The Role of Connexin 43 Gap Junction Formation in Monocytes

Trans-Endothelial Migration

By

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Abstract

Atherosclerosis is a chronic inflammatory disease characterised by the accumulation of monocytic cells and lipids within the sub-endothelial space by direct monocyte to endothelial cell contact through gap junctions (GJs). Both cell types express connexin 43 (Cx43) isoforms that permit the formation of GJs. This is enhanced by adhesion molecules in the presence of pro-inflammatory stimuli, such as tumour necrosis factor α (TNF-α). TNF-α is suggested to have a role in Cx43 expression mainly mediated through MAPK pathways over other intercellular pathways; however, to date the mechanism remains unclear.

Experiments were carried out in the absence and presence of 25ng/ml TNF-α and the functional integrity of human umbilical vein endothelial cell (HUVEC) monolayers was assessed by measuring the trans-endothelial electrical resistance (TEER). The trans-endothelial migration (TEM) assay used as a model for the transmigration of monocytes to the sub-endothelial space. Monocytes were added to HUVEC monolayers, and cells which passed from one chamber to another were collected, marked with CD14+ and measured by flow cytometry. The efficiency of TEER and TEM was measured in: 1) the presence of the GJ inhibitor, 43GAP27; 2) HUVECs transfected with Cx43-siRNA; and 3) MAPK inhibitors, chelerythrine chloride (CHE). TEER measurements showed a reduction in the presence of TNF-α compared to the control cells (13.58±2.82Ω.cm², 43.94±3.32Ω.cm²; n=6); however, when the protein kinase C (PKC) inhibitor CHE was added, the resistance was the same as in the control cells (48.64±3.62Ω.cm², 45.76±4.83Ω.cm²; n=6). TNF-α enhanced the migration of monocytes (66.3±2.1%; n=3) compared to the control cells (9.9±4.5%; n=3; P<0.001), but this was prevented in monolayer also treated with CHE (13.70±2.45%; n=3; P<0.001). Migration of monocytes was attenuated by the GJ blocker 43GAP27 and was comparable to HUVECs transfected with Cx43-siRNA (8.33±4.66%; n=3). High expression of total PKC in the HUVECs treated with TNF-α was shown compared to the control cells, but this effect was reduced in HUVECs treated with TNF-α when CHE was applied.

These results show that GJs are formed between ECs and monocytes, and that TNF-α increased TEM, reduced the TEER, and had no effect in HUVECs transfected with Cx43-siRNA. The effect of TNF-α is mediated through PKC as its effect was attenuated by the PKC inhibitor CHE. This study has demonstrated the important role of GJ communication as an underlying mechanism during the early stages of atherosclerosis development, enhanced by the action of TNF-α.
Declaration

This thesis and the work to which it refers are the results of my own efforts. Any ideas, data, images or text resulting from the work of others (whether published or unpublished) are fully identified as such within the work and attributed to their originator in the text, bibliography or in footnotes. This thesis has not been submitted in whole or in part for any other academic degree or professional qualification. I agree that the University has the right to submit my work to the plagiarism detection service TurnitinUK for originality checks. Whether or not drafts have been so-assessed, the University reserves the right to require an electronic version of the final document (as submitted) for assessment as above.

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Date.....................................................
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Publications Arising from this Thesis

Conference abstracts:


Conference attendance and presentations:


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<th>Description</th>
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<tbody>
<tr>
<td>2-ME</td>
<td>2-mercaptoethanol</td>
</tr>
<tr>
<td>AJ</td>
<td>Adherans Junction</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>AP-1</td>
<td>Activator Protein 1</td>
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<tr>
<td>APC</td>
<td>Antigen-Presenting Cell</td>
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<tr>
<td>BBB</td>
<td>Blood Brain Barrier</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
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<tr>
<td>Ca^{2+}</td>
<td>Calcium</td>
</tr>
<tr>
<td>CAM</td>
<td>Cell Adhesion Molecule</td>
</tr>
<tr>
<td>CD14</td>
<td>Cluster of Differentiation 14 (monocyte marker)</td>
</tr>
<tr>
<td>c-GMP</td>
<td>Cyclic Guanosine Monophosphate</td>
</tr>
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<td>CHE</td>
<td>Chelerythrine Chloride</td>
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<tr>
<td>CNS</td>
<td>Central Nervous System</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic Cell</td>
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<td>DMEM</td>
<td>Dulbecco's Modified Eagle Medium</td>
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<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>dsRNA</td>
<td>Double-stranded RNA</td>
</tr>
<tr>
<td>EBM</td>
<td>Endothelial Basal Medium</td>
</tr>
<tr>
<td>ECs</td>
<td>Endothelial Cells.</td>
</tr>
<tr>
<td>EDHF</td>
<td>Endothelium-Derived Hyperpolarising Factor</td>
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<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal Growth Factor</td>
</tr>
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<td>EGM</td>
<td>Endothelial Growth medium.</td>
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<tr>
<td>EGTA</td>
<td>Ethylene Glycol Tetraacetic Acid</td>
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<tr>
<td>eNOS</td>
<td>Endothelial Nitric Oxide Synthase</td>
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<tr>
<td>ER</td>
<td>Endoplasmic Reticulum</td>
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<tr>
<td>ERK</td>
<td>Extracellular Signal–Regulated Kinases</td>
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<tr>
<td>FBS</td>
<td>Foetal Bovine Serum</td>
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<tr>
<td>FGFR</td>
<td>Fibroblast Growth Factor Receptors</td>
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<tr>
<td>GJ</td>
<td>Gap Junction</td>
</tr>
<tr>
<td>GJIC</td>
<td>Gap Junctional Intercellular Communication</td>
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<tr>
<td>HRP</td>
<td>Horseradish Peroxidase</td>
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<td>HUVECs</td>
<td>Human Umbilical Vein Endothelial Cells.</td>
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<td>Intercellular Adhesion Molecule 1</td>
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<td>Interferon-Gamma</td>
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<tr>
<td>IgA</td>
<td>Immunoglobulin A</td>
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<td>IGF</td>
<td>insulin-like growth factors</td>
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<td>Immunoglobulin G</td>
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<td>IgM</td>
<td>Immunoglobulin M</td>
</tr>
<tr>
<td>IKK</td>
<td>IκB Kinase</td>
</tr>
<tr>
<td>Acronym</td>
<td>Full Form</td>
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<tr>
<td>---------</td>
<td>-----------</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>JNK</td>
<td>c-Jun N-terminal kinase</td>
</tr>
<tr>
<td>K⁺</td>
<td>Potassium</td>
</tr>
<tr>
<td>LDL</td>
<td>Low Density Lipoprotein</td>
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<tr>
<td>LPS</td>
<td>Lipopolysaccharides</td>
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<tr>
<td>MAPK</td>
<td>Mitogen-Activated Protein Kinase</td>
</tr>
<tr>
<td>MHC</td>
<td>Major Histocompatibility Complex</td>
</tr>
<tr>
<td>miRNA</td>
<td>Micro RNA</td>
</tr>
<tr>
<td>MNC</td>
<td>Mono-Nuclear Cell</td>
</tr>
<tr>
<td>NK</td>
<td>Natural killer Cell</td>
</tr>
<tr>
<td>NPPB</td>
<td>5-nitro-2-(3-phenylpropylamino)-benzoic acid (NPPB5-nitro-2-(3-phenylpropylamino)-benzoic acid)</td>
</tr>
<tr>
<td>oxLDL</td>
<td>Oxidized Low-Density Lipoprotein</td>
</tr>
<tr>
<td>PBMC</td>
<td>Peripheral Blood Mono-Nuclear Cells</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
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<tr>
<td>PECAM-1</td>
<td>Platelet Endothelial Cell Adhesion Molecule</td>
</tr>
<tr>
<td>PKA</td>
<td>Protein Kinase A</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein Kinase C</td>
</tr>
<tr>
<td>RISC</td>
<td>RNA-Induced Silencing Complex</td>
</tr>
<tr>
<td>RNAi</td>
<td>RNA interference</td>
</tr>
<tr>
<td>RPMI</td>
<td>Roswell Park Memorial Institute medium</td>
</tr>
<tr>
<td>RT</td>
<td>Room Temperature</td>
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<tr>
<td>SDS-PAGE</td>
<td>Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard Error of the Mean</td>
</tr>
<tr>
<td>shRNA</td>
<td>Small Hairpin RNA or Short Hairpin RNA</td>
</tr>
<tr>
<td>siRNA</td>
<td>Small Interfering RNA</td>
</tr>
<tr>
<td>SMCs</td>
<td>Smooth Muscle cells</td>
</tr>
<tr>
<td>TCR</td>
<td>T Cell Antigen Receptor</td>
</tr>
<tr>
<td>TEER</td>
<td>Trans-Endothelial Electrical Resistance</td>
</tr>
<tr>
<td>TEM</td>
<td>Trans-Endothelial Migration</td>
</tr>
<tr>
<td>TEMED</td>
<td>Tetramethylethylenediamine</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming Growth Factor</td>
</tr>
<tr>
<td>THP-1</td>
<td>Human monocytic cell line derived from an acute monocytic leukaemia patient</td>
</tr>
<tr>
<td>TJ</td>
<td>Tight Junction</td>
</tr>
<tr>
<td>TNFR</td>
<td>Tumour Necrosis Factor Receptor</td>
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<tr>
<td>TNF-α</td>
<td>Tumour Necrosis Factor – Alpha</td>
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<td>Untranslated Region</td>
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<td>Vascular Cell Adhesion Protein 1</td>
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<td>VEGF</td>
<td>Vascular Endothelial Growth Factor</td>
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<tr>
<td>vWF</td>
<td>von Willebrand factor</td>
</tr>
<tr>
<td>WB</td>
<td>Western blot</td>
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</table>
Chapter 1 - Introduction
1.1 Cellular Communication

The basic principle of any biological system is communication with the environment but due to the increasing complexity of higher organisms cells have had to evolve, leading to a diversification in mechanisms involving the exchange of spatial and temporal information crucial for tissue organisation and the maintenance of the organism as a whole. Furthermore, tissues are not simply a loose accumulation of individual cells; rather they are a highly organised population of interacting cells. Thus in various types of tissues, cell to cell communication is important for maintaining the supracellular organisation.

Cell signalling has been most extensively studied in the context of human diseases and signalling between cells of a single organism; however, cell signalling may also occur between the cells of two different organisms. In mammals early embryo cells exchange signals with cells of the uterus (Mohamed et al., 2005), while in the human gastrointestinal tract, bacteria exchange signals with each other and with human epithelial and immune system cells (Clarke and Sperandio, 2005). During mating, some Saccharomyces cerevisiae yeast cells send a peptide signal (mating-factor pheromones) into their environment, which may bind to a cell surface receptor on other yeast cells and induce them to prepare for mating (Lin et al., 2004).

Signalling within, between, and among cells can be subdivided into the following classifications: intracrine, autocrine, juxtacrine, paracrine and endocrine. Intracrine signals are produced by the target cell and stay within that target cell, whereas autocrine signals are produced by the target cell, are secreted, and affect the target cell itself via receptors. Sometimes autocrine cells can target nearby cells if they are the same type of cell as the emitting cell, for example immune cells. Juxtacrine signals target adjacent cells, via signals which are transmitted along cell membranes via protein or lipid components integral to the
membrane, and are capable of affecting either the emitting cell or cells immediately adjacent. Paracrine signals target cells in the vicinity of the emitting cell, and neurotransmitters represent an example of this type of signalling. Finally, endocrine signals target distant cells, through endocrine cells producing hormones that travel through the blood to reach all parts of the body (Clarke and Sperandio, 2005).

Cells communicate with each other via direct contact (juxtacrine signalling), over short distances (paracrine signalling), or over large distances and/or scales (endocrine signalling). Some cell–cell communication requires direct cell–cell contact, and some cells can form gap junctions (GJs) that connect their cytoplasm to the cytoplasm of adjacent cells. In cardiac muscle, GJs between adjacent cells allow action potential propagation from the cardiac pacemaker region of the heart to spread and co-ordinately cause contraction of the heart (Rohr, 2004).

Typically, cell-to-cell communication is carried out by signalling in the form of soluble chemical messengers, reception of the signal by target cell receptors, and propagation of the signal via second messengers amongst receiving cells (Figure 1.1). The immune system is a classic example exhibiting virtually all types of cell-cell interactions, homotypic and heterotypic communication among lymphoid and stromal cells.
Figure 1.1: Overview of cellular communication.
Extracellular stimuli carry out their effect by acting on specific cell receptors that transduce signals into the cell through a cascade of second messengers. Gap junction channels, an example of an intercellular communication system, are a major group of cell surface specialisations that allow the bi-directional exchange of signalling molecules between cells.

1.1.1 Direct Intercellular Communication

In order to establish intercellular communication, the process of interaction primarily involves the participation of a range of adhesion molecules, such as cadherins, integrins and selectins, located on the cell surface that ensure intercellular adhesion and enable cell-cell signalling (Mroue et al., 2011). Specialised close contacts, such as GJs, tight junctions (TJs), adherens junctions (AJs), desmosomes, hemi-desmosomes and focal adhesions, are also considered complex multi-unit plasma membrane structures that participate in this process (Mroue et al., 2011).

Over 50 years have elapsed since the discovery of a low resistance electrical transfer (insufficient to elicit an action potential) between adjacent crayfish axons by Furshpan and Potter (1959). Subsequent work demonstrated the existence of minute cellular junctions.
between non-neuronal cells that did not act as barriers but instead aided in the diffusion of small molecules (Figure 1.2) (Revel and Karnovsky, 1967). These junctions later came to be known as ‘gap junctions’, distinct from TJs (Goodenough and Revel, 1970).

Figure 1.2: Electron microscopic identification of gap junctions
(A) Liver plasma membranes negatively stained after isolation showing the hexagonal pattern of GJ channels, (B) high magnification view of a thin section. The intercellular space (ics) is reduced to a gap of ~2nm (g) in the junctional area (Revel and Karnovsky, 1967).

1.2 Gap Junction Intercellular Channels

GJs can be defined as intercellular membrane bound protein channels comprised of tightly packed aggregated particles that directly connect the cytoplasms of adjacent cells, allowing the bidirectional exchange of electrical currents, ions and small molecules such as metabolites, second messengers and peptides of up to 1kDa, for example inositol trisphosphate and calcium (Ca^{2+}) (Neijssen et al., 2005; Goodenough et al., 1996). These different molecules that can be transferred via GJ allow the electric, metabolic and immunological transfer of information and can direct processes including development, inflammation and cell death (Montecino-Rodriguez et al., 2000; Matsue et al., 2006).
1.2.1 Structure of Gap Junction Channels

A functional GJ channel is assembled from six connexin subunits arranged around a central pore that together constitute a single multimeric transmembrane structure (hexamer) called a connexon. These connexons may dock with other connexons in neighbouring cells or remain as hemichannels. Hemichannels are the building blocks of GJs but also function as free unapposed channels, and were previously thought to exist only transiently during the formation of GJs (Unger et al., 1999; Hemler, 2005; Sáez and Leybaert, 2014) (Figure 1.3). The composition of a connexon may be either homomeric or heteromeric, meaning that a connexon can be composed of either six identical connexin subunits (homomeric) or may consist of more than one isoform of connexin (heteromeric) (He et al., 1999) (Figure 1.4).

