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IMPLICATIONS OF PANNEXIN 1 AND PANNEXIN 3 IN KERATINOCYTE DIFFERENTIATION

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Abstract:

Pannexin (Panx) 1 and Panx3 are integral membrane proteins with sequence homology to the invertebrate gap junctions, innexins, and are expressed in mammalian skin. Panxs form functional single-membrane channels but their importance in regulating cellular function is poorly understood. In this study, the expression of Panx1 and Panx3 was assessed in mouse skin and the role of these Panxs in keratinocyte differentiation was investigated using differentiation-competent rat epidermal keratinocytes (REKs) engineered to over-express Panx1, Panx1-GFP or Panx3. Endogenous levels of a unique 70 kDa species of Panx3 increased in organotypic epidermis, while Panx1 remained unchanged compared to monolayer REKs. Ectopic Panx1, Panx1-GFP and Panx3 predominantly localized to the cell surface in monolayer cultures, but re-localized to intracellular compartments in organotypic epidermis. Furthermore, ectopic Panx1 in organotypic epidermis dysregulated cytokeratin 14 expression, disrupted the architecture of the epidermis and reduced the vital layer thickness while ectopic Panx3 had no effect on the epidermis. In summary, Panx1 and Panx3 are co-expressed in mouse epidermis and play distinct roles in regulating keratinocyte differentiation.

Keywords: Pannexin, Panx1, Panx3, differentiation, proliferation, migration, keratinocyte, REK, organotypic, epidermis

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List of Abbreviations and Symbols

293T	human embryonic kidney 293 cell line modified to constitutively express the simian virus-40 T antigen
ANOVA	analysis of variance
ATP	adenosine triphosphate
BICR-M1R$_K$	fibroblastoid rat Marshall mammary tumor cell line
BLAST	basic local alignment search tool
BrdU	5-bromo-2-deoxyuridine
BSA	bovine serum albumin
°C	degrees Celsius
cDNA	complementary deoxyribonucleic acid
CK	cytokeratin
CMV	cytomegalovirus
Cx	connexin
DMEM	Dulbecco's Modified Essential Medium
ER	endoplasmic reticulum
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GFP	green fluorescent protein
GJIC	gap junctional intercellular communication
H&E	hematoxylin and eosin stain
Inx	innexin
IP_3	inositol triphosphate

kDa	kilodalton
Mb	mega base pairs; 1,000,000 base pairs
MC3T3-E1	mouse osteoblastic cell line
MDCK	Madin-Darby canine kidney cells
mRNA	messenger ribonucleic acid
NO	nitric oxide
OPUS	acronym that describes the original founding members of the innexin family of gap junction proteins: ogre, Passover, unc-7 and shaking-B
Panx(s)	pannexin(s)
PBS	phosphate buffered saline
PDI	protein disulfide isomerase
pKi	isoelectric point/pH
REK	rat epidermal keratinocytes
s.d.	standard deviation
s.e.m.	standard error of the mean
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
TBLASTN	compares a protein query against a nucleotide sequence database dynamically translated in all reading frames
U/ml	units per milliliter

CHAPTER 1

INTRODUCTION

1.1 The history of pannexins

1.1.1 Pannexins share genomic lineage with innexin, but not connexin channels

A family of vertebrate proteins, termed pannexins (Panxs), that are homologous in sequence to invertebrate innexins, and share 36-46% similarity and 25-34% identity over 81-255 amino acid residues, were originally proposed to have functions similar to both innexin and connexin gap junctions (Panchin et al., 2000; Yen and Saier, 2007). However, as the pannexin story continues to unfold, pannexins may in fact have functions unrelated to gap junctional intercellular communication. Gap junctions are tight clusters of intercellular protein channels that allow direct cytoplasmic connections between apposing plasma membranes, serving as one type of intercellular communication in multi-cellular organisms (Sohl et al., 2005). Gap junctions mediate cell-cell communication through the passage of electrical signals, second messengers and small metabolites of less than 1 kDa in size, and provide a means to coordinate and regulate physiological processes including organ development, cell differentiation, and proliferation (Meier and Dermietzel, 2006).

One half of an intercellular gap junction channel results from the oligomerization of six innexin (Inx) or connexin (Cx) protein subunits (hemichannel, or also called a connexon in vertebrates) that dock to innexin or connexin hemichannels from apposing plasma membranes. These intercellular channels then aggregate to form gap junction plaques (Laird, 2006; Landesman et al., 1999; Norman and Maricq, 2007). One connexin subunit contains four transmembrane domains, two extracellular loops, one intracellular loop, and intracellular amino and carboxy termini. Connexins typically contain 3 cysteine residues per extracellular loop, except in the case of Cx23 which contains 2

cysteine residues per extracellular loop (Iovine et al., 2008; Kumar and Gilula, 1992). Since the discovery of connexins and the first cloning of Cx32 cDNA from human and rat liver (Kumar and Gilula, 1986; Paul, 1986), the connexin family has grown to 21 members in the human and 20 members in the mouse (Laird, 2006; Sohl and Willecke, 2003). Despite a large family size, no connexin homologues were identified in the fully sequenced genomes of *Caenorhabditis elegans* (nematode), or *Drosophila melanogaster* (arthropod; fly) suggesting that alternative gap junction candidates (which turned out to be innexins) needed to be investigated in invertebrates (Barnes, 1994; Landesman et al., 1999; Panchin, 2005; Phelan, 2005).

1.1.2 Innexin gap junctions

The membrane-associated protein family responsible for invertebrate gap junctions was first designated OPUS, which identifies these genes of the original founding members, ogre, Passover, unc-7 and shaking-B (Barnes, 1994; Krishnan et al., 1993; Phelan et al., 1998). Shaking-B (lethal) was the first invertebrate gap junction protein to be identified when Phelan et al. (1998) microinjected mRNA transcripts encoding shaking-B (lethal) into *Xenopus* oocyte pairs resulting in functional homotypic gap junction channels. In fact, the expression of innexins and connexins in *Xenopus* oocyte pairs had already been successfully utilized to characterize many of these protein family members (Willecke and Haubrich, 1996). The acronym OPUS of invertebrate gap junction family was quickly renamed to innexins, the invertebrate analogues of the connexins, since functional evidence had revealed their cellular role and an increasing number of innexins were being discovered (Landesman et al., 1999; Phelan et al., 1998).

The innexin family is spread over multiple animal phyla including Platyhelminthes, Nematoda, Arthropoda and Mollusca, and currently consists of at least 61 members, 6 of which form functional homotypic channels and 2 that form functional heterotypic channels using the *Xenopus* oocyte model (Dykes et al., 2004; Landesman et al., 1999; Panchin et al., 2000; Phelan, 2005; Phelan et al., 1998; Stebbings et al., 2000). Homotypic channels exist when the same innexin or connexin channels dock to form one intercellular channel, while heterotypic channels are assembled when different innexin or connexin protein hemichannels channels dock to form the intercellular channel.

1.1.3 Pannexin family members are related to innexins, but not connexins

Soon after innexins were renamed, the genomes from mollusk and flatworm sequences were subjected to a basic local alignment search tool (BLAST) (Altschul et al., 1997) and compared against the human genome for homology. Interestingly, BLAST revealed two human proteins, MRS1 with no known function (GenBank accession number AF093239, and a novel protein similar to MRS1 (Accession number AL022328) (Panchin et al., 2000). Furthermore, a position specific iterative BLAST search using unc-7 for comparison, detected the same two MRS1 proteins in humans, extending the innexin family to include the Chordata phyla (Baranova et al., 2004; Panchin et al., 2000).

Now that innexin family members extended to vertebrates, innexins were renamed pannexins, from the Latin pan—all, throughout, and nexus—connection, bond to appropriately define this superfamily based on their ubiquitous distribution and putative function (Panchin et al., 2000). Thus, the human MRS1 gene was designated by

the symbol PANX1, while the AL022328 relative was later designated human PANX2 (Baranova et al., 2004; Panchin et al., 2000). Finally, a protein-nucleotide 6-frame translation (TBLASTN) query revealed a third human pannexin, designated PANX3, as the third member of the pannexin family (Baranova et al., 2004).

Three distinct motifs are shared by innexins and pannexins that support their homology. First, the longest and most conserved sequence that has identified most innexins, YYQWV, plus a 23 amino acid flanking region shows 39% identity and 61% similarity between innexins and pannexins (Barnes, 1994; Yen and Saier, 2007). The second best conserved region occurs in the first extracellular loop, between the first and second transmembrane domain sequences, as two fully conserved cysteine residues along with 37% identity and 58% amino acid similarity when flanked by 24 additional amino acid residues (Phelan, 2005; Phelan and Starich, 2001; Yen and Saier, 2007). The third motif occurs in the second extracellular loop where only 1 of 2 cysteine residues are conserved between innexins and pannexins, and when flanked by an additional 17 residues, shows 20% identity and 45% similarity (Yen and Saier, 2007).

Although sequence homology does not exist between pannexins and connexins, PANX1, PANX2 and PANX3 have predicted topologies similar to that of the well-characterized connexins (Figure 1.1), containing four transmembrane domains, intracellular amino and carboxy termini, two cysteine-containing extracellular loops and one intracellular loop (Baranova et al., 2004; Krishnan et al., 1993; Laird, 2006; Starich et al., 1993; Watanabe and Kankel, 1990).

Figure 1.1 Panx1, Panx2 and Panx3 have similar predicted topologies to Cx43

Schematic diagrams showing the predicted topologies of Panx1 (A), Panx2 (B), Panx3 (C) and the topology of Cx43 (D). All molecules have four transmembrane domains with intracellular amino and carboxy termini.

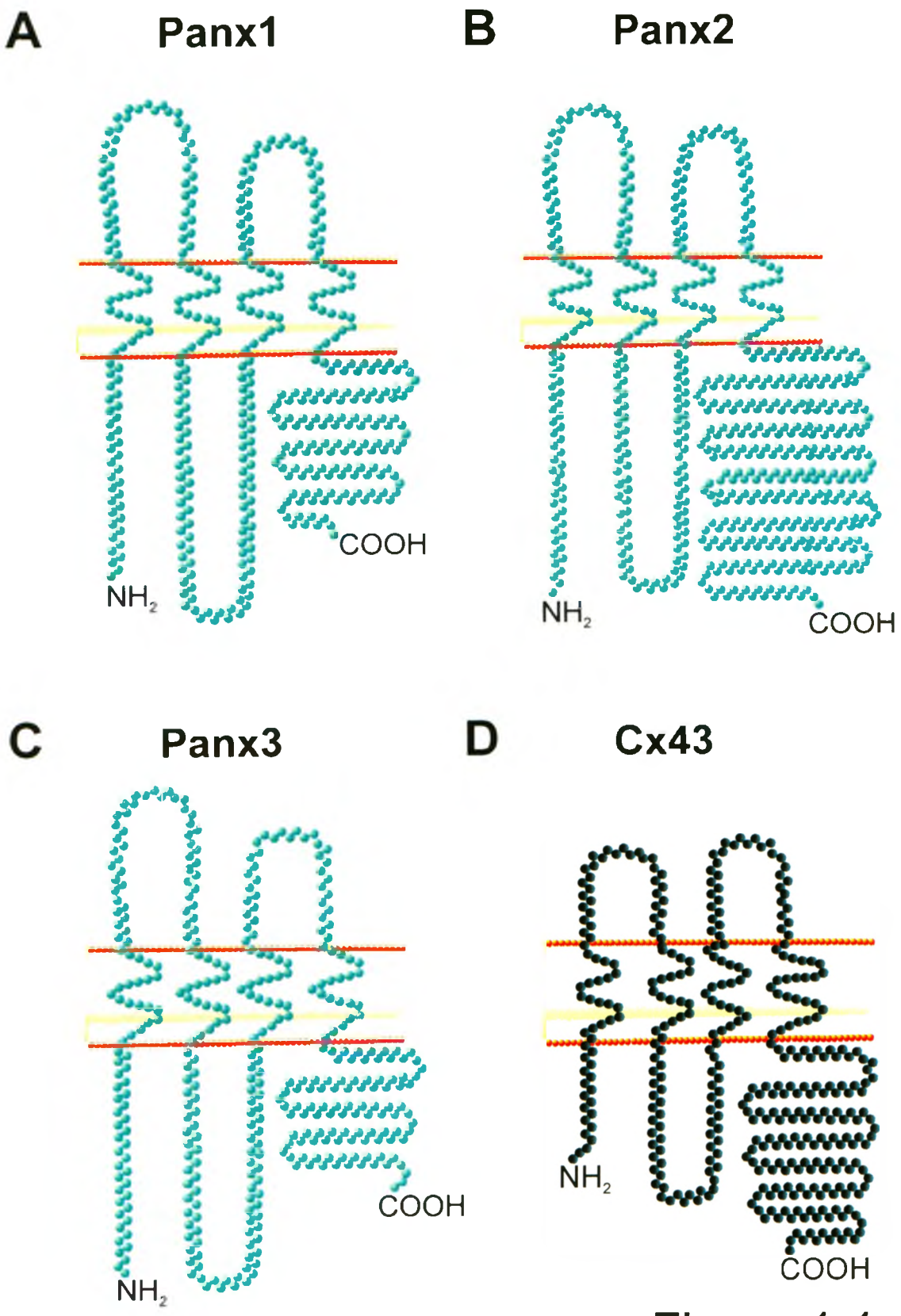

Figure 1.1

1.2 Molecular characteristics of pannexins

1.2.1 Human pannexins

Human PANX1 is located on chromosome 11q14.3 between the genes CRSP6 (cofactor for Sp1 transcriptional activation; also known as vitamin D3 receptor-interacting protein DRIP80) and MRE11 (meiotic recombination 11 homolog A) (Baranova et al., 2004; Panchin et al., 2000). The genomic sequence was compared to its cloned cDNA sequence and revealed that human PANX1 contains five exons, four introns, and encodes a 426 amino acid protein (Accession number NP_056183) with an expected molecular mass of 47.6 kDa (Baranova et al., 2004). On the other hand, human PANX2, is located on chromosome 22q13.31-13.33, and contains four exons. The human PANX2 transcript encodes two protein isoforms, isoform 1 which contains 677 amino acids (Accession number NP_443071) and isoform 2 which contains 643 amino acids (Accession number NP_001153772) with a predicted weight of ~70-80 kDa (Baranova et al., 2004; Yen and Saier, 2007). Human PANX3 is more similar in chromosome location and size to human PANX1 and is found on chromosome 11q24.2 between the olfactory gene family 8 cluster and TBRG1, which encodes a putative orthologue of mouse transforming growth factor-beta-regulated gene 1. Furthermore, human PANX3 contains 4 exons and an open reading frame that has significant similarity to that of PANX1. Finally, human PANX3 encodes a 392 amino acid protein (Accession number NP_443191) and has a predicted molecular weight of 44.7 kDa (Baranova et al., 2004).

1.2.2 Rodent pannexins

All three human pannexin-encoding genes, PANX1, PANX2 and PANX3 were subjected to an amino acid similarity screening tool (BLAST), which revealed high amino acid sequence equivalency percentages between human and mouse (Table 1.1). For the remainder of this thesis, murine pannexins will adapt the nomenclature Panx1, Panx2 and Panx3, respectively. Panx1 is located on chromosome 9 at map position A2 and, similar to human PANX1, contains five exons and four introns. The Panx1 transcript (Accession number AF209817) encodes a 426 amino acid protein product (Accession number NP_062355), with a molecular weight of ~41-48 kDa in rodents (Penuela et al., 2007). Murine Panx1 shows high similarity to the amino terminal portion of human PANX1, and gradually decreases its homology towards the carboxy terminus (Baranova et al., 2004; Bruzzone et al., 2003; Yen and Saier, 2007).

Murine Panx2 is also similar to its human counterpart since it is located on a different chromosome than that of Panx1 and Panx3, and can be found on murine chromosome 15 at map position E3/15. Panx2 transcript (Accession number BK000624) encodes 4 exons, and translates into a 677 amino acid protein product (Accession number NP_001002005), with a molecular weight of ~80 kDa in rodents. Similar to Panx1, Panx2 has striking similarity to human PANX2, which decreases towards the carboxy terminus (Baranova et al., 2004; Bruzzone et al., 2003).

Finally, murine Panx3 (Accession number BK000662) is located on chromosome 9 at map position A4, the same chromosome as Panx1. Murine Panx3 is encoded on four exons resulting in a 392 amino acid product (Accession number NP_766042), with a molecular weight of ~43 kDa in rodents (Penuela et al., 2007). Interestingly, both human

and mouse Panx1 genes spans greater chromosome intervals than both the Panx2 and Panx3 genes. On the other hand, Panx1 and Panx3 may be more closely related since they share 41% identity and 59% conservation at the amino acid level and are located on the same chromosomes, separated by only 30 Mb in the human and 22 Mb in the mouse (Baranova et al., 2004). Thus, in conjunction with their intron-exon similarities, it has been speculated that Panx1 and Panx3 may have arisen from chromosomal duplication, since several innexins have been located in clusters of two or three (Baranova et al., 2004; Curtin et al., 1999).

Table 1.1 Percentage of amino acid sequence similarity between human and mouse pannexins

	PANX1	PANX2	PANX3	Panx1	Panx2	Panx3
PANX1	-	51	62	94	51	61
PANX2	51	-	52	49	94	50
PANX3	62	52	-	62	51	93
Panx1	94	49	62	-	50	59
Panx2	51	94	51	50	-	50
Panx3	61	50	93	59	50	-

PANX: human; Panx: mouse. Table adapted from "The mammalian pannexin family is homologous to the invertebrate innexin gap junction proteins" (Baranova et al. 2004).

1.3 Expression profiles of pannexins

Currently, the genes of all three pannexin family members have been detected in at least 7 species, including *Homo sapiens* (human), *Mus musculus* (mouse), *Rattus norvegicus* (rat), *Canis familiaris* (dog), *Bos taurus* (cow), *Danio rerio* (zebrafish) and *Tetraodon nigroviridis* (green spotted puffer fish) (Yen and Saier, 2007). However, the pannexin expression profiles are most characterized in human and rodent tissues.

