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Cx43 Reduces Melanoma Growth Within a Keratinocyte Microenvironment and During Tumorigenesis in vivo

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A thesis submitted in partial fulfillment of the requirements for the Master of Science degree in Anatomy and Cell Biology

Cx43 REDUCES MELANOMA GROWTH WITHIN A KERATINOCYTE MICROENVIRONMENT AND DURING TUMORIGENESIS *IN VIVO*

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by

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Abstract

Connexins have been frequently identified as tumor suppressors in many cancers, however, their role in melanoma tumorigenesis remains controversial. Here, we show that B16-BL6 mouse melanoma cells express low levels of Cx26 and Cx43, rendering them gap junctional intercellular communication (GJIC) deficient. Following ectopic expression of Cx26 and Cx43, gap junction-like plaques were evident at the cell surface and the incidence of dye transfer was significantly increased similar to connexin-rich keratinocytes. The expression of Cx43, but not Cx26, significantly reduced proliferation and anchorage-independent growth relative to controls, whereas migration was unaffected. Additionally, Cx43-expressing melanoma cells displayed significantly reduced growth amongst keratinocytes, despite a complete lack of heterocellular GJIC. Furthermore, when grown *in vivo* in the chicken embryo, Cx43-expressing melanoma cells formed significantly smaller primary tumors compared to controls, whereas Cx26 expression did not alter primary tumor size. Collectively, these results suggest that Cx43, but not Cx26, acts as a tumor suppressor during melanoma tumorigenesis.

Keywords

The following keywords can be used to describe the thesis entitled " Cx43 reduces melanoma growth in a keratinocyte microenvironment and during tumorigenesis *in vivo*"

B16-BL6 mouse melanoma cells, rat epidermal keratinocytes (REK), gap junction, connexin, connexin26 (Cx26), connexin43 (Cx43), cell proliferation, migration, anchorage-independent growth, *in situ*, microenvironment, co-culture, *in vivo*, chick chorioallantoic membrane (CAM), tumor growth.

Co-Authorship Statement

Dr. Silvia Penuela was an invaluable resource throughout the course of the project as an expert in melanoma biology. She was integral in the implementation and setup of the chick-CAM assay used in this study.

Jack Lee began this project as a Master's student in the Laird lab and was involved in the design and rationale of the project.

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List of Abbreviations

AMP	adenosine monophosphate
ANOVA	analysis of variance
ATP	adenosine triphosphate
BCA	bicinchoninic acid
BCC	basal cell carcinoma
BSA	bovine serum albumin
CAM	chorioallantoic membrane
cAMP	cyclic adenosine monophosphate
CDKN2A	cyclin-dependent kinase inhibitor 2A gene
Cx	connexin
GJIC	gap junctional intercellular communication
DMEM	Dulbecco's modified Eagle's medium
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
EdU	5-ethynyl-2′-deoxyuridine
EGTA	ethylene glycol tetraacetic acid
ERK	extracellular signal-regulated kinase
ET_RB	endothelin receptor B
GFP	green fluorescent protein
HCl	hydrogen chloride
HeLa	Henrietta Lacks cervical cancer cell line
IP_3	inositol-1,4,5-triphosphate
kDa	kiloDalton
MAPK	mitogen-activated protein kinase

MCC	Merkel cell carcinoma
MEK	mitogen-activated protein kinase kinase
MEM	minimal Eagle's medium
MITF	microphthalmia-associated transcription factor
MMP-2	matrix metalloproteinase-2 gene
mRNA	messenger ribonucleic acid
NaCl	sodium chloride
NAD+	nicotinamide adenine dinucleotide
NMSC	non-melanoma skin cancer
NOV/CCN3	nephroblastoma overexpressed protein
PBS	phosphate buffered saline
PGE_2	Prostaglandin E2
PTEN	phosphatase and tensin homolog gene
qPCR	quantitative polymerase chain reaction
REK	rat epidermal keratinocyte
S-phase	synthesis phase of cell cycle
SCC	squamous cell carcinoma
SDS/PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	standard error of the mean
Tris	tris(hydroxymethyl)aminomethane
TRPM1	transient receptor potential cation channel subfamily M member 1
TSG101	tumor susceptibility gene 101
UTR	untranslated region
UV	ultraviolet
WT	wild-type
ZO	zonula occludens

Chapter 1

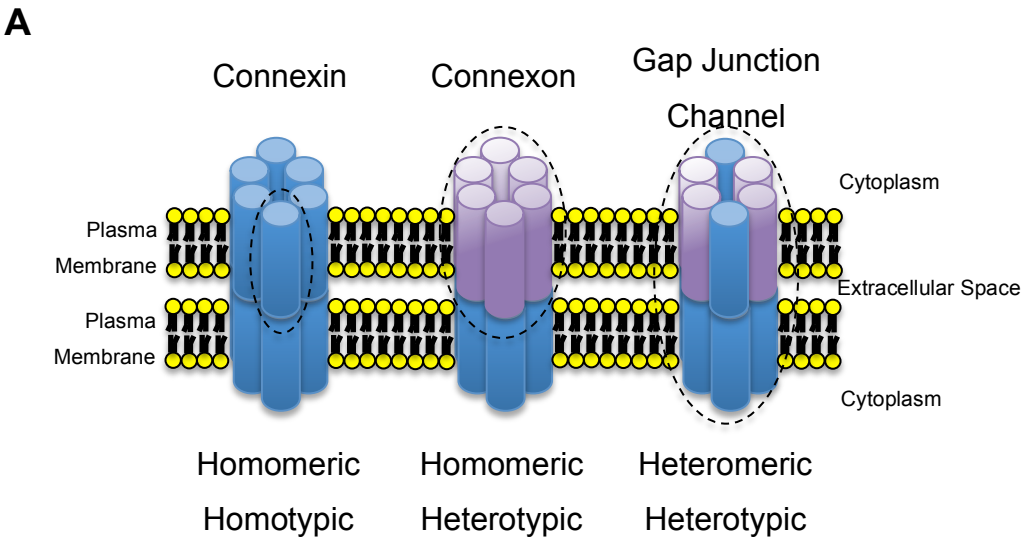
1 Literature Review

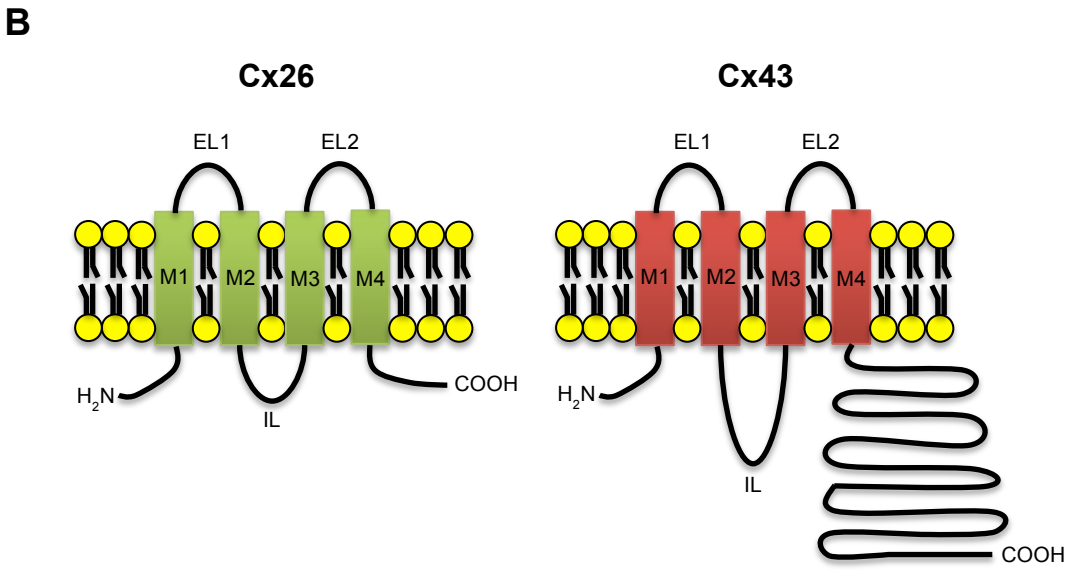
1.1 Connexins and Gap Junctions

Connexins (Cx) encompass a large family of integral membrane proteins that are expressed across all vertebrates. To date, 21 distinct connexins have been identified in the human genome and 20 connexins are expressed in rodents (Sohl and Willecke, 2003). Interestingly, of these connexins, 19 are homologous and highly conserved, allowing for functional insights on one connexin to be extended to other connexin family members and across different species (Sohl and Willecke, 2004). Although the lifecycle for each connexin family member has not been studied in depth, the well-characterized lifecycle for Cx43 often serves as a model for other connexins. Cx43 is co-translationally inserted into the endoplasmic reticulum as a tetra-membrane spanning protein, which creates several distinct domains, including two extracellular loops, one cytoplasmic loop, and cytoplasmic amino- and carboxy-terminal tails. Once properly folded, Cx43 is trafficked to the Golgi apparatus, where individual connexin proteins oligomerize into a hexameric arrangement, known as a connexon. Following oligomerization, connexons are subsequently trafficked to the cell surface where they can function as single-membrane 'hemichannels' permitting the passage of small molecules between the intracellular and extracellular environment. Additionally, these connexin hemichannels possess the ability to dock with compatible connexons from apposing cells forming gap junction channels, thereby permitting the passage of small molecules, second messengers and ions between apposing cells (Fig. 1.1A) (Laird, 2006). Although the majority of gap junctions are composed of identical connexin subunits, non-identical connexins can oligomerize into heteromeric connexons and form functional gap junctions (Fig. 1.1A) (Wagner, 2008). For example, Cx26 and Cx32 are known to intermix in hepatocytes while Cx46 and Cx50 co-oligomerize in the lens (Jiang and Goodenough, 1996; Sosinsky, 1995). To add to this complexity, non-identical connexons may also dock at cell-cell interfaces, forming heterotypic gap junction channels, which greatly increases the variety of distinct gap junction channels that can form (Fig. 1.1A) (Wagner, 2008). However, not all connexins

Figure 1.1 Connexin topology and organization into gap junction channels

Connexins oligomerize into hexamers, known as connexons, and dock with connexons from opposing cells to form gap junction channels. Typically, homomeric connexons form from one connexin isoform and dock with an identical connexon into a homotypic gap junction channel. However, non-identical connexons can dock forming heterotypic gap junction channels, and similarly, non-identical connexins can oligomerize into heteromeric connexons (A). All connexins share a similar topology, composed of four transmembrane domains (M1-M4), two extracellular loops (EL1 and EL2), an intracellular loop (IL) and intracellular amino-terminal (NH_2) and carboxy-terminal (COOH) tails. The transmembrane domains, extracellular loops and amino-terminal tails are highly conserved across connexin family members. Conversely, the intracellular loop and carboxy-terminal tails are the most diverse domains between connexins, both in amino acid sequence and length (B).





are compatible with each other and capable of oligomerizing into mixed connexons, and similarly, not all connexons are capable of docking and forming heterotypic gap junction channels. For example, when expressed in the same cell, Cx26 and Cx43 are incapable of intermixing (Gemel et al., 2004). Although connexins have been divided into two broad subgroups (denoted as alpha and beta groups) which is generally based on size, there does not appear to be an obvious criteria to predict connexin compatibility (White and Bruzzone, 1996).

All connexins share a common topology, including four transmembrane domains, two extracellular loops, one intracellular loop, and intracellular amino- and carboxy-terminal tails (Fig. 1.1B) (Goodenough et al., 1996). Of these regions, the transmembrane domains, extracellular loops and amino-terminal tail are highly conserved across connexin family members (Sohl and Willecke, 2004). Specifically in the extracellular loops, three highly conserved cysteine residues exist allowing for stabilizing intramolecular disulfide linkages (John and Revel, 1991). Conversely, the carboxy-terminal tail is the most diverse region across connexin family members, not only in sequence, but also in length (Laird, 2006). Some connexins possess phosphorylation sites within the carboxy-terminus that have been reported to regulate the connexin life cycle and channel permeability (Lampe and Lau, 2004).

1.2 Gap Junctions and Intercellular Communication

The ability to transfer small molecules, second messengers and ions between cells through gap junctions (known as gap junctional intercellular communication, or GJIC) is essential for the regulation of many cellular processes including proliferation, differentiation and apoptosis (Goodenough et al., 1996). Although the exact knowledge of which molecules are passing through gap junctions in any given tissue remains mostly unknown, for a few connexins, some work has been done on the identity of these transjunctional molecules and their relative permeabilities. The second messengers inositol-1,4,5-triphosphate (IP_3) and cyclic adenosine monophosphate (cAMP) have been extensively studied and have been shown to pass though gap junctions consisting of Cx26, Cx26/Cx30, Cx26/Cx32, Cx32, Cx43 and Cx26, Cx32, Cx36, Cx43, Cx43/Cx46,

Cx45, Cx47, respectively (Harris, 2007). Calcium ions have been shown to pass through Cx26, Cx32, Cx37, Cx37/Cx43 and Cx43 gap junction channels, whereas adenosine diphosphate (ADP)/adenosine triphosphate (ATP) has only been shown to pass though channels consisting of Cx32 and Cx43 (Alexander and Goldberg, 2003; Harris, 2007). It is important to note that although gap junction channels composed of different connexins possess the ability to pass many of the same constituents, the relative permeabilities of these channels is distinct for each molecule (Harris, 2007). For example, IP_3 has been shown to pass through various gap junction channels, but it appears to preferentially pass through Cx32-based channels compared to channels consisting of Cx43, which in turn is more permeable than Cx26-based gap junction channels (Harris, 2007). It was initially postulated that pore size and molecular charge may dictate the passage of molecules through distinct gap junctions, however, this generalization was later shown to be false (Harris, 2007). For example, adenosine passes through Cx32 channels 10-fold better than Cx43 channels, but adenosine with the addition of one, two, or three phosphate groups (resulting in AMP, ADP and ATP, respectively) exhibits a shift in permeability favoring passage through Cx43-based gap junction channels (Alexander and Goldberg, 2003).

1.3 GJIC-Independent Roles for Connexins

Although the ability to form gap junctions allowing for the transfer of molecules between apposing cells is commonly believed to be the primary function of connexins, these proteins also possess GJIC-independent roles. Once at the cell surface, connexons typically dock forming gap junction channels, however, connexons have been reported to act as single membrane hemichannels exchanging molecules with the extracellular environment (Wang et al., 2013). Hemichannels are typically closed under normal physiological conditions, but can be opened due to several stimuli including voltage changes, extracellular and intracellular ion changes, mechanical strain and post-translation modifications (Wang et al., 2013). Similar to their gap junction counterparts, the identity of molecules able to pass through hemichannels remains limited to small molecules, but includes ATP, IP_3, cAMP, nicotinamide adenine dinucleotide (NAD^+), glutamate, glutathione and prostaglandin E2 (PGE_2) (Harris, 2007; Wang et al., 2013).

In addition to the formation of undocked hemichannels, connexins may also affect cellular physiology through direct connexin binding partners and indirect interactions as part of a junctional complex. Connexins have been reported to bind to various post-translational modifiers involved in phosphorylation (Solan and Lampe, 2009), scaffolding and cytoskeletal proteins including various zonula occludens (ZO) and ZO-binding proteins (Gilleron et al., 2008; Hunter et al., 2005), trafficking proteins necessary for delivery to the cell surface and internalization, including caveolin proteins (Langlois et al., 2008) and other growth regulators including nephroblastoma overexpressed protein (NOV or CCN3) (Fu et al., 2004). It should be noted, however, that this collective gap junction proteome, which now includes over 40 distinct proteins, appears to be connexin-specific (Laird, 2010). Each connexin family member possesses a unique subset of this proteome, which may in large part be due to difference amongst connexin isoforms, primarily within the carboxy-terminal domain (Fig. 1.1B) (Laird, 2010; Naus and Laird, 2010). For example, Cx43 has 14 known serine and tyrosine phosphorylation sites on its large carboxy-terminus (Lampe and Lau, 2004), whereas Cx26 has not been reported to be phosphorylated (Traub et al., 1989).

