s in 95% alcohol, l min in each of two 100% alcohol baths and 5 min in each of two xylene baths while it was acceptable that slides were kept in the last xylene bath for up to 2 h.

LCM was carried out with a P.A.L.M. microlaser systems (P.A.L.M. Microlaser Technologies AG, Germany) including PALM® Robo Software microlaser systems version 3.0-0804 (EN) (P. A. L. M. Microlaser Technologies AG, Germany) and PALM® Micro Beam version 1104 Z (P. A. L. M. Microlaser Technologies AG, Germany). Sections were viewed under 200 x magnification. The barb ridge area, where MC are located at the interface of the pulp and the barb ridge (MC-area) and the area where keratinocytes are located at the interphase of the outer sheath and the barb ridge (keratinocyte-area), and the pulp area were premarked using the Freehand Tool. MC-, keratinocyte- and pulp-area were cut and catapulted into Adhesive Cap (Carl Zeiss Microimaging GmbH, Germany) using the function of AutoLPC. Parameters for laser functions were as follows: UV energy 72; UV focuses 69; LPC energy 91; Distance of autoLPC shots 12 um; stage movement 100steps/sec. Collecting tubes were kept in -80 ºC before next step of DNA extraction.

DNA extraction and quantiation from LCM samples:

The Pico Pure^{TM} DNA Extraction Kit (Applied Biosystems, Foster City, CA) was used to extract DNA from LCM collected MC-, keratinocyte- and pulp- area cells. Samples were incubated with 30 µL of PicoPure DNA Extraction Buffer containing Proteinase K at 65°C

for 16 h, followed by 95°C for 10 min to inactivate Proteinase K. Then, sample tubes were

transferred on ice for use in qPCR, either directly after collection or after storage at -80°C.

Taqman® quantitative PCR (qPCR):

qPCR was performed on the cDNA samples (primers and probes are shown in Table 1) in

25 μL reaction volume on a ABI PRISM 7300 sequence detection system (Applied

Biosystems, Foster City, CA) using the Universal PCR Master Mix (Applied Biosystems,

Foster City, CA) and following manufacturer's instruction. In each plate, a calibrator

sample (a pool of cDNA from age-matched BL birds), a non-template control, and sample

cDNA were included. 28S ribosomal RNA was the endogenous control gene. Cycling

profiles used were 94°C for 15 min, and 50 cycles of denaturation at 94°C for 60 s, and

annealing/extension at 60°C for 60 s. The relative gene expression was determined by the

delta delta Ct *(ΔΔCt)* method (Wong and Medrano, 2005). DNA quality and concentration

were determined by NanoDrop 1000 Spectrophotometer from Thermo Scientific.

Statistical Analysis

Statistical analysis was carried out using JMP Pro 9 (JMP Software Inc., Cary, NC).

Two-way ANOVA was used to examine the effects of line of chicken, time, and line by

time interaction on the relative expression of each target gene, HVT viral load, area and

density of leukocyte infiltration. In the presence of significant main effects for line and/or

time, and the absence of line by time interactions, Fisher's LSD multiple means

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comparison was used to determine differences among group means. P values ≤ 0.05 were

considered significant. Data were reported as means $\pm$ SEM.

Results

SLV incidence following HVT-administration at different ages post-hatch

Ninety-two % of SL chicks injected with live HVT at hatch (one day old) and raised under conventional farm conditions developed SLV, whereas HVT-negative (not injected with HVT at hatch) siblings raised in floor pens in an isolation room equipped with a HEPA filter had an SLV incidence of <10%. The SLV incidence of SL siblings injected with live HVT at 2-, 4-, 6-, and 10- weeks post-hatch were 72.5%, 52.5%, 35%, and 0% respectively at 20 weeks of age (Figure 3).

Leukocyte infiltration into the pulp of HVT injected growing feathers in HVT-vaccinated and -unvaccinated 18-week-old chickens

Leukocyte infiltration in response to a primary or second exposure of HVT was found to be mononuclear in nature. For the primary response to HVT injection, compared to pre-injection levels, the area score of mononuclear infiltration (lymphocytes and macrophages) in SL feathers increased ($P < 0.05$) at 1 d, reached peak levels ($P < 0.05$) at 5 d, and dropped ($P < 0.05$) to 1 d levels at 7 d, and remained at this level at 10 d. By comparison, in BL and LBL feathers, area score increased ($P < 0.05$) at 1 d and remained at this high level throughout the 10 d sampling period (Figure 4A). Compared to BL and LBL controls, area scores for the primary response were higher in SL feathers at 5 d (Figure 4A).

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In the primary response, density score of SL, BL, and LBL feathers increased (P < 0.05) at 1 d compared to pre-injection levels and remained at this high level throughout the 10 d sampling period (Figure 4B). There was no difference in density score among lines (SL, BL, and LBL; Figure 4B).

For the secondary (recall) response to HVT injection, compared to pre-injection levels, the mononuclear cell area score and density score in SL, BL and LBL feathers increased (P < 0.05) at 1 d stayed at this high level on 2 d, dropped (P < 0.05) at 3 d, and remained at this still elevated level throughout the 10 d sampling period. With the exception of higher (P < 0.05) area and density scores for BL on day 10, there were no line differences in leukocyte infiltration during the recall response to HVT 18-weeks post-primary HVT administration at hatch (Figure 4C and 4D).

The relative HVT viral load in cells from the whole- and MC-, keratinocyte- and pulp-area of feather sections from SLV, SLnoV and BL chickens post-HVT injection at hatch

Due to the dramatic change of HVT viral load at different observation time, data were converted to base 10 logarithmic values.

There were no differences in the relative HVT viral load in SL (SLV and SLnoV) and BL feathers before 4 weeks post-HVT vaccination at hatch. Specifically, compared to viral

load levels at 2.5 week, the relative HVT viral load in SL and BL decreased (more than

1000 fold change; P < 0.01) at 3 wks post-HVT vaccination, increased (P < 0.01) at 3.5 wks,

and reached peak-levels at 4 wks (Figure 5). The relative HVT viral load in SLV chickens

remained at a constant high level after 4 wks until a drop (P < 0.01) at 7 wks, and remained

at the lower level thereafter (Figure 5); whereas, in SLnoV and BL feathers, HVT levels

decreased (P < 0.05) from 4 wks to 4.5 wks (Figure 5), and remained at lower level until a

further drop at week 7.5 (Figure 5). Generally, compared to BL and SLnoV feather viral

load, the levels in the SLV chickens were higher from week 4.5 to week 7 (Figure 5).

With each vitiliginous chicken developing SLV at different weeks of age, data for each

SLV bird were aligned with respect to time of visible SLV onset for time course

comparisons. As data from SLV and BL feathers collected at the ages as SLV data

(corresponding to -4.5 wks and +1.5 wks with respect to SLV onset in SL) did not fluctuate

significantly (P > 0.1), the mean ± SEM across age was used for comparison to SLV data

(Figure 6 and 9). With respect to vitiligo onset in SLV feathers, the relative HVT viral load

in SLV increased (P < 0.05) starting at 4 wks, reached peak levels at -1 and -0.5 wks, then

decreased (P < 0.05) starting at onset (0 wks) and reached the lowest level at 1.5 wks. HVT

levels in SLV feathers were higher (P < 0.05) than the mean HVT levels in BL and SLnoV

feathers (Figure 6). The expression of HVT-gp could only be detected in the spleen and not

in feathers from all three lines of chicken (Figure 8). The pattern of expression of HVT-gp

in the spleen tended to be biphasic in SL and BL spleens (Fig. 8B). Specifically in SL

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spleens, relative HVT-gp expression levels peaked at 8 d, drop to low levels at 15 d then increased again at 21 d reaching higher levels compared to controls (BL and LBL; $P < 0.05$) at 28 to 42 days of age. Due to large variation in HVT-gp expression there was no statistical difference over time in HTV-gp expression levels in BL and LBL spleens. Correlation analysis of HVT load in different areas of feather sections with that from entire feather sections revealed a stronger relationship between relative amount of HVT in the MC-area and whole feather cross-sections for SLV ($R^2 = 0.69$) and SLnoV ($R^2 = 0.68$) chickens than for BL ($R^2 = 0.43$) chickens (Figure 7). The viral load in most of the samples collected from the keratinocyte-area and the pulp-area could not be determined due to undetected levels of HVT DNA in the qPCR assay (data not shown).

Profiles of cytokine and tyrosinase (TYR) expression at different weeks post-HVT vaccination at hatch and throughout SLV development

Owing to limited availability of chicken-specific antibodies, cytokine and TYR expression was examined at the transcriptional level using quantitative reverse-transcription real-time PCR (qRT-PCR). The relative expression was calculated by *ΔΔCt* method using a cDNA pool made from RNA of 16 growing feather of BL chickens from each time point as the calibrator and chicken 28S as the endogenous control gene. Data were expressed as fold change at different weeks post HVT vaccination. Starting at 6 weeks of age, IL-8, IL-10, IL-21, and IFN-gamma showed elevated expression in feathers from SLV chickens and

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reached peak levels near 8 weeks of age, whereas, in SLnoV and BL feathers expression of

these cytokines did not change significantly during the observation period (Appendix 1).

The expression of IL-1 in SLnoV and SLV was slightly increased at 6 weeks, and then

remained elevated level until 9 weeks of age, whereas, the expression of IL-1 in BL was

similar with the exception of increased expression at 8 weeks (Appendix 1). The

expression level of IL-4 was similar over the entire observation period in BL, SLnoV, and

SLV feathers, except a high value at 8 weeks in BL chickens. TYR expression in feathers

from all birds fluctuated with age but did not differ significantly between lines of chickens

(Appendix 1).

As each vitiliginous chicken developed SLV at different weeks of age, SLV data were

expressed with respect to onset (0 time) to demonstrate the general trend of cytokine and

TYR expression in SLV development and progression. Mean data, pooled across time for

BL and SLnoV samples were used for comparison with SLV data (see HVT section above).

In the feather tips collected from SLV chickens, IL-1, IL-4 and TYR were similar to those

of BL and SLnoV samples at all time-points, except a peak increase of IL-1 at 1 wk after

the onset of vitiligo and extremely low levels of TYR in white SLV feathers (complete

SLV). Relative expression of IL-8, IL-10, IL-21 and IFN-γ in SLV samples exhibited

greater than 3 fold higher levels than BL and SLnoV samples (Figure 9). During or near the

onset of SLV, parallel increases in the relative expression of IL-8, IL-10, IL-21 and IFN-γ

could be observed in the SLV feathers, with higher expression levels than those in

age-matched BL and SLnoV feathers. These four cytokines simultaneously reaching peak

levels and remaining elevated until 3 wks post SLV onset (Figure 9). The relative

expression of chemokine IL-8 and cytokines IL-10, IL-21 and IFN-γ emerged as the

signature cytokine profile during the early stage and active progression of SLV in feathers

from all vitiliginous chickens. Comparison of SLnoV and BL main effects means

(averaged over time) revealed higher levels of IL-21 and IFN-γ in feathers of SLnoV

chickens (Table 2).

Table 1. Primer[1] and probe[1,2] sequences for target genes examined in the relative expression analysis

Target	Primer/Probe	Sequences (5' to 3')	Accession NO.
28S	Forward	GGCGAAGCCAGAGGAAACT	X59733
	Reverse	GACGACCGATTTGCACGTC	
	Probe	AGGACCGCTACGGACCTCCACCA	
IL-1	Forward	GCTCTACATGTCGTGTGTGATGAG	AJ245728
	Reverse	TGTCGATGTCCCGCATGA	
	Probe	CCACACTGCAGCTGGAGGAAGCC	
IL-4	Forward	AACATGCGTCAGCTCCTGAAT	AJ621735
	Reverse	TCTGCTAGGAACTTCTCCATTGAA	
	Probe	AGCAGCACCTCCCTCAAGGCACC	
IL-8	Forward	GCCCTCCTCCTGGTTTCA	AJ009800
	Reverse	TGGCACCGCAGCTCATT	
	Probe	TCTTTACCAGCGTCCTACCTTGCGACA	
IL-10	Forward	CATGCTGCTGGGCCTGAA	AJ621614
	Reverse	CGTCTCCTTGATCTGCTTGATG	
	Probe	CGACGATGCGGCGCTGTCA	
IL-21	Forward	GTGGTGAAAGATAAGGATGTCGAA	NM_001024835.1
	Reverse	TGCCATTCTGGAAGCAGGTT	
	Probe	TGCTGCATACACCAGAAAACCCTGGG	
IFN-γ	Forward	GTGAAGAAGGTGAAAGATATCATGGA	Y07922
	Reverse	GCTTTGCGCTGGATTCTCA	
	Probe	TGGCCAAGCTCCCGATGAACGA	
TYR	Forward	ATAATGCCCTTCACATCTACATGAAT	NM_204160.1
	Reverse	GCTCAAAAATGCTGTCAACAAATG	
	Probe	CTCAATGTCCCAAGTACAAGGCTCTGCG	

[1] Primer and probe oligos were synthesized by MWG Biotech, Hight Point, NC.