1.3.1 Pannexin 1

Panx1 transcripts are ubiquitously expressed in normal rodent and human tissues, and appear to be enriched in the central nervous system, especially in embryonic stages E13.5-E18 of the mouse (Baranova et al., 2004; Penuela et al., 2007). Specifically, Panx1 transcript was detected in the brain, eye, heart, skeletal muscle, testis, ovary, placenta, spleen, kidney, liver, lung, thymus, prostate, small intestine, blood endothelium, erythrocytes and skin (Baranova et al., 2004; Bruzzone et al., 2003; Locovei et al., 2006a; Panchin et al., 2000; Ray et al., 2006; Ray et al., 2005; Vogt et al., 2005). Furthermore, Panx1 was recently detected in the rodent cochlea, including the supporting cells of the Organ of Corti, which include pillar, Hensen, Claudius and Boettcher cells, as well as in cells of the spiral limbus, inner and outer sulcus, spiral prominence, cochlear lateral wall, Reissner's membrane and strial blood vessels (Wang et al., 2009).

In the central nervous system, Panx1 transcripts were detected in retina (retinal ganglion, amacrine and horizontal cells), lens (fiber cells), cortex, pyramidal cells and interneurons of the hippocampus and neocortex, cerebellum, inferior olive, amygdala, substantia nigra, mitral cells of the olfactory bulb and spinal cord, with predominant

expression in neurons, but also in Bergman glial cells and endothelial cells (Baranova et al., 2004; Bruzzone et al., 2003; Dvoriantchikova et al., 2006a; Dvoriantchikova et al., 2006b; Ray et al., 2006; Ray et al., 2005; Shestopalov and Panchin, 2008; Sohl et al., 2005; Vogt et al., 2005; Zappala et al., 2007; Zoidl et al., 2007). Interestingly, murine Panx1 had high expression levels in embryonic and postnatal brain but was decreased in adult brain (Ray et al., 2006; Ray et al., 2005; Vogt et al., 2005). Importantly, we demonstrated strong, but variable glycosylated Panx1 protein expression (~41-48 kDa) which was detected in brain, spleen, cartilage and skin of 3-week old mice and in thymus of neonatal mice, while we observed weaker signals in lung, kidney and heart ventricle of 3-week old mice (Penuela et al., 2007).

1.3.2 Pannexin 2

In contrast to Panx1, Panx2 expression has been reported to be more restricted and strong expression was detected in many cell types of the brain, spinal cord, cochlea and retina (Baranova et al., 2004; Wang et al., 2009; Zappala et al., 2007). However, Panx2 transcript was also reported to be expressed in the testis, thyroid, prostate, kidney, and liver (Baranova et al., 2004; Bruzzone et al., 2003; Dvoriantchikova et al., 2006a; Dvoriantchikova et al., 2006b). Strong Panx2 transcript expression in the brain was revealed in the occipital pole and cerebral cortex, Purkinje cells in the cerebellum, hippocampus, dentate gyrus, medial and lateral mammillary nuclei, cortical amygdaloid nuclei, and was shown to be co-expressed with Panx1 in the retina, mitral cells of the olfactory bulb, neurons of the piriform cortex, amygdala, substantia nigra, adult pyramidal cells and interneurons of the hippocampus and neocortex. However, in

contrast to Panx1, Panx2 transcript expression was low in embryonic brain but subsequently increased as the brain developed (Baranova et al., 2004; Bruzzone et al., 2003; Ray et al., 2006; Ray et al., 2005; Shestopalov and Panchin, 2008; Vogt et al., 2005; Weickert et al., 2005). Panx2 was also recently found to be expressed in the stria vascularis and spiral ganglion neurons of the cochlear system (Wang et al., 2009). Since Panx2 was not reported to be found in the skin, this thesis will not continue to discuss this member of the pannexin family.

1.3.3 Pannexin 3

Initial characterization of Panx3 revealed transcript expression in mouse skin and cartilage (Bruzzone et al., 2003; Litvin et al., 2006) and low levels in the hippocampus (Baranova et al., 2004). Based on expressed sequence tag data, Panx3 was also predicted to be expressed in osteoblasts, synovial fibroblasts, whole joints of mouse paws, and cartilage of the inner ear (Baranova et al., 2004). Recently, Panx3 was also reported to be expressed in cochlear bone (Wang et al., 2009). Although a splice variant for Panx3 has not yet been identified, a murine tissue survey detected two species of Panx3 protein at ~43 kDa and 70 kDa that were both found to be expressed in the skin, cartilage and heart ventricle, while only the 70 kDa species was detected in lung, liver, kidney, thymus and spleen (Penuela et al., 2007). However, this 70 kDa species may represent a dimer of Panx3, a possibility that has not been ruled-out. Overall, while our understanding of the diverse expression profiles of pannexins is beginning to unfold, the cellular functional role(s) of pannexins remain relatively obscure.

1.4 Status of pannexin channel function

1.4.1 Pannexins do not form intercellular channels in mammalian cells

Despite the ability of innexins and connexins to form gap junctions in invertebrates and vertebrates, respectively, questions surrounding pannexin channel capabilities in vertebrates continue to be debated (Dahl and Locovei, 2006; Landesman et al., 1999; Phelan and Starich, 2001). *Xenopus* oocyte studies revealed the ability of homotypic Panx1 and heterotypic Panx1/Panx2 to form intercellular channels after 24 – 48 hours of pairing, while homotypic Panx2 channels could not (Bruzzone et al., 2003). Later studies would confirm that Panx1 could indeed form functional intercellular channels in *Xenopus* oocytes after 24 hours (Boassa et al., 2007). However, the length of oocyte pairing was considered excessive, causing these findings to be highly controversial to many researchers in the field. For example, Panx1 could not cause significant junctional conductance within 6 hours of pairing, while Cx46, a well-established gap junction protein, formed robust intercellular channels within 1 hour of oocyte pairing (Boassa et al., 2007).

The observed slow dynamics of pannexin coupling is a clear indication that pannexins may in fact not readily form gap junctions (Bruzzone et al., 2005). Boassa et al. (2007) also reported that communication-deficient Madin-Darby Canine Kidney cells that express Panx1 were unable to pass Lucifer yellow dye via scrape loading, a typical assay used to assess intercellular communication. Furthermore, functional studies in various mammalian cells including erythrocytes, neuronal and glial cells have shown no evidence of gap junction formation suggesting that pannexins are not redundant to connexins and do not provide conduits for communication through intercellular channels

(Huang et al., 2007; Locovei et al., 2006a; Penuela et al., 2007). Therefore, it remains difficult to accept the premise that pannexins act as gap junction proteins unless at some point in the future they are found to form intercellular channels in endogenous systems.

1.4.2 Pannexins form functional single-membrane channels

Pannexin studies were conducted by injecting pannexin transcript into *Xenopus* oocytes, similar to those used in studies that revealed connexin and innexin intercellular channel formation (Phelan et al., 1998; Willecke and Haubrich, 1996). In 2003, Bruzzone et al. (2003) used two-electrode voltage clamp to demonstrate the ability of Panx1 to form functional single-membrane channels in single *Xenopus* oocytes, while Panx2 could not. Panx1 and Panx2 mRNA were also co-injected in these oocytes and the conductance of heteromeric Panx1/Panx2 single-membrane channels was found to be reduced, suggesting that Panx2 attenuated the function of Panx1. Nevertheless, these results suggest that pannexins indeed form single-membrane channels that communicate with the extracellular environment and this notion has been supported by various groups (Boassa et al., 2007; Dahl and Locovei, 2006; Huang et al., 2007; Locovei et al., 2006a; Penuela et al., 2007; Schenk et al., 2008; Thompson et al., 2008).

Despite the controversy surrounding whether pannexins form intercellular channels, reports continue to support the role of pannexins playing a critical role in cellular communication with the extracellular environment through single-membrane channels. Essential to this claim is the fact that Panx1 has been shown to be able to oligomerize into hexameric structures that traffic to the cell surface (Boassa et al., 2007; Penuela et al., 2007) to form single membrane channels that can be blocked by

pharmacological reagents. Panx1 is sensitive to blockage by the common channel blockers carbenoxolone and probenecid, however in contrast to connexins, remain relatively insensitive to blockage by flufenamic acid (Bruzzone et al., 2005; Silverman et al., 2008). Panx1 channel closure has also been shown to occur via CO_2-mediated intracellular acidification (Locovei et al., 2006b), and ATP (Qiu and Dahl, 2009). On the other hand, Panx1 channel activation has been shown to be induced by mechanical stimulation, membrane depolarization, extracellular ATP and increased intracellular Ca^{2+} concentrations (Bao et al., 2004; Locovei et al., 2006a; Locovei et al., 2006b; Pelegrin, 2008; Reigada et al., 2008; Romanov et al., 2008; Schenk et al., 2008; Thompson et al., 2008). Activated Panx1 channels are capable of passing dye, Ca2+, ATP, bacterial toxins and antigens (Schenk et al., 2008). Thus, these various modes of Panx1 activation have subsequently implicated Panx1 in a diverse range of functions including Ca^{2+} wave propagation, vasodilation, ischemic cell death, inflammation, tumor-suppression and the release of neurotransmitters in taste buds (Huang et al., 2007; Locovei et al., 2006a; Locovei et al., 2006b; Pelegrin and Surprenant, 2006; Pelegrin and Surprenant, 2007; Romanov et al., 2007; Thompson et al., 2006).

1.5 Putative roles for pannexin single-membrane channels

1.5.1 Pannexin 1

Activation of Panx1 single-membrane channels has been shown in ischemic conditions and has been implicated in inflammation and tumorigenesis. For example, ATP release resulted in paracrine signaling and Ca^{2+} wave propagation through ATP-induced purinergic receptor activation upon mechanical stress, ischemia, increases in

intracellular Ca^{2+} concentrations and membrane deoplarization (Bao et al., 2004; Locovei et al., 2006a; Locovei et al., 2006b). This was determined through initial studies in *Xenopus* oocytes that demonstrated slow ATP release when Panx1 was co-expressed with P2Y1 receptors and a rapid, transient release with P2Y2 receptors (Locovei et al., 2006b). Furthermore, ATP-induced ATP release was also demonstrated when Panx1 was co-expression with the $P2X_7$ receptor. In fact, the $P2X_7$ receptor ($P2X_7R$) is the only known molecule to date to physically interact with Panx1, and this complex has been associated with the release of the pro-inflammatory cytokine interleukin-1-beta upon purinergic stimulation of macrophages, via caspase-1 activation which is independent of the Panx1-mediated dye uptake pathway (Brough et al., 2009; Pelegrin and Surprenant, 2006; Pelegrin and Surprenant, 2009; Schenk et al., 2008). Although the $P2X_7$/Panx1 complex is directly activated by purinergic stimulation of the $P2X_7$ receptor, indirect Panx1 channel opening can also occur via intracellular Ca^{2+} release through the stimulation of IP_3 (Locovei et al., 2006b). Moreover, these types of purinergic receptor activations resulting in the stimulation of Panx1 single-membrane channels have also been demonstrated in taste buds and macrophages (Huang et al., 2007; Pelegrin and Surprenant, 2006; Pelegrin and Surprenant, 2007; Romanov et al., 2007). Furthermore, Silverman et al. (2009) revealed that Panx1 responds to high extracellular K^+ levels and was associated with $P2X_7R$-mediated activation of the caspase-1 cascade involved in neural inflammasome activation. Finally, this Panx1-mediated ATP release has also been implicated in the elevation of pressure across the retina, however the neuropathological role of this finding has not yet been fully elucidated (Reigada et al., 2008).

In addition to ATP-induced ATP release through Panx1 channels, ATP release has also been shown to occur via mechanical stimulation and ischemia in blood capillary endothelial cells and erythrocytes causing vasodilation (Dvoriantchikova et al., 2006b; Locovei et al., 2006a). Since Panx1 has been shown to be expressed in these cell types, the role of ATP release via Panx1 single-membrane channels in capillary endothelium and erythrocytes has subsequently been implicated in Ca^{2+} wave propagation causing NO-mediated smooth muscle vasodilation (Locovei et al., 2006a). Panx1-mediated Ca^{2+} wave propagation has been hypothesized to occur when Panx1-mediated ATP release in erythrocytes and endothelial cells initiates purinergic activation of metabotropic P2Y receptors complexed with Panx1 in neighboring endothelial cells. Activation of these complexes subsequently increase intracellular Ca^{2+} concentration via IP_3-mediated Ca^{2+} release and influx of extracellular Ca^{2+} through Panx1 channels, propagating the Ca^{2+} wave and stimulating NO-mediated vasodilation (Boitano et al., 1992; Locovei et al., 2006a; Locovei et al., 2006b).

In addition to Panx1-mediated regulation of Ca^{2+} homeostasis, Ca^{2+} wave propagation, vasodilation, ischemic cell death and inflammation is the ability of Panx1 expression to induce tumor-suppressive properties in C6 gliomas (Lai et al., 2007). Although C6 glial cells do not endogenously express pannexins, exogenous Panx1 expression was shown to reduce cell proliferation, motility and anchorage-independent growth as well as flattened morphology which are key properties of tumor-suppression (Hanahan and Weinberg, 2000). Combined, it can be speculated that the putative loss or down-regulation of endogenous Panx1 in gliomas may contribute to tumor formation,

while Panx1 expression may serve to reduce the susceptibility of cells to tumor transformation.

1.5.2 Pannexin 3

Although much less is known about the channel capabilities of Panx3, both Panx1 (Boassa et al., 2007; Penuela et al., 2007) and Panx3 (Penuela et al., 2007) were determined to be N-linked glycosylated proteins that are capable of dye uptake supporting their role as functional single-membrane channels. Glycosylation of pannexins has also been shown to mediate pannexin trafficking. This conclusion was based on the impaired ability of N-linked glycosylated mutants to traffic to the cell surface (Boassa et al., 2007; Penuela et al., 2007). Glycosylation was determined to occur at asparagine 251 in the second extracellular loop of Panx1, and in Panx3 at asparagine 71 in the first extracellular loop (Penuela et al., 2007). Furthermore, two distinct glycosylated species of Panx1 (~41-48 kDa) and one glycosylated-doublet species of Panx3 (~43 kDa) were detected by immunoblotting, which migrate to a single band following treatment with N-glycosidase (Penuela et al., 2007). Glycosylation of pannexins is a distinct post-translational modification not found on any connexin, further supporting the notion that these protein families are distinct.

1.6 Pannexins are localized at the cell surface and in intracellular compartments

As pannexins continue to be better understood as single-membrane channels, distinct subcellular and tissue expression profiles of endogenous and ectopically-

expressed Panx1 and Panx3 are beginning to emerge. Penuela et al. (2007) showed that exogenously expressed Panx1 and Panx3 localized predominantly at the cell surface in normal rat kidney and BICR-M1R_K cell lines as well as formed functional single-membrane channels in 293T cells. In MDCK cells, endogenous Panx1 and Panx3 were localized to the cell surface (Boassa et al., 2007; Penuela et al., 2008) while Panx1 was found to be uniformly distributed throughout intracellular compartments in the mouse spleen (Penuela et al., 2007).

In fact, numerous studies have shown the localization of Panx1 protein in the Golgi apparatus and endoplasmic reticulum that suggest distinct roles for pannexins when localized to intracellular compartments (Dvoriantchikova et al., 2006a; Dvoriantchikova et al., 2006b; Huang et al., 2007; Lai et al., 2007; Penuela et al., 2008; Vanden Abeele et al., 2006; Zappala et al., 2006). In fact, Vanden Abeele et al. (2006) have suggested that endoplasmic reticulum localized Panx1 contributes to endoplasmic reticulum Ca^{2+} leak involved in intracellular Ca^{2+} homeostasis. Moreover, the degree of Ca^{2+} load in the endoplasmic reticulum has been shown to affect cellular processes including apoptosis and proliferation since the endoplasmic reticulum is a primary storage compartment of intracellular Ca^{2+} (Bao et al., 2004). This suggests that at least Panx1 plays a second role unrelated to communication with the extracellular environment through cell surface single-membrane channels.

1.7 Pannexin 1 and Pannexin 3 demonstrate distinct tissue localization profiles

As pannexins continue to establish their role as single-membrane channels, distinct subcellular and tissue expression profiles of endogenous Panx1 and Panx3 continue to emerge. Tissue specific localization of endogenous Panx1 includes post-synaptic sites in cortical and hippocampal neurons suggesting a potential role in regulating neuronal post-synaptic activity (Zoidl et al., 2007). Unlike the localization pattern that has been reported in cell culture, endogenous Panx1 was found to be uniformly distributed throughout intracellular compartments in cells of mouse spleen (Penuela et al., 2007). Similarly, diffuse intracellular staining of Panx3 was observed in mammalian cochlear bone at the lateral wall and modiolus (Wang et al., 2009). Both Panx1 and Panx3 were also endogenously detected in intracellular compartments of a mouse osteoblastic cell line, MC3T3-E1 (Penuela et al., 2008). Finally, distinct tissue-specific localization of Panx1 and Panx3 was seen in normal human facial skin (Penuela et al., 2007). While punctate Panx1 expression was detected chiefly in the stratum granulosum and spinosum, Panx3 expression boasted a more scattered distribution, present throughout all epidermal layers, and appeared to have an intracellular subcellular distribution.

1.8 Pannexin 1 and Pannexin 3 are expressed in keratinocytes of the skin

Panx1 and Panx3 were found predominantly at cell surface and intracellular locations, respectively, in human facial skin keratinocytes suggesting that these pannexins may have distinct roles in skin differentiation, maintenance or regeneration (Penuela et al., 2007). While punctate Panx1 localization was detected chiefly in the stratum granulosum and spinosum layers of human facial epidermis, Panx3 expression exhibited a more scattered distribution throughout all epidermal layers, and appeared to have an intracellular subcellular distribution (Penuela et al., 2007). Together, the distinct cellular and subcellular localization profiles of Panx1 and Panx3 provides evidence to suggest that the spatio-temporal expression of pannexins may exist in skin and have implications in fundamental keratinocyte processes.

