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The Differential Effects of Connexin26 and Connexin43 in Modulating Epidermal Health in Response to UV Injury

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Abstract

Using two disease-linked connexin mutant mice that possess significantly reduced connexin function ($Cx43^{I130T/+}$ and $Cx26^{K14\text{-}S17F/+}$) this investigation is the first to address the roles of Cx26 and Cx43 in modulating epidermal health in response to UV radiation. Viable $Cx26^{K14\text{-}S17F/+}$ mice were successfully created in-house to express the mutant Cx26 S17F protein in keratinocytes and possess scaly skin, rapidly develop epidermal desquamation, and die soon after UV exposure. Conversely, $Cx43^{I130T/+}$ mice did not possess any skin abnormalities before or after UV exposure. We also identified that primary murine melanocytes are not homocellularly coupled and do not express Cx43. Furthermore, we show that Cx43 may act as a tumor facilitator to promote tumor cell survival in human melanoma metastases in a variety of distant organ sites. Taken together, these studies strongly suggest that Cx26 is critical in protecting keratinocytes from acute UV damage, while Cx43 may provide melanomas with a survival advantage in distant metastases.

Keywords

The following keywords can be used to describe the thesis entitled “The differential roles of Connexin26 and Connexin43 in modulating epidermal health in response to UV injury”.

Gap junction, connexin, connexin26 (Cx26), connexin43 (Cx43), melanocyte, keratinocyte, primary culture, UV radiation, mutant mouse, Cx26 S17F, Cx43 I130T, human melanoma, melanoma metastasis, epidermis, skin, homeostasis.

Co-Authorship Statement

Eric R. Press was an excellent partner in the breeding of the $Cx26^{K14\text{-}S17F/+}$ mouse line, and the toluidine blue assay performed on unchallenged mice from this same line.

Melissa Crawford, from the laboratory of Dr. Lina Dagnino, was fundamental in establishing the techniques required to culture the primary murine melanocytes cultures.

Kevin Barr assisted in establishing both mouse lines used for this investigation and was an excellent mentor for mouse related work.

Dale W. Laird is the principal investigator, and was fundamental in project design.

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Dedication

This thesis is dedicated in memory of my Grandmother,

Clair Alagha

(1937 - 2012)

"Energy is neither created or destroyed, simply transformed from one form to another"

First Law of Thermodynamics

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List of Abbreviations

ATP	adenosine triphosphate
ANOVA	analysis of variance
BCA	bicinchoninic acid
BCC	basal cell carcinoma
BSA	bovine serum albumin
BM	basement membrane
CAM	chorioallantoic membrane
cAMP	cyclic adenosine monophosphate
CaRTT	Cancer Research and Technology Transfer
CO_2	carbon dioxide
Cx	connexin
DNA	deoxyribonucleic acid
DMEM	Dulbecco's modified Eagle's medium
EDTA	ethylenediaminetetraacetic acid
FBS	fetal bovine serum
GJ	gap junction
GJIC	gap junction intercellular communication
HCl	hydrogen chloride
I130T	isoleucine to threonine at position 130
IP_3	inositol-1,4,5-triphosphate
K14	keratin14
KID	keratitis ichthyosis deafness
KSFM	keratinocyte serum free media
kDa	kiloDalton

L-DOPA	L-3,4-dihydroxyphenylalanine
MITF	microphtalamia-associated transcription factor
MGM-4	melanocyte growth medium
NaCl	sodium chloride
NOV/CCN3	nephroblastoma overexpressed protein
ODDD	oculodentodigital dysplasia
OTB	Ontario Tumor Bank
OICR	Ontario Institute of Cancer Research
PBS	phosphate buffered saline
PPK	palmoplantar keratoderma
REK	rat epidermal keratinocytes
S17F	serine to phenylalanine at position 17
SDS/PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	standard error of the mean
SCC	squamous cell carcinoma
TEWL	transepidermal water loss
Tris	tris(hydroxymethyl)aminomethane
TRP1	tryosinase related protein-1
TSG101	tumor susceptibility gene 101
UV	ultraviolet
WT	wild type
ZO	zona occludens

Chapter 1

1 Literature Review

1.1.1 Connexins

Connexins encompass a family of integral membrane proteins that have been extensively studied at all levels of cellular, molecular, and disease biology. Members of the connexin family share similar overall structural topology: the polypeptide backbone spanning the plasma membrane four times resulting in two extracellular loops, one intracellular cytoplasmic loop, and intracellular amino- and carboxy-terminals (Goodenough and Paul, 1996). Many of the functional differences among members of the connexin family can be sourced to the variable length and sequence of the intracellular cytoplasmic loop and carboxy-terminus of the connexin protein (Elfgang et al., 1995). For example, the carboxy-terminus of some connexins contain phosphorylation sites that regulate channel permeability, in addition to connexin life cycle and intercellular trafficking (Lampe and Lau, 2010). As such, connexins are categorized by differences in sequence and structural homology, and are commonly distinguished in nomenclature by their respective molecular weights in kDa (i.e. Cx43 is approximately 43 kDa) (Scott and Kelsell, 2011).

Most importantly, connexins gain functional significance by oligomerizing into hexamer units, commonly known as connexons or hemichannels. The majority of connexons are composed of identical connexin subunits, however specific members within the connexin family can interact to form heteromeric connexon channels (Wagner, 2008). These connexons are trafficked to the plasma membrane where they can either act as a hemichannel, allowing for communication between a cell and the surrounding microenvironment, or they can form a communication channel by docking to another connexon from a neighbouring cell. These communication channels are termed gap junctions (GJs), which often form GJ aggregates on the plasma membranes of both cells termed GJ plaques (Laird, 2006). At this level, the docking of two identical or two non-identical connexon channels further increases the variety of GJ channels that can form (Wagner, 2008). In addition, each connexin member is specific in its compatibility to interact with other connexins, in both connexons and GJ channels. For example, under

standard conditions, Cx26 and Cx43 will not typically intermix when co-expressed in a cell (Gemel et al., 2004), whereas Cx26 and Cx30 are compatible (Di et al., 2005).

GJs are present in almost all tissues in the body, however, not all connexins are ubiquitously distributed. Specific tissues will express certain connexin proteins that presumably possess selective channel properties that are favorable to maintain the normal function and health of cells that reside within that tissue. For example, both Cx43 and Cx26 are expressed in the skin (Churko and Laird, 2013), however Cx43 is expressed in the heart and Cx26 is not (Severs et al., 2008). This is an indication of the putative roles connexins play in different physiological processes, including cellular proliferation, differentiation, and apoptosis (Dbouk et al., 2009; Alexander and Goldberg, 2003). As such, it is not surprising that the vast majority of members within the connexin family are homologous and highly conserved (Söhl and Willecke, 2003; Söhl and Willecke, 2004). Minus a few exceptions, almost all connexins share a similar gene structure, which is made up of two exons separated by an intron that can vary in length (Willecke et al., 2002). However, extensive regulatory processes (Söhl and Willecke, 2004), including tissue-specific promoters, allow connexin isoforms to be expressed differentially in an overlapping array with both spatial and temporal specificity (Dbouk et al., 2009).

1.1.2 Gap Junction Dependent Roles for Connexins

The most prominent role of GJ channels is to facilitate the passage of ions (i.e. Ca^{2+}, Mg^{2+}) and small molecules less than 1 kDa (i.e. ATP, cAMP) between neighbouring cells, which is termed gap junctional intercellular communication (GJIC) (Dbouk et al., 2009). The importance of GJIC in the normal development and physiology of vertebrates has been demonstrated through different connexin-deficient mice (Schütz et al., 2011; Kalcheva et al., 2007; Yamakage et al., 2000), in addition to the discovery of a variety of human diseases that have been linked to connexin mutations (Laird, 2008; Kelsell et al., 1997; Bergoffen et al., 1993). For example, Cx43 and Cx26 null mice are not viable due to severe deficits in ventricular flow (Reaume et al., 1995) and placental development (Gabriel et al., 1998), respectively, illustrating the prominent role of Cx43-based GJIC in the heart, and Cx26-based GJIC in the developing placenta.

Macroscopic gap junctional conductance has been determined by a variety of techniques including voltage clamp assessment of two coupled cells and the intercellular diffusion of fluorescent dyes (Herve & Derangeon, 2013). However, it is important to note that while GJ channels can typically pass many of the same constituents, they are not passive conduits for molecular intercellular transfer, but have distinct gating mechanisms that include their selective permeability to specific ions and molecules (Harris, 2007). For example, the secondary messenger inositol -1,4,5-triphosphate (IP_3) can pass through various GJ channels, however it tends to preferentially pass through Cx32-based GJ channels as opposed to Cx43- or Cx26- based GJ channels (Goldberg et al., 2004). Furthermore, the wide array of biological consequences linked to improper GJ mediated intercellular signaling have been partially sourced to the distinct permeability properties of each GJ channel (Harris, 2007). Unfortunately, while the functional conduit of ions through GJ channels was demonstrated many years ago (Lawrence et al., 1978), their specific permeability properties have been difficult to determine (Harris, 2007). This is primarily due to the vast variability between GJ channels, which is a result of the unique pore properties of each connexin isoform, the ability of these isoforms to interact with non-identical connexin/connexon units forming a wide variety of GJ channels, and further because the permeability of those GJ channels can be dynamically modulated (Harris, 2007). However, recent work has determined that while pore size is important, GJ channels possess a high degree of molecular charge selectivity, with the ability to discern between monovalent ions (Herve & Derangeon, 2013).

1.1.3 Gap Junction Independent Roles for Connexins

In addition to the central dogma that connexins act as the building blocks of GJs to facilitate the intercellular transfer of molecules, recent evidence has shown that they possess GJ independent roles (Goodenough and Paul, 2003; Wang et al., 2013). One important function that has been identified is the ability of connexons to function as hemichannels, the unpaired halves of GJ channels, at the cell surface to exchange ions and molecules between the cell and the extracellular environment (DeVries and Schwartz, 1992; Wang et al., 2013). Evidence for these extra-junctional connexin channels has come from a variety of surface labeling, sucrose gradient fractionation, and cross linking studies (as reviewed

in Goodenough & Paul, 2003). For example, antibodies are commonly too large to fit between the extracellular space within the two connexons that comprise a GJ channel (Goodenough and Revel, 1971), yet antibodies conferred against the extracellular epitopes of a connexon channel can block GJ formation (Meyer et al., 1992). This indicates that connexons are present at the cell surface even before they assemble into GJ channels (Goodenough and Paul, 2003). What has been raised into question is whether or not these hemichannels are active, originally it was presumed that they only could exist in a closed state, however channel opening appears to be dynamically regulated. For example, similar to other ion channels, hemichannels seem to be activated by common stimuli such as depolarization and changes in extracellular calcium (DeVries and Schwartz, 1992). Strikingly, these channels also seem to be activated by similar stimulants to that of their gap junctional counterparts, including changes in voltage, pH, cAMP etc. (DeVries and Schwartz, 1989). In addition, similar to gap junctions, hemichannels also allow for the passage of similar small molecules, including ATP, IP3, cAMP, glutathione etc. (Wang et al., 2013; Harris, 2007). Furthermore, hemichannels have been shown to form and be activated in a variety of different cell types, including oocytes (Paul et al., 1991), ventricular myocytes (Kondo et al., 2000), and astrocytes (Hofer and Dermietzel, 1998), and have been linked to important physiological processes (Goodenough and Paul, 2003). For example, the presence of connexons has been identified as an important tool for osteocyte survival, and osteogenesis (Orcel and Beaudreuil, 2002; Plotkin et al., 2002; Goodenough & Paul, 2003). However, uncontrolled hemichannel activity can have detrimental cellular effects. For example, the overexpression of Cx46 in oocytes caused cell lysis through hemichannels (Paul et al., 1991; Ebihara and Steiner, 1993).

Connexins have also been shown to have functions independent of molecular passage that is seen in both GJs and hemichannels. Connexins can also affect different physiological processes by binding to direct partners within a protein interactome, activating or inactivating different downstream signaling cascades (Vinken et al., 2012; Laird, 2010). Each connexin member has its own distinct protein interactome, most likely due to differences in carboxy terminal length (Giepmans, 2004). For example, over 30 binding partners have been identified for Cx43, whereas only 5 have been identified for Cx26 which has a short carboxy terminal (Laird, 2010). This has raised the notion that the carboxy

terminal tail of connexin proteins is particularly important in specific protein interactions that can actually modulate the genetic programming of a cell (Giepmans, 2004). For example, in transformed cells, full length Cx43 and Cx32 induced GJIC but did not affect cell proliferation. However, transformed cells with Cx43 and Cx32 mutants lacking the carboxy terminal tail were able to inhibit proliferation (Omori and Yamasaki, 1999).

Connexins also be interact with different post-transcriptional modifiers, including kinases, different scaffolding and cytoskeletal proteins (i.e. microtubules, tight junctions, adherens junctions), and other transcription factors and growth regulators (i.e. Wnt pathway modulators, NOV, CCN3, c-SRC, v-SRC etc.) (Giepmans, 2004; Laird, 2010). More importantly the interactions with these different binding partners, most notably with Cx43, have also been shown to have a variety of physiological effects. In addition, the role of Cx43 as a tumor suppressor in keratinocytes has been proposed to possibly be linked to its interaction with another known tumor suppressor, caveolin 1 (Langlois et al., 2010). In addition, Cx43 has also been shown to interact with other proteins, such as tumor susceptibility gene (TSG101; (Leithe et al., 2009) and NOV (Fu et al., 2004), that all have relationships to cell cycle control and furthermore to tumorigenesis (Naus and Laird, 2010). Furthermore, the interaction of connexons with different adhesion molecules, has also been shown to affect processes such as cellular migration and adhesion. For example, the interaction of Cx43 and N-cadherin has been shown to affect cell motility (Wei et al., 2005), and the interaction of Cx43 and zona occludens (ZO) has been shown to modulate the cellular cytoskeleton (Olk et al., 2010; Laird, 2010). As such, as we continue to resolve the importance of connexins as modulators of cellular homeostasis and tissue health, it is important that we keep their non-junctional functions in mind.

1.1.4 Connexins in the Epidermis

While the general function of GJ channels is conserved, their expression profile is tissue-specific, which is representative of their myriad of functions in both health and disease. In both humans and mice, numerous connexin members are expressed (21 and 20, respectively) in an overlapping array to maintain normal cellular function and tissue homeostasis (Söhl and Willecke, 2003; Alexander & Goldberg, 2003). In fact, many tissues express more than one connexin family member. For example, cardiomyocytes of the heart

express Cx40, Cx43, and Cx45, while hepatocytes of the liver express Cx26 and Cx32. Among these tissues, the epidermis of the skin is of great interest for its ability to express up to 10 connexins at the transcript level, and 7 at the protein level (as reviewed by Laird, 2006).

The epidermis of the skin is made of two primary cell types, keratinocytes and melanocytes. Melanocytes, which make up 5-10% of total cells in the epidermis, reside within the basal layer of epidermal keratinocytes (Figure 1.1). They distribute melanin pigment to surrounding keratinocytes in the epidermis, which helps prevent damage from environmental carcinogens, such as ultraviolet (UV) radiation (Li and Herlyn, 2000; Zaidi et al., 2008; Klein-szanto et al., 1994). The keratinocytes, which make up 90% of cells in the epidermis, are organized into four different layers: the basal layer, spinous layer, granular layer, and the uppermost stratified corneum. As the keratinocytes divide and differentiate they migrate from the basal layer to the stratified corneum, which is composed of terminally differentiated keratinocytes (Richard, 2000). The differentiation of keratinocytes into the insoluble stratified corneum is dependent on GJIC in addition to the expression of involucrin and loricrin and the incorporation of lipids (Scott et al., 2012). The functional capacity of the corneum is to protect the body from external pathogens by conferring an epidermal barrier (Hardman et al., 1998). The permeability barrier is maintained, in part, by the complement of connexins expressed throughout the different layers of the epidermis (Hardman et al., 1998; Schütz et al., 2011; Djalilian et al., 2006). As such, it is not surprising that multiple reports describe a prominent role for connexins in maintaining skin physiology (Churko & Laird, 2013; Martínez et al., 2009).

Within the strata of keratinocytes, Cx43 is the most prominent connexin expressed in the epidermis and it acts in establishing a baseline of GJIC. However, Cx43 alone is not sufficient to maintain the normal epidermal function. As such, approximately 9 other Cx species are expressed, including Cx26 (Laird, 2006; Richard, 2000; Kretz et al., 2004). Current studies suggest that both of these connexins play functional roles in the regulation of epidermal homeostasis (Haass et al., 2006; Haass et al., 2010). Moreover, mutations in genes encoding Cx43 (*GJA1*) or Cx26 (*GJB2*) are associated with a variety of skin diseases,

including palmoplantar keratoderma (Scott et al., 2012; Churko et al., 2010) and keratitis-ichthyosis-deafness syndrome (Scott et al., 2012; Schütz et al., 2011).

It is also important to note that the expression of connexins throughout the epidermis can vary between humans and rodents. For example, in humans Cx43 is predominately expressed in the suprabasal layers of the interfollicular epidermis (Figure 1.1) (Di et al., 2001), as opposed to the basal layers of rodent epidermis (Butterweck et al., 1994; Churko and Laird, 2013). In addition, in humans Cx26 exists at low levels in the granular layer of the epidermis (Figure 1.1) (Di et al., 2001) and in the more suprabasal layers of rodent epidermis (Butterweck et al., 1994; Churko and Laird, 2013). However, for the purpose of this investigation these slight differences in expression are not expected to affect the analyses of Cx43 and Cx26 in modulating epidermal health in response to UV radiation or skin cancer onset and progression.

In contrast to keratinocytes, the connexin status of epidermal melanocytes is still widely unknown. While limited, one immunolabeling study has provided evidence for the expression of Cx43 in epidermal primary human melanocytes, and melanocytic cell lines (Hsu et al., 2000). Furthermore, some evidence does suggest that primary human melanocytes and keratinocytes in co-culture exhibit intercellular communication that is presumably through connexin-mediated GJs (Hsu et al., 2000, Haass and Herlyn, 2005). However, further investigation is required to confirm the identity of Cx43 in melanocytes and ultimately the importance of GJIC. In addition, while Cx26 has been shown to be expressed in melanocytes of the vestibular dark area of the inner ear, there has been no published reports of Cx26 being expressed in mouse or human epidermal melanocytes (Masuda et al., 2001).

While keratinocytes and melanocytes make up the majority of epidermal cells, other cell types have been identified. Most prominently, Merkel and Langerhans cells have been identified, and reside within the stratum basal and the suprabasal layers, respectively (Maricich et al., 2009; Wolff and Stingl, 1983). Merkel cells make synaptic connections with somatosensory neurons to transmit sensory information (Maricich et al., 2009) while Langerhan cells act as the immune cells of the epidermis (Wolf and Stingl, 1983). Similar

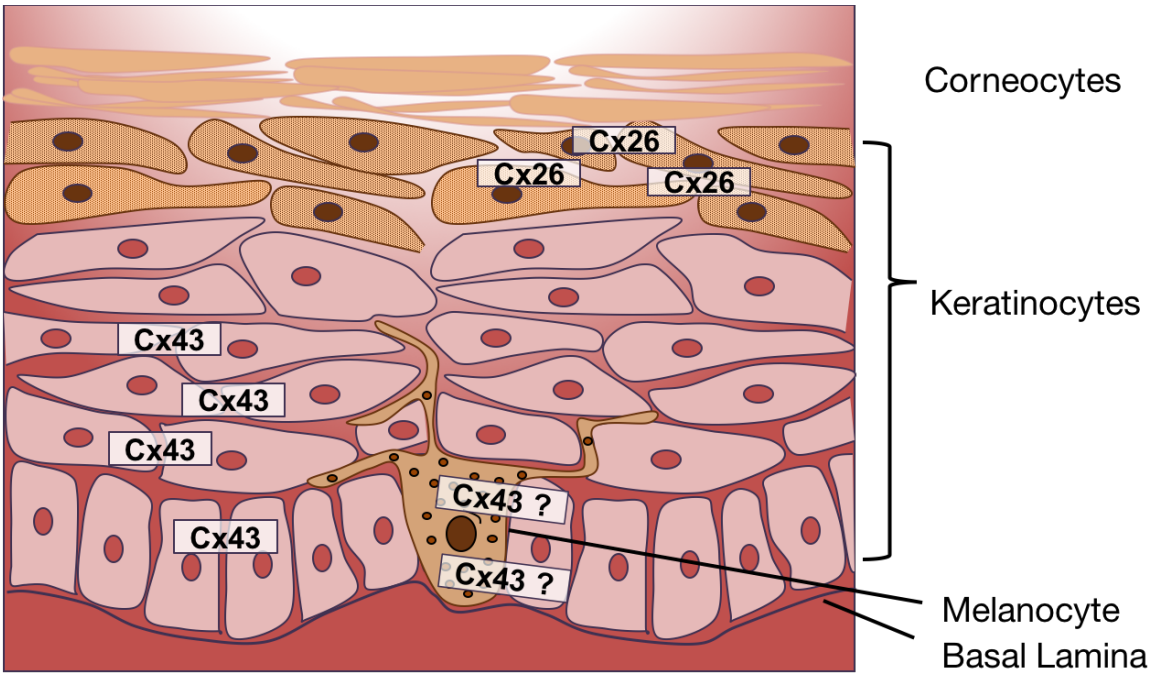

Figure 1.1: Cx43 and Cx26 are differentially expressed within the stratified human epidermis.

to melanocytes, the connexin profile of these cells has been extremely limited. While Langerhan cells have been shown to be uncoupled with their keratinocyte neighbours (Goliger and Paul, 1994; Zimmerli et al., 2007), Merkel cells have been shown to express Cx43 (Woo et al., 2010).