Figure 1.3: Structure of Gap Junction intercellular channels.
Structure of a GJ channel formed by opposing connexons present in the plasma membrane of two adjoining cells. A connexon is a multimeric transmembrane structure composed of six connexin subunits (Laird, 2006).
Figure 1.4: Putative potential combinations of connexons and gap junction channels in adjoining cells

Six connexon subunits oligomerise into a connexon or hemichannel that dock into different arrangements, including homotypic channels that are made from two identical homomeric connexons, heterotypic/homomeric channels made from two homomeric connexons of different connexins, and heteromeric/heterotypic channels made from a combination of either heteromeric or homomeric connexons with a heteromeric connexon (Laird, 2006).

1.2.2 Connexins

1.2.2.1 Nomenclature of connexin proteins and genes

The family of connexin genes is comprised of 20 members in the mouse and 21 in humans (Table 1.1) (Sohl and Willecke, 2004; Laird, 2006; Beyer and Berthoud, 2009). Connexin family members serve the common purpose of permitting the intercellular exchange of small molecules, but functional channel properties are attributed to the subset of connexins expressed in any one cell type (Laird, 2006). For example, in the myocardium, Cx43 plays a key role in action potential propagation (Severs et al., 2008), whereas in the lens, which is avascular, Cx43 has the responsibility for maintaining homeostasis and transparency (White et al., 1994; Shakespeare et al., 2009).

A new nomenclature system for connexin genes was agreed at the International Gap Junction Conference in 2007, which recognises the sub-divisions of the gene family based on DNA sequence comparisons (Kidder, 2009). A key feature of this system is that orthologous genes in all mammalian species have the same symbols, whereas non-orthologous genes are assigned unique numbers. In brief, the new terminology retains the
use of the connexin protein name written as the abbreviation Cx, followed by a suffix indicating the molecular mass in kDa. Many of the connexin genes have identifiable orthologous pairs in both human and mouse (Table 1.1). There are exceptions for certain connexins expressed only in the mouse (e.g. Cx33) or humans (e.g. Cx25). Furthermore, orthologous connexins in different species may not only differ in their molecular mass (e.g. mouse Cx30.2 is the orthologue of human Cx31.9) but also in expression (e.g. human Cx31.9 cannot be detected in the atrioventricular node whereas its mouse counterpart Cx30.2 is expressed) (Sohl and Willecke, 2004; Kreuzberg et al., 2009).

For identification of connexin genes a Greek letter classification is used. The genes begin with a three letter code starting with Gj (gap junction) and a sub-group derived from the Greek letter classification (α, β, γ, δ and ε). This is then followed by a number according to the order of discovery (e.g. Cx43 the first identified connexin gene of the α-group is encoded as Gja1). Although the connexin genes carry the same abbreviation irrelevant of the species in human and mouse, to distinguish human connexins from other species connexin genes and proteins are given capital letters, such as GJA1 and CX43 respectively.

Throughout my thesis, for clarity and convenience, the term Cxn is used, where n indicates the molecular mass defining the connexin, where the species is irrelevant.
Table 1.1: Nomenclature of connexin proteins and genes in humans and mice

The introduction of the new system led to the same gene names having the same abbreviation; these were changed and their former names are in brackets. The table is adapted from (Laird, 2006; Beyer and Berthoud, 2009). *Cx43 is the most ubiquitously expressed connexin in mammals and is present in more than 35 different cell types, including astrocytes, smooth muscle cells, myocytes, and endothelial cells.

<table>
<thead>
<tr>
<th>Human Protein</th>
<th>Gene</th>
<th>Mouse Protein</th>
<th>Gene</th>
<th>Tissue expressing the connexin</th>
</tr>
</thead>
<tbody>
<tr>
<td>CX43*</td>
<td>GJA1</td>
<td>Cx43</td>
<td>Gja1</td>
<td>Heart, Skin, Lung, Lens,</td>
</tr>
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<td>CX46</td>
<td>GJA3</td>
<td>Cx46</td>
<td>Gja3</td>
<td>Lens</td>
</tr>
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<td>Cx37</td>
<td>Gja4</td>
<td>Blood vessels</td>
</tr>
<tr>
<td>CX40</td>
<td>GJA5</td>
<td>Cx40</td>
<td>Gja5</td>
<td>Heart, Skin</td>
</tr>
<tr>
<td>-----</td>
<td>-----</td>
<td>Cx33</td>
<td>Gja6</td>
<td>Testes</td>
</tr>
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<td>Cx50</td>
<td>Gja8</td>
<td>Lens</td>
</tr>
<tr>
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<td>GJA9 (GJA10)</td>
<td>-----</td>
<td>-----</td>
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</tr>
<tr>
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<td>GJA10</td>
<td>Cx57</td>
<td>Gja10</td>
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<td>Gjb1</td>
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<td>Skin</td>
</tr>
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<td>GJB4</td>
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<td>Gjb4</td>
<td>Skin</td>
</tr>
<tr>
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</tr>
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<td>Gjb6</td>
<td>Skin</td>
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<td>-----</td>
<td>-----</td>
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<td>Cx45</td>
<td>Gjc1</td>
<td>Heart, Skin</td>
</tr>
<tr>
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<td>Cx47</td>
<td>Gjc2</td>
<td>Nervous system</td>
</tr>
<tr>
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<td>Cx29</td>
<td>Gjc3</td>
<td>Brain</td>
</tr>
<tr>
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</tr>
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<td>GJE1</td>
<td>Cx23</td>
<td>Gje1</td>
<td>N/A</td>
</tr>
</tbody>
</table>

1.2.2.2 Connexin Gene Structure

Initial identification of connexin genes suggested a relatively simple structure, with a typical gene consisting of a 5′-untranslated region (UTR), designated exon 1, which contains the transcription initiation site. This structure is followed by an intron of variable size, a second exon encoding the full coding region of the connexin and a 3′ UTR (Sohl and Willecke, 2004) (Figure 1.5).
Figure 1.5: Connexin gene structure

Gene organisation is shown with the exons in dark blue and the introns in light blue. Most connexin genes contain the complete, uninterrupted coding region in a single exon (A, B and C), with variation occurring in the 5′-UTR. (A) The initially described connexon gene structure contained only one 5′-UTR exon. (B) Some genes contain two or more 5′-UTR exons (1A and 1B) that are alternatively utilised for transcription via tissue specific promoters. (C) Other genes contain two or more 5′-UTR exons (1 and 2) that may be present with a coding exon 3 in the mature mRNA or may be alternatively spliced to generate multiple mRNA variants. For these genes multiple mRNAs can be generated after transcription driven from a single promoter. (D) In a few connexin genes the coding region is interrupted by an intron (Paolo et. al, 2006).

Studies by the Pfeifer group indicated that the mouse gene encoding Cx43 contains six exons (exons 1A to 1E), five of which correspond to 5′-UTRs and one containing the coding region (exon 2) (Pfeifer et al., 2004). Three alternate promoter regions (P1 to P3) were also identified. Similarly, the genes encoding Cx40, Cx45 and other connexins appear to contain multiple exons allowing the generation of alternatively spliced transcripts containing differing 5′-UTRs (Baldridge et al., 2001; Jacob and Beyer, 2001).

1.2.2.3 Connexin Protein Structure

Based on cloning studies, hydropathy plots were generated and used to predict the locations of transmembrane helices and to produce a topological model for connexins. This model has been tested and verified through the use of immunocytochemistry to determine the cytoplasmic or extracellular reactivity of antibodies directed against peptides
corresponding to subdomains within the connexin polypeptides (Goodenough et al., 1988; Beyer et al., 1989; Yancey et al., 1989). This strategy has been applied to several connexins, and the model has been extrapolated to all family members. The topological model reveals that connexins are comprised of four membrane spanning regions or transmembrane domains (M1-M4), two extracellular loops (E1 and E2) which comprise the gap-facing extracellular side, with characteristically spaced cysteine residues, three cytoplasmic portions which include a single intercellular loop, and the amino and carboxy terminal domains (Martin and Evans, 2004; Yeager and Gilula, 1992; Zhang and Nicholson, 1994) (Figure 1.6). Functionally, when assembled into connexons the transmembrane domains anchor the protein in the plasma membrane, while the cytoplasmic domains regulate the physiological properties of the channel, and the extracellular domains account for cell-cell compatibility and docking between the channel pairs (White and Bruzzone, 1996; Laird, 2006).
The protein contains four transmembrane domains (M1 to M4) with the amino terminal and carboxyl terminal domains (NT and CT respectively) located on the cytoplasmic side of the membrane. The protein also contains two extracellular loops (E1 between M1 and M2, and E2 between M3 and M4) and an intercellular loop (IL) between M2 and M3. The IL and CT domains are the most variable regions while E1 and E2 are more conserved regions (Laird D, 2006).

1.2.2.3.1 Transmembrane Domains

Topological studies have shown that the four transmembrane domains (M1-M4) are composed of approximately 20 amino acids. Further analysis of the transmembrane sequences led to a hypothesis which stated that the M3 transmembrane domain contains a series of well-spaced polar amino acids forming an amphipathic α-helix. Therefore, it was proposed that the M3 transmembrane domains of each connexin subunit form the innermost boundary of the transmembrane pore (Milks, 1988).

1.2.2.3.2 Cytoplasmic Domains

Compared to the transmembrane and extracellular domains, the cytoplasmic domains diverge in their amino acid composition. The differences among connexin family members are due to structural differences within the cytosolic areas (Vinken et al., 2006). X-ray diffraction and acidification studies have shown that although the cytoplasmic domains are
structurally variable throughout the connexin family, they exhibit a high degree of flexibility within the intercellular channels that are functionally involved in the opening and closing of the channel pore (Makowski et al., 1977). It was originally proposed that the carboxyl tail of the cytoplasmic domains, in particular for Cx43, acts as an independent binding particle that recognises and non-covalently binds to a specific peptide sequence at or near the pore (particle-receptor hypothesis) resulting in the closing and opening of the channel pore (Morley et al., 1996). The cytoplasmic carboxyl tail of connexin is also considered to be a major target for post-translational events, such as phosphorylation, which further regulates connexon assembly into GJs and channel gating (Warn-Cramer et al., 1998).

1.2.2.3.3 Extracellular Domains

The extracellular loops (E1 and E2) display the highest sequence conservation, and are characterised by three cysteine residues in a characteristically conserved spacing that follows the pattern CX₆CX₃C in E1 and CX₄CX₅C in E2, respectively (Hoh et al., 1991; Elfgang et al., 1995), where ‘X’ represents the integration of any amino acid and the subscript number signifies the number of residues.

The extracellular loop cysteine residues are also known to engage in intramolecular disulphide bridge formation, and it has been established that while disulphide bonds do not form between two opposing connexons in a GJ channel, they do form between the two extracellular loops of a connexin (Dupont et al., 1989; John and Revel, 1991; Rahman and Evans, 1991; Foote et al., 1998). Disruption of these motifs has been shown to abolish GJ function, indicating the importance of secondary and tertiary structures imposed on connexin topology by disulphide bonds (Dahl et al., 1991; Dahl et al., 1992).
1.3 Assembly of Gap Junction Intercellular Channels

The assembly of GJ intercellular channels can be broken down into two major parts: the formation and removal of GJ channels. The formation process involves the transcription of the connexin gene, the translation of the protein, the non-covalent oligomerisation of sets of six connexin monomers into connexons, the trafficking of connexons to the plasma membrane, their docking with neighbouring connexons and aggregation into GJ plaques. The removal process involves internalisation of either portions of a GJ or entire plaques, followed by degradation of connexons and constituent connexin proteins (Koval, 2006; Evans et al., 2006a; Laird, 2006).

1.3.1 Half-Life of Plasma Membrane Gap Junction Plaques

Pulse-chase analysis of metabolically labelled connexin in tissue culture cells and whole organs has revealed that a pool of newly synthesised, immunoprecipitable connexin protein has a half-life of only 1.5 to 3.5 hours (Traub et al., 1989; Musil et al., 2000; Beardslee et al., 1998; Darrow et al., 1995; Thomas et al., 2005). Although highly unusual for plasma membrane proteins, which typically have turnover rates greater than 24 hours, rapid degradation kinetics are common characteristics of cytosolic and nuclear proteins involved in signal transduction. For several regulatory molecules, a decrease in their turnover rate is a physiologically important mechanism, whereby their function is prolonged or enhanced. Because GJ assembly appears to be a cooperative self-assembly process, reducing the rate of connexin degradation would lead to a large increase in GJ formation and intercellular communication. It has been found in treatments that a decrease in connexin turnover rapidly induces the assembly of cell surface Cx43 into long lived, functional GJs (VanSlyke and Musil, 2002; Musil et al., 2000; Laird, 2006).
1.3.2 Biosynthesis and Assembly of Connexins into Gap Junction Intercellular Channels

Connexins are synthesised and co-translationally inserted within the endoplasmic reticulum (ER) as four transmembrane integral membrane proteins (Ahmad et al., 1999; Falk et al., 1994; Falk and Gilula, 1998; Zhang et al., 1996). The precise location of their oligomerisation remains the subject of debate; however, some results indicate that connexins assemble into connexons whilst in ER-Golgi intermediates. An alternative suggestion is that assembly to connexons occurs in the trans-Golgi network. The only identified exception is Cx26, which has been demonstrated to bypass the Golgi network and directly trafficked to the plasma membrane (Zhang et al., 1996; Ahmad and Evans, 2002). Recent studies by VanSlyke et al. (2009) have indicated that the expression level of Cx32 and Cx43 influences assembly into connexons during trafficking through the trans-Golgi network, leading to the hypothesis that folding, trafficking and oligomerisation occurs gradually.

The transport of connexons to the cell surface generally follows the conventional secretory pathway. In other words, connexons pass from the ER to the cis-medial- and trans-Golgi cisternae, the trans-Golgi network, and finally to the plasma membrane (Figure 1.7) (Musil and Goodenough, 1993; Laird et al., 1995; Koval et al., 1997; Lauf et al., 2002; Thomas et al., 2003; VanSlyke and Musil, 2000).
Figure 1.7: Life cycle of connexins
The GJ life cycle is a complex multi-step pathway which involves connexin biosynthesis and insertion into the ER, connexon assembly and trafficking to the plasma membrane, channel docking, GJ internalisation and connexin degradation. Connexins interact with many protein partners, some of which are indicated in this figure. Different connexin proteins may follow different secretion and degradation pathways. TGN – trans-Golgi network; ERAD – endoplasmic reticulum-associated degradation (Laird, 2009).

Studies in living cells using fluorescently tagged connexins have shown that they enter a variety of transport intermediates of different sizes and shapes en-route to the plasma membrane (Jordan et al., 1999; Lauf et al., 2002; Thomas et al., 2005). Connexin transport may be mediated in part by microtubules, which appear to act by improving the efficiency
of delivery. This role has been identified using nocodazole, a drug that disrupts microtubules (George et al., 1999; Johnson et al., 2002; Martin et al., 2001; Lauf et al., 2002; Thomas et al., 2005). Brefeldin A, a drug that disrupts the Golgi apparatus, has also been used to identify an alternative pathway in which connexins bypass the Golgi apparatus (George et al., 1998a; George et al., 1999; Martin et al., 2001). Once within the lipid bilayer of the plasma membrane, connexons diffuse laterally and dock with connexons from adjacent cells to form GJs under the guidance of specific N- and E- cadherin based events (Musil et al., 1990; Jongen et al., 1991; Meyer et al., 1992; Wei et al., 2005). Pulse chain and fluorescent recovery after photo-bleaching experiments have shown that new GJ channels form at the edge of GJ plaques, whereas older ones accumulate in the centre of a plaque (Gaietta et al., 2002; Lauf et al., 2002).