1.9 Skin

1.9.1 Sebaceous glands

During development, multipotent stem cells of the skin generate the keratinocytes of the epidermis, hair follicles and sebaceous gland (Merrill et al., 2001). The cushioning dermis is largely comprised of collagen and houses skin appendages including piloerector muscles, hair follicles and sebaceous glands. Sebaceous glands consist of differentiating keratin-expressing cells called sebocytes and in humans, are most abundant in skin of the face and scalp, but are not present in the palms and soles (Rizvi et al., 2003; Zouboulis et al., 1993). Sebocytes produce sebum, a mixture of wax monoesters, triglycerides, free fatty acids and squalene lipid molecules that coat the hair or fur of mammals and serve as

hydrophobic protection against overhydration and a method of heat insulation (Pochi, 1982). Sebocytes contain at least cytokeratins (CK) 4, 5, 6, 10, 14, 16, 17 and 18 and targeted CK10 knockout results in increased proliferation, differentiation and sebum secretion of cells at the periphery of the gland (Kaufman et al., 2002; Reichelt et al., 2004). Sebocytes are a cutaneous endocrine gland and are regulated under the influence of various steroid hormones including corticotrophin-releasing hormone, growth hormone, adrenocorticotropin and alpha-melanocyte-stimulating hormone (Zouboulis et al., 2002). Under hormonal influence, sebocytes play key roles in skin processes including the transport of antioxidants to the skin surface, natural photoprotection, anti-inflammation of the skin and antimicrobial activity (Marques et al., 2002; Wille and Kydonieus, 2003; Wrobel et al., 2003; Zouboulis et al., 2002).

1.9.2 Epidermis

Protecting the dermis, the outermost water-resistant epidermal barrier serves to protect the body from adverse external stimuli. The epidermis, a stratified squamous epithelium primarily composed of keratinocytes (Figure 1.2), continually renews itself in order to maintain appropriate epidermal thickness and repair wounds caused by environmental irritants (Eckert and Rorke, 1989). This epidermal regeneration is accomplished by mitotic divisions of a single cell layer termed the stratum basale. The highly proliferative keratinocyte basal layer of rodent epidermis is characterized by high levels of Cx43 and high levels of the intermediate filaments, CK5 and CK14 (Langlois et al., 2007; Moll et al., 1982; Presland and Dale, 2000).

Figure 1.2 The epidermis is a keratinocyte-rich stratified squamous epithelium

As keratinocytes residing in the stratum basale detach from the basement membrane, they begin to stratify as they differentiate towards the stratum corneum. The proteins listed on the right are markers of keratinocyte differentiation that are expressed in the indicated stratum (Adapted from Presland and Dale, 2000).

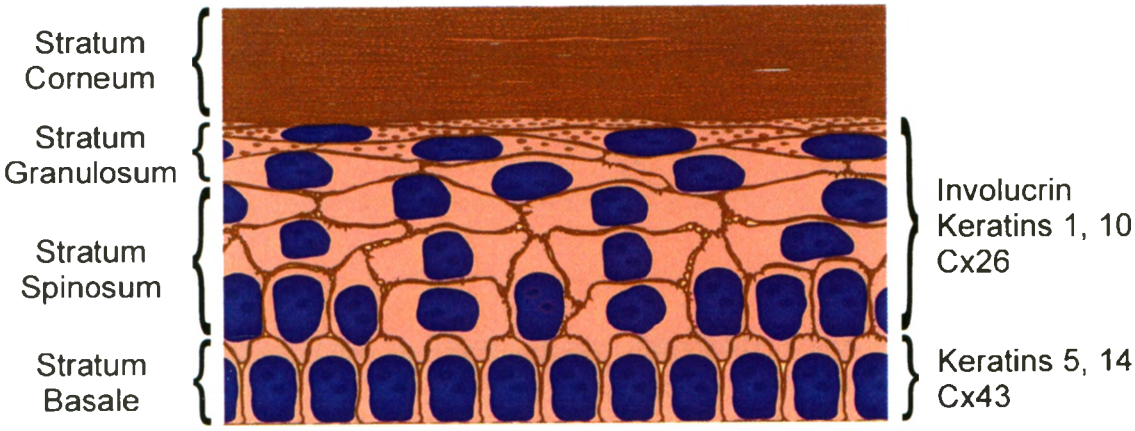
Stratum Corneum
Stratum Granulosum
Stratum Spinosum
Stratum Basale
Involucrin
Keratins 1, 10
Cx26
Keratins 5, 14
Cx43

Figure 1.2

1.9.3 Cytokeratins

Cytokeratins are fibrous, structural intermediate filament proteins that are encoded by a large, well-conserved multigene family of at least 49 proteins that form 8-12 nm filaments within the cytoplasm of epithelial cells (Hesse et al., 2004). The cytokeratins form a three-dimensional framework, connecting to desmosomes at cell-cell interfaces, and to hemidesmosomes at the junctions between the epidermis and the dermis (Cheng and Koch, 2004). The cytokeratins are subdivided into two groups, type I and type II keratins, depending on their sequences, isoelectric point, pH and size (Hesse et al., 2001). Type I keratins (CK9-CK23) are small (40-56.5 kDa), acidic (pKi = 4.5-5.5) proteins found in clusters on chromosomes 17q21 in the human and 11D in mice. On the other hand, type II keratins (CK1-8) are larger (52-67 kDa) and more basic (pKi = 5.5-7.5) found in clusters on chromosomes 12q13 in the human, and 15F in mice (Moll et al., 1982; Waterston et al., 2002a; Waterston et al., 2002b). CK5 and CK14 (50 kDa) are loosely bundled biochemical markers of the mitotically active basal layer of the epidermis and other stratified squamous epithelia (Dakir et al., 2008; Eichner et al., 1986).

As keratinocytes from the basal layer detach from the basement membrane, they begin to stratify as they migrate toward the surface of the skin and commence terminal differentiation (Eckert and Rorke, 1989). Since the skin is the largest organ of the body, it is essential that differentiation of keratinocytes is organized and highly regulated. This requires precise spacio-temporal regulation of various protein families, including but not limited to, bone morphogenic proteins (Takahashi and Ikeda, 1996), connexins (Langlois et al., 2007), caspases (Caulin et al., 1997) and cytokeratins (Koster and Roop, 2004),

which are critical in the development, differentiation, maintenance and regeneration of skin (Botchkarev and Sharov, 2004; Koster and Roop, 2007; Oshima, 2002).

The differentiating suprabasal layer is further sub-divided into the spinosum and granulosum layers of the epidermis. The stratum spinosum contains coarse bundles of tonofilaments and correlates with increased expression levels of Cx26, CK1 and CK10 (Bakirtzis et al., 2003; Mehrel et al., 1990; Nirunsuksiri et al., 1995; Presland and Dale, 2000). Differentiation specific CK1 and CK10 (56.5 kDa) leads to a reorganization of the keratin filament network, which is associated with an increase in the density of the bundling of keratin filaments (Eichner et al., 1986; Elias et al., 1988).

1.9.4 Involucrin

As differentiation continues, the granular layer is marked by keratinocytes that express high levels of loricrin and involucrin that begin to flatten as they continue to migrate towards the outermost cornified layer of the epidermis (Manabe and O'Guin, 1994; Presland et al., 2000). In the epidermis, involucrin (~80-110 kDa) is a precursor protein of the cross-linked cornified envelope (Beck et al., 2007; Phillips et al., 2000). It is an elongated alpha-helical rod-shaped protein, with the ability to cross-link multiple proteins, essential for cornified envelope formation and scaffolding (Beck et al., 2007; Eckert et al., 1993). In addition to increased expression of involucrin, the final stages of terminal differentiation undergoes a process called cornification where anucleated corneocytes become extremely flat and highly cross-link keratin filaments within a protein envelope (Eckert and Rorke, 1989).

1.10 Strict spatio-temporal regulation of keratinocyte processes

It is now known that the epidermis expresses Panx1, Panx3 and as many as ten connexins, which is likely representative of a complex regulation of single membrane channels and gap junctional intercellular communication (GJIC) required for development, differentiation and proliferation (Kretz et al., 2003; Laird, 2006; Langlois et al., 2007; Penuela et al., 2007; Wiszniewski et al., 2000). Although there exists functional redundancy between epidermal connexins, expression and localization patterns become differentially regulated during wound healing, various skin disorders and cancers including basal cell carcinoma and squamous cell carcinoma (Kretz et al., 2003; Kretz et al., 2004; Mese et al., 2007; Richard, 2000). Thus, it is possible that the spatio-temporal regulation of pannexins may also exist when co-expressed in the same tissue. For example, Cx43 is downregulated during wound healing and in both basal and squamous cell carcinomas (Kretz et al., 2004; Langlois et al., 2007; Mori et al., 2006). Interestingly, the loss of GJIC in tissues has been associated with increased susceptibility to tumorigenesis while increased levels of Cx43 have been shown to suppress cellular transformation when induced with carcinogenic agents (Haass et al., 2004; King and Bertram, 2005). On the other hand, Cx26 has been shown to be upregulated in hyperproliferative epidermis and carcinogenic conditions (Budunova et al., 1996; Goliger and Paul, 1994; Kretz et al., 2004; Richard, 2000). Although the specific cellular functions of pannexins remain obscure, the distinct localization of Panx1 and Panx3 recently observed in the epidermis provides evidence to suggest that spatio-temporal

expression of pannexin may exist in skin and have implications in fundamental keratinocytes processes.

1.11 Organotypic epidermis as a model of keratinocyte differentiation

Our study utilizes rat epidermal keratinocytes (REKs) which possess the ability to spontaneously differentiate when exposed to an air-liquid interface. REKs are grown to confluency on a collagen-coated transwell filter and upon exposure to air, are triggered to differentiate, and cultured for three-weeks. Organotypic epidermis can be easily manipulated and closely mimics newly generated rodent epidermis, with a defined proliferative basal layer, several suprabasal layers and a thick cornified layer. Similar to other techniques, such as gene knockout or silencing, used in various studies that investigate keratinocyte differentiation (Mildner et al., 2006; Wolff et al., 2009), the REK model expresses all the key markers of keratinocyte differentiation including CK14, CK10 and involucrin. In fact, REKs grown in both monolayer and three-dimensional organotypic epidermis have already proven to be useful models for investigating the role of connexins in keratinocyte differentiation (Ajani et al., 2007; Langlois et al., 2007; Maher et al., 2005; Marjukka Suhonen et al., 2003; Pasonen-Seppanen et al., 2003; Tammi et al., 2000; Thomas et al., 2007).

1.12 Rationale, Objectives and Hypothesis of the Research

1.12.1 Rationale

Although considerable evidence exists for Panx1 and Panx3 acting as membrane channels, the functional role of Panx1 and Panx3 channels at the cellular level remain unclear. In the current study, we characterize rodent pannexin localization in epidermal keratinocytes and analyze their putative roles in differentiation, proliferation and migration. We characterize the expression of Panx1 and Panx3 in rat epidermal keratinocytes (REKs) and in mouse epidermis under normal conditions and assess their role in differentiation. In fact, REKs grown in both monolayer and three-dimensional organotypic epidermis are useful models for investigating the role of various proteins in keratinocyte differentiation (Ajani et al., 2007; Langlois et al., 2007; Maher et al., 2005; Marjukka Suhonen et al., 2003; Pasonen-Seppanen et al., 2003; Tammi et al., 2000). Through over-expression studies in REKs, we reveal the putative roles of Panx1 and Panx3 as they pertain to the process of epidermal differentiation. Up until the beginning of this study, nothing was known about the endogenous localization of Panx1 and Panx3 in murine epidermis or the role of Panx1 and Panx3 in epidermal keratinocytes and their processes.

1.12.2 Hypothesis

It is hypothesized that Panx1 and Panx3 are differentially regulated in keratinocytes and play distinct roles in the differentiation, proliferation and/or maintenance of the epidermis.

1.12.3 Objectives

1. To characterize the endogenous expression and localization of Panx1 and Panx3 in murine epidermis.
2. To characterize the endogenous expression and localization of Panx1 and Panx3 in monolayer REKs and organotypic epidermis.
3. To assess the putative roles of Panx1 and Panx3 in keratinocyte proliferation, migration and differentiation.

1.13 References

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CHAPTER 2

IMPLICATIONS OF PANNEXIN 1 AND PANNEXIN 3 IN KERATINOCYTE DIFFERENTIATION[1]

[1] A similar version of this chapter is currently being reviewed for publication.

2.1 Summary

Pannexin (Panx) 1 and Panx3 are integral membrane proteins that have sequence homology to the innexin family of invertebrate gap junctions and are expressed in mammalian skin. Panxs form functional mechano-sensitive, single membrane channels but their importance in regulating cellular function is poorly understood. In the current study, we demonstrate reduced Panx1 expression in both thin and thick murine skin while Panx3 expression remained unchanged, when compared to newborn mice. To investigate the role of Panxs in keratinocyte differentiation, we employed rat epidermal keratinocytes (REKs) that have the capacity to differentiate into organotypic epidermis and engineered them to over-express Panx1, Panx1-GFP or Panx3. Compared to monolayer REKs, endogenous Panx1 levels remained unchanged while the 70 kDa species of Panx3 greatly increased in organotypic epidermis. In monolayer cultures, ectopic Panx1 and Panx1-GFP localized to the plasma membrane while Panx3 displayed both intracellular and plasma membrane profiles. While ectopic expression of both pannexins reduced cell proliferation, only Panx1 disrupted the architecture of organotypic epidermis and markedly dysregulated cytokeratin 14 expression and localization. Furthermore, expression of only Panx1 reduced the vital layer thickness of organotypic epidermis. In summary, Panx1 and Panx3 are co-expressed in the mammalian epidermis and play distinct roles in regulating keratinocyte proliferation and differentiation.

2.2 Introduction

A family of vertebrate proteins, termed pannexins (Panxs), are homologous in sequence to invertebrate innexins and thus were originally proposed to have functions similar to both innexin and connexin gap junctions (Panchin et al., 2000). Three pannexin family members, Panx1, Panx2 and Panx3, were identified in humans, rats and mice but their functions within mammalian cells remain poorly understood (Baranova et al., 2004). Panx1 transcripts are ubiquitously expressed in normal rodent and human tissues, and appear to be enriched in the central nervous system (Baranova et al., 2004; Penuela et al., 2007). Recently, the Panx1 glycoprotein was detected in several murine tissues including brain, spleen, cartilage and skin (Penuela et al., 2007). On the other hand, Panx2 expression has been reported to be more restricted to many cell types of the brain and retina. Two species of Panx3 of ~43 kDa and 70 kDa were found to be expressed in skin, cartilage and the heart (Penuela et al., 2007). Overall, while our understanding of the diverse localization patterns of pannexins is beginning to unfold, the cellular role(s) of pannexins remain relatively obscure.

Despite the controversy surrounding whether pannexins form intercellular channels, reports continue to support the role of pannexins playing a critical role in cellular communication with the extracellular environment through single-membrane channels. Panx1 has been shown to be able to oligomerize into hexameric structures that traffic to the cell surface (Boassa et al., 2007) to form single membrane channels that can be blocked by pharmacological reagents (Bruzzone et al., 2005). Upon mechanical stress, Panx1 single-membrane channels have been implicated in the release of ATP, resulting in Ca^{2+} wave propagation through ATP-induced purinergic receptor activation

(Bao et al., 2004). Additionally, Silverman et al. (2009) revealed that Panx1 responds to high extracellular K^+ levels and was associated with $P2X_7R$-mediated activation of the caspase-1 cascade involved in neural inflammasome activation. Although much less is known about the channel capabilities of Panx3, Penuela et al. (2007) showed that both Panx1 and Panx3 are glycoproteins capable of dye uptake supporting their role as functional single-membrane channels.

As pannexins continue to be better understood as single-membrane channels, distinct subcellular and tissue expression profiles of endogenous and ectopically-expressed Panx1 and Panx3 are beginning to emerge. Penuela et al. (2007) showed that exogenously expressed Panx1 and Panx3 localized predominantly at the cell surface in normal rat kidney and BICR-M1R_K cell lines as well as formed functional single-membrane channels in 293T cells. Tissue specific localization of endogenous Panx1 includes post-synaptic sites in cortical and hippocampal neurons suggesting a potential role in regulating neuronal post-synaptic activity (Zoidl et al., 2007). In MDCK cells, endogenous Panx1 and Panx3 were localized to the cell surface (Boassa et al., 2007; Penuela et al., 2008) while Panx1 was found to be uniformly distributed throughout intracellular compartments in the mouse spleen (Penuela et al., 2007). On the other hand, Panx1 and Panx3 were found predominantly at cell surface and intracellular locations, respectively, in human facial skin keratinocytes suggesting that these pannexins may have distinct roles in skin differentiation, maintenance or regeneration (Penuela et al., 2007). While punctate Panx1 localization was detected chiefly in the stratum granulosum and spinosum layers of human facial epidermis, Panx3 expression exhibited a more scattered distribution throughout all epidermal layers, and appeared to have a subcellular

distribution localized to intracellular compartments (Penuela et al., 2007). Together, the distinct cellular and subcellular localization profiles of Panx1 and Panx3 provide evidence to suggest that distinct spatio-temporal expression of pannexins may exist in skin and have implications in fundamental keratinocyte processes.

The strict spatio-temporal regulation of bone morphogenic proteins (Takahashi and Ikeda, 1996), connexins (Langlois et al., 2007), caspases (Caulin et al., 1997) and cytokeratins (Koster and Roop, 2004) are critical in the development, differentiation, maintenance and regeneration of skin (Botchkarev and Sharov, 2004; Koster and Roop, 2007; Oshima, 2002). For example, high levels of connexin 43 (Cx43) and intermediate filaments cytokeratin 14 (CK14) and CK5 are expressed in the basal layer of rodent epidermis and denote the keratinocytes that are highly proliferative (Langlois et al., 2007). The subsequent detachment of basal keratinocytes from the basement membrane initiates differentiation and correlates with increased expression levels of Cx26, CK1 and CK10 (Bakirtzis et al., 2003). As differentiation continues, high levels of loricrin and involucrin expression are detected in keratinocytes of the granulosum layer and the highly cross-linked cornified envelope (Presland et al., 2000). Similar to other techniques, such as gene knockout or silencing, used in various studies that investigate keratinocyte differentiation (Mildner et al., 2006; Wolff et al., 2009), our REK model closely mimics the differentiation of normal rodent epidermis by expressing all the key markers of keratinocyte differentiation. In fact, REKs grown in both monolayer and three-dimensional organotypic epidermis have already proven to be useful models for investigating the role of various proteins in keratinocyte differentiation (Ajani et al.,

2007; Langlois et al., 2007; Maher et al., 2005; Marjukka Suhonen et al., 2003; Pasonen-Seppanen et al., 2003; Tammi et al., 2000; Thomas et al., 2007).

