The Role of Gap Junctions in Cellular Migration

by

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The body of work presented here focuses on characterizing the role that gap junction intercellular channels play in regulating cellular migration. Cell migration is a ubiquitous process that is required for embryonic development and for maintaining the integrity of tissues and organs. Yet, the status of gap junction channels, with regard to structure and function, in migrating cells is not completely understood. We hypothesized that, “Gap junction channels, as mediators of intercellular communication, play a role in cell migration,” and have investigated and characterized gap junctions in migrating cells. Accordingly, the aims of this dissertation were: (i) to characterize gap junctions and their function during migration, (ii) to determine the effect of altering the status of gap junction expression on cell migration and to (iii) characterize the mechanism of gap junction internalization in migrating cells.

With combined molecular and imaging approaches we have demonstrated that in cells migrating as a sheet, gap junction plaque structures are retained on the plasma membrane surface; that gap junction communication is continuous in migrating cells and that interrupting this communication with connexin 43 specific mimetic peptides reduces migration. We have established a system in a human adrenal cortical cell line (SW13) wherein we significantly reduce gap junction protein expression with siRNA and show that cellular migration is inhibited. We have also demonstrated that gap junction plaque size and orientation are modified during migration. We also discovered that sometimes, migrating cells will spontaneously detach from one another at cellular processes and that during this event the gap junction plaques are internalized. Analysis of gap junction plaque internalization in migrating cells revealed that clathrin and several adaptor proteins associate with surface gap junctions and cytoplasmic annular gap junction structures and possibly regulate the unique mechanism of gap junction plaque removal from the plasma membrane. Within the field of gap junction research this work expands our understanding of gap junctions in living cells as dynamic structures that may play a key role in coordinating the migration of entire cell populations.
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1.0 INTRODUCTION

The fundamental survival of multicellular organisms requires that tissues respond to specific signals as integrated systems (Peracchia and Girsch, 1985). Much of this cooperation is attributed to intercellular gap junction communication channels that provide for the mutual exchange of electrical and chemical information between adjacent cells (Subak-Sharpe et al., 1969; Lo and Gilula, 1979; McNutt and Weinstein 1970). The majority of cells that comprise the tissues of our bodies are able to communicate directly with one another via gap junction channels (Kumar, 1999). A number of these cell populations are also capable of migration, an event that is thought to rely on the ability of individual cells, in a group, to communicate with one another (Pepper and Meda, 1992; Matic et al., 1997; Nadarajah et al., 1997; Saitoh et al., 1997, Civitelli et al., 1998, Winterhager et al., 1999; Ashton et al., 1999; Cina et al., 2007; Leaphart et al., 2007). According to the current migration model as proposed by the Horwitz group, the coordination of events for a single cell requires converging intracellular signaling pathways that regulate cytoskeletal turnover along with contraction/retraction processes (Horwitz and Parsons, 1999; Ridley et al., 2003, Horwitz and Webb, 2003; Vincente-Mazanares et al., 2005). Integrating these responses such that an entire population migrates in a regulated and coordinated manner, that is biologically relevant, would likely require direct cell-cell communication via gap junction intercellular channels. Based on this model it would be assumed that gap junctions would either be retained as cells migrate, or that they are
disassembled and reassembled in migrating populations such that information could be transmitted between cells. Yet, issues regarding the status of gap junction communication channels in cell populations that migrate in a cluster or sheet-like manner have not been addressed. The hypothesis, “gap junctions and intercellular communication play a pivotal role in migration,” was tested. Accordingly, the aims of this dissertation are: (i) to characterize gap junctions and their function in migrating cells, (ii) to determine the effect of altering the status of gap junction expression on cell migration and to (iii) characterize the mechanism of gap junction internalization in migrating cells.

1.1 GAP JUNCTION CHANNELS

To better understand intercellular communication between migrating cells it is necessary to discuss gap junction channels in detail. Gap junction channels are specialized membrane pores that allow mutual and direct exchange of electrical and chemical information between adjacent cells in tissues and organs (Loewenstein, 1966; Gilula et al., 1971; Sheridan, 1971). Each channel is comprised of 12 connexin monomers (Hand and Gobel, 1972). Connexins are four-pass transmembrane proteins that contain two extracellular loops and cytoplasmically localized N- and C- termini with amino acid sequences that are targeted for phosphorylation and other regulatory enzymes (Milks et al., 1988; Musil et al., 1990; Yeager and Gilula, 1992; Torok et al., 1997) (Fig. 1).

There are twenty-one connexin proteins that have been identified in mammals which vary by their molecular weight (e.g. Cx32, Cx43, Cx50 etc.) (Kumar and Gilula, 1996 rev. Yeager and Harris, 2007 rev.). Their expression is tissue specific with most cells expressing more than a
Figure 1 Model of Gap Junction Formation and Turnover

Monomeric connexin proteins (shown in the lower right-hand) oligomerize to form hemichannels/connexons. Following assembly in the Golgi the connexons are inserted into the plasma membrane. Gap junction channels are formed when connexons of neighboring cells come together or ‘dock’ and form an aqueous pore that spans across the two nanometers of extracellular space that now separates the plasma membranes. Gap junction channels aggregate to form ‘plaques’ and are hereafter capable of intercellular communication. An entire or portion of a plaque is internalized form an annular gap junction which will ultimately be targeted for degradation.
single connexin isotype (Lagree et al., 2003). Most cell types initially express the Cx43 isoform however; following development hepatocytes, for example, express only Cx32 and Cx26 whereas cardiomyocytes express only Cx43 and Cx26 isotypes (Paul, 1986; Zhang and Nicholson, 1989; Beyer et al., 1987; Dermietzel et al., 1989). Gap junctions are formed when connexins are initially assembled into cylindrical, hexameric connexons (hemichannels) (Musil and Goodenough, 1993) (Fig. 1). It is the characteristics of the individual connexin isotypes that contributes to the unique specificity of the connexon pore (e.g. size, charge and permeability) (Oyamada et al., 2005).

Connexons are either: homomeric (same connexin monomers) or heteromeric (different connexin monomers) and gap junctions may be homocellular (both cells contribute connexons that are identical) or heterocellular (both cells contribute connexons that are different) in nature (Diez et al., 1999; Elfgang et al., 1995; Sosinsky, 1995; Larsen, 1977). A single connexon loaded onto the plasma membrane of a cell will “dock” via non-covalent interaction with its hemichannel counterpart on the apposing cell membrane and form a single dodecameric gap junction channel (Foote et al., 1998; Perkins et al., 1998; Yeager et al., 1998) (Fig. 1). The newly formed intercellular channel contains a hydrophilic central pore and spans across a 2-3 nanometer “gap” that separates the apposing plasma membranes, hence the term “gap junction” (Revel and Karnovsky, 1967; Goodenough and Revel, 1971) (Fig. 1). Once on the plasma membrane, gap junction channels form aggregates or ‘plaques’ which are composed of hundreds or thousands of individual channels and extend several micrometers in length (Goodenough and Revel, 1970, McNutt and Weinstein, 1970) (Fig. 1). Gap junction channels are typically only thought to function in cell-cell communication once aggregated into plaques, and the central pore is virtually leak proof and can accommodate a range of transiting molecules including ions,
secondary messengers, amino acids, nucleic acids etc. (Simpson et al., 1977; Lowenstein, 1978; Saez et al., 1989; Kumar and Gilula, 1996 rev.) (Fig. 1). Incidentally, it has been reported that connexons/hemichannels may also play a role in molecular transport (Jiang et al., 2007; Krysko et al., 2005; Bennett et al., 2003). The average turnover for connexin proteins ranges between 1-5 hours, depending on the cell type (Musil et al., 2005; Fallon and Goodenough, 1981). Factors that regulate gap junction function, trafficking or dynamics are likely to alter gap junction channels and thereby affect physiological processes such as cellular migration. The key events associated with the regulation of gap junction function including: channel assembly, plaque assembly, channel gating and the removal of the gap junction plaque from the cell surface, will be discussed in distinct sections.

1.2 GAP JUNCTION CHANNEL ASSEMBLY

The assembly of gap junction channels is a multi-step process, and like many integral proteins is highly regulated and begins in the biosynthetic pathway (Musil and Goodenough, 1993; Hurtley and Helenius, 1988). Translation of mRNA and subsequent membrane insertion of connexin monomers occurs in the endoplasmic reticulum (ER) (Falk et al., 1994). The formation and transport of connexons through the biosynthetic pathway is differentially regulated and is thought to be based on the cell type (Musil and Goodenough, 1993; Koval et al., 1997). The oligomerization step, in which six connexins are bound together to form a single
Figure 2. Model of Cell-Cell Communication

(A) Small molecules (>1 Kd: e.g. ions, second messengers) transit through gap junction intercellular communication channels that have formed an aggregate or ‘plaque’ between cell 1 and cell 2. (B) Transmission Electron Microscopy of a gap junction plaque.
connexon/hemichannel, may take place in the ER or the Golgi apparatus depending on the type of cell or connexin (Diez et al., 1999; Koval et al., 1997; Kumar et al., 1995; Musil and Goodenough, 1993; Musil and Goodenough, 1991). The functional association of phosphorylation with connexins during assembly, has yet to be determined, though it is thought that phosphorylation may suffice for connexon trafficking through the Golgi or export to the plasma membrane (Solan and Lampe, 2004). Once assembled, some connexons are thought to follow the membrane trafficking route through the Golgi, trans-Golgi Network and on to the plasma membrane where they are inserted (VanSlyke and Musil, 2000; Falk et al., 1997; Wang et al., 1995) (Fig. 1).

1.3 GAP JUNCTION PLAQUE FORMATION

Gap junction plaques are specialized membrane domains wherein clusters of gap junction channels function in intercellular communication (Fig. 1). In an important work from the Falk group, it was demonstrated that plaque formation occurs via the lateral movement of hundreds or thousands of individual channels on the plasma membrane into a single cluster (Lauf et al., 2002). The actual regulation of channel aggregation into plaques has been demonstrated to coincide with physiological processes such as parturition and therefore, may involve hormones and downstream signaling mechanisms (Garfield, 1980; Risek et al., 1990; Risek et al., 1995). For example, gap junction formation was increased in smooth muscle (myometrium) cells following the exposure of uterine tissue to exogenous estrogen and prostaglandins (Garfield et al., 1980). The prostaglandin PGE in particular has been shown to increase intracellular cAMP levels which are thought to increase gap junction clustering into plaques on the plasma
membrane (Wang and Rose, 1995; Brueggemeier et al., 2003; Faucheux et al., 2002). Interestingly, prostaglandins have also been demonstrated to increase estrogen biosynthesis; the activation downstream targets of the estrogen receptor includes cAMP and protein kinases (e.g. PKA) which have been demonstrated to phosphorylate the connexin monomers of gap junction channels and may be involved in plaque formation (Garfield et al., 1980; Driggers and Segars, 2002). Gap junction clustering is a regulated event that correlates with physiological processes and formation of a distinct plaque compartment may also be regulated.

The creation of a plaque domain is thought to involve the plasma membrane lipids components as well as tight junction proteins (Abney et al., 1987; Singh et al., 2005). Biophysical analyses reveal that the interactions ‘holding’ the plaques together may involve electrostatic, contact repulsion and lipid mediated forces (Abney et al., 1987). The creation of this membrane compartment is thought to involve the PDZ domains of tight junction proteins ZO-1 and 2 and their interaction with the C-terminus of connexin 43 (Singh et al., 2005). It is thought that ZO-1 and 2 may function by stabilizing the gap junction plaques on the membrane (Singh et al., 2005). In addition it is has been demonstrated that the α and β catenin proteins may also play a role in mediating the interaction between ZO-1 and Cx43 during gap junction plaque assembly specifically, in cardiomyocytes (Wu et al., 2003). Once the plaques are formed, it is thought that connexons are continuously added to the periphery of existing gap junctions (Gaietta et al., 2002; Lauf et al., 2002). Once it is part of the plaque, it is thought that the gap junction channel is capable of opening and closing (gating).
1.4 GAP JUNCTION CHANNEL GATING

Gap junction communication consists of the mutual transit of small molecules including ions, secondary messengers, amino acids and nucleic acids via intercellular channels that can be gated in response to several stimuli including voltage change, intracellular pH, hormones and connexin phosphorylation (Solan and Lampe, 2004; Simpson et al., 1977; Lowenstein, 1978). Gap junction proteins are actually targets of a variety of protein kinases that have been reported to affect the opened and closed status of the channel pore including: protein kinase C, serine/threonine and tyrosine kinases (Cruciani and Mikalsen, 2002 rev). In fact, the down regulation of gap junction communication, i.e. open status, has been correlated with increased connexin phosphorylation (Solan et al., 2003). Several connexin proteins, including Cx32, Cx26 and Cx43 have been characterized as likely phosphorylation targets of protein kinase C (PKC) activity (Takeda et al., 1987; Mazzoleni et al., 1996). The phorbol ester (TPA) induced activation of PKC has been associated, in several cell types, with the inhibition of gap junction intercellular communication (GJIC) of fluorescent dyes and single channel conductances (Kwak et al., 1995; Mazzoleni et al., 1996; Rivedal and Liethe, 2005). Growth factors such as the epidermal growth factor (EGF) bind and activate specific membrane receptors and set into motion the activation of mitogen activated phosphorylating kinases (MAPK) intracellular signaling cascades that have been directly associated with reduced or increased gap junction communication, depending on the cell type (Rivedal et al., 1996; Lau et al., 1992). Disruption of gap junction communication and phosphorylation of Cx43 serine residues were reported in cells treated with the epidermal growth factor (Lau et al., 1992; Kanemitsu and Lau, 1993). Reduced gap junction communication was also reported along with the tyrosine phosphorylation of Cx43 src homology domains (SH2 and SH3) in v-src transformed cells (Azarnia et al., 1988; Kanemitsu et al., 1997). Alternatively, a
MAP kinase dependent signaling pathway is thought to be the cause of increased gap junction communication observed in human kidney epithelial cells, following the addition of epidermal growth factor (Rivedal et al., 1996). Under hypoxic conditions, PKA mediated targeting of connexin 43 has been shown to affect the open status of gap junction channel and thereby promote electrical coupling of cardiac myocytes (Matsumura et al., 2006). The regulation of gap junction communication may affect the transit of factors necessary for maintaining normal cell physiology and it is thought that the regulation of communication may also precede or coincide with gap junction removal.