Undocked connexons or hemichannels that are trafficked to the plasma membrane remain closed under physiological conditions (Sáez et al., 2005; Evans et al., 2006b). However, they are reported to open in low extracellular Ca\(^{2+}\) or Mg\(^{2+}\) solutions (Valiunas and Weingart, 2000; Valiunas et al., 2002). Whether a connexon becomes docked into a GJ channel or remains a hemichannel is likely to depend upon the physiological conditions and the correlating phosphorylation status of the connexins (Sáez et al., 2005).

### 1.3.3 Gap Junction Degradation

The short half-life of GJs may be mediated by an efficient mechanism which internalises and degrades them. Unlike most other types of protein assembly, the turnover of a GJ plaque involves two cells. Previous studies have documented the formation of ‘annular junctions’ in which an intact GJ plaque is internalised as a double membrane structure into the cytosol of one of the two partner cells (Jordan et al., 2001). The factors that determine which of the two cells receive the vesicle remain unknown.
Electron microscopic imaging has suggested that ‘aged’ channels that reside in the centre of a plaque are internalised, which suggests that not only can large membrane areas be endocytosed, but that connexin degradation can occur without destroying entire GJs (Falk et al., 2009; Gaietta et al., 2002). Two pathways have been suggested to regulate GJ quantity by endocytosis: (1) the internalisation of entire GJ plaques in order to be able to regulate GJ communication; and (2) the internalisation and degradation of small vesicles derived from the centre of a GJ for renewal of channels in an individual plaque (Falk et al., 2009).

Degradation of connexins either follows the lysosomal or the proteasomal ubiquitin-mediated pathway. Evidence supporting the lysosomal degradation pathway comes from electron microscopy data and studies performed in cells treated with inhibitors of lysosomal enzymes, such as leupeptin and ammonium chloride (Qin et al., 2003; Leithe and Rivedal, 2004; Berthoud et al., 2004). These studies indicate that internalised connexin-enriched fragments localise and fuse with lysosomes. In the case of proteasomal degradation, there is a substantial body of evidence resulting from the use of proteasomal inhibitors and from the finding that Cx43 is a suitable substrate for ubiquitin (Laing and Beyer, 1995; Musil et al., 2000; Leithe and Rivedal, 2004). An emerging hypothesis from these findings is that proteasomes may act primarily in ER-associated degradation as a form of quality control for protein folding and assembly, whereas lysosomes play a major role in degrading connexins from the plasma membrane (Salameh, 2006; Laird, 2006).

1.3.4 Connexin Mutations in Human Disease

Germline mutations of several different connexins are known to cause a number of human diseases. Disruptions in intercellular communication due to faulty connexin folding, oligomerisation, trafficking or docking have been identified as the underlying factors that
lead to the manifestation of these diseases. A few selected samples will be briefly introduced, with more comprehensive information and detailed clinical features available in recent reviews (e.g. Laird, 2008).

Mutations can occur anywhere in the connexin gene, and mutations in the non-coding regions can affect elements that regulate transcription or interfere with splicing to generate mature mRNA. An example of this is chromosome X-linked Charcot-Marie-Tooth disease, an inherited neurological disorder characterised by a loss of sensation and progressive weakening and atrophy of the distal limbs. This is associated with mutations in \textit{GJB1} which encodes Cx32 (Bergoffen et al., 1993; Bondurand et al., 2001; Martin et al., 2000). Cx32 is expressed in Schwann cells and oligodendrocytes and provide cell-to-cell communication in conjunction with other myelin-associated proteins essential for the functional and structural integrity of the myelin sheath and underpin nervous conduction (Martin et al., 2000). Site-directed mutagenesis of Cx32 has resulted in aberrant trafficking and disrupted oligomerisation (Martin et al., 2000; VanSlyke et al., 2009), leading to the failure of transfer of ions and small molecules across myelin sheaths and demyelination (Abrams and Rash, 2009). The hemichannel open probability can also be affected by mutations. For instance, a mutation that increases the probability of hemichannel opening may cause cell death similar to that suggested for Cx32 (Abrams et al., 2002).

The majority of known connexin mutations are located within the protein coding region (Beyer and Berthoud, 2009). Non-sense mutations or frameshift mutations result in the loss of normal full-length protein due to mRNA translation ending prematurely. An example is the 35delG mutation of \textit{GJB2}, which encodes Cx26, resulting in deafness (Carrasquillo et al., 1997). Over 90 mutations in the \textit{GJB2} gene are associated with inherited skin diseases and/or hearing loss (Laird, 2006; Aasen and Kelsell, 2009). Transfection of corresponding
mutated forms of Cx26 all lead to reduced intercellular-coupling, either due to faulty assembly (Martin et al., 2000; Forge et al., 2003) or the failure to dock (Thomas et al., 2003). GJ channel properties, including permeability, selectivity and gating, can also be altered by mutations (Beyer and Berthoud, 2009). Dominant-negative mutations may alter the specificity and compatibilities of co-expressed connexins. In the skin, Cx43 and Cx26 do not form heteromeric channels, but several Cx26 mutants associated with skin disease can inhibit Cx43 function via heteromerisation (Rouan et al., 2001). More than 20 autosomal dominant mutations in the GJA1 gene are known to cause the rare disorder, oculodentodigital dysplasia, in which patients show craniofacial abnormalities and malformations in their fingers and teeth (Paznekas et al., 2009). In patients with oculodentodigital dysplasia, GJ communication is thought to be markedly reduced despite a functional Cx43 wild-type allele (Roscoe et al., 2005). In addition to germline mutations, disease may be produced by the development of connexin mutations within somatic cells (Beyer and Berthoud, 2009). Several mutant forms of GJA5 (encoding Cx40) have been isolated from the hearts of patients with atrial fibrillation, suggesting that somatic mutations of GJA5 may be responsible for some cases of arrhythmia (Gollob et al., 2006).

Taken together, these findings highlight that mutated connexins can cause disease through disrupting the connexin life cycle, resulting in markedly reduced intercellular communication.

### 1.4 Functional Regulation

#### 1.4.1 Interactions between Gap Junctions and Adhesion Molecules in the Cell Membrane

The relationship among AJs, TJs and GJs has been explored in many cell types, yielding generally consistent but occasionally conflicting results (Fujimoto et al., 1997; Wu et al.,
AJs are known to provide strong mechanical attachments between cells mediated by cadherins. Cadherins contain an extracellular domain that can homodimerise, thereby bringing cells into close contact. Through interactions with catenins (a family of proteins found in complexes with cadherin cell adhesion molecules of animal cells) via their intracellular domains, they can remain anchored to the actin cytoskeleton. TJs perform a barrier function in epithelial cells, creating a tight seal between cells thereby defining and restricting the movement of integral membrane proteins between the apical and basolateral surfaces.

In many, although not all cases, the formation of AJs are necessary for and to precede GJ formation. This association is generally thought to reflect the physical need for membranes to come into close proximity for hemichannels in opposing cells to dock (Xu et al., 2001; Luo and Radice, 2003). In addition, AJ proteins are likely to be involved in signaling pathways that promote GJ assembly. This is consistent with the fact that the ability of cadherins to enhance GJ formation can be dependent on cell type (Meyer et al., 1992; Paul et al., 1995; Lee et al., 1987).

Several studies have shown consistently that AJs formation or cross-linking of cadherins activates a signalling pathway that promotes the delivery of connexion-containing vesicles to the plasma membrane so that GJs form in the vicinity of AJs (Fujimoto et al., 1997). This is supported by time-lapse studies in cardiomyocytes, where a Ca^{2+} switch was used to induce junction formation. In this study, the accumulation of adherens junctional components, such as α-catenin, β-catenin and ZO-1, occurred at the plasma membrane before Cx43 accumulation at the same sites (Wu et al., 2003). Another time-lapse study utilising live cell imaging and fluorescence microscopy on transiently transfected HeLa cells showed the increased delivery of Cx43 containing vesicles to the plasma membrane in cells
in which N-cadherin was cross linked (Shaw et al., 2007). The study also showed that both GJs and AJs may provide tethering sites for carrying Cx43 cargo, leading to directed delivery of Cx43 to cell-cell contact sites (Shaw et al., 2007).

Other studies using biochemical techniques and immunofluorescence microscopy have shown that the interaction between adherens junctional components and Cx43 occur intracellularly, and that catenins, cadherins, connexins and ZO-1 form complexes in the secretory pathway that are delivered to the plasma membrane together (Wei et al., 2005; Wu et al., 2003). In addition, one study showed that N-cadherin was quickly internalised after arrival at the plasma membrane, leading to a decrease in AJs, whereas GJs were not affected, and it was argued that important interactions occur before connexins reach the plasma membrane (Wei et al., 2005).

GJs, especially those containing Cx32, have also been convincingly shown to be associated and intermingled in TJ strands by freeze fracture electron microscopy (Kojima et al., 2001; Fujimoto et al., 1997) and can be co-immunoprecipitated along with several TJ proteins, such as occluding and claudins (Kojima et al., 2001; Nagasawa et al., 2006). Thus, there are substantial and convincing data showing that adherens and tight junctional components interact with and affect the connexin life cycle, both at the plasma membrane and in secretory compartments. Additionally, there are common proteins found in cell-cell junctions, also known as scaffolding proteins, including ZO-1 and ZO-2. ZO-1, which is binding partner of Cx43, interacts with all connexins that contain a post-synaptic density protein, Drosophila disc large tumour suppressor and ZO-1 (PDZ) binding motif in their carboxyl terminal domains (Giepmans, 2004).

As a scaffolding protein, ZO-1 provides a linkage between the different cell-cell junctions, junctional components and the cytoskeleton. Several studies have reported that the
interaction between Cx43 and ZO-1 is regulated. In rat kidney cells, G₀ phase cells increased co-localisation and the interaction of ZO-1 and Cx43 was demonstrated by immunofluorescence and Western blot analysis (Singh et al., 2005). This correlates with the report that G₀ is the phase in which rat kidney cells are most efficient at GJ assembly (Solan et al., 2003). However, in spite of being a well described connexion interacting protein, the functional significance of the interactions of connexins with ZO-1 remain unclear.

1.4.2 Regulation of Gap Junction Channels

1.4.2.1 Electrical Properties

GJ channels are voltage-sensitive; and, depending on species, the macroscopic gap-junctional current (Iₒ) is sensitive to: (1) the trans-junctional voltage, defined as the potential difference between the two cell interiors; and (2) the transmembrane potential, which is the voltage difference between the cell interior and the external medium (Bukauskas and Verselis, 2004; Gonzalez et al., 2007).

Development of the dual whole-cell voltage clamp technique has allowed the accurate resolution of the electrical properties of GJ channels. This technique permits the cells to be clamped at a fixed membrane potential and the GJ current to be recorded (Desplantez et al., 2004). To begin with the membrane potential of both cells is clamped to the same value, but thereafter the membrane potential of one of the cells is depolarised or hyperpolarised in a stepwise fashion to establish a voltage gradient or gap-junctional potential. From the current recorded it is possible to calculate the macroscopic junctional conductance. This value is influenced by: (1) the number of GJ channels (N); (2) the open probability (Pₒ); and (3) the channel specific unitary conductance (Van Veen et al., 2006; Moreno and Lau, 2007).

Dual voltage-clamp analysis has shown that in homotypic channels, steady-state junctional conductance is usually maximal when trans-junctional voltage is zero. As one cell of a cell
pair is depolarised or hyperpolarised, conductance reduces from the main-state to lower values. The junctional current is rarely abolished, and even at high voltages a voltage-insensitive component of junctional conductance remains. This relationship of macroscopic voltage sensitivity is commonly visualised by plotting all normalised gap-junctional current values measured at each depolarising and hyperpolarising voltage step, and fitting to a Boltzman equation.

To establish the unitary conductance, the number of channels between cell pairs has to be reduced, which can be achieved through treatment with gap-junctional communication inhibitors, such as heptanol, octanol or halothane, which permits the recording of single channel behaviours. After removing the agent, the channels gradually open enabling single currents to be recorded at a given voltage over time. Analysis of these single channel recordings can be used to determine unitary open conductances for individual channels, which are connexin isotype and channel-specific. The electrical properties of connexins (e.g. Cx40, Cx43 and Cx45) have been studied most commonly in Xenopus oocytes and cultured mammalian cells, such as HeLa, N2a and RIN cells transfected with different connexin subtypes (Dahl et al., 1987; Elfgang et al., 1995; Valiunas et al., 2000; 2001; Elenes et al., 2001). From these experiments it has been determined that homotypic channels composed of Cx45 exhibit the smallest unitary conductance at 30-40pS, whereas Cx43 and Cx40 homotypic channels have values of 60-120pS and 150-200pS (Gros and Jongsma, 1996; Moreno, 2004).

The electrical properties of heteromeric and heterotypic channels have also been studied in co-transfected cells to identify the functional significance of connexin co-expression patterns in vivo. Heterotypic docking of Cx43-Cx45 channels produced conductances that were intermediate between that of the single unitary conductance for homotypic Cx43 and
Cx45 (Elenes et al., 2001; Desplantez et al., 2004; Moreno, 2004). Similar results were also obtained for Cx40-Cx45 heterotypic channels (Moreno, 2004) and Cx30.2-Cx45 (Kreuzberg et al., 2006; Rackauskas et al., 2007). The formation of heterotypic channels between Cx40 and Cx43 remains controversial due to conflicting results (Elfgang et al., 1995; Haubrich et al., 1996; Rackauskas et al., 2007). However, Valiunas et al. (2000) and Cottrell et al. (2002) have reported the presence of heterotypic channels in cell pairs with considerably reduced coupling compared to the homotypic conductances for Cx43 or Cx40.

It has also been shown that Cx45, Cx43 and Cx40 can form heteromeric connexons producing channels with intermediate conductances or lower that what was observed for their respective homotypic conductances. For example, Cx40 and Cx43 form heteromeric channels in co-expressing HeLa cells with an average conductance that is lower than the values for homotypic channels of either connexin (Valiunas et al., 2001). In the A7r5 smooth muscle cell (SMC) line that naturally co-expresses Cx40 and Cx43, a range of unitary conductances distinct from homomeric-homotypic Cx40 or Cx43 channels were observed (He et al., 1999). Thus there is considerable scope for electrical properties to vary according to the precise connexin make-up of a channel.