1.1.5 Connexins in Epidermal Pathology

The overlapping array of connexins expressed within the epidermal strata play a variety of roles in regulating cellular behaviour to maintain overall tissue health and function (Laird, 2006). The differential expression of connexins throughout the epidermis indicates the critical role of GJIC in epidermal physiology, and in more recent investigations, their effects on epidermal biology through non junctional binding proteins (Goodenough and Paul, 2003; Scott et al., 2012). Moreover, aberrant connexin protein expression has shown to result in abnormal keratinocyte proliferation, migration, and differentiation (Dbouk et al., 2009; Scott et al., 2012). As such, it is not surprising that a range of skin pathologies can be attributed to mutations in connexin-encoding genes resulting in impairments in epidermal barrier, wound healing, and/or syndromic skin disease. Among these Cx43, Cx26, and Cx30 have been the primary focus due to their identified role in human skin pathology, but other connexins including Cx31 and Cx31.1 have also been examined (Scott et al., 2012).

Studies in both humans and mice report that a transient decrease in Cx43 expression is necessary for epidermal healing after wounding (Kretz et al., 2003; Brandner et al., 2004), and has been identified as a regulator of keratinocyte proliferation (Pollok et al., 2011; Churko et al., 2012). In chronic non-healing wounds from humans and diabetic mice, Cx43 is found to be expressed abnormally at the wound margins (Wang et al., 2007; Brandner et al., 2004). Furthermore, the application of mimetic peptides that reduce Cx43 GJIC or hemichannel function have been shown to decrease wound healing time, collectively demonstrating the need for regulated Cx43 expression to maintain normal keratinocyte behaviour (Pollok et al., 2011; Scott et al., 2012). In addition, truncating the c-terminal tail of Cx43 results in aberrant murine skin barrier formation and keratinocyte differentiation (Maass et al., 2005). Mutations in the gene encoding Cx43 (*GJA1*) have also been linked to the development of oculodentodigital dysplasia (ODDD), where a subset of patients have

been shown to present with palmoplantar keratoderma and hyperkeratosis (Scott et al., 2012; Avshalumova et al., 2014). This indicates that the transient decrease in Cx43 expression is required for certain epidermal physiological processes, such as wound healing, but that its chronic loss can also be detrimental to other processes involving keratinocyte differentiation.

Cx26 is also a critical regulator of epidermal health, and has been identified in the epidermal response to wounding, regulating processes such as proliferation and differentiation. For example, a transient increase in Cx26 expression is necessary in epidermal healing after mechanical wounding (Goliger and Paul, 1995; Kretz et al., 2003). Human chronic non-healing wounds have been shown to persistently express Cx26 and Cx30 at wound margins throughout the healing process (Brandner et al., 2004), and persistent Cx26 expression in murine epidermis resulted in keratinocyte hyperproliferation, hindering wound closure (Djalilian et al., 2006). Collectively, these studies outline the critical role of Cx26 in regulating keratinocyte proliferation. The critical role of Cx26 in keratinocyte differentiation is highlighted by the fact that its regulated expression is required for epidermal barrier acquisition in murine development (Djalilian et al., 2006).

Autosomal dominant mutations in the gene encoding Cx26 (*GJB2*) have also been linked to a multitude of skin pathologies that are often characterized by epidermal thickening and keratinocyte hyperproliferation. For example, *GJB2* has been identified as a psoriasis susceptibility locus in Chinese populations (Sun et al., 2010), and Cx26 expression was found to be upregulated in human hyper-proliferative psoriatic lesions (Labarthe et al., 1998); Scott et al., 2012). In addition, autosomal dominant mutations in *GJB2* have also been directly associated with the development of human skin diseases such as, palmoplantar keratoderma (PPK), Vohwinkel syndrome, keratitis ichthyosis deafness (KID) syndrome, Bart-Pumphrey syndrome, and hystrix-like ichthyosis deafness syndrome (Scott et al., 2012). However, it is interesting to note that while mutations in *GJB2* result in a wide array of skin pathologies, patients that are homozygous for some mutations that completely abolish Cx26 associated GJIC do not present with skin disease (D'Andrea et al., 2002). It has been postulated that this is due to compensation from the wide array of other connexin proteins expressed amongst the epidermal strata (Scott et al., 2012). As

such, a multitude of connexin-disease linked mechanisms have been identified, especially for mutations in *GJB2*. These mechanisms include mutations that not only possess loss of function and impaired GJIC (Vohwinkel Syndrome), but also include trafficking defects, aberrant or leaky hemichannels, and dominant negative effects on WT connexins or other connexin types (Scott et al., 2012). Collectively, these studies demonstrate the crucial role that connexins play in maintaining overall keratinocyte health and function.

1.1.6 UV-induced Epidermal Pathogenesis

Despite the well-established importance of Cx43 and Cx26 in epidermal health, their roles in the epidermal response to common environmental insults, including UV radiation, are poorly understood. This is of concern as exposure to UV radiation can lead to both acute and chronic skin damage, ranging from sunburn and epidermal desquamation to UV-induced tumorigenesis (DeGruijl, 1999). Solar UV rays that reach the earth's surface can be primarily broken down into two different wavelengths, UVA (320-400 nm) and UVB (290 – 320 nm) (Bernerd et al., 2000). Exposure to both UVA and UVB have been widely implicated in the induction of both long term and acute epidermal pathogenesis (Bernerd et al., 2000; Ichihashi et al., 2003). UVB has been deemed the primary carcinogen linked to the development of skin cancers, causing significant DNA damage in the form of cyclobutane pyrimidine primers. In addition, both UVA and UVB radiation have been shown to damage skin by stimulating the production of reactive oxygen species (Ichihashi et al., 2003). This has been linked to long term skin damage including photoaging and skin cancer development (Ichihashi et al., 2003; DeGruijl, 1999). In addition, exposure to UV radiation has been shown to disrupt the epidermal permeability barrier (Haratake et al., 1997).

To delineate the mechanisms behind UV-induced epidermal pathogenesis, current studies have started to investigate the role of Cx43 and GJIC in the epidermis' acute response to UV exposure (Bellei et al., 2008; Gambichler et al., 2008). However only two reports have discussed this gap in knowledge. For example, in human keratinocytes, Cx43 and GJIC have been proposed to be downregulated in response to UVA radiation (Bellei et al., 2008), but upregulated in response to UVB (Gambichler et al., 2008). Furthermore, not only does

the significance of these changes in Cx43 expression remain unclear, but the role of Cx26 in the epidermal response to UV radiation is completely unknown.

1.1.7 Connexins in Skin Cancer

Normally, to prevent skin cancer formation, cells are maintained under tight homeostatic control to maintain the overall function and health of their residing tissue (Dbouk et al., 2009). Accordingly, the disruption of this process can lead to the development of diseases, such as cancer. Specifically, skin cancers can arise from the uncontrolled proliferation of keratinocytes and melanocytes. Common keratinocyte derived skin cancers can be broken down into either squamous or basal cell carcinomas, depending on which layer of the epidermis the cancer originates (Seebode et al., 2016). If keratinocytes that comprise the basal layer experience uncontrolled growth and proliferation, the resulting cancer is termed basal cell carcinoma (BCC). However, if it is the squamous suprabasal keratinocytes that undergo this change, the resulting cancer is termed squamous cell carcinoma (SCC) (Seebode et al., 2016). Accordingly, one non-keratinocyte derived cancer, termed melanoma, can develop if the melanocytes in the epidermis undergo uncontrolled proliferation and growth (Bandarchi et al., 2010). In addition, the long-term effects of UV-induced epidermal damage have been extensively categorized and identified as the primary carcinogen responsible for the development of skin cancers. For example, more than 90% of human keratinocyte derived-tumors (Koh et al., 1996), and more than 86% of human melanomas have been directly attributed to UV-induced mutations (Parkin et al., 2011).

Cancer cells have long since been reported to lack normal intercellular communication, including GJIC (Loewenstein and Kanno, 1966). This may be a consequence of GJIC being able to spread cell-killing signals (i.e. Ca^{2+}). Therefore, its loss may be a mechanism of tumor cell survival (Haass et al., 2004). As such, the potential regulation of connexins and GJIC in tumor progression has become an area of focus (Brandner et al., 2004). In this pursuit, studies have reported a loss or down-regulation of connexins and/or GJIC in an array of different cancers, including hepatocellular carcinoma (Eghbali et al., 1991), colon cancer (Friedman & Steinberg, 1982), prostate cancer (Tsai et al., 1996), and mammary carcinomas (Hirschi et al., 1996), in addition to SCC, BCC and melanoma (Haass et al., 2006; Tada and Hashimoto, 1997; Haass et al., 2010). However, while connexins often

appear to act as tumor suppressors in this respect, the role of connexins is highly dependent on the tumor type and stage of disease. Considerable evidence suggests that Cx43 and Cx26 can act as either tumor facilitators or tumor suppressors, depending on the disease state (Brandner and Haass, 2013). However, the mechanisms responsible for the biphasic effects of connexins have not been clearly delineated. Furthermore, there are gaps in our understanding of how altered cell-cell communication in the skin affects keratinocyte or melanocyte homeostasis resulting in skin cancer development (Brandner and Haass, 2013; Haass and Herlyn, 2005). Therefore, further research is required to delineate the possible mechanisms behind skin cancer progression, to ultimately develop preventative strategies.

1.1.8 Connexins in Squamous and Basal Cell Carcinomas

While the role of connexins in melanocyte regulation is still in question, GJIC has been extensively linked to the regulation of keratinocyte growth, differentiation, and migration (Haass et al., 2004). Reductions in connexin function or GJIC are correlated with keratinocyte deregulation and the development of SCCs or BCCs (Tada & Hashimoto, 1997). Furthermore, studies have reported that keratinocyte-derived tumors have reduced Cx43 function, which in turn results in increased keratinocyte proliferation and migration (Haass et al., 2010; Mori et al., 2006; Tada and Hashimoto, 1997). This suggests a protective role for Cx43 in the early stages of keratinocyte tumorigenesis. In contrast, reports show that Cx26 is expressed in hyper-proliferative skin, as well as up-regulated in both SCCs and BCCs, predominately in the more invasive areas (Haass et al., 2006). These findings suggest that Cx43 and Cx26 may have complicated roles in the initiation and progression of keratinocyte-derived skin cancers, and further studies are required to define the potential of these proteins as therapeutic targets in SCCs and BCCs.

1.1.9 Connexins in Melanomas

Under basal conditions, melanocytes form an epidermal melanin unit with surrounding keratinocytes. The keratinocytes within this unit have been suggested to provide homeostatic regulatory signals to assist proper melanocyte division (Haass and Herlyn, 2005; Haass et al., 2004). These include the release of paracrine growth factors, secondary messengers, as well intercellular connections including cell-cell adhesions, cell-matrix

adhesions, and possibly GJIC (as reviewed by Haass et al., 2005). Furthermore, in melanoma progression, melanoma cells have been proposed to escape interactions with keratinocytes as they undergo uncontrolled cellular division and invade across the basement membrane (Haass and Herlyn, 2005). Surprisingly, only a handful of studies define keratinocyte-melanocyte intercellular communication (Hunter and Pitts, 1981; Hsu et al., 2000), and only one suggests that this could possibly be mediated by Cx43 associated GJs (Hsu et al., 2000). This report does not show evidence of prominent Cx43 associated GJ plaques at the keratinocyte-melanocyte interface (Hsu et al., 2000), which would implicate this connexin in the heterocellular interaction between these two primary cell types. Interestingly, Cx43 has been proposed to be downregulated during normal melanocyte division, as melanocytes separate from surrounding keratinocytes to divide (Haass and Herlyn, 2005), and in immunofluorescence of human primary cutaneous melanoma compared to the surrounding epidermis (Haass et al., 2010). However, while Cx43 has been identified as a possible mediator of melanocyte homeostasis, studies have shown that Cx43 can act as both a tumor suppressor (Naus and Laird, 2010; Ableser et al., 2014) and a tumor facilitator (Elzarrad et al., 2008; Rezze et al., 2011; Sargen et al., 2013). Therefore, the role of Cx43 in epidermal melanocyte homeostasis and human melanoma progression remains unclear.

As such, recent studies have begun to address the controversial role of Cx43 and Cx26 function in the epidermis. For example, Ableser et al. (2014) showed that the ectopic expression of Cx43 in the B16-BL6 mouse melanoma cell line significantly reduced uncontrolled melanocyte proliferation and growth, while ectopic Cx26 expression did not (Ableser et al., 2014). In addition, there are few studies that have investigated the expression of Cx43 in human primary cutaneous melanomas (Haass et al., 2010; Sargen et al., 2013). However, these reports are controversial, where one study reported the absence of Cx43 expression in primary cutaneous melanomas (Haass et al., 2010), others reported its increased expression (Sargen et al., 2013; Rezze et al., 2011). Furthermore, neither of these investigations assessed the localization patterns of Cx43 to determine if its localization was consistent with Cx43 playing a role in cell-cell communication as fully assembled gap junctions, which is a focus of our current study. In addition, existing reports have found an absence of Cx26 in primary melanoma tumors (Haass et al., 2006; Sargen

et al., 2013), which may not be surprising as there is no evidence that human epidermal melanocytes express Cx26 normally. Therefore, in order to address outstanding questions regarding the role of connexins in melanocytes, and human melanoma progression we began to assess the potential biphasic role of Cx43 in melanocyte homeostasis in vivo and it possible role(s) in tumorigenesis.

1.1.10 Mouse Models to Assess the Roles of Cx26 and Cx43 in the Skin

The first mouse model that we employed in our current study, $Cx43^{I130T/+}$, globally expresses an autosomal dominant missense mutation that results in an isoleucine to threonine mutation located in the cytoplasmic loop of the Cx43 protein, as previously described (Kalcheva et al., 2007). However, the characterization of the skin in the $Cx43^{I130T/+}$ mouse model has been limited even though Cx43 is prominently involved in epidermal maintenance and function (Dbouk et al., 2009; Scott et al., 2012). Previous studies have shown that while the expression of $Cx43^{I130T/I130T}$ is embryonic lethal, murine $Cx43^{I130T/+}$ heterozygotes possess aberrant Cx43 phosphorylation and trafficking, with a resultant decrease in GJ functional conductance (Kalcheva et al., 2007; Stewart et al., 2013; (Seki et al., 2004). Therefore, this mutant mouse is useful to examine how decreased Cx43 associated GJIC affects skin homeostasis, including its susceptibility to skin tumor initiation and progression. Furthermore, this mutant mouse mimics the mutated Cx43 protein that is also found to cause human oculodentodigitial dysplasia (ODDD) (Kalcheva et al., 2007; Laird, 2008). As such, it can also provide insight on Cx43 function in skin cancer that can be extrapolated to the human ODDD population.

In addition, we generated a novel Cx26 mutant mouse to define the role of Cx26 in the regulation of keratinocytes. $Cx26^{CK14\text{-}S17F/+}$ mice express the autosomal dominant S17F mutant, located within the amino terminus of the Cx26 protein, specifically in keratinocytes where keratin14 (K14) is expressed (Figure 1.2). A constitutive $Cx26^{S17F/+}$ transgenic mouse had previously been created and analyzed as a model mimicking the Cx26 S17F mutation observed in human patients suffering from keratitis–ichthyosis–deafness (KID) syndrome (Schütz et al., 2011). While this study found that the global expression of $Cx26^{S17F/S17F}$ was embryonic lethal, surviving murine heterozygotes displayed many

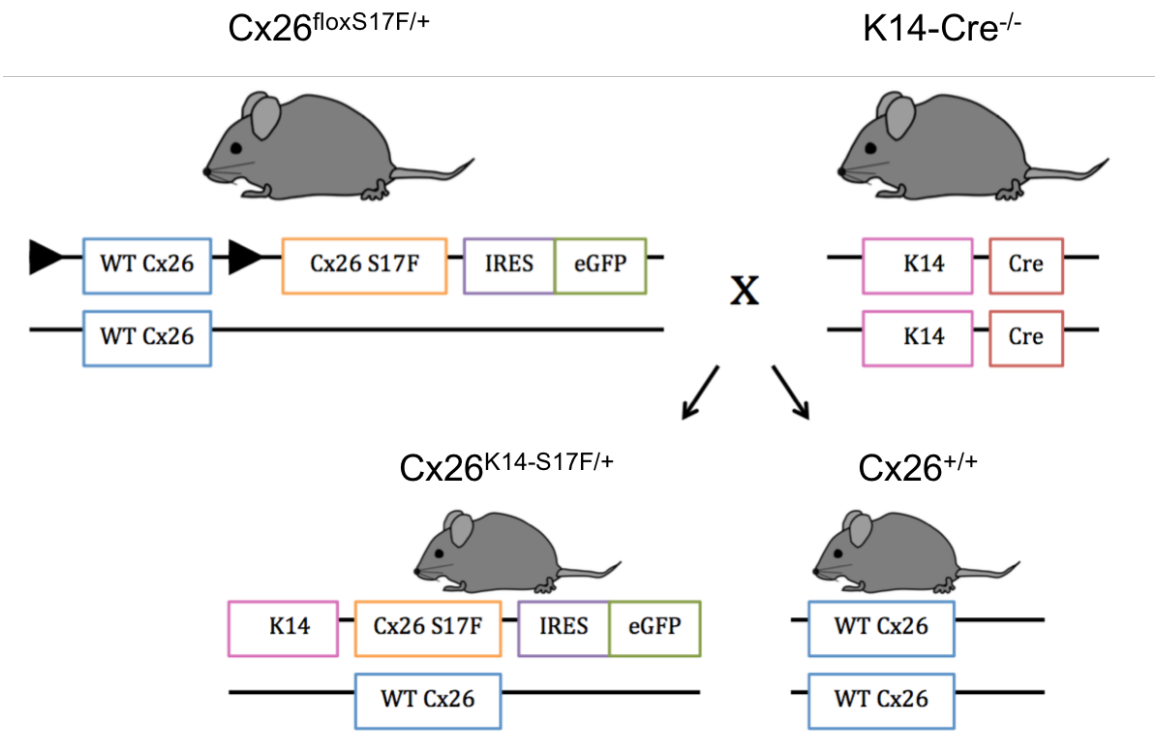

Figure 1.2: Breeding structure for the creation of the tissue specific $Cx26^{K14\text{-}S17F/+}$ mouse model and WT littermates.

similar symptoms to that of human KID patients, including skin hyperplasia and hearing impairments (Schütz et al., 2011). In addition, the Cx26 S17F protein has been shown to traffic normally to the plasma membrane and can successfully form gap junction plaques (Richard et al., 2002). However, in comparison to WT protein function, dye-coupling studies showed a significant reduction in gap junctional conductance (Richard et al., 2002), in addition to a complete loss in biochemical coupling and hemichannel activity (Lee et al., 2009). Furthermore, the Cx26 S17F mutant has also been shown to exhibit aberrant interactions, including a strong transdominant-negative effect on WT Cx43 when co-expressed in HeLa cells, in addition to increased hemichannel currents when co-expressed with WT Cx26 (Garcia et al., 2016). As such, we anticipate that $Cx26^{K14\text{-}S17F/+}$ mice possess a major reduction in gap junction conductance in keratinocytes. Moreover, we can examine how a reduction in Cx26 associated GJIC affects the regulation of the skin, and it's possible role in tumor onset and progression.

1.1.11 Hypothesis

We hypothesize that Cx43 and/or Cx26 play protective roles against UV induced damage to maintain the health of the epidermis. Therefore, impairments in Cx43 or Cx26 GJIC will result in impaired skin health and homeostasis, including increased susceptibility to UV radiation and skin cancer development.