1.5 GAP JUNCTION REMOVAL

It is thought that the removal of plaques from the cell surface may be a way of regulating intercellular communication; and thereby affecting physiological processes (Merk et al., 1973; Albertini et al., 1974; Dewey and Barr, 1974; Perissel et al., 1976; Larsen et al., 1979; Murray et al., 2004). The removal of gap junction channels is thought to occur from the center of the plaque and consists of the internalization of gap junction channel plaques into one of the two cells for the formation of a double membrane cytoplasmic vesicle referred to as annular gap junctions (Gaietta et al., 2002) (Fig. 1). Annular gap junctions are vesicular structures that are formed when gap junction plaques are removed from the plasma membrane by being internalized into the cytoplasm of one the two contacting cells (Murray et al., 1981). Annular vesicles have been characterized with light and transmission electron microscopy in several cell types including, rabbit granulosa, human adrenal cortical adenocarcinoma and HeLa cells (Larsen et al., 1979; Murray et al., 1981; Piehl et al., 2007). The Larsen group initially demonstrated the
association between annular gap junctions and bristle coats at the transmission electron microscopy level (Larsen et al., 1979). This bristle coat was similar in appearance to the coat that surrounds clathrin coated pits and vesicles. This has lead to the hypothesis that clathrin may facilitate gap junction plaque internalization. Further support of this hypothesis was provided by immunocytochemical studies in which Cx43-GFP was expressed in HeLa cells. It was suggested that clathrin colocalized with Cx43-GFP positive annular gap junction vesicles, but this could not be confirmed at the light microscopic level (Piehl et al., 2007). The possibility that clathrin could be involved in gap junction plaque removal is supported with the finding of several putative clathrin adaptor protein (AP2) binding sites (YXXΦ) within the C-terminal of the connexin 43 sequence. However, a direct association between clathrin and/or mediators of clathrin driven internalization of gap junction plaques and annular gap junctions has yet to be demonstrated. Furthermore, clathrin mediated endocytosis of integral membrane proteins has been implicated in cellular migration (Kamiguchi and Lemmon, 2000; Rappoport and Simon, 2003). To better understand the role of gap junctions in cell migration, there is a need to demonstrate whether there is an association between clathrin and gap junction structures and assess the nature of the bristle coat and internalized annular gap junction vesicles.

1.6 CELLULAR MIGRATION

To understand the possible role of gap junction intercellular channels in migratory cell populations, it is useful to begin with a discussion of the physiology of migration. Cell migration is an evolutionarily conserved mechanism that underlies numerous physiological processes including; embryonic development, functioning of unicellular and multicellular organisms,
maintenance of injured tissues, the inflammatory immune response, angiogenesis and metastasis (Horwitz and Parsons, 1999; Kurosaka and Kashina, 2008). The cell types that participate in migration vary, however the process is virtually consistent for each. The stimulus for cell motility begins with external signals that are converted into intracellular gradients which establish the cellular polarity necessary to promote migration (Horwitz and Webb, 2003; Bretscher, 1996 rev.). Protrusion at the leading edge of the plasma membrane is directed by signaling mediators that organize actin-myosin polymerization into protrusive lamellipodia or filipodia (Vincente-Manzanares et al., 2005, Jaffe and Hall, 2005) (Fig. 3). Stabilization of the protruding membrane involves the establishment of large and dynamic focal adhesion protein complexes which relay signals between the cytoskeleton and extracellular matrix (ECM) (Ridley et al., 2003; Horwitz and Webb, 2003) (Fig. 3). Contraction and forward motion arise from the focal adhesion complexes via integrins which serve as traction sites and transmit information about the physical status of the ECM into the cell and thereby alter the assembly and disassembly of focal adhesions (Ridley et al., 2003; Vincente-Manzanares et al., 2005) (Fig. 3). In response to the extracellular signal that is transduced through the integrins, actin polymerization produces a myosin force at the leading edge and propels the cell body forward (Horwitz and Webb, 2003). In the final and rate-limiting step, the rear release of adhesion complexes occurs, allowing the forward progression of migrating cells (Fig. 3). This sequence of events is highly conserved, and perturbations affecting any one of the aforementioned steps may lead to developmental or physiological deficiencies.
Figure 3. The steps of cell migration.

Protrusion of the leading edge of the plasma membrane (step 1). The leading edge is stabilized with dynamic focal adhesions that relay signals from the external environment to the cell (step 2). Contraction arises from integrins which are part of the focal adhesion complex and serve as traction sites to the ECM (step 3). Rear release of focal adhesions from the trailing edge allows forward progression of the cell (step 4).
1.7 THE ROLE OF GAP JUNCTIONS IN CELL MIGRATION

By regulating the passage of small molecules, gap junction channels have been suggested to play a pivotal role in orderly and effective cell migration, which includes the regulation of proper direction and rate of cell movement (Fushiki et al. 2003; Huang et al. 1998; Kwak et al. 2001; Lo 1999; Richards et al. 2004; Tate et al. 2006; Xu et al. 2006; Xu et al. 2001). For example, the trajectory of glioma cell migration in a two-dimensional culture appears to be dependent on cell-cell communication through gap junction channels (Aubert et al. 2006). Enhanced gap junction communication in neural crest cell populations is associated with an increased rate of cell migration (Huang et al. 1998) and inhibition of gap junctions has the opposite effect, in reducing the rate of cell migration (Ashton et al. 1999; Huang et al. 1998; Lo et al. 1999). The Menezes group, in particular, have shown that inhibition of gap junction communication reduced migration of striatal subventricular zone cells to the olfactory bulb (Menezes et al. 2002; Marins et al., 2009) which would suggest the need for gap junction communication during coordinated cell movement. The communication of molecules as cells migrate though has not yet been previously demonstrated. It has however been demonstrated that cell motility was reduced when cells were treated to decrease communication (Pepper et al., 1989). In contrast, it has been suggested that the process of uncoupling may actually trigger migration in some cell types (Batten and Haar, 1979). Based upon these observation it is likely that gap junctions play a pivotal role in cell migration.

The proper coordination of migratory cell clusters poses a distinct set of challenges and numerous migration studies have shown that mutations affecting gap junction-mediated intercellular communication are associated with migration defects which results in aberrant development (Huang et al, 1998; Reaume et al., 1995; Ruangvoravat and Lo, 1991).
These defects alter normal physiology and include heart malformations (viscerointrial heterotaxia) (Britz-Cunningham., 1995) and oculodentodigital dysplasia with a phenotype that includes limb syndactyly, craniofacial and skeletal anomalies (Paznekas et al., 2003). It is suggested, based on such studies, that gap junction channels may facilitate the coordination of cells for migration, and there is a need for greater understanding of gap junction function and behavior in migratory cell populations particularly since the coordinated movement of cell populations appears to be essential for development, wound healing, and other physiological responses and intercellular communication via gap junctions may be required for the mediation of coordinated cellular migration. Characterizing the status of gap junctions and the capacity for communication in migrating cell populations may also further our understanding of the mechanisms that underlie physiological processes that require migration.
Figure 4. Migration and Gap Junctions

(A) Cells grow to confluency and form a monolayer in culture and a micropipette tip is used to create a scratch down the center of the monolayer and stimulate cells to migrate. (B) Cells migrate in a sheet-like formation toward the leading edge and maintain intercellular gap junction channels which may aid in coordinating migration of a group of cells. In order for sheet migration to proceed, the leading cell releases its rear focal adhesions while the plasma membrane of the trailing cell protrudes and establishes focal adhesions that stabilize the cell.
It has been suggested that gap junctions play a pivotal role establishing body plan during embryogenesis and mutations affecting connexin proteins may hinder proper development (Khalimi and Lo, 1988). Connexin 43 (Cx43) is the primary isoform that is expressed in all germ cell types, and mutations or knock-out models affecting Cx43 gap junction expression or function have been shown to affect migrating primordial cells. Mouse embryonic neural crest cells typically express high levels of Cx43 transcripts and are capable of direct cell-cell communication and sheet-like migration, however connexin 43 null (Cx43−/−) mice exhibit open cranial and conotruncal heart malformations due to neural crest cells that fail to migrate to their proper developmental targets ((Ruangvoravat and Lo, 1992; Lo et al., 1997; Lecanda et al., 2000; Reaume et al., 1995). Epithelial cells, isolated from Drosophila, fail to migrate during development when the connexin homologue that is expressed in invertebrates, innexin 2, is mutated (Bauer et al., 2002, Phelan, 2005 review). Pioneering work from the Lo group showed that neural crest cells isolated from Cx43 over expressing mouse embryos exhibit increased migration rates and directionality while the speed and directionality are significantly reduced in migrating primordial germ cells isolated from Cx43 mouse embryonic knock-outs (Huang et al., 1998; Francis and Lo, 2006). Furthermore, when Cx43 levels were altered in mouse models and cardiac neural crest cells (CNCs) were analyzed to determine the effects on polarized cell migration and directional movement: the speed and directionality of migration were both reduced in Cx43 knock-out cells, whereas both speed and directionality were increased in cells that over expressed Cx43 (Xu and Lo, 2006). Multiple mutations of the Cx43 gene affecting regulation of gap junction mediated communication were associated with heart malformations in patients with congenital heart defects (visceroatrial heterotaxia) (Britz-Cunningham., 1995). Gap junction
mediated communication and the speed of migration were significantly reduced in cellular explants isolated from Cx43 hemizygotes and knockout mice (Li et al., 2002). It has been demonstrated with the aid of both molecular and genetic analyses that a missense substitution in the Cx43 gene results in primordial cells that fail to establish left/right asymmetry during development (Gebbia et al., 1997). Gap junctions thus may play an important role in cell migration during the establishing the tissue architecture of the body and may also aid in preserving tissue structures.

1.9 GAP JUNCTION EXPRESSION AND METASTATIC CELLULAR MIGRATION

Connexin expression is reported to have tumor suppressive properties that may prevent the uncontrolled proliferation and migration of cancer cells (Mehta et al., 1986; Shao et al., 2005). For instance, the overexpression of Cx26 in the MCF-7 breast cancer cell line was reported to significantly reduce migration (Momiyam et al., 2003). The migration of MDA-MB-231 breast cancer cells and underlying human vascular endothelial cells (HUVECs) were also reduced when both Cx26 and Cx43 were exogenously expressed in the cell lines (McLachlan et al., 2006). In a similar study, MDA-MB-435 breast tumor cells that expressed either functional or non-functional forms of Cx26, exhibited significantly slower the rates of cell migration, and reduced cell invasion (Kalra et al., 2006). Furthermore, the exogenous expression of Cx43 inhibited breast cancer cell migration into a bone microenvironment (MacLachlan et al., 2007). Brain derived glioma cells that exhibited greater gap junction intercellular communication and expressed more surface Cx43 had slower migration and proliferation rates than cells with decreased Cx43 expression and gap junction communication (McDonough et al., 1999).
Conversely, connexin 43 expression in gliomas enhanced the formation of homo- and heterocellular type gap junctions and is thought to facilitate the invasion into co-cultures of astrocytes, brain slice cultures and parenchymal tissue in nude mouse brain (Oliviera et al., 2005). Similarly, gap junction deficient mammary epithelial tumor cells that expressed exogenous Cx43 were able to form heterocellular gap junctions with human endothelial cells and exhibited enhanced diapedesis (Pollmann et al., 2005). Thus, it appears that the lack of gap junction communication may facilitate the out of control proliferation and enhanced metastatic potential of cancerous cells; and that the same cancerous cells may in turn, be able to use gap junctions to facilitate invasion and subsequent transformation of normal tissues.

1.10 THE ROLE OF GAP JUNCTIONS IN CELL MIGRATION DURING THE INFLAMMATORY IMMUNE RESPONSE

Migration that occurs during the immune response involves the cooperation of one or more cell types, and may require gap junction mediated communication. Early transmission electron microscopy studies revealed the presence pentilaminar intercellular junctions, or gap junctions, between vascular endothelial cells and migratory lymphocytes (Campbell., 1983). Similarly, intercellular communication was detected between endothelial cell layers that formed heterocellular-typic gap junctions with trans-endothelial migrating lymphocytes (Orvieto-Orda et al., 2002). Both enterocyte migration and gap junction communication were inhibited by the release of nitric oxide from macrophages during the inflammatory immune response (Anand et al., 2008). The migration of enterocytes was also significantly inhibited when gap junction communication was interrupted in cells that were treated with the proinflammatory cytokine,
interferon γ (Leaphart et al., 2007). Alternatively, leukocytes and neutrophils treated with Cx43 inhibiting mimetic peptides displayed enhanced transmigration through endothelial cells (Zahler et al., 2003). Generally speaking, it is reasonable to assert that gap junction communication allows for the necessary cooperation between multiple cell types that takes place during the immune response and may also facilitate the reestablishment of tissues following trauma.