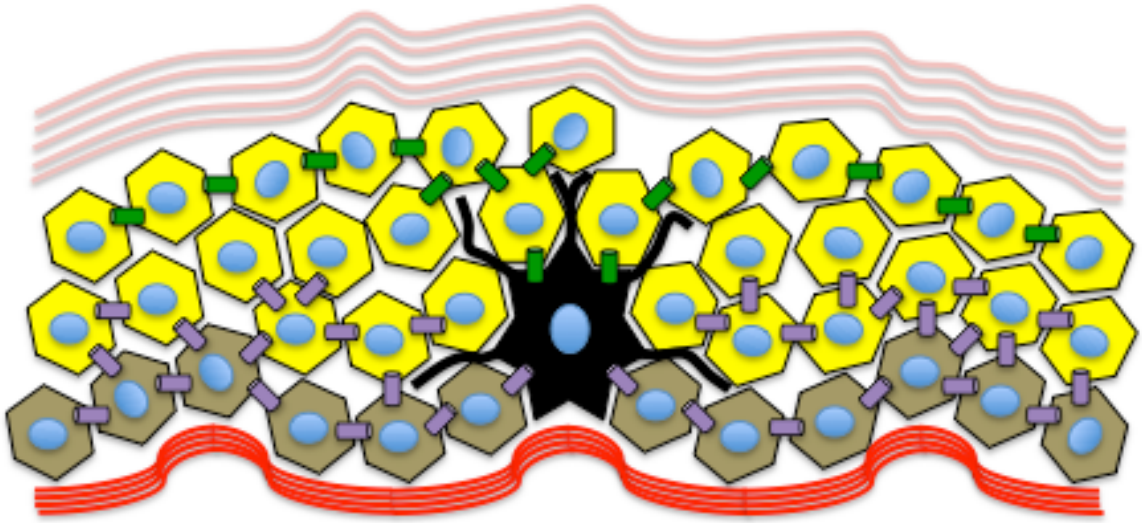
1.4 Cells of the Epidermis

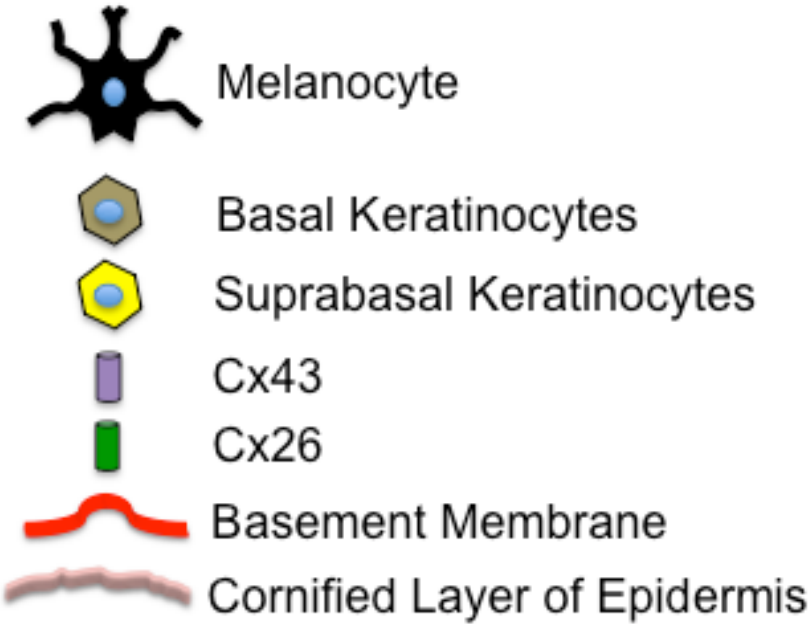
The epidermis covers the outermost layer of the human body as the most superficial layer of the skin and acts as a physical barrier to environmental stresses (Cartlidge, 2000). Keratinocytes make up the vast majority of the epidermis, and are divided into five epidermal strata. A single layer of basal keratinocytes, which rests atop a basement membrane separating the epidermis from the underlying dermis, is the deepest of the epidermal layers and acts as the site for epidermal stem and progenitor cells which can proliferate and replenish keratinocytes in the layers above (Eckert et al., 1997). These basal keratinocytes differentiate sequentially to form the stratum spinosum, stratum granulosum and stratum lucidum, collectively termed the suprabasal layers, before finally undergoing apoptosis to form the stratum corneum (Fig. 1.2) (Eckert et al., 1997).

The second most abundant cell type of the epidermis is the melanocyte. Melanocytes reside in the basal layer of the epidermis and typically form the ‘epidermal-melanin unit’, where one melanocyte forms connections with approximately 30 keratinocytes within the

Figure 1.2 Connexins involved in the epidermal-melanin unit

Melanocytes, which reside in the basal layer of the epidermis, extend their dendrites into the suprabasal layers and form connections with approximately 30 keratinocytes. Through their expression of Cx43 and Cx26, melanocytes are believed to be capable of GJIC with basal and suprabasal keratinocytes, respectively, which helps to maintain the epidermal-melanin unit. Cx43 is highly expressed amongst basal keratinocytes and also found within the stratum spinosum. Conversely, Cx26 appears to be confined to the stratum granulosum. Additionally, suprabasal keratinocytes express Cx30, Cx31, Cx37, Cx40 and Cx45 (not shown in figure).





basal and suprabasal layers (Fig. 1.2) (Haass and Herlyn, 2005). Melanocytes are vital to the epidermis and underlying tissues due to their production and release of the pigment melanin which protects cells from ultraviolet (UV) radiation damage (Lin and Fisher, 2007). Epidermal melanocytes produce two types of melanin; pheomelanin and eumelanin, which are transported to surrounding keratinocytes through dendritic processes and released via exocytosis. These pigment-containing granules, known as melanosomes, are ingested by keratinocytes where they form protective 'caps' above the nucleus to prevent DNA damage from UV radiation (Lin and Fisher, 2007). Interestingly, melanocytes tend to maintain a symbiotic relationship with keratinocytes, exhibiting controlled replication such that they maintain a ratio of approximately 1:5-8 with basal keratinocytes (Hsu et al., 2000b). It is commonly believed that melanocyte growth and proliferation is controlled by these surrounding keratinocytes through various mechanisms including paracrine signaling, intercellular communication and intracellular communication (Haass et al., 2004).

Although keratinocytes and melanocytes account for nearly all cells within the epidermis, two additional cell types are found in this tissue. Merkel cells reside within the stratum basale, acting as sensory receptors to light touch through synaptic connections with somatosensory neurons (Maricich et al., 2009). Additionally, Langerhans cells are present within the suprabasal layers. These dendritic cells possess immune function and act as antigen-presenting cells when exposed to foreign microbial agents (Wolff and Stingl, 1983).

1.5 Connexins Expressed within the Epidermis

The epidermis is one of the most diverse connexin environments across all mammals. In human epidermis, as many as 10 connexins are expressed at the messenger ribonucleic acid (mRNA) level, with 7 of these being translated into detectable levels of proteins, primarily within epidermal keratinocytes. Interestingly, these connexins are differentially regulated across the different epidermal strata. Cx43 is highly expressed in the stratum basale as well as trace levels of Cx26. As many as 7 connexins are expressed in the suprabasal layers, including Cx26, Cx30 Cx31, Cx37, Cx40, Cx43 and Cx45, although Cx26 appears to be confined to the stratum granulosum (Di et al., 2001; Richard, 2000).

Similarly, in rodent epidermis, Cx31.1, Cx40 and Cx43 are expressed in the basal layer, Cx31.1, Cx37, Cx43 are expressed in the stratum spinosum, and Cx31, Cx37, Cx26 and Cx30 are expressed in cells within the stratum granulosum (Churko and Laird, 2013). Across all mammals, the cornified layer is devoid of connexins, as this stratum contains no living cells. Overall, the selective and spatial specific expression of connexins within the epidermis is vital to proper development, proliferation and differentiation of the epidermis (Richard, 2000).

In contrast to keratinocytes, the connexins status of melanocytes has been much less studied. Two studies suggest that Cx26 and Cx43 are the predominant connexins expressed in human melanocytes (Hsu et al., 2000a; Masuda et al., 2001), while connexins have yet to be reported in rodent melanocytes. Additionally, Cx32 has also been implicated in melanocyte biology, although the evidence for a role for Cx32 is not strong (Bondurand et al., 2001). Importantly, melanocytes have been shown to be capable of GJIC with keratinocytes, possibly through channel formation with complementary connexins in both cell types (Fig. 1.2) (Hsu et al., 2000a). It has been proposed that melanocyte growth and proliferation is, in part, mediated by this heterocellular GJIC with keratinocytes (Fig. 1.2) (Haass et al., 2004).

A very limited amount of work has been done on the connexin profile in Merkel cells and Langerhans cells. Woo et al. (2010) have shown that mouse Merkel cells express Cx43. Conversely, connexins have not been reported in Langerhans cells. Goliger and Paul (1994) showed that rat Langerhans cells do not couple with adjacent keratinocytes through dye transfer studies, which was later supported in human Langerhans cells (Zimmerli et al., 2007).

1.6 Cancers Derived from the Epidermis

Several cancers can arise within the epidermis depending on the lineage of the initial transformed cell. In general, epidermal skin cancers can be classified as either non-melanoma skin cancer (NMSC) or melanoma skin cancer (Gallagher et al., 1990). Non-melanoma skin cancers are derived from keratinocytes, and can be further subdivided into two groups depending on the subclass of keratinocyte that becomes transformed.

Basal cell carcinomas (BCC) are cancers derived from basal keratinocytes and account for the vast majority of reported skin cancers (Christenson et al., 2005). These cancers are typically found on areas of high sun exposure, primarily the head and neck, and are generally believed to be benign, metastasizing less than 0.1% of the time (Netscher et al., 2011). Squamous cell carcinomas (SCC) are derived from suprabasal keratinocytes and are more aggressive than BCCs as they can metastasize due to malignant precursors, potentially depending on *in situ* subtypes (Netscher et al., 2011). Melanoma skin cancers are derived from pigment-producing melanocytes, and are the result of the vast majority of skin cancer-related fatalities (Miller and Mihm, 2006). In addition to these two classes of epidermal skin cancers, Merkel cells have also been recently discovered to form tumors within the epidermis, although Merkel cell carcinomas (MCC) are classified as neuroectodermal tumors (Feng et al., 2008). Similar to melanomas, MCCs are highly aggressive with an overall 5-year survival rate of ~60% (Schrama et al., 2012).

1.7 Melanoma as a Disease

Although melanoma only accounts for ~4% of reported skin cancers, it remains the most lethal form of skin cancer accounting for ~80% of skin cancer-related fatalities as of 2004 (Tsao et al., 2004). In fact, the global incidence of melanoma appears to be continually increasing, and has been consistently doing so for over 60 years (Linos et al., 2009). Primarily, the high mortality rate that accompanies melanoma is the result of a high incidence of metastasis to the liver, brain, lung and bone, which can significantly decrease the survival rate to as low as 7%, compared to a 99.9% survival rate when identified early *in situ* within the epidermis (Balch et al., 2001a; Miller and Mihm, 2006).

Melanomas are typically identified within the epidermis as pigmented lesions. As a general rule of thumb to predict severity, clinicians use the mnemonic “ABCDE”. Lesions with **A**symmetrical borders, irregular **B**orders, variegated **C**olours, **D**iameter of >6mm, and that are continuously **E**volving over time are characterized as potential melanomas and are usually removed and analyzed (Netscher et al., 2011).

1.8 Melanocyte Transformation and Melanoma Progression

The symbiotic relationship between melanocytes and keratinocytes is defined as the epidermal-melanin unit, where one melanocyte forms connections with approximately 30 keratinocytes (Haass and Herlyn, 2005). The association with keratinocytes through the formation of the epidermal-melanin unit causes melanocytes to become dendritic, suppress melanoma-associate cell surface markers and control melanocyte proliferation (Valyi-Nagy et al., 1993). Melanocyte transformation disrupts the epidermal-melanin unit, thereby freeing melanocytes from the control of keratinocytes and facilitates the progression towards malignant melanoma (Miller and Mihm, 2006). The Clark progression model remains one of the most commonly used staging classifications for melanoma progression. This model tracks the transformation of melanocytes to precancerous nevi through to radial and vertical growth phases of the melanoma, and finally to the metastatic stage of the melanoma (Clark et al., 1984).

The development of a benign nevus, or common mole, initiates the dissociation of melanocytes from the control of keratinocytes and exhibits partially disrupted growth and proliferation (Clark et al., 1984). This proliferation, however, remains limited and does not lead to immediate tumorigenesis, likely due to oncogene-induced senescence (Braig and Schmitt, 2006), and further transformation is required to progress the nevus towards a more malignant phenotype (Miller and Mihm, 2006). For example, *BRAF* mutations are associated with 50% of melanomas, however, this frequency appears to be similar between benign nevi, primary, and metastatic melanoma, suggesting other combinatorial mutations, such as inactivating p53, are necessary to further the disease (Michaloglou et al., 2005; Pollock et al., 2003). Dysplastic nevi can form from pre-existing benign nevi or arise as new lesions and exhibit further aberrant growth. Additional mutations may accumulate at this stage, including to the tumor suppressor phosphatase and tensin homolog gene (*PTEN*), which occurs in 25-50% of non-familial melanomas (Wu et al., 2003). Inactivation of *PTEN* alone is not sufficient to cause melanoma, however, when combined with mutations to the cyclin-dependent kinase inhibitor 2A gene (*CDKN2A*), which occurs in 25-40% of familial melanomas, tumors can arise (Thompson et al., 2005; You et al., 2002). Collectively, it is understood that no singular mutation results in

melanoma tumorigenesis, but rather, a collection of detrimental mutations are necessary to further the disease.

The next stage of melanoma tumorigenesis, as proposed by Clark et al. (1984), occurs as radial growth phase melanoma. Here, melanoma cells display further increased proliferation, however, they only proliferate within the confines of the epidermis as they lack the ability to penetrate through the epidermal basement membrane and into the underlying dermis (Miller and Mihm, 2006). The ability of melanomas to penetrate the basement membrane and invade the dermis, thereby entering the vertical growth phase, is accompanied by the expression of αvβ3 integrin, resulting in the induction of matrix metalloproteinase-2 (MMP-2); an enzyme necessary to weaken the basement membrane (Brooks et al., 1996; Danen et al., 1994). Transition from radial to vertical growth phase is also accompanied by a concomitant shift in expression from E-cadherin to N-cadherin (Danen et al., 1996; Hsu et al., 1996). This 'cadherin shift' is commonly associated with invasive carcinomas of epithelial origin, and allows for further spread of the disease by associating with other N-cadherin-expressing cells, including fibroblasts and endothelial cells (Hazan et al., 2004; Hsu et al., 2000a). The absolute depth, spread and progression of melanoma is often further characterized using the Breslow index which is the best method for predicting prognosis for melanoma lesions in the skin (Balch et al., 2001b). Once melanoma cells have entered the vertical growth phase and spread into the dermis, melanoma cells can acquire metastatic potential, which has been correlated with decreased expression of the transient receptor potential cation channel subfamily M member 1 (TRPM1) (Miller et al., 2004). Metastatic melanomas primarily spread to the liver, bone, brain and lungs which greatly worsens overall prognosis (Miller and Mihm, 2006).