To address this hypothesis, we examined the physiological response of two mutant mouse lines with greatly reduced connexin function when unchallenged and when challenged with UV radiation. Furthermore, using primary murine keratinocytes and melanocytes we assessed connexin expression and coupling status to better elucidate the roles of Cx43 in epidermal homeostasis. Finally, we addressed the proposed tumor suppressor role of Cx43 by examining its expression throughout all stages of human melanoma disease progression.

1.1.12 Objectives

My specific aims are as follows:

Aim 1: Determine the significance of Cx43 and Cx26 in epidermal homeostasis using two mouse models ($Cx43^{I130T/+}$ and $Cx26^{K14\text{-}S17F/+}$) in unchallenged and UV challenged dorsal skin.

Aim 2: Determine the expression levels of Cx43 in melanocytes and assess if mice with compromised Cx43 function are more susceptible to skin tumors upon UV insult.

Aim 3: Determine the expression and localization of Cx43 in primary and metastasized human melanomas.

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Chapter 2

2 The differential roles of Connexin26 and Connexin43 in modulating the epidermal response to UV injury

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Eric R. Press: Assisted in breeding the $Cx26^{K14\text{-}S17F/+}$ mouse line and partnered to perform the first toluidine blue assay on unchallenged $Cx26^{K14\text{-}S17F/+}$ neonates (Figure 5a).

Kevin Barr: Assisted in establishing mouse lines used in this study.

Melissa Crawford: From the lab of Dr. Lina Dagnino, provided all protocols for the successful isolation of primary murine melanocytes, including the TRP1 antibody used for their identification in culture (Figure 7b).

Dale W. Laird: Principal investigator, co-designed all experiments, and consulted on their interpretation.

2.1 Summary

The roles of connexins in modulating epidermal homeostasis in response to UV radiation and UV - induced skin cancer remain largely undefined. In order to address the role of Cx26, we successfully created the $Cx26^{K14\text{-}S17F/+}$ mouse model that conditionally expresses the Cx26 S17F loss-of-function mutant protein in keratinocytes. We showed that $Cx26^{K14\text{-}S17F/+}$ mice are viable and mimic the skin disease seen in patients suffering from keratitis-ichthyosis-deafness syndrome. We analyzed the dorsal skin of $Cx26^{K14\text{-}S17F/+}$ and $Cx43^{I130T/+}$ neonates (with reduced connexin function), when unchallenged and when exposed to UV radiation. $Cx26^{K14\text{-}S17F/+}$ neonates did not survive after a single exposure to 14 kJ/m^2 of UV radiation or exposure of 6.2 kJ/m^2 every day for 5 consecutive days. In addition, while both mouse lines did not show any significant differences in epidermal permeability or basement membrane integrity, $Cx26^{K14\text{-}S17F/+}$ neonates showed increased susceptibility to UV-induced skin damage, as evidenced by rapid epidermal desquamation compared to controls post exposure. In contrast, $Cx43^{I130T/+}$ mice were relatively unaffected by UV insult, did not develop tumors of melanocyte or keratinocyte origin within 6 months after UV exposure, and their skin appeared normal. In addition, while primary keratinocyte cultures express Cx43, primary murine melanocytes show no evidence of Cx43 expression and were not functionally coupled by gap junctions. However, human melanoma metastases to distant organ sites showed increased Cx43 expression, but little evidence of Cx43 gap junction assembly. Collectively, these studies strongly suggest that Cx26 is critical in protecting keratinocytes from acute UV damage, and that Cx43, although not evident in primary melanocytes, is expressed in metastasized human melanomas.

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2.2 Introduction

Skin is the largest organ in the body where the stratified layers of differentiated keratinocytes constitute the major component of the epidermis (Scott et al., 2012). Melanocytes reside within the basal layer of the epidermis and distribute melanin pigment to surrounding keratinocytes to protect against UV-induced DNA damage (Klein-szanto et al., 1994). The dynamic coordination of keratinocytes and melanocytes enables the epidermis to protect the body against environmental insults, dehydration, and external pathogens (Baroni et al., 2012). Deregulation of these cells has been directly linked to the development of a wide variety of skin diseases, including loss of barrier function, altered wound healing, and cancers (as reviewed by Scott et al., 2012). Thus, epidermal homeostatic control is critical and often involves dynamic homocellular and heterocellular interactions, facilitated by gap junction (GJ) channels assembled from connexins (Haass et al., 2004).

Connexins encompass a 21-member family of integral membrane proteins that form gap junction channels to allow for the intercellular transfer of ions and small molecules (< 1 kDa), termed gap junctional intercellular communication (GJIC) (Söhl and Willecke, 2003). Connexin proteins oligomerize in the endoplasmic reticulum or Golgi apparatus into hexameric channels (termed connexons), which then translocate to the plasma membrane where they can dock with a connexon from an adjacent cell to form a gap junction channel for direct exchange of cytosolic signaling molecules (Dbouk et al., 2009). This exchange is essential for regulating controlled cellular events, such as proliferation, differentiation, and migration (Scott and Kelsell, 2011). In addition to their primary function in GJIC, connexins also provide regulatory control via an extensive network of protein interactions (Vinken et al., 2012; Giepmans, 2004; Laird, 2010). Furthermore, the expression profile of connexins is tissue-specific, and can be altered in many pathologies, highlighting their complex regulation during both health and disease.

Specifically, as many as 10 connexins are differentially expressed throughout the strata of epidermal keratinocytes and play a variety of roles in regulating cellular behaviour to maintain overall tissue health and function (Scott and Kelsell, 2011; Laird, 2006). Among

these, connexin43 (Cx43) and connexin26 (Cx26) are expressed predominately in the stratum basale and spinosum, and the suprabasal layers of the epidermis, respectively (Richard, 2000; Kretz et al., 2004; Laird, 2006). Studies in both humans and mice report that a transient decrease in Cx43 expression is necessary for epidermal healing after wounding (Kretz et al., 2003; Brandner et al., 2004), and keratinocyte proliferation (Pollok et al., 2011; Churko et al., 2012). However, truncating the c-terminal tail of Cx43 results in aberrant murine skin barrier formation and keratinocyte differentiation (Maass et al., 2005). Similarly, a transient increase in Cx26 expression is necessary in epidermal healing after mechanical wounding (Goliger and Paul, 1995; Kretz et al., 2003) and its regulated expression is required for epidermal barrier acquisition in murine development (Djalilian et al., 2006). Furthermore, germline mutations in the genes encoding Cx43 (*GJA1*) or Cx26 (*GJB2*) are associated with a variety of human skin diseases, including palmoplantar keratoderma (PPK), and keratitis-ichthyosis-deafness (KID) syndrome (Scott et al., 2012; Avshalumova et al., 2014). Collectively, these studies demonstrate the crucial role that connexins play in maintaining overall epidermal health and function. However, despite the vast array of connexin species expressed throughout keratinocyte layers, little is known about the connexin profile of melanocytes. Some preliminary evidence suggests that melanocytes express Cx43 and exhibit GJIC both in melanocyte monoculture, and in melanocyte-keratinocyte co-culture (Hsu et al., 2000). However, other than one study that reports Cx26 expression in melanocytes of the vestibular dark area of the ear, there is no information on whether Cx26 is expressed in epidermal melanocytes (Masuda et al., 2001).

Despite the well-established importance of Cx43 and Cx26 in epidermal health, their roles in the epidermal response to common environmental insults, including UV radiation, are poorly understood. This is of concern as exposure to UV radiation can lead to both acute and chronic skin damage, ranging from sunburn and epidermal desquamation to UV-induced tumorigenesis (IARC, 2012; DeGruijl, 1999). Current studies have begun to delineate the role of Cx43 and GJIC in the acute epidermal response to UV radiation (Bellei et al., 2008; Gambichler et al., 2008), however these reports are limited. In human keratinocytes, Cx43 and GJIC have been proposed to be downregulated in response to UVA radiation (Bellei et al., 2008), but upregulated in response to UVB (Gambichler et al., 2008). Furthermore, not only does the significance of these changes in Cx43 expression

remain unclear, but the role of Cx26 in the epidermal response to UV radiation is completely unknown.

The long-term effects of UV-induced epidermal damage have been extensively categorized and identified as the primary carcinogen responsible for the development of skin cancers. For example, more than 90% of human keratinocyte derived-tumors (Koh et al., 1996), and more than 86% of human melanomas have been directly attributed to UV-induced mutations (Parkin et al., 2011). However, the role of connexins in human squamous cell carcinomas (SCCs), basal cell carcinomas (BCCs), and melanomas remain controversial. In the few characterizations of connexins in human skin cancers, Cx43 and Cx26 have been proposed to act as either tumor facilitators or as tumor suppressors depending on the stage and type of disease, suggesting a complex role for these proteins in disease progression (Laird, 2010). For example, immunohistochemistry revealed that Cx26 was upregulated in human keratinocyte-derived tumors (Haass et al., 2006), but was not expressed in melanocyte-derived tumors (Haass et al., 2006; Sargen et al., 2013), but no subcellular localization of Cx26 was provided raising questions as to whether Cx26-based gap junctions were formed. However, Cx26 was also found to be upregulated in the keratinocytes directly adjacent to malignant melanomas, suggesting a possible role in the tumor soil (Haass et al., 2010). In addition, human keratinocyte-derived tumors expressed low levels of Cx43, which was postulated to allow increased keratinocyte proliferation and migration (Haass et al., 2006). The role of Cx43 in human melanoma remains controversial. Tissue microarray analysis, and immunohistochemistry of primary cutaneous human melanoma revealed increased Cx43 expression in comparison to melanocytic nevi, suggesting it may have a possible role as a tumor facilitator (Rezze et al., 2011; Sargen et al., 2013); while another study reports its decreased expression (Haass et al., 2004). Despite these initial investigations, a thorough characterization of the expression and localization of Cx43 throughout the different stages of human melanoma progression remains unknown.

To better elucidate the roles of Cx43 and Cx26 in epidermal homeostasis, two mutant mouse models ($Cx43^{I130T/+}$, $Cx26^{K14\text{-}S17F/+}$), that possess a significant reduction in GJIC, were analyzed when unchallenged and when exposed to UV radiation. The $Cx43^{I130T/+}$

mouse has been previously described (Kalcheva et al., 2007), and has been shown to possess aberrant Cx43 phosphorylation and trafficking, with a resultant systemic decrease in GJ functional conductance by approximately 50% (Stewart et al., 2013; Seki et al., 2004). Furthermore, these mice mimic the same mutation seen in a subset of human oculodentodigital dysplasia (ODDD) patients (Kalcheva et al., 2007). In addition, we have generated a novel Cx26 mutant mouse ($Cx26^{K14\text{-}S17F/+}$) to better define the role of Cx26 in the regulation of keratinocytes. $Cx26^{K14\text{-}S17F/+}$ mice harbor the autosomal dominant S17F missense mutation in tissues that also express keratin14 (K14), which includes keratinocytes. Previous studies have shown that the Cx26 S17F protein is trafficked normally to the plasma membrane and assembles into gap junction-like structures, but the resulting channels are not functional (Lee et al., 2009). Furthermore, the expression of the Cx26 S17F protein has also been directly linked to KID syndrome in human patients. Therefore, both mouse lines can not only be used as models of inherited channelopathy, but also provide insight into the role of Cx43 and/or Cx26 in the health of ODDD and/or KID patient skin upon exposure to a common environmental insult. In addition, to further address the roles of connexins in skin health and cancer, we analyzed Cx43 expression and cellular localization in human melanoma samples from primary cutaneous tumor, nodal metastases, and metastases to distant organ sites.

Using both connexin mutant mouse lines and a cohort of human melanomas, we sought to address the possible protective roles of Cx43 and Cx26 in the maintenance of unchallenged and UV-challenged epidermis. $Cx26^{K14\text{-}S17F/+}$ mice were viable but exhibited impaired epidermal health when unchallenged, and this effect was exacerbated in response to UV injury, resulting in high mortality soon after UV exposure. $Cx43^{I130T/+}$ mice did not show any adverse effect from UV exposure, nor did they exhibit increased propensity to UV-induced skin cancers of keratinocyte or melanocyte origin. Although keratinocytes abundantly express both Cx43 and Cx26, primary murine melanocytes did not exhibit connexin expression or GJIC in primary cultures. In addition, Cx43 was poorly expressed in primary melanoma tumors as well as in nodal metastases, but was prominently expressed intracellularly in melanoma metastases found in a variety of distant organ sites. Taken together, these studies strongly suggest that Cx26 is critical in protecting keratinocytes

from immediate UV damage, while Cx43 may play a more complex role in the progression of late stage melanomas.

2.3 Materials and Methods

2.3.1 Animals

Heterozygote mice (*Gja1*^{tm3GFi}, Cx43$^{I130T/+}$) expressing the disease-causing I130T amino acid substitution that results in reduced Cx43 associated GJ function, were generated by Kalcheva and colleagues (2007) and bred on a background of CD1 and C57BL/6 (generously provided by Dr. Glenn Fishman). All Cx43$^{I130T/+}$ (also referred to as I130T/+ mice in this study) mice and their wild-type (WT) littermates were used at generation 6-10 of backcrossing on to C57BL/6. Heterozygote mice (Cx26$^{K14\text{-}S17F/+}$, also referred to as S17F/+ mice in this study) expressing the disease-causing S17F mutation, that reduces Cx26 associated GJ function, were created in-house. Mice heterozygous for loxp floxed WT *Gjb2* (Cx26$^{floxS17F/+}$, C57BL/6 and 129Sv) were generously provided by Dr. Klaus Willecke (Schütz et al., 2011) and mated with homozygous keratin14 (K14) - Cre (*Gjb2*$^{tm2.2Kwi/Cnrm}$, Jackson Labs) mice to conditionally express the Cx26 S17F mutant protein in the epidermis, oral ectoderm, and dental epithelium (Dassule et al., 2000). For both mouse lines, both male and female littermates were used as controls, kept on a 12h/12h light-dark cycle, and given food (2018 Teklad 18% Global Diet, Harlan) and water ad libitum. The Animal Care Committee at The University of Western Ontario has approved all experiments performed in this study (Appendix D).

2.3.2 Human Melanoma Samples

Human melanoma samples and normal controls were provided by the Ontario Tumor Bank (OTB), which is funded by the Ontario Institute of Cancer Research (OICR). All cases were verified by a pathologist at the OICR before use. Paraffin embedded sections (5 μm) of primary cutaneous tumor (N = 14), nodal melanoma metastases (N = 15), and melanoma metastases from distant organ sites (N = 7), in addition to normal controls (N = 3, N =1, N =3) were selected from the OTB database (Appendix A) for analysis. Distant tumors represent metastasized melanomas isolated from either lung, kidney, bone, or pelvic wall (Appendix A). To avoid the possible inclusion of adjacent normal tissue in analysis, the

core and its surrounding radius of all tumor samples was analyzed for Cx43 expression using immunofluorescence. All work with human specimens was approved by The Human Science Research Ethics Board at the University of Western Ontario (Appendix E).

2.3.3 Histology

Mice (3 days of age) were euthanized by decapitation and dorsal skin sections were collected and fixed in 10% neutral buffered formalin overnight at 4°C. Tissues were processed, paraffin embedded, and cut into transversal sections (6 μm) for further analysis. Paraffin embedded sections were deparaffinized in xylene and rehydrated in decreasing ethanol concentrations (100%, 95%). Sections were stained with 1% Harris Hematoxylin for 1 minute, followed by 1% eosin for 2 minutes (Lerner Laboratories). Stained tissues were then hydrated in increasing ethanol concentrations (100%, 95%), followed by xylene baths prior to mounting with Cytoseal (Richard-Allan Scientific), as previously described (Stewart et al., 2013). Stained tissue was imaged using a Leica DM IRE2 inverted epifluoresence microscope with ProgRes C5 camera (Jenoptik) and ProgRes Mac CapturePro 2.7.6 imaging software.

2.3.4 UV Radiation

UV radiation was performed using a 302 nm UV lamp with filter (UVP, Upland, CA) at a fixed distance of 45 cm, and dosage controlled using a digital radiometer and sensor (UVX-31; UVP) with a peak sensitivity calibrated to 302 nm (Appendix B). $Cx43^{I130T/+}$, $Cx26^{K14\text{-}S17F/+}$ neonates, and their WT littermates (3 days of age) were placed in plastic containers and irradiated either once (14 kJ/m^2), or every day for 5 consecutive days (6.2 kJ/m^2). Sham treated neonates (3 days of age) were placed in the holding apparatus for the same dosage time but not exposed. The survival of these mice was recorded daily during the first two weeks, and subsequently on a weekly basis, until they were euthanized at 6 months using CO_2. At 6 months of age, the dorsal hair of surviving mice was shaved, and chemically removed with Nair, prior to the collection of dorsal skin sections that possessed low and high levels of pigment. Dorsal skin from unchallenged and UV challenged (24 hours post exposure to 14 kJ/m^2) neonates was cryopreserved in 30% sucrose overnight at 4°C, before being embedded in 18% sucrose and 1% UltraPure Low Melting Point Agarose

(Invitrogen, 15517-022). Tissues were sectioned at -25°C into transversal sections (14 µm) for analysis. Dorsal skin from mice 6 months after UV exposure were fixed overnight at 4˚C with 10% neutral buffered formalin prior to being embedded in paraffin, cut into 6µm transverse sections, and analyzed for evidence of keratinocyte and/or melanocyte tumorigenesis using immunofluorescence.

2.3.5 Toluidine Blue Penetration

The epidermal permeability barrier to water was assessed for both mouse lines and their WT littermates before and 8 hours after exposure to 14 kJ/m^2 of UV radiation. Both unchallenged and challenged neonatal mice (1 day of age) were euthanized with carbon dioxide (CO_2) for 30 minutes. To assess epidermal permeability barrier function, mice were placed in increasing methanol concentrations for one minute (25%, 50%, 75%, 100%, diluted in PBS), equilibrated in PBS for one minute, and subsequently stained using 0.02% toluidine blue solution (diluted in distilled water) for 15 minutes. Mice were then washed in 90% ethanol (diluted in distilled water) for one minute three times, and once in distilled water for the same duration (Schütz et al., 2011). After staining, mice were allowed to air dry and imaged using digital photography. As a positive control, a small section on the side of a WT littermate was washed with 2 ml of acetone or lacerated to artificially break down the epidermal barrier prior to the staining procedure.

2.3.6 Primary Murine Cell Cultures

The dorsal skin was collected from WT mouse neonates (2 – 3 days of age) as previously described (Churko et al., 2012). Neonates were obtained and euthanized by inhalation of CO_2 for 30 minutes, immersed in 70% ethanol for 5-10 seconds for sterilization, and washed with PBS (without Ca^{2+} or Mg^{2+}). Neonatal dorsal skin was excised and washed twice in PBS (without Ca^{2+} or Mg^{2+}) and remaining pieces of muscle were removed. Skin was isolated from each pup, placed in separate wells that contain 2 ml of dispase (5000 CU, Corning, 354235) and rocked overnight at 4°C to separate the epidermis from the rest of the skin.

Isolation of primary murine melanocytes was accomplished as previously described (Kumar et al., 2012), with the following modifications. Following dispase incubation, 6-8

epidermises were separated, minced, and transferred to tubes containing 0.5 µl of 0.25% Trypsin/EDTA solution per epidermis. The epidermis was then incubated on a rocker for 10 minutes at 37°C, and then double the amount of Trypsin Neutralizing Solution (Gibco, R002100) was added. The suspension was re-suspended continuously to release single cells from epidermis, passed through a 70-micron strainer, and centrifuged for 10 minutes at 1000 rpm. Supernatant was then removed and the pellet was re-suspended in 5 ml of Clonetics Melanocyte Growth Medium-4 (MGM-4 Bullet Kit, CC-3249) with Endothelin 3 Lyophilized (ET3, Lonza, CC-4510). Cells were then counted with an automated cell counter (Countess, Life Technologies) and 1 x 10^6 cells were plated on 60mm dishes. Medium was changed the following day and subsequently every two days. Melanocytes were passaged when they were 80% confluent, which occurs approximately 1 – 2 weeks post-plating. Adherent cells are rinsed with PBS (without Ca^{2+} or Mg^{2+}) and released by digestion using 0.25% Trypsin/EDTA incubated at 37°C for varying times depending on the number of passages (P1 – 6 minutes, P2 – 7 minutes, P3 – 8 minutes, P4 – 9 minutes). After incubation, Trypsin Neutralizing Solution was added and the suspension was centrifuged at 1000 rpm for 10 minutes. The pellet was re-suspended in MBM-4 + ET3 medium and cells were counted with an automated cell counter (Countess, Life Technologies) and plated. Cells analyzed by immunohistochemistry were plated on coverslips coated with rat-tail collagen type 1 as previously described (BD Biosciences, 354236).