[2] Probes had FAM (6-carboxyfluorescein) at 5' end and TAMRA (6-carboxytetramethylrhodamine) at 3' end.

Table 2. The line of chicken main effects on the relative expression[1] of target genes from BL and SL chickens without vitiligo.

Gene	BL	SL without vitiligo	Main effect (P-value)
IL-1	1.40 ±0.26	1.73 ± 0.20	0.31
IL-4	1.62 ± 0.43	1.15 ± 0.34	0.39
IL-8	1.00 ± 0.16	1.13 ± 0.12	0.49
IL-10	0.83 ± 0.49^a	2.40 ± 0.39^b	0.01
IL-21	0.11 ± 0.09^a	0.62 ± 0.07^b	<0.001
IFN-γ	0.58 ± 0.11^a	1.38 ± 0.09^b	<0.001
TYR	2.85 ± 0.29	2.50 ± 0.23	0.35

[1]Relative expression of targets was calculated by the delta delta Ct method of data obtained by real-time PCR, where S28 served as endogeneous control gene and a pool of cDNA from 16 growing feather of BL chickens from each time point was used as the calibrator sample. Data were analyzed by Fit Y by X of the JMP 9.0 software and were shown as mean ± SEM.

[a,b] For each target gene, means without a common letter are significantly different (P < 0.05).

Table 3. The line of chicken main effect on relative HVT viral load[1] in whole feather sections and melanocyte-areas from SL and BL chickens.

HVT Load Location	BL	SL	Main Effect (P-value)
Whole Feather Sections	0.21 ± 0.14	0.43 ± 0.09	0.18
MC Areas	-1.60 ± 0.25	-1.36 ± 0.14	0.41

[1]Relative amount of HVT DNA was calculated using the delta delta Ct method of data obtained by Real-time PCR, where S28 served as endogeneous control gene and a pool of cDNA from 11 whole feather sections of BL chickens from each time point was used as the calibrator sample. Data were analyzed by Fit Y by X of the JMP 9.0 software and were shown as mean $\pm$ SEM.
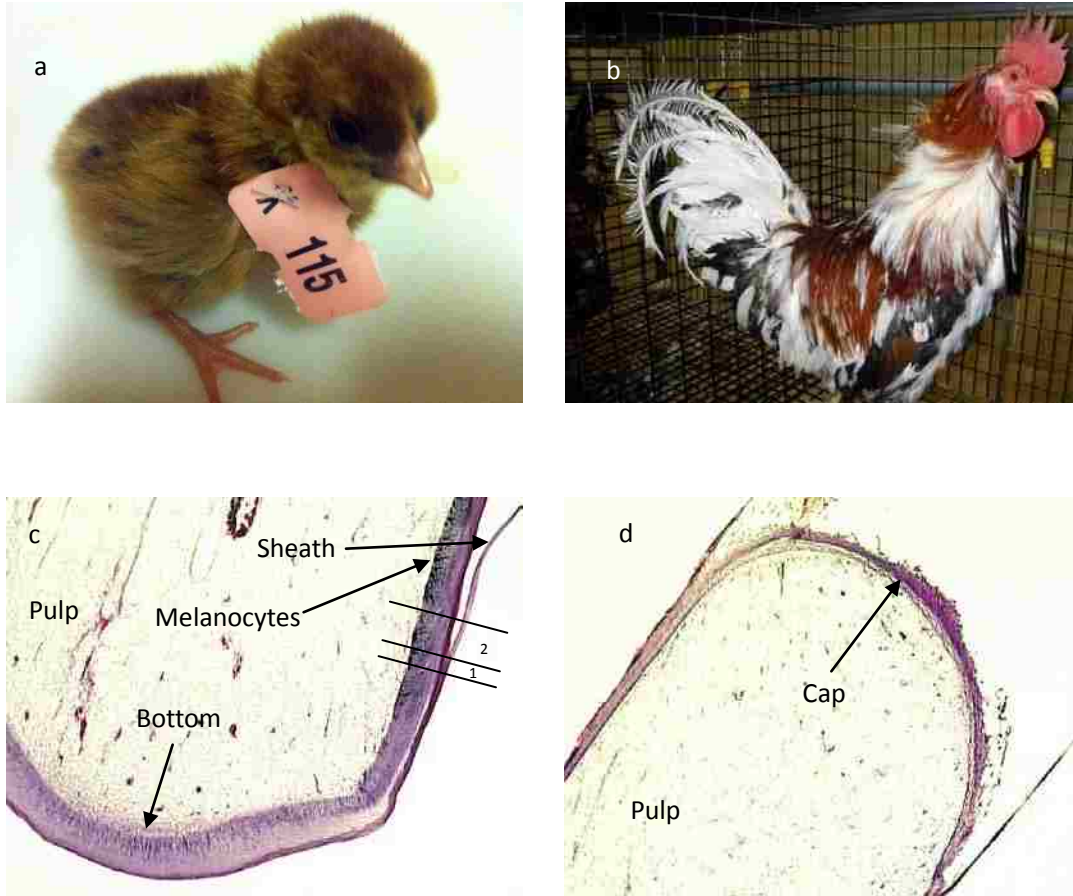
Figure 1. SL chickens and morphology of their 2- to 3-week-old growing feathers. a) SL chicks hatch with normal pigmentation. b) SL chicken with vitiligo; loss of feather pigmentation due to loss of melanocytes (MCs) in the feather. The ensheathed unit of living tissue of growing feathers is approximately 8-10 mm in length and 1-3 mm in width (depending on the age of the chicken). c) bottom newest growth of feather, d) oldest growth of feather with epidermal cap. Most of the tissue consists of the inner pulp (dermis). Sections were stained with Hematoxylin/Eosin stain and viewed at 50x magnification using a bright-field microscope. c) also shows 1) the portion of the feather tip where12 µm cross sections were taken for the LCM study and 2) where the 100 µm cross sections for HVT viral load determination (whole feather sections) were cut.