1.9 Current Therapeutic Targets for Melanoma/Personalized Medicine

Currently, very few therapies and therapeutic targets exist to treat advanced melanoma, which is part of the reason this cancer remains so lethal. Activating *BRAF* mutations have been associated with 50% of melanomas, which cause constitutive activation of the extracellular signal-regulated kinase (ERK) - mitogen-activated protein kinase (MAPK)

pathway resulting in melanoma growth (Miller and Mihm, 2006). Because of this, small molecules have been designed to target BRAF, however, these were met with limited success in the clinic (Lyons et al., 2001). Additionally, the growth of BRAF-mutated melanomas can be suppressed through the inhibition of mitogen-activated protein kinase kinase (MEK), an enzyme downstream of BRAF, making MEK a suitable therapeutic target (Solit et al., 2006), and is currently undergoing clinical investigation. Furthermore, genomic analysis using a database of melanoma samples found that the microphthalmia-associated transcription factor (*MITF*) locus frequently displays increased copy number leading its increased protein expression (Garraway et al., 2005). Although increased MITF expression alone has not been reported to transform melanocytes, melanoma can arise as a result of increased MITF and BRAF. Given this, MITF has been identified as another potential therapeutic target in the ERK-MAPK pathway for individuals harboring activating *BRAF* mutations (Garraway et al., 2005). Due to the limited identification of therapeutic targets for melanoma, it is imperative to identify new targets with the hopes of bettering the bleak prognosis for melanoma patients with advanced disease. In our study, we assessed the role of two connexins, Cx26 and Cx43, in melanoma tumorigenesis with the goal of elucidating new therapeutic targets.

1.10 Connexins in Cancer

The exchange of molecules between cells through GJIC has been implicated in cancer for nearly 50 years (Loewenstein and Kanno, 1966). Primarily, connexins have been implicated as tumor suppressors in many cancers due to the fact that they are frequently downregulated in tumor cell lines and histological sections of solid tumors in mammals (Cronier et al., 2009; Laird et al., 1999; Loewenstein and Kanno, 1966; Mesnil et al., 2005). Additionally, connexin-knockdown studies, gap junction blockers which promote tumorigenesis, and oncogenes which reduce gap junction permeability or connexin expression further support the notion that connexins act as tumor suppressors (Atkinson et al., 1981; Azarnia and Loewenstein, 1984a; Azarnia and Loewenstein, 1984b; Azarnia and Loewenstein, 1984c; Trosko et al., 1990). However, perhaps the most compelling evidence that connexins have tumor suppressive capabilities lie in the findings that ectopically reintroducing connexins into tumor cell lines reduces their tumorigenic

behavior and connexin-knockout mice are more prone to developing chemically- and radiation-induced tumors (Eghbali et al., 1991; Hellmann et al., 1999; Hirschi et al., 1996; King and Lampe, 2004a; King and Lampe, 2004b; Loewenstein and Rose, 1992; McLachlan et al., 2006; Temme et al., 1997; Zhu et al., 1991). Connexins may act as tumor suppressors via GJIC-dependent mechanisms through the passage of transjunctional molecules, or through GJIC-independent mechanisms, including tumor suppressive connexin binding partners within the large gap junction proteome (Laird, 2010; Naus and Laird, 2010). However, to think all connexins can act as tumor suppressors across all cancers may be naïve, not only due to the various types of cancer, but also due to the different stages of tumorigenesis where connexins may play a role. In fact, recent evidence has shown that connexins may actually facilitate the later stages of tumorigenesis, including extravasation and metastasis (Ezumi et al., 2008; Ito et al., 2000; Naoi et al., 2007; Pollmann et al., 2005; Saito-Katsuragi et al., 2007; Stoletov et al., 2013), leading to their reclassification in breast cancer as 'conditional tumor suppressors' (Naus and Laird, 2010).

1.11 Connexins in Human and Mouse Melanomas

Although connexins have been implicated as tumor suppressors in many types of cancers, the role of connexins in melanoma tumorigenesis, and their status during tumor onset and progression remains controversial and poorly understood. In human melanoma biopsies, Cx26 and Cx43 have been inconsistently detected. Cx26 has been shown to be expressed in melanomas residing within the epidermis (Ito et al., 2000), and has also been reported to be upregulated in melanomas that invade the dermis (Ito et al., 2000; Saito-Katsuragi et al., 2007). Additionally, using the Oncomine database, Stoletov et al. (2013) reported that increased Cx26 expression within primary melanomas correlates to increased metastasis and poor prognosis, and others have detected ~65% of metastatic lesions expressing Cx26 (Saito-Katsuragi et al., 2007). Conversely, Saito-Katsuragi et al. (2007) and others did not report any Cx26 expression in melanomas *in situ* within the epidermis, when assessing primary and metastatic melanomas (Haass et al., 2004; Haass et al., 2006; Sargen et al., 2013). Similar to the discrepancies of Cx26 expression in melanomas, the expression of Cx43 at various stages of melanoma tumorigenesis remains controversial,

as some show its expression increases as tumorigenesis progresses (Rezze et al., 2011; Sargen et al., 2013), whereas others do not observe its expression across various stages of melanoma (Haass et al., 2004; Haass et al., 2006).

Limited work has been done assessing the status of connexins in human melanoma cell lines and their role during tumorigenesis, and similar to the mixed results obtained from human melanoma biopsies, a consensus has yet to be reached. Saito-Katsuragi et al. (2007) showed that human HMY-1 melanoma cells express Cx26 which renders them GJIC-competent with endothelial cells which share similar connexin and cadherin complements. The expression of Cx26 was believed to facilitate tumor cell extravasation and promote a more malignant phenotype. Similarly, Hsu et al. (2000a) showed at least basal levels of Cx43 expression across various primary (WM115) and metastatic (WM164 and 1205Lu) melanoma cells which allowed them to exhibit GJIC between themselves and fibroblasts which also express comparable connexins and cadherins. Conversely, some have suggested that Cx43 expression decreases throughout melanoma tumorigenesis. Bagnato et al. (2004) showed that Cx43 and GJIC decreases in cutaneous (1007) and metastatic (SK-Mel28, M10 and Mel120) melanoma cells due to increased endothelin signaling through endothelin receptor B (ET_RB). Additionally, it has been reported that the Cx43 locus, located on chromosome 6, is frequently altered causing reduced connexin expression, as found in the malignant and genetically-linked UACC903 and SRS3 melanoma cell lines (Su et al., 2000; Trent et al., 1990).

Fewer studies have assessed the status of connexins in mouse melanoma models which can serve to provide insights into melanoma tumorigenesis. Primarily, these studies utilized cells derived from the melanoma B16 parental cell line, which can be subdivided into 4 isogenic lines; F0, F1, F10 and BL6, which are characterized by increasing cell aggressiveness *in vivo* (Nakamura et al., 2002). Ito et al. (2000) showed that Cx26 progressively increases from F10 to BL6 melanoma cells. They reciprocally show that functional Cx26 mediates an aggressive phenotype in F10 melanoma cells and a dominant-negative Cx26 mutant decreases metastasis in BL6 cells. The metastatic phenotype of Cx26-expressing F10 and BL6 melanoma cells was attributed to an increase in GJIC with endothelial cells. Similarly, Stoletov et al. (2013) showed that metastatic

B16 cells also express Cx26, rendering them capable of heterocellular GJIC with endothelial cells. Although Cx43 has been detected in human melanomas, both of these studies did not report the expression of this connexin isoform in B16 melanoma cells, nor did they assess the potential role of Cx43 in melanoma tumorigenesis. In our studies, we utilize the aggressive BL6 melanoma cell line to assess the role of Cx26 and Cx43. It is possible that each connexin may behave differently during the different stages of tumorigenesis, and similarly, Cx26 and Cx43 may have opposing effects within the same stage of tumorigenesis.

Several groups have assessed the effects of ectopic connexin expression or connexin downregulation within model melanoma cell lines. Interestingly, it has been reported that chromosome 6 is frequently altered in malignant human melanoma, including deletions to the long arm, 6q, containing the *GJA1* locus encoding Cx43 (Su et al., 2000; Trent et al., 1990). Although the coding region of Cx43 harbors no mutations in UACC903 cells, mutations were detected in the 3' untranslated region (UTR), which reduced Cx43 expression (Su et al., 2000). Ectopically expressed Cx43 significantly reduced anchorage-independent growth of these cells, similar to the UACC903(+6) microcell hybrid which harbors a normal chromosome 6 (Su et al., 2000). Conversely, Ito et al. (2000) demonstrated that Cx26 facilitates metastasis in mouse melanoma cells. When F10 melanoma cells were engineered to express Cx26, metastasis increased similar to Cx26-expressing BL6 melanoma cells. Similarly, when the Cx26 function of BL6 melanoma cells was abrogated via the expression of a dominant-negative Cx26 mutant, metastasis was reduced. Given this, it is possible that Cx26 and Cx43 act differently during melanoma tumorigenesis, thus it is important to study these connexins individually in a unifying model.

It is maybe not surprising that the role of connexins in melanoma tumorigenesis is unclear and complicated when we consider the complexity of cancer. It is possible that connexins may act differently throughout the stages of tumorigenesis, potentially acting as conditional tumor suppressors, as seems to be the case in breast cancer (Naus and Laird, 2010). In melanoma, the majority of data implicating Cx26 in tumorigenesis would suggest a role for this connexin as a facilitator of metastasis though the interaction

with endothelial cells. However, Cx43 may have a tumor suppressive role in human melanomas. Due to these differences, it is imperative to study both connexins together to assess their role throughout the different stages of melanoma tumorigenesis.

1.12 Assessing Melanomas in Complex Environments

It is important to assess tumorigenesis in multicellular, complex systems as the tumor microenvironment often plays a crucial role in cancer progression (Allinen et al., 2004). This concept is further emphasized when studying the role of connexins in tumorigenesis. Tumor cells in isolation may transmit signals via GJIC that are drastically different than the intercellular signals transmitted when grown in mixed cell cultures or tissue settings. Furthermore, growing tumor cells in multicellular environments allows for potential heterocellular GJIC with surrounding untransformed cell types. A few investigators have assessed GJIC in melanoma cells when co-cultured with keratinocytes (Hsu et al., 2000a; Hsu et al., 2000b) as well as fibroblasts and endothelial cells (Hsu et al., 2000a; Ito et al., 2000; Saito-Katsuragi et al., 2007; Stoletov et al., 2013). Collectively, these studies show that melanoma cells exhibit GJIC with fibroblasts and endothelial cells, mediated through Cx43 and Cx26, respectively. Alternatively, melanoma cells were incapable of heterocellular GJIC with keratinocytes, perhaps due to reduced or absent Cx43 expression. In the present study, we used a co-culture model where melanoma cells were cultured with rat epidermal keratinocytes (REK) in an attempt to mimic the *in situ* microenvironment in order to assess the effects of ectopic Cx26 and Cx43 expression in melanoma cells on heterocellular GJIC with keratinocytes and tumor cell growth.

Although it is important to assess the phenotype of tumor cells *in vitro* and within *in situ*-like models, ultimately one must implement *in vivo* models to assess tumorigenicity. Only two groups have examined the role of connexins in melanoma tumor formation and metastasis *in vivo*. Using immunodeficient mice, Ito et al. (2000) showed that Cx26-transfected F10 and Cx26-expressing BL6 mouse melanoma cells exhibited increased metastasis to the lung. Similarly, Cx26-expressing B16 mouse melanoma cells were found to be more capable of colonizing the brain compared to GJIC-deficient cells blocked with carbenoxolone (Stoletov et al., 2013). In both cases, Cx26 was suggested to facilitate tumor cell extravasation mediated by heterocellular GJIC with endothelial cells.

Because of the scarcity of *in vivo* data on the role of connexins in melanoma and the complete lack of Cx43-based studies, we utilized the chick chorioallantoic membrane (CAM) assay to investigate primary tumor growth of connexin-expressing and connexin-deficient melanoma cells. This inexpensive and efficient tumor formation assay has been used by many as a model to study primary melanoma tumor growth and metastasis (Arpaia et al., 2012; Chambers et al., 1982; Penuela et al., 2012; Stoletov et al., 2013; Zijlstra et al., 2002). The immunosuppressed chicken embryo acts as a host for foreign tumor cell engraftment, and its extensive vasculature network provides tumor cells with *in vivo* growth factors as well as offering a pathway for metastasis to developing organs (Karnofsky et al., 1952; Knighton et al., 1977; Leighton, 1964; Scher et al., 1976).

1.13 Hypothesis

We hypothesized that ectopic expression of Cx26 and Cx43 in connexin-deficient melanoma cells would increase gap junctional intercellular communication (GJIC), thereby decreasing their tumorigenic properties. To address this hypothesis, we used replication- defective retroviruses to stably express GFP-tagged Cx26 and Cx43 in an aggressive, metastatic BL6 mouse melanoma cell line that exhibited compromised GJIC. Using these connexin-expressing melanoma cell lines, we assessed GJIC using a microinjection dye transfer assay. Furthermore, we assessed for several *in vitro* tumorigenic behaviors including cell proliferation, migration and anchorage-independent growth. Using connexin-rich rat epidermal keratinocytes, we developed a co-culture model to assess for heterocellular GJIC between melanoma cells and keratinocytes and evaluated melanoma cell growth within this *in situ*-like microenvironment. Finally, we measured tumorigenesis *in vivo* using the chick CAM assay.

1.14 Objectives

1.) Examine the effect of ectopic Cx26 and Cx43 expression in melanoma cells on GJIC *in vitro.*

2.) Assess the tumorigenic behavior of Cx26- and Cx43-expressing melanoma cells *in vitro* and within the *in situ*-like microenvironment of keratinocytes.

3.) Assess the tumorigenicity of Cx26- and Cx43-expressing melanoma cells within the *in vivo* chicken embryo model.

1.15 References

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Chapter 2

2 Connexin43 Reduces Melanoma Growth in a Keratinocyte Microenvironment and During Tumorigenesis *in vivo*

Connexins have been identified as tumor suppressors and enhancers, a distinction that appears to be dependent on the type and stage of disease. However, the role of connexins in melanoma tumorigenesis and their status during cancer onset and progression remains controversial and unclear. Here, we show that the aggressive B16-BL6 mouse melanoma cell line expresses low basal levels of Cx26 and Cx43 rendering them gap junctional intercellular communication (GJIC) deficient as elucidated by immunofluorescence, Western blotting and dye transfer studies. Following ectopic expression of green fluorescent protein (GFP)-tagged Cx26 and Cx43 in these connexin-deficient melanoma cells, punctate gap junction-like plaques were evident at sites of cell-cell apposition and the incidence of dye transfer was significantly increased similar to connexin-rich keratinocytes. We found that the expression of Cx43, but not Cx26, significantly reduced cellular proliferation and anchorage-independent growth from control melanoma cells, whereas migration was unaffected. Additionally, melanoma cells expressing Cx43 displayed significantly reduced growth within the *in situ*-like microenvironment of keratinocytes, despite a complete lack of heterocellular GJIC between the two cell types. Furthermore, when grown *in vivo* in the chicken embryo, primary tumors that formed in the chorioallantoic membrane derived from Cx43-expressing melanoma cells were significantly smaller than controls, whereas Cx26-expressing melanoma cells produced tumors similar to controls. Collectively, these results suggest that Cx43, and not Cx26, can act as a tumor suppressor during melanoma tumorigenesis.