For primary keratinocyte cultures, isolated skin was incubated overnight in dispase at 4°C, subsequently the epidermis was separated, minced, and 1 ml of 0.25% Trypsin/ EDTA was added per tissue and incubated for 10 minutes at 37°C. Trypsin/cell solution was then removed and centrifuged at 1000 rpm for 3 minutes. Thereafter, the pellet was first suspended in 2 ml of Trypsin Neutralizing Solution and 2 ml of cold 1.4 mM calcium diluted in Keratinocyte - Serum Free Media (K-SFM, Gibco, 10724-011) with human recombinant factor and bovine pituitary extract (Gibco, 37000-015). The pellet was centrifuged again and then re-suspended in 2 ml of cold 0.05 mM calcium diluted in K-SFM. Suspension was then filtered through a 70µm strainer, and cells were counted with an automated cell counter (Countess, Life Technologies) prior to being plated on dishes coated with rat-tail collagen type 1. To induce keratinocyte differentiation, primary cells

were placed in 1.4mM calcium K-FSM 24 hours after isolation, and maintained for an additional 24 hours before use (Modified from Churko et al., 2012).

2.3.7 L-DOPA Staining

Coverslips with attached melanocytes were removed, washed in PBS (pH 6.8) and fixed in 4% PFA for 20 minutes at 25˚C. PFA was removed and fixed cells were washed three times in PBS (pH 6.8), prior to incubation with 0.1% L-3,4-dihydroxyphenylalanine (L – DOPA) (Alfa Aesar, A11311) for 4 hours at 37°C, as previously described (Wang et al., 2013). Coverslips were subsequently washed with PBS (pH 6.8) before being mounted on glass slides with permount and five images were taken for each passage using a LSM 800 Zeiss confocal microscopic using a 40x lens. Images were blinded to the investigator and melanocyte purity was calculated by measuring the percent yield of L-DOPA positive cells (dark pigmented cells) verses total cells in an image area.

2.3.8 Calcein-AM Dye Transfer

P2 primary murine melanocytes and rat epidermal keratinocytes (REKs), which were previously isolated and characterized by Baden and Kubilus (1983), were grown to confluence on glass bottom dishes in Dulbecco's Modified Eagle Medium (DMEM, 10% FBS), and GJIC was evaluated using a modified dye transfer assay from what has been previously described (Goldberg et al., 1995). Calcein-AM (ThermoFisher Scientific, C3100MP) dye was diluted in 10 µl of DMSO, and 2 µl of DMSO + Calcein-AM solution was added to 1 ml of isotonic solution. Culture media was removed and Calcein-AM in isotonic solution was added to plates and incubated for 10 minutes at 37°C. Cells were then washed twice in media and then cells that were surrounded by other neighboring cells (N = 30) were photo-bleached to 20% of original staining intensity using a 488 nm argon laser (80% power). Images were captured every 3 seconds until maximum recovery was reached (15 minutes) using a LSM 800 Zeiss confocal microscopy using a 40x lens. Ten cytosolic areas (2 mm^2) in bleached cell and unbleached non-adjacent cells were selected and mean fluorescence intensity was measured over the time course of the experiment using ImageJ Time Series Analyzer V3.

2.3.9 Immunofluorescence

Paraffin embedded sections were deparaffinized in xylene and rehydrated in descending concentrations of ethanol baths (100, 95, and 70%) before being placed in a sub-boiling solution of sodium citrate buffer (0.01M, pH 6.0) for 20 minutes, and cooled in the same solution for 10 minutes. Sections were then rinsed with water and PBS for 5 minutes each. Both paraffin and cryo-embedded sections were outlined with a hydrophobic marker and blocked using 3% blocking serum (3% BSA, 0.2% Triton X-100, 10 ml PBS) for 60 min. Slides were probed with rabbit anti-Cx43 (1:300; Sigma-Aldrich, C6219), mouse anti-E-cadherin (1:200; BD Transduction Labs, 610182), rabbit anti-Ki67 (1:200; Abcam, 15580), mouse anti-K14 (1:100; Neomarkers, LL002), or rabbit anti-MITF (1:200; Abcam, 20663), overnight at 4°C. Subsequently, cells were incubated with Alexa Fluor 555-conjugated anti-rabbit (1:400; Molecular Probes) and/or Alexa Fluor 488-conjugated anti-mouse (1:400; Molecular Probes) for 1 hour at 25ºC. Slides were then incubated with nuclear Hoescht for 10 minutes at 25ºC (1:1000; Molecular Probes, H3570) prior to mounting using Airvol and imaged with confocal microscopy (Stewart et al., 2013).

Keratinocytes and melanocytes were washed in PBS, and fixed in 4% PFA for 20 minutes at 25ºC. Cells were permeabilized with 0.1% Triton X-100 for 10 minutes, prior to blocking using a 2% solution of BSA in PBS for one hour at 25ºC. Thereafter, cells were probed with either mouse anti-tyrosinase related protein1 (TRP1, 1:200; Abcam 3312), rabbit anti-Cx43 (1:400), and/or mouse anti-E-cadherin (1:200) overnight at 4°C. Cells were then probed with aforementioned secondary antibodies for 1 hour at 25ºC, labeled with nuclear Hoescht stain (1:1000), and images were taken using confocal microscopy.

2.3.10 Immunoblotting

Following decapitation, dorsal skin was collected from $Cx43^{I130T/+}$, $Cx26^{K14\text{-}S17F/+}$ neonates and their WT littermates both at 3 days of age, and 24 hours post 14 kJ/m^2 of UV exposure. Tissues were homogenized using liquid nitrogen in 2X immunoprecipitation buffer (2% Triton X-100, 300 mM NaCl, 20 mM Tris/HCL (pH 7.4), 2mM EDTA, and 1% Nonidet P40, supplemented with complete mini proteinase inhibitor (Roche Diagnostics), and 100 mM sodium fluoride and 100 mM sodium orthovanadate). Lysates were run on 10%

polyacrylamide gels using SDS-PAGE, and protein was transferred to a nitrocellulose membrane using iBlot dry transfer. Membranes were then blocked for 1 hour with 3% BSA in PBS + Tween 20, and subsequently probed using rabbit anti-Laminin (1:750, Abcam, 11575) and anti-GAPDH (1:5000, Millipore, MAB374) overnight at 4°C. Blots were then washed and stained with Alexa Fluor 680-conjugated anti-rabbit (1:10,000, Molecular Probes) and Alexa Fluor 800-conjugated anti-mouse (1:10,000, Molecular Probes) secondary antibodies for 1 hour at 25ºC, prior to imaging and quantification with an Odyssey infrared imaging system (LiCor) (Stewart et al., 2013).

2.3.11 Statistical Analysis

Statistical analyses were performed using GraphPad Prism 6.0 and statistical significance was determined ($P<0.05$). Statistical tests included the unpaired two tailed *t* test, one-way ANOVA with Tukey's multiple comparison test, or Kaplan Meir Mantel-Cox test. Values are presented as means ± S.E.M. N≥3 for all experiments.

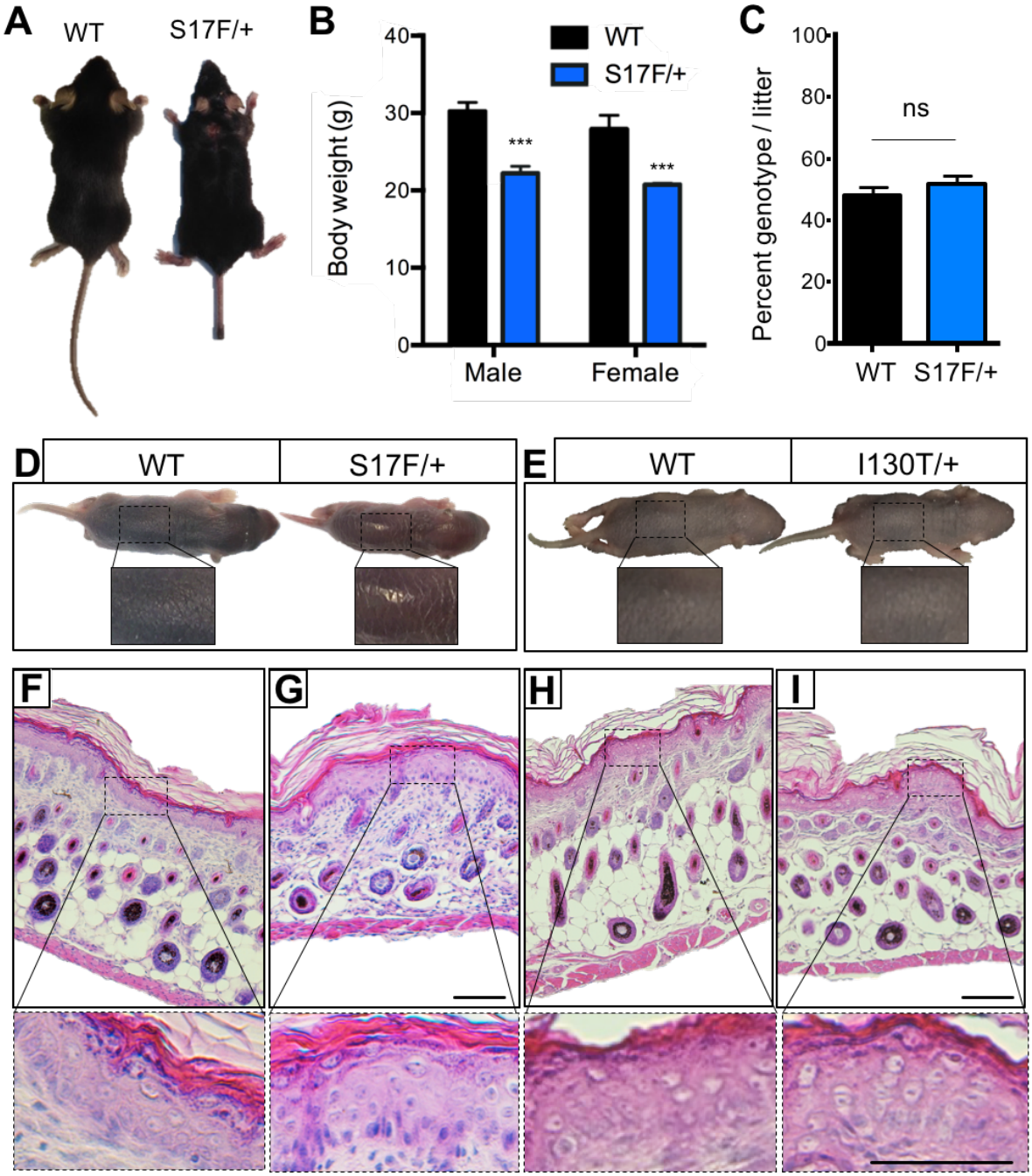
2.4 Results

2.4.1 $Cx26^{K14\text{-}S17F/+}$ mice are viable, exhibit annular tail restrictions, reduced body weight and erythrokeratoderma.

To characterize the effects of the mutant Cx26 or Cx43 protein in the epidermis we first examined $Cx26^{K14\text{-}S17F/+}$ and $Cx43^{I130T/+}$ mice for any apparent anatomical abnormalities in comparison to their WT littermates. Adult $Cx26^{K14\text{-}S17F/+}$ (3 months of age) mice presented with annular tail restrictions (Figure 2.1A), and weighed significantly less than their WT littermates at 3 months of age (Figure 2.1B) (N = 6). To mitigate sex-biased weight differences, bodey weight was calculated for both male and female cohort (Figure 2.1B). In addition, the keratinocyte-specific expression of the mutant Cx26 S17F in $Cx26^{K14\text{-}S17F/+}$ heterozygotes did not result in any significant impairment in neonatal survival compared to WT (Figure 2.1C) (WT: N = 157; $Cx26^{K14\text{-}S17F/+}$: N = 148). Anatomical images of $Cx43^{I130T/+}$ neonates (3 days of age) illustrate the lack of distinct skin abnormalities (Figure 2.1E). Whereas, full body images of $Cx26^{K14\text{-}S17F/+}$ neonates (3 days of age) show a red furfuraceous epidermal phenotype and were distinctively smaller than their WT littermates (Figure 2.1D). In

Figure 2.1: $Cx26^{K14\text{-}S17F/+}$ mice are viable, exhibit annular tail restrictions, reduced body weight, erthyokeratoderma and ichythosis.

(A) Representative image of annular tail restrictions in $Cx26^{K14\text{-}S17F/+}$ mice compared to its WT littermates at 3 months of age. In addition, (B) adult $Cx26^{K14\text{-}S17F/+}$ mice weighed significantly less at 3 months of age compared to WT controls (N = 6, ** $P < 0.05$; two-way ANOVA), in both male and female cohorts. (C) $Cx26^{K14\text{-}S17F/+}$ mice do not exhibit deficits in neonatal survival compared to WT littermates (WT: N = 157, S17F/+: N = 148). (D) Representative full body image of red scaly skin phenotype that was observed in $Cx26^{K14\text{-}S17F/+}$ neonates (3 days of age) in comparison to their normal WT littermates. In contrast, (E) the dorsal skin of $Cx43^{I130T/+}$ neonates (3 days of age) was not distinctively different from their WT littermate controls. (F-I) Dorsal skin sections stained with hematoxylin and eosin from (F) $Cx26^{K14\text{-}S17F/+}$ neonates and their (G) WT counterparts (3 days of age), and (H) $Cx43^{I130T/+}$ neonates, and their (I) WT counterparts (3 days of age), showed no distinct differences in skin or epidermal (insets) integrity. Scale bar = 100 μm.



addition, neonatal (3 days of age) dorsal skin stained with hematoxylin and eosin revealed no gross abnormalities in overall skin structure for both mouse lines. Higher magnification of the epidermis also showed no ultrastructural differences in the keratinocytes of either mouse line (N = 5) (Figure 2.1F - I).

2.4.2 $Cx26^{K14\text{-}S17F/+}$ neonates do not survive after UV exposure.

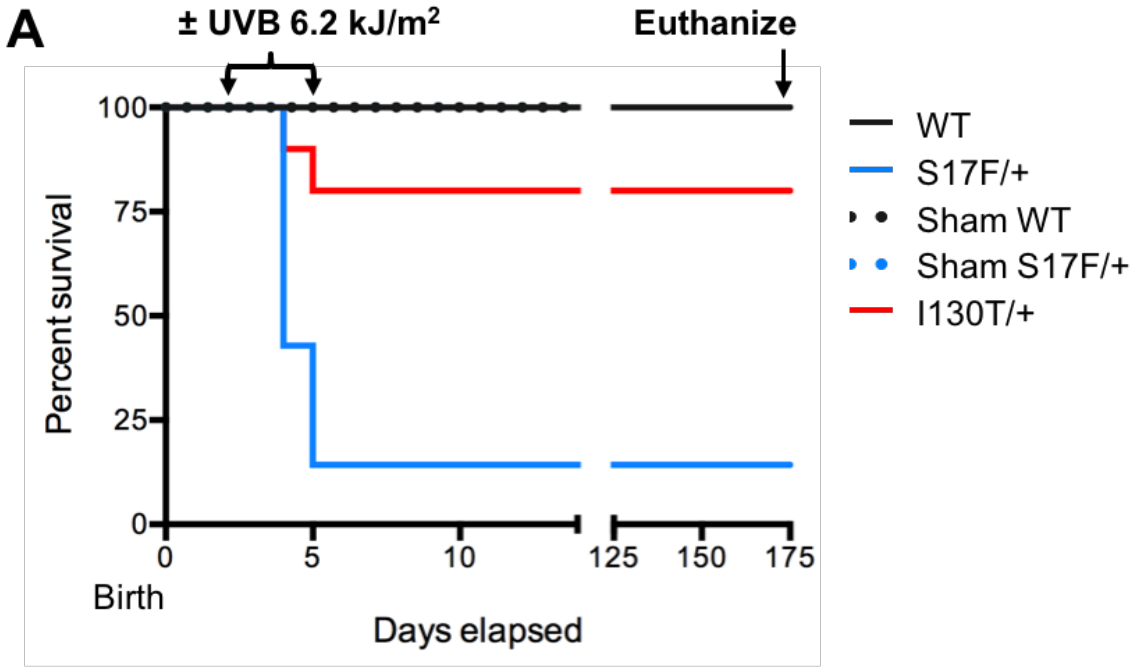
To assess the possible protective role of Cx26 and Cx43 associated GJIC in protecting the epidermis from UV radiation both mouse lines were exposed to either 6.2 kJ/m^2 of UV radiation every day for five consecutive days ($Cx43^{I130T/+}$ n = 9, WT n = 12; $Cx26^{K14\text{-}S17F/+}$ n = 14, WT n = 22), or 14 kJ/m^2 once ($Cx43^{I130T/+}$ n = 12, WT n = 9; $Cx26^{K14\text{-}S17F/+}$ n = 14, WT n = 24) starting at three days of age. Interestingly, compared to exposed WT littermates and unexposed sham controls, 86 and 85% of $Cx26^{K14\text{-}S17F/+}$ mice died within 72 hours of exposure to either 6.2 kJ/m^2 or 14 kJ/m^2 of UV radiation, respectively. In contrast, $Cx43^{I130T/+}$ mice possessed no significant differences in survival in response to UV radiation (Figure 2.2A, B).

2.4.3 UV radiation does not destroy barrier function in $Cx26^{K14\text{-}S17F/+}$ and $Cx43^{I130T/+}$ neonates.

It has been well documented that exposure to UV radiation can alter epidermal permeability and ultimately disrupt barrier function (Lee et al., 2014). Thus to determine whether reduced survival of $Cx26^{K14\text{-}S17F/+}$ mice was due to impairments in epidermal barrier, toluidine blue dye uptake was studied. Interestingly, $Cx26^{K14\text{-}S17F/+}$ and $Cx43^{I130T/+}$ mice showed no evidence of dye uptake, and therefore no loss in barrier function, both when unchallenged and when challenged with 14 kJ/m^2 of UV radiation (Figure 2.3A, B). Since an intact basement membrane has also been defined as an unequivocal requirement for skin barrier function (Breitkreutz et al., 2013), the structure and expression of laminin, an integral component of the basement membrane, was also examined. Immunohistochemistry revealed similar epidermal-dermal localization of laminin in both mouse lines compared to their controls before and 24 hours after exposure to 14kJ/m^2 of UV radiation (Figure 2.4A, B). In addition, immunoblotting revealed no significant

Figure 2.2: $Cx26^{K14\text{-}S17F/+}$ but not $Cx43^{I130T/+}$ neonates die after UV radiation.

(A) $Cx26^{K14\text{-}S17F/+}$ (N = 14) and $Cx43^{I130T/+}$ (N= 9) neonates and their WT littermates (N = 22, N = 9, respectively) were exposed to 6.2 kJ/m^2 of UV radiation for 5 consecutive days starting at 3 days of age and survival was recorded until remaining mice were euthanized at 175 days (6 months). Kaplan Meir survival plot illustrates that $Cx26^{K14\text{-}S17F/+}$ mice died quickly (**** $P < 0.0001$, Log-rank (Mantel-Cox) test) in comparison to WT littermates and unexposed sham controls (N = 5). $Cx43^{I130T/+}$ mice tolerated UV exposure as only two mice died. (B) $Cx26^{K14\text{-}S17F/+}$ (N = 14) and $Cx43^{I130T/+}$ (N= 12) neonates and their WT littermates (N = 24, N = 9, respectively) were exposed to 14 kJ/m^2 of UV radiation once at 3 days of age and survival was recorded. Similarly, $Cx26^{K14\text{-}S17F/+}$ mice died sooner than their WT littermates, and $Cx43^{I130T/+}$ neonates survived (**** $P < 0.0001$, Log-rank (Mantel Cox) test).