In the current study, we hypothesize that Panx1 and Panx3 are differentially regulated in keratinocytes and play distinct roles in the differentiation, proliferation and/or maintenance of the epidermis. To that end, we characterized the expression of Panx1 and Panx3 in mouse epidermis under steady-state conditions and also assessed their role in rat epidermal keratinocyte (REK) differentiation into organotypic epidermis.

2.3 Materials and Methods

2.3.1 Generation of Panx1, Panx1-GFP and Panx3 Stable Cell Lines

All cell culture reagents and labware were obtained from Invitrogen (Burlington, ON) and Becton-Dickenson (BD; Franklin Lakes, NJ). Commercially available REK cell lines were cultured in Dulbecco's Modified Essential Medium (DMEM) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100 ug/ml streptomycin and 2 mM L-glutamine with a humid environment at 37°C and 5.0% CO_2 as described in Maher et al. (2005). Prior to reaching confluency, cells were passed by enzymatic dissociation with 0.25% Trypsin/EDTA solution at 37°C for 5-15 minutes. Mouse Panx1 and Panx3 expression constructs were generated as described in Penuela et al. (2007). Briefly, Panx1 and Panx3 was isolated from 3-week old mouse osteoblasts and/or brain and PCR products were amplified using primers designed to include the full-length coding regions of the two genes. The PCR products were cloned by pGEM-T Easy vector systems (Promega, Madison, WI) and cloned into expression vectors. REK cell lines were engineered to express an empty vector (V), Panx1, green fluorescent protein-tagged Panx1 (Panx1-GFP) or Panx3 using sequence-validated cDNA constructs incorporated into AP2 replication-defective retroviral vectors as described in Galipeau et al. (1999). The GFP tag was fused to the carboxy-terminal of Panx1. Infection protocols were performed serially 3 times as described in Mao et al. (2000). Briefly, OptiMEM1 medium containing Lipofectamine2000 was used to transfect each of the constructs into the 293 GPG packaging cell line to produce replication-defective virus-containing supernatant. Supernatants were filtered through 0.45 mm filters then incubated on low density plated wild-type (WT) REKs in 35 mm dishes at 37°C as previously described

(Mao et al., 2000; Thomas et al., 2007). Up to passage 4, pannexin expression was 85-95% efficient as determined by immunofluorescent labeling for pannexins and assessing the percentage of cells (n>2000 cells) expressing pannexins in comparison to cells positive for the Hoechst 33342 nuclear stain (data not shown). For all experiments, passage 2-4 cells were used.

2.3.2 Organotypic Epidermis as a Model of Keratinocyte Differentiation

REK cell lines were used to grow organotypic epidermis as described by Maher et al. (2005) and Thomas et al. (2007). Briefly, $5x10^5$ cells were plated on 24 mm transwell filter inserts containing 3 μm high density pores (BD Labware, Franklin Lakes, NJ) coated with 1 ml type I rat tail collagen (BD Biosciences, Bedford, MA) and bathed in 2 ml DMEM above and below the filter. When the cells grew to confluency after 3 days, the top media was removed exposing the REKs to air. This stimulated REK differentiation which was allowed to proceed for 21 days, while the media below the filter was changed daily.

2.3.3 Dissection of Murine Skin

Mouse skin was obtained and handled in accordance with the standard operating procedures outlined by the University of Western Ontario and the International Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). In order to identify the localization of Panx1 and Panx3 in skin, 1 cm^2 biopsies were obtained from newborn and 3-week old (adult) C57/BL6 mice. Specifically, dorsal portions of the trunks of newborn and shaved

dorsal (thin) and paw (thick) adult skin were collected and subsequently used for histochemistry and Western blot studies.

2.3.4 Immunocytochemistry

In order to study the localization of Panx1 and Panx3 at the cellular level in keratinocytes, REKs were grown on coverslips and immunolabeled as previously described in Thomas et al. (2007) with some modifications. Briefly, cells were fixed in an 80% methanol/20% acetone solution for 20 minutes at 4°C and blocked with 3% bovine serum albumin (BSA; Sigma-Aldrich, St. Louis, MO) in PBS at room temperature for 45 minutes. Cells were incubated with 2 μg/ml anti-Panx1 or -Panx3 affinity-purified primary antibodies, generated and described by Penuela et al. (2007), protein disulfide isomerase (PDI; 1:500; Stressgen, Vancouver, BC), or the anti-G_3G_4 directed against bromodeoxyuridine (BrdU; 1:100; Developmental Studies Hybridoma Bank; Iowa City, IA) primary antibody for 1 hour at room temperature. Cells were then incubated with secondary antibodies AlexaFluor® 555, AlexaFluor® 488 (1:500; Invitrogen, Burlington, ON) or Texas Red (1:200; Jackson ImmunoResearch, West Grove, PA) for 1 hour at room temperature, followed by counterstaining with 10 μg/ml Hoechst 33342 to denote nuclei, and mounted with Airvol. As a negative control, peptide pre-adsorption assays were performed exactly as described in Penuela et al. (2007) in order to account for any non-specific staining and fully appreciate the specificity of our purified Panx1 and Panx3 antibodies. Briefly, a 10:1 molar ratio of Panx1 or Panx3 peptide to its respective antibody was incubated for 30 minutes at room temperature. Using identical conditions to primary antibody alone, peptide competitions were incubated on cell samples in

parallel immunolabeling studies. All immunolabeling experiments were analyzed using a Zeiss (Thornwood, NY) LSM 510 Meta inverted confocal microscope system as described in Thomas et al. (2007) except that a 63x oil objective lens was used to assess the localization patterns. Each experimental group was imaged under identical instrument configuration settings.

2.3.5 REK Proliferation Assay

To assess the proliferation status of our engineered REK cell lines, $2x10^4$ cells were plated on coverslips in 35 mm dishes for 24 hours and subsequently incubated with 10 μM BrdU (Sigma-Aldrich, St. Louis, MO) for 4 hours at 37°C. Cells were processed for immunocytochemistry as described above, except that DNA was subsequently denatured using 2 M HCl for 20 minutes at room temperature following fixation. At least 2000 cells per cell line (n=5) were counted and the percentage of BrdU-positive staining nuclei versus total nuclei denoted by Hoechst 33342 staining was assessed.

2.3.6 REK Migration Assay

The migration capabilities of REK cell lines were assessed using a scrape assay, commonly used in wound healing studies, and described by Thomas et al. (2007) with the following modifications. Cells were plated at a concentration of $2x10^5$ on 35 mm dishes with grid. When cells were confluent and appeared cobblestone-like after 36 hours, a 16 cm rubber cell scraper (Sarstedt, Newton, NC) was used to carefully scrape cells off the center of the dish. Cells were allowed to migrate in OptiMEM1 medium for 48 hours then fixed with 3.7% formaldehyde for 30 minutes at room temperature and viewed using

a Leica microscope. At least 50 images per cell line (n=5) that displayed clear cell migration were blindly selected and measured in 3 predetermined locations using Adobe Photoshop CS2 software and averaged.

2.3.7 Organotypic Epidermis and Murine Skin Histochemistry

In order to determine the architecture and localization of Panx1 and Panx3 in organotypic epidermis and murine skin, samples were preserved and processed as previously described in Thomas et al. (2007) with some modifications. Approximately 1 cm^2 samples were preserved in 10% neutral buffered formalin overnight at 4°C. Samples were dehydrated in a graded series of ethanol, then xylene and embedded in paraffin wax. Using the Leica RM2125RT microtome (Leica Microsystems, Nussioch, GmbH), 5-7 μm sections were prepared on glass slides. Slides were then independently stained with Harris Hematoxylin and Eosin-Y (H&E) for 5 minutes each and mounted with Cytoseal (Canadawide Scientific, Ottawa, ON) to reveal the epidermal architecture. Stained sections were subsequently viewed with an AxioPlan2 microscope (Carl Zeiss, GmbH) equipped with a 63x lens and imaged using an AxioCam camera via AxioVision LE Rel. 4.3 imaging software (Carl Zeiss, GmbH).

To assess the differentiation capabilities of pannexin over-expressing cell lines, vital layer thickness was measured using H&E stained organotypic epidermis slides using the Axiovision software. Three measurements, at pre-determined locations (60, 120, 180 μm along the x-axis ruler) were taken per image at right angles from the bottom of the basal layer to the top of the suprabasal layer and averaged. At least 50 images were used per cell line collected from at least 5 epidermal preparations (n=5).

Immunohistochemistry of tissue samples were also used to examine the localization of Panx1 and Panx3 as described by Penuela et al. (2007) with some modifications. Briefly, antigen retrieval was performed on all slides using 0.01 M sodium citrate buffer, pH 6.0 and microwaved for 2.5 minutes at 80% power. Non-specific labeling was blocked with 3% BSA and 0.1% Triton-X-100 (Sigma-Aldrich, St. Louis, MO) for 45 minutes at room temperature. Affinity purified anti-Panx1 and anti - Panx3 antibodies (4 μg/ml; Penuela et al., 2007), as well as, anti-CK14 (1:100; Neomarkers, Fremont, CA) or anti-CK10 (1:100; Neomarkers, Fremont, CA) primary antibodies were gently shaken and incubated at 4°C overnight. Samples were incubated with secondary antibodies AlexaFluor® 555 or AlexaFluor® 488 (1:500; Invitrogen, Burlington, ON) for 1 hour at room temperature and counterstained with 10 μg/mL Hoechst 33342 to denote nuclei prior to mounting with Vectashield (Vector Laboratories, Burlingame, CA). As a negative control, peptide pre-adsorption assays were used in parallel as described above. Analysis was performed using the Zeiss (Thornwood, NY) LSM 510 Meta inverted confocal microscope system.

2.3.8 Immunoblotting

In order to determine the levels of Panx1 and Panx3, lysates were extracted from cell lines, organotypic epidermis and murine skin using a Triton-based extraction buffer as described by Penuela et al. (2007), homogenized physically and sonication for 10 seconds on ice. Protein was quantified using a bicinchonic acid assay (Pierce, Rockford, IL). Twenty-five μg of cell lysates and 35 μg of tissue lysates were boiled for 10 minutes, separated using 10% SDS-PAGE and then transferred to a nitrocellulose

membrane. Membranes were blocked with 5% Blotto, non-fat dry milk (Santa Cruz Biotechnology, Santa Cruz, CA) and 0.05% Tween®20 in PBS and gently shaken for 30 minutes at room temperature. Affinity purified anti-Panx1 or anti-Panx3 antibody (0.4 μg/ml; Penuela et al., 2007), as well as, anti-CK14 (1:1000; Neomarkers, Fremont, CA) or anti-involucrin (1:1000; Covance Research Products, Princeton, NJ) primary antibodies were gently rocked in the presence of the antibody solution and incubated at 4°C overnight. Anti-GAPDH antibodies (1:20000; Chemicon/Millipore, Temecula, CA) were used to assess equal loading. Following washes with PBS-Tween®20, either AlexaFluor® 680 (1:5000; Invitrogen, Burlington, ON) or IRDye™ 800 (1:5000; Rockland, Gilbertsville, PA) secondary antibodies were used, and membranes were visualized with an Odyssey infrared imaging system (LiCor, Lincoln, NE). As a negative control, peptide pre-adsorption assays were used as described above. Densitometry of unsaturated images was analyzed using Odyssey 2.0.4 application software (LiCor, Lincoln, NE). Quantified protein levels were normalized to GAPDH and expressed relative to control samples (newborn skin or monolayer wild-type REKs). Western blot results were averaged for at least 3 mouse skin experiments and 5 monolayer or organotypic epidermis culture experiments.

2.3.9 Statistical Analysis

For all quantified experiments, the raw data was subjected to a one-way analysis of variance (ANOVA) to determine if significance existed between the experimental groups. For the proliferation, migration and vital layer measurements, a Tukey's multiple comparison post-hoc test was used to indicate if significant differences existed between

any of the groups ($p<0.05$). Western blot analysis was coupled to the Dunnett's multiple comparison post-hoc test to indicate significant differences, if any, between the control group (newborn skin or wild-type monolayer REKs) and the experimental groups ($p<0.05$). All tests were performed and graphed using GraphPad Prism 4.03 software.

2.4 Results

2.4.1 Differential Panx1 and Panx3 localization and expression levels in murine skin

The integrity of the mouse tissue sections was first evaluated by H&E staining of newborn skin, 3-week old thin skin (dorsal), three week old thick skin (paw) and skin containing sebaceous glands (Figure 2.1A). Immunohistochemistry revealed that Panx1 was localized to intracellular compartments of suprabasal layer cells of newborn skin and throughout all vital layers of epidermal thin skin. In contrast, Panx1 expression was below detectable levels in thick skin and sebaceous glands (Figure 2.1B). On the other hand, Panx3 exhibited diffuse intracellular localization throughout all vital epidermal layers of thin skin, most notably in the basal layer and keratin-expressing sebocytes (Figure 2.1D). Unlike Panx1, a diffuse intracellular staining profile of Panx3 was revealed in all vital epidermal layers of thick skin, but went undetected in newborn skin. These results suggest that Panx1 and Panx3 may play diverse roles in murine skin, temporally and spatially. To ensure that all immune-staining was specific, a peptide pre-adsorption assay was used to block anti-Panx antibody binding (Figure 2.1C, E).

Figure 2.1 Panx1 and Panx3 are differentially expressed within murine skin

Images of murine skin biopsies stained with H&E (A) reveal the epidermal architecture of newborn, as well as thin and thick epidermis of 3-week old mice with the basal layer located above the dotted line and the cornified layer above the dashed line. An image of a sebaceous gland (arrowheads) next to hair follicles was obtained from the dorsal area of a 3-week old mouse. Tissue sections were stained with either anti-Panx1 or anti-Panx3 antibodies (B, D) or the same antibodies in the presence of their respective cognate peptides (C, E) and counterstained with Hoechst (blue). Staining profiles revealed intracellular Panx1 (B; red) localization in the suprabasal layer of newborn skin and throughout all layers of thin epidermis. In contrast, Panx1 was not detectable in thick skin and barely detectable in sebaceous glands. On the other hand, Panx3 (D) was detectable in all thin and thick epidermal layers, but favored an intracellular localization profile in the basal layer and sebaceous glands (arrowheads) of thin skin. In contrast to Panx1, Panx3 was barely detectable in newborn skin. Bars, 20 μm.

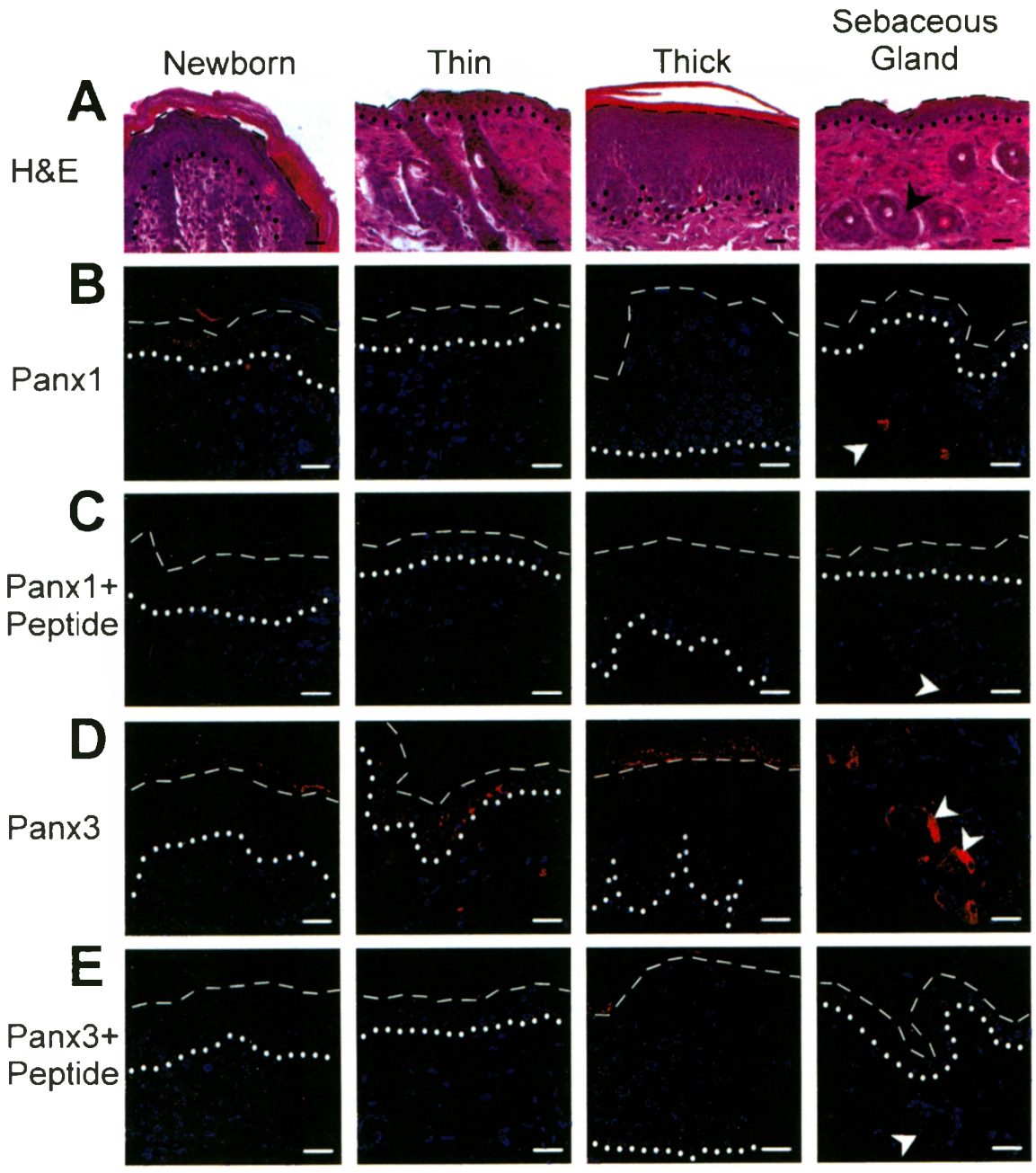

Newborn
Thin
Thick
Sebaceous Gland
A
H&E
B
Panx1
C
Panx1+ Peptide
D
Panx3
E
Panx3+ Peptide

Figure 2.1

To further confirm the expression of Panx1 and Panx3, skin samples were subjected to immunoblotting (n=3). Since Panx1 is extensively glycosylated (~41-48 kDa) it resolves as multiple bands within a Western blot (Penuela et al., 2007). Panx1 levels were significantly less in 3-week old thin and thick murine skin compared to newborn ($p<0.05$; Figure 2.2A, B). On the other hand, Panx3 levels were similar between newborn and 3-week old skin which included both the ~43 kDa species and the 70 kDa species ($p>0.05$; Figure 2.2C, D). These results support the notion that Panx1, but not Panx3, may be temporally regulated. Somewhat expected, involucrin, but not CK14, had significantly increased expression levels in thick skin compared to newborn ($p<0.05$; Figure 2.2E, F). In all cases, GAPDH was used as a housekeeping loading control.