1.11 GAP JUNCTIONS AND WOUND HEALING

Cell migration is critical for wound repair in a variety of tissues, and the role of gap junction mediated communication has been hypothesized to influence this process. For example, increased gap junction plaque numbers were observed in epithelial and myofibroblast cell populations during wound healing (Gabbiani et al., 1978). Furthermore, increased Cx43 protein and mRNA levels were associated with sustained cell-cell communication during wound healing (Larson and Haudenschild, 1988; Pepper et al., 1989; Pepper et al., 1992). Conversely, gap junction communication was not detected during wound healing in migrating cells that were isolated from the imaginal wing disc of adult Drosophila (Bryant and Fraser, 1988). Additionally, cell migration rates were increased in mouse fibroblasts that lacked the capacity for gap junction intercellular communication (Lin et al., 2006). Interestingly, regulated increases and decreases in the spatio-temporal expression of specific connexin monomers in-situ are also thought to affect wound healing (Goliger and Paul, 1995; Richards et al., 2004). For example, in wounded epidermis Cx26 was up-regulated in cells proximal to the wound, but was down-regulated in cells located at the wound edge; in contrast, Cx31.1 and Cx43 were down-regulated in cells both peripheral to and at the wounded edge (Goliger and Paul, 1995). In human foreskin
keratinocytes, migration was coupled with an initial increase in Cx43 immunostaining and gap junction intercellular communication (GJIC) at the wound edge followed by a 24 hour period of restriction of Cx43 expression to the basal epidermal layer and reduced cell-cell communication (Richards et al., 2004). Thus, while it appears that gap junction expression and communication during wound healing may be cell type specific, determining the status and behavior of gap junctions in specific cell types will greatly increase our understanding of the role that gap junctions play in migrating cells.

1.12 SUMMARY

Cell migration is a ubiquitous event that is necessary for embryonic development and maintaining the integrity of tissues and organs (Locascio and Nieto, 2001; Kurosaka and Kashina, 2007). Integrating extra and intra-cellular responses such that an entire population migrates in a regulated and coordinated manner, which is also biologically relevant, has been hypothesized to require direct cell-cell communication via gap junction intercellular channels. Gap junction channels facilitate the direct exchange of electrical and chemical information between adjacent cells in tissues and organs (Loewenstein, 1966; Gilula et al., 1971; Sheridan, 1971). It is thought that factors that regulate gap junction function or dynamics are likely to alter gap junction channels and thereby affect physiological processes such as cellular migration.

Cell migration underlies numerous physiological processes including; embryonic development, the inflammatory immune response, metastasis and wound healing, (Horwitz and Parsons, 1999; Kurosaka and Kashina, 2008). The proper coordination of migratory cells poses a distinct set of challenges and numerous migration studies have shown that mutations affecting
gap junction mediated intercellular communication are associated with migration defects may lead to and diverse array of developmental and/or physiological deficiencies. Yet the status of gap junctions in migrating cells is not completely understood.

Furthermore, while the pattern of gap junction plaque distribution and coupling in adult and embryonic tissues has been extensively studied (Andries et al. 1985; Becker et al. 2002), and the life cycle of gap junction plaques in non-migrating cell populations has been described (Laird 1996; Lauf et al. 2002; Lopez et al. 2001), the presence and functionality of gap junction plaques in migrating populations has not been demonstrated. It is clear, however, that communication during cell movement requires that the gap junctions must be retained or continually reassembled at new sites of cell-cell contact as cells migrate.

The presence and functional status of gap junctions in migrating cells have been assessed with a combination of light and transmission electron microscopy, immunocytochemistry, fluorescence recovery after photobleaching (FRAP) and live cell imaging of migrating cells. The affect of altering gap junctions have been measured with pharmacological agents and siRNA techniques on cell migration have been demonstrated. And finally, the fate of gap junctions in migrating cells and the molecular mechanisms involved in gap junction processing have been demonstrated with live cell imaging, immunocytochemical and transmission immuno-electron microscopy. The methods that haven been used to conduct these studies will be discussed in the following section and the presentation of the data supporting the hypothesis will ensue.

In this study, we provide the first characterization of the relationship between cell movement and gap junction plaque dynamics revealed by time-lapse microscopy in migrating cells. The internalization of gap junction plaques to form cytoplasmic annular gap junction packets was demonstrated during cell migration when cells detached from one another. The
morphology, origin and fate of these annular cytoplasmic structures have been previously studied by us and others (Jordan et al. 2001; Jordan et al. 1999; Lopez et al. 2001) but not during cell detachment and migration. Our findings establish for the first time the presence of gap junction communication during migration, the retention of gap junction plaques at the initial sites of formation, and the internalization of plaques during cell process detachment. This is also the first study to demonstrate that the bristle coat found surrounding annular gap junctions is composed of clathrin; and that clathrin accumulates on one side of the gap junction plaque, indicating its role in mediating gap junction plaque internalization. We have termed this internalization process, endoexocytosis.
2.0 METHODS

2.1 CELL CULTURES

The human adrenal cortical tumor cell line (SW-13) was obtained from American Type Cell Culture (ATCC), (Manassas VA). SW-13 cells were utilized because these cells form numerous and large gap junction channels, and in addition have been demonstrated to have annular gap junction structures. Cells were maintained Leibovitz’s L-15 media prepared to a final volume of 500 mL (Gibco, Grand Island, NY) that contained 10% fetal calf serum, 2.0% fungizone (250 µg/mL amphotericin B + 205 µg sodium deoxycholate) and 1.0% Pen Strep (10,000 Units/mL Penicillin + 10,000 µg/mL Streptomycin) (Gibco, Grand Island, NY; Gemini BioProducts, West Sacramento, CA). Cell cultures were maintained at 37°C and 5% CO₂.

The SBAC bovine adrenal cell line was cultured in DMEM/F-12 media (Gibco, Grand Island, NY) containing 10% fetal calf serum (Gibco, Grand Island, NY), 2.0% fungizone (250 µg/mL amphotericin B + 205 µg sodium deoxycholate) and 1.0% Pen Strep (10,000 units/mL Penicillin + 10,000 µg/mL Streptomycin) (Gibco, Grand Island, NY), and basic fibroblast growth hormone (BD biosciences, Bedford, MA). Cells were cultured at 37°C and 5% CO₂. This cell line is ACTH responsive, capable of assembling gap junctions and secreting steroids similar to adrenal cortical cells in vivo.
Y-1 mouse adrenocortical cells were obtained from ATCC and cultured in DMEM media supplemented with 15% horse serum, 2.5% fetal bovine serum (Gibco, Grand Island, NY), 2.0% fungizone (250 µg/mL amphotericin B + 205 µg/mL sodium deoxycholate) and 1.0% Pen Strep (10,000 Units/mL Penicillin + 10,000 µg/mL Streptomycin) (Gibco, Grand Island, NY). Cells were maintained at 37°C and 5% CO₂. Some Y-1 subclones express gap junction proteins while other subclones do not.

HeLa epithelial carcinoma cells were obtained from ATCC. Cell were cultured in DMEM (Gibco, Grand Island, NY) supplemented with 10% fetal calf serum, 2.0% fungizone (250 µg/mL amphotericin B + 205 µg sodium deoxycholate) and 1.0% Pen Strep (10,000 Units/mL Penicillin + 10,000 µg/mL Streptomycin). Cells were maintained in 5% CO₂ at 37°C. HeLa cells lack gap junction proteins and therefore, are ideal for studies examining the role of gap junctions with connexin cDNA expression techniques.

### 2.2 TRANSFECTION

To visualize gap junction dynamics in migrating cells, SW-13 cells were transfected cDNAs encoding for fluorescent Cx43-GFP or GFP control vector (kindly provided by Dr. Matthias Falk, Lehigh University). The Cx43-GFP was constructed by linking the green fluorescent protein (GFP) to the C-terminus of the rat connexin 43 cDNA. The Cx43-GFP fusion protein has been previously characterized in and demonstrated to assemble into gap junction plaque structures similar in kind to endogenous Cx43 (Falk 2000; Lopez et al., 2001). The Lipofectamine 2000 (Invitrogen, Carlsbad, CA.) transfection reagent was used to transiently transfect cell populations with Cx43-GFP.
Cell populations were grown in 35 mm tissue culture dishes until 70-80% confluency was reached. Cells were transfected in 2 ml of sterile Opti-MEM media (GIBCO, Carlsbad, CA.) that contained 10 µL Lipofectamine 2000 and 4 µg of plasmid cDNA (Cx43-GFP or GFP cDNA). Cells were maintained at 37°C and 5% CO2 for six hours. The transfection medium was then removed by gentle aspiration and cells were washed with phosphate buffered saline (PBS). Fresh L-15 complete growth media was added to the dishes, and cells were incubated at 37°C for 48 hours prior to imaging.

2.3 IMMUNOCYTOCHEMISTRY

Immunocytochemistry of proteins was performed as previously described (Oyoyo et al., 1997). Briefly, cells were grown on sterile glass coverslips and then fixed with 4% paraformaldehyde for 20 minutes and rinsed 3 times with 1X PBS. Cells were permeabilized with ice-cold acetone for 7 minutes and then rinsed with 1X phosphate buffered saline (PBS) solution. Primary antibodies: Cx43 (1:100) (0.5mg/mL) monoclonal and polyclonal (Zymed, South SanFrancisco, CA); Clathrin (1:10) mouse, AP2 (1: 10) mouse, Dab2 (1:10 ) rabbit, Epsin (1:10 ) rabbit (were all kindly provided by Dr. Linton Traub); Dynamin (1:50) (5 µg/mL) rabbit (Transduction Labs), and secondary antibodies: AlexaFluor goat anti mouse (GAM) or goat anti rabbit (GAR) conjugated to fluorophore 488 or 594 (1:1000) were prepared in blocking buffer containing: 1X PBS, bovine serum albumin (BSA) and goat serum. Coverslips containing the cells were inverted onto primary antibody and incubated at 4°C, overnight, or for 1 hour at 37°C. The cells were washed for 10 minutes in 1X PBS, 3-4 times, placed in secondary antibody for 1 hour at 37°C and then washed for 10 minutes 3-4 times in 1X PBS. In some studies, the cells were
incubated with phalloidin conjugated to AlexaFluor 488 (prepared in blocking buffer) for 1 hour at 37°C and then washed 3-4 times for 10 minutes in 1X PBS before placing in the secondary antibody. To visualize nuclei, cells were incubated with Hoescht nuclear dye (1:1000 in 1X PBS) for 3 minutes at room temperature before drying and mounting in Fluormount G anti-quenching agent (Southern Biotechnologies Associates, Birmingham, AL) on glass slides. Fluorescence microscopy imaging was characterized with either an Olympus IMT-2 or Nikon Microphot FXA fluorescence phase contrast microscope and quantifications were made with the MetaMorph® software program.

2.4 WESTERN BLOT

Cells were lysed in 0.5% SDS lysis buffer and sonicated for 20-30 seconds. Samples were dried under vacuum overnight and reconstituted in sterile water. Samples were diluted with 4X Laemmli sample buffer for a final 1X sample buffer concentration. The protein concentrations of samples were quantified with the Bicinchoninic Acid (BCA) (Pierce, Rockford, IL) method and spectrophotometry. Samples were quantified with a Softwarepro® software program.

Samples were loaded and run on 10% precast or 12% polyacrylamide gels in buffer (1X Tris/Glycine/SDS) for 1-2 hours at 200 mV. Samples were transferred to a PVDF membrane (Millipore, Bedford, MA.) in (Tris-HCl/Glycine/Methanol (20%) buffer for 1 hour at 100 mV. To verify that proteins were successfully transferred, the PVDF membrane was stained with Ponceau S and then destained with acetic acid (1%). To limit non-specific binding, membranes were placed in a blocking solution containing 1X TBST and 5% non-fat dry milk (NFDM) or 1-
2% BSA and placed on a rocking apparatus for 1 hour at room temperature. Membranes were then placed in a primary antibody solution containing anti-Cx43 (1:1000) or anti-α-tubulin (1:1000) antibody overnight at 4 °C with continuous rocking and washed extensively with 1X TBST buffer the following morning. A horseradish peroxidase (HRP) conjugated secondary antibody (1:10,000) in blocking solution was applied for 1 hour at room temperature. The membrane was washed with 1X TBST 3 times (10 min.) and an ECL substrate (Millipore, Bedford, MA.) was applied for 1 minute. Protein bands were imaged with either phosphorimager or on x-ray film (Fuji).

2.5 MIGRATION ANALYSIS

Cellular migration was assessed with a wound healing assay (Rodriguez et al., 2005). Briefly, cells were plated and then grown until cells reached confluency in 6 or 12 well tissue culture plates. Cell populations that became overgrown with a secondary layer were discarded. To initiate migration, a mechanical scratch was created in the monolayer with a sterile 0.5 µL micropipette tip, leaving behind a denuded space. Cells were washed three times with 1X PBS to remove any debris or loosened cells and fresh media was applied thereafter. The underside of the tissue culture was marked in order to locate the same exact area of the denuded space over time. The progression of cells into the open space was monitored at the same location over a time course: (0 min., 2, 4, 6 and 24 hours) post wounding with phase contrast microscopy (Olympus IMT-2 microscope). The cells were maintained at 37 °C for the duration of the time course. Multiple fields were acquired for each time point and the width or area of the denuded space was measured for each time point with the computer-assisted MetaMorph® data analysis software.
program. The distance that cell migrated was calculated by measuring the area of the denuded space at each experimental time interval. The experiment was performed in triplicate and a minimum of two fields from three separate wells at each time point were analyzed. The data were expressed as mean area ± SEM. A statistical comparison of the means was calculated with the Student’s T-test and a p-value of ≤0.05 was considered statistically significant.