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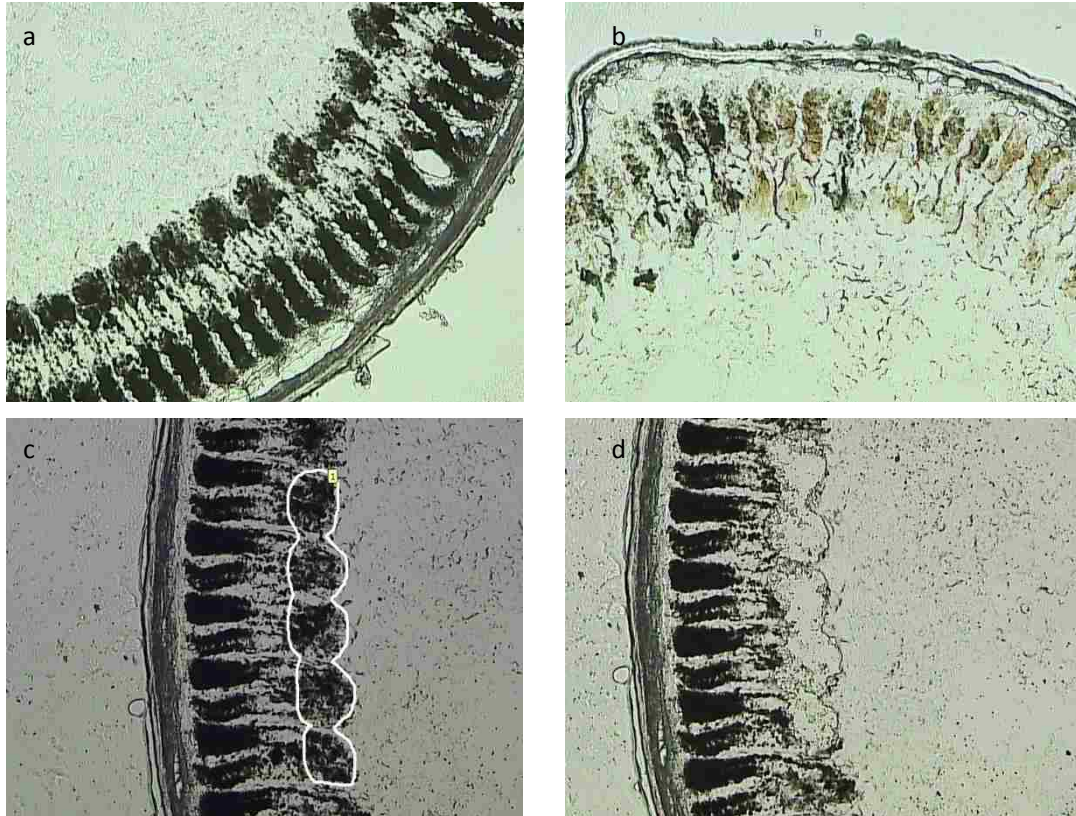
Figure 2. Morphology of melanocytes (MCs) in feather cross-section from SL chickens. The unstained cross-sections of snap frozen feathers showing MC morphology in a) the normally pigmented and b) active vitiligo SL barb ridge area of a feather tip cross section. c) and d) shows the dehydrated feather cross-sections before and after removal of MC-area in the barb ridge where the MC cell bodies are located. Sections were viewed at 200x magnification under a bright field laser capture microscope.
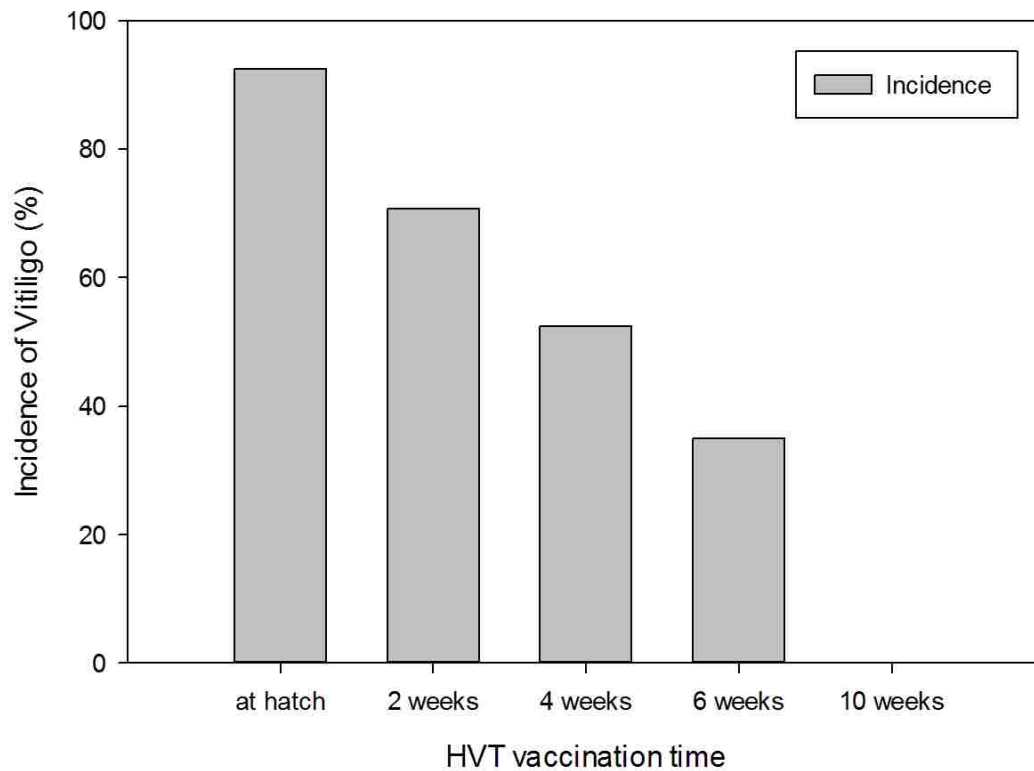
Figure 3. SLV incidence following HVT-administration at different ages post-hatch. The sibling of SL chicks (N = 40/group) were injected with live HVT at hatch or at 2-, 4-, 6-, and 10 weeks post-hatch. Non-vaccinated chicks were raised in isolation room (BSL 2) equipped with a high efficiency particulate air filter until HVT administration. Following HVT vaccination SL chicks were then moved to conventional farm conditions. SLV incidence was monitored until chickens were 20 weeks of age.
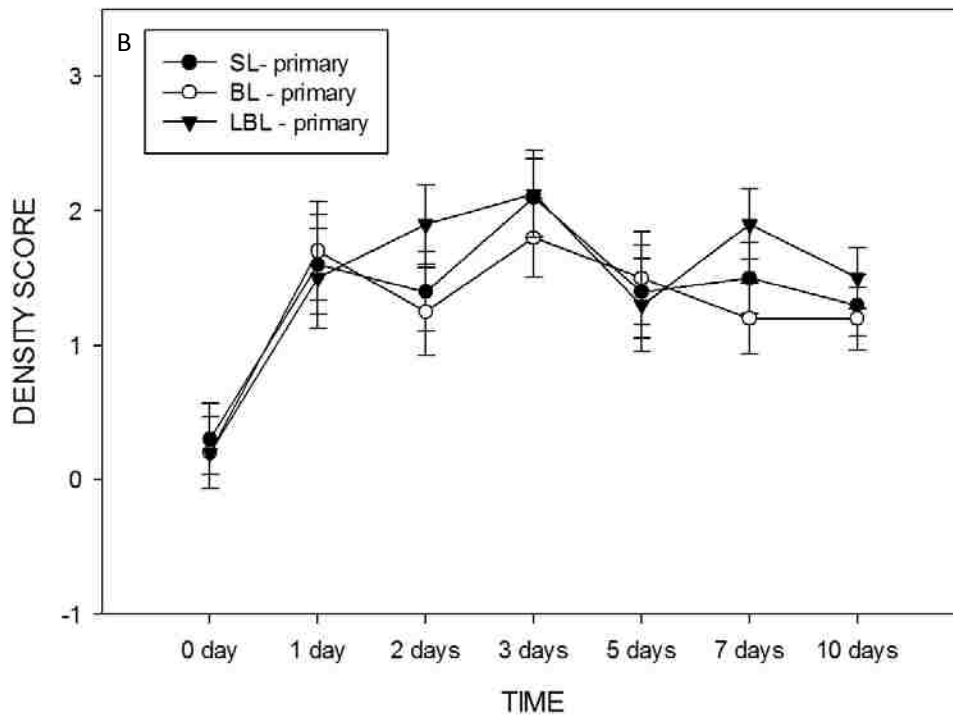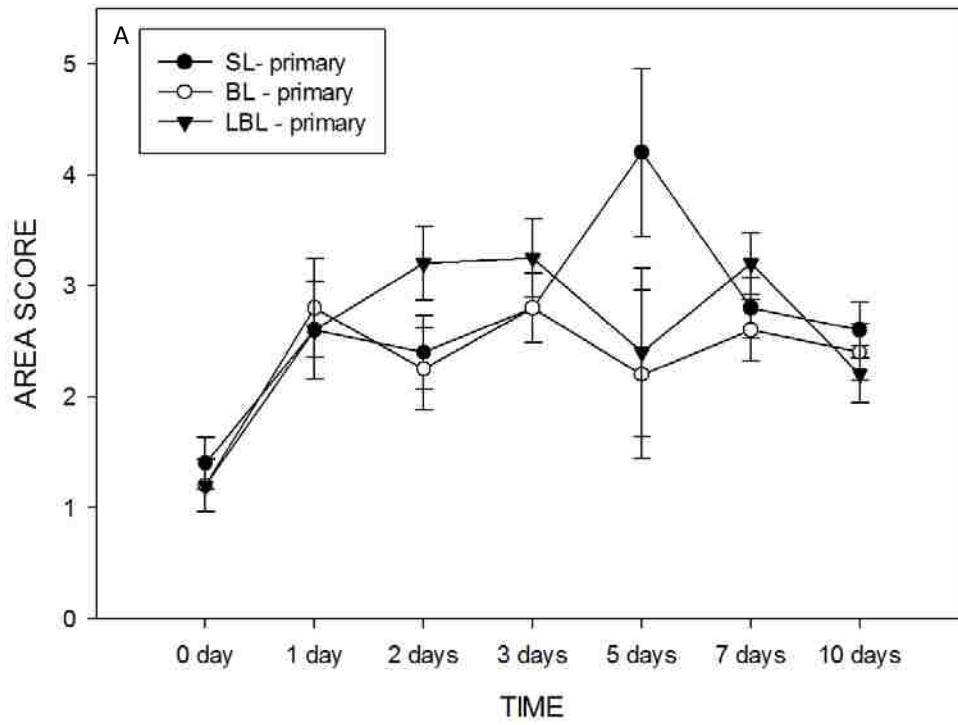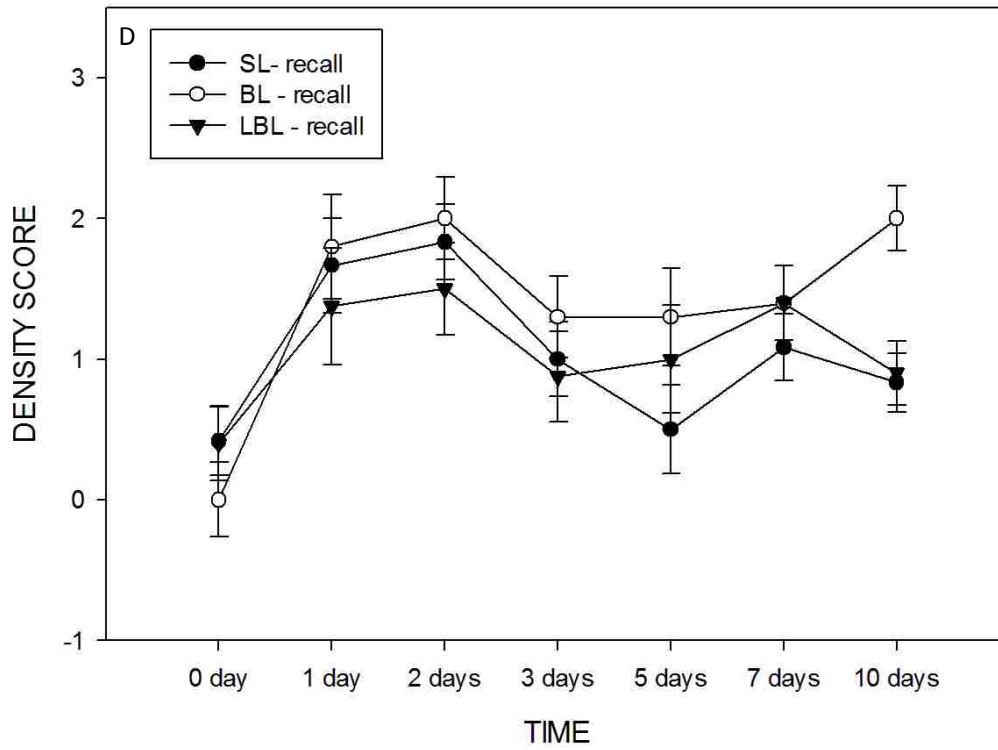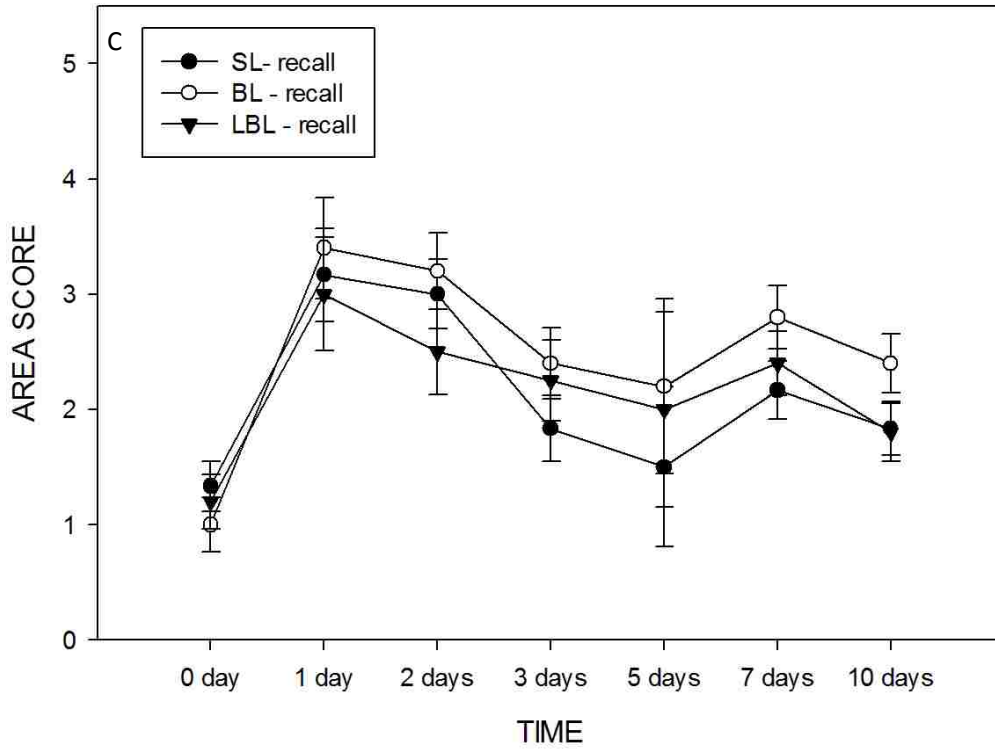
Figure 4. Mononuclear cell infiltration in the pulp of growing feathers injected with HVT. Growing feathers of Smyth line (SL), Brown line (BL), and Light Brown Leghorn (LBL) chickens were injected with 10 µL HVT (16000 pfu) into the feather pulp. Injected feather were collected at 1-, 2-, 3-, 5-, 7-, and 10-days post-HVT vaccination and preserved in 10% buffered formalin for conventional histology. Sections were stained with Hematoxylin/Eosin stain. The area scores ranging from one to five was given to each feather after visually examination according to the following infiltration: <20% (score 1); >20%,<40% (score 2); >40%,<60% (score 3); >60%,<80% (score 4); and >80%,<100% (score 5). Density scores ranging from zero to three were given depending on the number of leukocytes in 10 squares of a 100 square (10 mm by 10 mm) ocular insert viewed at 50 x magnification under a microscope. The density scores were assigned according to the following criteria: 0-20 cells (score 0), 20-100 cells (score 1), 100-150 cells (score 2), and >150 cells (score 3). Data were expressed as mean ± SEM.
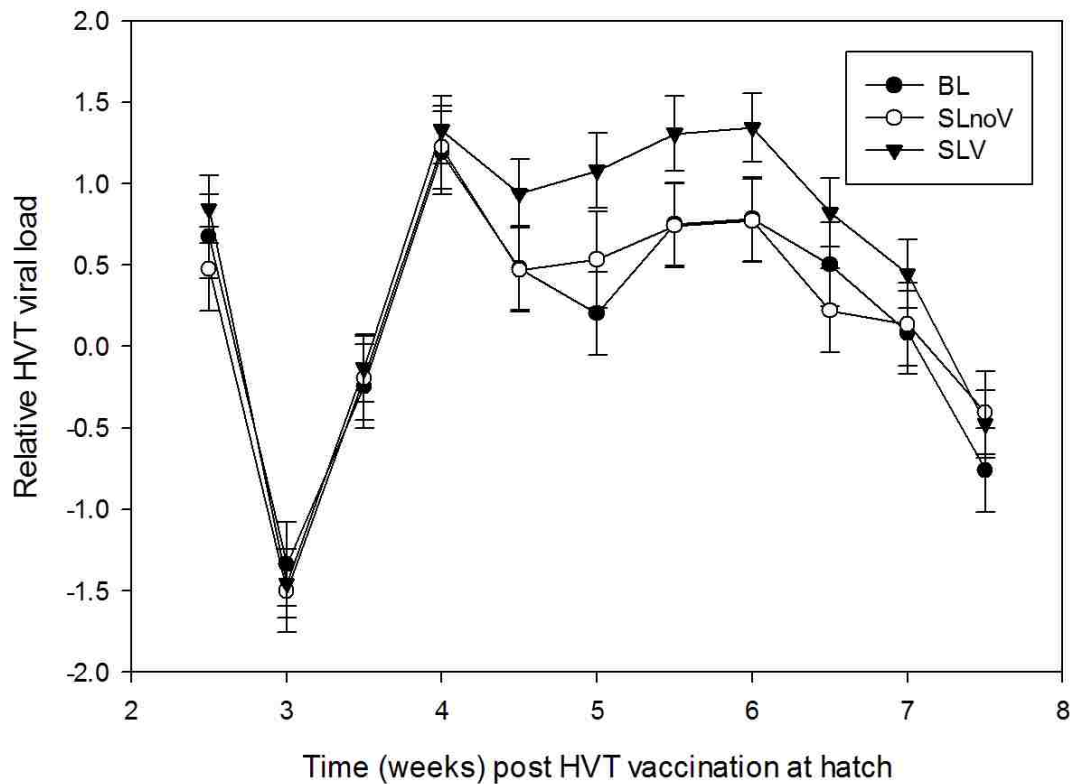
Figure 5. The effect of time (weeks post-HVT vaccination) on the relative amount (fold change) of HVT DNA in whole feather sections from HVT vaccinated SLV, SLnoV, and BL chicks. One-day-old chicks from the vitiligo-prone SL and parental BL chickens were vaccinated with HVT and feather samples were collected at 2.5, 3, 3.5, 4, 4.5, 5, 5.5, 6, 6.5, 7, and 7.5 weeks post-vaccination. DNA from three whole 100 µm cross-sections of feather sample was extracted using Pico Pure^{TM} DNA Extraction Kit (Applied Biosystems, Foster City, CA). The relative amount of HVT DNA present in each sample was then quantified by real-time PCR using an ABI PRISM 7700 sequence detection system (Applied Biosystems, Forest City, CA). For each sample, the relative amount of HVT DNA was calculated using the *ΔΔCt* method described by Applied Biosystems (Forest City, CA) and data expressed as the fold difference with respect to a calibrator sample from 11 whole feather cross-sections collected from BL chickens from each time point. For each time point, data shown are mean ± SEM each BL (N = 4), SLnoV (N = 4), SLV (N = 6) chickens