Figure 2.2 Panx1, but not Panx3, expression levels decrease in aging and thickened murine skin compared to newborn

Western blots of Panx1, Panx3, CK14 and involucrin from newborn, thin and thick skin (A). GAPDH was used as a loading control. As a negative control, peptide pre-adsorption assays were used to determine antibody specificity while over-expression of Panx1 or Panx3 in REK cell lines served as a positive control. Panx1 expression was less in 3-week old thin and thick skin compared to newborn (B) while the expression levels of both the ~43 kDa and 70 kDa species of Panx3 remained relatively unchanged (C, D). Asterisks denote statistical significance ($p<0.05$). Although the ~43 kDa species appeared to increase in thick skin, this was not statistically significant. CK14 exhibited no change in expression between the different mouse skin types (E), however, involucrin expression did increase in thick skin compared to newborn (F). Bar graphs represent normalized means ± s.e.m. The results shown here are representative of samples obtained in parallel and repeated at least 3 times.

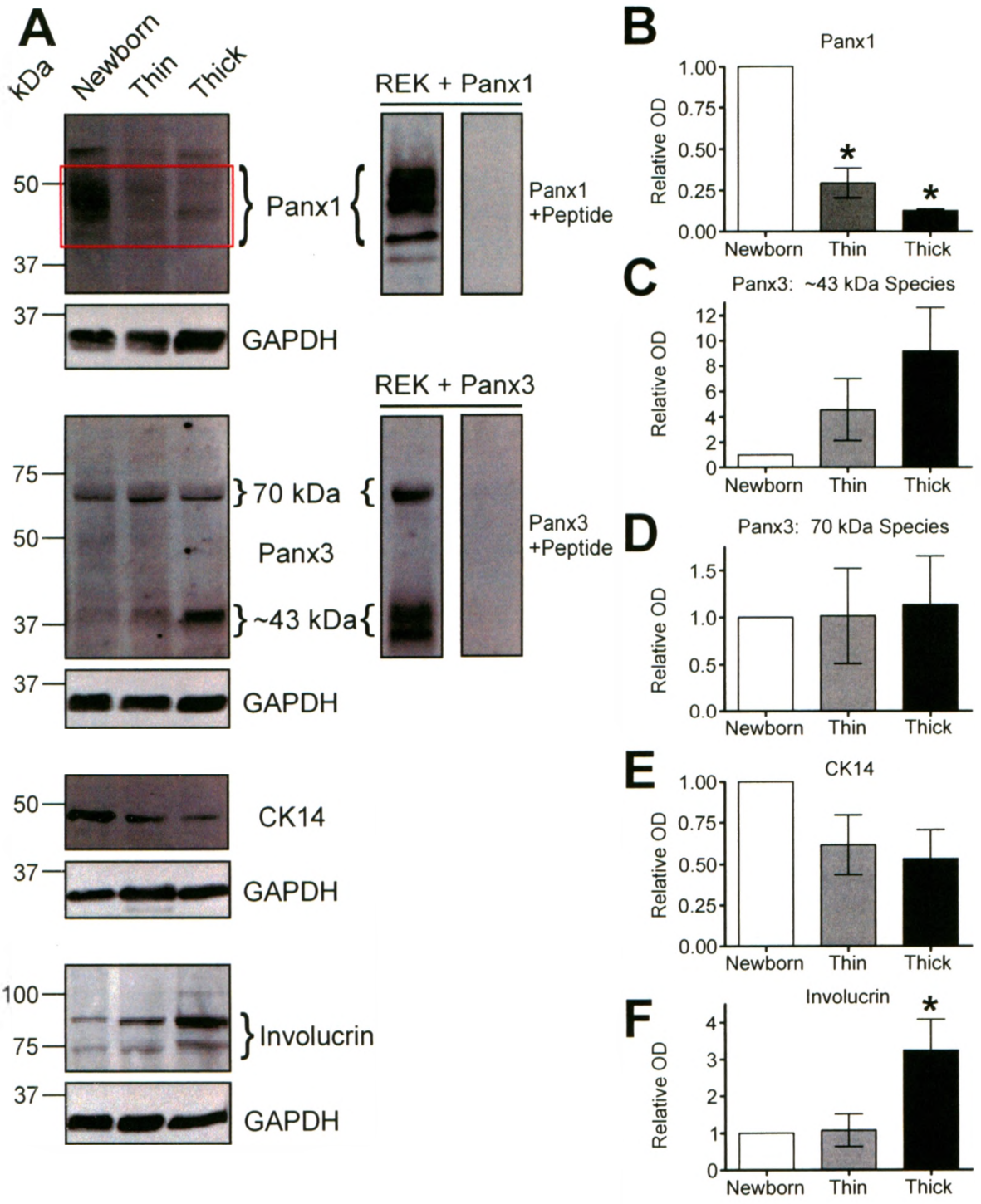

A
kDa
Newborn
Thin
Thick
REK + Panx1
50
37
Panx1
Panx1
+Peptide
37
GAPDH
REK + Panx3
75
50
37
70 kDa
Panx3
~43 kDa
Panx3
+Peptide
37
GAPDH
50
CK14
37
GAPDH
100
75
Involucrin
37
GAPDH
B
Panx1
Relative OD
1.00
0.75
0.50
0.25
0.00
Newborn
Thin
Thick
C
Panx3: ~43 kDa Species
Relative OD
12
10
8
6
4
2
0
Newborn
Thin
Thick
D
Panx3: 70 kDa Species
Relative OD
1.5
1.0
0.5
0.0
Newborn
Thin
Thick
E
CK14
Relative OD
1.00
0.75
0.50
0.25
0.00
Newborn
Thin
Thick
F
Involucrin
Relative OD
4
3
2
1
0
Newborn
Thin
Thick

Figure 2.2

2.4.2 Localization of Ectopically Expressed Panx1 and Panx3 in REKs

Rat epidermal keratinocytes (REKs), which did not express detectable levels of Panx1 or Panx3 by immunocytochemistry (Figure 2.3A) were engineered to express these Panxs. When Panx1 or Panx1-GFP were expressed in REKs, they predominantly localized to the cell surface (Figure 2.3B; green) with little co-localization with the endoplasmic reticulum resident protein, PDI (Figure 2.3B, C). On the other hand, Panx3 localized to the cell surface in an estimated 70% of the cell population (Figure 2.3D; green) while exhibiting an intracellular co-localization pattern with PDI in ~30% of the cell population (Figure 2.3E; yellow). The differential localization patterns observed between Panx1 and Panx3 suggest that these two pannexins may play distinct roles within keratinocytes as they enter different states of differentiation. Anti-Panx labeling was confirmed by peptide pre-adsorption assays (Figure 2.3B, D; inserts).

Figure 2.3 Ectopically expressed Panx1 and Panx3 are predominantly localized to the cell surface in REKs

Endogenous Panx1 and Panx3 were undetectable by immunocytochemistry in REKs (A). When over-expressed, Panx1 (B) and Panx1-GFP (C; green) were localized to the cell surface in REKs, and did not co-localize with PDI (red). Similar to Panx1 and Panx1-GFP, about 70% of Panx3 over-expressing cells (D) localized Panx3 to the cell surface while the remaining cells revealed an intracellular distribution pattern that co-localized with PDI (E, yellow). Peptide pre-adsorption assays eliminated Panx1 and Panx3 immunolabeling (B, D inserts). Hoechst nuclear stain is noted in blue. Bars, 20 μm.

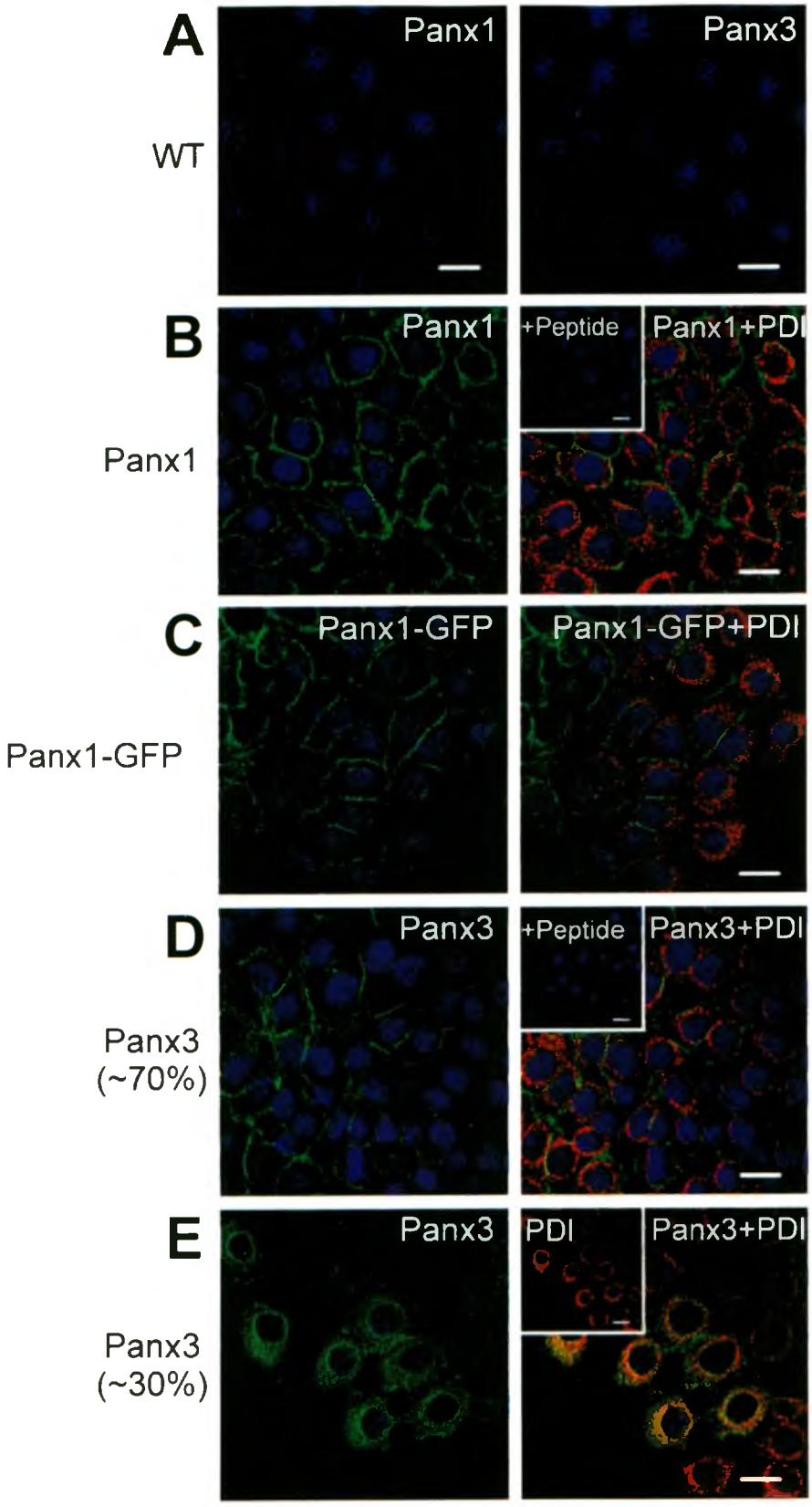

A
WT
Panx1
Panx3
B
Panx1
Panx1
+Peptide
Panx1+PDI
C
Panx1-GFP
Panx1-GFP
Panx1-GFP+PDI
D
Panx3
(~70%)
Panx3
+Peptide
Panx3+PDI
E
Panx3
(~30%)
Panx3
PDI
Panx3+PDI

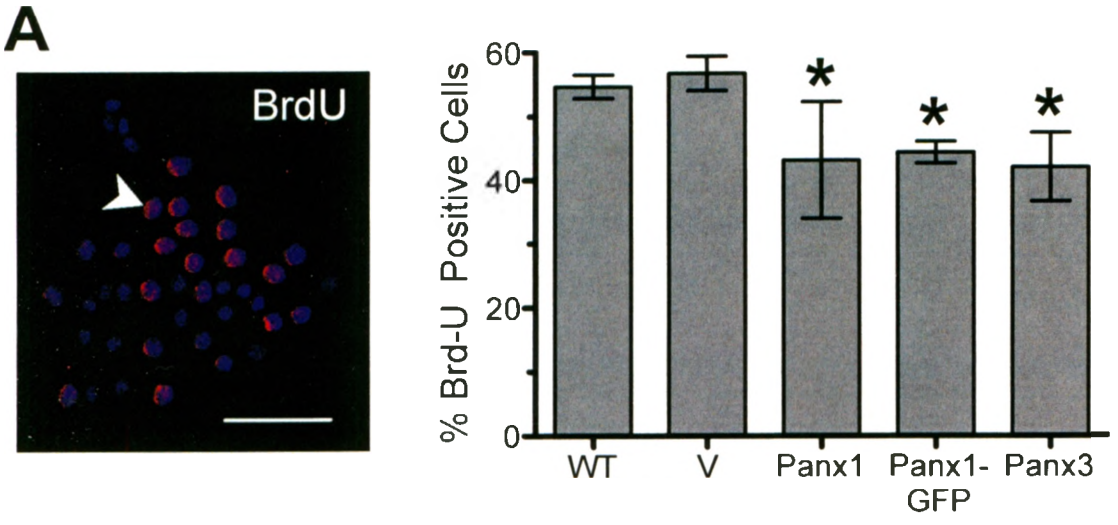
Figure 2.3

2.4.3 Pannexins decreased cell proliferation while migration properties remained unchanged

To further characterize pannexin expressing REKs, we assessed the proliferative status of our REKs using a BrdU incorporation assay (n=5). Over-expression of Panx1, Panx1-GFP or Panx3 decreased the percentage of BrdU positive REKs by ~15-20% compared to both wild-type and empty vector controls ($p<0.05$; Figure 2.4A). However, a cell migration assay (n=5) revealed that pannexin-expressing REKs migrated equally well as controls over a 48 hour time period ($p>0.05$; Figure 2.4B).

Figure 2.4 Panx1 and Panx3 reduced keratinocyte proliferation but had no effect on cell migration

BrdU incorporation revealed that the proliferation rate of REKs over-expressing Panx1 and Panx3 was reduced. BrdU-positive nuclei (pink) were expressed as a percentage of total Hoechst-stained nuclei (blue) per viewing field (A). Asterisks denote statistical significance compared to both wild-type and empty vector controls ($n=5$, $p<0.05$). A scrape assay revealed that Panx1 or Panx3 did not influence the migration abilities of REKs after 48 hours (B; $n=5$, $p>0.05$). Bars, 100 μm (A) and 0.2 mm (B). Bar graphs show means ± s.d.


A
BrdU
% Brd-U Positive Cells
60
40
20
0
*
*
*
WT
V
Panx1
Panx1-GFP
Panx3

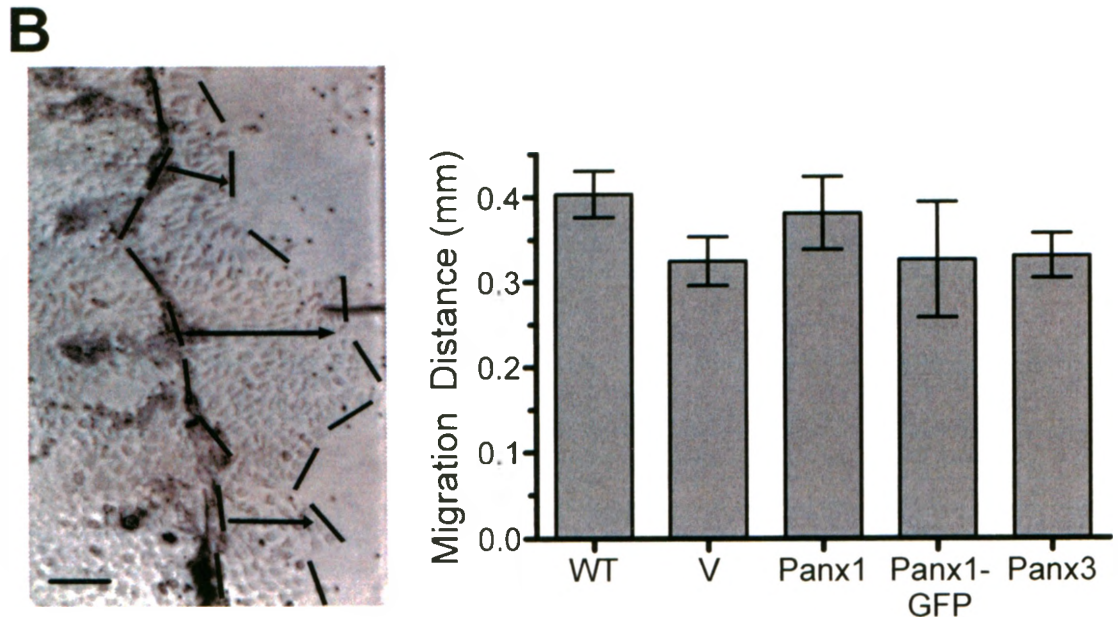

B
Migration Distance (mm)
0.4
0.3
0.2
0.1
0.0
WT
V
Panx1
Panx1-GFP
Panx3

Figure 2.4

2.4.4 Panx1 expression is maintained in organotypic epidermis

In order to assess the role of pannexins in REK differentiation, we employed a liquid-air chamber that induced the differentiation of wild-type REKs into a 3-5 cell thick vital layer and an overlying cornified layer (Figure 2.5). In monolayer cultures, multiple glycosylated species of endogenous Panx1 were only revealed in wild-type REKs when the gels were over-exposed (Figure 2.6A; WT (2) lane). On the other hand, REKs exhibited a high expression of exogenously expressed Panx1 or Panx1-GFP when grown in either monolayer or as organotypic cultures and the pannexin labeling pattern could be competed with cognate peptide (Figure 2.6A-C). Quantification of these data normalized to GAPDH, revealed no significant difference in the expression levels of Panx1 in organotypic epidermis as compared to monolayer culture ($p>0.05$).