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2.1 Introduction

Connexins (Cx) are a family of integral membrane proteins that can oligomerize into hexamers, known as connexons, which dock with connexons from apposing cells forming gap junction channels (Sohl and Willecke, 2003). The gap junction-mediated exchange of small molecules (< 1 kDa) between cells is known as gap junctional intercellular communication (GJIC), and this process is critical for the maintenance of cellular homeostasis and regulation of controlled cellular events including proliferation, differentiation and apoptosis (Sohl and Willecke, 2003). Although it is commonly accepted that the primary function of connexins is to assemble into gap junctions to allow for GJIC, connexons also possess the ability to act as single-membrane hemichannels allowing for the exchange of signaling molecules with the extracellular environment (Wang et al., 2013). The human genome encodes 21 distinct connexins, which are expressed in some combination in virtually all cell types of the body (Sohl and Willecke, 2003). Interestingly, as many as 10 connexins, including Cx26 and Cx43, are expressed in human epidermis with a similar subset expressed in rodent epidermis (Richard, 2000). Primarily, these connexins are expressed by keratinocytes and are differentially regulated within the different epidermal strata (Richard, 2000). Additionally, melanocytes, which reside in the basal layer of the epidermis, have been reported to only express Cx26 and Cx43 (Hsu et al., 2000a; Masuda et al., 2001). Typically, one melanocyte establishes contacts with approximately 30 keratinocytes within the basal and suprabasal layers forming the 'epidermal-melanin unit' (Haass and Herlyn, 2005) and the connexin compatibilities of the two cell types render them capable of heterocellular GJIC (Hsu et al., 2000a). It is commonly believed that melanocyte growth is controlled by keratinocytes through GJIC (Haass and Herlyn, 2005) as well as the exchange of other regulatory signals (Seiberg et al., 2000; Shih et al., 1994).

Gap junctions were first implicated as being important in tumorigenesis nearly 50 years ago (Loewenstein and Kanno, 1966). Since then, many investigators have reported that connexins are down-regulated or even absent in cancer cell lines resulting in compromised GJIC (Cronier et al., 2009; Krutovskikh et al., 1994; Loewenstein and Kanno, 1966; Mesnil et al., 2005; Yamaoka et al., 1995), and also observed reductions in

tumorigenic behaviors when connexins were ectopically re-introduced into tumor cells (Eghbali et al., 1991; Hirschi et al., 1996; Loewenstein and Rose, 1992; McLachlan et al., 2006; Zhu et al., 1991). These findings, combined with the observations that Cx32-knockout mice are more prone to developing chemical- and radiation-induced tumors (King and Lampe, 2004a; Temme et al., 1997), support the notion that connexins have tumor suppressor properties. However, more recently, connexins have been shown to facilitate tumor progression and metastasis in late-stage disease leading to their reclassification as conditional tumor suppressors (Naus and Laird, 2010). Given this paradox, it is not surprising to find that the role of connexins during the onset and progression of melanoma tumorigenesis is controversial and remains poorly understood. Although the majority of research suggests that melanomas exhibit poor GJIC due to connexin deficiencies (Bagnato et al., 2004; Hsu et al., 2000a; Ito et al., 2000; Su et al., 2000), some studies suggest that connexins may be facilitators of late stage disease (Ito et al., 2000; Saito-Katsuragi et al., 2007; Stoletov et al., 2013), alluding to a potential biphasic role of connexins during melanoma tumorigenesis.

When studying tumorigenesis, it is important to consider the *in situ* tumor cell microenvironment in order to account for contact-dependent and diffusible factors that may influence tumor growth and progression. In particular, when studying the role of connexins in tumorigenesis, the *in situ* microenvironment takes on further importance as direct cell-to-cell contact is needed to facilitate GJIC. In the case of melanoma, the loss of the ability to form heterocellular contacts and exhibit GJIC with keratinocytes may be a contributor to melanoma growth within the epidermis (Hsu et al., 2000a; Hsu et al., 2000b) whereas GJIC between melanoma cells and fibroblasts or endothelial cells may enhance tumor progression and metastasis (Ito et al., 2000; Saito-Katsuragi et al., 2007; Stoletov et al., 2013). Thus, it is imperative to study connexins during melanoma tumorigenesis in a multidimensional context that encompasses *in situ* and *in vivo* environments to better understand how tumor cells behave during cancer progression while also in the setting of normal cell types.

Using this approach, we sought to further assess the role of connexins and GJIC during melanoma tumorigenesis in a unifying model mindful of the melanoma

microenvironment. We found that the metastatic B16-BL6 mouse melanoma cell line (BL6) expressed only low basal levels of Cx26 and Cx43 rendering them GJIC-deficient. Following ectopic expression of both connexins, we established GJIC amongst melanoma cells, but this failed to restore GJIC with keratinocytes. The expression of Cx43 in melanoma cells significantly reduced proliferation and anchorage-independent growth *in vitro*, reduced growth within the *in situ*-like microenvironment of keratinocytes and reduced primary tumor size within the *in vivo* microenvironment of the chicken embryo. Conversely, the expression of Cx26 did not affect the aggressive behavior and tumorigenic properties of melanoma cells. Taken together, these studies strongly suggest that Cx43, but not Cx26, is capable of suppressing melanoma tumorigenesis.

2.2 Materials and Methods

2.2.1 Cell culture

The murine melanoma cell line B16-BL6 (kindly provided by Dr. Moulay Alaoui-Jamali, McGill University) was cultured in Minimal Eagle's Medium (MEM) containing 2 mM L-glutamate, 10% FBS, 100 units/mL penicillin, 100 µg/mL streptomycin, 0.1 mM MEM non-essential amino acids, MEM vitamin solution and 1 mM MEM sodium pyruvate (Invitrogen, Carlsbad, CA). Rat epidermal keratinocytes (REK, kindly provided by Dr. Vincent Hascall, Cleveland Clinic Foundation) were cultured in Dulbecco's Modified Eagle's Medium with 4.5 g/L glucose 1X (DMEM) supplemented with 2mM L-glutamate, 10% FBS, 100 units/mL penicillin and 100 µg/mL streptomycin (Invitrogen). All cells were incubated at 37°C and 5.0% CO_2.

2.2.2 Generation of connexin-expressing melanoma cell lines

BL6 cells were stably infected with an empty AP2 replication-defective vector or vectors encoding Cx43 or Cx26 fused to monomeric GFP (Cx43-GFP or Cx26-GFP, respectively) as previously described (Thomas et al., 2005). Due to the heterogeneous nature of the retroviral infection, cells were grown into isolated colonies in order to select for cells which displayed strong Cx26-GFP and Cx43-GFP expression with characteristic cell surface localization. Briefly, newly engineered Cx26-GFP and Cx43-GFP expressing cells were plated at very low density in 10 cm dishes and allowed to grow into

isolated colonies for two weeks. Using an inverted epifluorescence microscope (Leica Microsystems, Wetzlar, Germany), isolated colonies enriched in GFP expression were identified and subsequently picked and allowed to expand in 12-well plates. Of the isolated colonies selected, the Cx26- and Cx43-expressing colonies that were most enriched in GFP expression were determined and used for the remainder of the experiments.

2.2.3 Immunocytochemistry, confocal microscopy and western blotting

Control and connexin over-expressing melanoma cells were cultured on coverslips, grown to confluence and fixed in 10% formalin. Fixed cells were blocked and permeabilized with 3% bovine serum albumin (BSA)/0.1% Triton X-100 in phosphate buffered saline (PBS) for 1 h and immunolabeled with rabbit anti-Cx26 (1:200 dilution, Invitrogen 51-2800), rabbit anti-Cx43 (1:500 dilution, Sigma) and/or mouse anti-N-cadherin (1:200 dilution, BD Transduction Laboratories, Mississauga, ON) antibodies for 1 h at room temperature, followed by incubation with Alexa Fluor® 488-conjugated anti-rabbit (1:500 dilution, Molecular Probes, Eugene, ORz) or Alexa Fluor® 555-conjugated anti-mouse (1:1000 dilution, Molecular Probes) secondary antibodies. Nuclei were stained with Hoechst 33342 dye prior to mounting on glass slides with Airvol. Immunolabeled coverslips were imaged using a Zeiss LSM 510 inverted confocal microscope (Carl Zeiss AG, Oberkochen, Germany) using a 63X objective lens to determine connexin localization.

In the case of western blotting, protein lysates were collected from confluent monolayers (or over-confluent cultures of REKs to stimulate keratinocyte differentiation leading to the expression of Cx26) in a lysis buffer [1% Triton X-100, 150mM sodium chloride (NaCl), 10mM tris(hydroxymethyl)aminomethane (Tris)/hydrogen chloride (HCl) (pH 7.4), 1mM ethylenediaminetetraacetic acid (EDTA), 1mM ethylene glycol tetraacetic acid (EGTA) and 0.5% Nonidet P40, supplemented with protease inhibitor mixture (Roche Applied Sciences, Penzberg, Germany) and phosphatase inhibitors (1 mM sodium fluoride and 1 mM sodium orthovanadate)]. Protein concentrations were determined using a bicinchoninic acid (BCA) protein determination kit (Pierce, Rockford, IL). Total

protein lysates (35 or 50 µg) were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS/PAGE) and transferred onto nitrocellulose membranes using the iBlot Dry Blotting System (Invitrogen). Membranes were blocked using 5% Blotto, Nonfat Dry Milk (Santa Cruz Biotechnology, Dallas, TX) for 1 h before being immunolabeled with rabbit anti-Cx26 (1:1000 dilution, Invitrogen 71-0500), rabbit anti-Cx43 (1:5000 dilution, Sigma-Aldrich, St. Louis, MO), mouse anti-GFP (1:7500 dilution, Millipore) or mouse anti-β-tubulin (1:7500 dilution, Sigma) antibodies at 4°C. Primary antibodies were detected using the fluorescently conjugated anti-rabbit Alexa Fluor® 680 (1:10,000 dilution, LI-COR Biosciences, Lincoln, NB) or anti-mouse IRdye 800 (1:10,000 dilution, LI-COR Biosciences) antibodies and scanned using the Odyssey Infrared Imaging System (LI-COR Biosciences).

2.2.4 Microinjection and dye transfer

Cells grown to confluence were microinjected with 10mM Alexa Fluor® 350 hydrazide (Molecular Probes), which is known to permeate through gap junctions (Harris, 2007), using an Eppendorf Femojet automated pressure microinjector. Images were collected using a Leica DM IRE2 inverted epifluorescence microscope using a 20X objective lens equipped with Openlab 5.5.3 imaging software. The percentage of cells that passed dye to at least one neighboring cell was recorded. For each biological replicate, 15 injections were performed on each cell line and statistical analysis was performed on the average of three biological replicates.

2.2.5 Growth curves and proliferation

Cells were plated into individual wells of a 6-well plate at a density of 1 x 10^4 cells/well. On days 2, 4, 5 and 6, cells were lifted from the plate with 500 µL of 0.25% trypsin-EDTA (Invitrogen) and counted using a Countess Automated Cell Counter (Invitrogen) or supplemented with fresh media. Statistical analysis was performed on the average of two technical replicates of four biological replicates. In parallel, cells were plated on coverslips at a density of 1 x 10^4 cells/well. On day 4, cells were incubated with fresh media containing 1 µg/mL 5-ethynyl-2′-deoxyuridine (EdU) for 3 h and subsequently fixed in 3.7% formalin for 15 minutes at room temperature. Cells were labeled using the

Click-It® EdU Alexa Fluor® 488 Cell Proliferation Kit (Molecular Probes) according to the manufactures instructions. The fluorescence of GFP within the engineered cells was quenched due to the intensity of the fluorescence from the conjugated Alexa Fluor® 488 azide. As such, in order to visualize the presence of GFP, cells were countered-stained with a mouse anti-GFP (1:500 dilution, Millipore) antibody followed by an Alexa Fluor® 555-conjugated anti-mouse (1:1000 dilution, Molecular Probes) antibody. Images were collected using a Leica DM IRE2 inverted epifluorescence microscope equipped with Openlab 5.5.3 imaging software. For each biological replicate, 10 blinded, randomized images were taken using the 63X objective lens and the percentage of EdU-positive cells compared to total nuclei were counted using ImageJ 1.46r (NIH, Bethesda, MD). Statistical analysis was performed on the average of four biological replicates.

2.2.6 Anchorage-independent growth

BL6 cells were plated at a density of 1 x 10^4 cells/well in 0.3% soft agar suspension atop a polymerized 0.4% soft agar layer. For each biological replicate, BL6 cells were plated in six wells for each treatment. Cultures were grown for 10 days under normal conditions. Images were collected using a Leica DM IRE2 inverted epifluorescence microscope and Openlab 5.5.3 imaging software. For each well, 10 randomized images were taken with a 5X objective lens. Colonies that were greater than 50 μm in diameter were counted and the average number of colonies/mm^2 was determined. Statistical analysis was performed on the average of four biological replicates.

2.2.7 Migration scratch assay

Control and engineered BL6 cells were plated on etched grid-plates and grown to confluence prior to treating with 25 μg/mL Mitomycin C (Sigma), a mitotic inhibitor, for 30 min in normal media. Cells were scraped along a straight line down the midline of the grid-plate with a rubber cell scraper, clearing half the plate of cells. Cells were then supplemented with serum-free MEM. Five intersecting grid-points were marked along the scrape edge as reference points. Images were collected using a Leica DM IRE2 inverted epifluorescence microscope equipped with Openlab 5.5.3 imaging software. Images were taken at each reference point (0, 24 and 48 hours) using a 5X objective lens.

For each reference point, the distance from the scrape edge (0 h) to the leading migratory edge (24 and 48 hrs) was measured using ImageJ 1.46r (NIH). Statistical analysis was performed on the average of four biological replicates.

2.2.8 Co-culture melanoma growth

REKs were plated at a density of 5 x 10^4 cells/well and co-cultured with BL6 cells at a density of 1 x 10^4 cell/well. Mixed cell cultures were supplemented with DMEM and MEM at a 4:1 ratio, respectively, and were grown for 6 days to confluence. Clusters were identified under phase contrast due to their morphological differences, and their melanoma phenotype was confirmed by the presence of GFP-tagged connexins in engineered cells. For each biological replicate, 10 blinded, randomized images were taken using a 5X lens. Under phase contrast, melanoma clusters were outlined using ImageJ 1.46r (NIH) and the total area of all melanoma clusters for a given field was determined. Statistical analysis was performed on the average area occupied by BL6 cells of four biological replicates.

2.2.9 Co-culture microinjections and dye transfer

REKs were co-cultured with BL6 cells at a 10:1 ratio, respectively. Mixed cell cultures were supplemented with DMEM and MEM at a 4:1 ratio, respectively, and allowed to grow to confluence. Melanoma clusters were identified under phase contrast due to their morphological differences, which was confirmed by the presence of GFP-tagged connexins in engineered cells. BL6 cells or REKs at the melanoma-keratinocyte boundary were microinjected with 10nM Alexa Fluor® 350 hydrazide (Molecular Probes) using an Eppendorf Femojet automated pressure microinjector. Images were collected using a Leica DM IRE2 inverted epifluorescence microscope using a 20X objective lens equipped with Openlab 5.5.3 imaging software. The percentage of injected BL6 cells that passed dye to at least one neighboring BL6 or REK was recorded. Similarly, the percentage of injected REKs that passed dye to at least one neighboring REK or BL6 was recorded. For each biological replicate, at least 6 REKs and 6 BL6 cells were injected. Statistical analysis was not performed due to a complete lack of heterocellular dye transfer between different cell types over three biological replicates.

2.2.10 *In vivo* chick chorioallantoic membrane (CAM) assay

Primary tumor properties of BL6 cells were assessed in the chicken embryo as previously described (Penuela et al., 2012). Briefly, fertilized chicken eggs (McKinley Hatchery, St. Mary's, ON) were incubated in an automated rotary incubator (GQF Manufacturing Company, Savannah, GA) under 40% humidity at 37°C for 3 days, rotating once every hour. Eggs were removed from their shell and placed in covered dishes. Chicken embryos were incubated for an additional 7 days in stationary incubators (GQF Manufacturing Company) under 60% humidity at 37°C. On day 11 of development, 3.0 x 10^5 control or connexin-expressing BL6 cells in 20 µL of serum-free MEM were topically applied to a site of major branching vessels on the CAM distal to the embryo and covered with a coverslip. The embryos were returned to a humidified 37°C stationary incubator for 7 days to allow tumor growth. Primary melanoma tumors that formed at the site of topical application were excised and weighed. Statistical analysis was performed on the primary tumor weights of 18 control, 15 Cx43-GFP, and 12 Cx26-GFP expressing melanoma tumors.