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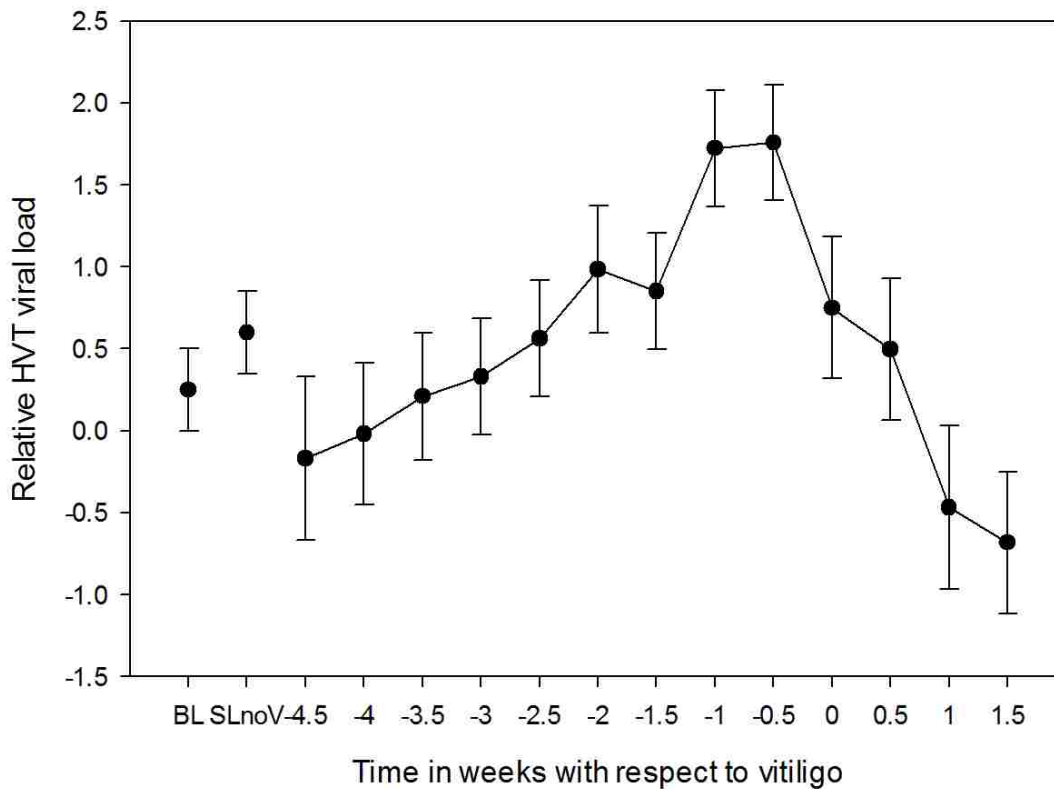
Figure 6.Time course with respect to vitiligo onset of the relative amount (fold change) of HVT DNA in the whole feather sections from HVT vaccinated SLV, SLnoV, and BL chicks. One-day-old chicks from the vitiligo-prone SL and parental BL chickens were vaccinated with HVT and feather samples were collected at 2.5, 3, 3.5, 4, 4.5, 5, 5.5, 6, 6.5, 7, and 7.5 weeks post-vaccination. DNA from three whole 100 µm cross-sections of feather sample was extracted using Pico Pure^{TM} DNA Extraction Kit (Applied Biosystems, Foster City, CA). The relative amount of HVT DNA present in each sample was then quantified by real-time PCR using an ABI PRISM 7700 sequence detection system (Applied Biosystems, Forest City, CA). For each sample, the relative amount of HVT DNA was calculated using the *ΔΔCt* method described by Applied Biosystems (Forest City, CA) and data expressed as the fold difference with respect to calibrator sample from 11 whole feather sections of BL chickens from each time point. The X-axis represents the time (weeks) of feather cross-section collection with respect to vitiligo onset (0), BL and SLnoV represent the pooled data from feather cross-sections collected from age-matched BL and SLnoV throughout the course of the study. Data are mean ± SEM of relative amount of HVT in feathers from 6 SLV, 4 SLnoV and 4 BL, chickens.
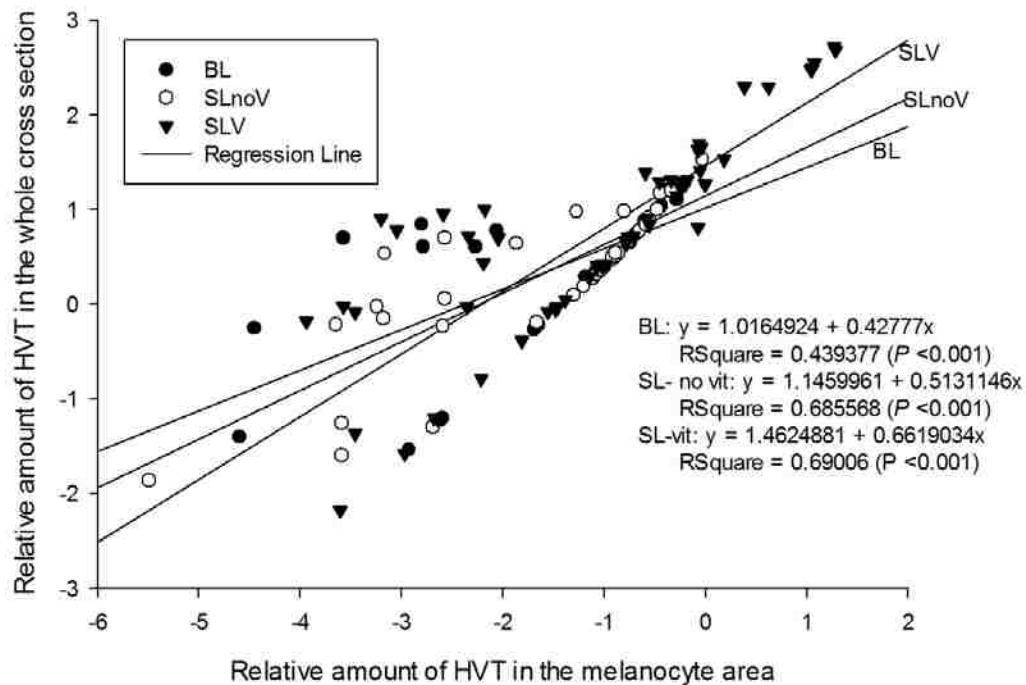
Figure 7. Relationship between relative amount of HVT in the melanocyte (MC) area and in the whole cross section of feather samples. One-day-old SL and BL chicks (N=10 and 4 per time point, respectively) were vaccinated with HVT at hatch and feather samples were collected at 2.5, 3, 3.5, 4, 4.5, 5, 5.5, 6, 6.5, 7, and 7.5 weeks post-vaccination. DNA from three whole 100 µm cross-sections of each feather sample and from MCs collected by LCM (three 12 µm cross sections) was extracted using Pico Pure^{TM} DNA Extraction Kit (Applied Biosystems, Foster City, CA). The relative amount of HVT DNA present in each sample was then quantified by real-time PCR using an ABI PRISM 7700 sequence detection system (Applied Biosystems, Forest City, CA). For each sample, the relative amount of HVT DNA was calculated using the $\Delta\Delta Ct$ method described by Applied Biosystems (Forest City, CA) and data expressed as the fold difference with respect to a calibrator sample from 11 whole feather cross-sections of BL chickens from each time point. The data were transformed to Log_{10}.
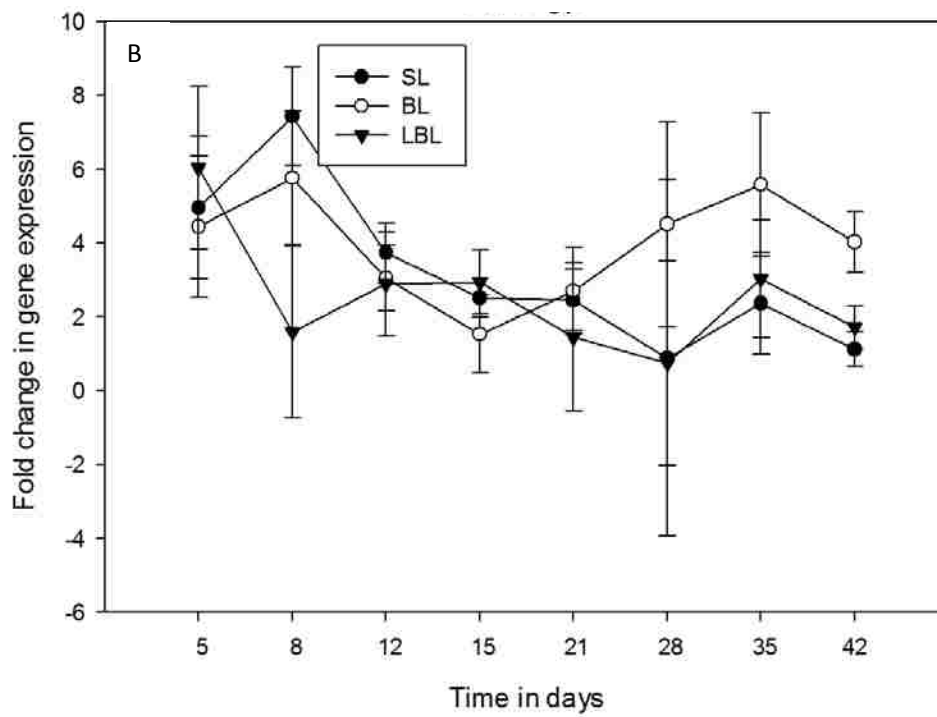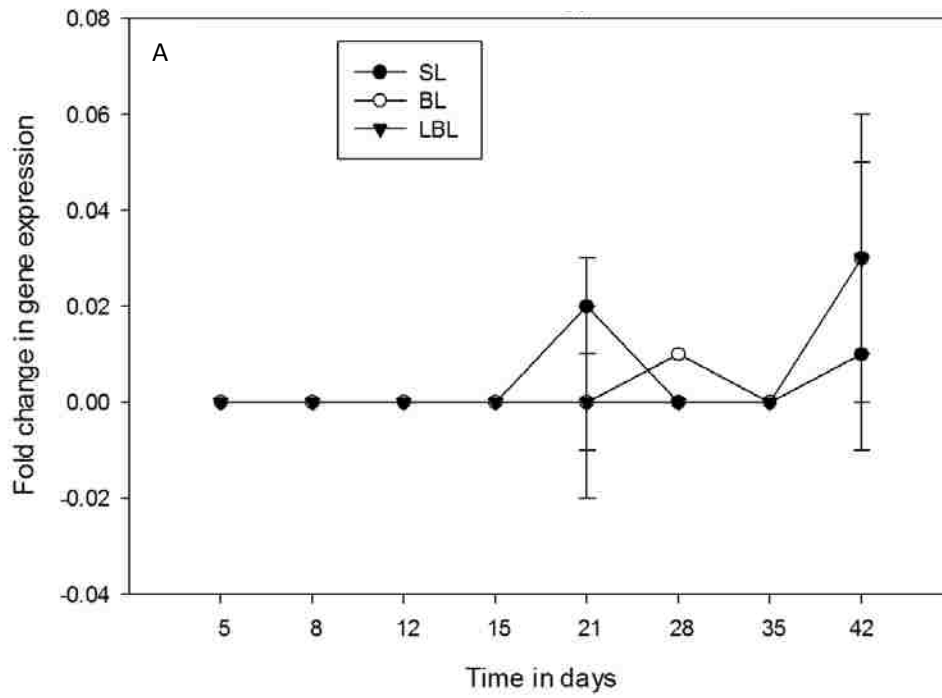
Figure 8. Time course of HVT glycoprotein (HVT-gp) gene expression in A) feather and B) spleen collected from SL, BL, and LBL chickens post-HVT vaccination at hatch. One-day-old chicks from the vitiligo-prone SL, parental BL and LBL chickens (N=40) were vaccinated with HVT at hatch. Feather and spleen samples were collected from 5 euthanized birds from each line at 5, 8, 12, 15, 21, 28, 35, and 42 days post-vaccination. Relative expression was calculated by the $\Delta\Delta C_t$ method, using a cDNA pool made from 8 growing feather of BL chickens from each time point as the calibrator and chicken 28S as the endogenous control gene. The *X*-axis represents the time (days) of feather tip collection post HVT vaccination. Data were reported as means ± SEM.