Figure 2.5 REKs differentiate into organotypic epidermis modeling murine skin

When grown on a collagen-coated filter and exposed to air, REKs are able to differentiate into organotypic epidermis within 3 weeks complete with 3-5 vital layers and a cornified layer. Bar, 10 μm.

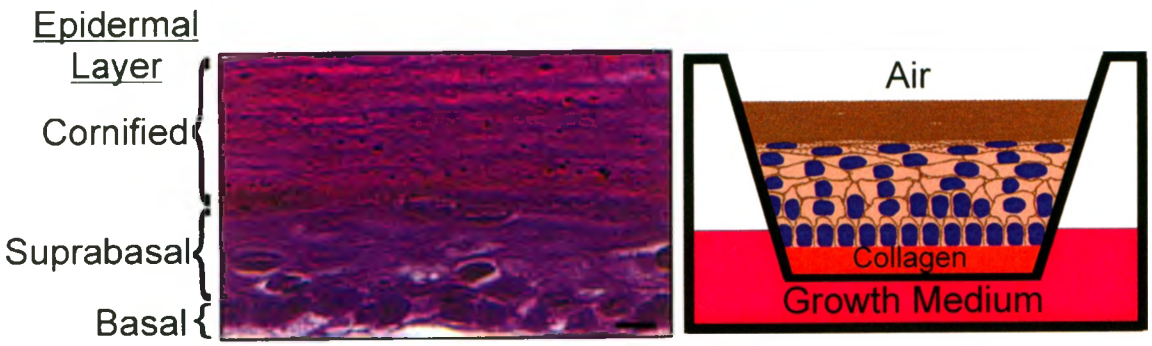

Epidermal Layer
Cornified
Suprabasal
Basal
Air
Collagen
Growth Medium

Figure 2.5

Figure 2.6 Exogenous and endogenous expression of Panx1 in monolayer cultures and organotypic epidermis

Western blotting (A) and subsequent densitometric quantification (C) revealed that over-expression of Panx1 (~41-48 kDa), and Panx1-GFP remained stable after multiple passages and when grown into organotypic epidermis. Wild-type REKs expressed low levels of Panx1 as revealed by over-exposing the immunoblots (A; WT (2) lane). GAPDH was used as a loading control and asterisks denote statistical significance compared to monolayer wild-type REKs ($p<0.05$). Pre-adsorption of the anti-Panx1 antibody with its cognate peptide eliminated all antibody binding to Panx1 and Panx1-GFP (B). Bar graphs show normalized means ± s.e.m. The results shown here are representative of experiments that were repeated at least 5 times.

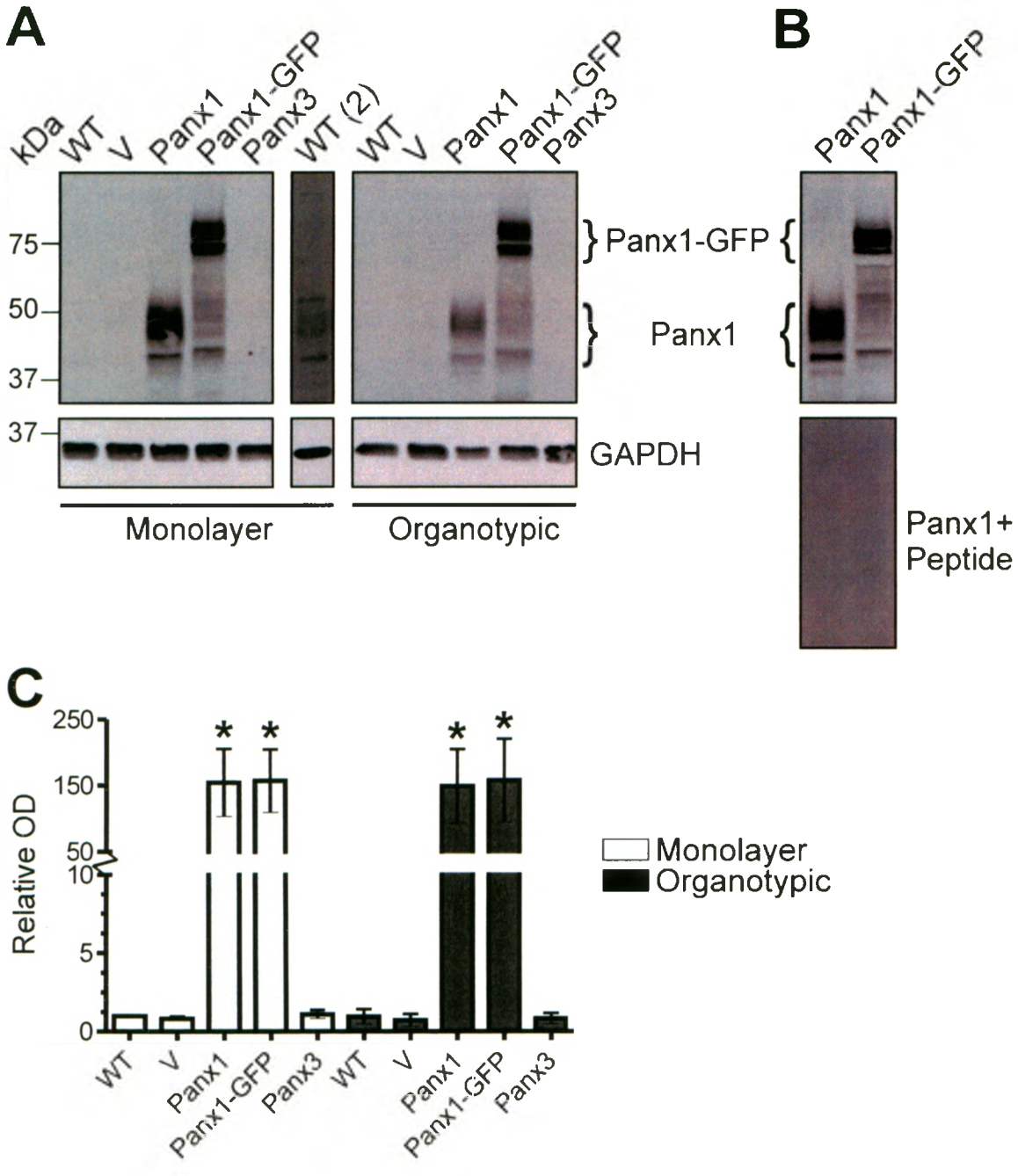

A
kDa
WT
V
Panx1
Panx1-GFP
Panx3
WT (2)
WT
V
Panx1
Panx1-GFP
Panx3
75
50
37
37
Panx1-GFP
Panx1
GAPDH
Monolayer
Organotypic
B
Panx1
Panx1-GFP
Panx1+
Peptide
C
Relative OD
250
150
50
10
5
0
*
*
*
*
WT
V
Panx1
Panx1-GFP
Panx3
WT
V
Panx1
Panx1-GFP
Panx3
Monolayer
Organotypic

Figure 2.6

2.4.5 The 70 kDa species of endogenous Panx3 increases when REKs are grown as organotypic epidermis

Low levels of the 70 kDa species of endogenous Panx3 were detected by Western blotting in monolayer REKs (Figure 2.7A) and this species greatly increased when cells were grown as organotypic epidermis suggesting it may be important for keratinocyte differentiation ($p<0.05$; Figure 2.7A, D). In contrast, the well-characterized doublet species of Panx3 at ~43 kDa exhibited no observable change in expression levels in organotypic epidermis compared to monolayer ($p>0.05$; Figure 2.7A, C). When Panx3 was ectopically expressed in REKs, the doublet at ~43 kDa continued to be highly expressed when cells were grown in monolayers or as organotypic epidermis (Figure 2.7A, C). To ensure that both the ~43 kDa and 70 kDa species of Panx3 were bona fide Panx3 species, both species were eliminated when competed with cognate peptide (Figure 2.7B).

Figure 2.7 Increased levels of the 70 kDa species of Panx3 in organotypic epidermis

Western blotting (A) and subsequent densitometric quantification revealed undetectable levels of the doublet Panx3 species at ~43 kDa (C) in wild-type REKs. GAPDH was used as a loading control and asterisks denote statistical significance compared to wild-type monolayer REKs ($p<0.05$). In contrast, endogenous levels of the 70 kDa species of Panx3 (D) were significantly increased when REKs were allowed to differentiate into organotypic epidermis. Panx3 expression remained high when REKs were engineered to express Panx3 and grown in monolayer culture or as organotypic epidermis (A). Pre-adsorption of the anti-Panx3 antibody with its cognate peptide eliminated specific antibody binding to both the ~43 kDa and 70 kDa species (B). Bar graphs show normalized means ± s.e.m. The results shown here are representative of experiments that were repeated at least 5 times.

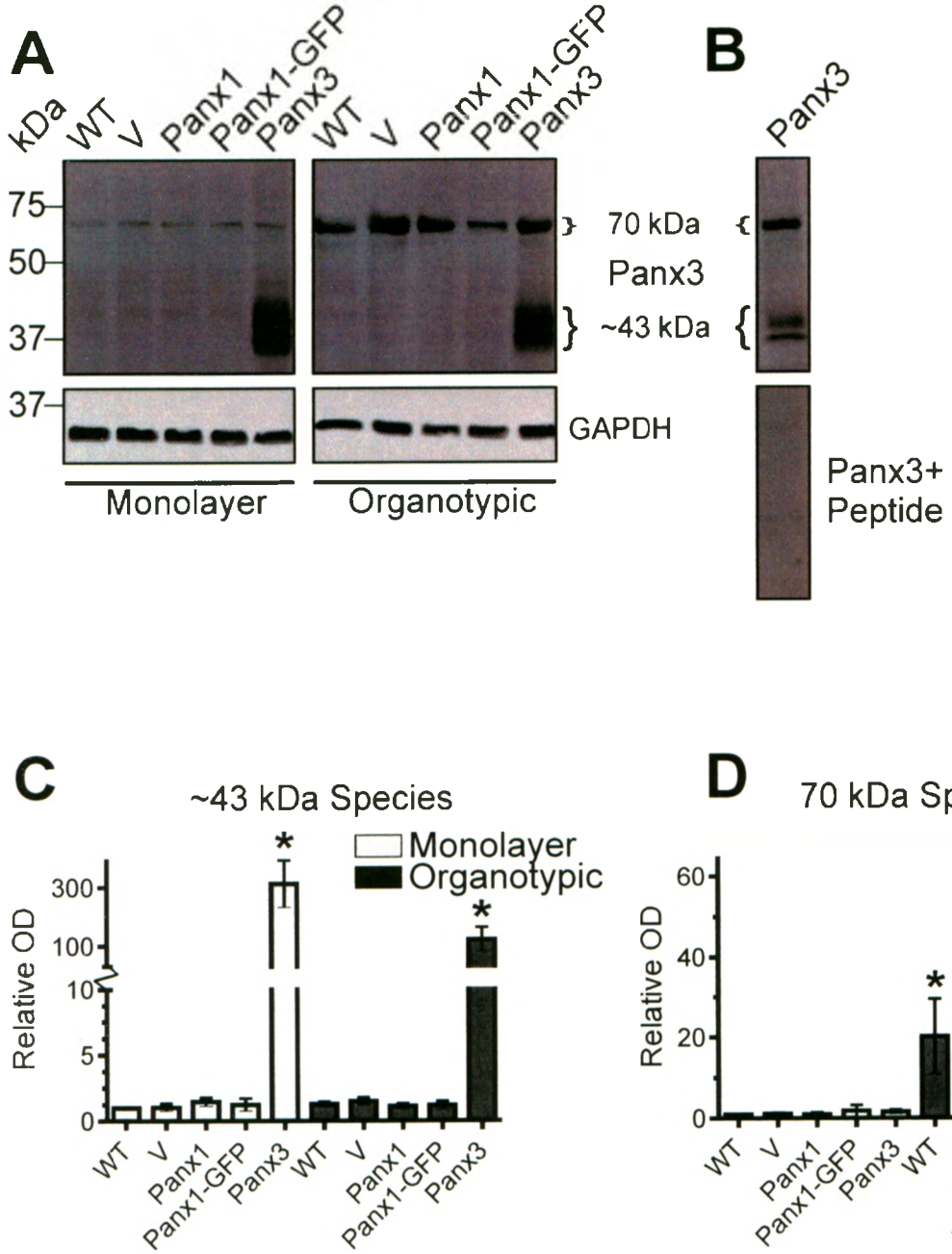

A
kDa
WT
V
Panx1
Panx1-GFP
Panx3
75
50
37
37
70 kDa
Panx3
~43 kDa
GAPDH
Monolayer
Organotypic
B
Panx3
Panx3+
Peptide
C
~43 kDa Species
Monolayer
Organotypic
Relative OD
300
100
10
5
0
D
70 kDa Species
Relative OD
60
40
20
0

Figure 2.7

2.4.6 Increased CK14 in Panx1-expressing organotypic REKs suggestive of epidermal dysregulation

In parallel to immunoblotting for Panx1 and Panx3 as shown Figures 2.6 and 2.7, CK14 and involucrin expression levels were assessed as indices of keratinocyte differentiation. As would be expected for normal epidermis differentiation, in all cases, involucrin expression increased when REKs were grown into organotypic epidermis ($p<0.05$; Figure 2.8A, C). Surprisingly, CK14 expression increased in Panx1-expressing organotypic epidermis compared to wild-type monolayer REKs suggesting that differentiation was at least partially dysregulated in this case ($p<0.05$; Figure 2.8B).

Figure 2.8 An aberrant increase in CK14 in Panx1-expressing cells when grown as organotypic epidermis

Western blotting revealed the levels of CK14 and involucrin in monolayer and organotypic epidermal models (A). GAPDH was used as a loading control. Densitometric quantification revealed increased CK14 expression in Panx1 over-expressing organotypic epidermis (B). Involucrin expression was increased in all organotypic cultures indicative of cell differentiation (C). Asterisks denote statistical significance compared to wild-type monolayer REKs ($p<0.05$) and bar graphs represent normalized means ± s.e.m. The results shown here are representative of experiments that were repeated at least 5 times.

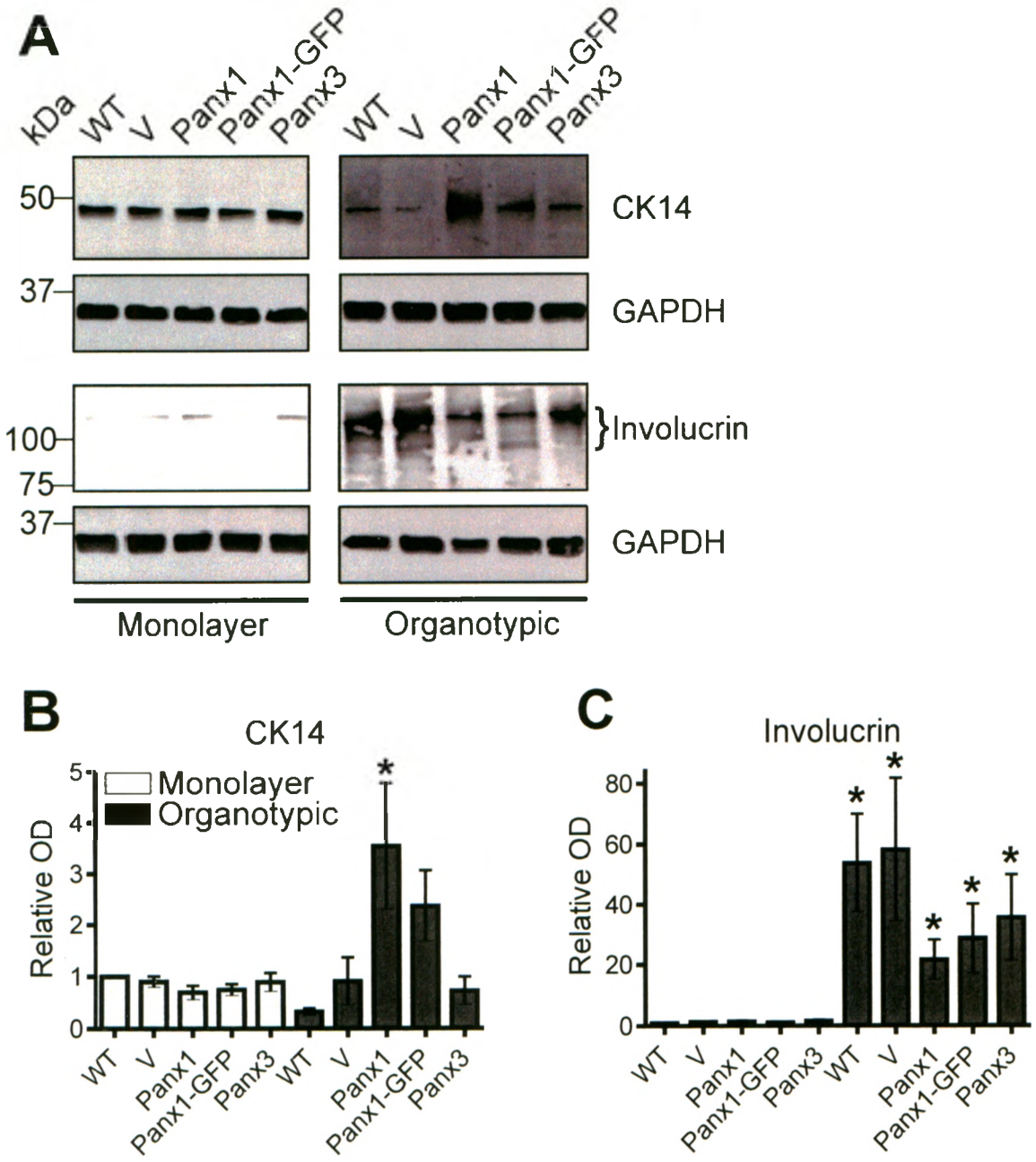

A
kDa
WT
V
Panx1
Panx1-GFP
Panx3
50
37
CK14
GAPDH
100
75
Involucrin
Monolayer
Organotypic
B
CK14
Monolayer
Organotypic
Relative OD
C
Involucrin