2.6 TIME-LAPSE IMAGING

SW-13 cells expressing Cx43-GFP were grown to confluency on 40 mm. round glass coverslips. The coverslip was placed into a temperature controlled FCS2 Bioptechs chamber (Butler, PA) and maintained at 37°C on a Zeiss Axovert 135 microscope stage. The cells remained in culture media supplemented with 10 mM HEPES at pH 7.2. Differential interference contrast (DIC) and/or fluorescence microscopic images were obtained using standard fluorescein-isothiocyanate (FITC) filters and a 40X or 63X oil objectives. Images were collected at 3 or 5 minute intervals with a Hamamatsu ORCA ER camera. In some experiments the cell population was wounded before being placed in the chamber.
2.7 FUNCTIONAL GAP JUNCTION COMMUNICATION ASSAYS

2.7.1 Lucifer yellow functional dye-coupling assay

Cells were seeded on glass coverslips or tissue culture plates and cultured until a confluent monolayer was formed. The culture media was removed and Lucifer yellow dye (Invitrogen, Carlsbad, CA) (5mg/ml) or rhodamine dextran (5mg/ml) prepared in 1X PBS or EBSS was added to the cells. To load the dye into the cells, a scratch was created in the monolayer with a sterile 0.5 µl micropipette tip. Cells were incubated with the dye for 5-7 minutes at 37 °C and then rinsed with 1X PBS and immediately imaged or fixed with 4% paraformaldehyde and then imaged. Functional dye coupling was assessed with phase contrast and fluorescence microscopy (Olympus IMT-2 fluorescence microscope). Green fluorescence (Lucifer Yellow) in cells away from the cut edge was considered to be gap junction mediated communication of Lucifer yellow dye while red fluorescence in cells at the cut edge was considered to be (rhodamine dextran). Since rhodamine dextran is too large to move via gap junctions, red fluorescence away from the cut edge was used as a measure of non-gap junction mediated dye movement (most likely the result of cell population damage).

2.7.2 Fluorescence Recovery after Photobleaching (FRAP)

SW-13 adrenal cells were grown to confluency on glass coverslips and loaded with 2.0 µM green-fluorescent- calcein-AM (Molecular Probes, Eugene, PR.) for 15 min. at 37 °C. Calcein-AM is a membrane permeable dye which, upon entering a viable cell, is cleaved into a fluorescent membrane impermeable form that is capable of transiting gap junction channels but
not through other plasma membrane channels (Pollmann et al., 2005). To initiate migration, calcein-AM loaded cells were scratched with a 0.5 μL pipette tip and rinsed extensively with complete L-15 media to remove loosened cells and debris. The cells were maintained at 37°C in an open chamber microincubator (Harvard Apparatus) in media. In some experiments, cells that were preteated with connexin 43 specific siRNA were monitored. In these studies, cells were transfected according to transfection protocol and analyzed 24 hours later. The FRAP assay was performed on cells visualized with a 40X objective on an inverted Olympus Fluoview 1000 confocal microscope. Real time photobleaching was performed with a 405 nm line on a laser diode and excited with a 488 nm line on the argon laser. Regions of interest were drawn around cells that were either at the wound edge or located several cells away from the wound edge. The areas were photobleached for 10 seconds with 100% laser output and bleached and nonbleached (control) regions were sequentially imaged (3 second scan, every 10 seconds for 3 minutes). Images were captured with FluoView software. FRAP was performed in duplicate at multiple times over a six hour period following the initiation of migration. The extent of bleaching and post-bleach recovery of fluorescence was analyzed with MetaMorph® software by measuring change in fluorescent intensity over time within the bleached region and a control region far removed from the bleached region. For statistical analysis, 20 cells located 4 or more cells away from the wound margin, 20 bleached cells at the margin and 40 unbleached cells (controls) were monitored. In addition, three cells that were not in contact with other cells and hence had no gap junctions (negative controls) were also bleached and monitored. The data are expressed as the percent fluorescent recovery ± SEM after bleaching.
2.8 TRANSMISSION ELECTRON MICROSCOPY

Cells were processed according to Murray et al., 1981. Briefly, cell monolayers were fixed with 2.5% glutaraldehyde buffered with 0.05M cacodylate followed by 1% osmium in 0.05M cacodylate for 20 minutes and then dehydrated with ethanol and embedded in Araldite (Cedar Grove, NJ.). Ultra thin sections were mounted on grids and imaged with a JEOL 1210 electron microscope.

2.9 QUANTUM DOT

Quantum dots were used to specifically immnolabel molecules (e.g clathrin) that mediate gap junction plaque internalization. Sw-13 cells grown on coverslips were fixed with 2% paraformaldehyde and 0.1% glutaraldehyde in PBS. Cells were incubated with Cx43 or clathrin primary antibodies, followed by biotin-xx IgG conjugated antibody, and then in a QD conjugated secondary antibody (Streptavidin conjugates with QD 655). To test the specificity of the QD immuno-staining cells were either: incubated in quantum dot-linked anti-rabbit immunoglobulin without preincubation of the thin sections in primary antibody or; binding of QD complexes to embedded resin was assessed in area that were free of cells. Analysis of quantum dot immuno-staining was made with TEM at 60,000 or 100,000X on micrographs that were taken from each of three preparations. The number of annular gap junction vesicles coated with quantum dots was assessed and the data are expressed as a percent of the total annular gap junction vesicles counted.
2.10 MIMETIC PEPTIDES

Cx43 specific memetic peptides, Gap 26 (HVCYDKSFPISHVR) and Gap27 (HSRPTEKTIFII), were obtained from Sigma Genosys (St. Louis, MO). Peptide stock solutions were prepared in 1X PBS and then added to media for a final concentration of 600 µM. Confluent cells (~90%) were incubated with the peptides for 3 hours prior to the migration assay. Cells were wounded, rinsed and cultured in the presence of the mimetic peptide during migration. Cells were assayed for functional dye coupling and migration analysis with phase contrast microscopy.

2.11 CONNEXIN 43 SIRNA KNOCKDOWN

Pre-designed connexin 43 siRNA oligos were obtained from Ambion (Austin, TX), resuspended in DEPC-treated, nuclease free water (Ambion, Austin, TX) and then stored at -20°C until use (siRNA sense strand sequence Gja1 siRNA sequence 1: 5’-UAGGCAAAACUCCUUGACAAGGUUCA-3’; Gja1 siRNA sequence 2: 3’-UUAUCCGUUUGAGGAACUGUUCCAAGU-5’; and Gja sense strand sequence 3: 5’-CAGTCTGCTTTTCGTTGTA-3’). The Gja1 siRNA sequence 1 was chosen because it effectively reduced connexin 43 expression. The Cx43 siRNA oligo (3 µL) was transferred into 497 µL of OptiMEM media (Invitrogen, Carlsbad, CA) and 10 µL of Lipofectamine 2000 (Invitrogen, Carlsbad, CA) (10 µL) for a final oligo concentration of 10 nM. and then added to cells. For transfection SW-13 cells were cultured in either 6 or 12 well tissue culture plates at 75-85% confluency. Cells were incubated in the presence of the transfection media (0.5 mL in 6-well or 1 mL in 12-well) for six hours. At the end of the incubation, the transfection media...
was removed and replaced with complete L-15 culture media for an additional 12 – 18 hours before analysis of Cx43 protein expression was assessed with western blot and immunocytochemistry.

2.12 STATISTICAL ANALYSIS

Comparisons of the mean ± standard error of the mean (SEM) area of the denuded space over the course of migration were made with the Student’s T-test or ANOVA. Comparisons of the mean ± SEM number of cells from the edge with regard to the fluorescent dye, Lucifer Yellow transfer were made with the Student’s T-test. Comparisons of the percentage ± standard error of the percent dye recovery after photobleaching were made with the Student’s T-test. A p-value of $p \leq 0.05$ or less was considered a significant difference between the compared means or percentages. An asterisk indicates that the difference between the compared values is significant ($p \leq 0.05$ or less). Experiments were performed in duplicate or triplicate.
3.0 MIGRATING CELLS RETAIN GAP JUNCTION PLAQUE STRUCTURE AND FUNCTION

3.1 ABSTRACT

Cell migration is an essential process in organ development, differentiation and wound healing, and it has been hypothesized that gap junctions play a pivotal role in these cell processes. However, the changes in gap junctions and the capacity for cell communication as cells migrate are unclear. To monitor gap junction plaques during cell migration, adrenocortical cells were transfected with cDNA encoding for the connexin 43-green fluorescent protein. Time lapse imaging was used to analyze cell movements and concurrent gap junction plaque dynamics. Immunocytochemistry was used to analyze gap junction morphology and distribution. Migration was initiated by wounding the cell monolayer and diffusional coupling was demonstrated by monitoring Lucifer yellow dye transfer and fluorescence recovery after photobleaching (FRAP) in cells at the wound edge and in cells located some distance from the wound edge. It was demonstrated that gap junction plaques were retained at sites of contact while cells migrated in a “sheet-like” formation, even when cells dramatically changed their spatial relationship to one
another. Consistent with this finding, cells at the leading edge retained their capacity to communicate with contacting cells.

### 3.2 INTRODUCTION

Gap junction channels are cylindrical units composed of proteins called connexins (Goodenough et al. 1996). The protein sequences of several connexin gap junction proteins have been determined (Kumar and Gilula 1986; Kumar and Gilula 1996), and it is now evident that many cells express more than one of the 20 known members of the connexin family (Goodenough et al. 1996). Connexin 43 gap junction protein (Cx43), the most abundant gap junction protein in mammalian vertebrates (Goodenough et al. 1996; White and Paul 1999), is synthesized in the endoplasmic reticulum, oligomerized into hexameric hemichannels (connexons) in the Golgi, and then transported to the cell surface (Evans 1994; Goodenough and Musil 1993; Musil and Goodenough 1993). They unite with similar connexons from apposing cells to form gap junction channels which, in turn, aggregate to form gap junction plaques. Gap junction channels provide pathways for the direct exchange of small molecules between adjacent cells, and it is also thought that gap junctions play a role in synchronizing entire cell populations during migration (Bedner et al. 2003; Bruzzone et al. 1996; Loewenstein 1981).

Gap junctions are expressed in nearly all tissues of the body and may be required for synchronized cell activities such as migration. For example, gap junctions are required for proper targeting of migrating precursor cells during development (Huang et al., 1998; Li et al., 2002; Xu et al., 2006) and mutation of the gap junction gene or altered protein expression can result in malformations affecting the heart, brain and limbs (Lecanda et al., 2000; Reaume et
Similarly, a missense substitution in the Cx43 gene characterized in primordial cells resulted in failure of the cells to migrate properly and establish left/right asymmetry (Gebbia et al., 1997). The migration of enterocytes was significantly inhibited when gap junction communication was interrupted in cells that were treated with the proinflammatory cytokine, interferon γ (Leaphart et al., 2007). Observations of gap junctions during migration are consistent with contributing to cell migration, yet the status of and dynamics of gap junctions in migrating cells has yet to be characterized. The aim of this study therefore, is to characterize gap junctions and intercellular communication in living and migrating cell populations.

3.3 RESULTS

3.3.1 Characterization of Cell Migration

Cell migration was characterized in human adrenal cortical derived (SW-13) cells which form numerous and large gap junction plaques as well as annular structures. Migration was induced by wounding the cell monolayer with a sterile micropipette tip and migration of the remaining cells into the denuded space was captured with phase contrast microscopy at specified times (0, 2, 4, 6, 10 and 24 hours) over a twenty-four hour period. The cells appeared to remain in contact with one another and to move in a ‘sheet-like’ manner, rather than as a single cell or loosely associated cell clusters, and eventually closed the denuded space within the twenty-four period as demonstrated with phase contrast imaging and graphically (Fig. 5 A and B). The suggestion that cells at the wound edge were in contact as they migrated into the wound area was supported by visualizing cortical actin in cells near the wound edge (Fig. 6). In these
populations, the intimate contact between migrating cells was demonstrated and even in cases where the cell bodies of the cells were somewhat separated from one another, the cells still remained in contact at cell processes.

To assess the presence of gap junction plaques between migrating cells, cells were fixed and processed for immunocytochemistry with a Cx43 specific antibody at specific time points (Fig. 7). Gap junction plaques were seen, throughout the migration period, between cells at the wound edge and their adjacent neighbors (Fig. 7 a-c). Furthermore, there was no statistically significant change in the number of plaques in cells at the leading edge or recessed (3-5 cells from the edge) areas (Fig. 8).

3.3.2 Monitoring Cx43-GFP in Transfected Cells

Based on the immunocytochemical analysis of fixed cells, gap junction plaques appear to persist in migrating cells, however the dynamics of the gap junctions is not known. To determine whether gap junction plaques are retained as cells migrate, multimode time-lapse imaging was used to analyze Cx43-GFP gap junction protein dynamics in migrating cells. As anticipated from the immunocytochemical data, the fluorescent Cx43-GFP protein localized to areas of cell-cell contact or within cytoplasmic Cx43-GFP puncta (Fig. 10) in cells visualized 24 hours after Cx43-GFP cDNA transfection with live cell imaging gap junction plaques initially appeared as small puncta on the cell surface. Small Cx43-GFP particles (< 150 nm) were observed to stream toward these existing plaques throughout the observation period. It is thought that the gap junction plaques might be remodeled, either by fusion with one another or fragmentation.

In cell cultures wounded 24 hours after Cx43-GFP transfection, gap junction plaques were observed to be retained at sites of cell-cell contact in migrating populations, even though
throughout migration, the cells shifted their relationship to one another. As can be seen in figure 3-7 for example, the Cx43-GFP gap junction plaque between Cell #1 and Cell #2 can be followed as the cells move to fill the denuded space. Following the initiation of migration Cell #1 was adjacent to Cell #2, and as migration continued the relative position of the cells had changed such that by the end of migration Cell #1 had assumed an inferior position with respect to Cell #2 (Figure 3-7 a-d). Importantly, the gap junction plaque between the two cells was retained even as the cells continued to move toward and finally fill in the denuded area. Once the denuded space was filled with cells, migration halted and the cells no longer changed their relative spatial positions (Fig. 11d and h). In some cases, cells that invaded the denuded space did not shift their respective spatial positions and the gap junction plaques were also retained in these instances (Fig. 11 e-h). Multimode time-lapse analyses of Cx43-GFP gap junction dynamics are clearly indicative of retained gap junction plaques between cells throughout migration.