A

BL SLnoV

B

BL SLnoV

175

D

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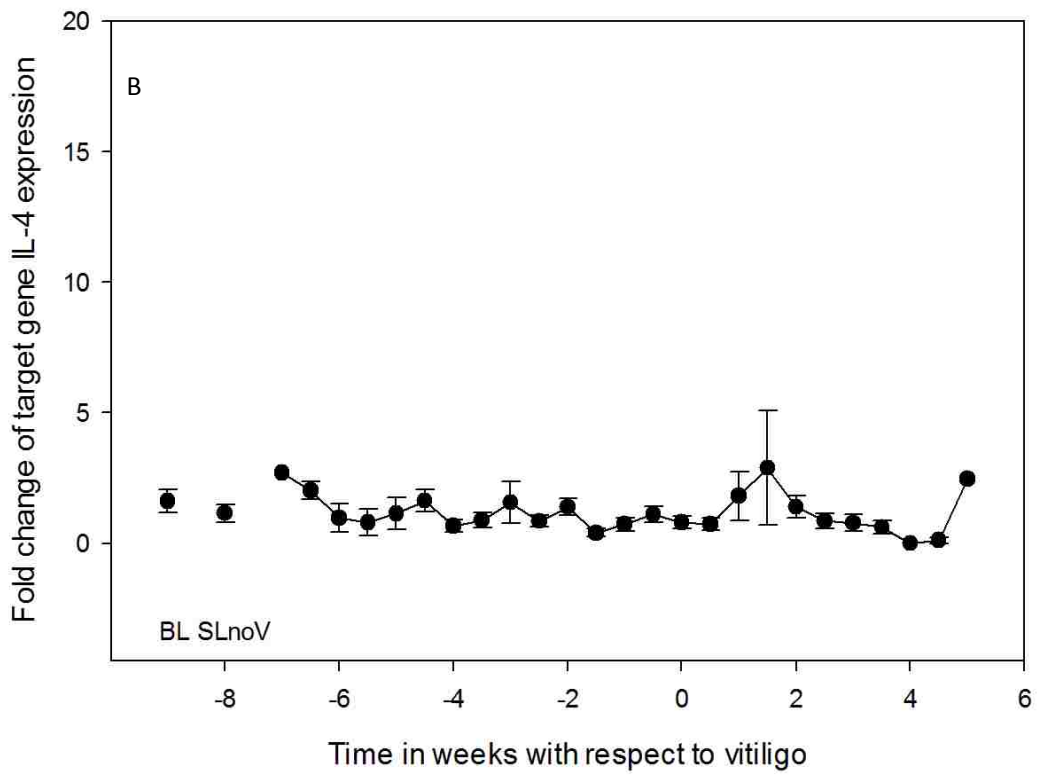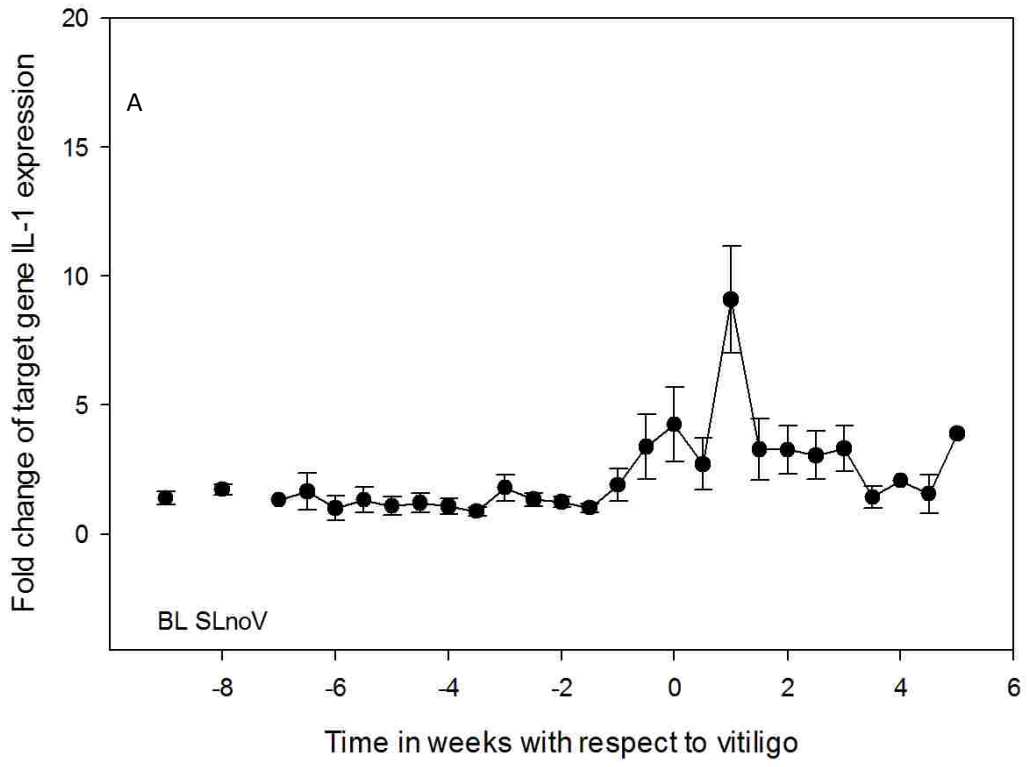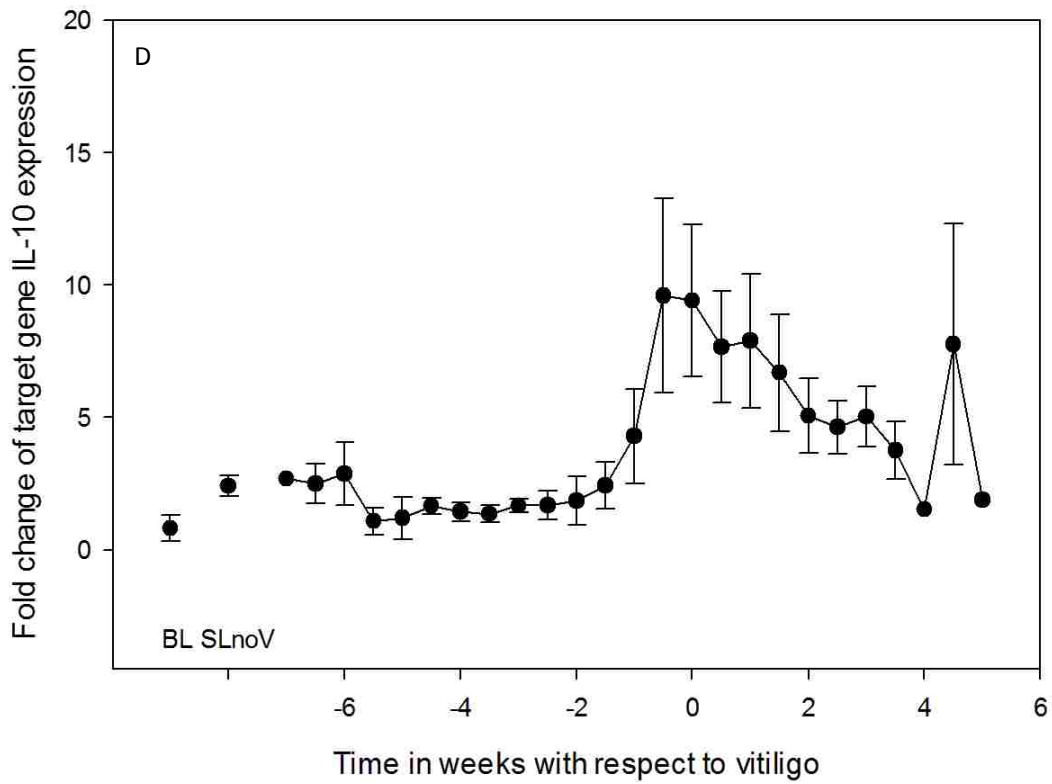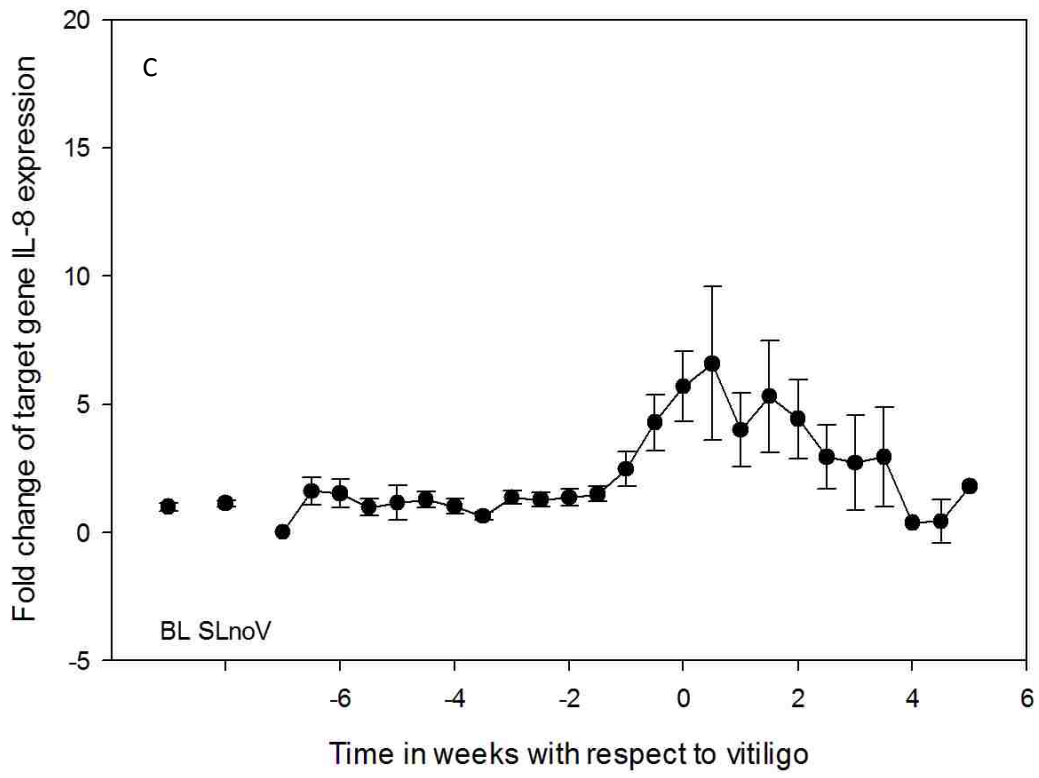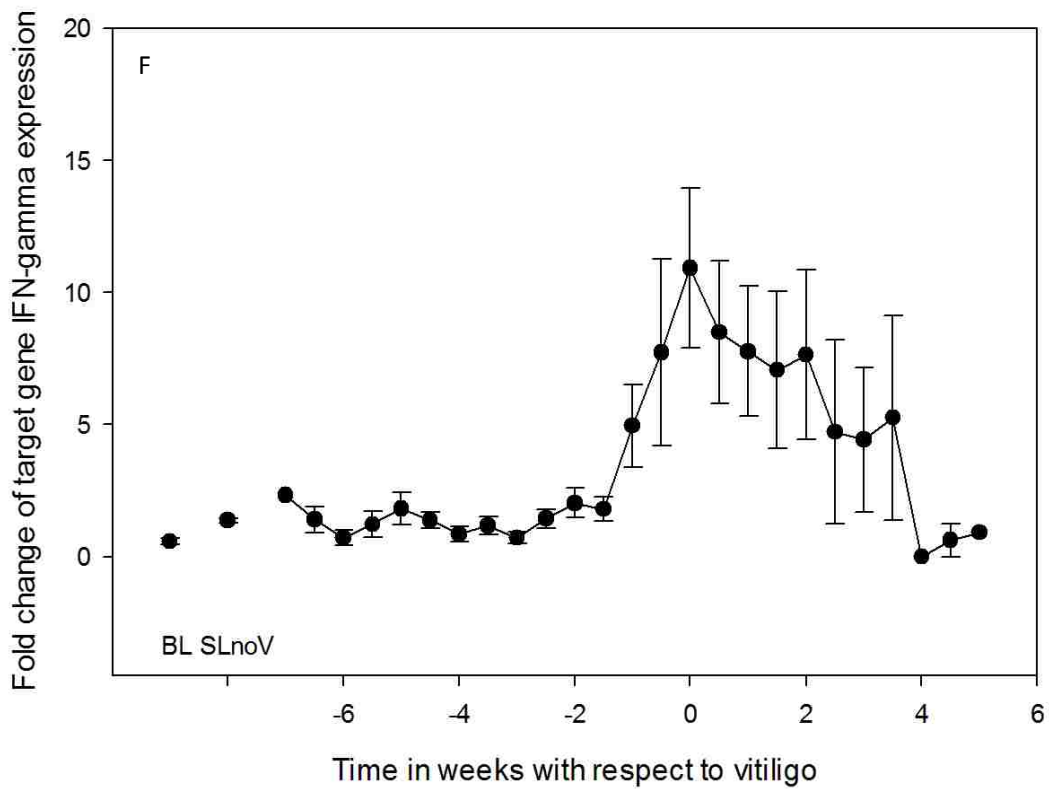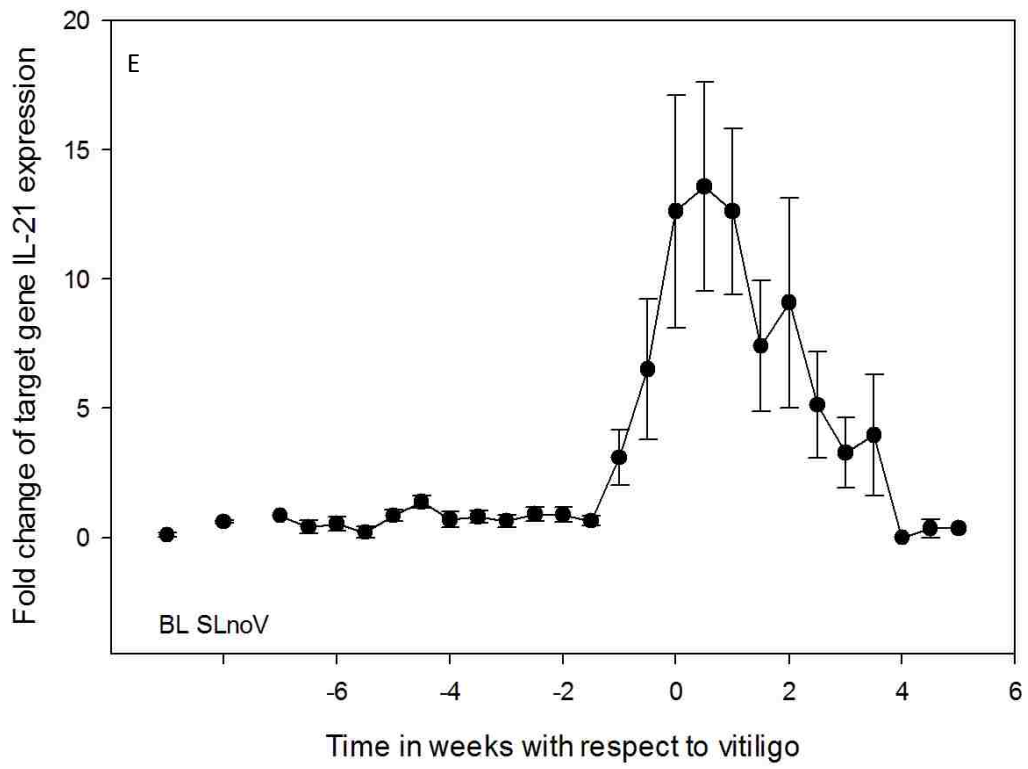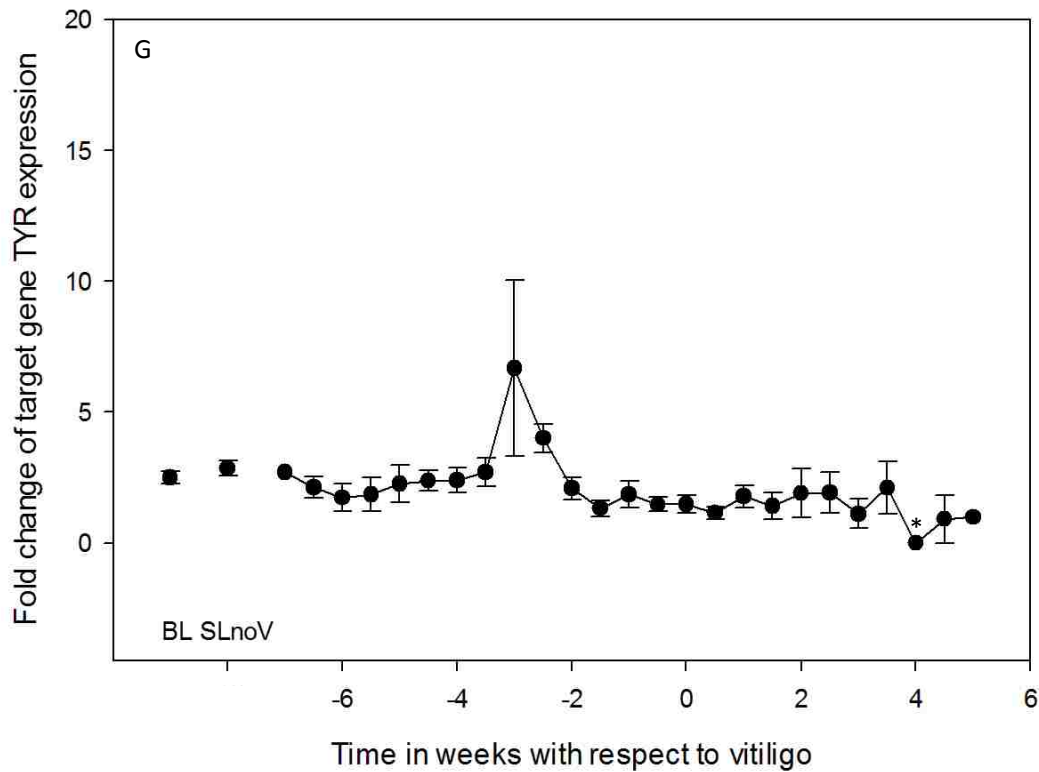
Figure 9. Time course of relative cytokine and tyrosinase gene expression with respect to SLV onset in growing feathers from HVT-vaccinated SLV chicks. A) IL-1, B) IL-4, C) IL-8, D) IL-10, E) IL-21, F) IFN-γ and G) tyrosinase (TYR). Relative expression was calculated by the $\Delta\Delta C_t$ method from 10 SLV birds, using a cDNA pool made from 16 growing feather of BL chickens from each time point as the calibrator and chicken 28S as the endogenous control gene. The *X*-axis represents the time (weeks) of feather tip collection post HVT vaccination. Weeks -7 to -0.5 represent early vitiligo and weeks 0-4 correspond to active vitiligo. BL and SLnoV data are the mean expression level overtime in feathers from BL and SL without vitiligo. *shows TYR expression in completely amelanotic feathers.