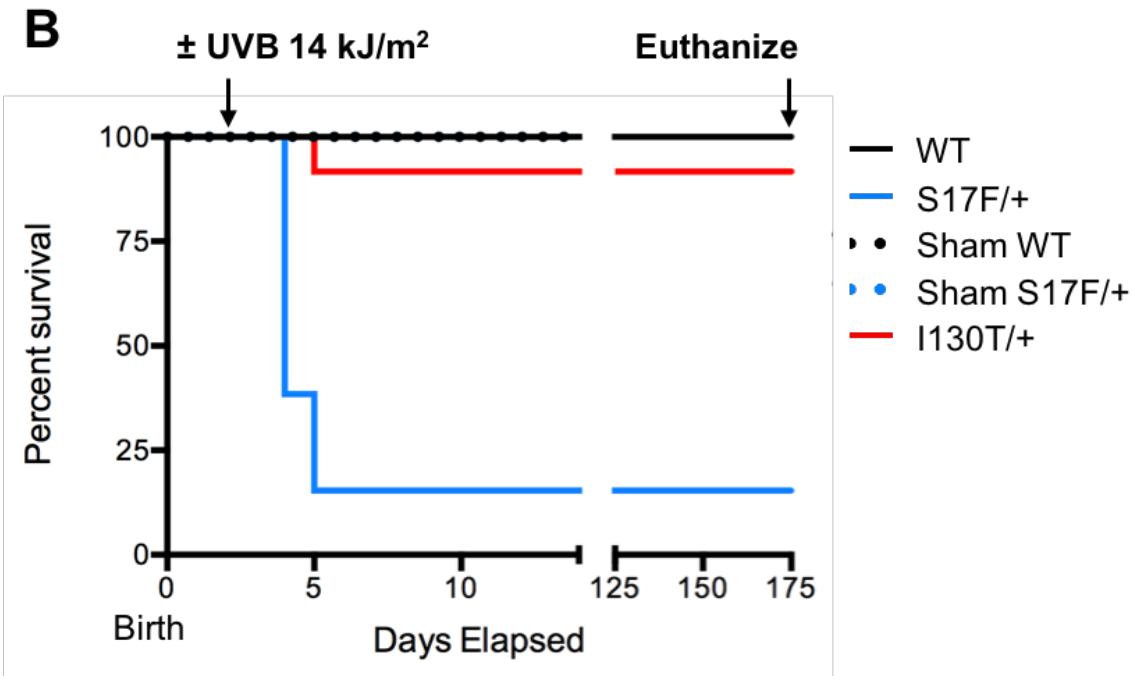
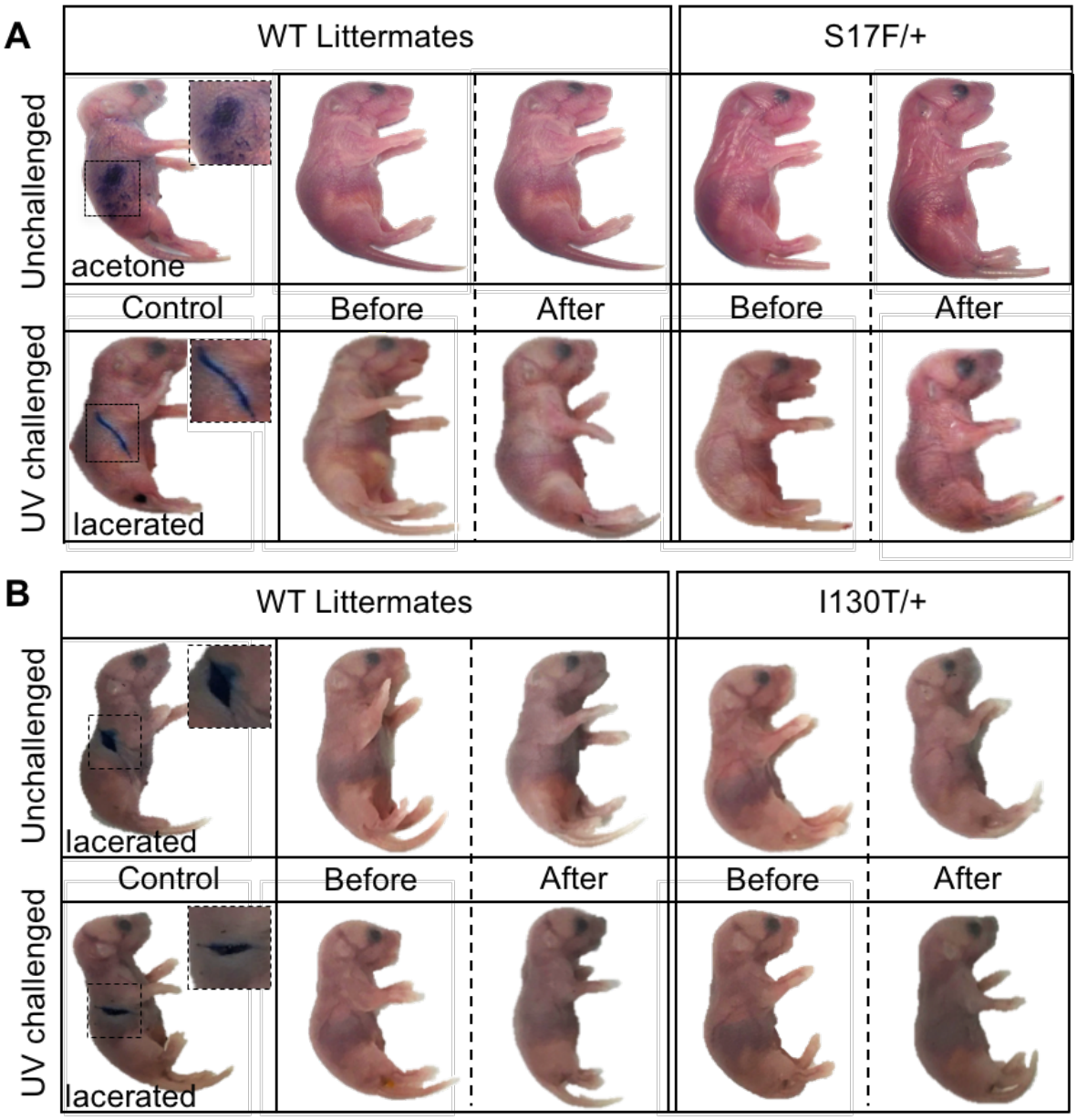


Figure 2.3: UV radiation did not perturb the epidermal permeability barrier in mutant mice.

(A) Similar to unchallenged and UV challenged WT littermates (N = 6 and N = 7, respectively), negative toluidine blue uptake in unchallenged $Cx26^{K14\text{-}S17F/+}$ (N = 5) and challenged $Cx26^{K14\text{-}S17F/+}$ (N = 4) mice indicates an intact epidermal barrier. WT controls display positive toluidine blue dye uptake when the epidermal barrier was disrupted with acetone (top left panel) or lacerated (bottom left panel). (B) Similarly, the skin of unchallenged $Cx43^{I130T/+}$ (N = 7), UV challenged $Cx43^{I130T/+}$ mice, and their WT littermates (N = 5, N = 6 respectively) did not take up toluidine blue dye.



differences in total laminin expression across all experimental groups (Figure 2.4C, D) indicating that laminin proteins of the basement membrane are equally expressed in mutant mice. Therefore, neither mouse line experienced impairments in barrier function when unchallenged or when challenged with UV radiation.

2.4.4 $Cx26^{K14\text{-}S17F/+}$ develop UV-induced epidermal desquamation

Another acute injury often incurred by exposure to UV radiation is a sunburn-like condition, which is often followed by epidermal desquamation, otherwise known as epidermal peeling. Increased degree and rate of epidermal desquamation, has been used as a tool to classify UV-reactivity of the skin (Fitzpatrick, 1988). As such, epidermal desquamation in both mouse lines and their WT littermates was measured. Interestingly, $Cx26^{K14\text{-}S17F/+}$ mice exhibited macroscopic evidence of UV-induced epidermal peeling within 48 hours of exposure, while exposed skin from their WT littermates and $Cx43^{I130T/+}$ mice did not show any evidence of epidermal peeling within the same time frame (Figure 2.5). Collectively, this suggests an increased UV-reactivity in $Cx26^{K14\text{-}S17F/+}$ neonatal skin.

2.4.5 $Cx43^{I130T/+}$ mice show no evidence of skin tumors of keratinocyte or melanocyte origin after UV insult

To determine the roles of connexins in UV-induced tumor incidence, surviving mice (6 months of age) that were exposed to either 6.2 kJ/m^2 of UV radiation ($Cx43^{I130T/+}$ n = 7, WT n = 12) or 14kJ/m^2 ($Cx43^{I130T/+}$ n = 11, WT n = 9) at 3 days of age were analyzed for keratinocyte and melanocyte tumors. At 6 months no distinct macroscopic abnormalities or growths were observed (Appendix C) for either experimental cohort. In addition, low pigmented and high pigmented dorsal skin sections of $Cx43^{I130T/+}$ mice and their WT littermates showed similar K14 expression 6 months post exposure to 14 kJ/m^2 (Figure 2.6A), thus indicating a lack of early stage keratinocyte transformation. Dorsal sections from both experimental groups did not express the melanocytic specific microphtalmia associated transcription factor (MITF) or show evidence of hyper-proliferation (Ki67) in immunohistochemistry post exposure to 14 kJ/m^2 of UV radiation (Figure 2.6B, C). Dorsal sections also showed prominent E-cadherin expression across all

Figure 2.4: Unchallenged and UV-challenged mutant mice have an intact basement membrane.

Dorsal skin samples of 3-day-old neonates from both mouse lines were examined for the integrity of laminin when unchallenged or 24 hours post exposure to 14 kJ/m^2 of UV radiation. Unchallenged and UV challenged (A) Cx26$^{K14\text{-}S17F/+}$ and (B) Cx43$^{I130T/+}$ mice and their WT littermates (N = 4) showed no apparent differences in laminin localization or expression at the epidermal – dermal interface ($P > 0.05$, One-way ANOVA with Tukey's multiple comparisons) (C, D). Dotted lines outline stratum corneum, solid line approximates epidermal-dermal interface. Scale bar = 20 μm.

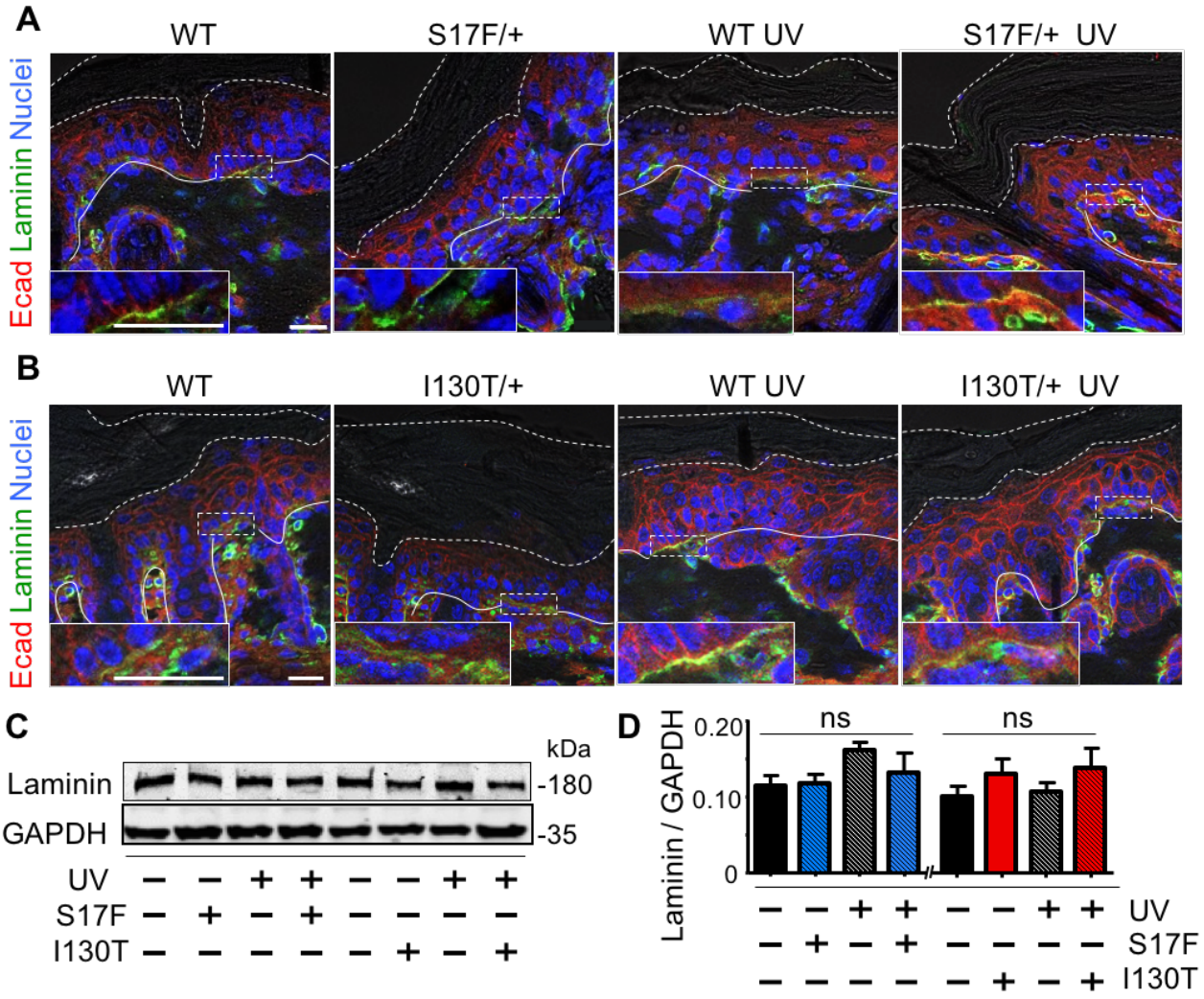
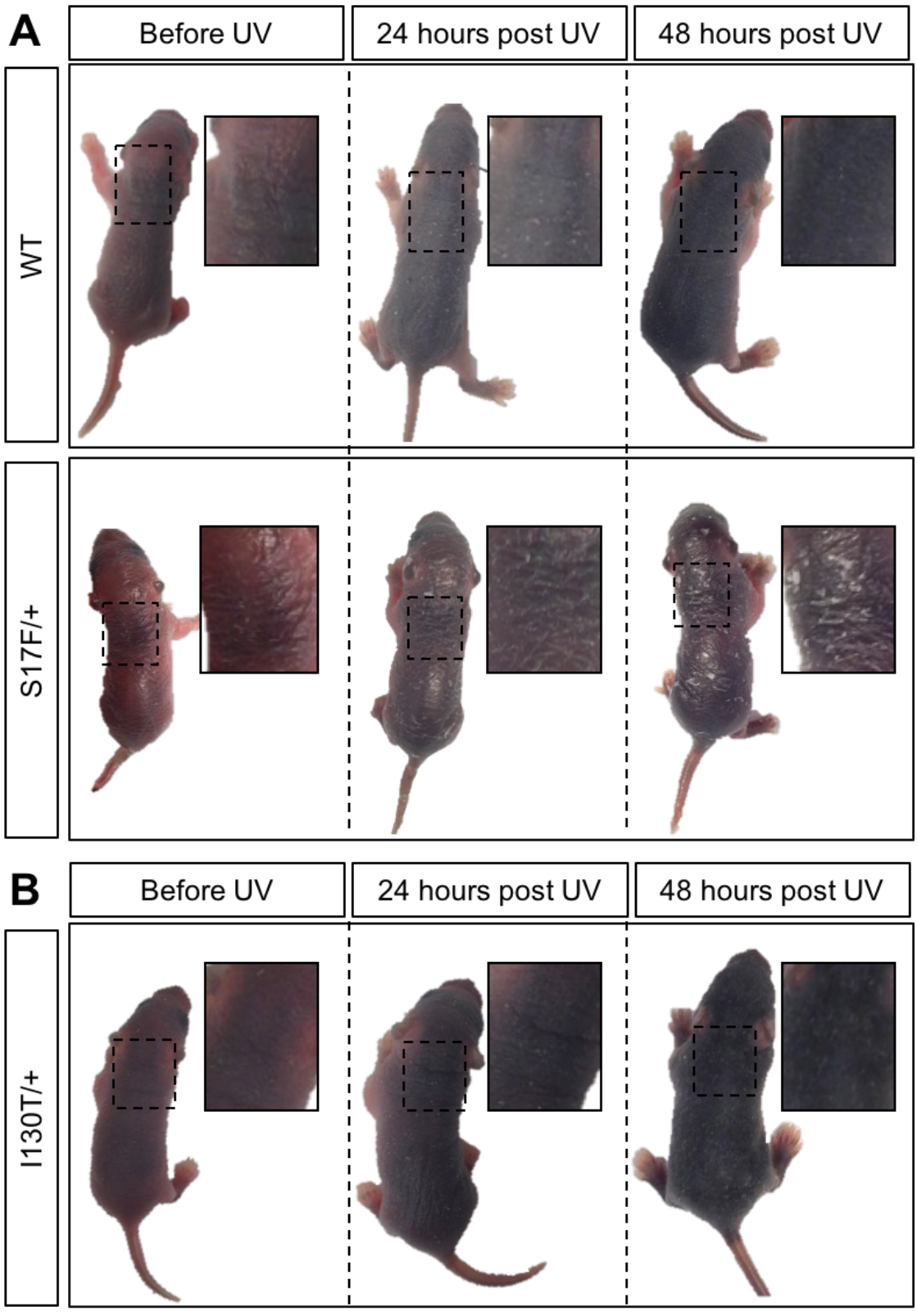


Figure 2.5: $Cx26^{K14\text{-}S17F/+}$ mice exhibit UV-induced epidermal desquamation compared to $Cx43^{I130T/+}$ mice and WT littermates.

(A) In comparison to WT littermates, images of $Cx26^{K14\text{-}S17F/+}$ neonates show evidence of epidermal desquamation, or sunburn-like peeling, as soon as 48 hours post exposure to 14 kJ/m^2 of UV radiation. In contrast, (B) $Cx43^{I130T/+}$ mice did not show any evidence of skin damage within the same time frame.



experimental groups, which is normally lacking in melanoma tissue (Hsu et al., 2000) (Figure 2.6C). Collectively, the loss-of-function mutation in the gene encoding Cx43 (a putative tumor suppressor) and a carcinogenic insult (UV radiation) did not lead to any evidence of skin tumors.

2.4.6 Primary murine melanocytes show no evidence of Cx43 expression or homocellular coupling.

To better elucidate the role of GJIC in the homeostatic regulation of the epidermal cells in the skin, we examined mouse melanocytes for connexin expression and/or intercellular coupling as this remains poorly understood. Primary melanocytes were successfully isolated as evidence by positive expression for the melanogenesis markers, L-DOPA and TRP1 (Figure 2.7A, B). The percent yield of L-DOPA positive melanocytes (dark pigment) was calculated across all passages (P2-P4), and the cultures were identified as essentially pure melanocyte monocultures (Figure 2.7A).

In contrast to a previous report (Hsu et al., 2000), while Cx43 positive plaques were readily detectable at the cell surface of REKs (Figure 2.7C) and primary murine keratinocytes (Figure 2.7D), primary murine melanocytes showed no evidence of Cx43 expression. To assess for intercellular coupling that could be caused by the presence of other connexin family members, keratinocytes and primary (P2) melanocytes were incubated with calcein-AM dye and dye recovery after photobleaching was measured. Primary melanocytes did not show any evidence of calcein-AM dye recovery compared to REKs, which are connexin rich (Figure 2.8A, B). Collectively, this indicates that mouse melanocytes are devoid of functionally relevant levels of connexins.

2.4.7 Intracellular Cx43 expression is high in human melanoma metastases to distant organ sites compared to nodal metastases and primary melanomas.

To better characterize the role of Cx43 in human melanoma, the tumor core of primary melanomas (N = 14), in addition to nodal (N = 15) and distant (N = 7) metastases were evaluated for Cx43 expression using immunohistochemistry. Cx43 expression was categorized as low or high, and protein localization as either intracellular or forming

Figure 2.6: $Cx43^{I130T/+}$ mice did not develop tumors of melanocyte or keratinocyte origin 6 months post UV exposure.

Dorsal skin sections from skin regions of low and high pigmentation were taken from $Cx43^{I130T/+}$ mice and their WT counterparts 6 months post exposure to 14 kJ/m^2 of UV radiation. (A) Low pigmented and high pigmented dorsal skin areas expressed K14 in the basal keratinocyte layer of the epidermis. (B) Low-pigmented and high pigmented dorsal skin sections from $Cx43^{I130T/+}$mice and their WT littermates exhibited little to no expression of MITF, or (C) Ki67, but prominently expressed E-cadherin (Ecad). In comparison, (B, C) primary human melanomas expressed MITF and Ki67. Dotted lines outline stratum corneum, solid line approximates epidermial-dermal interface. Scale bar = 20 μm.