3.3.3 Characterization of Gap Junction Function

Gap junction functionality (capacity to transfer dye) during migration was demonstrated with Lucifer yellow dye communication studies wherein the cells were scrape loaded and transfer of the dye was monitored (Fig. 12). In these studies rhodamine dextran, which was too large to move through the gap junctions, did not spread to adjacent cells (data not shown). The cell-cell communication of Lucifer yellow dye was robust in cells at the leading edge in migrating cell populations evaluated at three distinct time points post wounding ranging from 5-40 minutes for dye scrape loading analysis (Fig. 12 a-c). Because the kinetics of dye movement and possible
changes over time were difficult to monitor with the scrape techniques, dye transfer was monitored with fluorescence recovery after photobleaching.

Following the photobleaching of cells pre-loaded with a green fluorescent and gap junction permeable dye, calcein-AM, the recovery of fluorescence in was observed in migrating cells (Fig. 13) and at eleven different times ranging from 36 – 375 minutes (Fig. 14). During this monitoring period dye communication was maintained and there was no evidence that cell-cell communication was significantly reduced in cells at the leading edge of the migrating population. For example, the average percent recovery of fluorescence in cells at the leading edge 180 seconds after photobleaching was not significantly different from that in cells that were located some distance from the leading edge (78.76 ± 0.04% vs. 81.32 ± 0.05% at two hours and 79.19 ± 0.04 % vs. 84.47 ± 0.18% at six hours). As expected, cells lacking gap junction plaques (single cells that were isolated from neighboring cells) did not recover fluorescence following photobleaching (not shown). Fluorescence recovery following photobleaching therefore clearly indicates that cell-cell communication via gap junctions occurs in migrating cells and this is in agreement with the immunocytochemical analyses of gap junctions maintained at sites of cell contact, both at the leading edge and in cells some distance from the edge.
Figure 5. Characterization of SW-13 Migration

Cell Migration occurs gradually over 24 hours as shown with DIC microscopy (A) and graphical representation (B). Cell migration over time into the denuded space was assessed at the same point of reference with phase contrast microscopy. The size of the open wound area was measured with the Metamorph® imaging software package. For each of the time point represented n = 12.
Figure 6. Connexin 43 gap junctions in SW-13 cells.

Immunocytochemistry and fluorescence microscopy of connexin 43 gap junction plaques and annular gap junctions (red) in Sw-13 cells. Note the cortical actin cytoskeleton (green) is stained with phallodin outlining and distinguishing individual cells. Nuclei (n) are stained blue.
Figure 7. Fluorescence microscopy of SW-13 cells.

SW-13 cells were fixed and immunostained with anti-Cx43 antibodies at specified time points after wounding. Note the gap junction plaques (red) are present between migrating cells that are at the wound edge throughout migration (a-c).
Figure 8. The average number of gap junction plaques per cell.

The average number of plaques per cell at the wound margin and recessed areas (4-6 cell layers away from the edge) are represented at specified time points during SW-13 cell migration. The average numbers of plaques between cells at the margin and recess were not significantly different. For each experimental group 10 fields analyzed.
Figure 9. Average Plaque Size

The average gap junction plaque size between SW-13 cells that were at the wound margin and cells in the recessed areas did not significantly differ from one another. For this set of experiments 10 fields were analyzed per group.
Figure 10. Cx43-GFP expression in cells.

Cx43-GFP was expressed in SW13 cells and formed gap junction plaques and annular vesicles as seen here with fluorescence microscopy (A) and with corresponding DIC - fluorescent overlay (B).
Figure 11. Gap Junction Dynamics during SW-13 Migration.

Cx43-GFP gap junction plaques were retained throughout cell migration even as the relative positions of cell 1 and cell 2 shifted with respect to one another (a-d) or stayed the same (e-h). Bar, 30µM.
Figure 12. Functional coupling during migration

Intercellular communication of the gap junction permeable dye Lucifer Yellow (green) occurred between migrating SW-13 cells 5 – 40 minutes following wounding (a-c).
Figure 13. Fluorescence Recovery after Photobleaching (FRAP) in SW-13 cells.

Fluorescence recovery was assessed shortly after the initiation of migration (A-C). Note calcein AM dye recovery following photobleaching in the leading edge cells (Cell #1 and #2) as well as in the cell distal to the edge (Cell #3) (A-C and D). Also, calcein AM dye fluorescence remained constant in cells that are not photobleached as shown in Cell #4 (A-C and D). Bar, 30 µM.
Figure 14. Gap junction communication during cell migration.

Intercellular communication of fluorescent dye was maintained between migrating cells for over six hours following the initiation of migration, as assessed with FRAP. The fluorescence recovered by SW-13 cells at each time point is expressed as the percentage of dye that was recovered by the photobleached cell compared to its pre-bleach status. Fluorescence recovery was assessed in two cells per each time point.
3.4 DISCUSSION

Previous studies are consistent with the importance of cell-cell communication in regulating the
directionality, speed and sequence of events that are required for cell migration and it is therefore
a reasonable suggestion that the mediators of direct intercellular communication must persist as
the cells migrate (Fushiki et al., 2003; Huang et al., 1998; Kwak et al., 2001; Lo 1999; Richards
et al. 2004; Tate et al., 2006; Xu et al., 2006; Xu et al., 2001; Ashton et al., 1999; Menezes et al.
2002; Marins et al., 2009). In this study, immunocytochemistry and time-lapse imaging were
utilized to monitor the presence and position of gap junction plaques at various time points
during in vitro two-dimensional migration. Sheet migration was observed throughout the
population, and extended from cells proximal to the wound edge to cells that bordered the edge
of the tissue culture surface. Gap junction plaques were also evident between cells leading edge
as well as their contacting neighbors. In fact, the average number of gap junction plaques was
not significantly different in cells at the leading edge when compared to cells that were some
distance from the leading edge. This finding was somewhat unexpected since cells at the leading
edge had fewer neighboring cells with which to potentially form cell-cell contacts. This finding
may be indicative of an increase in gap junction formation or a decrease in gap junction turnover
in cells located at the leading edge. One possible explanation is suggested by observations of
monolayer cultures wherein the number of gap junction plaques increases as the number of
neighboring cells increases; however the gap junctions do not occur at every site of possible
contact. It is possible that the number and size of gap junctions are regulated by factors that
interact with the connexin molecule. For example, it has been demonstrated that gap junction
plaques formed with the green fluorescent protein tagged Cx43 (Cx43-GFP) which fail to bind
ZO-1, are larger than those formed from native Cx43 in which ZO-1 binding is intact (Girao and
This observation has lead to the suggestion that ZO-1 plays a role in gap junction size and turnover (Giepmans et al., 2001). Each cell may be therefore capable of autonomously regulating gap junction plaque numbers and size and independently of neighboring cells. Regardless of these considerations, our data are consistent with migrating cells retaining gap junction plaques that are in position to facilitate cell migration.

The observation of retained of gap junction gap junctions in both live and fixed migrating cells addresses the possibility that dividing rather than migrating cells were the source of cells that filled in the denuded space. Live cell imaging of SW-13 cells revealed that the majority of the cells extended membrane followed the movement of the cell body into the denuded space. The retention of gap junction plaques by migrating cells, also observed with live imaging, suggest that the plaques were not removed, which is what would have been the case if cells had been dividing. Gap junction plaques were also seen on the surface of cells that were fixed and processed for immunocytochemistry at specified times during migration. Furthermore, previous characterizations of several cell models describe collective migration as a process wherein cells of the leading edge migrate into an open area and are followed by adjacent cells that are modulated by contact inhibition (Weijer, 2009 rev.) Thus, the contribution of dividing cells, to the fill in the denuded space, is a plausible concern. Nevertheless, the findings presented are evidence that the source of cells comprising the denuded space is largely from migrating cells.

The necessity for gap junctions has been previously demonstrated in migrating cells however these studies fail to clarify their functional status. It might be suggested, for example, that cells simply tolerate or utilize gap junctions as structural components during migration, while the junctions themselves do not participate in intercellular communication to
coordinate multicellular migration (Elias et al., 2007). We report, however, that molecules continue to diffuse between cells during migration, as assessed with FRAP analysis. Therefore, the transfer of regulatory molecules between cells while migrating is possible, and even at the leading edge where the cells are the most active in altering their relative positions. It should be noted that calcein-AM dye used in the FRAP analysis is a membrane permeable substrate that is hydrolyzed by non-specific esterases into a non-membrane permeable fluorescent polyanionic calcein that is retained in the cytoplasm (Czyz et al., 2000). This fluorescent form of calcein is gap junction channel permeable, but cannot permeate through other plasma membrane channels and is therefore widely used to study gap junction-mediated communication (Czyz et al., 2000; Pollmann et al., 2005; Tenopoulou et al., 2007; Wade et al., 1986). In our study, all of the cells in the population were initially labeled with the calcien and all cells including those at the leading edge were able to recover fluorescence after photobleaching. Cells that weren’t in contact with other cells (thus lacking the capacity for gap junction mediated communication) did not recover fluorescence following photobleaching. The failure of such isolated cells to recover fluorescence confirms the need for cell-cell contact as determined from our FRAP studies and continued gap junction mediated communication between migrating cells.

The gap junction protein Cx43 has the potential to interact with numerous cytoskeletal and signaling molecules, and the physical structure of gap junctions therefore may serve as a platform for assembly of cytoplasmic elements for cell migration. In support of such a possibility the cytoplasmic domains of gap junction proteins have been demonstrated to interact with: the actin-binding protein, drebrin; microtubules; as well as the tight junction, ZO-1; PKC and src (Butkevich et al., 2004; Giepmans et al., 2004; Giepmans, 2006; Majoul et al., 2007; Giepmans et al., 2001; Levy and Holzbaur, 2007; Giepmans and Moolenaar, 1998; Li et al.,
2004; Chung et al., 2007, DeVuyst et al., 2007; Duffy et al., 2007; Giepmans et al., 2001; Lin et al., 2006). In addition to gap junction interactions with cytoskeletal and membrane structures to produce a platform for binding components needed for migration, gap junction hemichannels in the membrane can be opened or closed by a number of factors, and have been shown to release molecules such as ATP from the cell (Ramachandran et al., 2007; Retamal et al., 2007; Spray et al., 2006; Srinivas et al., 2006; Stout et al., 2002; Zhao et al., 2005). The release molecules from hemichannels combined with the physical structure of gap junctions and the well-known role of gap junction mediated intercellular communication could all play a pivotal role in providing information about the structural platforms needed by migrating cells. Our data do not rule out the possibilities of hemichannels or gap junction physical structured as the mechanism. However the data are consistent with gap junction channels as being capable of facilitating cell-cell communication as cells migrate.

In summary, the results of our correlated immunocytochemical, time-lapse and migration studies are consistent with gap junction plaques persisting between cells that migrate as a sheet. The fact that migrating cells retain their gap junctions would suggest that the capacity for cell-cell movement of regulatory molecules is critical in physiological processes such as wound healing. The consequence of altering gap junctions was investigated and will be presented in Chapter Four.
4.0 ALTERED CONNEXIN 43 EXPRESSION AND GAP JUNCTION COMMUNICATION REDUCS CELL MIGRATION

4.1 ABSTRACT

To investigate if gap junction structure and intercellular communication are required for coordinating migrating cells we utilized an in vitro, wound healing, model for initiating and monitoring cell movement. To assess the effect of altering intercellular communication on cell migration, we introduced, into cell cultures, mimetic peptides (Gap 26 and 27) that specifically inhibit gap junction coupling. Functional dye coupling was confirmed with the diffusion of the gap junction permeable dye, Lucifer yellow and the assessment of fluorescence recovery after photobleaching (FRAP). Gap junction plaques were reduced with siRNA treatments, specifically targeting the mRNA of the gap junction protein connexin 43 (Cx43). Cx43 protein expression and gap junction plaque formation were confirmed with western blot and immunofluorescence, respectively. Reduced gap junction mediated dye coupling was observed in cells treated with mimetic peptides. In communication impaired cells migration was slowed, thus confirming the necessity for gap junction communication in efficient cell migration. Furthermore, cell motility was drastically hindered when Cx43 gap junction plaque structures
were reduced. Our results are consistent with gap junction plaques and intercellular communication playing a pivotal role in cell migration.

4.2 INTRODUCTION

The majority of cells that comprise the tissues of our bodies are capable of migration, an event that is thought to rely on the ability of individual cells, in a group, to communicate with one another (Pepper and Meda, 1992; Matic et al., 1997; Nadarajah et al., 1997; Saitoh et al., 1997, Civitelli et al., 1998, Winterhager et al., 1999; Ashton et al., 1999; Cina et al., 2007; Leaphart et al., 2007). A number of these cell populations also often express one or more types of proteins that can potentially form gap junction intercellular communication channels that may function in relaying signals that help guide the cells during migration (Davis and Trinkhaus, 1981; Lo et al., 1997; Bruzzone et al., 1996; Kumar, 1999). Developmental defects, associated with mutation of gap junction proteins, may result from cells that failed to communicate and therefore, could not migrate properly to target sites (Lecanda et al., 2000; Reaume et al., 1995; Law, 2002). Thus, it is possible that perturbation of gap junction expression and/or channel function will alter cellular migration.

Ideally, the need for gap junction mediated communication, may be straightforward, e.g. gap junctions function solely for the purpose of communicating vital spatial and temporal cues during migration, and therefore, mutations that inhibit connexin expression or alter protein structure would, hypothetically, impair migration. This possibility was suggested from studies in which the directionality and rate of cell migration were significantly reduced in cardiac neural crest cells (CNC) that were isolated from Cx43 +/- mice compared to wild type (WT) and Cx43
restored (CMV43) mutants (Xu et al., 2006; Huang et al., 1998). Accordingly, gap junction communication may facilitate cell motility and therefore altered gap junction communication and/or expression may inhibit migration.