Discussion

Vitiligo in SL chickens is an acquired idiopathic dermatological disorder characterized by postnatal loss of pigment-producing cells (MCs) in the feather and the choroid which results in white feathers (Smyth, 1989). Genetic predisposition (inherent MC defect), immune system components, as well as environmental factors are known to play a role in the pigmentation loss (Smyth, 1989; Erf, 2006). Previous observation showed that SL chickens without HVT administration at hatch had a substantially lower incidence of vitiligo (< 20%) compared to 80 - 95% of SL chickens vaccinated with live HVT at hatch (Erf *et al.,* 2001). Recent development in housing conditions, reliably reduced the incidence of SLV in unvaccinated SL chickens to < 10% (no HVT administration and rearing of chicks in a HEPA filtered room; Erf personal communication). The large difference in SLV incidence in HVT-positive and HVT-negative SL chickens provides unique opportunity for study of HVT and other environmental triggers of SLV expression in susceptible SL chickens.

Live HVT is not the only MDV serotype that can trigger SLV expression. Live MDV1 (oncogenic) and live vaccine MDV2 (non-oncogenic chicken MDV), like MDV3 (HVT), were found to trigger SLV expression to a similar extent. This finding, together with reports that inactivated HVT and other live vaccine viruses (NDV, IBV), an mycoplasma infection, did not influences SLV expression (Erf *et al*., 2001; Erf, 2010), strongly suggests

that the ability of MDV viruses to influence SLV is unique to these α-herpesviruses. In this study, the strong association of HVT administration at hatch and the incidence of SLV was confirmed. However, with later time of HVT administration, SLV incidence decreased substantially by 6 weeks and SLV was no longer observed when HVT was administered at and after 10 weeks of age (Figure 3). This indirect relationship between age at HVT administration and SLV incidence needs further investigation, but may be related to a more fully developed, better regulated adaptive immune system and/or more robust MCs in older birds. A feature of MDV viruses, such as HVT, that may be related to SLV expression is the fact that these viruses translocate to the feather follicle where they replicate in the feather follicle epithelium. It has been our hypothesis (Erf , 2010) that the presence of the HVT-infection and the resulting immune response activity at this location, causes cellular stress to the inherently fragile MC of SL chickens. Stressed or apoptotic MC then draw the attention of the adaptive immune system to MC and the MC specific immune response develops (Erf, 2010). The observation in the current study further supports this hypothesis.

To examine whether SL chickens respond differently to a first and/or repeat HVT infection, a study was carried out to observe the mononuclear cell infiltration in feathers directly injected with HVT into the feather pulp area. Typically both SL and control chickens showed mononuclear cell infiltration in the feather pulp post HVT injection (Figure 4). The general mononuclear cell infiltration pattern was similar for the primary as well as the recall response in feathers from SL, BL and LBL chickens. While SL exhibited more

extensive infiltration at several time-points post-HVT injection, the differences were not so large as to suggest that heightened reactivity of SL to HVT is a factor in triggering SLV. However, this study was conducted when chicks were 18 weeks of age and it is possible that immune activity, both innate and adaptive, to HVT at a very young age may be substantially different in vitiligo-prone SL and control chickens.