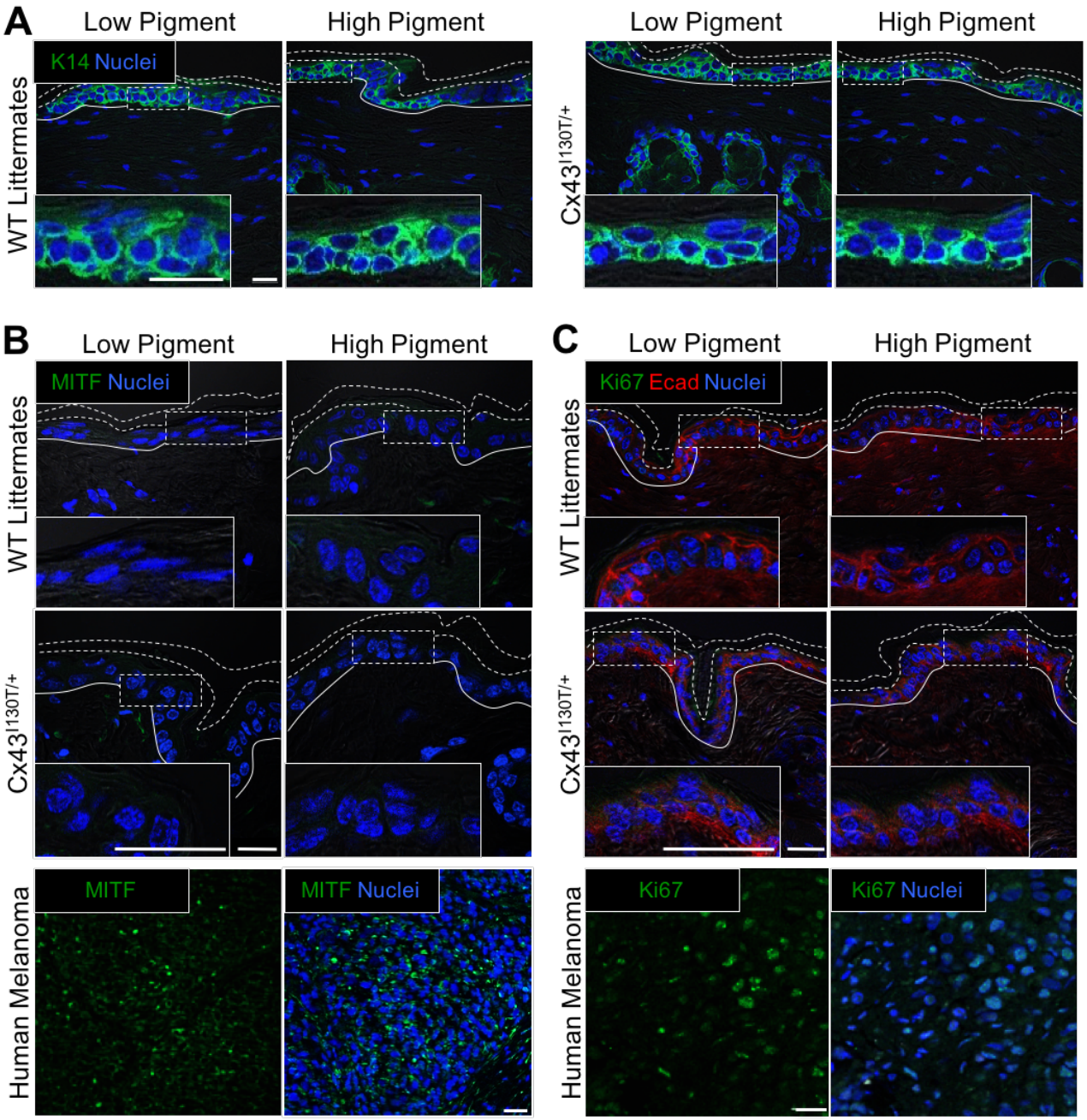


Figure 2.7: In contrast to keratinocytes, primary murine melanocytes show no evidence of Cx43 expression.

(A) Primary murine melanocytes were positive for L-DOPA (black pigment) across all passages (P) 2 – 4. L-DOPA positive cells were counted as a percentage of total cells to indicate mono culture purity and were essentially pure across all passages (P2-P4) (N = 3, n = 15). (B) Primary murine melanocytes also stained positive for TRP1. Interestingly, (C) P2 primary melanocytes show no evidence of Cx43 expression, in comparison to connexin rich REK controls. (D) Both undifferentiated and calcium-induced differentiated primary murine keratinocytes show evidence of Cx43 GJs at the junctional membrane identified by E-cadherin. Arrows indicate the expression of Cx43 plaques at cell-cell interface. Scale bar = 20 μm.

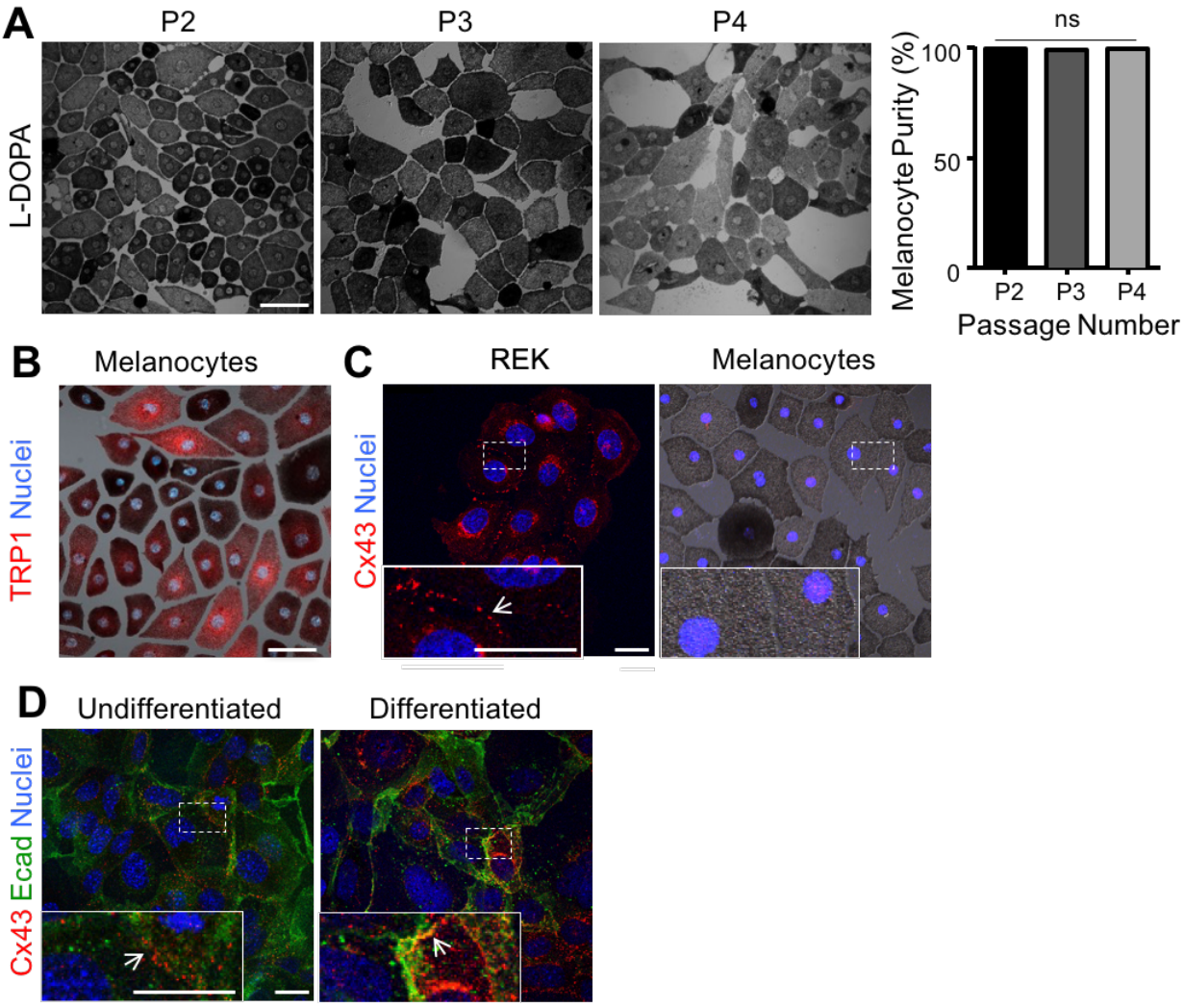
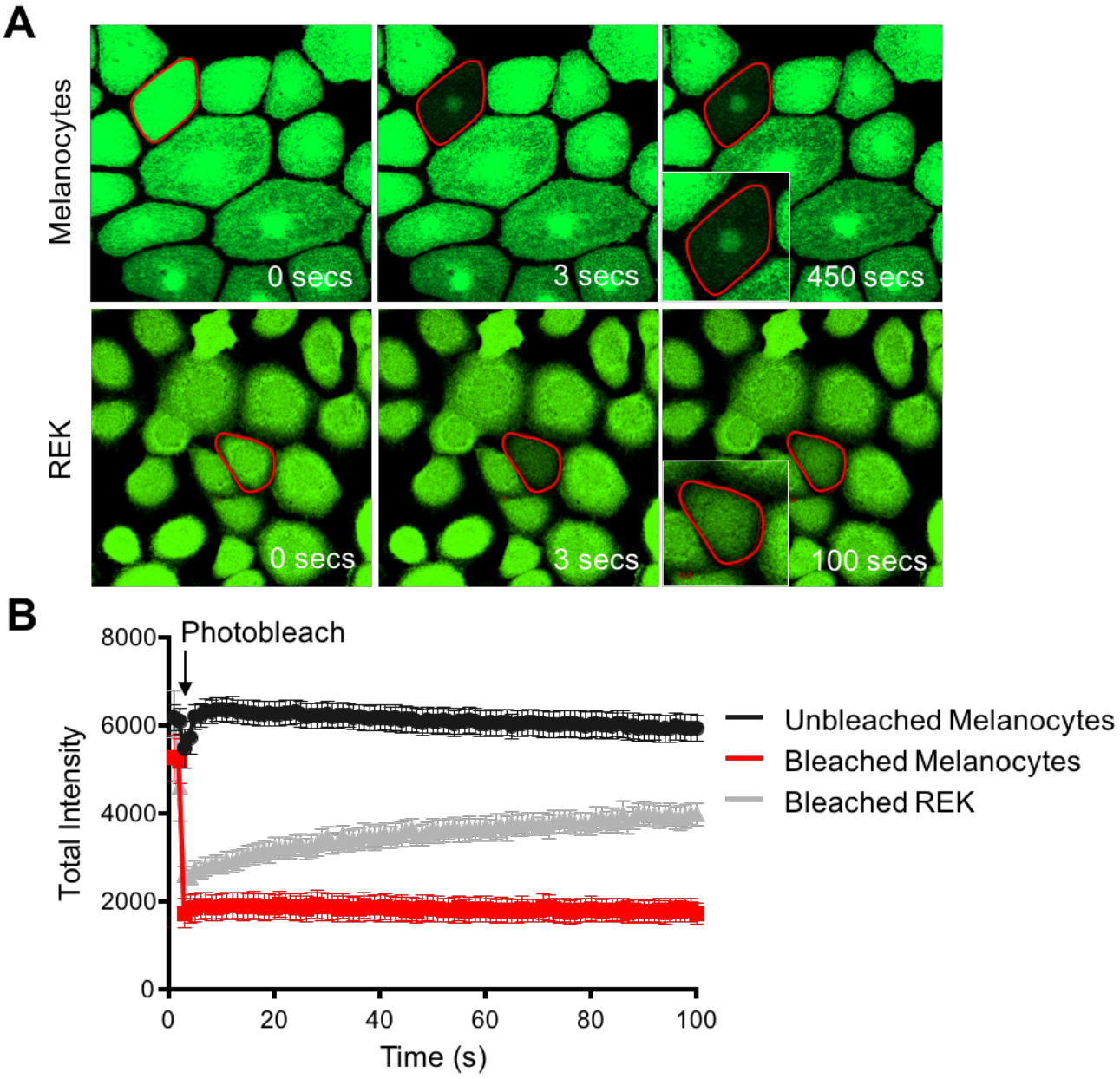


Figure 2.8: Primary murine melanocytes do not communicate through homocellular GJ channels.

(A) Outlined melanocytes (red) loaded with gap junction permeable calcein dye were photobleached and dye recovery was subsequently measured every 3 seconds for 450 seconds. Photobleached melanocytes did not receive calcein dye (450 seconds) indicating lack of GJIC. In contrast, photobleached connexin-rich REKs (keratinocytes) were able to recover dye from neighbouring cells within 100 seconds of bleaching. (B) Quantified data illustrates fluorescent recovery in photobleached REKs (N = 3, n = 30) but not in photobleached primary melanocytes (N = 3, n = 30). Scale bar = 20 μm.



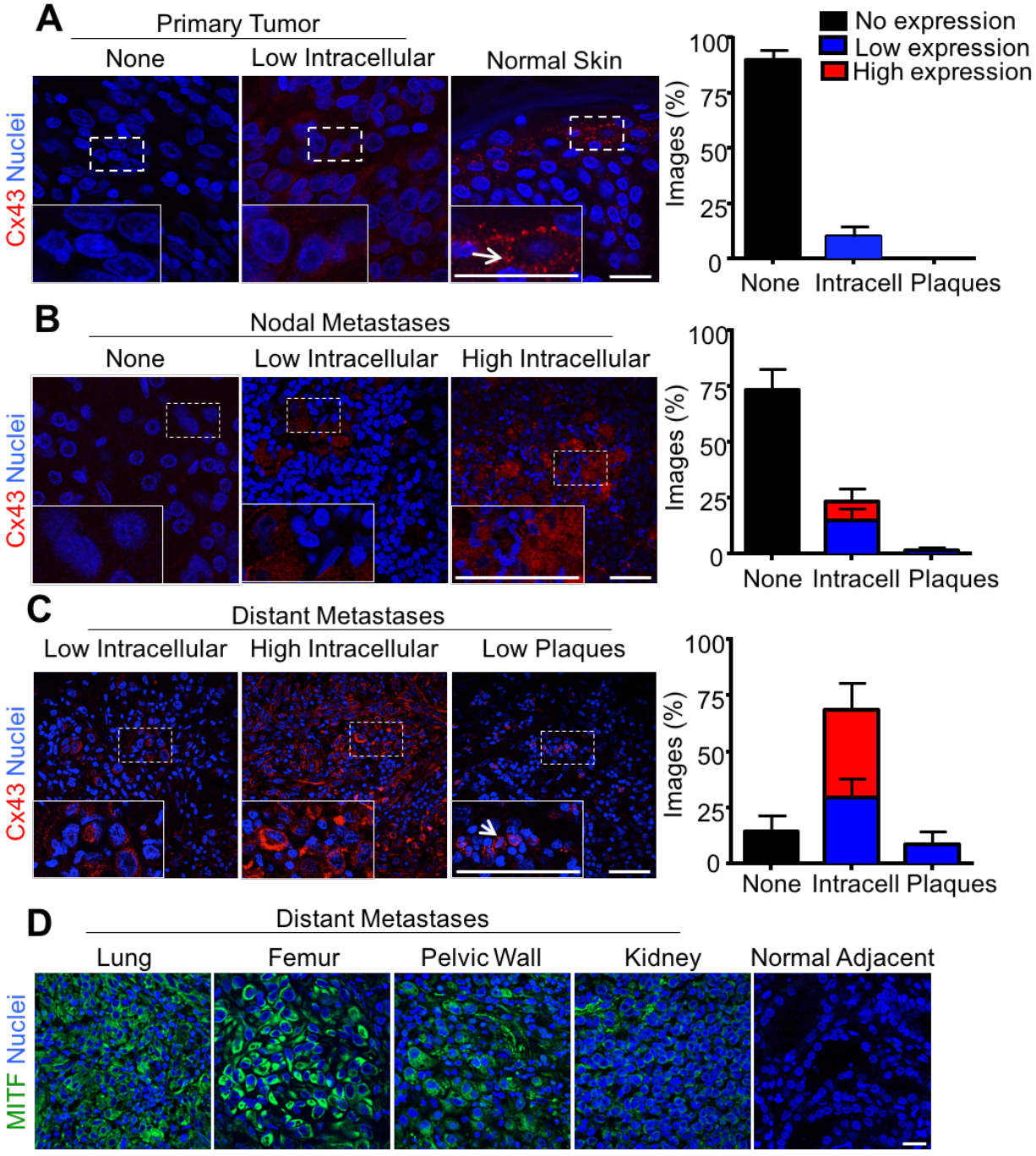
punctate gap junction plaques. Primary melanomas showed no evidence of Cx43 expression (90%), and in the rare cases where Cx43 was evident, it did not localize to gap junction plaques (Figure 2.9A). In addition, the majority of melanomas that metastasized to the lymph nodes did not express Cx43 (75%), and while more Cx43 was localized to intracellular regions in tumors defined as expressing high (9%) and low (14%) levels, little to no gap junction plaques could be identified (2%) (Figure 2.9B). Interestingly, the majority of samples collected from melanoma metastases to distant organs expressed Cx43 within intracellular compartments at both high (37%) and low (34%) levels. However, consistent with other stages of the disease, very few gap junction plaques were identified (8%) (Figure 2.9C). Furthermore, the tumor core of melanomas that metastasized to distant organs (lung, femur, pelvic wall, kidney) stained positive for MITF confirming the tumor core, which was analyzed for Cx43 expression, was of melanocytic origin (Figure 2.9D).

2.5 Discussion

The role of gap junctions in human physiology has been of great interest particularly due to the sheer frequency of connexin mutations that have been linked to human diseases (Scott et al., 2012; Laird, 2008). Specifically, mutations in genes encoding Cx26 (*GJB2*) and Cx43 (*GJA1*) have been linked to the onset and progression of skin pathologies, including skin cancers (Naus and Laird, 2010; Ableser et al., 2014). Therefore, in the current study we used genetically modified mice that lacked a full complement of Cx26 or Cx43 function to address the potential roles of Cx26 and Cx43 in normal epidermis homeostasis and their putative protective roles in UV-induced skin damage. Furthermore, we assess Cx43 as a putative tumor suppressor in skin cancer onset and progression. Interestingly, $Cx26^{K14\text{-}S17F/+}$ mice that mimic keratosis ichthyosis deafness (KID) syndrome had high mortality after UV-induced damage to the skin. $Cx43^{I130T/+}$ mutant mice that mimic oculodentodigital dysplasia (ODDD) had normal skin that was resistant to UV-induced damage and did not develop UV-induced skin tumors of keratinocyte or melanocyte origin. In addition, cultured primary mouse melanocytes, primary human melanomas and nodal metastases of melanomas did not show significant expression of Cx43. However, human distant melanoma metastases to different vital organs exhibited

Figure 2.9: Human melanoma metastases to distant organ sites express Cx43 intracellularly compared to nodal metastases and primary cutaneous melanomas.

(A) The majority of images analyzed from primary melanoma (N = 14), and (B) nodal metastases (N = 15) did not show evidence of Cx43 expression. In contrast, (C) the majority of images analyzed from melanoma metastases to distant organ sites (N = 7) showed evidence of Cx43 expression, however, this expression was predominately intracellular and did not form gap junction plaque-like structures indicative of potentially functional gap junction channels. Arrow indicate punctate Cx43 gap junction structure at the cell-cell interface. Scale bar = 20 μm. (D) Regardless of the distant organ site of melanoma metastases, tumor cores from lung, femur, pelvic wall, and kidney stained positive for MITF, indicating the tumor was from a melanocytic cell lineage.



high levels of intracellular Cx43. Taken together, the current study provides evidence for a critical role of Cx26 in protecting the epidermis from acute UV damage and suggests that, even though melanocytes are devoid of Cx43, high levels of Cx43 in metastatic melanomas at distant sites may provide the tumor with a survival advantage in vital organs.

Over the past two decades, mutations in five connexin encoding genes have been linked to skin diseases (Scott et al., 2012). In particular, mutations in the *GJB2* gene encoding Cx26 are prevalent in the human population, and are routinely screened for in the clinic in cases where children present with hearing loss and/or skin disease (Chan and Chang, 2014). Among these, autosomal dominant mutations have been identified that are associated with both deafness and skin disorders (Scott et al., 2012). For example, the serine to phenylalanine substitution at position 17 (S17F) in Cx26 has been described to cause keratitis-ichthyosis deafness (KID) syndrome in humans (Richard et al., 2002). KID patients present with hearing deficits, as well as hyperkeratotic skin lesions and an increased propensity to develop skin cancer (Mazereeuw-Hautier et al., 2007). When expressed in connexin-deficient HeLa cells and *Xenopus laevis* oocytes, the Cx26 S17F mutant localizes at cell-cell contacts, but cells remained completely uncoupled indicating that the mutant has no capacity to make functional gap junction channels (Richard et al., 2002; Lee et al., 2009). In addition, the Cx26 S17F mutant exhibited aberrant interactions, including a strong transdominant-negative effect on WT Cx43 when co-expressed in HeLa cells, in addition to increased hemichannel currents when co-expressed with WT Cx26 (García et al., 2015). Considering that Cx26 and Cx43 are co-expressed in numerous keratinocytes in the epidermis, these effects begin to explain the severity of the disease observed in KID patients.