1.4.2.2 The Nature of Connexins Dictates Gap Junction Permeability

GJs are permeable to many biologically relevant molecules, and using a technique called transport specific fractionation, Bevans et al. (1998) demonstrated the different permeabilities of connexin channels to biological molecules. Cyclic nucleotides, such as c-GMP and c-AMP, readily pass from one cell to the next through GJ channels. However, depending on the connexin type, certain molecules are more efficiently transferred than others. For instance, c-GMP passes more efficiently through heteromeric channels composed of Cx32 and Cx26 than channels composed of Cx32 alone (Bevans et al., 1998).
Components of cellular signalling pathways are also spread via GJs. The second messenger inositol 1,4,5-triphosphate (Niessen et al., 2002) is transferred with connexin subtype specific permeabilities. GJs are also permeable to small interfering RNA (siRNA) molecules of a specific connexin subtype from cell-to-cell, thereby influencing gene expression in neighbouring cells (Valiunas et al., 2005). Wallner et al. (2009) also demonstrated the transfer of micro RNA (miRNA) via GJs in HeLa cells which potentially represents a new mechanism for the control of gene expression by the cellular environment.

Differences in ion selectivity by the connexin subtypes to dyes of varying molecular mass and charge (e.g. Lucifer yellow, ethidium bromide and Alexa dyes) were shown in HeLa cells and *Xenopus* oocytes transfected with different connexins (Elfgang et al., 1995; Cao et al., 1998; Valiunas et al., 2002; Weber et al., 2004). The flux of fluorescent dye through GJs was measured by light microscopy to determine whether the cells are coupled by GJs, and revealed that channels composed of each connexin display distinctive permeability properties.

GJs are non-ion selective for channels with specificity for certain anions and cations, and function as an aquatic pore that enables bidirectional ion movement. For example, Cx45 channels have equal permeability to caesium and potassium cations, but low permeability to chloride ions (Veenstra et al., 1995). Further studies have demonstrated that Cx40 channels are cation-selective (Beblo and Veenstra, 1997), while channels composed of Cx43 exhibit no overall selectivity for either anions or cations (Wang and Veenstra, 1997).

1.4.2.3 Effect of Intracellular Calcium on Gap Junction Permeability and Conductance

The role of Ca$^{2+}$ in modulating cell-cell coupling was initially established from experimental data correlating the loss of electrical and dye coupling to increased intracellular Ca$^{2+}$
monitored by the Ca\textsuperscript{2+} indicator aequorin (Rose and Loewenstein, 1975; 1976). This was later confirmed in a number of cell types, including cardiac cells, cultured lens cells, and osteoblasts (Peracchia, 2004). Its role in uncoupling may be particularly important in protecting intact cells from membrane depolarisation and leakage of metabolites and ions, in particular K\textsuperscript{+}, through GJs (Rackauskas et al., 2010), and this process is called ‘healing-over’. Ca\textsuperscript{2+}-mediated inhibition of intercellular communication has been confirmed in a number of cell models (De Mello, 1975; Dahl and Isenberg, 1980; Neyton and Trautmann, 1985). Although it has been established that Ca\textsuperscript{2+} affects channel gating, how intracellular Ca\textsuperscript{2+} activates the gating mechanism remains unclear and may depend on the connexin and cell type, with effective concentration in the range 500 nM – 1.5 μM (Peracchia, 2004). This wide concentration range could be due to variation in the effectiveness of internal buffering systems and sometimes the increase in intracellular Ca\textsuperscript{2+} is too brief to activate the relatively slow gating mechanism. To enable a functional interaction between Ca\textsuperscript{2+} and connexins, clusters of negatively charged amino-acid residues would be required to face the cytoplasm, and connexins possess only a single amino acid residue (glutamate) which faces the cytoplasm, located at the transition between the fourth transmembrane (M4) domain and carboxyl-terminus. Despite there being six of these residues per connexon to bind to Ca\textsuperscript{2+} this may not be sufficient to mediate channel gating. It is likely that Ca\textsuperscript{2+} acts through an intermediate and one potential candidate is calmodulin (Peracchia et al., 1983; Torok et al., 1997).

1.4.2.4 The Effect of Intracellular pH on Gap Junction Formation

Alterations to intracellular pH can also affect junctional conductance (Turin and Warner, 1977; Spray et al., 1981), and cellular uncoupling through pH dependent gating would protect neighbouring cells from the potentially damaging effects of intracellular acidification. Sensitivity to pH varies among cell types and is in part related to the type of
connexin expressed. By using a method that monitors junctional conductance and pH at different levels of acidification, studies have demonstrated that Cx32 is the least sensitive to changes in pH and Cx50 the most sensitive (Liu et al., 1993; Stergiopoulos et al., 1999). Connexin co-expression could also affect pH sensitivity through properties unique to individual connexins. For example, Gu et al. (2000) demonstrated that mixing Cx40 and Cx43 yields channels with increased sensitivity to pH. The molecular mechanism of pH gating is likely to involve the connexin cytoplasmic loop and carboxyl-terminal. Cx43 truncation abolishes pH sensitivity (Liu et al., 1993) but co-expression of mutant connexin with the missing carboxyl-terminal sequence restores pH sensitivity and gating (Morley et al., 1996). The histidine residue at position 95 in the cytoplasmic loop has been proposed to be involved in pH gating, since substitution with a basic or uncharged residue reduces pH sensitivity (Ek et al., 1994). The proposed mechanism is that low-pH facilitates the interaction of the carboxy-terminal with a receptor domain elsewhere in the connexin molecule causing the channel to close (Liu et al., 1993; Ek et al., 1994; Morley et al., 1996).

1.4.2.5 Connexin Phosphorylation

A significant mechanism of alteration to GJ properties is through changes to the phosphorylation state of connexins. This provides a relatively rapid and reversible alteration in comparison to altered protein turnover and redistribution. Phosphorylation and/or dephosphorylation occurs predominantly at serine residues but also some at threonine sites, through the action of serine/threonine protein kinases and phosphatases, such as tyrosine phosphatase (Abdelmohsen et al., 2004), alkaline phosphatase (Lampe and Lau, 2004) protein kinase A (PKA), protein kinase C (PKC), mitogen activated protein kinase (MAPK), cyclin dependent kinase 2, and casein kinase 1, as shown in Figure 1.8 (Herve and Dhein, 2006). Changes to the phosphorylation state exhibits diverse roles, encompassing both physiological and pathophysiological functions of connexins and includes regulation...
of the life-cycle, turnover and open probability, GJ conductance, and intercellular communication (Herve et al., 2004; Solan and Lampe, 2005; Herve and Dhein, 2006; Burstein et al., 2009).

![Connexin topology and phosphorylation sites](image)

**Figure 1.8: Connexin topology and phosphorylation sites**
Cdc2, cyclin dependent kinase 2; CK1, casein kinase 1; MAPK, mitogen-activated protein kinase; PKA, protein kinase A; PKC, protein kinase C; PKG, protein kinase G.

Phosphorylation of connexins has been primarily identified through shifts in their electrophoretic mobility when analysed by SDS-PAGE and the direct incorporation of $^{32}$P (Musil and Goodenough, 1991; Laird, 1991; Laing et al., 1994; Cooper et al., 2000).
Cx43 phosphorylation has been the most extensively described to date and is typically detected as three bands derived from different phosphorylation states; the fastest migrating band represents the newly synthesised connexin which is non-phosphorylated (NP) and two slower migrating bands (P₁ and P₂) represent the phosphorylated forms (Musil and Goodenough, 1991). Treatment with alkaline phosphatase confirmed that the differences in molecular weight were due exclusively to phosphorylation. Pulse-chase experiments showed that the NP form is trafficked to the membrane where it is then phosphorylated and integrated into functional GJ plaques. It should be noted that phosphorylation does not exclusively take place at the membrane (Puranam et al., 1993; Solan et al., 2003). Using phosphospecific and epitope-specific antibodies it has been established that differences in the P₁ and P₂ mobility forms are derived from phosphorylation at different sites on the protein (Musil et al., 1990; Solan et al., 2003; Solan et al., 2007; Solan and Lampe, 2009). Regulators of phosphorylation, such as c-AMP, can mediate gap junctional communication via an increase in synthesis, trafficking and assembly at the plaque (Atkinson et al., 1995; Darrow et al., 1996; Paulson et al., 2000). c-AMP mediates its effects via adenylyl cyclase and PKA, leading to enhanced assembly of GJ plaques (Paulson et al., 2000).

Activation of kinases is cell-type and cell-stage specific and is highly sensitive to altered physiological conditions, such as ischaemia or the presence of growth factors including platelet-derived growth factor and vascular endothelial growth factor (Warn-Cramer and Lau, 2004). Phosphorylation has been correlated with an increase or decrease in hemichannels and GJ channels (Moreno, 2005). For instance, the opening of a variety of hemichannels (e.g. Cx43, Cx46) can be blocked by the activation of protein kinases (e.g. PKC) (Saez et al., 2003). Phosphorylation of rat Cx43 by PKC caused a shift in the channel unitary conductance favouring a lower conductance and lower permeability to Lucifer.
yellow (Moreno et al., 1992; 1994; Kwak et al., 1995; Lampe et al., 2000). Phosphorylation of connexins is thus a rapid regulator of intercellular communication.

Cx43 does not contain serine residues in the intercellular loop and there are no reports on phosphorylation of the Cx43 N-terminus. However, the total number of phosphorylation sites at the C-terminus of Cx43 is 21 (19 serine residues and two tyrosine residues), many of which have been studied with varying results. Site-specific assessment of phosphorylation states has been enabled through the employment of (co-)immunoprecipitation, Western blotting and SDS-PAGE techniques, and the development of phospho-specific antibodies. For example, kinase activation can cause phosphorylation of S368 by PKC, S255, S279 and S282 by MAPK, S325, S328 and S330 by casein kinase CK1, S247 and S265 by Src tyrosine kinase, and S364, S365, S369 and S373 by PKA at the C-terminus. This has enabled the analysis of the association between the phosphorylation state of specific residues and particular interventions or pathologies (Lampe et al., 2000; Beardslee et al., 2000; Bokník et al., 2001; Lampe et al., 2006; Burstein et al., 2009; Srisakuldee et al., 2009; Jeyaraman et al., 2012).

1.5 Gap Junction Modulators

Despite the universality in multicellular organisms of gap junctional intracellular communication (GJIC), relatively few reagents are known that block GJIC in an acceptably specific manner. The GJ channel is well insulated from the extracellular space, and access to allow direct channel modulation appears to be restricted.

1.5.1 Chemical Inhibitors

GJ blockers are chemicals that disrupt GJ communication, defined for example, as the electrical coupling of neighbouring cells or dye transfer from one cell to another (Cruikshank et al., 2004 and Guan et al., 1997). To date, GJ blockers have been used most
often to investigate the functioning of electrical synapses in \textit{in vitro} experiments. Limited data are available on the effects of the systemic administration of these compounds on brain physiology and the behaviour of laboratory animals. Nonetheless, some compounds, that quite recently turned out to have the ability to block the GJs, have been used for a long time to treat a wide range of diseases, including malaria, ulcer disease or cardiac arrhythmia in humans. Therefore, the human side effects of these compounds are well known. Furthermore, there is an abundance of data on their toxicity from studies in laboratory animals, although the effects of the compounds on GJs have not been considered in these studies.

\subsection*{1.5.1.1 Glycyrrhetinic Acid Derivatives}

\subsubsection*{1.5.1.1.1 Carbenoxolone}

Carbenoxolone is a glycyrrhetic acid derivative with a steroid-like structure, similar to substances found in the root of the licorice plant. Carbenoxolone is used for the treatment of peptic, oesophageal and oral ulceration and inflammation. Electrolyte imbalance is a serious side effect of carbenoxolone when used systemically (Spray et al., 2002).

Carbenoxolone has been shown to block GJs in human mutant fibroblast cell lines (IC$_{50}$ = 3 µM) but it has not been reported to display specificity for any particular type of connexins (Davidson et al., 1986; Davidson and Baumgarten, 1988). Carbenoxolone also blocks voltage-gated Ca$^{2+}$ channels (IC$_{50}$ = 48 µM) (Vessey et al., 2004). Furthermore, carbenoxolone is known to enhance the effects of endogenous glucocorticoid hormones by inhibiting 11 beta-hydroxysteroid dehydrogenase (Bujalska et al., 1997).

\subsubsection*{1.5.1.1.2 Glycyrrhetinic Acid}

Glycyrrhetic acid is a potent non-selective blocker of GJ channels (18α,GA — IC$_{50}$ = 1.5 µM; 18β,GA — IC$_{50}$ = 2 µM) (Davidson et al., 1986; Davidson and Baumgarten, 1988) and
hemichannels (Cx46 — 250 µM and Cx50 — 100 µM) (Eskandari et al., 2002). Glycyrrhetinic acid is also an inhibitor of 11 beta-hydroxysteroid dehydrogenase (IC$_{50}$ = 300 nM) (Su et al., 2007) and has been found to inhibit voltage-sensitive Ca$^{2+}$ currents with a significant effect at 10 µM (18β,GA) (Matchkov et al., 2004). Moderate chronic or high acute exposure to glycyrrhetinic acid has been demonstrated to cause increased potassium excretion, sodium and water retention, body weight gain, alkalosis, suppression of the renin-angiotensin-aldosterone system, hypertension, and muscular paralysis (Panel, 2007). Glycyrrhetinic acid is a natural constituent of liquorice used as a flavouring agent in foods, beverages and some drugs (Asl and Hosseinzadeh, 2008). Excessive liquorice ingestion can cause transient visual loss, headache, and hypermineralocorticoidism, leading to sodium retention, potassium loss, oedema, hypertension, suppression of the renin-angiotensin-aldosterone system, and hypokalaemia paralysis (Dobbins and Saul, 2000; van den Bosch et al., 2005; Asl and Hosseinzadeh, 2008). Glycyrrhetinic acid has been applied in vitro to study the role of GJs in neuronal oscillations, epileptic discharges and glutamate-induced neurotoxicity (de Curtis et al., 1998; Hughes et al., 2004; Leznik and Llinas, 2005; Proulx et al., 2006).

1.5.1.2 Derivatives of Quinine

1.5.1.2.1 Quinine

It has been demonstrated, using the dual whole cell voltage clamp technique applied to N2A cells transfected with connexins, that quinine is a selective and reversible GJ blocker (Srinivas et al., 2001; Cruikshank et al., 2004). Quinine selectively blocked Cx36 (IC$_{50}$ = 32 µM) and Cx50 (IC$_{50}$ = 73 µM) GJs, and at higher concentrations moderately affected Cx45 junctions (300 µM), but did not substantially block GJs formed by other connexins, namely Cx26, Cx32, Cx40, and Cx43 (300 µM) (Srinivas et al., 2001). Quinine can also act as blocker of ATP-sensitive and voltage-dependent K$^+$-channels (IC$_{50}$ = 3 µM and IC$_{50}$ = 8 µM, respectively) (Lin et al., 1998; Gribble et al., 2000). Although quinine does not seem to cross
the human blood brain barrier (BBB) freely (Silamut et al., 1985), it does cross it in rats and mice (Mikov et al., 2004 and Pussard et al., 2007). The BBB permeability of quinine in cats has not been tested directly, but it can be concluded indirectly from the central nervous system (CNS) effects of this drug observed following intraperitoneal (i.p.) injections (Golebiewski et al., 2006). Quinine is used for treatment of malaria and benign nocturnal leg cramps, and at low concentrations it is also present in tonic water (Bateman and Dyson, 1986 and Townend et al., 2004).