2.2.11 Statistics

Statistical analysis was performed on all data using a one-way ANOVA followed by a Tukey's post-hoc test in which $p < 0.05$ was considered significant. Values are presented as means ± S.E.M. All statistics were performed using GraphPad Prism Version 4.03 for Windows.

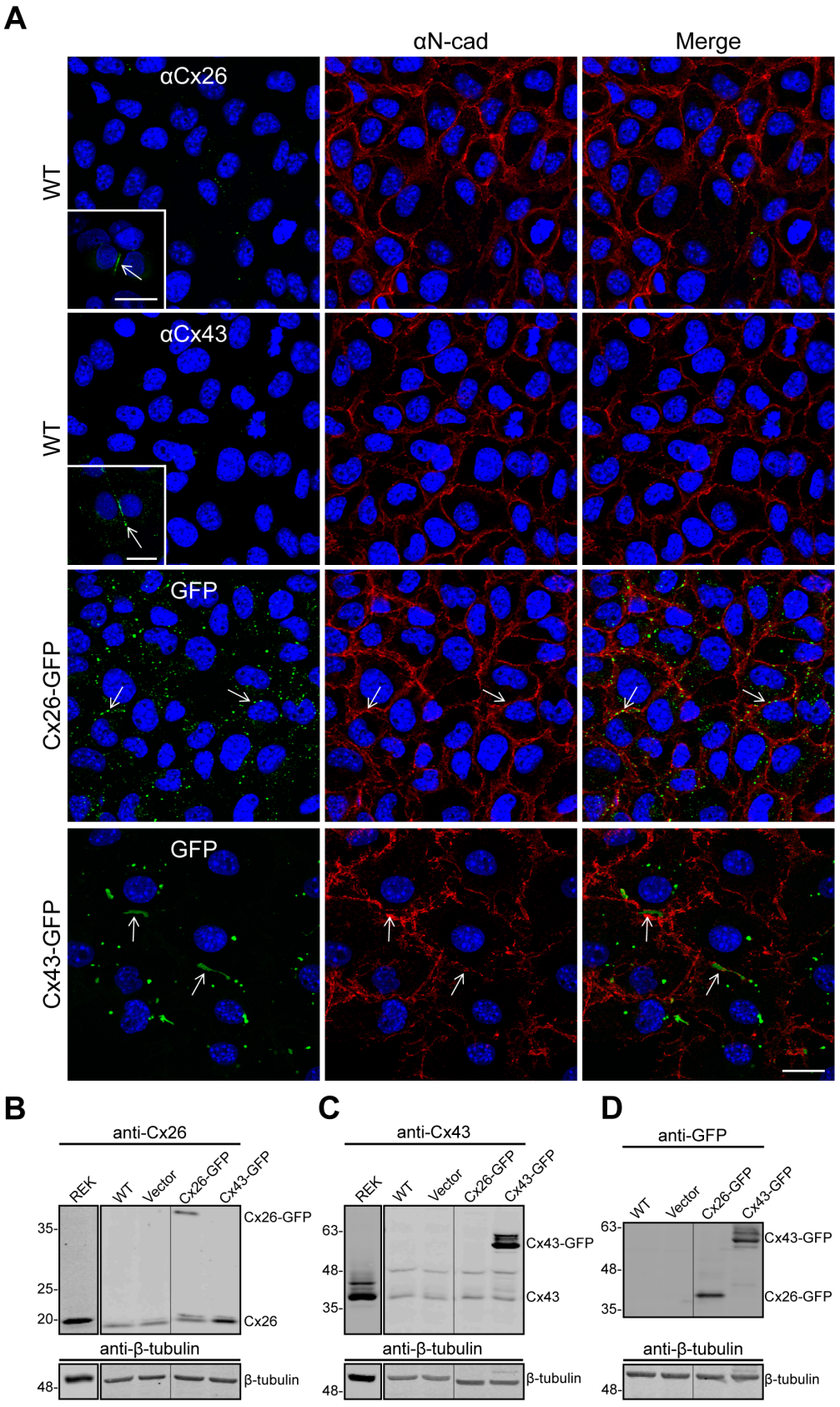
2.3 Results

2.3.1 Expression of Cx26 and Cx43 increases gap junction plaque formation and GJIC in mouse melanoma cells

The mouse melanoma cell line B16-BL6 expressed low levels of both Cx26 and Cx43 as indicated by immunofluorescence (Fig. 2.1A) and western blot analysis (Fig. 2.1B,C). Cx26 was observed only in over-confluent REKs while Cx43 was readily detectable in these cells (Fig. 2.1A, inserts). Following ectopic expression of Cx26-GFP and Cx43-GFP, BL6 cells exhibited punctate gap junction-like structures that localized to the cell surface as indicated by their co-localization with the cell adhesion molecule N-cadherin

Figure 2.1 BL6 mouse melanoma cells express low levels of Cx26 and Cx43.

Immunofluorescence revealed that wild-type (WT) BL6 cells endogenously express low levels of Cx26 and Cx43 (A) compared to REKs which display typical gap junctional plaques at sites of cell-cell apposition (A, inserts). Western blots confirmed low levels of Cx26 (B) and Cx43 (C) in melanoma cells compared to lysates collected from over-confluent and confluent REK cultures, respectively. Following ectopic expression of Cx26-GFP or Cx43-GFP, punctate gap junction-like plaques were evident at the cell surface as denoted by the cell adhesion molecule N-cadherin (red) (A, arrows; blue, nuclei) and the expression of GFP-tagged connexins were readily detected by western blots immunolabeled for Cx26 (B) or Cx43 (C). Additionally, both fusion proteins were expressed at similar levels as detected by western blots immunolabeled for GFP (D). β-tubulin was used as loading control. The vector control represents cells transfected with a construct that did not encode connexins. Lanes separated by vertical lines were run on the same blot but spliced together. Lanes loaded with REK lysates were run as independent experiments. Scale bars = 20 μm.



(Fig. 2.1A, arrows). Western blots revealed low levels of Cx26 and Cx43 in BL6 cells compared to REKs, while both Cx26-GFP and Cx43-GFP were readily detected following their ectopic expression (Fig. 2.1B,C). Additionally, both fusion proteins displayed similar expression levels when probed for GFP (Fig. 2.1D).

To assess the GJIC capacity of control and engineered cells, all cell lines were microinjected with the small molecular dye Alexa350 and the incidence of dye transfer to neighboring cells was measured. Wild-type and vector control BL6 cells were found to be GJIC-deficient, only passing dye from ~25% of the microinjected cells (Fig. 2.2A, arrows). Conversely, Cx26-GFP and Cx43-GFP expressing melanoma cells displayed significantly increased dye transfer resulting in 80% and 100% of the microinjected cells passing dye to neighboring cells, respectively ($p < 0.05$). The Cx26 and Cx43 engineered cells acquired a GJIC status that was statistically similar to Cx43-rich and well coupled REKs (Fig. 2.2B). Thus, our findings show that both Cx26 and Cx43 are able to establish functional GJIC in BL6 cells.

2.3.2 Cx43 expression in melanoma cells significantly reduces cell proliferation

When subjected to a 6-day growth curve analysis, Cx43-expressing BL6 cells displayed significantly reduced total cell number compared to controls as early as day 4 ($p < 0.05$), which was further amplified on days 5 and 6 (2.5-fold; $p < 0.001$). Conversely, Cx26 did not impart as profound an effect on total cell number but there was a slight reduction of ~20% in cell number on day 6 ($p < 0.05$) (Fig. 2.3A).

To determine if the effect of Cx43 on total cell numbers was due to changes in cell proliferation, we used an EdU labeling assay at day 4 to determine the number of cells actively cycling through the synthesis phase (S-phase) of the cell cycle. We found that the expression of Cx43 in BL6 cells significantly reduced the percentage of EdU+ cells by ~25% from controls ($p < 0.01$) (Fig. 2.3B,C). Consistent with our growth curve analysis, the expression of Cx26 did not significantly reduce the percentage of EdU+ cells (Fig. 2.3C). Collectively, Cx43 was found to reduce total cell numbers by reducing the rate at which cells pass through S-phase of the cell cycle.

Figure 2.2 Ectopic connexin expression significantly increases GJIC.

Alexa350 dye transfer studies revealed that wild-type and control melanoma cells were poorly coupled, as dye rarely spread from the microinjected cell (arrows) to neighboring cells (A). Following ectopic expression of Cx26-GFP or Cx43-GFP, the incidence of dye transfer was significantly increased to 80% and 100%, respectively, statistically similar to Cx43-rich REK controls ($N = 3$, $p < 0.05$) (B). Phase contrast images depict cellular morphology prior to microinjection while the GFP fluorescence denotes the expression of ectopic connexins. Letters depict statistical significance among groups. Scale bars = 40 μm.

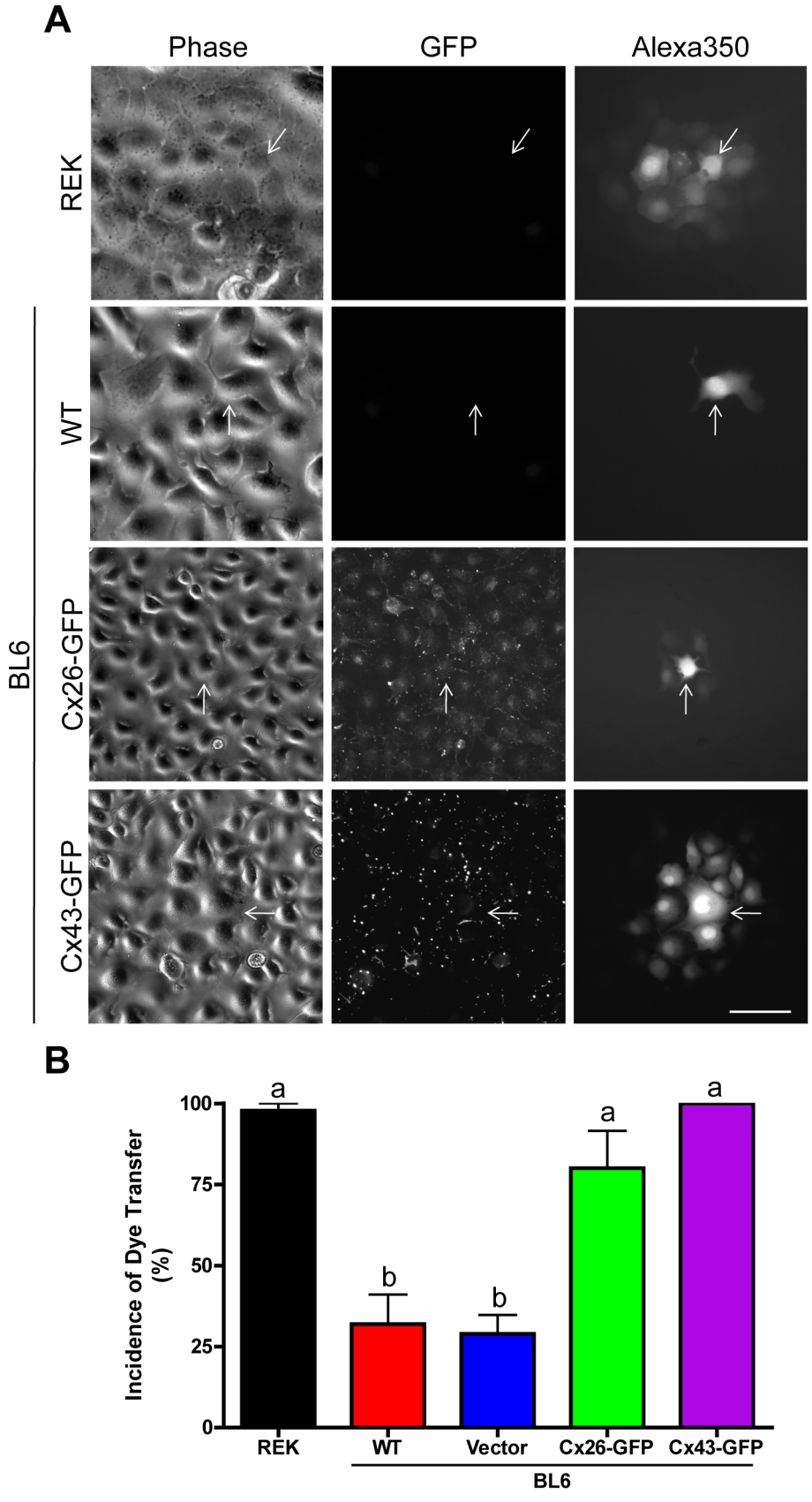
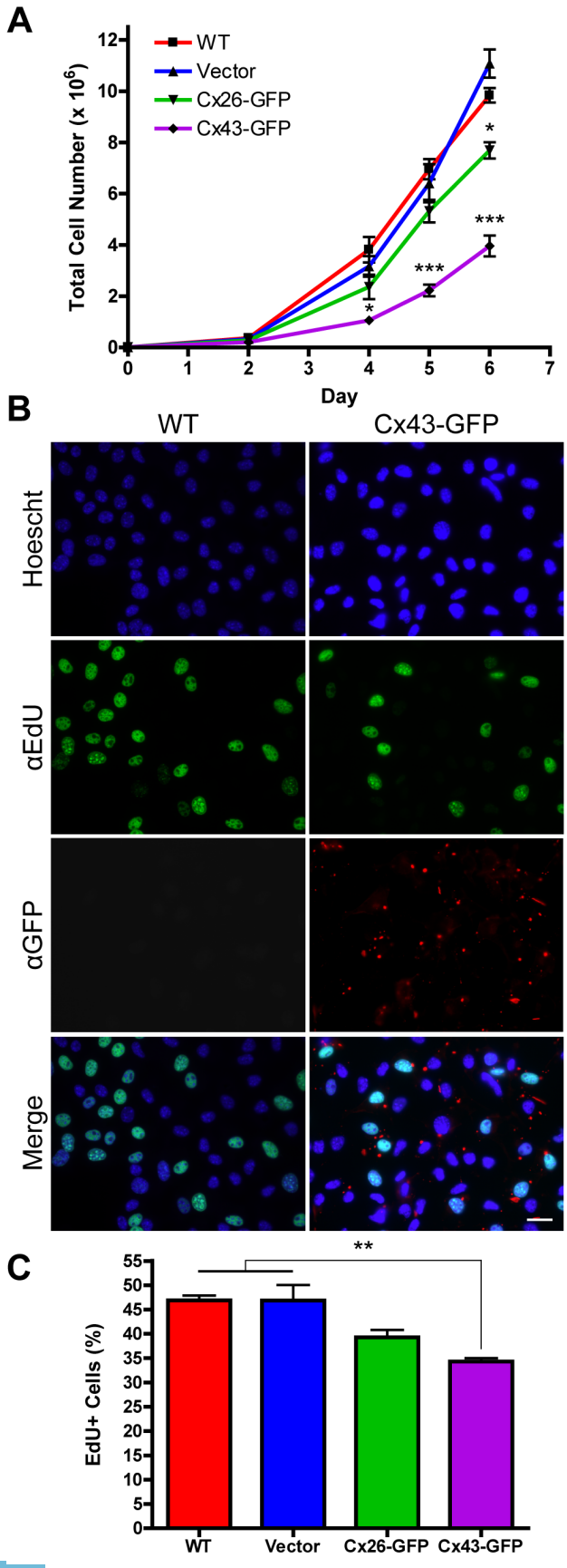


Figure 2.3 Expression of Cx43 significantly reduces total cell number and cell proliferation.