These findings suggest that both Cx26 and Cx43 are critically important for the health of the epidermis and raises the possibility that genetically-modified mice that mimic connexin-linked diseases could be used to provide insights into the role of specific skin connexins. Previously, a constitutive $Cx26^{S17F/+}$ mutant mouse was created and analyzed as a model mimicking the Cx26 S17F mutation observed in human patients suffering from KID syndrome. However, $Cx26^{S17F/+}$ mouse survival was poor as only a few mice lived into adulthood (Schütz et al., 2011). To mitigate low mouse viability and to eliminate

confounding effects from the multiple organs where Cx26 is expressed, we created a novel, tissue-specific mouse that expressed the S17F mutant in the epidermis as driven by the keratin 14 (K14) promoter. Our study revealed that the novel $Cx26^{K14\text{-}S17F/+}$ mouse mimicked skin disease observed in KID patients and mutant mice typically survived into adulthood. In addition, autosomal dominant mutations in the *GJA1* gene that encodes Cx43 have been directly linked to development of oculodentodigital dysplasia (ODDD), which is typically characterized by craniofacial abnormalities, enamel loss, digit syndactyly, and in select cases, palmoplantar keratoderma and hyperkeratosis. Mutant mice ($Cx43^{I130T/+}$) harboring the Cx43 I130T mutant protein have been previously characterized for abnormalities of the heart (Kalcheva et al., 2007), mammary gland (Stewart et al., 2013), and bladder (Huang et al., 2014) but no skin defects or abnormalities have been reported. In all instances, there was a significant reduction in overall Cx43 expression, including a significant dominant-negative effect on co-expressed WT Cx43, resulting in an approximate 50% reduction in overall GJIC (Kalcheva et al., 2007; Stewart et al., 2013). Thus, in addition to mimicking the human ODDD condition, $Cx43^{I130T/+}$ mutant mice may shed insights into the role of Cx43 in the epidermis where it is abundantly expressed.

As predicted, $Cx26^{K14\text{-}S17F/+}$ neonates exhibited erythrokeratoderma and ichthyosis of the skin, further implicating the necessity of a fully functional complement of Cx26 in the epidermis. Interestingly, unchallenged $Cx26^{K14\text{-}S17F/+}$ mice did not possess any deficits in overall survival, whereas UV-exposed $Cx26^{K14\text{-}S17F/+}$ neonates frequently died soon after UV exposure. It has been well documented that exposure to UV radiation can alter epidermal permeability (Lee and Lee, 2014), possibly compromising mouse survival. Furthermore, the constitutive $Cx26^{S17F/+}$ mutant mouse exhibited significant impairments in epidermal barrier, associated with the disarrangement of lipids that normally form a tight seal within the stratum corneum (Bosen et al., 2015; Schütz et al., 2011). However, $Cx26^{K14\text{-}S17F/+}$ neonates did not exhibit any impairment in epidermal barrier when unchallenged or when challenged with 14 kJ/m^2 of UV radiation. As such, we can extrapolate that the injury incurred by the keratinocyte-specific expression of the S17F mutant and/or by UV exposure does not dramatically affect the outer epidermal barrier, or that any alterations in the barrier were below our threshold of sensitivity. On first pass, one might predict that the keratinocyte-specific expression of the Cx26 S17F protein would

possess a similar barrier impairment to the $Cx26^{S17F/+}$ mutant mouse. Particularly because the embryonic development of barrier function would occur after the K14-cre induction of the Cx26 S17F mutation in our mouse model (Byrne et al., 1994). It is likely that these mouse specific differences are related to the conditional versus global expression of the Cx26 S17F protein throughout development, such that the impairments seen in $Cx26^{S17F/+}$ mice could be due to other impeding factors. However, the exact mechanism(s) resulting behind these differences in barrier function are still unclear.

Exposure to UV radiation has also been shown to disrupt the basement membrane (BM), and increase the basal lamina thickness, in both aged humans (Lavker, 1979) and mice (Feldman et al., 1990), leading to a fragile interface that reduces epidermal capacity to respond to external forces (Amano, 2009). The BM exists at the epidermal-dermal junction and is dynamically regulated for the survival and maintenance of the epidermis, acting as both a stabilizing interface and a diffusion barrier (Breitkreutz et al., 2013; Amano, 2009). Furthermore, increases in basal lamina thickness, has been noted as a nonspecific response to trauma (Lavker, 1979). However, consistent with our findings that $Cx26^{K14\text{-}S17F/+}$ and $Cx43^{I130T/+}$ unchallenged and UV challenged neonates possessed uniform laminin localization and distribution, the severe UV-induced disruption and increased thickness of the basal lamina beneath epidermal keratinocytes was also not observed in young individuals, and was instead identified as a phenomena localized to aged skin (Amano, 2009). Therefore, the additive insult of reduced Cx43 or Cx26 function alongside UV exposure does not impede the structural integrity of the epidermal-dermal basal lamina, thus indicating an intact inner permeability barrier in both mouse lines.

Interestingly, despite an intact permeability barrier in both mouse lines, $Cx26^{K14\text{-}S17F/+}$ neonates incurred a higher degree of UV-injury compared to WT controls, as evidenced by the rapid development of UV-induced epidermal desquamation. This indicates that Cx26 is critical in modulating epidermal health in response to UV radiation, such that individuals that harbor aberrant Cx26 function may possess increased UV-reactivity of the skin. Unfortunately, due to the high level of mortality in exposed $Cx26^{K14\text{-}S17F/+}$ mice, we were not able to analyze the incidence of UV-induced tumorigenesis. However, KID patients have been noted to possess increased propensity to develop squamous cell carcinomas

(SCCs) (Mazereeuw-Hautier et al., 2007), and our findings are the first to suggest that this could possibly be due to an increased sensitivity to the common environmental carcinogen, UV radiation.

Surprisingly, little is known on how important Cx43 in the epidermis is to protecting humans from skin diseases and cancers of the skin that are prevalent in society and often linked to UV-damage. Here, we found that $Cx43^{I130T/+}$ mice did not present with any adverse skin defects or abnormalities which suggests that the epidermis may produce a surplus of Cx43 or there is sufficient compensation from the 8 or more other connexins co-expressed in the epidermis. Consistent with our findings, $Cx43^{+/-}$ heterozygotes, which have a 50% reduction in Cx43 function (Yamakage et al., 2000), and ODDD patients who harbor the I130T mutation (Paznekas et al., 2003), do not possess any known skin abnormalities or defects. However, Cx43 mutations that result in a much greater reduction in Cx43 function possess aberrant keratinocyte proliferation and differentiation (Churko et al., 2012), defective epidermal barrier (Maas et al., 2004), and palmoplantar keratoderma and hyperkeratosis (Gong et al., 2006; Van Steensel et al., 2005). Therefore, we also examined for any defects that would result from combining $Cx43^{I130T/+}$ mice with exposure to UV radiation. Interestingly, we discovered that $Cx43^{I130T/+}$ mice were not more susceptible to acute UV injury compared to their WT littermates. Thus, we can also infer that partial (50%) Cx43 function is sufficient to maintain the skin's resiliency to environmental insults. Consistent with this notion, $Cx43^{I130T/+}$ mice did not develop keratinocyte or melanocyte tumors 6 months after exposure to UV radiation. Taken together, our findings provide evidence that partial (50%) Cx43 function is sufficient to appropriately modulate epidermal homeostasis and maintain the skin's resiliency against UV-induced pathogeneses in settings that can be extrapolated to the human ODDD population.

Knowledge regarding the fundamental role of Cx43 in the epidermis has been hampered by a limited understanding in which cell types it is expressed. While it is abundantly expressed in the keratinocytes (Churko et al., 2012; Langlois et al., 2007), its expression and role in melanocytes is ill-defined. In keratinocytes, Cx43 has been shown to regulate proliferation (Pollok et al., 2011), differentiation (Churko et al., 2012), and migration

(Wright et al., 2009). However, its role in melanocyte regulation is still largely unknown, primarily due to the low yield of melanocytes in the epidermis (Lin and Fisher, 2007). Previous work has found that primary melanocytes are heterocellularly coupled with keratinocytes (Haass and Herlyn, 2005), and another study has suggested that this heterocellular coupling may be mediated by Cx43 (Hsu et al., 2000). Interestingly, the downregulation of Cx43 has been shown to facilitate normal melanocyte division (Haass and Herlyn, 2005), and if this occurs chronically, has been linked to the onset and progression of melanomas (Hsu et al., 2000; Haass et al., 2004). However, the proposed down regulation of Cx43 upon the onset of melanocyte transformation is dependent on the proposition that melanocytes actively produce meaningful levels of Cx43, which has never been firmly established. Furthermore, it is interesting to note that both benign and cancerous human melanocytic nevi were also found to possess low levels of Cx43 (Sargen et al., 2013; Rezze et al., 2011). In our study, we found that primary murine melanocytes in monoculture did not express Cx43 and were also not GJIC coupled with neighboring cells. This questions the putative role of Cx43 as potential regulator of melanocyte proliferation and tumor suppressor at early stages of melanocyte dysregulation. Our studies would argue that Cx43 plays little or no role in mouse melanocytes as primary cultures were completely devoid of Cx43 and were, in fact, not coupled by gap junctions in stark contrast to our findings in keratinocytes. This is in fact not surprising as melanocytes are typically dispersed in the epidermis with little opportunity for homocellular interactions. However, we cannot dismiss the possibility that the *Gja1* gene encoding Cx43 is only activated upon signaling stimuli from surrounding keratinocytes that contact melanocytic processes *in vivo*. Since our studies revealed that Cx43 was absent from mouse melanocytes, the question remained as to its role in human melanocyte tumorigenesis where primary tumors form within the confines of the epidermis only to later break through the basement membrane and invade the lymphatic system and, eventually, vital organs.

Currently, evidence exists that supports a role for Cx43 as both a tumor suppressor and as a tumor facilitator which appears to be dependent on the type of tumor and the stage of the disease (Ableser et al., 2014; as reviewed by Naus and Laird, 2010). In human melanoma, studies regarding Cx43 expression are limited and controversial. Two studies report that Cx43 is expressed in primary human melanoma (Sargen et al., 2013; Rezze et al., 2011)

while another reports the lack of its expression (Haass et al., 2004). Furthermore, the role of Cx43 in human melanoma metastasis remains largely unexplored. Given the evidence that Cx43 may not be highly expressed in melanocytes, the question remained as to whether Cx43 would be upregulated at any stage of tumorigenesis and whether it would be properly assembled into gap junctions. Using a library of human melanomas, we found that Cx43 was minimally expressed in primary cutaneous tumors and melanoma metastases to nodal tissue, but was surprisingly expressed at moderate to high levels in melanoma metastases to distant organ sites. However, Cx43 expression remained largely intracellular, suggesting that any causal role Cx43 played in tumor progression was GJ-independent. Many GJ-independent roles for Cx43 have been proposed but most involve Cx43 interactions to a human interactome, which exceeds over 30 proteins, many of which have been linked to cancer or cell proliferation pathways, including NOV/CCN3 and caveolin-1 (Naus and Laird, 2010; Zhou and Jiang, 2014). The lack of the ability of the tumor cells to form Cx43-based GJ may be linked to a tumor survival mechanism, particularly to prevent the passage of molecules such as glutathione that assist in detoxifying carcinogens (Balendiran et al., 2004). Furthermore, high intracellular expression of Cx43 in late stage disease could occur because of an identified GJ-independent role of Cx43 to resist cellular injury (Lin et al., 2003). Nevertheless, our findings provide no direct evidence that Cx43 is a tumor suppressor in melanomas but supports the position that Cx43 may provide the tumor cell a survival advantage when its metastasized to vital organs. Importantly, this unveils the potential of Cx43 as a therapeutic target in late stage disease.

In conclusion, a full complement of functional Cx26 in mice plays a critical role in protecting the epidermis from a common environmental insult (UV) suggesting that Cx26 is critical for skin homeostasis. However, mice can tolerate a reduction in Cx43 function with little detrimental effects to the skin and no increase in susceptibility to skin tumors. Contrary to what we expected, mouse melanocytes were found to be devoid of Cx43 and not surprisingly, primary human melanomas also lacked Cx43. But as disease progressed and melanomas metastasized to vital organs, Cx43 expression was abundant suggesting that Cx43 may provide the tumor with a survival advantage. Finally, the lack of Cx43 forming gap junction plaques in late stage tumors would suggest that its role in cancer progression would be restricted to a GJ-independent mechanism likely linked to its

interactome. In the future, it would be of interest to determine if drugs targeting Cx43 in late stage melanoma disease would have therapeutic value.

2.6 Additional Remarks

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Chapter 3

3 General Overview

Connexins have been extensively implicated in maintaining epidermal health (Scott et al., 2012; Laird, 2010), but their role during epidermal homeostasis in response to the common environmental insult, UV radiation, remains poorly understood. Connexins, specifically Cx43, have also been proposed to act as tumor suppressors against the development of skin cancers (Naus and Laird, 2010; Ableser et al., 2014), of which overexposure to UV radiation is the primary cause (Parkin et al., 2011; Koh et al., 1996). However, their status during human melanoma progression, from melanocyte to melanoma metastases, remains controversial and poorly understood. To add to this uncertainty, the connexin status of human melanoma tissues throughout the late stages of disease progression (metastasis) is relatively nonexistent. In our study, we assessed the possible protective roles of Cx43 and Cx26 in modulating epidermal health in response to UV radiation using two disease-linked connexin mutant mice. We found that a partial reduction in Cx26, but not Cx43, was critical in maintaining epidermal health both when unchallenged and when challenged with UV radiation. Furthermore, we found that similar to primary melanocytes, primary cutaneous melanoma and nodal metastases did not show prominent Cx43 expression, whereas, human melanoma metastases to distant organ sites expressed Cx43 intracellularly.

3.1 Limitations, Future Studies, and Conclusions

The use of disease-linked genetically engineered mouse models are excellent tools to investigate the role of connexins in UV-induced pathogeneses *in vivo*. As such, in this investigation two disease-linked connexin mutant mice ($Cx26^{K14\text{-}S17F/+}$ and $Cx43^{I130T/+}$) that possessed a significant reduction in connexin function were analyzed to discern the roles of Cx26 and Cx43 in epidermal health. However, for the study of UV-induced injury, the genetic background and the degree of pigmentation within the skin is an important consideration (Mäkinen and Stenbäck, 1998). For example, past work has noted that UV-reactivity and UV-induced skin tumor incidence was higher in mice with genetic backgrounds that possessed little to no skin pigment (SKH-1) compared to those that were highly pigmented (C57BL/6) (Mäkinen and Stenbäck, 1998). Furthermore, individuals

with darker skin have also been shown to possess increased resistance to skin tumor development and photoaging (IARC, 1992). However, while both of our mutant mouse lines ($Cx43^{I130T/+}$ and $Cx26^{K14\text{-}S17F/+}$) were bred on highly pigmented genetic backgrounds, which is commonly referred to as tumor-insensitive, they still provided excellent context to the roles of Cx26 and Cx43 in modulating epidermal homeostasis. In addition, it also should be noted pigment-causing melanocytes are localized primarily in the interfollicular epidermis in humans, and primarily in the follicular skin of mice after 4 days of age (Chou et al., 2013). This may limit our extrapolation of melanocyte behaviour to human diseases such as melanoma. Therefore, in this study we have combined our analysis to include both mutant mice and human melanoma samples, and have ensured that exposure to UV in mice occurs at 3 days of age.

On first pass, one might suggest that it would be useful to analyze these mouse models on a genetic background with a lower level of skin pigmentation, such as SKH-1 or DBA/2, that is presumably more susceptible to UV radiation (Mäkinen and Stenbäck, 1998). However, it is interesting to note that additional work has reported that the degree of erythema and skin tumor incidence is also heavily related to factors other than pigment, including immunological and genetic differences (Mäkinen and Stenbäck, 1998; IARC, 1992; Noonan and Hoffman, 1994; Klein-szanto et al., 1994). In addition, the use of a UV-insensitive genetic background further implicates the mutations in these mice as being primarily responsible for any observed UV-induced changes. For example, despite the high pigmented genetic background of $Cx26^{K14\text{-}S17F/+}$ mice ($Cx26^{floxS17F/+}$: 129Sv; K14-cre: C57BL/6), they still presented with a dramatic degree of UV-reactivity, and an intolerance to UV-insult, further implicating the severity of a partial reduction in Cx26 function in the skin. Furthermore, in cases such as this one, where a genetic mutation results in a high degree of UV-susceptibility, a less pigmented genetic background may actually compromise the survival of an otherwise viable mouse model. However, one of the major questions left unanswered in our investigation is whether or not the lack of UV-susceptibility and UV-induced tumor incidence in $Cx43^{I130T/+}$ (C57 BL/6) mice was fully representative of a partial reduction in Cx43 function, or if the innate insensitivity of the pigmented genetic background possibly masked subtler changes in epidermal health. As such, future studies should examine if partial Cx43 function (50%) affects epidermal

homeostasis in a model that has been sufficiently backcrossed onto a genetic background with a lower level of skin pigmentation.

Mutant mice bred on a C57BL/6 background have been used in prior analyses of UV-induced melanoma incidence when paired with a genetic alteration that significantly impairs the function of a tumor suppressor (i.e. Cdkn2a) (Gaffal et al., 2011), or induces the expression of an oncogenic sequence (i.e. SV40) (Klein-szanto et al., 1994). These genetic changes significantly increased the susceptibility of C57BL/6 mice such that they often spontaneously developed skin tumors (Gaffal et al., 2011; Klein-szanto et al., 1994). However, the $Cx43^{I130T/+}$ (C57BL/6) mutant mouse has never been reported to spontaneously develop tumors of any kind (Kalcheva et al., 2007; Stewart et al., 2013). Accordingly, $Cx43^{I130T/+}$ mice did not show evidence of skin tumor development after exposure to UV radiation, and did not possess any dramatic epidermal abnormalities when unchallenged. Therefore, a partial (50%) reduction in Cx43 function was not sufficient to significantly affect its proposed role as a tumor suppressor, or as a regulator of epidermal homeostasis. Interestingly, the development of palmoplantar keratoderma and hyperkeratosis in human ODDD patients (Paznekas et al., 2003; Avshalumova et al., 2014), in addition to aberrant murine keratinocyte differentiation and barrier formation (Maass et al., 2005; Churko et al., 2012) all occurred when mutations in Cx43 reduced its function by more than 50%. Among these, the $Cx43^{G60S/+}$ ($Gja1^{Jrt/+}$) mouse line has been shown to possess a greater than 50% reduction in Cx43 (Stewart et al., 2013), which resulted in keratinocyte hyperproliferation (Churko et al., 2012). Moving forward, to further elucidate the proposed role of Cx43 in maintaining epidermal health of the skin, specifically in response to UV insults, we could examine $Cx43^{G60S/+}$ mice for UV-induced epidermal changes. In addition, the $Cx43^{G60S/+}$ mice are bred on to a mixed background of C3H/HeJ and C57BL/6 that is lighter in pigment (Stewart et al., 2013). Thus, such a study would also help elucidate the potential role of Cx43 as a tumor suppressor in response to a carcinogenic UV insult, in a model that is presumably more susceptible to UV radiation.

In addition, the implications of Cx26-induced impairments in epidermal health need to be further explored physiologically. In our studies, $Cx26^{K14\text{-}S17F/+}$ neonates did not show evidence of gross impairments in epidermal permeability when unchallenged or when

challenged with UV radiation. However, the assays we used to assess epidermal permeability and integrity (toluidine blue dye penetration and laminin expression) only detect drastic alterations in epidermal health, and future studies are required to isolate the possible occurrence of significant, but subtler changes, in epidermal physiology. The main function of the epidermis is to confer a barrier against the diffusion of molecules from the external environment, in addition to the excessive diffusion of molecules out of the skin (Hardman et al., 1998). However, the epidermal barrier is not absolute, and also facilitates the normal movement of water, termed transepidermal water loss (TEWL) (Baroni et al., 2012; Lee and Lee, 2014). Interestingly, measuring the amount of TEWL has been identified as a useful tool to detect skin damage, as the rate of TEWL generally increases in proportion to the level of damage (Baroni et al., 2012). As such, future studies could examine if the dramatic skin phenotype seen in both unchallenged and UV challenged $Cx26^{K14\text{-}S17F/+}$ mice can be explained by excessive water loss through the skin. Commonly, TEWL is analyzed using a probe that measures water vapor pressure on the surface of the skin (Hardman et al., 1998), and can be used to measure TEWL within 6 – 24 hours after UV- exposure (Bergeron et al., 2012). Thus, future studies can measure TEWL in $Cx26^{K14\text{-}S17F/+}$ neonates and their observed decrease in survival after UV exposure should not interfere.

In addition, the epidermal layers beneath the stratum corneum confer a second line epidermal barrier (Baroni et al., 2012). Within these layers, tight junctions play an important role in controlling the paracellular movement of molecules between keratinocytes (Sugawara et al., 2013). UV radiation has also been shown to disrupt tight junction organization in keratinocytes (Yuki et al., 2011), and tight junction disruption or dysfunction has also been shown to result in impaired barrier function (Yuki et al., 2011; Sugawara et al., 2013). As such, future studies should also assess for transient impairments in tight junctions *in vivo* and in primary keratinocytes from both $Cx26^{K14\text{-}S17F/+}$ neonates and their WT littermates. Collectively, this data will help further unravel the physiological role of Cx26 in maintaining epidermal health, and how this could be perturbed during UV exposure.