1.5.1.2.2 Quinidine

Using the dual whole cell voltage clamp technique applied to N2A cells transfected with connexins, quinidine, a stereoisomer of quinine, has also been found to block GJs (Cruikshank et al., 2004). However, the specificity of this compound for the different subtypes of GJs has not been tested, although quinidine potently blocks Cx50 junctions (300 µM) (Cruikshank et al., 2004). Due to the structural similarity of quinidine to quinine, it can be expected that quinine and quinidine have similar properties, but this cannot be taken for granted because stereoisomers can differ in their biological activity. Other possible effects of quinidine include the blockade of Na⁺ (IC₅₀ = 20 µM) and K⁺ channels (IC₅₀ = 6 µM) (Koumi et al., 1991; Yatani et al., 1993; Grace and Camm, 1998). Quinidine crosses the BBB in rats, mice, dogs, and humans (Harashima et al., 1985; Kusuhara et al., 1997; Fromm et al., 1999; Dagenais et al., 2001; Vasovic et al., 2007).

1.5.1.2.3 Mefloquine

Another potent GJ blocker is mefloquine (Cruikshank et al., 2004), which has been found to be relatively selective for certain subtypes of GJs. At concentrations sufficient to cause the complete blocking of Cx36 and Cx50 junctions (3 µM), mefloquine had no effect on Cx26, Cx32, Cx43, and Cx46 (Cruikshank et al., 2004). When a higher concentration was
used (30 µM), mefloquine also blocked, almost completely, Cx43 junctions and significantly blocked Cx26 and Cx32 junctions (Cruikshank et al., 2004). Mefloquine has been commonly used in the prophylaxis and treatment of malaria and it is known to produce a number of neuropsychiatric adverse side effects. The most common are vivid dreams, nightmares, light-headedness (described as an inability to concentrate), and dizziness (Kukoyi and Carney, 2003 and Tran et al., 2006). Less frequently observed are severe schizophrenia-like delusions and visual or auditory hallucinations (Javorsky et al., 2001; Kukoyi and Carney, 2003; Tor et al., 2006; Tran et al., 2006).

1.5.1.3 Octanol and Heptanol
Long-chain alcohols, octanol (0.5–1 mM) and heptanol (1–3 mM), have been reported to block GJs in crayfish giant axons, rat glial cells and insect cells (Johnston et al., 1980; Guan et al., 1997; Weingart and Bukauskas, 1998). There is no evidence for a strong selectivity of these alcohols for any particular subtype of GJ channels in mammalian cells (Spray et al., 2002). Octanol (1 mM) blocks Cx46 and Cx50 GJ hemichannels (unpaired connexons) with a similar potency to GJ channels (Eskandari et al., 2002). Heptanol activates Ca\(^{2+}\)-activated potassium channels (150 µM) and inhibits voltage-sensitive Ca\(^{2+}\) currents (150 µM) (Matchkov et al., 2004), whereas octanol has been found to block T-type calcium channels (IC\(_{50}\) = 122 µM) (Todorovic and Lingle, 1998). At higher doses, octanol and heptanol, like other alcohols, produces intoxication (McCreery and Hunt, 1978; Lyon et al., 1981)

1.5.1.4 Fenamates
Fenamates, used as nonsteroidal anti-inflammatory drugs and analgaesics, have been demonstrated to block GJs in rat fibroblasts and neuroblastoma cells. Harks et al. (2001) reported that fenamates inhibited GJs composed of Cx43 with the potency decreasing in the order: meclofenamic acid (IC\(_{50}\) = 25 µM) > niflumic acid > flufenamic acid (IC\(_{50}\) = 40 µM) acid. In another study it was found that these three drugs (flufenamic acid 100 µM;
meclofenamic acid 100 µM; niflumic acid 300–1000 µM), and also 5-nitro-2-(3-phenylpropylamino)-benzoic acid (NPPB) (100 µM), blocked GJs formed by Cx50 (Srinivas and Spray, 2003). A detailed study of the effects of flufenamic acid (100 µM) revealed that this drug inhibited with low selectivity GJs composed of Cx26, Cx32, Cx40, Cx43, Cx46, and Cx50 (Srinivas and Spray, 2003). Flufenamic acid, niflumic acid and NPPB have been also tested for their ability to block GJ hemichannels. All three drugs inhibited Cx50 hemichannels (flufenamic acid 100 µM; NPPB 100 µM, niflumic acid 10–100 µM), whereas NPPB (250 µM) and flufenamic acid (250 µM), in contrast to niflumic acid (250 µM), also blocked Cx46 hemichannels (Eskandari et al., 2002).

1.5.1.5 2-Aminoethoxydiphenyl Borate and Derivates

It was reported that 2-aminoethoxydiphenyl borate (2-APB), at a low concentration (20 µM) potently blocked GJs composed of Cx36 (IC₅₀ = 3 µM), Cx40 and Cx50 (IC₅₀ = 3.7 µM), and moderately disrupted communication through Cx26, Cx30 and Cx45 (IC₅₀ = 18 µM) junctions (Bai et al., 2006). The effects of this compound at these lower concentration on other types of connexins (Cx32, Cx43, IC₅₀ = 51 µM; Cx46, IC₅₀ = 29 µM) were small (Bai et al., 2006). However, when higher concentrations were used, 2-APB also blocked Cx32 GJs (100 µM) and Cx32 hemichannels (IC₅₀ = 49 µM) and heteromeric Cx32/Cx26 hemichannels (IC₅₀ = 44 µM) (Tao and Harris, 2007). Tao and Harris (2007) tested compounds structurally similar to 2-APB and found that phenytoin, diphenhydramine (DPDM) and diphenylboronic anhydride (DPBA) blocked Cx32 and heteromeric Cx26/Cx32 hemichannels with a potency similar or even higher than 2-APB. DPDM (100 µM), DPBA (100 µM) and 2,2-diphenyltetrahydrofuran (DPTHF) (100 µM) were also found to inhibit the dye coupling of cells expressing Cx32 (Tao and Harris, 2007).
1.5.1.6 Anaesthetics

Several anaesthetics, such as halothane, enflurane, isoflurane, propofol, and thiopental, inhibit GJ communication between cultured astrocytes (halothane, 0.1–1.0 mM; enflurane, 1–1.6 mM; isoflurane, 0.5–1 mM; propofol, 0.01–0.1 mM) (Mantz et al., 1993), crayfish axons (halothane, 28.5 mM and isoflurane, 23.6 mM) (Peracchia, 1991) and in hippocampal slices (halothane, 2.8 mM; propofol, 15 µM; thiopental, 10 µM) (Wentlandt et al., 2006). Halothane has also been widely applied to disrupt neuronal coupling in in vitro experiments (Draguhn et al., 1998 and Kohling et al., 2001) and in anaesthetised animals (Ylinen et al., 1995; Grenier et al., 2003; Grenier et al., 2001; Fuentealba et al., 2004). However, the use of these compounds in behavioural studies is problematic because they block GJs at concentrations comparable, or even higher, to their anaesthetic doses (Wentlandt et al., 2006).

1.5.1.7 Retinoic Acid

Retinoic acid, an active form of vitamin A, blocks GJs (50–100 µM/0.3–30 µM, IC50 = 2.4) (Weiler et al., 1999; Zhang and McMahon, 2000; Pan et al., 2007), but its specificity for different connexins has not been determined. Retinoic acid plays an important role in gene expression and has been implicated in synaptic plasticity, sleep, learning, memory, and in the pathogenesis of Alzheimer’s disease, schizophrenia and depression (Mey and McCaffery, 2004; Lane and Bailey, 2005; Bremner and McCaffery, 2008). Recently, it has been shown that retinoic acid possesses anti-epileptic properties (Sayyah et al., 2007). The acute toxicity of retinoic acid is characterised by respiratory distress, fever, pulmonary infiltrates, weight gain, pleural effusion, and renal failure (De Botton et al., 1998).
1.5.1.8 **Oleamide**

Oleamide is a sleep-inducing lipid originally isolated from the cerebrospinal fluid of sleep-deprived cats (Cravatt et al., 1995). Oleamide was shown to block GJ coupling (20–50 µM) but the connexin specificity has not been tested (Guan et al., 1997).

1.5.1.9 **Spermine**

Spermine is an aliphatic amine formed and stored in most eukaryotic cells. It plays different functions, ranging from growth-related effects to radical scavenging and immunomodulation (Seiler, 2005). Spermine was tested for its ability to block Cx40 and Cx43 GJs, and proved to be a Cx40 GJ blocker (0.1–2 mM) (Musa and Veenstra, 2003).

1.5.1.10 **Aminosulfonates**

Aminosulfonates, used as pH buffers (HEPS, 10 mM; TAPS, 10 mM), and taurine (10 mM) have been found to inhibit heteromeric connexin channels containing Cx26 GJs, without demonstrating any significant effects on homomeric Cx32 channels (Bevans and Harris, 1999).

1.5.2 **Connexin Mimetic Peptides**

Connexin mimetic peptides are short synthetic peptides corresponding to chosen sequences in the extracellular loops of connexins. They were developed by screening the efficacy of a range of short peptides covering most of external loop 1 (50 amino acids) and external loop 2 (60 amino acids) of Cx32 in delaying synchronised contraction by aggregating myocytes dissociated from chick hearts (Warner et al., 1995). These cells express mainly an unknown connexin homologous to Cx32 characterised in mammals (Barker and Gourdie, 2002). Similar short synthetic connexin peptides have been used to dissect the docking process of connexons expressed in paired *Xenopus* embryos (Bao et al.,
Once formed, GJs, especially when large numbers of units accrete laterally in the plasma membrane, are extremely difficult to tear apart into the constituent hemichannels.

Experimentally useful inhibitory peptides emerging from subsequent work correspond to mainly to extracellular sequences in Cx43 positioned next to the second and fourth transmembrane domains, which have been labelled GAP26 (EL 1; VCYDKSFIPISHVR) and GAP27 (EL 2; SRPTEKTIIFII and SRPTEKNVFIV) (Figure 1.9) (Chaytor et al., 1997; Dahl et al., 1994; Oviedo-Orta et al., 2000; Oviedo-Orta et al., 2001). GAP27 incorporates the sequence SRPTEK present in most connexins, and the utility of this potentially universal GJ-inhibitory peptide was found to be enhanced significantly by including amino acids that are located in the fourth transmembrane region (Chaytor et al., 1999).

The utility of these benign and reversible inhibitors of GJIC acting from the outer aspect of cells has been documented in a number of systems. They block the intercellular transfer of fluorescent dyes in various cultured cells (Oviedo-Orta et al., 2000, 2001) and also the propagation of Ca\(^{2+}\) waves across groups of confluent cells and electrical communication (Isakson et al., 2001; Boitano and Evans, 2000). The effect of the connexin mimetic peptides on communication efficiency in tissues has so far proved difficult to demonstrate for technical reasons. However, the inherent specificity of the inhibition of GJIC by connexin mimetic peptides makes them ideal candidates for the eventual modulation of tissue and organ function.
Figure 1.9: Position of GAP26 AND GAP27 connexin mimetic peptides in the intercellular region of the gap junction.

GAP26 binds to extracellular loop 1 and GAP27 binds to extracellular loop 2, disrupting the formation of functional GJ channels (Evans et al., 2001).

1.6 Intercellular Communication across Gap Junctions in the Immune System

1.6.1 Connexin Expression in Immune System Cells

Multiple cell types of the immune system have been shown to express connexins. These cells usually have different functions and control immune responses or execute them in a series of different ways (Oviedo-Orta et al., 2001). The phagocytic cells of the innate immune system are dendritic cells (DCs) and monocytes/macrophages. These cells express connexins and can form functional GJs between identical (Matsue et al., 2006; Eugenan et
al., 2003), as well as different cells (Krenacs et al., 1997; Martin et al., 1998b). Connexins are expressed by almost all immune cells and can be upregulated upon exposure to inflammatory factors (Eugenan et al., 2003).

Immune cells originate in the bone marrow, where immune stem cells may communicate with their surrounding stromal cells, since both express Cx43 (Cancelas et al., 2000). These stem cells can differentiate into the nine types of defined circulating blood cells, including all members of the cellular immune system. In this early phase of haematopoiesis, Cx43-containing GJs appear to be critical for terminal differentiation of primary T and B cells, as tested in Cx43-deficient mice (Montecino-Rodriguez et al., 2000).

T and B cells further develop or are activated in the lymphoid organs, such as the thymus and lymph nodes. In the thymus, progenitor T cells have extensive contact with surrounding thymic epithelial cells and thymic DCs. These cell types express Cx43, possibly allowing homo- and heterotypic interactions (Montecino-Rodriguez et al., 2000). In secondary lymphoid organs, T cells encounter antigens presented by DCs. T cells that are not deleted during negative selection in the thymus migrate into peripheral tissues to search for antigenic information in the form of peptides. The expression of Cx43 by these T cells may allow communication with the surrounding tissues, possibly allowing proper activation and differentiation.

B cells also express Cx43 and may interact via GJs with follicular DCs in secondary lymphoid organs. The coupling of these cells may mediate synchronising germinal centre events or facilitate the transfer of anti-apoptotic molecules to rescue B cells from apoptosis (Krenacs et al., 1997). T, B and natural killer (NK) cells isolated from tonsils express low levels of Cx40, but the function of GJs in these cells and their connected tissue is unknown. It has been proposed that these cells form hemichannels composed of Cx40 and that these
facilitate ATP-mediated propagation of Ca\textsuperscript{2+} waves (Oviedo-Orta et al., 2001, 2002). B cells ultimately produce antibodies and T helper cells control this process. Oviedo-Orta et al. (2002) studied the role of GJs in a mixed lymphocyte culture and showed that GJ communication is required for efficient antibody secretion by B cells. Although the exact intracellular signals being exchanged are unclear, the results show that GJ mediated intercellular signalling to B cells is required for optimal antibody production.

DCs also express GJs to communicate with their environment. In addition, human monocytes as well as DCs upregulate Cx43 and form GJs when detecting inflammation, suggesting that they contact the environment in order to sample metabolic information from neighbouring cells in response to infection (Eugenan et al., 2003; Neijssen et al., 2005). It has been shown that GJ communication between DCs is required for their activation, but the specific signals involved/transferred between cells are unclear (Matsue et al., 2006).

1.6.2 Pathogenesis of Inflammation

The inflammatory response is an important homeostatic mechanism to counter tissue dysregulation and malfunction, and is classically viewed as a reaction to infection or tissue injury (Medzhitov, 2008). In this context, inflammation is an important physiological process resulting from a complex network of cellular and molecular events. The inflammatory response is initiated through the recognition of a proinflammatory stimulus by immune sensors, which is followed by the release of proinflammatory mediators and leads to the activation of effector cells and tissues. However, several different factors may promote inflammation and contribute to vastly different outcomes, ranging from tissue repair to detrimental chronic inflammation that underpins diseases with an auto-immune component, such as type II diabetes and atherosclerosis (Barton, 2008; Pickup, 2004).
Leukocyte infiltration to the site of infection or injury is one of the key steps in the initiation of an inflammatory response. It has been shown that the pro-inflammatory cytokine interleukin 6 (IL-6) plays an important role in modulating this process through the upregulation of adhesion molecules and chemokine secretion by endothelial cells (ECs) (Hurst et al., 2001). In addition, the secretion of chemokines, vasoactive amines and eicosanoides by tissue resident macrophages and mast cells mediates the extravasation of leukocytes and plasma proteins at the site of inflammation (Medzhitov, 2008; Cailhier et al., 2006). Neutrophils are the first and most abundant cell type at the site of inflammation and these cells are activated by proinflammatory cytokines secreted by tissue resident cells or through direct contact with pathogens. This leads to degranulation and the release of anti-microbial compounds, such as reactive oxygen species, as well as enzymes including proteinase 3, cathepsin G and elastase (Hurst et al., 2001; Segal, 2005). Non-specific effector proteins are also released that cause damage to host tissues while destroying pathogens. However, removal of the initial instigator of inflammation leads to the initiation of the resolution and repair phase. This process is largely mediated by macrophages that remove apoptotic cells and debris through phagocytosis (Khanna et al., 2010) and results in macrophage polarisation towards an anti-inflammatory profile (Chan et al., 2011). These cells secrete several other anti-inflammatory molecules, including IL-10, resolvins, protectins and growth factors. In this way, lasting damage as a result of inflammation is avoided. Lipoxins are also important anti-inflammatory lipid mediators that promote monocyte recruitment while inhibiting neutrophil recruitment (Segal, 2005; Serhan et al., 2008).