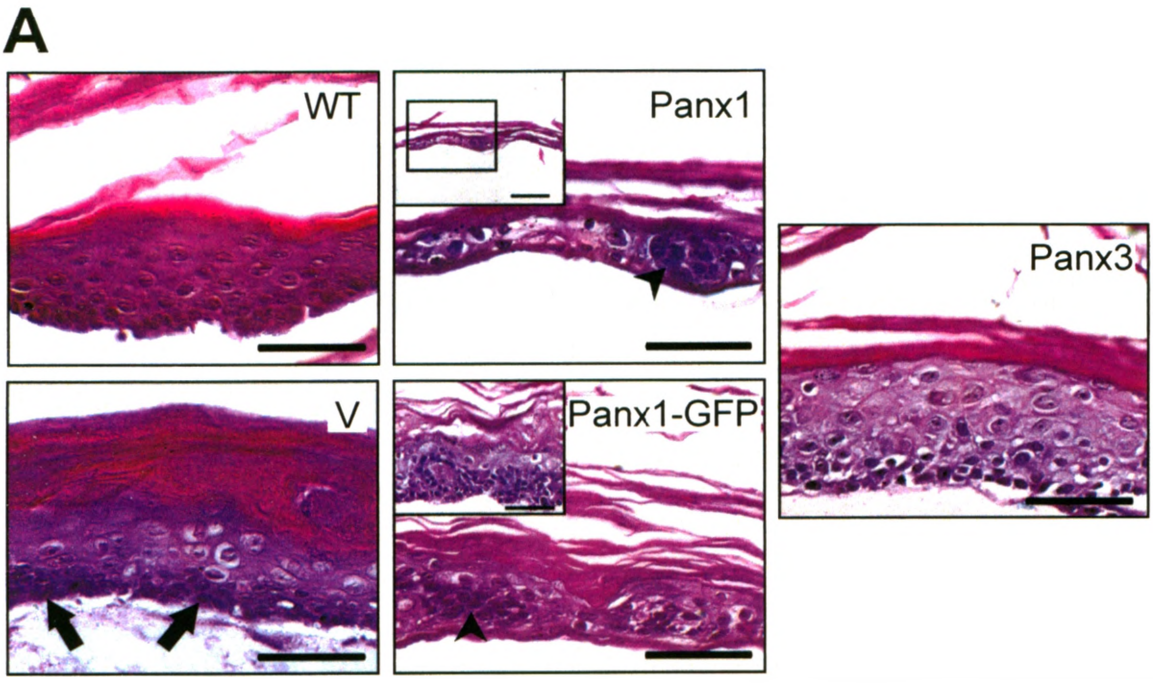
Figure 2.8

2.4.7 Panx1 expression dysregulates keratinocyte differentiation while elevated Panx3 expression maintains epidermal architecture

Organotypic epidermis grown from REKs expressing Panx1, Panx1-GFP or Panx3 was stained with H&E to reveal the epidermal architecture (Figure 2.9A). Wild-type, empty vector and Panx3-expressing organotypic models resembled newborn murine skin that displayed an organized basal layer, 3-5 suprabasal layers of differentiated keratinocytes and a thick cornified layer. On the other hand, Panx1 and Panx1-GFP expressing organotypic epidermis appeared highly dysregulated and disorganized. In addition to Panx1 dysregulating keratinocyte differentiation, Panx1 was found to significantly reduce the vital layer thickness ($p<0.05$; Figure 2.9B). Although Panx1-GFP expressing organotypic epidermis also appeared dysregulated, vital layer thickness was similar to that of controls and the Panx3-expressing model. This suggests that the GFP-tag on Panx1 may in fact partially alter the Panx1 function in keratinocytes.

Figure 2.9 Over-expression of Panx1 disrupted the architecture of the epidermis and decreased the vital layer thickness

Hematoxylin and eosin stained images of organotypic epidermis revealed similar differentiation profiles between wild-type, vector and Panx3 over-expressing organotypic cultures (A). Nuclei of undifferentiated keratinocytes appeared dark purple (arrows), while the basophilic cornified layer was brightly stained pink. In contrast, Panx1 and Panx1-GFP revealed differentiation phenotypes that were highly dysregulated as indicated by the pockets of undifferentiated-appearing keratinocytes (arrowheads). Vital layer thickness of Panx1-GFP cultures revealed both thin and thick phenotypes (A; insert). In addition to the dysregulated phenotype, Panx1 over-expressing organotypic epidermis revealed that vital layer thickness was reduced (B). Asterisks denote statistical significance compared to wild-type monolayer and empty vector controls ($p<0.05$) and bar graphs show means ± s.d. Bars, 100 μm. The results shown here are representative of experiments that were repeated at least 5 times.


A
WT
Panx1
Panx3
V
Panx1-GFP

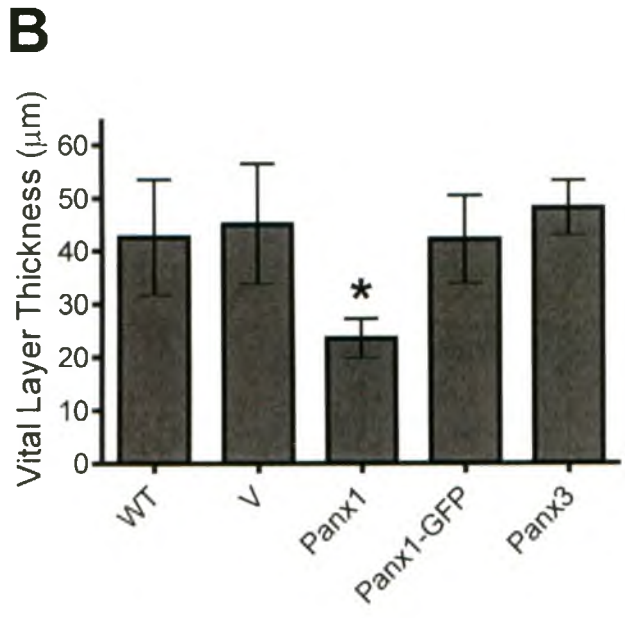

B
Vital Layer Thickness (μm)
60
50
40
30
20
10
0
*
WT
V
Panx1
Panx1-GFP
Panx3

Figure 2.9

2.4.8 Panxs and cytokeratins are differentially localized in organotypic epidermis

In keeping with the weak Panx1 signal observed in Western blots, Panx1 was not readily detectable by immunofluorescence in wild-type (not shown) or vector control organotypic epidermis (Figure 2.10A). Surprisingly, Panx3 was only detected in the cornified layer of organotypic epidermis (Figure 2.10A). As expected, CK14 was primarily localized to the basal layer with weak evidence of its presence in suprabasal layers, while CK10, a marker of differentiated keratinocytes, localized mainly to the suprabasal layers. Consistent with the fact that ectopic pannexin expression is driven from the CMV promoter, we were not surprised to find that Panx1, Panx1-GFP and Panx3 were found in all keratinocyte differentiation strata regardless of the state of cell differentiation (Figure 2.10B-D). Interestingly, the pannexins were observed more readily within intracellular compartments as opposed to the cell surface suggesting that they re-localize upon cell growth into organotypic epidermis. Occasionally, Panx1 was observed at the cell surface suggesting that re-localization did not occur in at least some cells (Figure 2.10B; arrowheads). Importantly, Panx1-expressing keratinocytes exhibited abnormal CK10 and CK14 localization patterns throughout all vital layers, providing additional evidence that REK differentiation is dysregulated when Panx1 is over-expressed (Figure 2.10B). Although CK10 was predominantly localized to suprabasal layers in Panx1-GFP organotypic epidermis, CK10 still appeared dysregulated in this cultured environment compared to wild-type and vector controls. Whether cells expressed Panx1 or Panx1-GFP, CK14 was found throughout all epidermal layers supporting the premise that Panx1, either untagged or tagged with GFP, dysregulates

keratinocyte differentiation (Figure 2.10B, C). In cells expressing Panx3, CK10 localization appeared similar to that seen in vector controls, supporting the notion that Panx3 does not disrupt differentiation of REKs (Figure 2.10D).

Figure 2.10 Exogenous Panx1 and Panx3 differentially affect the differentiation of the epidermis as exhibited by the localization of CK14 and CK10

Immunofluorescent images of organotypic epidermis grown from empty vector expressing REKs revealed the detection of Panx3 (red) in the cornified layer while Panx1 expression was below detectable levels in the vital layer (A). As expected, CK14 (green) was mainly localized to the basal layer, while CK10 (green) was found in suprabasal layers of control epidermis. When Panx1 was ectopically expressed, it was localized throughout the epidermis in intracellular compartments (B) and occasionally at the cell surface (B; arrowheads). CK14 expression was detected in irregular patterns and the cells exhibited weak CK10 expression in both Panx1 (B) and Panx-GFP (C) expressing cells. In cells engineered to over-express Panx3, Panx3 and CK14 were found throughout all epidermal layers, while CK10 was localized only to suprabasal layers. The basal layer is denoted above the dotted line and the cornified layer is located above the dashed lines. Hoechst nuclear stain is shown in blue. Bars, 20 μm.

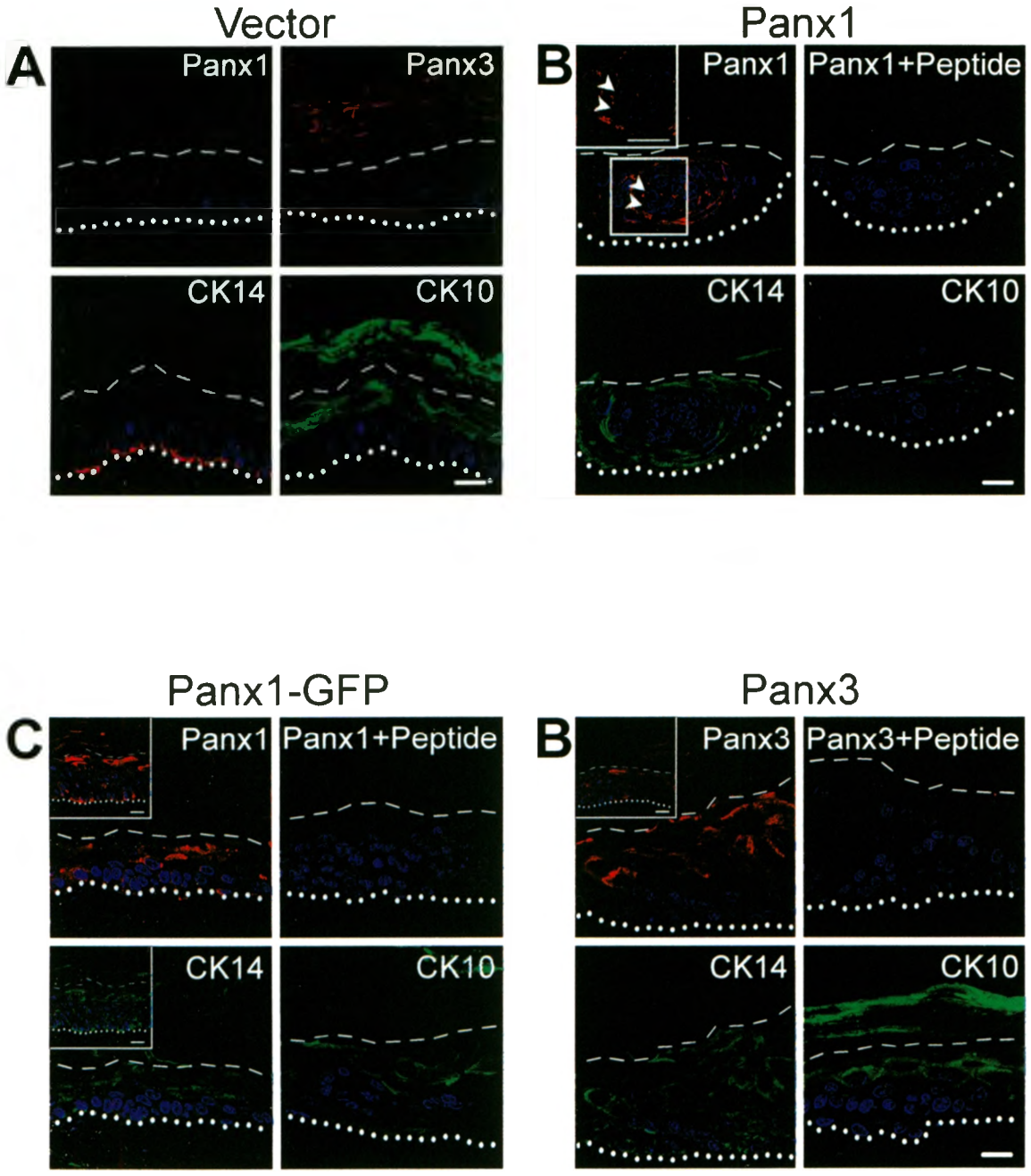

Vector
A
Panx1
Panx3
CK14
CK10
Panx1
B
Panx1
Panx1+Peptide
CK14
CK10
Panx1-GFP
C
Panx1
Panx1+Peptide
CK14
CK10
Panx3
B
Panx3
Panx3+Peptide
CK14
CK10

Figure 2.10

2.5 Discussion

Almost a decade after their discovery in the mammalian genome (Panchin et al., 2000), pannexins have been classified as single-membrane channels with a broad range of tissue expression patterns which include the skin (Baranova et al., 2004; Penuela et al., 2007). However, while the debate on whether pannexins only form single membrane channels continues, the diverse expression profiles of Panx1 and Panx3 observed in several cell and tissue types highlights the complexity of their possible cellular roles. At the subcellular level, ectopic Panx1 and Panx3 expression revealed their preferential localization to the cell surface in several cell types including REKs (Penuela et al., 2008). In contrast, the subcellular profile of endogenous Panx1 was found to be punctate in human facial epidermis (Penuela et al., 2007) and mouse lens and retina (Dvoriantchikova et al., 2006a; Dvoriantchikova et al., 2006b), while a diffuse cellular distribution of Panx3 was found throughout all layers of human facial epidermis. Adding to this complexity was the observed intracellular localization of Panx1 in mouse spleen and the intracellular distribution of both Panx1 and Panx3 in MC3T3-E1 cells (Penuela et al., 2007; Penuela et al., 2008). Thus, based on their complexity and differential expression profiles in skin, we hypothesized that Panx1 and Panx3 play key roles in epidermal keratinocyte differentiation. In this study we demonstrate that keratinocyte differentiation was dysregulated upon ectopic Panx1 expression, but not Panx3, suggesting that these pannexins play diverse roles when co-expressed in the same tissue.

2.5.1 Differential Panx1 and Panx3 protein expression in murine skin

In order to begin to understand the putative roles of Panx1 and Panx3 in murine skin, the differential distribution profiles of these pannexins were assessed. Panx1 was detected in all epidermal layers of thin epidermis but was only found in the suprabasal layer of newborn epidermis. Although a predominately punctate Panx1 staining was detected in human facial epidermis (Penuela et al., 2007), we observed a diffuse intracellular expression profile of Panx1 in murine epidermis suggesting that Panx1 localization may vary amongst animal species. On the other hand, Panx3 displayed an intracellular localization profile in all thin and thick epidermal layers, but appeared to favor localization in the basal layer and sebaceous glands of thin skin, and the cornified layer of thick skin. The diffuse intracellular Panx3 staining observed throughout all epidermal layers in murine epidermis was in keeping with that of human facial epidermis, and of immunoblotting studies revealing glycosylated Panx3 species at ~43 kDa and a second 70 kDa species, as seen in Penuela et al. (2007). Interestingly, intense Panx3 staining was revealed in what appears to be keratin-rich sebocytes raising the possibility that Panx3 may be involved in sebocyte function or regulation. Sebum production appears to be the main function of sebaceous glands, however sebocytes are the primordial relatives of epidermal keratinocytes that are continuously turning over, differentiating and expressing involucrin (Zouboulis, 2004).

Panx1 and Panx3 have already been implicated in cellular communication with the extracellular environment through single-membrane channels (Locovei et al., 2006; Penuela et al., 2007). However, the diffuse intracellular localization profiles revealed in murine epidermis suggest that they have several cellular functions that may be tissue and

species specific. In fact, an alternative function of Panx1 has been proposed by Vanden Abeele et al. (2006) suggesting that Panx1 may regulate intracellular calcium homeostasis through endoplasmic reticulum calcium leak channels. Of further note, decreased expression of Panx1 in 3-week old thin and thick skin compared to newborn suggests that Panx1 may play an important role early in skin generation, but be of less importance in skin maintenance. This concept is not without precedence as cytokeratin composition changes in aged and dry epidermis exemplifying the adaptation of skin to changes in the external environment (Engelke et al., 1997). Nevertheless, the spatio-temporal regulation of Panx1 and Panx3 and differential localization patterns at both the cellular and subcellular levels suggest that these pannexins may play distinct roles within the skin.