Six day growth curves revealed that Cx43-expressing melanoma cells displayed significantly reduced total cell number as early as day 4 ($N = 4$, *, $p < 0.05$), which was further reduced on days 5 and 6 ($N = 4$, ***, $p < 0.001$). Cx26-expressing melanoma cells displayed a slight reduction in total cell number only on day 6 ($N = 4$, *, $p < 0.05$) (A). Proliferation at day 4 was assessed using an EdU assay, which labels nuclei passing through S-phase of the cell cycle, where the proportion of EdU labeled nuclei (green) to total nuclei labeled with Hoechst (blue) was determined (B). Cells were counter-stained with an anti-GFP antibody (red) as the normal fluorescence of GFP-tagged Cx43 was quenched by the EdU signal. Expression of Cx43 significantly reduced the percentage of EdU-positive cells by ~25% in comparison to the controls ($N = 4$, **, $p < 0.01$) (C). Scale bars = 20 μm.



2.3.3 Cx43-expressing melanoma cells display a reduced malignant phenotype

When grown in soft agar suspension to assess for anchorage-independent growth, the amount of colonies formed from Cx43-expressing melanoma cells was significantly reduced by ~4.5-fold from controls ($p < 0.001$). Conversely, the expression of Cx26 in BL6 cells did not significantly reduce anchorage-independent growth (Fig. 2.4A and B). Using a scratch assay, the migratory distance from the wound edge at 0 h (Fig. 2.4C, left panel, red line) to the leading edge at 48 h (Fig. 2.4C, right panel, red line) was not statistically different between control and Cx-expressing melanoma cells (Fig. 2.4D).

2.3.4 Cx43 expression in melanoma cells reduces growth in an *in situ*-like microenvironment independent of heterocellular GJIC with keratinocytes

BL6 cells were co-cultured at a low density with REKs and allowed to grow to confluence over 6 days. Identifiable clusters were observed under phase contrast (Fig. 2.5A, left column, dashed lines) and their melanoma phenotype was confirmed due to the expression of GFP-tagged connexins within the outlined areas in engineered cells. Wild-type and control melanoma cells were found to occupy approximately 14% of the 2-dimensional field of view. Melanoma cells expressing Cx26 did not grow as robustly as control melanoma cells in the context of the keratinocytes and were found to occupy slightly reduced area, although this was not statistically significant. However, the expression of Cx43 significantly reduced the total area occupied by melanoma cell clusters by ~4.5-fold compared to controls ($p < 0.05$) (Fig. 2.5A and B).

Using this co-culture model, we assessed for heterocellular GJIC between melanoma cells and keratinocytes by microinjecting both cell types at the melanoma-keratinocyte interface. Again, when BL6 cells were co-cultured with Cx-rich REKs, identifiable melanoma cell clusters were observed under phase contrast (Fig. 2.6, left column, dashed lines). As expected, connexin-deficient melanoma cells were poorly coupled to each other and did not transfer Alexa350 dye to the Cx43-rich keratinocytes. While connexin-expressing melanoma cells displayed high incidences of dye transfer with neighboring melanoma cells, in all cases, they failed to pass dye to adjacent keratinocytes. Similarly,

Figure 2.4 Cx43 expression significantly reduces anchorage-independent growth while cell migration remains unaffected.

When grown in soft agar suspension for 10 days, colonies were evident under phase contrast and those formed from Cx-expressing melanoma cells were GFP-positive (A). The number of colonies formed was significantly reduced in melanoma cells expressing Cx43 by ~4.5-fold in comparison to controls (N = 4, ***, $p < 0.001$), whereas Cx26 expression did not significantly alter the number of formed colonies (N = 4, p = ns) (B). Using a migration scratch assay, the migratory ability of Cx-deficient and Cx-expressing melanoma cells was assessed. Cells were grown to confluence, pulsed with 25 μg/mL of Mitomycin C for 30 minutes, scratched (C, red line at 0 hrs) and supplemented with serum-free media. The distance migrated from the initial scratch edge to the leading edge after 24 and 48 hours (C, red line at 48 hrs) was not statistically different between Cx-deficient and Cx-expressing melanoma cells (N = 4, p = ns) (D). Scale bars = 200 μm.

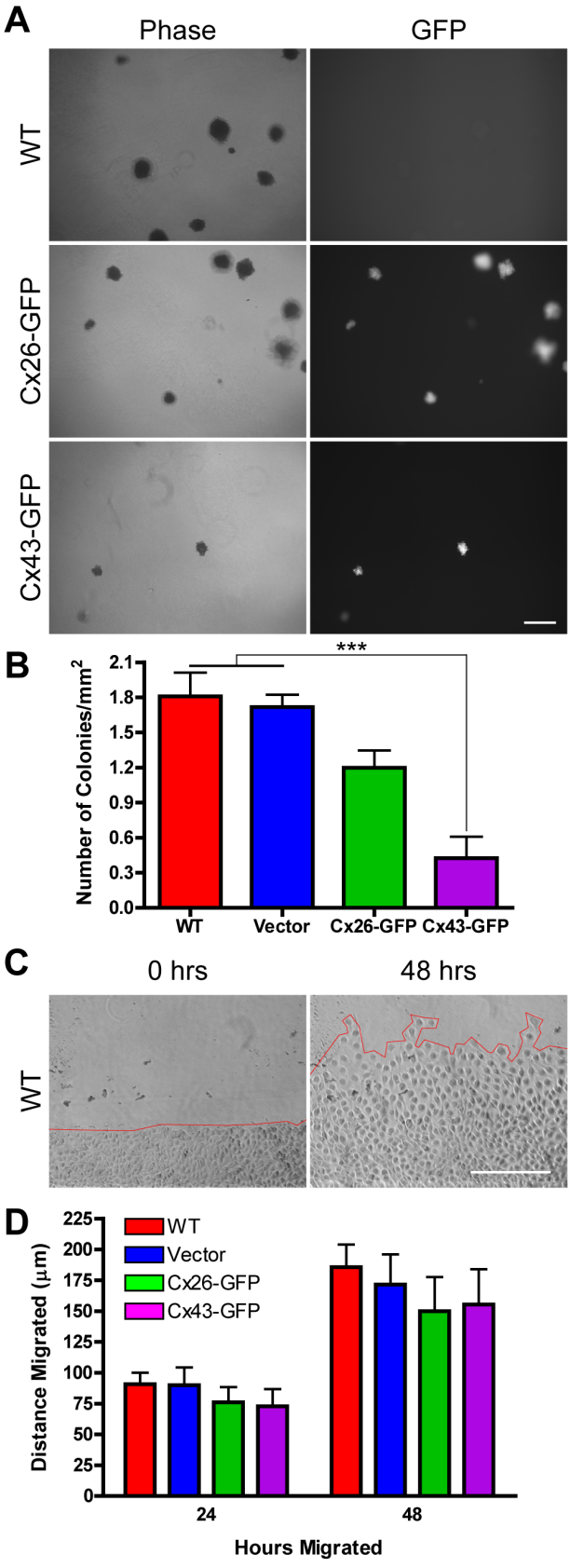
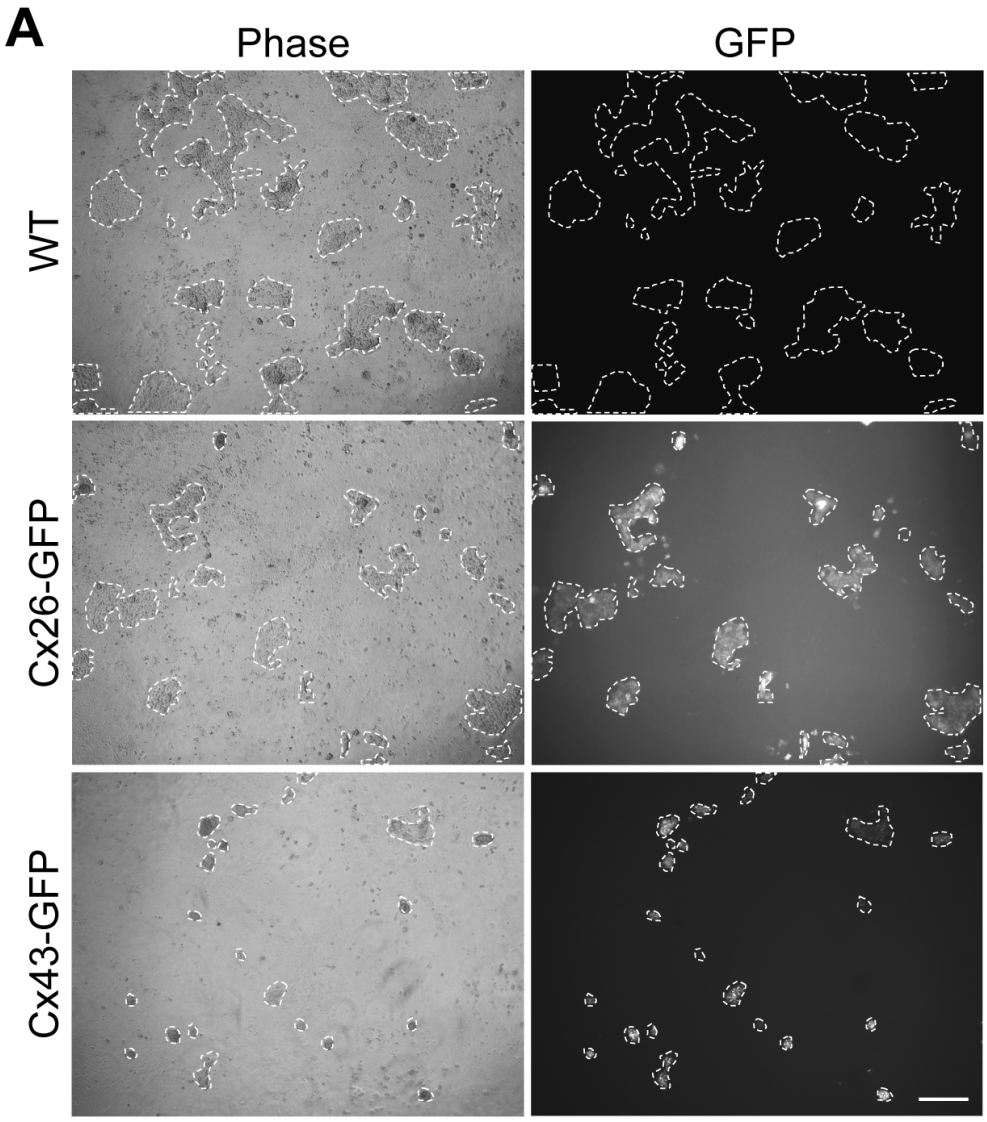


Figure 2.5 Expression of Cx43 significantly reduces melanoma cell growth within a keratinocyte-based microenvironment.

Melanoma cells were co-cultured with keratinocytes at a 1:5 ratio and allowed to grow for 6 days. Under phase-contrast imaging, melanoma cell clusters were identifiable (dashed lines) and their melanoma phenotype was confirmed in engineered cell lines due to the expression of GFP (A). The area of all melanoma cell clusters for a given field was measured and Cx43-expressing melanoma cells were found to occupy ~4.5-fold less area than controls ($N = 4$, *, $p < 0.05$) (B). Scale bars = 200 μm.



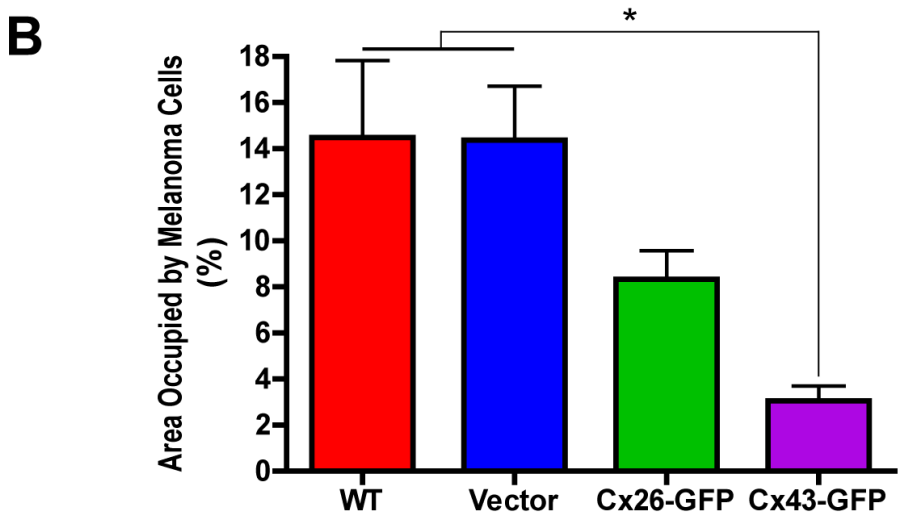
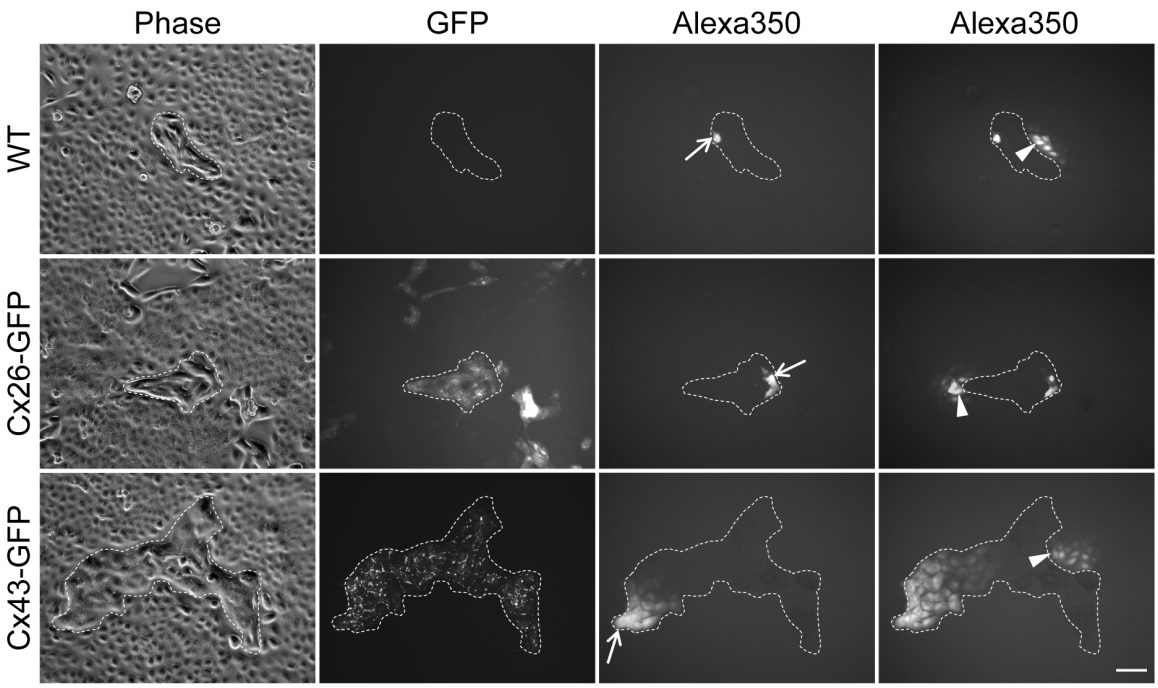


Figure 2.6 Ectopic connexin expression in melanoma cells does not affect heterocellular GJIC with keratinocytes.