1.6.3 Gap Junction Communication and Inflammation

Cell-cell interactions are of major importance for expanding the competency of cells in the immune system to control infections and maintain tolerance. Activation of adaptive
immune response involves the interaction between T cell antigen receptors (TCR) and major histocompatibility complex (MHC) molecule-peptide complexes. The nanometer scale gap between a T lymphocyte and antigen-presenting cell (APC) is referred to as the immunological synapse (Bromley et al., 2001). The specificity of these recognitions is critical, since reactions to microbial peptides are required for clearance of many infections and responses to self-derived peptides on APCs can give rise to autoimmunity. Despite the early observation of GJs in lymphocytes and the extensive characterisation of connexins in primary and secondary lymphoid organs, understanding of the potential role of GJs in the immune system is limited.

GJs composed of Cx43 between antigen-presenting Langerhans cells and T lymphocytes have been observed both in vitro and in vivo (Concha et al., 1988; Concha et al., 1993; Sáez et al., 1999; Brand et al., 1995). Furthermore, it was demonstrated that disruption of GJIC influenced fundamental aspects of lymphocyte function, including immunoglobulin secretion and cytokine production (Oviedo-Orta et al., 2001). Inhibition of GJIC by synthetic peptides homologous to the first and second external loop of Cx43 markedly reduced the secretion of immunoglobins M, G and A (IgM, IgG and IgA) in mixed cultures of activated purified human B and T lymphocytes. Additionally, complex temporal inhibitory effects on cytokine synthesis, in particular interleukin-10 (IL-10), were observed in human B and T lymphocytes. These results have led to the suggestion that connexins and GJIC may be an important component of the molecular mechanism underlying lymphocyte activation and function in the immune response (Oviedo-Orta and Evans, 2002).

1.6.3.1 Gap Junction Communication in Leukocyte Recruitment

Cx37, Cx40 and Cx43 are the three connexins that have been detected in the vascular endothelium, and the precise distribution of these connexins within the vessel wall is
known to be species and vessel specific (van Kempen and Jongsma, 1999). Studies on human umbilical vein endothelial cells (HUVECs) have shown the expression of these three vascular connexins and their location is confined at cell-cell contacts (van Rijen et al., 1998). Furthermore, tumour necrosis factor α (TNF-α) altered the connexin expression pattern and reduced GJIC between ECs (van Rijen et al., 1998). This reduction in GJIC within the endothelium might protect the endothelium by restricting the spread of injurious signals via EC GJs, thus limiting the area of inflammation. Additionally, as more connexins from ECs become available for docking, they might form heterocellular GJs with leukocytes to control leukocyte migration across the endothelium. There have been recent indications of the presence of GJs between ECs and leukocytes, and that GJIC may play a role in leukocyte extravasation.

Oviedo-Orta et al. (2002) first described cell communication via GJs during transmigration. Using dye transfer experiments, it was demonstrated that lymphocytes and ECs generate functional heterocellular GJ channels during extravasation in vitro. In addition, blocking GJIC with pharmacological agents or connexin mimetic peptides caused only a modest reduction in the transmigration of lymphocytes across an EC monolayer (Oviedo-Orta and Evans, 2002). Neutrophils and HUVECs also formed functional GJ channels in vitro, but this bidirectional coupling was reduced when HUVECs were stimulated with TNF-α but not with interferon γ (IFN-γ). This indicates that coupling between neutrophils and HUVECs is selectively modulated during an inflammatory reaction. Neutrophil transmigration was also enhanced when GJIC was inhibited, thereby suggesting a negative regulatory role for this coupling during the transmigration process, and it was also shown that strongly adherent neutrophils were more coupled than weakly adherent ones, although the adhesive properties between connexons played no role in this strengthened cell adhesion process (Zahler et al., 2003). This lead to the hypothesis that the tight adhesion mediated by
integrins and their ligands between leukocytes and ECs may be modulated by signalling through GJs.

Human monocytes form GJs with ECs in a BBB model during the process of transmigration (Eugenan et al., 2003). The blockade of GJIC using the connexin mimetic peptide GAP27, reduced the number of monocytes that transmigrated, suggesting that cell-cell signalling through GJ channels might even affect the efficiency of the transmigration process across a tight endothelium. Trans-endothelial migration (TEM) of different leukocytes appears to be differentially regulated by GJIC, such that inhibition of GJIC increases the TEM of neutrophils but decreases the TEM of monocytes and has modest effects on lymphocyte TEM.

1.7 Endothelium

1.7.1 Role of the Endothelium in Determining Physiological Vascular Tone

The largest blood vessels are arteries and veins, which have a thick, tough wall of connective tissue and many layers of SMCs. The wall is lined by an exceedingly thin single cell layer known as the endothelium, which is separated from the surrounding outer layers by a basal lamina.

Although only a simple monolayer, the healthy endothelium is optimally placed and is able to respond to physical and chemical signals through the production of a wide range of factors that regulate vascular tone, cellular adhesion, thromboresistance, SMC proliferation, and vessel wall inflammation. The importance of the endothelium was first recognised by its effect on vascular tone. This is achieved by the production and release of several vasoactive molecules that relax or constrict the vessel, as well as by responses to and modification of circulating vasoactive mediators, such as bradykinin and thrombin. This vasomotion plays a direct role in the balance of tissue oxygen supply and metabolic
demand through the regulation of vessel tone and diameter, and is also involved in the remodelling of vascular structure and long-term organ perfusion (Schechter and Gladwin, 2003).

The pioneering experiments of Furchgott and Zawadzki (1980) first demonstrated an endothelium-derived relaxing factor that was subsequently shown to be nitric oxide (NO) NO is generated from L-arginine by the action of endothelial NO synthase (eNOS) in the presence of cofactors such as tetrahydrobiopterin. The gas diffuses into the vascular SMCs and activates guanylate cyclase, which leads to c-GMP-mediated vasodilatation. Shear stress is a key activator of eNOS during normal physiology, and this adapts organ perfusion to changes in cardiac output (Corson et al., 1996; Forstermann and Munzel, 2006). In addition, the enzyme may be activated by signalling molecules, such as bradykinin, adenosine, vascular endothelial growth factor (in response to hypoxia), and serotonin (released during platelet aggregation) (Govers and Rabelink, 2001). The endothelium also mediates the hyperpolarisation of vascular SMCs via an NO-independent pathway, which increases potassium conductance and subsequent propagation of the depolarisation of vascular SMCs to maintain vasodilator tone (Busse et al., 2002).

The endothelium modulates vasomotion, not only by the release of vasodilator substances, but also by an increase in constrictor tone via the generation of endothelin and vasoconstrictor prostanoids, as well as via the conversion of angiotensin I to angiotensin II at the endothelial surface (Saye et al., 1984; Kinlay et al., 2001). These vasoconstrictor agents predominantly act locally, but may also exert some systemic effects and have a role in the regulation of the arterial structure and remodelling (Kinlay et al., 2001).

In normal conditions, NO plays a key role in maintaining the vascular wall in a quiescent state through the inhibition of inflammation, cellular proliferation, and thrombosis. This is
in part achieved by s-nitrosylation of cysteine residues in a wide range of proteins, which reduces their biological activity (Stamler et al., 2001). Target proteins include the transcription factor NFκB, cell cycle–controlling proteins, and proteins involved in the generation of tissue factor (Ghosh and Karin, 2002). Laminar shear stress is probably the major factor that maintains this quiescent, NO-dominated, endothelial phenotype (Gimbrone, 1999).

1.7.2 Endothelial Activation and Atherosclerosis
What is generally referred to as endothelial dysfunction can mainly be considered as endothelial activation, which may eventually contribute to arterial disease when certain conditions are fulfilled. Endothelial activation represents a switch from a quiescent phenotype towards one that involves the host defence response. Indeed, most cardiovascular risk factors activate the molecular machinery in the endothelium which results in the expression of chemokines, cytokines, and adhesion molecules designed to interact with leukocytes and platelets, and targets inflammation to specific tissues in order to clear microorganisms (Hansson, 2005).

Prolonged and/or repeated exposure to cardiovascular risk factors, such as hypercholesterolemia, hypertension, and diabetes (Celermajer et al., 1994; D’Aiuto et al., 2006), can ultimately lead to endothelial dysfunction. As a consequence, the endothelium not only becomes dysfunctional, but ECs can also lose integrity, progress to senescence, and detach into the circulation (Woywodt et al., 2002). Circulating markers of such EC damage include endothelial microparticles derived from activated or apoptotic cells, and whole ECs (Mallat et al., 2000). These markers have been found to be increased in both peripheral and coronary atherosclerosis disease, as well as other inflammatory conditions.
associated with increased vascular risk, such as rheumatoid arthritis and systemic lupus erythematosus (Rajagopalan et al., 2004).

Endothelial integrity depends not only on the extent of injury, but also on the endogenous capacity for repair. Two mechanisms by which the process of repair occurs have been recently identified. Adjacent mature ECs can replicate locally and replace lost and damaged cells. A recent modelling study suggested that although local ECs would be sufficient to maintain vascular integrity throughout life during healthy circumstances, in the presence of risk factors, the loss of endothelial integrity would rapidly develop if local replication were the only repair mechanism available (Buijs et al., 2004). More recently, it has become clear that circulating endothelial progenitor cells, which are cells recruited from the bone marrow circulating in peripheral blood, can differentiate into mature cells with endothelial characteristics, thus representing an alternative mechanism for the maintenance and repair of the endothelium (Asahara et al., 1997).

Recent evidence has indicated that risk factors not only interfere with the recruitment of circulating endothelial progenitor cells, but also with the differentiation and function of these cells. For example, important cellular properties, such as migration, adhesion, and the formation of tubules under culture conditions, can be impaired in the presence of risk factors and atherosclerotic disease (Vasa et al., 2001). The importance of the balance between exposure to risk factors and the capacity for repair in the determination of the clinical endothelial phenotype has been highlighted by the demonstration that subjects with increased numbers of circulating endothelial progenitor cells have preserved endothelial function, despite exposure to high levels of risk factors (Hill et al., 2003).

Endothelial cells adhere to one another through junctional structures formed by transmembrane adhesive proteins that are responsible for haemophilic cell-to-cell
adhesion. In turn, transmembrane proteins are linked to specific intracellular partners, which mediate anchorage to the actin cytoskeleton, and as a consequence, stabilise junctions (Dejana et al., 1999). Junctions in endothelial and epithelial cells share common features and in both cell types two major types of junctions have been described; AJs and TJs. However, epithelial cells also form desmosomes, which are absent in the endothelium (Bazzoni et al., 1999). In addition to cell-to-cell adhesion, GJs mediate cell-to-cell communication. Three of connexins proteins, Cx43, Cx40, and Cx37, are expressed in the endothelium. Connexins are organised into connexons, which act as channels for the intercellular passage of ions and small-molecular-weight molecules (Simon and Goodenough, 1998). Junctions present in the endothelium control different features of vascular homeostasis. For instance, permeability to plasma solutes is controlled to a considerable extent by junction permeation. In addition, leukocyte extravasation and infiltration into inflamed areas requires finely regulated opening and closing of cell-to-cell contacts (Johnson-Leger et al., 2000; Müller, 2003). Notably, junctional proteins can also transfer intracellular signals, which modulate EC growth and apoptosis (Lampugnani and Dejana, 1997).

The organisation of endothelial junctions varies along the vascular tree in function due to organ-specific requirements. For instance, in the brain where strict permeability control between blood and the nervous system is required, junctions are well developed and rich in TJs (Rubin and Staddon, 1999). In contrast, post-capillary venules which allow dynamic trafficking of circulating cells and plasma proteins, display poorly organised TJs. These morphological features may also account for the high sensitivity of post-capillary venules to permeability-increasing agents, such as histamine and bradykinin. The endothelium of large arteries, which tightly controls permeability, has a well-developed system of TJs.
Finally, lymphatic endothelium displays specific junctional structures, *complexus adhaerentes* (Schmelz and Franke, 1993; Schmelz et al., 1994).

### 1.8 Connexins and Atherosclerosis

#### 1.8.1 Pathogenesis of Atherosclerosis

A healthy artery consists of three layers; the tunica intima, tunica media, and tunica adventitia. The tunica intima is the layer closest to the arterial lumen and consists of connective tissue covered by a layer of ECs. Atherosclerotic lesions can be initiated by injury to ECs through mechanisms such as increased shear stress due to the disruption of laminar flow and hypertension, infection or increased oxidative stress. This leads to the activation of ECs and increased permeability, resulting in the increased accumulation of low density lipoprotein (LDL) particles within the intima. LDL accumulation in the intima leads to aggregation and retention through ionic interactions between proteoglycans produced by SMCs and apoB-100 in LDL (Daugherty et al., 2008; Haka et al., 2009; Hermansson et al.).

Oxidation of LDL in the vascular intima is a critical step in the initiation of atherosclerotic lesions and occurs through the presence of transition metal ions, hemin and other cell-derived free radicals. In addition, several enzymes, including lipooxygenase and myeloperoxidase, as well as reactive oxygen species produced by activated macrophages, catalyse LDL oxidation (Yoshida and Kisugi, 2010). LDL oxidation occurs in stages and minimally modified LDL results from partial oxidation of lipid moieties while apoB-100 remains intact. The term oxidised LDL (oxLDL) includes all LDL particles that have undergone oxidative modification, with much variation observed in terms of size, charge and lipid content (Daugherty et al., 2008; Bobryshev et al., 2007).

Activated ECs also show increased expression of vascular adhesion molecules, such as VCAM-1, ICAM-1 and P-selectin, as well as the secretion of chemokines, including
monocyte recruitment protein 1 and interleukin 8 (IL-8), which facilitate adhesion and migration to the sub-endothelial layer of the intima (Yuri, 2006; Bobryshev, 2005). Once inside the intima, the majority of monocytes differentiate into macrophages but some develop into DCs. In the lipoprotein rich environment, most macrophages take up large amounts of oxLDL, to form foam cells, mainly through scavenger receptors (Bobryshev et al., 2007; Libby et al., 2011). It was also found that macrophages take up aggregated LDL complexes through the formation of an acidic extracellular hydrolytic compartment, and minimally modified LDL through the LDL receptor (Haka et al., 2009). oxLDL is broken down in the lysosome to cholesteryl-fatty acid esters and stored in cytoplasmic droplets associated with the cell membrane. However, at advanced stages of foam cell formation, not all the oxLDL is broken down and some can be found in cytoplasmic microvesicles (Bobryshev et al., 2007; Yuri, 2006). oxLDL uptake activates macrophages, and foam cells are known to produce reactive oxygen species, thus promoting LDL oxidation and forming a self-propagating loop between lipid accumulation, oxidation and immune activation (Libby, 2002).