The implications of this specific response to gap junction communication, is indicative of the complex role of gap junction channels play in cell physiology, particularly migration. However, there has yet to be a systematic assessment of the effect of gap junction communication on cell migration. In the previous chapter we demonstrated that gap junction structure and function were retained in migrating cells. To assess the effect of altering the status of gap junctions on migrating cells, chemical and molecular approaches were utilized to either inhibit intercellular communication or reduce Cx43 expression. To determine whether gap junctions and or intercellular communication are required for cell migration, Cx43 expression was altered and the effects on cell migration were assessed.

4.3 RESULTS

4.3.1 Migration in Cells with Varied Amounts of Connexin 43 Expression

Three adrenal cortical derived cell lines were examined: human (SW-13), bovine (SBAC) and mouse (Y-1) in order to determine the migration characteristic of different cell types that express differing levels of connexin and to select which of the cell types to use for the various studies. The SW13 and SBAC cell populations form numerous and large gap junction plaques and have an abundance of cytoplasmic staining presumed to be annular gap junction vesicles. The clone of Y-1 cells, in our possession do not express connexin proteins and therefore do not form gap
junction plaques. The cell populations were subject to a wound healing assay and migration was monitored with phase contrast microscopy, over a time course (0, 2, 4, 6 and 24 hours). As described in Chapter Three, the SW13 cells migrated in sheet-like, gradual manner, and closed the denuded space within the twenty-four hour period (Fig. 5 A and B). There was an initial lag of six hours observed after wounding before SBAC migration occurred into the denuded area (Fig. 15 A and B). The Y-1 mouse cells did not migrate into the denuded space after twenty-four hours (Fig. 16 A and B) or after forty-eight hours (not shown). Since, SW-13 has the most abundant gap junction plaques this cell line was chosen for further studies of the effects of decreasing or increasing gap junctions on cell migration.

4.3.2 Altered Gap Junctions and Cell Migration

To effectively decrease connexin protein expression, siRNA specifically targeted against Cx43 mRNA were introduced into SW-13 cells. Decreased Cx43 protein expression was confirmed quantitatively with western blot (Fig. 17 B) and qualitatively with immunofluorescence (Fig. 17 A). Gap junction mediated intercellular dye coupling was assessed with Lucifer yellow dye and fluorescence recovery after photobleaching (FRAP) analyses. Both Lucifer Yellow (Fig. 18) and FRAP (Fig. 19) analyses were consistent with a significant decrease in dye coupling in cells with reduced Cx43 expression. A significant decrease in the percent change in migration was observed in cells with reduced Cx43 protein expression (Fig. 20).
4.3.3 Altered Gap Junction Communication and Cell Migration

In addition to the siRNA approach, to determine the effect of reduced gap junction communication on cell migration Cx43 specific mimetic peptides (Gap 26 and 27) were used to inhibit gap junction intercellular communication (Fig. 21). Mimetic peptides are short protein sequences that bind to the extracellular loops of connexons, thereby inhibiting channel pore formation. Intercellular dye communication and cellular migration were assessed in cell cultures treated with either Gap 26 or Gap 27. Treatment with the mimetic peptides reduced gap junction mediated communication of Lucifer yellow dye (Fig. 21 G). Migration was slowed in these communication impaired cells compared to control cells (Fig. 22 G).

4.3.4 Increasing Gap Junctions and Cellular Migration

To address the relationship between gap junction expression and cell migration, SW13 cell cultures were pre-treated with an analog of cAMP, dibutyryl cAMP (dbcAMP). Cyclic AMP (cAMP) has been shown in SW-13 cells to increase the size of gap junction plaques while reducing the number of annular structures, thereby increasing the number gap junctions on the plasma membrane (Burghardt et al., 1995; Wang and Rose, 1995). In addition, cAMP increases connexin synthesis and assembly (Wang and Rose, 1995). A wound healing assay was used to assess migration of dbcAMP treated cells. Migration into the wound area was gradual for both control and dbcAMP treated cells (Figure 23 A and B). However, the rate of migration was reduced in cells treated with dbcAMP compared to control cells (Figure 23 B).
A

B

SBAC Cellular Migration

Wound Area (μM²)

0  5000  10000  15000  20000

0  2  4  6  24

Time (hours)
Figure 15. Characterization of SBAC cell migration.

An initial lag is followed by migration that is completed by 24 hours (A and B). Cell migration over time into the denuded space was assessed at the same point of reference with phase contrast microscopy. The size of the open wound area was measured with the Metamorph® imaging software package. For each time point represented n = 12.
Figure 16. Characterization of Y-1 cell Migration.

Y-1 cells, which do not express connexins to form gap junctions did not migrate into the open wound area.

For each time point represented n = 10
Figure 17. Connexin 43 Knockdown and Gap Junction Expression.

Immunocytochemistry (A and B) and Western Blot (C) of Cx43 siRNA transfected and control transfection (Mock) SW-13 cell cultures. Note the reduction in Cx43 protein expression in Cx43 siRNA treated cells (B, C).
Figure 18. Functional coupling in connexin 43 reduced cells.

Lucifer Yellow dye transfer was measured in control and Cx43 siRNA transfected SW-13 cells. Note the significantly reduced dye transfer in Cx43 siRNA treated cells compared to control cultures. For each group represented 12 fields were analyzed.
Figure 19. Fluorescent dye recovery following photobleaching.

The percent fluorescence recovery in SW-13 cells compared to initial fluorescence values was compared. The percent fluorescence recovery in cells with decreased Cx43 expression (43 ± 9%) was significantly reduced compared to Mock (69 ± 12%) and non-treated (NT) (70 ± 10%) controls. For assessment of FRAP: NT: n = 15; Mock: n = 21; Cx43 siRNA n = 31.
Figure 20. Comparison of percent change in cell migration.

The percent change in SW-13 cell migration was compared in control (Mock) and Cx43 siRNA transfected cell cultures. Note the significant decrease in percent change in migration of cells transfected with Cx43 siRNA compared to the control.
Figure 21. Functional dye coupling assessed in cells with gap junction inhibition

Phase contrast (A-C) and fluorescence microscopy (D-F) of Lucifer Yellow dye transfer in SW-13 cells. Dye transfer was compared in control cells (D) and cells treated with the mimetic peptides (Gap 26 and 27) (E-F). Note the reduced dye transfer compared to the control (as measured from the wound edge (E, F, G). For each group represented the communication of Lucifer Yellow dye was performed 10 -12 times.
Figure 22. Comparison of migration in cells with reduced communication

Cellular migration was assessed in control cells (A, D) and cells treated with Cx43 specific mimetic peptides (Gap 26 and 27) (B, E and C, F). Note migration into the denuded space was reduced with the mimetic peptides as compared to the control. The percent change in migration in cells treated with Gap 27 was significantly reduced compared to non-peptide treated controls. For each group represented, the assessment of cell migration was performed 6-8 times.
A

Control    DbcAMP

B

Cellular Migration

<table>
<thead>
<tr>
<th>Time (hours)</th>
<th>Control</th>
<th>DbcAMP</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>12000</td>
<td>13000</td>
</tr>
<tr>
<td>6</td>
<td>8000</td>
<td>9000</td>
</tr>
<tr>
<td>10</td>
<td>5000</td>
<td>6000</td>
</tr>
<tr>
<td>24</td>
<td>2000</td>
<td>2500</td>
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</tbody>
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Area (µm²)
Figure 23. Migration in cells with increased gap junctions

Migration of SW-13 cells treated with DbcAMP is gradual though reduced compared to the control. For each of the groups represented: control n = 12 and DbcAMP n = 6.
The goal of this study was to evaluate the role of gap junction communication channels in cell migration by altering the gap junctions. We initially characterized migration in cells that expressed varying levels of the gap junction protein connexin 43 (Cx43). SW13 and SBAC cells expressed Cx43, formed gap junction plaques and also migrated gradually within twenty-four hours. The migration of SW13 and SBAC cells which express an abundance of gap junction proteins and are therefore capable of intercellular communication suggests that gap junctions may have been involved in regulating cellular migration. The Y-1 cells did not express Cx43 and did not migrate. Y-1 cells are steroid producing cells that appear flat and adherent in culture (Whitehouse et al., 2002). It was observed that an increase in the cell population was marked by stacking or piling up of the cells on top of one another rather than spreading out and forming a monolayer. The failure of Y-1 cells to migrate does not necessarily indicate that the lack of motility was due to lack of gap junctions; it is known that the shape and function of the Y-1 cytoskeletal proteins are controlled by hormones (Han and Rubin, 1996). However, Y-1 cell responsiveness to activators of cyclic AMP –dependent kinase (PKA) signaling is associated with changes in cell morphology from flat and attached to round and non-adherent (Whitehouse et al., 2002). It is therefore unlikely that Y-1 cells grown in the presence of increased concentrations of hormones and growth factors in the culture media would be morphologically poised for migration.

The expression of Cx43 in Y-1 cells, however may influence the migration process. For example, it has been suggested from studies in Cx43 knockout mice that polarization required for epicardial cell migration may require interaction between connexin 43 and the cytoskeletal
element tubulin (Rhee et al., 2009). Furthermore, Cx43 has been demonstrated to interact with several cytoskeletal elements that aid in producing the force at the leading edge that propels the cell body forward during migration (Butkevich et al., 2004; Horwitz and Webb, 2003). It was also recently suggested by Elias and co-workers (2007) that the migration of radial glial cells may depend on gap junction interaction with cytoskeletal components. It is thus possible that the expression of exogenous connexins (whether or not they form functional gap junction channels) may stabilize or reorganize the cytoskeleton thereby affect the ability of Y-1 cells to migrate. These issues will be addressed in future experiments.

The migration of the gap junction expressing cells is, however, consistent with other in vivo and in vitro studies demonstrating increased Cx43 protein and mRNA levels associated with sustained cell-cell communication during wound healing (Larson and Haudenschild, 1988; Pepper et al., 1989; Pepper et al., 1992). While the migration of SW-13 and SBAC cells supports our hypothesis that gap junctions play an important role in cell migration, altering gap junctions by specifically targeting connexins will ultimately indicate the importance of gap junctions in cell migration.

In this study, we altered gap junction plaques and assessed the effects on SW-13 cell migration by either using siRNA techniques to reduce the availability of Cx43 protein to form gap junction channels and plaques, and also with mimetic peptides that inhibited the functional coupling of gap junction channels. Cell migration was reduced in cells cultures treated with mimetic compounds, and the decrease in migration was even more pronounced in cells treated with Cx43 specific siRNA. Importantly, SW-13 migration was inhibited in cells transfected with the Cx43 specific siRNA but it was not inhibited in cells transfected with control Cx43 (a non-knockdown sequence) or GAPDH specific siRNA (not shown). An important caveat considered
in using the siRNA approach was the possibility of creating a non-specific effect that could affect migration. To address this concern, the status of the actin cytoskeleton in control and Cx43 siRNA knockdown cells was assessed with immunocytochemistry and fluorescence microscopy. Preliminary analysis revealed control and knockdown with similar appearing cytoskeletal arrangement. The reduced migration in SW13 cells with decreased connexin 43 expression is consistent with studies showing reduced migration in neural crest cells isolated from connexin 43 null mouse embryos (Ruanvoravat and Lo, 1992; Lo et al., 1997). Furthermore, our findings that reduced cell migration correlates with decreased gap junction coupling suggests that intercellular communication may be necessary for efficient cell migration. One may suggest, on the other hand, that it is possible that treatment with connexin 43 specific mimetic compounds, while inhibiting cell-cell communication, may also inadvertently alter the protein conformation of connexin monomers and thus the interaction of connexin with the cytoskeleton and thereby, affect migration (Yamane et al., 2002). If so, one might suggest that gap junction channels may also play a role as structural components that aid in cell migration. In a recent study, connexin subunits incapable of pore function were expressed in neocortical radial glia and were found to be sufficient for cellular migration (Elias et al., 2007). On the other hand, reduced cellular migration of enterocytes and neural crest cells has been attributed to the disruption of gap junction coupling with non-specific inhibitors, such as oleamide (Huang et al., 1998; Leaphart et al., 2007; Bannerman et al., 2000). The suggestion that migration, in some cells, may be attributed to gap junctions as mediators of adhesion rather than mediators of communication reveals the complexities of the relationship between gap junction channels and the cells that they inhabit. For example, gap junctions may function solely as communication channels that relay important messages for migration or as adhesion factors or in both capacities.
The findings following siRNA treatment would not rule out either possibility. Nevertheless, our findings demonstrate, for the first time, that direct targeting of connexin 43 expression with siRNA results in decreased cell migration. In addition, the specific inhibition of gap junction coupling with mimetic compounds Gap 26 and 27 resulted in reduced cellular migration. These findings are consistent with the suggestion that altered gap junction channels and intercellular communication reduce cellular migration.

Hitherto we have demonstrated that decreased gap junctions and intercellular communication results in reduced cellular migration. Therefore, to determine the effect of increasing gap junctions on migration cells were treated with the compound dbcAMP. Accordingly, the migration of SW-13 cells was reduced in populations treated with the cAMP analog dibutyryl cAMP (dbcAMP). However, one limitation in interpreting this data is the non-specific effect of dbcAMP on adrenal cells that has been demonstrated to decrease proliferation, and increase steroid production (Oyoyo et al., 1997; Murray and Taylor, 1988). Furthermore, dbcAMP treatment may have effected cell migration by altering many factors such as focal adhesion contractile strength, inhibiting RHO kinase dependent adhesion or modifying the actin cytoskeleton (Leader et al., 1983; Pereyra et al., 2006; Laudanna et al., 1997). Treatment with dbcAMP has been demonstrated to sustain protein kinase A activation (PKA) and interestingly prolonged (PKA) activity in ameboid PKA mutants resulted in an inability to alter the cytoskeleton thereby and reduced chemotaxis (Zhang et al., 2003; Pereyra et al., 2006). Based on our studies we can conclude that, in cells treated with dbcAMP to increase gap junction plaques, cell migration is reduced though the relationship between these finding remains to be elucidated. Future studies designed to restore gap junctions in cells that either express few or no endogenous connexin are needed to further discern the role of gap junctions in cell migration. Recent studies
suggest that gap junction are key participants in cell migration via interaction with cytoskeletal components; thus, our understanding of the role of gap junctions in cellular migration may also be aided by assessing the influence of sustained PKA signaling on increased gap junction plaques and how this may correlate with cell shape and adhesion (Elias et al., 2007; as reviewed in Olk et al., 2009).