Melanoma cells were co-cultured with keratinocytes at a 1:10 ratio, respectively. Under phase contrast, melanoma cell clusters were identifiable (dashed lines) and their melanoma phenotype was confirmed, where possible, by the expression of GFP. In all cases, injected melanoma cells (arrows) failed to pass Alexa350 to adjacent keratinocytes, and only Cx-expressing melanoma cells exhibited sufficient dye transfer amongst each other (N = 3). Similarly, keratinocytes injected with Alexa350 (arrowheads) were highly coupled with neighboring keratinocytes, but in all cases, failed to pass dye to adjacent melanoma cell (N = 3). Scale bars = 50 μm.



when keratinocytes were injected at the melanoma-keratinocyte border, REKs were highly coupled with neighboring REKs, but never transferred dye to adjacent melanoma cells, regardless of connexin status (Fig. 2.6).

2.3.5 Cx43-expressing melanoma cells display reduced primary tumor size *in vivo*

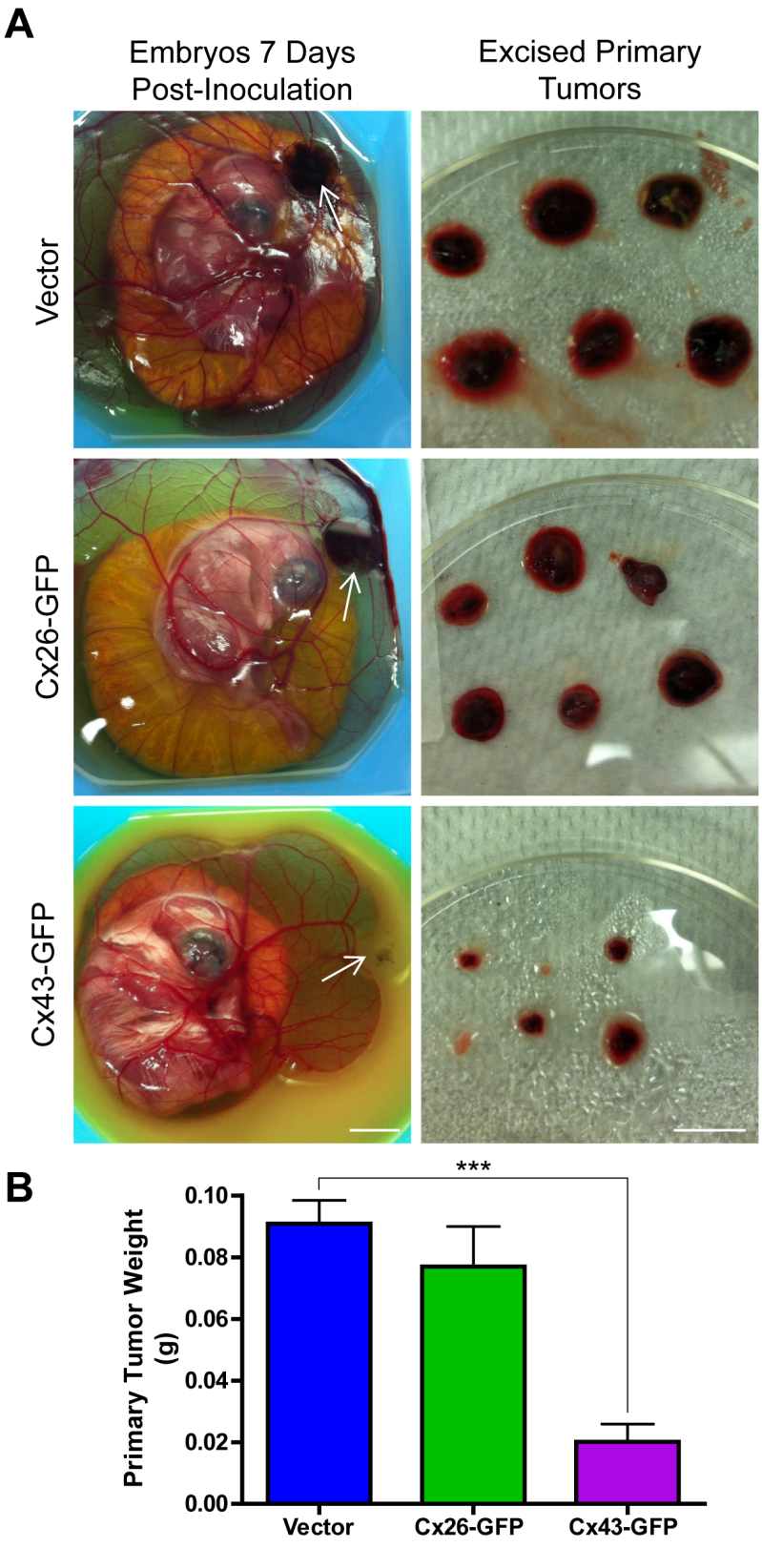
Using the *in vivo* chicken embryo model, control or connexin-expressing melanoma cells were topically applied to the CAM of 10-day old embryos and allowed to grow for one week. In all cases, primary tumors formed at the sight of topical application (Fig. 2.7A, arrows). Primary tumors were excised and excess CAM was removed from the tumor mass. Embryos inoculated with control melanoma cells produced large, highly vascularized tumors averaging 0.09 ± SEM g in weight. The expression of Cx26 within these tumors did not significantly reduce primary tumor weights in the chick-CAM assay. Conversely, Cx43-expressing melanomas exhibited significantly reduced primary tumor weight, producing tumors on average of 0.02 ± SEM g, which represents a reduction in tumor mass of ~4.5-fold compared to the vector control ($p < 0.001$) (Fig. 2.7A and B). These tumors appeared more diffuse, less vascularized, and occasionally less pigmented. Together, these studies suggest that Cx43 is a potent tumor suppressor in melanoma cells in the *in situ*-like microenvironment of keratinocytes as well as *in vivo*.

2.4 Discussion

The role of connexins in melanoma tumorigenesis remains poorly understood as some studies support the notion that connexins behave as tumor suppressors while other reports suggest that connexins in melanomas facilitate the spread of the disease during metastasis (Bagnato et al., 2004; Hsu et al., 2000a; Ito et al., 2000; Saito-Katsuragi et al., 2007; Stoletov et al., 2013; Su et al., 2000). While the connexin family is large, most evidence suggests that the two prominent connexins in melanocytes are Cx26 and Cx43, which enables GJIC with keratinocytes (Hsu et al., 2000a; Masuda et al., 2001). This direct cell-to-cell communication is often lost during melanoma transformation, which appears to be due to connexin down-regulation (Hsu et al., 2000a; Ito et al., 2000). Most studies pertaining to connexins in melanomas tend to focus on specific stages of tumorigenesis,

Figure 2.7 Cx43 expression significantly reduces primary tumor growth *in vivo.*

Control or Cx-expressing melanoma cells (3 x 10^5) were topically applied to the CAM of 10-day old chicken embryos. Primary melanoma tumors visible on the CAM 7 days post-inoculation (arrows) were excised, photographed and weighed (A). Control melanoma cells produced large, highly vascularized tumors of 0.09 ± SEM g in weight (N = 18). Cx26-expressing melanomas exhibited a slight reduction in primary tumor weight (0.075 ± SEM g) but this was not statistically significant (N = 12, $p > 0.05$). Conversely, melanoma cells expressing Cx43 produced significantly smaller tumors of 0.02 ± SEM g in weight (~4.5-fold reduction from controls) (N = 15, ***, $p < 0.001$) (B). Scale bars = 1 cm.



however, no previous studies have investigated the role of connexins in a unifying model system where connexin-expressing melanoma cells are evaluated *in vitro*, within the context of keratinocytes and *in vivo*. Here, we show that Cx43, but not Cx26, is an effective tumor suppressor in melanoma cells that reside in the context of keratinocytes and *in vivo*.

In the present study we chose to ectopically express GFP-tagged Cx26 and Cx43 in an aggressive, gap junction-deficient BL6 mouse melanoma cell line. Previously, we and others have shown that the GFP tag exhibited negligible changes to the function and localization of both these connexins (Laird et al., 2001; Thomas et al., 2005). As expected, both connexins readily assembled into gap junctions and established extensive functional GJIC as assessed by dye transfer assays. In our study, we assessed for the incidence of dye transfer, and not degree of dye spread, due to the intrinsic differences between the two connexins in question. Firstly, Cx26 channels are expected to be more restrictive in the passage of transjunctional molecules in comparison to Cx43 channels due to their smaller pore size (Weber et al., 2004). Additionally, it is possible that this feature may be more exacerbated by the attachment of GFP to the much smaller carboxy-terminal tail of Cx26 (Naus and Laird, 2010). Regardless, both connexins were shown to significantly increase dye transfer, similar to connexin-rich keratinocytes.

Since connexins have been defined as conditional tumor suppressors (Naus and Laird, 2010) it is possible that their effects on cell characteristics associated with tumorigenesis may vary in early and late stage disease as has been suggested to occur in melanomas (Ito et al., 2000). Many connexin family members have been reported to inhibit cell proliferation in several cancer cell types (Eghbali et al., 1991; Sato et al., 2007). To that end, ectopic expression of Cx43 has been shown to reduce cell proliferation in many distinct cancers (Huang et al., 1998; Ionta et al., 2009; Zhu et al., 1991). Here, we show that Cx43 expression in Cx-deficient melanomas reduces total cell number via delayed passage through the S-phase of the cell cycle. Likewise, the expression of Cx26 in cancer cells has also been reported to reduce cell proliferation (Kalra et al., 2006), as has the related and often co-expressed Cx30 isoform (Princen et al., 2001). However, here we found that Cx26 exhibited no significant effect on cell proliferation in melanoma

cells, suggesting tumor cell specific differences. Mechanistically, it has been suggested that Cx43 reduces proliferation in glioblastomas by inhibiting cell cycle progression through GJIC-independent mechanisms (Huang et al., 1998). These findings are consistent with later studies where Cx43 was suggested to control cell cycle progression at the first growth phase through increased expression of p27, a cyclin-dependent kinase inhibitor, as a result of the inhibition of the S-phase kinase associated protein, Skp2 (Zhang et al., 2003). Additionally, Cx43 has also been reported to decrease the expression of several cyclins and cyclin-dependent kinases in transformed kidney epithelium (Chen et al., 1995), although the requirement for channel function was not assessed.

Cx26 has been shown to reduce anchorage-independent growth in several cancer cell types (McLachlan et al., 2006; Omori and Yamasaki, 1999) as has Cx43 (Huang et al., 1998; McLachlan et al., 2006; Su et al., 2000). Importantly, Cx43 has been reported to reduce anchorage-independent growth in melanoma cells (Su et al., 2000) similar to our results, while Cx26 was not previously assessed. Interestingly, Omori and Yamasaki (1999) showed that in Henrietta Lacks cervical cancer (HeLa) cells, carboxy-terminally truncated Cx43 was capable of establishing GJIC and also reduced anchorage-independent growth. Thus, transjunctional passage of molecules is likely causing the cellular changes rather than binding of one of the many members of the interactome that have been reported to bind to the carboxy-terminus of Cx43 (Laird, 2010).

In some tumor cell types connexins have also been reported to affect migration. For example, Cx26, but not Cx43, has been shown to reduce migration in breast tumor cells (Kalra et al., 2006). Similarly, Zucker et al. (2013) reported that both wild-type Cx43 and a channel-dead Cx43 mutant did not alter migration in melanoma cells. Although Cx26 and Cx43 do not consistently play a role in melanoma cell migration, as our *in vitro* studies show, one must also consider the many factors of the *in vivo* microenvironment as connexins have been shown to be up-regulated and correlated to increased melanoma metastasis (Ito et al., 2000; Saito-Katsuragi et al., 2007; Stoletov et al., 2013). However, in addition to migration, this complex process encompasses other cellular events in which

connexins have been deemed important, including extravasation (Elzarrad et al., 2008; Pollmann et al., 2005).

In the epidermis, the growth and homeostasis of melanocytes (the precursors to melanoma) is controlled by epidermal keratinocytes which together with melanocytes, form the 'epidermal-melanin unit' (Haass and Herlyn, 2005; Hsu et al., 2000a; Seiberg et al., 2000). Heterocellular GJIC is established between keratinocytes and melanocytes for the putative passage of regulatory signals that coordinates the epidermal-melanin unit (Hsu et al., 2000a). As expected, connexin-deficient melanoma cell clusters that arise are not only isolated from each other, but also from keratinocytes as we discovered in mixed cell cultures of these two cell types. Somewhat to our surprise, connexin-expressing melanoma cells also remained functionally isolated from keratinocytes despite the ability of both cell types to express both Cx26 and Cx43. Their lack of ability to re-establish heterocellular GJIC may be due to the cadherin differences between melanoma cells and keratinocytes, expressing N-cadherin and E-cadherin, respectively, as previously reported (Hsu et al., 2000a). In support of this concept, it has been reported that melanoma cells which retained E-cadherin expression, as well as melanoma cells that were engineered to express E-cadherin, could establish GJIC with keratinocytes (Hsu et al., 2000a), producing a less malignant phenotype (Hsu et al., 2000b). In contrast to this, we show that increasing Cx43-based GJIC between melanoma cells can significantly inhibit melanoma cell growth when grown in the microenvironment of keratinocytes, despite not re-establishing heterocellular GJIC with keratinocytes. This inhibition of growth in co-culture was significantly higher than the moderate growth reduction observed in Cx43-expresing melanoma cells grown in isolation. We postulate that diffusible factors produced by keratinocytes or contact-dependent signaling acts to enhance the tumor suppression, which has been shown to control melanocyte growth and proliferation (Haass et al., 2004).

Since Cx43 reduced the tumorigenic behavior of melanoma cells *in vitro* and further in the context of keratinocytes, we sought to assess the tumorigenicity of connexin-expressing melanomas *in vivo*. The chick CAM avian embryo model has been widely used as a model for tumorigenesis *in vivo*, including the assessment of melanoma primary

tumor growth and metastasis (Penuela et al., 2012; Stoletov et al., 2013). We found that control melanoma cells produced relatively large, vascularized tumors, not unlike tumors produced by Cx26-expressing melanoma cells. Conversely, Cx43-expressing melanoma cells produced significantly smaller, more diffuse tumors, which at times appeared less vascularized (data not shown). In our *in vitro* studies, Cx43-expressing melanoma cells displayed a 2.5-fold reduction in total cell number. Comparatively, these cells produced 4.5-fold smaller tumors *in vivo*, suggesting again that the tumor suppressive effects of Cx43 are microenvironment dependent, similar to our mixed culture studies with keratinocytes. Interestingly, the epithelial cells of the CAM have been reported to express Cx43 (Girolamo et al., 2006), providing a compatible connexin microenvironment, although we have no evidence that heterocellular gap junctions form *in vivo*. Furthermore, we have shown that Cx43 can reduce angiogenesis in breast cancer in a 3-dimensional microenvironment (McLachlan et al., 2006). It is possible that these and other factors present within the *in vivo* microenvironment produce additive effects that reduce primary tumor size above the observed proliferative differences *in vitro*.