Foam cell deposition leads to fatty streak formation, the earliest form of an atherosclerotic lesion. Fatty streaks are asymptomatic and are present at all ages, and they can disappear with time or progress to structurally complex atheromas (Yoshida and Kisugi, 2010). Early in atherosclerosis, foam cell formation may have a protective effect through the removal of oxLDL, which is a potent proinflammatory stimulus. However, beyond fatty streak formation, foam cell formation contributes to lipid accumulation and plaque growth (Yuri, 2006; Bobryshev, 2005). SMCs normally reside in the arterial media, although increased numbers of SMCs are found in the intima in areas of intimal thickening, which are also predisposed to atherosclerotic lesion development. Initial SMCs play an important role in the initiation of atherosclerosis, and like macrophages, can also take up oxLDL to form
foam-like cells. In addition, these cells promote the accumulation of monocytic cells in the vascular intima through the expression of adhesion molecules such as VCAM-1 and ICAM-1 and by inhibiting apoptosis in these cells (Libby et al., 2011).

Lesion progression is further mediated by migration of SMCs from the media to the intima, and differentiation of SMCs into fibroblasts that secrete matrix proteins such as collagen proteoglycans that lead to the formation of a fibrous cap (Doran et al., 2008; Sherer and Shoenfeld, 2002). At this stage SMCs play a protective role by containing the lesion through the secretion of extracellular matrix proteins that form the fibrous cap (Doran et al., 2008). The lesion underneath the fibrous cap contains immune cells, including foam cells, macrophages, T cells, DCs, B cells, NK cells, mast cells and neutrophils (Libby et al., 2011). Excessive lipid uptake by macrophage foam cells leads to cell death due to extensive DNA damage caused by oxLDL, and the accumulation of extracellular lipid and cellular debris, which contributes to the formation of a necrotic core. Further lipid accumulation contributes to plaque growth and with time a plaque becomes increasingly acellular due to the necrosis of foam cells and SMCs within the core (Ira, 1997).

Most stable plaques with an intact fibrous cap remain asymptomatic, and more than 75% of clinical events associated with atherosclerosis result from plaque destabilisation and rupture due to degradation of the fibrous cap. The two main contributory factors to a thin fibrous cap are reduced collagen and proteoglycan synthesis due to decreasing SMC numbers and increased degradation by matrix metalloproteases secreted by macrophages and other cell types (Galis et al., 1994; Andrew, 2007). When the fibrous cap ruptures, it exposes the necrotic core to the blood and this may lead to thrombosis through the release of tissue factor-expressing microparticles derived from apoptotic cells in the core (Zoll et al., 2008). The formation of an occluding thrombus results in ischaemic damage to
downstream organs, causing a myocardial infarction or stroke. However, the formation of thrombi depends on the size and location of the plaque rupture and also on the systemic balance between coagulation and fibrinolysis. Therefore, plaque ruptures are often clinically silent and heal through the accumulation of SMCs that secrete collagen and glycosaminoglycans (Bentzon et al., 2007). This process of plaque rupture and healing may be repeated several times in the same plaque, and ultimately contributes to arterial stenosis (Hunninghake et al., 2001). Figure 1.10 is a graphical representation of the progression of atherosclerosis from fatty streak formation to plaque rupture.
Figure 1.10: Evolution of atherosclerosis

(A) Endothelial-cell dysfunction and activation under pro-inflammatory conditions of hyperlipidaemia leads to early platelet and leukocyte adhesion, and increased permeability of the endothelium. (B) Monocytes that are recruited to the intima and sub-intima accumulate lipids and transform into macrophages or foam cells, which make up fatty streaks. (C) Apoptosis of macrophages and other plaque cells create a necrotic core, and a fibrous cap that consists of matrix and a smooth-muscle-cell layer forms. (D) Thinning and erosion of the fibrous cap in unstable plaques, for example, owing to matrix degradation by proteases, ultimately results in plaque rupture, with the release of debris, activation of the coagulation system and plaque thrombosis of the artery (Weber et al., 2008).

It is well recognised that inflammation is central for all stages of atherosclerosis. Paracrine intercellular communication, involving cytokines, chemokines and growth factors, is known to play an important role in the development of atherosclerotic lesions (Natalia et al., 2008). However, another form of intercellular communication involving connexins has also been implicated in the development of the disease.

1.8.1.1 Connexin Expression during Atherogenesis

Connexin expression is modulated during atherosclerosis, and the expression patterns of Cx37, Cx40 and Cx43 change during plaque formation in murine and human atherosclerotic plaques (Kwak et al., 2002) (Figure 1.11).
1.8.1.1 Connexin37

Cx37 is expressed in healthy ECs but disappears from these cells in advanced atherosclerotic plaques (Kwak et al., 2002), and is also found in macrophages during early and late atheroma (Yeh et al., 2003). Taking into account that ECs and monocytes/macrophages have central roles in atherogenesis, Cx37 is expected to play a role during atherosclerotic lesion development (Wong et al., 2004; Kwak et al., 2002). Cx37 appeared to have a protective effect against atherosclerosis in ApoE⁻/⁻ mice subjected to a high cholesterol diet for 10 weeks. It was shown that a Cx37 deletion accelerated atherosclerotic lesion development in the thoracic abdominal aorta, aortic sinus, and increased the number of leukocytes in atherosclerotic plaques in comparison with Cx37⁺/⁺ApoE⁻/⁻ mice (Wong et al., 2006). Thus, the recruitment of leukocytes appeared to be dependent upon the presence of Cx37 in monocytes/macrophages but not between ECs, or on intercellular communication within the endothelium.
Inflammation is mediated in part by extracellular purines (ATP, ADP and adenosine) and ATP is known to pass through various types of GJs and hemichannels (Evans and Leybaert, 2007). The absence of Cx37 or the inhibition of Cx37 by blocking peptides reduced the release of ATP by monocytes/macrophages and increased their adhesion, indicating that Cx37 protects against atherosclerosis by regulating ATP-dependent monocyte adhesion (Wong et al., 2006).

Cx37 is also expressed in medial SMCs beneath advanced atherosclerotic lesions in mice (Kwak et al., 2002). Similar expression was also observed in advanced atherosclerotic plaques in a human carotid artery; however, the role of Cx37 in SMCs is yet to be fully elucidated.

1.8.1.1.2 Connexin40

Cx40 is present in the ECs of healthy vessels but is absent in the endothelium covering advanced atherosclerotic plaques (Kwak et al., 2002). It has been demonstrated that abrupt reoxygenation following hypoxia reduces GJ coupling between microvascular ECs of wild type but not of Cx40 deficient mice. A reduction in GJIC involves a PKA-dependent pathway and reactive oxygen species (Bolon et al., 2005).

Cx40 deficient mice are hypertensive, a major independent risk factor for atherosclerosis. To overcome this limitation, ApoE−/− mice with a specific Cx40 deletion in ECs were designed (de Wit et al., 2000), and these mice were not hypertensive and had a normal heart rate. Studies using these mice indicated that the EC-specific deletion of Cx40 induced increased atherosclerotic plaques compared to control mice (Chadjichristos et al., 2010), suggesting an atheroprotective role for Cx40; however, the mechanisms implicated remain to be investigated.
1.8.1.1.3 Connexin43

Cx43 is mostly expressed in SMCs of healthy vessels; however, coronary arteries of hearts removed from patients undergoing cardiac transplantation show markedly increased Cx43 expression in GJs between intimal SMCs compared with non-diseased vessels (Blackburn et al., 1995). In advanced atherosclerotic plaques the intimal expression for Cx43 declines. Cx43 expression was found to increase in intimal SMCs in early atherosclerotic lesions in LDLR\(^{-/-}\) mice fed a cholesterol rich diet. Cx43 expression was also shown in macrophage foam cells in the mouse aorta and human carotid artery, in ECs covering the shoulder region of atherosclerotic lesions, and in ECs at the branch points of large arteries (Gabriels and Paul, 1998).

Atherosclerotic plaques are generally formed at branch points or at curved areas of large arteries, regions associated with turbulent blood flow (Davies, 2009). Oscillatory shear stress induces a large and rapid increase of endothelial Cx43 expression (Kwak et al., 2005; Cowan et al., 1998), but the effects of unidirectional shear stress on endothelial Cx43 expression are less clear. This shear stress is associated with an increase or with no change in Cx43 expression dependent on the experimental conditions used. Increased hydrostatic pressure does not modify Cx43 levels in ECs (Kwak et al., 2005).

Cx43 knockout mice die at birth because of severe cardiac malformations (Reaume et al., 1995). As a result, studies implicating Cx43 in the development of atherosclerotic plaques have been performed by interbreeding atherosclerotic susceptible LDLR\(^{-/-}\) mice with heterozygous Cx43\(^{+/}\) mice. The expression of Cx43 was found to be reduced by half in Cx43\(^{+/}\) mice (Guerrero et al., 1997). Ten week old Cx43\(^{+/}\) LDLR\(^{-/-}\) that were fed a cholesterol rich diet for 14 weeks showed reduced atherosclerotic plaque development in the thoracic-abdominal aorta and in the aortic sinus by 50% in comparison to Cx43\(^{+/+}\) LDLR\(^{-/-}\) mice (Kwak
et al., 2003). Furthermore, it was also shown that atherosclerotic lesions in Cx43+/− LDLR−/− mice had smaller lipid cores and fewer macrophages, whereas leukocyte counts in peripheral blood were found to be similar between both groups of mice. Additionally, the fibrous cap of atherosclerotic plaques in Cx43+/− LDLR−/− mice was shown to contain more SMCs and interstitial collagen. However, synthetic SMCs have been described to express higher levels of Cx43 than the contractile phenotype (Chadjichristos et al., 2008; Rennick et al., 1993). The vulnerability of atherosclerotic lesions to rupture is dependent on the content of SMCs and macrophages, the amount of collagen within the lesion, and the size of the lipid core. As plaque rupture might lead to acute myocardial infarction, it has been hypothesised that targeting Cx43 may be promising for stabilisation of a plaque. However, the mechanism by which Cx43 influences atherosclerotic lesion formation and plaque stability are yet to be clearly identified (Wong et al., 2003).
1.9 Hypothesis and Aims

GJs have emerged as an important component in the pathology of atherosclerosis and inflammation. Among the 21 connexin isoforms present in the human genome, three are expressed by ECs, Cx37, Cx40 and Cx43, as well as by monocytes/macrophages/foam cells; however, Cx43 is the most predominantly expressed in both cell types. Therefore, it is hypothesised that (i) Cx43 expression in both monocytes and ECs is required for the formation of functional GJs which regulate the inflammatory response, and hence atherosclerosis, through the regulation of monocytes TEM, and (ii) migration is regulated by the cytokine TNF-α.

1.9.1 Aims and Objectives

The experimental objectives are to determine which connexins are specifically involved in the process of adhesion and migration of monocytes through the endothelium using an in vitro approach and using connexin-specific RNA interference (RNAi). In order to be as close as possible to the human situation adult human ECs from blood vessels known to be prone to atherosclerosis will be used together with human umbilical vein endothelial cells (HUVECs), which are widely used in most similar studies measuring TEM. Adult human monocytes from peripheral blood (CD14+) will be utilised, as these are the main infiltrating cells in atherosclerotic lesions and are present within lesions at all stages of the disease.

**Aim 1: Characterisation and functional assessment of Cx43 expression in HUVECs as an EC model and human peripheral monocytes (CD14+), experimental objectives are:**

i. Characterisation of HUVECs and monocytes (CD14+) obtained from peripheral blood using immunoconfocal microscopy and Western blotting techniques.
ii. Characterisation of functional Cx43 GJ formation through the use of the dye transfer technique in presence and absence of growth factors, GJ blockers and Cx43\(^{-/-}\) monocytes, via a human monocytic leukaemia cell line THP-1.

iii. Assessment of the role of Cx43 in the formation of functional GJs between monocytes and HUVECs using the following techniques:
- Assessment of gap junctional communication between monocytes and HUVECs using the dye transfer assay.
- TEM assay.
- Trans-endothelial electrical resistance (TEER) measurements.

Aim 2: Application of RNAi technology for the suppression of Cx43 expression, experimental objectives are:

i. Optimisation of electroporation conditions for sufficient and efficient delivery of siRNA into HUVECs.

ii. Determination of the most effective siRNA concentration for Cx43 down-regulation in HUVECs.

iii. Assessment of the role of Cx43 in the formation of functional GJs between monocytes and HUVECs using the following techniques:
- Assessment of gap junctional communication between monocytes and HUVECs using the dye transfer assay.
- TEM assay.
- TEER measurements.

Aim 3: To examine the effects of TNF-\(\alpha\) on monocytes/endothelial transmigration in the presence and absence of HUVECs Cx43 si-RNA, experimental objectives are:

i. Characterisation of TNF-\(\alpha\) dose and time response in HUVECs.
ii. Assessment of the role of Cx43 in the formation of functional GJs between monocytes and HUVECs +/- TNF-α using the following techniques:

- Assessment of gap junction communication between monocytes and HUVECs using the dye transfer assay.
- TEM assay.
- TEER measurements.

iii. Analysis of possible pathways for the action of TNF-α on HUVECs/Monocytes via TEM.
Chapter 2 - Material and Methods
2.1 Cell Culture

2.1.1 Culturing and Maintenance of HUVECs

All cell culture procedures were carried out in a laminar flow safety cabinet to ensure sterility and incubated at 37°C in a 5% CO₂ humidified incubator (Thermo Scientific, UK). HUVECs were commercially obtained from Lonza (HUVECs; Cat no. C2519A, Lonza, UK). Unless otherwise stated, these cells were maintained in complete endothelial growth medium (EGM-2) which is an endothelial basal medium (EBM-2; Cat no. CC-3156, Lonza, UK) supplemented with a growth factor kit (EGM-2 SingleQuot Kit Suppl. & Growth Factors; Cat no. CC-4176, Lonza, UK). For full details on the growth factor kit see Table 2.1.

HUVECs where received as a frozen cryo-tube (usually passage 1), and were cultured immediately in a 100 mm dish plate (Cell culture dish 100 mm cellBIND; Cat no. 3296, Fisher Scientific, UK) with EGM-2 media and incubated overnight at 37°C in a 5% CO₂ humidified incubator. The medium was changed each day and cells were examined microscopically daily to check for confluency.