It may also be possible that the extent of HVT infection is different in SL compared to BL and LBL controls which could influence the local MC environment and cause MC cellular stress. Interestingly, previous study showed no differences in relative HVT-viral load in the feathers from, SL, BL, and LBL chickens over a 42 day period post HVT vaccination, whereas SL had higher viral load in spleen than BL chickens. In both, feathers and spleen, HVT viral load levels exhibited a biphasic pattern in all three lines of chickens, with peak levels early during infection, a drop at 21 days, and another peak at 28-35 days post HVT vaccination (Bateman, 2008). The current study was conducted to further examine the association between HVT levels and expression of SLV. Specifically, the relative viral load of HVT in feathers was compared between HVT-vaccinated chicks from SLV, SLnoV (did not develop SLV) and BL chickens using a time course approach to examine feather tissue in the same individuals. It shows the dramatic decrease of HVT viral load in both SL and BL chickens from 2.5 to 3 weeks which is consistent with previous data (Bateman, 2008). The elevated HVT viral load in SLV feathers as compared with SLnoV and BL feathers starting from 4.5 weeks up to 7 weeks (the time when most SLV developed)

(Figure 6) suggests a link between HVT load in SLV birds and the expression of SLV. Moreover, expression of HVT levels in feathers with respect to SLV onset, revealed heightened HVT levels 0.5 to 1 week before SLV onset. The increase in HVT DNA levels may be due to viral replication. However, HVT gp expression could only be detected in spleen from SL and controls but not in feather tips (Figure 8). It appears that increased HVT load in the SL MCs environment just prior to visible onset of SLV may be a factor in SLV development. The LCM study examining HVT viral load in certain areas within the feather also revealed a stronger correlation of HVT viral load in the feather and the MC-area in SLV and SLnoV chickens compared to that in BL (Figure 7), which also supports this conclusion.

A role of the immune system has been well established in the autoimmune etiology of SLV (Wick *et al*., 2006). Both humoral and cell-mediated immunity play a part in SLV development and progression (Erf, 2008; Shi and Erf, 2012). In the current study, elevated expression of IFN-γ, IL-8, IL-10, and IL-21 just prior to and during the active phase of SLV strongly indicated immune response activation during the onset of vitiligo. Specifically, IFN-γ plays an important role in macrophage activation, phagocytic activity, cytokine and chemokine production, MHC I and MHC II expression (Abbas *et al*., 2010) and is the signature cytokine of a Th1-polarized cell-mediated immune response. In addition to driving cell-mediated immune activities, IFN-γ also has been shown to increase the formation of autophagosomes in human CD4+ T cells (Son *et al*., 2010) and macrophages

(Shi and Kehrl, 2010). The formation of autophagosomes in MCs from SLV chickens

(Boissy *et al*., 1983) has also been reported, which could be due to the heightened levels of

IFN-γ in the MC environment. Heightened proportions of MHC II-expressing cells in early

and active vitiligo samples (Shi and Erf, 2012; Erf *et al*., 1995) supports the role of IFN-γ

in antigen presentation and in macrophage, B-cell, and T-cell-activation. IL-8 is a

chemokine produced by macrophages during the inflammatory response (Abbas *et al*.,

2010). IL-8's primary function is to recruit phagocytic cells to the site of infection

(Mizunoe *et al*., 2012). The increased expression of IL-8 in SLV in this study is in support

of mononuclear cell infiltration observed during melanocyte loss. IL-10 is generally

known as a cytokine with anti-inflammatory and immune-suppressive activities (Abbas *et

al*., 2010). However, it also has been shown to have immunostimulatory effects by

increasing IFN-γ production and activation of natural killer cells and cytotoxic T cells (Tilg

et al., 2002). IL-10 and IFN-γ co-expression was also reported as a result of HVT infection

(Abdul-Careem *et al*., 2007) in feathers and HVT infection may have primed the

melanocyte-specific immune response in SLV in favor of immunostimulatory effects of

IL-10. Lastly, IL-21 has been shown to be required for sustainability of CD8+ T cells and

IFN-γ producing cells during chronic viral infection (Elsaesser *et al.*, 2009). In addition,

the elevated gene expression of IL-10, IL-21, and IFN-γ at 3 weeks in SL feathers post

HVT administration at hatch (samples from previous study described in Study 3; data not

shown) may be a key connection of SLV with HVT. Taken together, the source of immune

activation may be the routine vaccination at hatching with live HVT against

lymphoma-causing MDV which in SL may spiral out of control and focus on the stressed,

inherently fragile SL MC. The mechanisms underlying the interrelationship of cytokine

profile and HVT viral load in MC loss need to be further examined.