While the use of genetically modified mice presents distinct advantages to study the roles of connexins *in vivo*, the maternal behavior to commit pup infanticide can make determining the direct cause of death in neonates challenging. Primarily because as shown recently, post-natal pups can also die from a variety of causes other than maternal cannibalism initially and the mother simply eats the lost pups post-mortem (Weber et al., 2013). This makes it difficult to discern the initial cause of infant mouse death. Accordingly, one of the major questions left unanswered from our study is whether or not $Cx26^{K14\text{-}S17F/+}$ neonates die immediately after UV exposure and then the mother cleans up her dead offspring, or whether $Cx26^{K14\text{-}S17F/+}$ neonates are significantly weaker due to incurred UV insult and the mother then targets and eats weaker offspring. However, even without this knowledge the end inference is the same, $Cx26^{K14\text{-}S17F/+}$ neonates incur a significant degree of UV-injury, which ultimately compromises their fitness. In support of this notion, $Cx26^{K14\text{-}S17F/+}$ neonates developed epidermal desquamation, or sunburn-induced peeling, within 48 hours of UV exposure whereas $Cx43^{I130T/+}$ mice and the WT littermates from both mouse lines did not. Moving forward, it is important that we resolve the molecular mechanism(s) surrounding this occurrence to increase our understanding of Cx26 in modulating epidermal health. To do so, primary keratinocyte cultures isolated from $Cx26^{K14\text{-}S17F/+}$ neonates and their WT littermates are an excellent avenue, and circumvent any impairments in viability incurred by UV exposure. Past reports have used this model to examine molecular changes in keratinocytes in response to UV-insult (Sitailo et al., 2002; Rivas and Ullrich, 1992), and have identified an ideal dosage around 0.25 – 1 kJ/m^2 of narrowband UV radiation (Cho et al., 2008). After UV exposure, primary keratinocytes from $Cx26^{K14\text{-}S17F/+}$ neonates and their WT littermates can also be easily assessed for propensity to undergo UV-induced tumorigenesis, including epithelial to mesenchymal transition. This is of particular interest because our findings are the first to suggest that increased UV-reactivity, incurred by a reduction in Cx26 function, could be the reason why the skin of KID patients has been identified as significantly susceptible to the development of keratinocyte-derived tumors (Mazereeuw-Hautier et al., 2007).

In our studies we also show that unlike keratinocytes, primary murine melanocytes in monoculture do not express Cx43 and are not coupled. It is important to note that epidermal melanocytes are extremely difficult to analyze *in vivo*, primarily because they only make

up 5-10% of total cells in the epidermis in humans (Li and Herlyn, 2000). In particular, epidermal melanocytes of mouse skin are hard to analyze because not only do they only make up a small proportion of epidermal cells (Hirobe, 1995), but at 4 days of age the level of epidermal melanocytes dramatically decreases and are localized primarily to the hair follicles (Hirobe, 1995; Chou et al., 2103). As such using primary isolates of melanocytes in culture allow us to better analyze these cells under conditions that produce a higher cell yield. However, *in vivo* epidermal melanocytes (in humans and mice before 4 days of age) normally exist as a part of an epidermal melanin unit, where they are completely surrounded by keratinocytes, and rarely come into contact with one another (Haass and Herlyn, 2005). In fact, aggregates of melanocytes are considered precursors for melanoma progression, commonly existing as melanocytic nevi (Bandarchi et al., 2010). Past work has reported that melanocytes in monoculture possess some similarities to melanoma cells, expressing similar antigens such as melanoma adhesion molecule (Shih et al., 1994). Therefore, it is possible that the connexin profile of our primary melanocytes represent the very early stages prior to melanoma progression. Interestingly, when melanocytes were co-cultured with keratinocytes the expression of melanoma adhesion molecule was lost (Shih et al., 1994), thus identifying the keratinocyte-melanocyte co-culture as a useful model to mimic melanocytes *in vivo*. Therefore, as we continue to model the role of Cx43 in modulating epidermal melanocytes, it is important that we also examine for its expression when melanocytes are surrounded by keratinocytes in co-culture. One study has proposed that within these conditions melanocytes express Cx43 (Hsu et al., 2000), however there is no evidence that Cx43 forms GJ plaques at the melanocyte-keratinocyte interface, which would implicate Cx43 in heterocellular communication. Thus, to elucidate the role of Cx43 and GJIC in melanoma onset, future studies should examine the connexin profile of melanocytes in a keratinocyte microenvironment.

Our study also provides evidence that suggests Cx43 may act as a tumor facilitator in late stage human melanoma. Past work has elucidated that distant organ metastasis occurs in a non-random fashion, most likely due to the specific organ microenvironment (Fidler, 2003; Hart and Fidler, 1980). Unfortunately, due to the limited selection of distant melanoma tumors, we were not able to distinguish if there were any organ specific differences in Cx43 expression, that could possibly impact metastatic behaviour. As such, moving forward it is

important that we broaden our analysis of Cx43 in distant melanoma metastases to include an increased sample set from a variety of organ sites. Within this analysis, it is also important that we continue to discern Cx43 localization in addition to its expression using immunohistochemistry. While other tools, such as tumor microarrays, provide a higher throughput analysis of human tissue, they only analyze a small fraction of the tumor and are not representative of the entire heterogeneous lesion (Sargen et al., 2013). Furthermore, they do not provide information on protein localization, which is imperative as we continue to elucidate the mechanism behind the possible role of Cx43 as a late stage tumor facilitator. As such, moving forward, it is important that we continue to analyze for Cx43 expression and localization in larger sample sets of human melanoma using immunohistochemistry of whole tumor sections. Collectively, these studies will provide us with a fuller picture of the role of Cx43 as a potential tumor facilitator in late stage melanoma disease.

In addition, Cx43 localization in human melanoma metastases found in distant organ sites was primarily intracellular, indicating that if it is functioning as a tumor facilitator it is most likely due to GJ-independent mechanisms. Many GJ-independent roles for Cx43 in cancer have been identified, the majority surrounding around the interaction of Cx43 to proteins that are linked to cancer or cell proliferation pathways (Zhou and Jiang, 2014). Therefore, to increase our knowledge surrounding the role of Cx43 in distant organ metastases, future studies should also focus on elucidating the mechanism by which Cx43 possibly facilitates melanoma metastasis. In this regard the chick-chorioallantoic membrane (CAM) assay, which has been used as a highly vascularized *in vivo* system, is an excellent tool that can rapidly and inexpensively recapitulate melanoma metastasis (Stoletov et al., 2013; Penuela et al., 2012; Chambers et al., 1982; Chambers et al., 1992; Ableser et al., 2014). To determine whether or not Cx43 expressing human tumors direct metastasis or facilitate tumor cell survival in distant organs, future studies should isolate primary human melanoma cells from frozen specimens and implant them between branching vessels on the CAM as previously described (Welte et al., 2013; Penuela et al., 2012). Furthermore, Cx43 mimetic peptides, such as Gap27, that block Cx43 mediated intercellular communication can be applied to isolated human melanoma cells prior to their injection to determine if Cx43 can still facilitate end stage metastases, which would further

implicate a GJ- independent mechanism (Pollok et al., 2011). Collectively these studies will increase our knowledge of Cx43 in late stages of melanoma progression that will allow us to determine if we can add to the growing list of diseases where connexin inhibitors will provide a therapeutic advantage.

3.2 Overall Contributions

This investigation is one of the first to address important questions regarding the roles of Cx43 and Cx26 in modulating epidermal homeostasis in response to UV radiation. In addressing this question, we have successfully created a novel and viable mouse model ($Cx26^{K14\text{-}S17F/+}$) that can be used to continue to analyze the role of Cx26 in keratinocytes, that can be extrapolated to the human KID population. In our study the reduction of Cx26 function in keratinocytes lead to erythrokeratoderma and ichthyosis of the skin, and an increased susceptibility to UV radiation. Our work is the first to suggest a link between a reduction in epidermal Cx26 and increased UV-reactivity of the skin, which could possibly explain why KID patients, who harbor Cx26 mutations, have an increased propensity to develop keratinocyte-derived tumors. Furthermore, these studies will bring us closer to making recommendations to patients who suffer from Cx26-associated syndromic skin disease regarding safe UV-exposure practices. In addition, $Cx43^{I130T/+}$ mice were not more susceptible to UV-induced pathogenesis, indicating that a partial (50%) Cx43 function was sufficient to maintain epidermal health. Therefore, the epidermis expresses a surplus of Cx43 then what is actually required to maintain cellular behavior and tissue function. This is encouraging as it suggests that human patients who possess a similar reduction in Cx43 expression, as seen in certain subsets of ODDD patients, are not more susceptible to UV-induced injuries, including cancers.

Furthermore, this investigation is the first to identify that Cx43 may act as a tumor facilitator to promote tumor cell survival in human melanoma metastases in a variety of distant organ sites. The analysis of Cx43 expression in human melanoma progression represents the next vital step in translating our knowledge of these channel-forming proteins to the clinic, an important avenue to pursue in the investigation of cancer treatment. In particular, it is imperative that we continue to investigate the most fearsome aspect of human melanoma: its metastases to distant organ sites (Fidler, 2003). Despite

significant advancements in technology, surgical techniques, and adjuvant therapies, the majority of cancer deaths, including melanoma, are associated with metastatic tumor burden (Fidler, 2003). As such it is important that we continue to characterize the molecular profile of human melanoma to increase our understanding of mechanisms behind metastatic pathogenesis. In this regard, our work is also the first to suggest that Cx43 may be acting through GJ-independent mechanism in end stage human melanoma progression. Therefore, as we continue in pursuit of personalized therapeutics, we have unveiled the potential of Cx43 as a target in metastatic human melanoma, and have provided a direction for which future studies can begin to delineate the Cx43 related mechanism that facilitates metastatic tumor burden.

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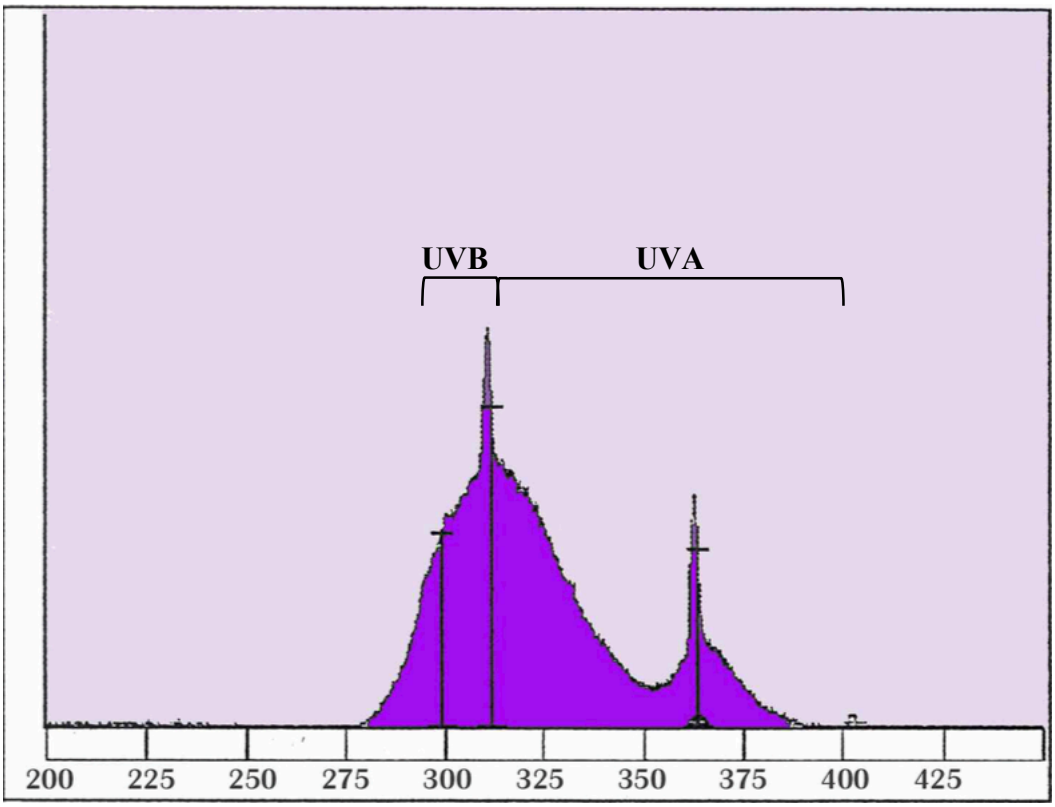
Appendices

Appendix A: Human melanoma sample set ordered from the Ontario Institute of Cancer Research

	Tissue Type	Number of Donors
Primary Tumour	Melanoma	N = 14, n = 56
	Normal	N = 3, n = 12
	Normal patient matched	N = 2, n = 8
Nodal Metastases	Melanoma	N = 15, n = 60
	Normal	N = 1, n = 4
	Normal patient matched	N = 1, n = 4
Distal Metastases	Melanoma	N = 7, n = 28
	Normal	N = 4, n = 16
	Normal patient matched	N = 3, n = 12

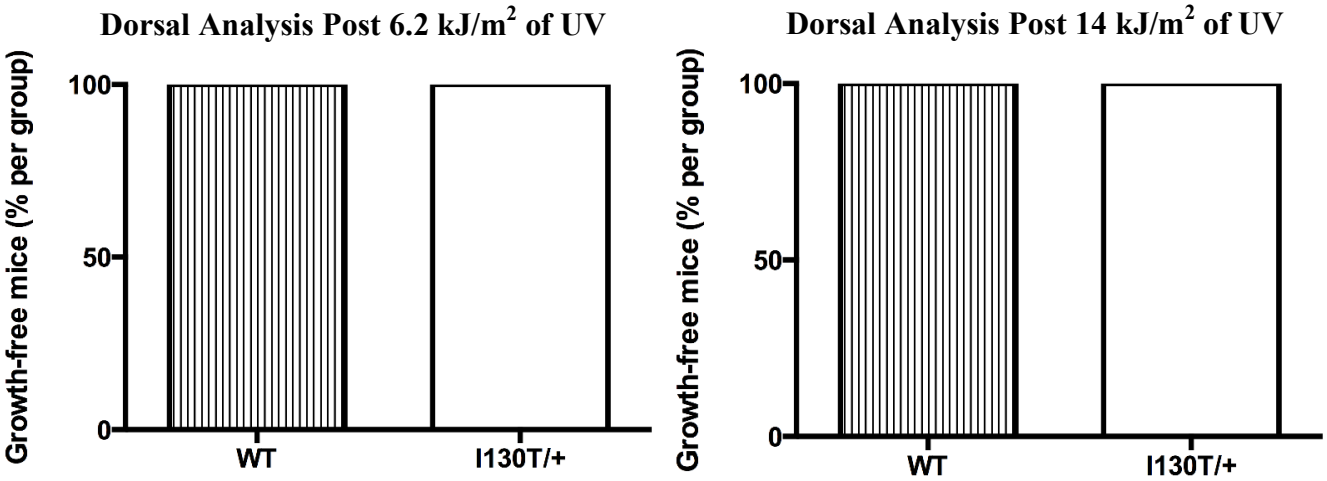
	Site of Distal Organ Metastases			
	Kidney	Lung	Bone	Pelvic Wall
Number of Donors	3	2	1	1

Appendix B: UV spectral chart of 302nm UV lamp with filter used in all UV exposure experiments.



Modified from: http://www.uvp.com/spectralcharts.html

Appendix C: Cx43$^{I130T/+}$ mice are free of any growths or abnormalities 6 months after UV exposure.



Appendix D: Animal Use Protocol approval for all animal work within this investigation.

Dale Laird - eSirius Notification - New Protocol Modification Has Been APPROVED2006-101-10::6

From:
To:
Date:
Subject:
CC:

Western

AUP Number: 2006-101-10
PI Name: Laird, Dale W
AUP Title: The Role of Gap Junction in Diseases

Official Notification of AUS Approval: A MODIFICATION to Animal Use Protocol 2006-101-10 has been approved.

The holder of this Animal Use Protocol is responsible to ensure that all associated safety components (biosafety, radiation safety, general laboratory safety) comply with institutional safety standards and have received all necessary approvals. Please consult directly with your institutional safety officers.

Submitted by: Kinchlea, Will D
on behalf of the Animal Use Subcommittee

The University *of* Western Ontario
Animal Use Subcommittee University Council on Animal Care
Health Sciences Centre • London, Ontario • CANADA – N6A 5C1
PH 519-661-2111 ext. 86761 • FL 519-661-2123
Email [illegible] • http [illegible]

Appendix E: Human Science Research Ethics Board approval for all work using human melanoma samples.

Western Research

Research Ethics

Western University Health Science Research Ethics Board
HSREB Amendment Approval Notice

Review Type: Expedited
HSREB File Number: 103381
Study Title: The novel channel forming protein pannexin1 as a viable target for melanoma treatment
Sponsor:

HSREB Amendment Approval Date: November 06, 2015
HSREB Expiry Date: March 08, 2016

Documents Approved and/or Received for Information:

Document Name	Comments	Version Date
Revised Western University Protocol		
Change in Study Personnel	Incoming Co-I (S. Penuela) Incoming Research Student (K. Alaga, T. Freeman) Incoming Research Technician (D. Johnston, C. Shao)	2015/06/23

The Western University Health Science Research Ethics Board (HSREB) has reviewed and approved the amendment to the above named study, as of the HSREB Initial Approval Date noted above.

HSREB approval for this study remains valid until the HSREB Expiry Date noted above, conditional to timely submission and acceptance of HSREB Continuing Ethics Review.

The Western University HSREB operates in compliance with the Tri-Council Policy Statement Ethical Conduct for Research Involving Humans (TCPS2), the International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use Guideline for Good Clinical Practice Practices (ICH E6 R1), the Ontario Personal Health Information Protection Act (PHIPA, 2004), Part 4 of the Natural Health Product Regulations, Health Canada Medical Device Regulations and Part C, Division 5, of the Food and Drug Regulations of Health Canada.

Members of the HSREB who are named as Investigators in research studies do not participate in discussions related to, nor vote on such studies when they are presented to the REB.

The HSREB is registered with the U.S. Department of Health & Human Services under the IRB registration number IRB 00000940.

Ethics Officer to Contact for Further Information: Erika Basile ___ Nicole Kaniki ___ Grace Kelly ___ Mina Mekhail ___ Vikki Tran ___

This is an official document. Please retain the original in your files

Curriculum Vitae

Name: Katanya Alaga

Post-secondary Education and Degrees:

University of Western Ontario
London, Ontario, Canada
2014-2016 MSc Candidate
Anatomy and Cell Biology

The University of Western Ontario
London, Ontario, Canada
2014-2016 BMSc
Honors Specialization in Medical Sciences

Honours and Awards:

Strategic Training Program in Cancer Research and Technology Transfer (CaRTT) - $25,700
2014-2016

Western Graduate Research Scholarship - $7500/year
Western University
2014-2016

Top 100 Poster
London Health Research Day
March 2016

Gabriel G. Altman Biological Research Award - $150
Anatomy and Cell Biology Research Day
October 2016

Poster Presentations

Katanya Alaga, Kevin Barr, and Dale W. Laird. (June 26th, 2016). Examining the Roles of Connexin26 and Connexin43 in Skin Cancer Onset and Progression. Oncology Research and Education Day. London, Ontario.

Katanya Alaga, Kevin Barr, and Dale W. Laird. (March 29th, 2016). Examining the Roles of Connexin26 and Connexin43 in Skin Cancer Onset and Progression. London Health Research Day. London, Ontario.

Katanya Alaga, Kevin Barr, and Dale W. Laird. (December 12-16th, 2015). Examining the Roles of Connexin26 and Connexin43 in Skin Cancer Onset and Progression. American Society of Cell Biology International Meeting. San Diego, California.

Katanya Alaga, Kevin Barr, and Dale W. Laird. (October 16th, 2015). Examining the Roles of Connexin26 and Connexin43 in Skin Health and Cancer Development. Anatomy and Cell Biology Research Day. London, Ontario.

Katanya Alaga, Kevin Barr, and Dale W. Laird. (June 26th, 2015). Examining the Roles of Connexin26 and Connexin43 in Skin Health and Cancer Development. Oncology Research and Education Day. London, Ontario.

Related Work Experience

Tutorial Instructor
Human Physiology, PHYS 2130
The University of Western Ontario
2015-2016

Teaching Assistant
Translational Models of Cancer, ACB 4461B
The University of Western Ontario
2015