Our findings are consistent with the hypothesis that gap junction channels and intercellular communication are necessary for cell migration. Cell migration was effectively reduced in cells with altered gap junction expression or intercellular communication. The reduced migration associated with increased gap junction plaques suggests that gap junction internalization may also be necessary for cell migration. The mechanism of gap junction internalization will be addressed in the following Chapter Five: The Mechanism of Gap Junction Channel Internalization.
5.0 THE MECHANISM OF GAP JUNCTION CHANNEL INTERNALIZATION

5.1 ABSTRACT

Gap junction-mediated intercellular communication has been implicated in the development, differentiation and function of most cells of the body. While gap junction channel assembly has been extensively studied, mechanisms involved in the disassembly of gap junction plaques are not well understood. Yet, the rate of gap junction plaque removal from the cell surface is thought to be critical to gap junction function, and alterations in removal may be related to a number of disease processes. This study focused on gap junction protein (connexin 43) removal from the cell membrane, specifically gap junction plaque internalization, which leads to the formation of cytoplasmic double-membraned structures referred to as “annular” gap junctions. The process of gap junction plaque removal is an “endoexocytic” process in which one cell internalizes membrane (endocytosis) and the the adjoining cell contributes membrane (exocytosis). To study the fundamental cellular mechanisms involved in gap junction plaque endoexocytosis, a combination of immunocytochemistry, live cell imaging, quantum dot immuno-electron microscopy and computer reconstruction techniques were used. A detailed examination of the spatial and temporal-dependent redistribution of connexin 43-GFP, revealed that gap junction
plaques internalized preferentially into one of the two contacting cells. Gap junction plaque endoexocytosis, rather than plaque component (hemichannel) dispersion within the membrane, appeared to be the dominant method of gap junction removal. Gap junctional invaginations and annular gap junction vesicles at the cell surface were coated with short electron-dense bristles similar in appearance to the clathrin coated vesicles of nonjunctional membrane. To determine if this associated clathrin-like coat was composed of molecules involved in facilitating endoexocytosis, clathrin and its adaptor proteins were imaged with quantum dot immunoelectron microscopic and immunocytochemical light microscopic techniques. Clathrin was observed to colocalize with some of the connexin 43 gap junction plaques, as well as, the annular gap junction profiles. The clathrin adaptor protein, AP-2, which is thought to play a major role in the formation of clathrin-coated vesicles, also colocalized with some of the annular gap junction vesicles and gap junction plaques. An important finding was that clathrin preferentially localized to the intracellular surface of the gap junction plaque in one of the two contacting cells. Our findings provide evidence that clathrin, with the assistance of AP-2, may serve as the machinery essential for gap junction plaque endoexocytosis.

5.2 INTRODUCTION

One important method of gap junction regulation involves the removal of gap junction plaques from the cell surface. Gap junctions differ from most cell surface ion channels by exhibiting high turnover rates (1-5 hrs. as opposed to > 20 hrs.) and it has been proposed that the removal of gap junctions from the plasma membrane may serve to regulate intercellular communication and thereby, physiological processes (Musil et al., 2005; Fallon and Goodenough, 1981; Piehl et al.,
For example, it was reported that the appearance of internalized gap junctions coincided with the spontaneous release of individual cells from a monolayer into the media (Murray et al., 1981, Larsen et al., 1983). The mechanism of gap junction removal is thought to involve several steps including: the endocytosis of channels and membrane components of both cells into one of the connected cells, the formation of double membrane annular vesicle and the movement of the annular vesicle to the interior of the receiving cell (Murray et al., 1981). During migration, the loss of gap junctions via internalization may serve a multitude of purposes including: freeing certain regions of the plasma membrane to participate in the migratory process (e.g. protrusion or adhesion) or an opportunity to replace the ‘older’ gap junctions with new ones. Spatial shifting of the plasma membrane and gap junction function and dynamics has been characterized in live cells during migration (DeFranco et al., 2008). However, the mechanism underlying gap junction removal from the surface of migrating cells has yet to be determined.

The observation of cytoplasmic vesicles with distinct pentalaminar profiles in many cell types has motivated presumptions that gap junctions at the cell surface may be targeted for internalization via an endocytic mechanism rather than dispersal within the plasma membrane (Larsen et al., 1979; Larsen and Tung, 1978). Early transmission electron microscopy (TEM) studies described short electron dense bristles, thought to be clathrin, emanating from invaginating surface gap junctions and cytoplasmic annular gap junction vesicles (Larsen et al., 1979); suggesting the possibility that gap junctions are removed from the cell surface via a clathrin dependent mechanism.

In a recent study, combined microscopy and immunocytochemistry approaches were used to demonstrate subcellular association between clathrin and Cx43-GFP transfected gap
junction molecules in HeLa cells (Piehl et al., 2006). However, neither a direct association between endogenously expressed Cx43 and clathrin nor any of the clathrin adaptor proteins has been shown. Thus the mechanism underlying gap junction endocytosis has yet to be clarified. Given the physiological importance of regulated intercellular communication, and our observations of gap junction puncta internalization in our live cell imaging studies, gap junction internalization in migrating cells was studied with molecular and microscopic techniques that include live imaging, fluorescence microscopy and and transmission electron microscopy.

5.3 RESULTS

5.3.1 Gap Junction Dynamics during Process Detachment

Migrating and non-migrating cells of the monolayer were connected to one another via relatively long processes. These processes were dynamic such that cells retracted the processes whenever connections between the cells were lost (Fig. 24). In the instances where Cx43-GFP gap junction plaques between cell processes and the cell body of contacting cells were monitored with time-lapse imaging techniques, gap junction plaques were observed to internalize as cells detached from one another at such processes (Fig. 24 g-j). As the processes were detached, the linear gap junction plaques were observed becoming cytoplasmic annular gap junction vesicles (Fig. 24 i and j). Gap junction plaque endocytosis, rather than plaque component dispersion within the membrane was the obvious method used for gap junction removal as the cells lost contact with one another. It appears that cells throughout the population were capable of removing gap junction plaques from the cell surface in this manner since the number of Cx43
cytoplasmic gap junction packets, measured in cells at the leading edge and cells located at a distance from the edge were not significantly different at 2, 4, 6 or 24 hours post wounding. For example at 4 hours, the number of cytoplasmic packets in cells at the leading edge was \((7.95 \pm 0.72)\) versus the number of packets in relatively distant cells, which was \((10.1 \pm 1.1)\). Furthermore, the Cx43-GFP cytoplasmic packets and plaques were similar in size to that of the endogenous Cx43 gap junction packets and plaques. Small puncta or long linear arrays of Cx43 gap junctions were observed at the sites of cell-cell contact throughout the population.

Previous analyses of internalized annular gap junction vesicles with TEM revealed pentalaminar profiles and in some cases faint bristle-like coats, thought to be clathrin, emanating from discrete locations along the outer membrane of the vesicle, similar to those described in earlier studies (Murray et al., 1981).

5.3.2 Immunocytochemistry Analysis of Gap Junction Internalization

To elucidate these internalized gap junction structures further, immunocytochemical techniques were used. Subcellular colocalization of gap junction structures and clathrin was assessed by probing fixed cells with specific antibodies for Cx43 and the clathrin heavy chain and then labeling specimens with fluorescent secondary antibodies. Clathrin expression was abundant in SW13 cells and co-localization with Cx43 molecules occurred at gap junction plaques and annular gap junction vesicles (Fig. 25).

Since clathrin does not directly bind to its cargo, but instead uses adaptor proteins, we investigated the association of gap junction proteins with clathrin adaptor proteins: AP2, Dab2 and Epsin. We have found that the clathrin adaptor protein 2 (AP-2) is abundant in SW13 cells and also colocalized with gap junction plaques and annular vesicles (Fig. 26). Co-
immunoprecipitation analysis, is not indicative of a direct protein-protein association between AP-2 and Cx43 (not shown). The status of clathrin adaptor proteins Disabled 2 (Dab2) and Epsin were also assessed and found in relatively less abundance than AP-2 (Figs. 27 and 28). Furthermore, there was no indication of any significant colocalization with Cx43 and Dab2 or Epsin.

5.3.3 Transmission Electron Microscopy Analysis of Gap Junction Internalization

Resolution at the light microscopy level was not sufficient for determining with certainty, the association between Cx43 and clathrin. To further characterize the relationship between clathrin and gap junctions fixed cells labeled with quantum dots that are specific for Cx43 or clathrin and then processed the samples for TEM analysis. Quantum dots (QD) are 10-50 nm nanocrystals that offer the advantage being excited by laser and emitting visible light and also being visible as spheres at the electron microscopic level. Clathrin specific quantum dots specifically labeled gap junctional regions of the plasma membrane and not non-junctional membrane (Fig. 29). Interestingly, clathrin specific quantum dots labeled plaques and annular gap junctions on one side of the membrane (Fig. 30). The clathrin label sometimes coated the entire annular vesicle or was seen in discrete areas along the vesicular membrane (Fig. 5-7).
Figure 24. Gap Junction internalization occurs during cell process detachment.

Pseudo-fluorescent (left) and phase contrast (right) imaging of SW-13 cells shows Cx43-GFP plaque invagination, annular gap junction formation and subsequent internalization into one of the two contacting cells.
Figure 25. Colocalization of connexin 43 gap junctions and clathrin

Subcellular localization of endogenous Cx43 (green) and clathrin (red) molecules (red) is shown at a gap junction plaque and annular gap junction (arrows). Merged image SW-13 cells immunostained with anti-Cx43 and anti-clathrin antibodies are visualized with fluorescence microscopy. Note the areas of Cx43 and clathrin colocalization appear yellow.
Figure 26. Subcellular localization of gap junctions and the clathrin adaptor protein AP-2

Colocalization of endogenous Cx43 (green) and the clathrin adaptor protein, AP-2 (red) is demonstrated at gap junction plaque and cytoplasmic annular structures (shown with arrows). Merged image SW-13 cells immunostained with anti-Cx43 and anti-AP-2 antibodies are visualized with fluorescence microscopy. Note the areas of Cx43 and AP-2 subcellular colocalization appear yellow.
Figure 27. Subcellular localization of connexin 43 gap junctions and Dab2

Immunofluorescence of Cx43 (green) gap junction proteins and the adaptor protein Dab2 (red) is shown. Merged image SW-13 cells immunostained with anti-Cx43 and anti-Dab2 antibodies are visualized with fluorescence microscopy. Note the lack of yellow as a sign of colocalization of Cx43 with Dab2.
Figure 28. Subcellular colocalization of gap junctions and Epsin

Immunofluorescence of Cx43 gap junction plaques and annulars (green) and the adaptor protein Epsin (red) is demonstrated. Merged image SW-13 cells immunostained with anti-Cx43 and anti-Epsin antibodies are visualized with fluorescence microscopy. Connexin 43 gap junctions and annulars are present however there is no sign of colocalization with Epsin.
Figure 29. Subcellular colocalization of gap junctions and clathrin assessed with transmission electron microscopy

Quantum dot immunoelectron-microscopy demonstrating the accumulation of clathrin on one side of the gap junction plaque (white arrow) (A) in SW-13 cells. Localization of Cx43 quantum dots at a gap junction plaque (B). Note: the Cx43 quantum dots do not label non junctional membrane (B).
Figure 30. Quantum dot transmission immunoelectron microscopy of SW-13 cells.

The arrows designate clathrin surrounding internalized annular gap junction vesicles. Bar, 0.1 µm.
5.4 DISCUSSION

The maintenance of physiological processes requires electrical and metabolic coupling cells and it is likely therefore, that the regulation of gap junction internalization would affect intercellular communication and thereby, physiological processes (Gumpert et al., 2008). Cells internalize gap junctions in response to numerous stimuli and gap junction internalization may serve a multitude of purposes including: freeing certain regions of the plasma membrane to participate in the migratory process, liberation of cells for the purpose of growth and/or mitosis and also to replace the ‘older’ gap junctions with new ones. For example, gap junctions are often targeted for degradation in cancerous cells such that ultimately, these cells are no longer in communication with neighboring cells and are therefore able to grow and proliferate out of control (Fiorini et al., 2008) Furthermore, increased gap junction internalization has also been reported to coincide with mitosis in SW13 cells (unpublished data). Connexin 43 and 31.1 gap junction expression were downregulated in epidermal cells during wound healing (Goliger and Paul, 1995). We have also demonstrated that gap junctions are internalized in regions of membrane detachment during cell migration (DeFranco et al., 2008); however the mechanism of plaque removal is not completely understood.

We show with live cell imaging of migrating cells that neighboring cells may detach partially from the adjacent cell and when a plaque is in the vicinity, portions (or even the entire structure) are removed and internalized into one of the two cells. The internalized gap junction plaque forms a double membrane or pentalaminar cytoplasmic vesicle that is referred to as an annular gap junction. Transmission electron microscopy (TEM) revealed putative clathrin association as a bristle-like coat at discrete segments along annular gap junction vesicles. Our analysis of plaque and annular structures, with immunocytochemistry and fluorescence
microscopy, revealed that the clathrin heavy chain colocalizes with both gap junction plaques and annular gap junction vesicles. Analyses of clathrin-specific quantum-dots/TEM are consistent with clathrin association with one side of the gap junction plaque and with the outermost membrane of annular gap junction vesicles. Based on findings that clathrin associates with gap junction plaques and annular vesicles, one might suggest that clathrin is a mediator of a unique endoexocytic mechanism that removes Cx43 gap junction plaques from the plasma membrane (Figure 5-8).