2.5.2 Characterization of exogenously expressed Panx1, Panx1-GFP and Panx3 in REKs

Since REKs possess the ability to differentiate into organotypic epidermis and express low levels of endogenous Panx1 and Panx3, they represent an excellent model to investigate the cellular role of pannexins. As might be expected, over-expressed Panx1, Panx1-GFP and Panx3 localized predominantly to the cell surface in the majority of REKs supporting the premise that the major functional role of these pannexins occurs at the cell surface presumably through communication with the extracellular environment. These findings are similar to that of Penuela et al. (2008), where untagged Panx1 and Panx3 were localized to the cell surface in REK, Madin-Darby canine kidney, and gap junction-deficient HeLa cells. It was also previously determined that N-linked

glycosylation of Panx1 and Panx3 aided in the trafficking of these pannexins to the cell surface (Boassa et al., 2007; Penuela et al., 2008). At the cell surface, Panx1 channels were found to pass electrical current and dyes and release ATP in a variety of cell types (Bruzzone et al., 2005; Penuela et al., 2007; Ransford et al., 2009). Intriguingly, Panx3 revealed a second distinct intracellular distribution pattern not unlike that found in human skin keratinocytes, mouse spleen, mouse osteoblasts and some rat neuroblastoma cells (Penuela et al., 2007; Penuela et al., 2008). The reason for this distribution pattern is not clear but may reflect changes in the differentiation status of individual keratinocytes even when grown in monolayer culture. This suggestion is supported by the fact that Panx3 is found almost exclusively in intracellular compartments, and not at the cell surface, when Panx3 expressing REKs are grown into organotypic epidermis. Intriguingly, ectopic Panx1 also acquires a predominant intracellular distribution when cells are grown as organotypic epidermis further suggesting that the differentiation program promotes keratinocytes to re-localize Panx1 and Panx3.

Considerable evidence has suggested that Panx1 and Panx3 single-membrane channels are capable of cellular communication with the extracellular environment. However, one emerging role of Panx1 and Panx3 at the cellular level may involve the regulation of cell proliferation. Based on BrdU incorporation, Panx1 and Panx3 decreased keratinocyte proliferation, which is consistent with the Panx1-induced decrease in the proliferation of C6 gliomas (Lai et al., 2007). It is possible the even small changes in the rate of cellular proliferation may have larger effects on disrupting the delicate balance between cell proliferation and cell differentiation. However, this is not likely the case here as Panx3 also decreased proliferation but the ability of REKs to differentiate, as

assessed by the architecture of the tissue and the markers of differentiation, remained relatively unchanged.

2.5.3 Panx1 dysregulates keratinocyte differentiation while Panx3 maintains epidermal architecture

Using our model of keratinocyte differentiation we revealed that ectopic Panx1, Panx1-GFP and Panx3 predominantly redistributed from the cell surface to intracellular compartments, which, in fact, more accurately reflects the distribution profile of endogenous Panx1 and Panx3 in mouse skin. However, several lines of evidence suggest that Panx1 over-expression dysregulates keratinocyte differentiation. First, the expression levels of CK14, a common marker of undifferentiated basal keratinocytes, was increased and expressed throughout all vital layers in organotypic epidermis compared to monolayer REKs. Second, the architecture of organotypic epidermis was highly disorganized. Third, there was a reduction in the vital layer thickness. Finally, the localization of CK10 was abnormal. Taken together with the in vivo finding that Panx1 is reduced in aging skin compared to newborn mice, Panx1 may be more important in epidermal development and generation rather than maintenance and renewal. In essence, it is possible that Panx1 expression may delay the differentiation of keratinocytes in newly forming epidermis, only to be down-regulated when keratinocytes signal to increase differentiation. Although GFP-tagged Panx1 displayed dysregulated epidermal architecture similar to that of untagged Panx1, the response appeared attenuated since vital layer thickness and CK14 levels remained similar to control. This may be explained

in part by the fact that the GFP tag has been shown to reduce Panx1 channel function (Ma et al., 2009).

In contrast to Panx1, Panx3 expression did not alter the integrity of the organotypic epidermis and our in vivo and in vitro evidence further suggests that Panx3 may in fact support keratinocyte differentiation. Differentiation of REKs into organotypic epidermis increased the expression levels of the 70 kDa species of endogenous Panx3. The localization of this unique species of Panx3 remains unclear, but it was notable that the cornified layer of wild-type organotypic epidermis was specifically stained with anti-Panx3 antibodies. One possibility that has yet to be ruled-out, is that the 70 kDa species is an aggregate of Panx3 which is associated with programmed cell death as the keratinocytes fully differentiate and form the dead cornified layer. This possibility is not without precedent, as classic differentiation markers CK10 and involucrin are known to increase their levels in differentiating keratinocytes and even localize to the cornified layer (Chen et al., 2006; Hara-Chikuma et al., 2009). Further supporting the role of Panx3 in epidermal maintenance, the integrity of organotypic epidermis did not appear dysregulated when the ~43 kDa species of Panx3 was over-expressed. Similar to endogenous Panx3 in human facial skin (Penuela et al., 2007), Panx3 primarily localizes to an intracellular compartment which is likely the site of its function in differentiated keratinocytes.

In summary, our two- and three-dimensional in vitro studies support the hypothesis that Panx1 and Panx3 play key and distinct roles in keratinocyte differentiation and proliferation. This study revealed that Panx1 must be exquisitely regulated to allow for new epidermis formation and to maintain healthy epidermis.

Furthermore, our studies suggest that both Panx1 and Panx3 acquire distinct subcellular localizations depending on the state of keratinocyte differentiation. Future research should be directed towards investigating how pannexin single-membrane channels fulfill their roles in keratinocyte differentiation and proliferation both at the cell surface and within intracellular compartments.

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CHAPTER THREE

GENERAL DISCUSSION

3.1 Summary and Conclusions

Objective #1: To characterize the endogenous expression and localization of Panx1 and Panx3 in murine epidermis.

Although we had previously revealed punctate Panx1 predominantly localized to suprabasal layers and diffuse intracellular Panx3 throughout all layers in human facial epidermis (Penuela et al., 2007), this study was the first to reveal distinct spatio-temporal expression and localization of Panx1 and Panx3 that was specific to murine epidermis. In contrast to human, a diffuse intracellular localization of Panx1 was revealed in newborn and thin epidermis, but remained predominantly localized to suprabasal layers suggesting that Panx1 expression may be species specific. We also found that Panx1 expression levels in 3-week old thin and thick skin were lower than that found in newborn mice suggesting that temporal regulation of Panx1 exists in skin. On the other hand, Panx3 displayed an intracellular localization profile in all thin and thick epidermal layers, in keeping with that of human skin, but seemed to favor localization to basal layer cells and sebaceous gland.

Objective #2: To characterize the endogenous expression and localization of Panx1 and Panx3 in monolayer REKs and organotypic epidermis.

Ectopically expressed Panx1 and Panx3 localized to the cell surface supporting the role of Panx1 and Panx3 as single-membrane channels capable of communication with the extracellular environment (Bao et al., 2004; Boassa et al., 2007; Boassa et al., 2008; Bruzzone et al., 2003; Dahl and Locovei, 2006; Lai et al., 2007; Locovei et al., 2006a; Locovei et al., 2006b; Ma et al., 2009; Penuela et al., 2007; Penuela et al., 2008;

Reyes et al., 2009; Silverman et al., 2008). On the other hand, about 30% of the Panx3 expressing cell population displayed an intracellular Panx3 profile. When grown into organotypic epidermis, both Panx1 and Panx3 re-localized to intracellular compartments. These findings support other reports that show intracellular populations of pannexins and suggest a second role for pannexins when localized to intracellular compartments (Dvoriantchikova et al., 2006a; Dvoriantchikova et al., 2006b; Huang et al., 2007; Lai et al., 2007; Penuela et al., 2007; Vanden Abeele et al., 2006; Zappala et al., 2006).

Objective #3: To assess the putative roles of Panx1 and Panx3 in keratinocyte proliferation, migration and differentiation.

This study was the first to reveal that over-expression of Panx1 results in dysregulated epidermis as assessed by cytokeratin expression and localization, as well as vital layer thickness. Interestingly, the addition of the GFP tag attenuated the effects of the Panx1-mediated epidermal dysregulation suggesting that the function of Panx1 may become compromised by the GFP tag. In contrast, over-expressed Panx3 did not disrupt epidermal architecture. However, endogenous expression of the 70 kDa species of Panx3 increased in organotypic epidermis compared to monolayer suggesting that upregulation of this Panx3 species may be important to support differentiation. This study also revealed that Panx1 and for the first time, Panx3 reduces keratinocyte proliferation. On the other hand, neither Panx1 nor Panx3 altered the migration capabilities of keratinocytes after wounding in a two-dimensional environment.

3.2 Contributions of the Research

3.2.1 General Significance

Prior to this study, the role of pannexins at the cellular level was obscure. Using keratinocytes as a model of cellular differentiation, proliferation and migration, the overall objective of this study was to determine the cellular roles of Panx1 and Panx3. One key finding in this study is that Panx1 levels need to be lowered in skin to allow for proper epidermal differentiation, while high levels of Panx3 appear to support epidermal differentiation. We propose that Panx1 may play a role in early epidermal development and generation, while Panx3 may be important for epidermal maintenance and support keratinocyte differentiation. Thus, we conclude that Panx1 and Panx3 acquire distinct subcellular profiles depending on the state of differentiation and play diverse and distinct roles when co-expressed in the same tissue.

3.2.2 Differential Panx1 and Panx3 protein expression in murine skin

The differential distribution profiles of Panx1 and Panx3 suggest that these pannexins have distinct roles in murine skin. Although we observed punctate Panx1 staining in human facial epidermis (Penuela et al., 2007), we discovered a diffuse intracellular expression profile of Panx1 in murine epidermis which suggests that Panx1 localization is variable amongst species. On the other hand, intracellular Panx3 staining was observed throughout all epidermal layers in murine epidermis which was a similar finding to our observations in human skin (Penuela et al., 2007). Panx3 was also detected in what appears to be keratin-rich sebocytes. Sebocytes are the primordial relatives of

epidermal keratinocytes which suggests that Panx3 may also be involved in sebocyte differentiation, regulation or even function (Zouboulis, 2004).

Although pannexins have already been implicated in cellular communication with the extracellular environment through single-membrane channels (Locovei et al., 2006; Penuela et al., 2007), the intracellular localization profiles revealed in murine and organotypic epidermis suggests that Panx1 and Panx3 may have other cellular functions. One alternative function of Panx1 was proposed by Vanden Abeele et al. (2006) which suggested that Panx1 may regulate intracellular calcium homeostasis through endoplasmic reticulum calcium leak channels. Thus, pannexins may in fact be playing multiple cellular roles while expressed at the cell surface and in endoplasmic reticulum. Nevertheless, the spatio-temporal regulation of Panx1 and Panx3 and differential localization patterns at both the cellular and subcellular levels suggest that these pannexins play distinct roles within the skin.

3.2.3 Characterization of exogenously expressed Panx1, Panx1-GFP and Panx3 in REKs

Since REKs express low levels of endogenous Panx1 and Panx3 and are able to differentiate into organotypic epidermis, they represent an excellent model to investigate the cellular role of pannexins. Over-expressed Panx1, Panx1-GFP and Panx3 primarily localized to the cell surface in the majority of REKs, supporting the notion that the primary function of pannexins occurs at the cell surface presumably through communication with the extracellular environment. These findings are similar to that of Penuela et al. (2008), where untagged Panx1 and Panx3 were localized to the cell surface

in REK, Madin-Darby canine kidney, and gap junction-deficient HeLa cells. It was also determined that Panx1 channels were found to pass electrical current and dyes, and also release ATP in a variety of cell types (Bruzzone et al., 2005; Penuela et al., 2007; Ransford et al., 2009). Interestingly, Panx3 revealed a second distinct intracellular distribution pattern, similar to that observed in human skin keratinocytes, mouse spleen, mouse osteoblasts and some rat neuroblastoma cells (Penuela et al., 2007; Penuela et al., 2008). The reason for this distribution pattern remains obscure, but may indicate that pannexins redistribute to intracellular locations depending on their state of differentiation, even in monolayer. This suggestion is supported by the fact that both Panx1 and Panx3 are found almost exclusively in intracellular compartments when grown into organotypic epidermis, and not at the cell surface as observed in monolayer grown cells. Another emerging role of Panx1 and Panx3 at the cellular level may involve the regulation of cell proliferation. Panx1 and Panx3 decreased keratinocyte proliferation, which is consistent with the Panx1-induced decrease in the proliferation of C6 gliomas (Lai et al., 2007). Minor changes in cell proliferation rates may have drastic effects on disrupting the balance between cell proliferation and cell differentiation, however, this is not likely the case here as Panx3 also decreased proliferation but the ability of REKs to differentiate remained relatively unchanged.

3.2.4 Panx1 dysregulates keratinocyte differentiation while Panx3 maintains epidermal architecture

Using our model of keratinocyte differentiation we revealed that ectopic Panx1, Panx1-GFP and Panx3 redistributed from the cell surface to intracellular compartments

reflecting the localization patterns of endogenous Panx1 and Panx3 found in mouse skin. However, there were several indications that suggested that Panx1 over-expression dysregulates keratinocyte differentiation. First, the expression levels of CK14, a frequently used marker of undifferentiated basal keratinocytes, was elevated and expressed throughout all vital layers in organotypic epidermis compared to monolayer REKs. Second, the architecture of organotypic epidermis was highly disorganized. Third, there was a reduction in the vital layer thickness. Finally, the localization of CK10 was abnormal.

Since Panx1 is reduced in aging skin compared to skin from newborn mice, we speculate that Panx1 may play a more important role in newly forming epidermis during development. It is possible that Panx1 may a part in delaying the differentiation of newly forming epidermis, and is subsequently down-regulated when differentiation is signaled to increase. Intriguingly, GFP-tagged Panx1 displayed dysregulated epidermal architecture similar to that of untagged Panx1; however the response appeared attenuated since vital layer thickness and CK14 levels remained similar to control. This may be explained in part by the fact that the GFP tag has been shown to reduce Panx1 channel function (Ma et al., 2009).

In contrast to Panx1, the integrity of organotypic epidermis was maintained when Panx3 was over-expressed, which suggests that Panx3 is compatible with keratinocyte differentiation. Differentiation of REKs into organotypic epidermis increased the expression levels of the 70 kDa species of endogenous Panx3, similar to other key markers of differentiation such as CK10, involucrin and loricrin (Ekanayake-Mudiyanselage et al., 1998). The localization and nature of this unique Panx3 species

remains obscure, but it was notable that Panx3 was detected in the cornified layer of wild-type organotypic epidermis. It is possible that the 70 kDa species is an aggregate of Panx3 and may be associated with the cornification process as keratinocytes terminally differentiate and form the dead cornified layer. This possibility is supported by the expression patterns of the differentiation markers CK10 and involucrin, which increase their levels in differentiating keratinocytes and also localize to the cornified layer (Chen et al., 2006; Hara-Chikuma et al., 2009). Furthermore, the architecture of organotypic epidermis did not appear dysregulated when the ~43 kDa species of Panx3 was over-expressed, supporting the notion that Panx3 is not pathological to the maintenance of the epidermis. The localization profile of ectopically expressed Panx3 was similar to that of endogenous Panx3 in human facial skin, suggesting that the primary site of Panx3 function may in fact be within intracellular compartments (Penuela et al., 2007).

3.3 Limitations of the Research and Suggestions for Future Studies

3.3.1 Limitations of in vitro models

These studies were limited to over-expression analysis since low endogenous expression levels of Panx1 and Panx3 existed in our REK model of keratinocyte differentiation making this organotypic model unstable for pannexin knockdown studies. If robust Panx1 and Panx3 expression did exist in REKs, then our studies could have included siRNA experiments to knockdown pannexins and examine the effect on keratinocyte differentiation. Utilizing a second experimental approach would have also increased the confidence in our salient findings.

While the use of the organotypic model system has the ability to be extensively manipulated and does not require breeding of rodents, it is extremely fragile and requires extensive, daily maintenance to ensure reproducibility. Furthermore, the processing of fragile organotypic epidermis can be challenging as histochemistry and immunolabeling relies on the epidermis remaining intact. This fragility hindered critical measurements of cornified layer thickness, which may have provided insight about the role of pannexins in the cornification process of terminally differentiated keratinocytes. Ideally, Panx1 and Panx3 knock-out mice would have been a valuable addition to our studies to assess the role of pannexins in skin differentiation and maintenance but these mice are not yet available.

3.3.2 Future Studies

It is clear that the true role of pannexins in any tissue or organ awaits further clarification. Part of the complexity in examining the functional role of pannexins arises from their differential tissue localization and subcellular profiles observed in a wide range of tissues and cells. The fact that Panx1 and Panx3 are expressed at the cell surface in monolayer keratinocytes and are redistributed to intracellular compartments in organotypic epidermis indicates that pannexins probably play multiple subcellular roles. Overall, Panx1 and Panx3 have diverse roles in keratinocyte differentiation, while both reducing keratinocyte proliferation which suggests that these family members play distinct roles when co-expressed. Future research should be directed towards investigating how pannexin single-membrane channels fulfill their roles in keratinocyte

differentiation and proliferation both at the cell surface and within intracellular compartments.

On a separate note, our studies show that over-expression of Panx1 dysregulates keratinocyte differentiation, but the mechanism remains unclear. Interestingly, the $P2X_7$ receptor complex, which is the only molecule known to date to physically interact with Panx1 (Pelegrin and Surprenant, 2009), is also expressed in human epidermis and increases upon differentiation in cultured keratinocytes (Inoue et al., 2005). This complex has been associated with the release of the pro-inflammatory cytokine interleukin-1-beta upon purinergic stimulation of macrophages, via caspase-1 activation which is independent of the Panx1-mediated dye uptake pathway (Brough et al., 2009; Pelegrin and Surprenant, 2009; Schenk et al., 2008). The activated caspase-1 pathway has been shown to induce apoptosis via catalytic activation of other caspase family members and through the cleavage of key intracellular structural and regulatory proteins (Tatsuta et al., 2000). Thus, it is possible that the combined increase in $P2X_7$ expression upon epidermal differentiation and over-expressed Panx1 in organotypic epidermis may have over-activated the caspase-mediated apoptosis pathway resulting in the dysregulated phenotype that was observed. Future studies should pursue this hypothesis to investigate how Panx1 mediates epidermal dysregulation.

In summary, our two- and three-dimensional in vitro studies support the hypothesis that Panx1 and Panx3 play key and distinct roles in keratinocyte differentiation and proliferation. This study revealed that Panx1 must be exquisitely regulated to allow for new epidermis formation and to maintain healthy epidermis.

Furthermore, our studies suggest that both Panx1 and Panx3 acquire distinct subcellular localizations depending on the state of keratinocyte differentiation.

3.4 References

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