Collectively, these data show that Cx43 is a tumor suppressor in melanoma cells found within the relevant microenvironment of keratinocytes as well as *in vivo*. Additionally, we show that Cx26-based GJIC within melanoma cells does not result in tumor suppression, but rather, tumor suppression occurs due to the specific expression of Cx43 and the formation of Cx43-based gap junction channels. This concept is not surprising when we consider the differences in transjunctional molecules that are able to pass through each respective gap junction channel. Not only are Cx43 channels capable of passing molecules excluded from Cx26 channels, but they also have different relative permeabilities to the same molecules (Harris, 2007). These differences in transjunctional molecules and molecular permeability properties undoubtedly lead to differences in gap junction-mediated cellular signaling and must be considered when evaluating the channel-dependent role of connexins during tumorigenesis. Additionally, Cx43 has been reported to interact with over 30 distinct proteins, compared to approximately 5 for Cx26 (Laird, 2010). In fact, several of these Cx43 binding partners have been implicated as tumor suppressors including caveolin-1 (Langlois et al., 2010), CCN3 (Fu et al., 2004), tumor susceptibility gene 101 (TSG101) (Leithe et al., 2009) and Src (Kieken et al.,

2009). Given this, as well as the many anti-proliferative proteins that have been reported to be co-regulated with Cx43, the tumor suppressive properties of Cx43 are likely specific to the cancer type and complex, involving both GJIC-dependent and independent mechanisms. Furthermore, although we did not re-established GJIC between melanoma cells and keratinocytes in an attempt to mimic GJIC between melanocytes and keratinocytes as part of normal skin homeostasis (Haass and Herlyn, 2005), we show that increasing Cx43 expression and GJIC within melanoma cells has tumor suppressive properties that are further enhanced in the presence of keratinocytes or other *in vivo*-like environments. Thus, Cx43 may prove to be a useful target for future drug therapeutics where Cx43 expression is selectively enhanced in melanomas.

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Chapter 3

3 General Overview

3.1 Discussion, Future Studies and Conclusion

Connexins have long been implicated as tumor suppressors, but the role of connexins in melanoma tumorigenesis, and their status during cancer onset and progression remains controversial and poorly understood. Some investigators suggest that connexins are downregulated in melanoma and act as tumor suppressors (Bagnato et al., 2004; Haass et al., 2004; Hsu et al., 2000a; Su et al., 2000), whereas others propose a tumor enhancing role (Ito et al., 2000; Saito-Katsuragi et al., 2007; Stoletov et al., 2013; Villares et al., 2009). To add to this uncertainty, the identity of which connexins are involved in melanoma tumorigenesis remains debated, as some suggest a role for Cx26, whereas others implicate Cx43. It is possible that these contradictions may actually depend on the stage of disease progression, potentially mimicking the biphasic, 'conditional tumor suppressor' and tumor promoting roles of connexins as proposed in breast cancer (Naus and Laird, 2010). However, none of these studies assessed multiple connexins in a comparative approach and typically only focus on specific stages of melanoma tumorigenesis. In our study, we assessed the role of two connexins, Cx26 and Cx43, on melanoma cell behavior *in vitro*, within the relevant *in situ*-like microenvironment of keratinocytes, and *in vivo*. In all cases, we found that Cx43 acts as a potent tumor suppressor, reducing proliferation and anchorage-independent growth *in vitro*, reducing growth amongst keratinocytes and reducing tumor size within the *in vivo* chicken embryo. Conversely, Cx26 did not affect melanoma cell behavior or tumorigenesis.

Connexins and GJIC are vital for the maintenance of tissue homeostasis as they regulate important cellular processes (Goodenough et al., 1996). Aberrant connexin expression has been shown to cause abnormal proliferation, as in the case of many skin diseases linked to connexin mutations (Gerido and White, 2004). Because connexins have been linked to cellular proliferation, it is not surprising that connexins are frequently dys-regulated in proliferating tumor cells. In fact, connexins have been shown to be

downregulated in many cancer cell types rendering them GJIC-deficient (Cronier et al., 2009; Loewenstein and Kanno, 1966; Mesnil et al., 2005), and interestingly, their proliferation is often suppressed following ectopic reintroduction into tumor cells (Huang et al., 1998; Ionta et al., 2009; Zhu et al., 1991). In our study, we showed that Cx43 reduced proliferation of melanoma cells by reducing the rate of passage through the synthesis phase of the cell cycle, whereas Cx26-expressing melanoma cells proliferated similarly to connexin-deficient melanoma cells. Cx43 has been previously been shown to reduce proliferation in various cancer cell types by altering several cell cycle-related proteins, including p27, Skp2, as well as other cyclins and cyclin-dependent kinases. Some of these proteins appear to be regulated by GJIC-independent mechanisms, whereas others may require channel function (Chen et al., 1995; Huang et al., 1998; Zhang et al., 2003). However, it is possible that these mechanisms may be cell type dependent. In the future, to assess the necessity of channel function, we could use a channel-dead Cx43 mutant (Cx43 T154A) to observe the effects on proliferation, similar to that performed by Zucker et al. (2013) using genetically-linked pre-metastatic (WM793B) and metastatic (1205Lu) human melanoma cells. Alternatively, we could use a C-terminally truncated Cx43 protein that retains channel function, similar to Omori and Yamasaki (1999) who showed that channel function, and not the carboxy-terminus, can reduce anchorage-independent growth in HeLa cells. Collectively, these two types of Cx43 mutants would allow us to determine if the tumor suppressive properties we observed in melanoma cells was due to GJIC-dependent or -independent mechanisms, possibly involving a member of the gap junction proteome.

Melanocytes form the epidermal-melanin unit *in situ* within the epidermis with approximately 30 keratinocytes, and it is commonly believed that melanocyte growth and proliferation is controlled by keratinocytes through GJIC, contact-dependent and diffusible factors (Haass et al., 2005; Seiberg et al., 2000; Shih et al., 1994; Valyi-Nagy et al., 1993). Interestingly, we showed that Cx43 expression reduced melanoma cell growth amongst keratinocytes beyond the expected reductions seen in our proliferation and growth curve studies. However, despite the fact that both cell types expressed Cx43, heterocellular GJIC was not established between melanoma cells and keratinocytes, perhaps due to incompatible cadherins between cell types, as previously hypothesized

(Hsu et al., 2000a). This would suggest that the additional growth suppression seen in Cx43-expressing melanoma cells when cultured in a keratinocyte microenvironment was due to signals received from keratinocytes in a GJIC-independent manner. Such signals could, in turn, potentially propagate throughout the tumor cells via GJIC. Going forward, to determine the mechanism by which keratinocytes reduced the growth of Cx43-expressing melanoma cells, we could culture these cells with conditioned media from keratinocytes and re-evaluate melanoma cell proliferation. Such a study would help elucidate the possible presence of a soluble or contact-dependent factor that may act on melanoma cells.

The *in vivo* chick CAM assay has been widely used as a model to study tumorigenesis in many cancers, including primary melanoma tumor formation and metastasis (Arpaia et al., 2012; Chambers et al., 1992; Chambers et al., 1982; Penuela et al., 2012; Stoletov et al., 2013; Zijlstra et al., 2002). This inexpensive and time-efficient model offers an immunosuppressed host that permits the growth of foreign tumor cells, but more importantly, provides a highly vascularized system that can provide *in vivo* factors that approximately recapitulate *in situ* tumor growth (Karnofsky et al., 1952; Knighton et al., 1977; Leighton, 1964; Scher et al., 1976). In our study, we showed that Cx43-expressing melanoma cells produced ~4.5-fold smaller tumors within the chick-CAM assay compared to controls, whereas the expression of Cx26 did not significantly alter primary tumor formation. Similar to our mixed culture studies with keratinocytes, the reduction in tumor size appeared to be further enhanced in the *in vivo* setting compared to the observed reductions in proliferation and cell number *in vitro*. It is clear that these differences can be attributed to the microenvironment, which is known to affect tumor cell growth and proliferation (Allinen et al., 2004). The CAM, formed from the fusion of the chorionic and allantoic membranes in the developing chicken, encompass portions of the three embryonic germ layers (Gabrielli et al., 2001). Interestingly, the epithelial cells within the ectoderm and endoderm of the CAM have been reported to express Cx43 (Girolamo et al., 2006), creating a connexin complement to Cx43 expressed in the melanomas. However, whether or not these epithelial cells are capable of GJIC with Cx43-expressing melanoma cells remains to be seen. Previously, it has been shown that Cx43 expression in breast cancer cells can reduce angiogenesis within a 3-dimensional

microenvironment (McLachlan et al., 2006), which could result in further reduced tumor growth within the vascularized CAM. It is possible that these and other mechanisms of Cx43-based tumor suppression *in vivo* may require channel function. To test this, we could employ the use of channel-dead and truncated Cx43 mutants, as outlined earlier. In addition, the nature of the topical tumor growth in the CAM lends itself to the use of pharmacological agents that could block gap junction channel function (Storgard et al., 2005), which could be further used to test the role of gap junction channels in tumorigenesis. To that end, we could use pharmacological connexin-channel blockers, such as carbenoxolone, to determine the effect of GJIC on *in vivo* tumor formation.

One of the major questions left unanswered from our studies is the role of connexins in melanoma metastasis. Recently, several studies suggest that connexins may actually facilitate the later stages of tumorigenesis (Elzarrad et al., 2008; Ezumi et al., 2008; Lin et al., 2002; Pollmann et al., 2005), leading to their reclassification as conditional tumor suppressors in breast cancer (Naus and Laird, 2010). This potential biphasic role of connexins has also been supported in melanoma tumorigenesis due to increasing evidence that Cx26 facilitates melanoma metastasis (Ito et al., 2000; Saito-Katsuragi et al., 2007; Stoletov et al., 2013). Uniquely, our model for primary tumor formation in the chicken embryo also allows for metastasis of tumor cells from the CAM into the developing embryonic organs, as demonstrated previously (Arpaia et al., 2012; Penuela et al., 2012; Zijlstra et al., 2002). Although the short duration of the tumor formation assay in the chicken embryo would not provide sufficient time for the development of marcometastasis, we can quantitatively assess for micrometastasis in the chicken organs using a qPCR-based method to detect the mouse *Alu* repeat sequence, as previously describe (Zijlstra et al., 2002). This data would help to further clarify the role of connexins throughout melanoma tumorigenesis, from primary tumor formation to metastatic melanoma.

In our studies, we showed that connexin-deficient melanoma cells not only fail to couple with each other, but also with adjacent keratinocytes in mixed cultures. Although melanoma cells have been previously shown to be incapable heterocellular GJIC with keratinocytes (Hsu et al., 2000a; Hsu et al., 2000b), increasing connexin expression in

melanoma cells was not previously evaluated as a means to re-establish GJIC with keratinocytes (Haass and Herlyn, 2005; Hsu et al., 2000a). Interestingly, we showed that providing melanoma cells with the same connexins as expressed in keratinocytes did not restore heterocellular GJIC with keratinocytes. Hsu et al. (2000a) suggested that cadherins mediate partner-specific binding of connexins. They showed that melanocytes, which normally express E-cadherin, exhibited GJIC with E-cadherin-expressing keratinocytes. However, N-cadherin-expressing melanoma cells did not demonstrate GJIC with keratinocytes, but rather with fibroblasts, which also expressed similar cadherins and connexins. Additionally, they showed that melanoma cells which retain E-cadherin expression and melanoma cells that were transfected with E-cadherin formed heterocellular gap junctions with keratinocytes and were capable of dye transfer between the two cell types. Furthermore, Hsu et al. (2000b) showed that melanoma cells which expressed E-cadherin display reduced malignancy, perhaps due to this heterocellular GJIC with keratinocytes. Taken together with our findings, these studies open the door to an interesting future study to assess the role of connexins and cadherins in melanoma tumorigenesis. Here, we could assess the tumor suppressive effects of E-cadherin and Cx43 alone, or in combination. Knowing that Cx43 and E-cadherin each can suppress melanoma tumorigenesis, it would be interesting to evaluate whether Cx43- and E-cadherin-expressing melanoma cells would display additive or synergistic effects on reducing tumor growth.

Throughout our studies, we found that both Cx43 and Cx26 were ectopically expressed at approximately equal levels based on immunoblotting for the GFP tagged added to each connexin. However, although immunofluorescence images revealed that both Cx26-GFP and Cx43-GFP trafficked and localized to the cell surface, Cx26-GFP appeared to display a much more intracellular profile compared to Cx43-GFP. Additionally, although dye transfer studies revealed that the incidence of dye transfer was statistically similar between engineered lines and connexin-rich keratinocytes, the degree of dye spread did not appear to be as robust in melanoma cells expressing Cx26. This may be due to the intrinsic difference between Cx26-based and Cx43-based channels, however, this may also be due to the amount of functional gap junctional channels that form in each cell line. If this is the case, the amount gap junction channels that form in Cx26-expressing

melanoma cells could, in theory, be significantly less than that obtained in Cx43-expressing melanoma cells. Thus we cannot exclude the possibility that a higher degree of gap junctional-coupling through Cx26 channels may inhibit or promote melanoma tumorigenesis.

Collectively, our results would strongly suggest that Cx43 acts as a tumor suppressor in melanoma cells, whereas Cx26 does not significantly change the overall tumorigenic properties of melanoma cells. These differences may be attributed to the identity of distinct transjunctional molecules that pass through each respective channel (Alexander and Goldberg, 2003; Harris, 2007), or potentially due to GJIC-independent mechanisms, including the interaction with connexin-binding partners to the carboxy-terminus of Cx43 (Laird, 2010; Naus and Laird, 2010). Future studies using mutant connexin proteins or pharmacological agents may further elucidate the difference between these two general mechanisms of tumor suppression. Regardless, these studies have clearly identified that Cx43 is a potent melanoma tumor suppressor, which lends itself to further study as a future therapeutic target.

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Supervisor: Dr. Dale Laird
Thesis: Connexin43 Reduces melanoma growth in a keratinocyte microenvironment and during tumorigenesis *in vivo*

Honors BSc, The University of Western Ontario, 2006-2010
London, Ontario, Canada
Honors Specialization in Biology

Honours and Awards:

Top Platform Presentation Award - $100
Oncology Research and Education Day
London, Ontario, Canada – June 2013

Graduate Thesis Research Award - $510
Department of Anatomy and Cell Biology, Western University
London, Ontario, Canada – January 2013

SOGS Travel Award - $300
Western University
London, Ontario, Canada – January 2013

Schulich Graduate Scholarship - $7500/year
Western University
London, Ontario, Canada – 2011-2013

Platform Presentations

Oncology Research and Education Day
"*Cx43 reduces melanoma growth in a keratinocyte microenvironment and during tumorigenesis in vivo"*
Mark J. Ableser, Silvia Penuela, Qing Shao, Dale W. Laird
London, Ontario, Canada – June 2013

London Health Research Day
"*The role of connexins in melanoma tumorigenesis as revealed in 2D and 3D microenvironments"*
Mark J. Ableser, Silvia Penuela, Qing Shao, Dale W. Laird

London, Ontario, Canada – March 2013

Gap Junction Research Forum
"*The role of connexins in melanoma tumorigenesis as revealed in 2D and 3D microenvironments*"
Mark J. Ableser, Silvia Penuela, Qing Shao, Dale W. Laird
London, Ontario, Canada – February 2013

Poster Presentations

American Society for Cell Biology
"*Cx43 reduces melanoma growth in a keratinocyte microenvironment and during tumorigenesis in vivo*"
Mark J. Ableser, Silvia Penuela, Qing Shao, Dale W. Laird
San Francisco, California, USA – December 2012

Physiology and Pharmacology Research Day
"*Ectopic expression of Cx43 reduces the tumorigenic potential of BL6 melanoma cells*"
Mark J. Ableser, Qing Shao, Dale W. Laird
London, Ontario, Canada – November 2012

Anatomy and Cell Biology Research Day
"*Ectopic expression of Cx43 reduces the tumorigenic potential of BL6 melanoma cells*"
Mark J. Ableser, Qing Shao, Dale W. Laird
London, Ontario, Canada – October 2012

Oncology Research and Education Day
"*The role of connexins in melanoma tumorigenesis as revealed in 2D and 3D microenvironments*"
Mark J. Ableser, Qing Shao, Dale W. Laird
London, Ontario, Canada – June 2012

Related Work Experience

Graduate Teaching Assistant
Mammalian Histology (ANAT3309)
Western University
2011-2013 (4 terms)

Publications:

Ableser MJ, Penuela S, Lee J, Shao Q, Laird DW. Cx43 reduces melanoma growth in a keratinocyte microenvironment and during tumorigenesis *in vivo*. Journal of Biological Chemistry. (submitted for review)