We have shown several clathrin associated adaptor proteins, including AP2, Dab2 and epsin are expressed in SW13 adrenal cells. The AP2 adaptor protein, in particular, colocalizes with gap junction plaques and annular vesicles. Interestingly, we also observed in a majority of cells that AP2 lies along one-side of the plaque, suggesting perhaps, a mechanism of gap junction plaque invagination and removal from the plasma membrane in which AP-2 would determine the direction of internalization. Co-immunoprecipitation analysis, however, was not indicative of a direct protein-protein association between AP2 and Cx43. This may be a reflection the fragile nature of the Cx43/AP2 interaction or the stringency of our methods to pull down the complex. Overall, our analyses made with clathrin-specific quantum-dots/TEM are consistent with clathrin association with one side of the gap junction plaque and with the outermost membrane of annular gap junction vesicles.

Early TEM studies of annular gap junctions identified a short electron dense bristle coat on the outermost membrane that was thought to be clathrin. Furthermore, the bristle coat was also associated with an invaginating gap junction plaque suggesting that the mechanism of gap junction internalization is clathrin dependent (Larsen and Tung, 1978; Larsen et al., 1979). Recently immunocytochemistry studies with clathrin specific antibodies were used to
demonstrate subcellular colocalization between clathrin and Cx43-GFP gap junction plaques in HeLa cells and at the TEM level electron dense patches identified with annular gap junction vesicles (Piehl et al., 2007). Furthermore, gap junction plaque internalization was inhibited with the depletion and/or inhibition of clathrin, AP2 and Dab2 (Gumpert et al., 2008). Overall, these studies are consistent with the internalization of gap junctions via a clathrin dependent mechanism.

Our studies suggest that the internalization of Cx43 gap junction plaques in migrating cells occurs via a clathrin dependent mechanism. This is first time that migration as the stimulus for gap junction endocytosis has been linked to a specific mechanism. Importantly, the association with gap junction plaques and annular vesicles indicates that clathrin may be an important mediator of a unique endoexocytic mechanism that removes Cx43 gap junction plaques from the plasma membrane.
Figure 31. Model of clathrin mediated gap junction plaque endocytosis.

Plaque invagination is stimulated and the adaptor protein complex, AP-2 is recruited to one side of the plaque followed by clathrin (1). A clathrin coated annular pit is formed (2) that ultimately becomes an annular gap junction vesicle (3). In the final step the annular vesicle is internalized into one of the two cells (4).
We demonstrate that cells that endogenously expressed the gap junction protein connexin 43 migrated as a sheet, i.e. the cells maintained connection with one another throughout migration. Our finding that gap junction structure and function are retained in migrating cells would be consistent with the suggestion that the communication of molecules between cells occurs during migration. The retention of intercellular communication is indicative of a conserved mechanism through which, cells may remain synchronized for proper migration, and it is, therefore, reasonable to suggest that gap junction plaques would be retained during migration, which is what we found. Furthermore, we found that when communication was decreased with connexin 43 specific inhibitory mimetic peptides, the migration was reduced. Consistent with this finding, when gap junction protein expression was targeted and significantly decreased with Cx43 specific siRNA, cell migration was significantly decreased. These findings are in agreement with studies that show reduced neural crest cell migration in cells treated with non-specific inhibitors of gap junction communication (e.g. glycyrrhetinic acid) (Bannerman et al., 2000). Importantly, the accumulation of evidence in this body of work and by others suggests that gap junction mediated communication is a necessary process for synchronizing cell populations during migratory events; and expands upon the current model of cell migration.
The preservation of gap junction channels and intercellular communication in migrating cells are significant findings in the field of cell migration research. Hitherto, cell migration has been characterized as a set of repeated steps, which are set in motion by an external stimulus (Horwitz and Webb, 2003; Jaffe and Hall, 2005). However, this characterization describes the migration of a single cell rather than groups of cells moving together in a cell population, which, in some cases may be more physiologically relevant. The question of how cells are able to migrate as a group is more complicated and must depend upon the cooperation among individual cells. Gap junction intercellular channels have been implicated as facilitators of cooperative (sheet-like) migration in several cell types including neural crest cell populations and epithelial cells (Xu and Lo, 2006; Richards et al., 2004; Pepper et al., 1989; Pepper et al., 1992). In fact, the lack of gap junctions has been linked to serious developmental deficiencies, including oculodentodigital dysplasia (OCDDD) and malformations of the heart and brain (Britz-Cunningham., 1995; Paznekas et al., 2003 ). While these studies provide evidence that supports the importance of gap junctions in cell migration, they do not address several important questions regarding the structure, dynamics and functional status of gap junction channels in migrating cells. The answers to these questions (do gap junction channels uncouple; do the plaques disperse into plasma membrane; or do they go away and return; are the channels even functional?) may provide evidence for recognizing the importance of gap junction channels and communication in cell migration.

This body of work provides evidence that supports the role of gap junction channels as mediators of cooperation between cells during migration. This study demonstrated that cells remain coupled via gap junctions that persist, though on occasion are modified, during migration. The inhibition of gap junction communication directly correlates with decreased cell migration.
and thus supports the evidence that intercellular communication is also necessary for gap junction communication. A significant decrease in cell migration was associated with the specific targeting and down regulation of Cx43 expression. A detailed examination of the spatial and temporal-dependent redistribution of connexin 43-GFP, revealed that gap junction plaques internalized preferentially into one of the two contacting cells. Our subsequent analysis also provides evidence that clathrin, with the assistance of AP-2, may serve as the machinery essential for gap junction plaque endoexocytosis. The fact that gap junction plaques and annulars associated with clathrin and AP-2 endocytic machinery, may suggest that the mechanism underlying plaque removal from the plasma membrane is clathrin mediated. Therefore, gap junction plaques are dynamic and resilient structures that are necessary for mediating intercellular communication during migration.

It has now been well established that gap junction channels are specialized membrane pores that facilitate the mutual and direct exchange of electrical and chemical information between adjacent cells in tissues and organs (Loewenstein, 1966; Gilula et al., 1971; Sheridan, 1971). The information that is exchanged is in the form of small molecules and includes secondary messengers, amino acids, nucleic acids and ions such as cyclic AMP (cAMP), calcium and nitric oxide (Simpson et al., 1977; Lowenstein, 1978; Saez et al., 1989; Kumar and Gilula, 1996 rev.). Based on our findings, one may propose that gap junction channels are conduits of such signals that are in turn capable of regulating gap junction communication and/or coordinating the steps of migration.
6.1 FUTURE STUDIES

There are questions yet to be addressed that will expand our knowledge of gap junctions and their role in cellular migration. Numerous potential binding partners, particularly the very same small molecules that transit the gap junction channels, and opportunities that exist for interaction with gap junction proteins imply that regulation of gap junctions may be targeted for therapeutic development. Furthermore, future characterization of connexin interaction with these small molecules may reveal unique mechanisms that are involved in regulating migration (Tribulova et al., 2008). Such possibilities to be addressed in future studies include connexin interactions with nitric oxide (NO), calcium and cyclic AMP. Our studies have now demonstrated that gap junctions are retained in migrating cells and perhaps the next level of questions may ask whether the sustained presence of gap junctions in migrating cells is regulated by intercellular communication.

Gap junction permeable small molecules are key participants in maintaining cellular physiology and therefore, may also affect cell migration (Anand et al., 2008; Ridley et al., 2003). The free radical gas, nitric oxide (NO) is one the molecules capable of transiting gap junction channels as well as affecting gap junctions. For example, NO was shown to not only increase connexin 43 expression, but also gap junction permeability in mesangial cells (Yao et al., 2005). It was recently demonstrated in vivo that NO increased the open status of gap junctions in vascular tissues, however, NO also inhibited gap junction communication between enterocytes (Anand et al., 2008, Gonczi et al., 2009). Nitric oxide signaling also plays a role in regulating numerous physiological processes including cell migration (Jadeski et al., 2003; Kang et al., 2004; Gupta and Vlahakis, 2009; Zhou and Zhu, 2009). For example, NO signaling is thought to
be involved in the regulation of α9β1 integrin dependent cell adhesion during migration (Gupta and Vlahakis, 2009). Accordingly, NO is a small molecule capable of transiting gap junction channels, as well as regulating the discrete steps involved in cell migration such as adhesion and intercellular communication. The sustained presence of gap junctions in migrating cells therefore may be regulated by the actual passage NO through gap junctions and subsequent targeting gap junction proteins. Such possibilities will be addressed in future studies.

Calcium is another small molecule that is able to transit gap junction channels and inhibit the channel pore at increased intracellular concentrations. Incidentally, mechanical stimulation has been shown to trigger an increased propagation of calcium through gap junction channels (Anand et al., 2008; Hahn et al., 1992). The process of cell migration during wound repair depends on calcium gradients and waves (Boitano et al., 1994). Importantly, directional movement during migration has been shown to require intracellular calcium that is well organized in space, time and concentration (Ridley et al., 2003 rev.; Hahn et al 1992; Wei et al., 2009). One might therefore suggest that the retention of functional gap junctions during migration is a way to regulate calcium concentrations of cells within a population and thereby coordinate the movement of contacting cells. While the role of calcium communication remains to be addressed it is necessary to assess the potential role other small signaling molecules in regulating gap junctions and cell migration.

The gap junction permeable second messenger cyclic adenosine monophosphate (cAMP), is an important intracellular signaling mediator and yet another small molecule that has the potential to regulate a number of target proteins including the gap junction protein, connexin (Pierre et al., 2009). For example cAMP was shown to increase the permeability of gap
junctions in human granulosa cells, on the other hand; cAMP suppressed the permeability of gap junctions in retinal cells (Furger et al., 1996; Hidaka, 2008). Increased gap junction plaque size along with reduced internalized packets has been demonstrated in cells treated with cAMP (Wynn et al., 2002). The migration of vascular smooth muscle cells and macrophages was reduced when the cells were treated with cAMP (Newman et al., 2003; Block et al., 1979). Alternatively, the directional migration of keratinocytes during wound healing was shown to require cAMP (Pullar and Isseroff, 2005). One may propose that, depending on the cell population and given function, small molecules are capable of regulating gap junction permeability and expression and in this manner affecting cellular migration. Furthermore, the communication of these molecules may be necessary not only to regulate gap junctions to remain on the cell surface in migrating cells but also synchronized the connected cells for mass migration.

6.2 SUMMARY

Cells do not generally act in isolation, but coordinate their activities with surrounding cells (Sanderson et al., 1994). Gap junctions have a unique and dual role as membrane channels and intercellular junctions, and both functions are necessary for coordinating the cells that comprise tissues or the purpose of maintaining proper physiology. Accordingly, altered gap junction expression, function and remodeling have been identified as underlying causes of diseases such as cancer and cardiac arrhythmias; deformities such as limb syndactyly and heart malformations; and even deafness (Paznekas et al., 2003; Britz-Cunningham, 1995; Tribulova et al., 2008; Leibovici et al., 2008). There are questions yet to be addressed that will expand our
knowledge of gap junctions and their role in cellular migration. Particularly, what signals are being communicated between migrating cells, and are the same signals capable of altering the very gap junction channels they transit, thereby having an effect on cell migration? As previously discussed, there are several small molecules that are capable of transiting gap junctions and potentially binding connexins. Opportunities for interaction with gap junction proteins suggests that gap junction proteins can be targeted for therapeutic development and future characterization of connexin interactions with these small molecules may reveal unique mechanisms that are involved in regulating migration (Tribulova et al., 2008).

Cell migration is a multistep process that requires integration of cytoskeletal and signaling components and coordination of multiple cells for forward movement. Gap junctions are necessary mediators of intercellular communication for the purpose of synchronizing cell populations. Gap junctions are retained and are functional throughout migration of cells that migrate in a sheet like manner. The sustained presence of gap junctions in migrating cells may be regulated by small molecules i.e. NO and calcium that pass through gap junctions and are involved in cell migration. Gap junction permeable small molecules are key participants in maintaining cellular physiology and therefore, may also affect cell migration. Accordingly, gap junctions should be included as key players in the discussion of cell migration, though there are still questions yet to be addressed that will expand our knowledge of gap junctions and their role as therapeutic targets.
Figure 32. Gap junctions and intercellular communication are retained as cells migrate. Leading and trailing cells are coupled through gap junctions (A). The gap junction plaque and intercellular communication of small molecules are retained as shown (B). As cells migrate, the plaque may be remodeled via clathrin-dependent endocytosis to form an annular gap junction in one of the two contacting cells.
APPENDIX A

LIST OF ABBREVIATIONS

CX    Connexin

DbcAMP Dibutyryl cyclic Adenosine Monophosphate

FRAP Fluorescence Recovery after Photobleaching

SBAC Bovine Adrenal Cortical derived Cell Line

siRNA small interfering Ribonucleic Acid

SW13 Human Adrenal Carcinoma Cell Line

TEM Transmission Electron Microscopy

Q-Dots Quantum Dots
APPENDIX B

LIST OF PUBLICATIONS AND ABSTRACTS


Decreased Connexin 43 and Gap Junction Communication Alter Cell Migration. B. Hewa DeFranco, Beth Nickel and Sandra A. Murray. 48th Annual American Society for Cell Biology, San Francisco, CA.


Activation of Phospholipase D through a Positive Feed-back Mechanism Promotes Migration of Epithelial Cells. BH Achebe, AR Matela, ER Block, and JK Klarlund. 44th Annual American Society for Cell Biology, Washington, D.C.
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