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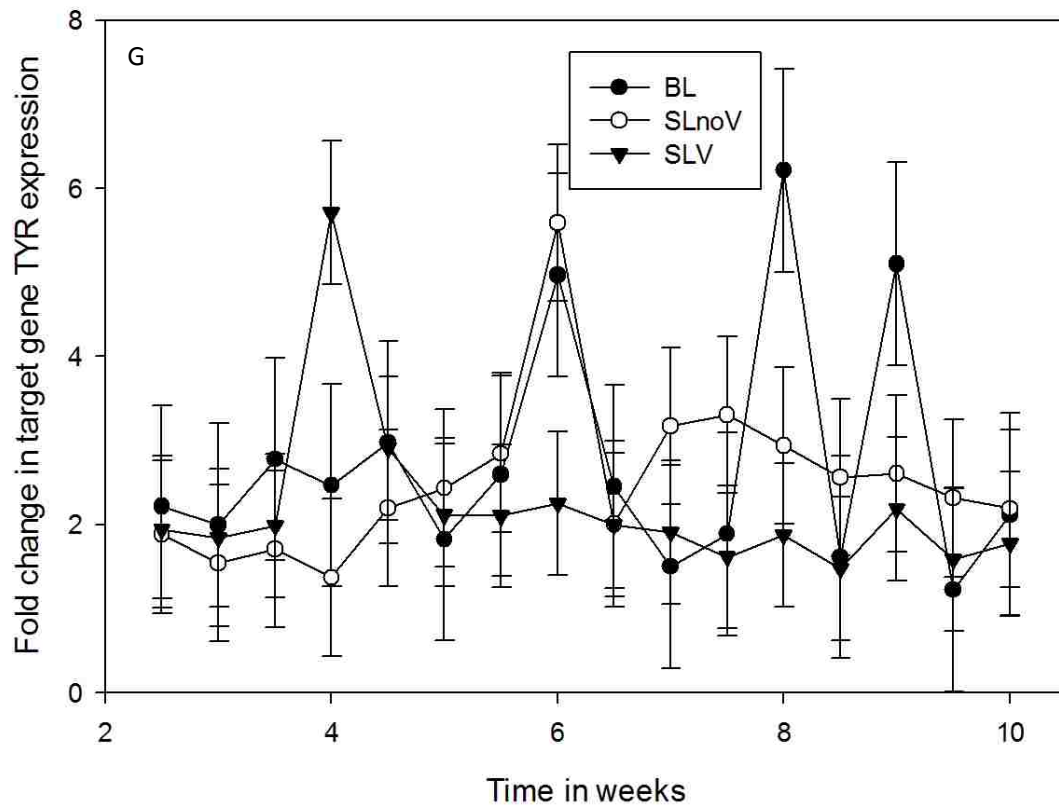
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APPENDIX 1
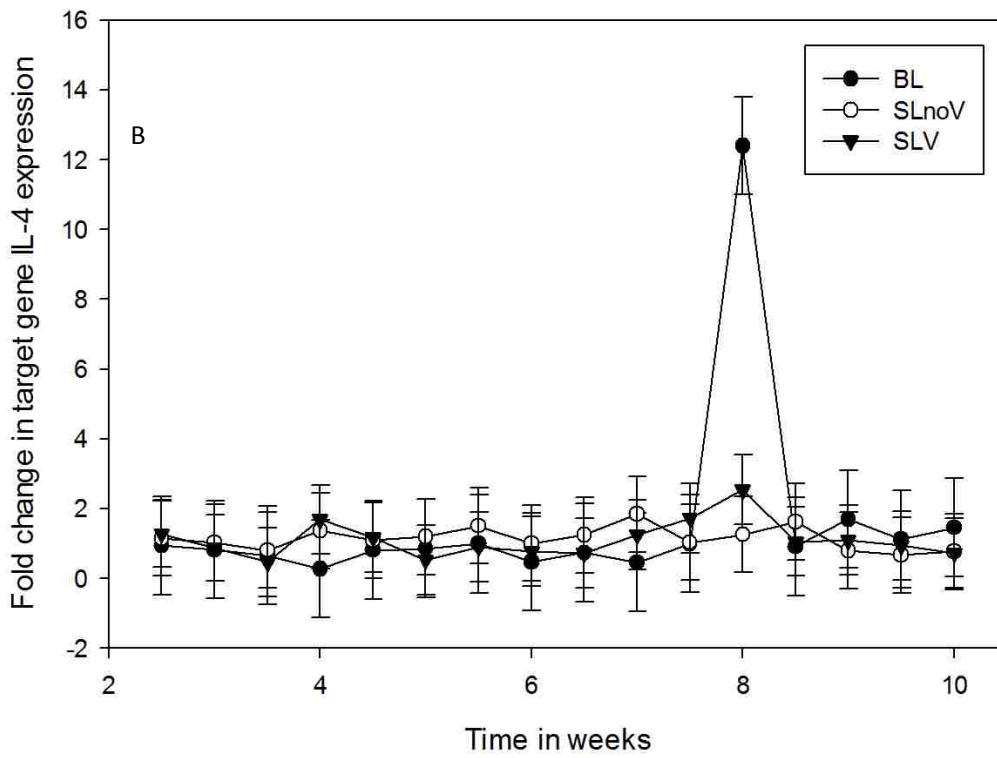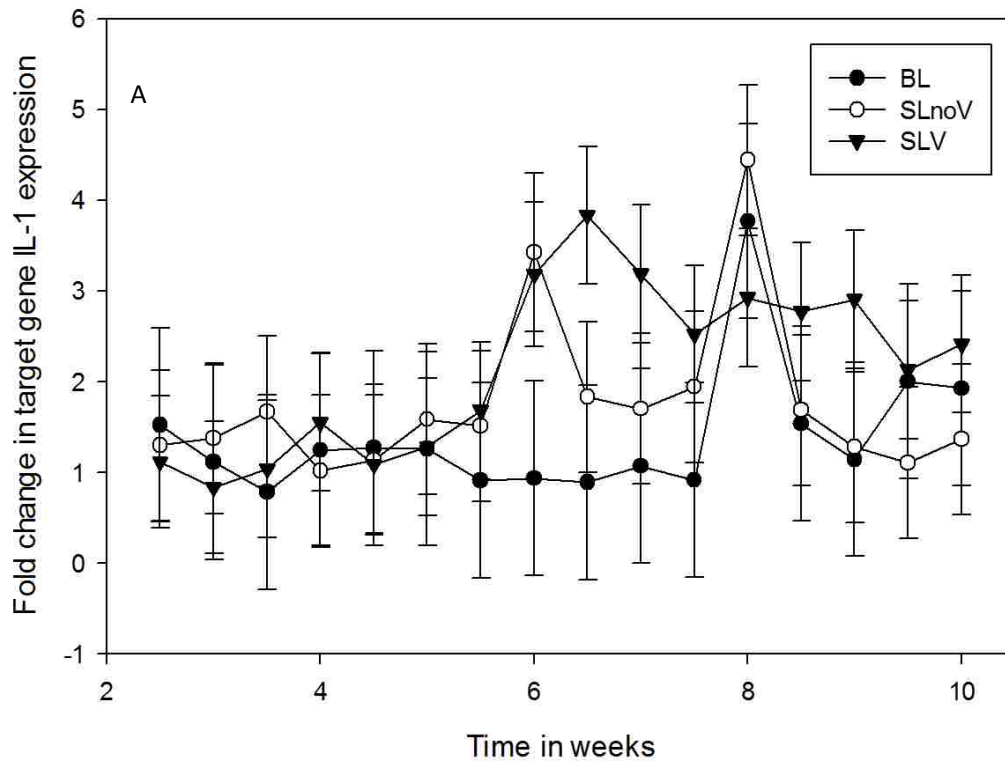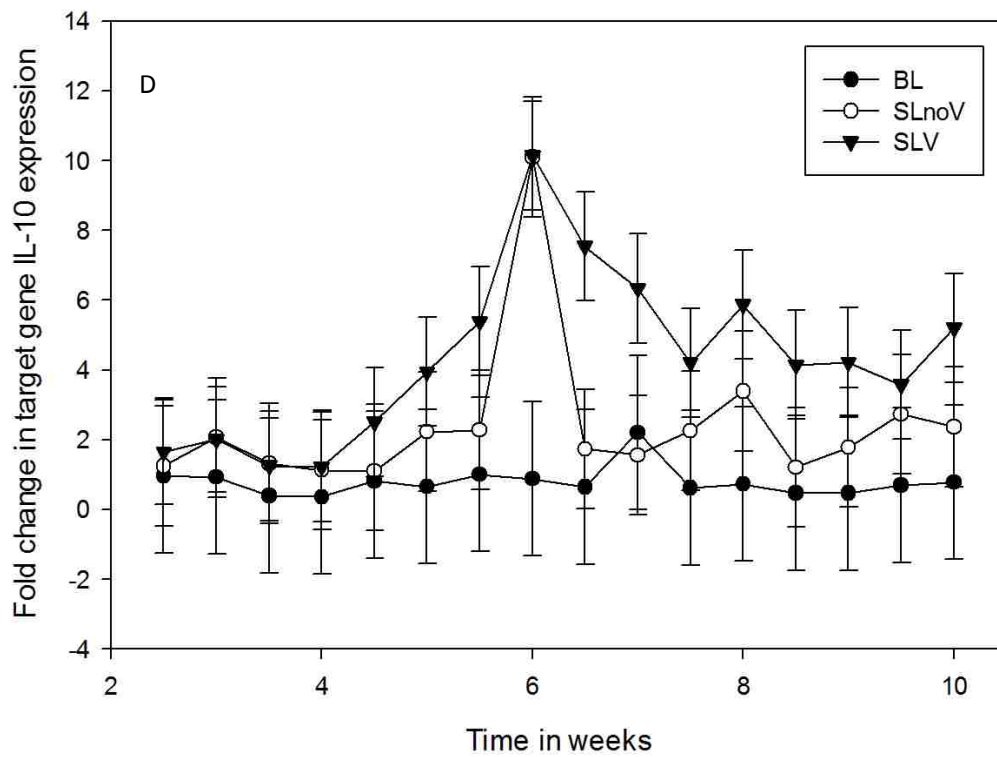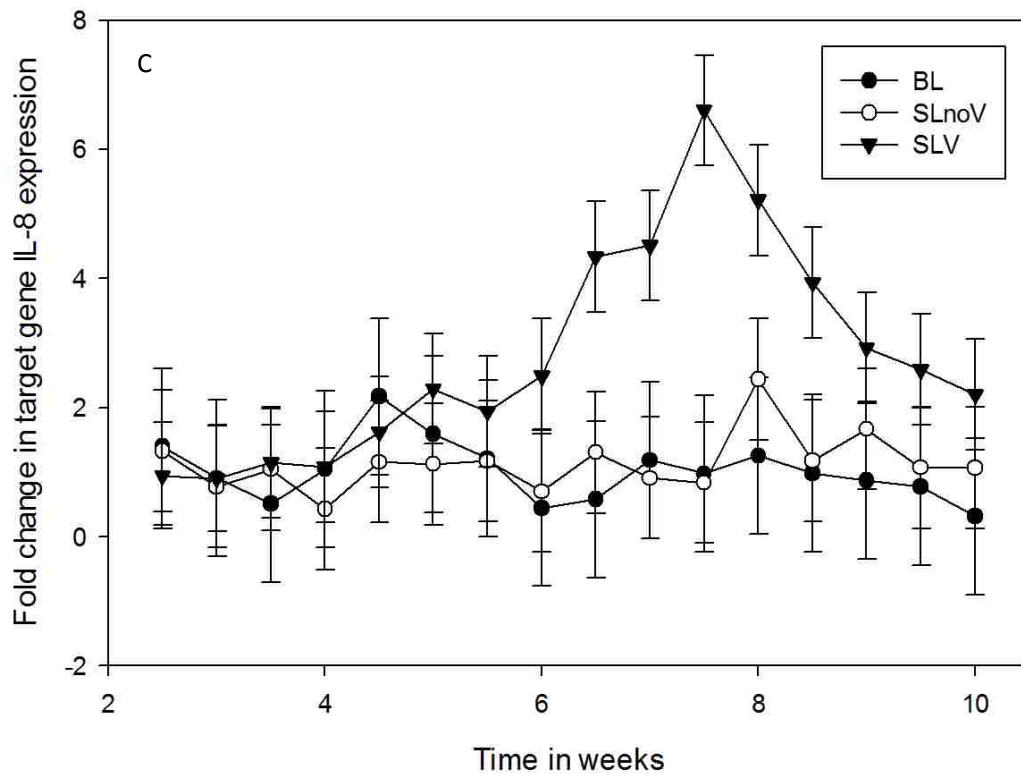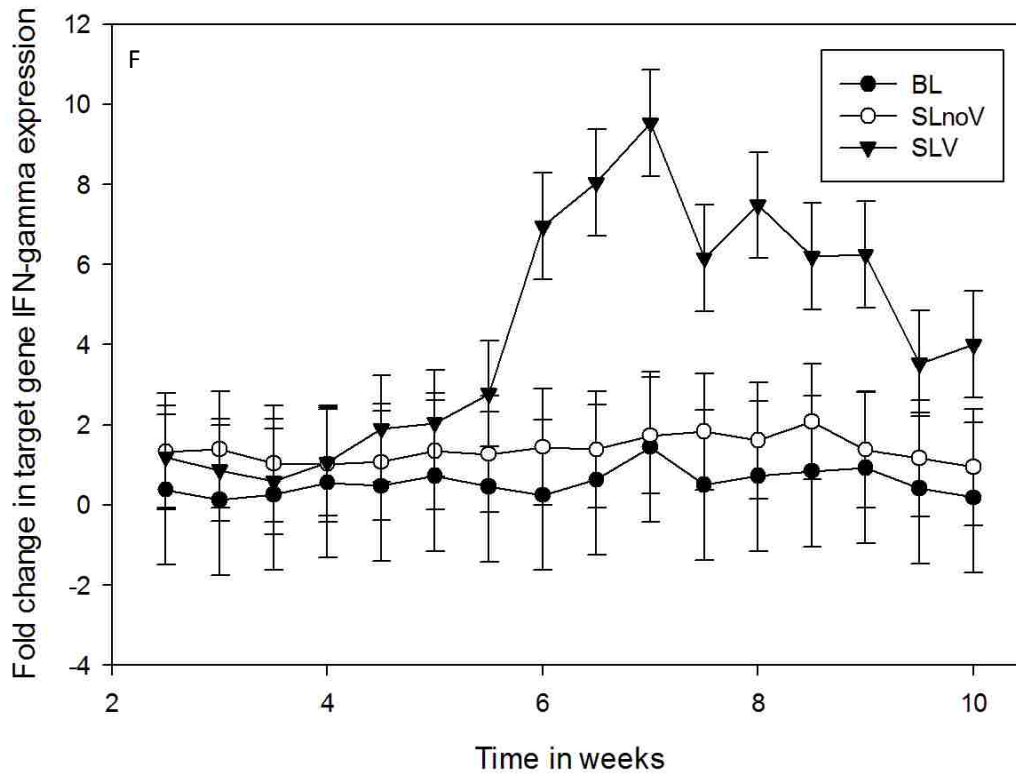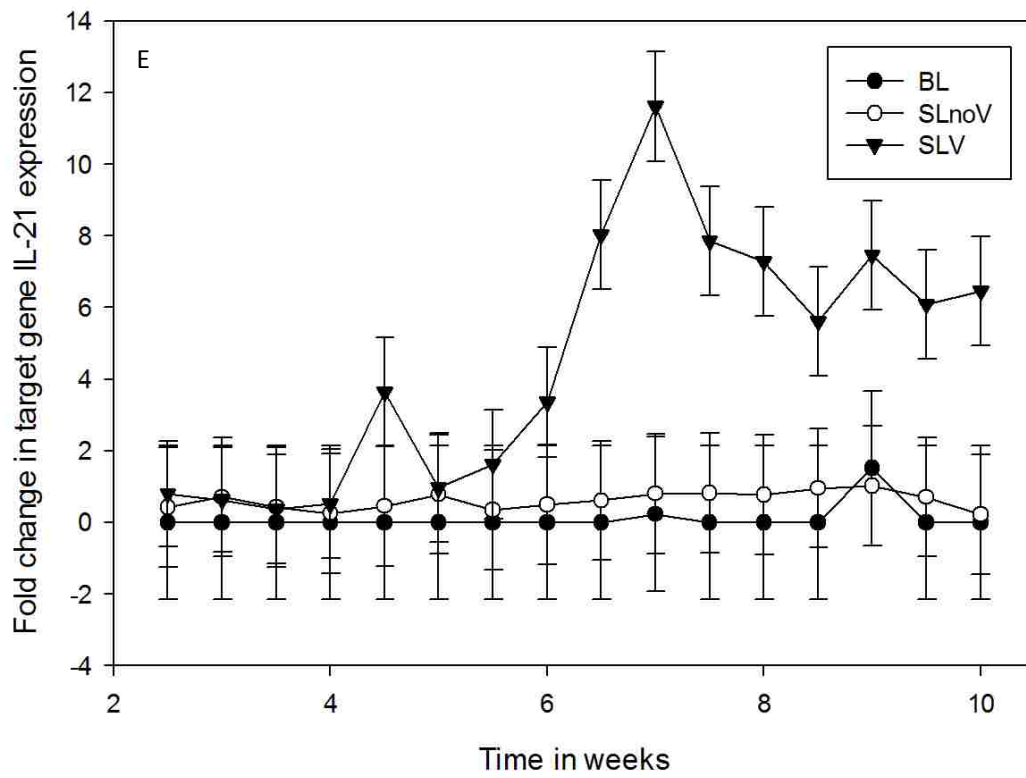
Appendix 1. Time course of relative cytokines A) IL-1, B) IL-4, C) IL-8, D) IL-10, E) IL21, F) IFN-gamma and G) tyrosinase (TYR) gene expression in the growing feather from HVT vaccinated SLnoV, SLV, and BL chicks. One-day-old chicks from the vitiligo-prone SL, parental BL chickens (N=10 and 4 per time point, respectively) were vaccinated with HVT and feather samples were collected at 2.5, 3, 3.5, 4, 4.5, 5, 5.5, 6, 6.5, 7, and 7.5 weeks post-vaccination. Relative expression was calculated by the $\Delta\Delta C_t$ method using a cDNA pool made from 16 growing feather of BL chickens from each time point as the calibrator and chicken 28S as the endogenous control gene. The X-axis represents the time (weeks) of feather tip collection post HVT vaccination. The mean ± SEM of relative gene expression averaged from each SLnoV, SLV, and BL chickens.

Conclusion

The Smyth line (SL) chicken is an excellent animal model for human autoimmune vitiligo. In addition to the spontaneous and predictably high incidence of vitiligo in the SL chicken, the advantages of using the SL chicken model to study the etiology of vitiligo include the location of melanocytes in growing feathers, providing repeated access to the evolving autoimmune lesions in the same individual for in *in vitro* and/or *in vivo* studies. Other advantages of the SL model include the regeneration characteristics of growing feathers, the availability of pure melanocytes derived from growing feathers and 72 h embryos, the identification of a reliable environmental trigger (herpesvirus of turkey; HVT) of SL vitiligo expression, as well as, the MHC-matched control lines of chickens with low or no expression of vitiligo. Results regarding the effects of 4-TBP induced oxidative stress on the response of SL melanocytes, reinforces the concept of an inherent melanocyte defect in SL chickens and also demonstrates the value and suitability of SL chicken as the avian model for human autoimmune vitiligo. The observed progressive reduction (90 to 0%) in HVT's ability to trigger SL vitiligo during the first 10 weeks of age suggests that HVT exerts its effect on vitiligo expression when adaptive immunity is not fully developed. The observation of elevated cytokine expression (IL-8, IL-10, IFN-γ and IL-21) during active SL vitiligo which was preceded by increased levels of HVT at 1 to 0.5 weeks before SL vitiligo onset, strengthens the association of HVT-infection and development of the melanocyte-specific autoimmune response. With the translocation of HVT to the feather,

its presence and resulting immune activity may cause cellular stress in the inherently defective SL melanocyte. Stress-associated melanocyte alterations may lead to immune recognition of SL melanocytes and development of melanocyte-specific autoimmune disease.