Cell passaging (passage 1-14) was carried out when the cells reached approximately 85-95% confluence to prevent contact inhibition and cell apoptosis. Cells were washed with sterile phosphate buffered saline (PBS; Cat no. D8537, Sigma-Aldrich, UK), detached from the plate by the addition of trypsin/EDTA solution (0.5 g porcine trypsin and 0.2 g EDTA per litre of Hanks’ Balanced Salt Solution with phenol red; Cat no. T4174, Sigma-Aldrich, UK) and incubated for 5 minutes at 37°C in a 5% CO₂ humidified incubator. Trypsin activity was stopped with trypsin inhibitor (Cat no. T6414, Sigma-Aldrich, UK). The cell number was counted using a haemocytometer (Bright-Line™ Hemacytometer; Cat no. Z359629, Sigma-Aldrich, UK) and cells were diluted with trypan blue solution (1:1 ratio) (Cat no. T8154, Sigma-Aldrich, UK) to check viability. Cells were then re-suspended in EGM-2 growth media.
and sub-cultured equally into culture dishes, usually in a 1:3 - 1:5 ratio of the original culture, or depending on the type of experiment, as some required a large number of cells. HUVECs were cultured for all the experiments, including immuno-confocal microscope and Western blotting, and siRNA experiments, while cells were cryopreserved and cultured in specific wells for the TEM assay and TEER measurements.

2.1.2 HeLa Transfected Cells

HeLa cells are an immortal cell line used in scientific research, and are the oldest and most commonly used human cell line. The line was derived from cervical cancer cells taken in 1951 from Henrietta Lacks, a patient who eventually died of her cancer. This cell line has been found to be remarkably durable and prolific, which has led to its contamination of many other cell lines used in research. HeLa wild-type cells and HeLa cells transfected with Cx37, Cx40 or Cx43 were used in this study as controls (kindly donated by Dr Emmanuel Dupont). HeLa cells were maintained in complete Dulbecco’s modified Eagle’s medium (DMEM; Cat no. D6429, Sigma-Aldrich, UK) supplemented with 10% (v/v) foetal bovine serum (FBS; Cat no. 10109-163, GIBCO, UK), 4 mM L-glutamine (Cat no. G7513, Sigma-Aldrich, UK) and 100 U/ml penicillin/streptomycin (Cat no. P4333, Sigma-Aldrich, UK). Cells were passaged at a split ratio ranging from 1:8 to 1:20, as they were able to recover with a highly proliferative phenotype even when very sparse. Confluent HeLa monolayers were split using the same procedure, as described for HUVECs.

2.1.3 Human Monocyte-like Cell Line Tamm-Horsfall Protein-1 (THP-1)

The human monocyte-like cell line Tamm-Horsfall Protein-1 (THP-1) was purchased from the European Collection of Cell Culture (ECACC, UK), and used as a negative control in some experiments as they lack the expression of Cx37, Cx40, and Cx43.
THP-1 cells were maintained in complete Roswell Park Memorial Institute (RPMI; Cat no. R8758, Sigma-Aldrich, UK) supplemented with 10% (v/v) FBS (Cat no. 10109-163, GIBCO, UK), 4 mM L-glutamine (Cat no. G7513, Sigma-Aldrich, UK) and 100 U/ml penicillin/streptomycin (Cat no. P4333, Sigma-Aldrich, UK). Cells were passaged at a split ratio ranging from 1:8 to 1:20, as they were able to recover as a highly proliferative phenotype. Confluent THP-1 cells were split differently from HUVECs and HeLa cells as they do not adhere and consequently are present as a suspension. Cells were collected in Falcon 50 ml conical centrifuge tubes (Cat no. 14-432-22, Fisher Scientific, UK) and subjected to centrifugation at 1000 ×g for 5 minutes (Cat no. LMC-4200R, Laboratory Refrigerated Centrifuge, BioSan, UK). The supernatant was discarded and cells were re-suspended in fresh RPMI, and the cell number counted using a haemocytometer (Bright-Line™ Hemacytometer; Cat no. Z359629, Sigma-Aldrich, UK). Cells were diluted with trypan blue solution (1:1 ratio) (Cat no. T8154, Sigma-Aldrich, UK) to assess viability. Cells were then re-suspended in RPMI and sub-cultured equally into culture dishes, usually at a 1:3 - 1:5 ratio of the original culture.
Table 2.1: Endothelial growth medium components.

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration (% w/v)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Foetal bovine serum (FBS)</td>
<td>2%</td>
</tr>
<tr>
<td>Hydrocortisone</td>
<td>0.04%</td>
</tr>
<tr>
<td>Fibroblast growth factor-basic (hFGF-B)</td>
<td>0.4%</td>
</tr>
<tr>
<td>Vascular endothelial growth factor (VEGF)</td>
<td>0.1%</td>
</tr>
<tr>
<td>Insulin-like growth factor (R3- IGF-1)</td>
<td>0.1%</td>
</tr>
<tr>
<td>Epidermal growth factor (hEGF)</td>
<td>10mg/ml</td>
</tr>
<tr>
<td>Gentamycin/amphotericin-B (GA-1000)</td>
<td>0.1%</td>
</tr>
<tr>
<td>Ascorbic acid</td>
<td>0.1%</td>
</tr>
<tr>
<td>Heparin</td>
<td>0.1%</td>
</tr>
</tbody>
</table>

2.1.4 Cryopreservation of HUVECs, HeLa and THP-1 Cells

Cells were grown to approximately 90% confluency, and the medium was changed 24 hours before freezing. Cells were detached with trypsin as described above (for HUVECs and HeLa cells), before re-suspending in medium appropriate for each cell line (EGM-2 for HUVECs, DMEM for HeLa, and RPMI for THP-1) and then harvesting by centrifugation (1000 ×g, 5 minutes). Pellets were re-suspended in the required amount of complete medium as follow: HUVECs were re-suspended in EGM-2 plus 5% FBS with 10% dimethyl sulphoxide (DMSO), HeLa cells were re-suspended in DMEM with 10% DMSO, and THP-1 cell line were re-suspended in RPMI with 10% DMSO. Cells aliquots of 1 ml were stored at -80°C overnight then transferred to storage in liquid nitrogen.

2.2 Immunofluorescence and Confocal Microscopy

2.2.1 Immunofluorescence Labelling

In order to determine the cellular localisation of Cx37, Cx40, and Cx43 in HUVECs, immunolabelling and immunohistochemical analysis of connexins was performed. Primary antibodies for specific unique sequences within the target protein were utilised (Table 2.2). Once the primary antibody is bound, a secondary, fluorescently conjugated, antibody designed to recognise the animal specific IgG of the primary antibody is allowed to bind.
This complex therefore detects a specific protein and where it is localised, whilst also possessing a fluorescent signal enabling visualisation. Labelled cells can be viewed via a confocal microscope, where the fluorescent tag is excited at its specific excitation wavelength and the emitted fluorescence wavelength captured and displayed on screen as a specifically coloured label.

Cells were grown to the desired density (85-90%) on sterile 13 mm borosilicate cover slips, 0.08 to 0.12 mm thick (Cat no. 12392128, Fisher Scientific, UK) in 24 well plates, with one cover slip in each well filled with 500 μl of EGM-2 for 48 hours at 37°C in a 5% CO₂ humidified incubator (Thermo Scientific, UK). Cells were rinsed twice with PBS for 5 minutes in PBS containing 1 mM CaCl₂ at room temperature (RT) to remove albumin and cell debris. The addition of Ca²⁺ ions maintains cellular adhesion through Ca²⁺-dependent adhesion molecules (Lodish et al., 2000; Sotomayor and Schulten, 2008).

Cells were then fixed and permeabilised with ice-cold methanol (-20°C), which permeabilises the cell membrane enabling dehydration of the cells by removing lipids and precipitating proteins, thereby allowing antibodies to bind. In contrast, paraformaldehyde only fixes cells by directly cross-linking proteins through the amino groups, thereby maintaining the cellular structure, however it does not permeabilise the membrane and consequently a permeabilisation step is needed (Yamanushi et al., 2015). Cells were incubated with ice-cold methanol for 10-15 minutes on ice before being washed with PBS three times for 5 minutes at RT. All fixed and permeabilised cells were incubated for 1 hour at RT with freshly prepared blocking buffer (1% (w/v) bovine serum albumin (BSA) in PBS with 0.1% (v/v) sodium azide). This was followed by incubating the cells with the appropriate primary antibody (Table 2.2), which was diluted in blocking buffer and left on
overnight at 4°C. Cells were rinsed three times for 5 minutes with PBS at RT and then incubated with the anti-species specific IgG secondary antibody (Table 2.4), diluted in blocking buffer for 1 hour at 37°C in a dry incubator (Thermo scientific, UK). This was followed by three washes with PBS for 5 minutes at RT.

Finally, cells were double stained with the fluorescent probe DRAQ5™ (20 μM; Cat no. 65-0880-96, EBioscience, UK), which is a far-red DNA stain for fluorescent cellular imaging applications with live or fixed cells. Because of its far-red excitation and emission, the DRAQ5 stain can be multiplexed with many other fluorophores and is commonly chosen to counterstain nuclei. Cells were washed twice with PBS for 5 minutes with a final wash with distilled water after counter staining.

At the end of the labelling procedure each cover slip was carefully lifted from the wells using forceps; excess water was removed, and they were mounted using a drop of FluorSave mounting medium (Cat no. 345789, Calbiochem, UK) onto labelled glass slides. Cover slips were sealed onto the slide using clear nail varnish. Mounted slides were kept in dark box (microscope slide boxes, Cat no. SB-100-BLACK, LabScientific, UK) at 4°C to protect fluorophores from bleaching until they were ready to be visualised by confocal microscopy.

2.2.2 Confocal Microscopy

Slides were viewed and analysed using a laser-scanning inverted confocal microscope (Zeiss LSM 510, Carl Zeiss, USA) and image acquisition software (Axiovision and LSM 510 Meta software, Carl Zeiss, USA). The microscope was fitted with helium, neon and argon lasers and suitable filter blocks for the detection of green fluorescence from fluorescein isothiocyanate (FITC)/Alexa Fluor 488 /Cy2, red fluorescence from tetramethyl rhodamine isothiocyanate (TRITC)/Alexa Fluor 568 /Cy3 and blue fluorescence from TO-PRO-3 /Cy5, measured at excitation/emission wavelengths of 492/520, 596/615 and 644/657 nm,
respectively. Gain and contrast were determined in each experiment through the use of appropriate negative controls; i.e. no primary antibody to ensure that only the antibody signal was being detected and not background non-specific signal. Slides were viewed and images taken under either ×40 or ×63 objectives, indicated in each image within the results sections. Fluorescence assigned by wavelength/colour to a specific antibody was not detected with either primary or secondary antibody alone, confirming the specificity of the labelling.
Table 2.2: Primary antibodies used in immunofluorescence (IF) and Western blotting (WB).

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Clone/ Cat. No.</th>
<th>Host</th>
<th>Isotype</th>
<th>Stock Concentration.</th>
<th>Working Concentration.</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cx37</td>
<td>C-20/ sc-27712</td>
<td>Goat</td>
<td>Affinity Isolated</td>
<td>100 μg/ml</td>
<td>1:100 IF 1:100 WB</td>
<td>Santa Cruz, UK</td>
</tr>
<tr>
<td>Cx40</td>
<td>C-20/ sc-20466</td>
<td>Goat</td>
<td>Polyclonal IgG</td>
<td>200 μg/ml</td>
<td>1:100 IF 1:100 WB</td>
<td>Santa Cruz, UK</td>
</tr>
<tr>
<td>Cx43</td>
<td>C6219</td>
<td>Rabbit</td>
<td>Affinity Isolated</td>
<td>500 μg/ml</td>
<td>1:500 IF 1:500 WB</td>
<td>Sigma Aldrich, UK</td>
</tr>
<tr>
<td>vWF (Von Willebrand Factor)</td>
<td>A0082</td>
<td>Rabbit</td>
<td>Purified Immunoglobulin</td>
<td>5 mg/ml</td>
<td>1:250 IF 1:400 WB</td>
<td>Dako, UK</td>
</tr>
<tr>
<td>PECAM-1 (Platelet endothelial cell adhesion molecule)</td>
<td>M-20/ sc-1506</td>
<td>Goat</td>
<td>Polyclonal IgG</td>
<td>200 μg/ml</td>
<td>1:200 IF</td>
<td>Santa Cruz, UK</td>
</tr>
<tr>
<td>SMC-α (smooth muscle cells- actin)</td>
<td>1A4/ A2547</td>
<td>Mouse</td>
<td>Monoclonal purified IgG2a</td>
<td>2 mg/ml</td>
<td>1:200 IF</td>
<td>Sigma Aldrich, UK</td>
</tr>
<tr>
<td>Goat neg. control</td>
<td>011001G</td>
<td>Goat</td>
<td>IgG</td>
<td>5 mg/ml</td>
<td>1:200 IF</td>
<td>AbD Serotec, UK</td>
</tr>
<tr>
<td>Rabbit neg. control</td>
<td>Ab 37415-5</td>
<td>Rabbit</td>
<td>IgG</td>
<td>5 mg/ml</td>
<td>1:200 IF</td>
<td>Abcam, UK</td>
</tr>
<tr>
<td>Mouse neg. control</td>
<td>MCA929</td>
<td>Mouse</td>
<td>IgG2a</td>
<td>5 mg/ml</td>
<td>1:200 IF</td>
<td>AbD Serotec, UK</td>
</tr>
<tr>
<td>Anti-ZO1 tight junction protein</td>
<td>ab59720</td>
<td>Rabbit</td>
<td>Polyclonal IgG</td>
<td>100 μg/ml</td>
<td>1:100 IF</td>
<td>Abcam, UK</td>
</tr>
</tbody>
</table>
Table 2.3: Primary antibodies and inhibitors/blockers used in Western blotting (WB).

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Clone/ Cat. No.</th>
<th>Host</th>
<th>Isotype</th>
<th>Stock Concentration.</th>
<th>Working Concentration.</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-Erk1/2</td>
<td>05-1152</td>
<td>mouse</td>
<td>IgG1</td>
<td>100 µg/ml</td>
<td>1:5,000 WB</td>
<td>Emdmillipore, UK</td>
</tr>
<tr>
<td>Anti-phospho-Erk1/2</td>
<td>05-797R</td>
<td>Rabbit</td>
<td>IgG</td>
<td>100 µg/ml</td>
<td>1:5,000 WB</td>
<td>Emdmillipore, UK</td>
</tr>
<tr>
<td>Anti-JNK/SAPK1</td>
<td>06-748</td>
<td>Rabbit</td>
<td>IgG</td>
<td>100 µg/ml</td>
<td>1:5,000 WB</td>
<td>Emdmillipore, UK</td>
</tr>
<tr>
<td>Anti-p38/SAPK2A</td>
<td>ABS29</td>
<td>Rabbit</td>
<td>IgG</td>
<td>100 µg/ml</td>
<td>1:5,000 WB</td>
<td>Emdmillipore, UK</td>
</tr>
<tr>
<td>Anti-PKC Antibody (α, β, γ)</td>
<td>05-983</td>
<td>Mouse</td>
<td>IgG1</td>
<td>100 µg/ml</td>
<td>1:5,000 WB</td>
<td>Emdmillipore, UK</td>
</tr>
<tr>
<td>IκBα</td>
<td>sc-371</td>
<td>Mouse</td>
<td>Monoclonal purified IgG2a</td>
<td>200 µg/ml</td>
<td>1:100 WB</td>
<td>Santa Cruz, UK</td>
</tr>
<tr>
<td>IKKα/β</td>
<td>sc-7184</td>
<td>Rabbit</td>
<td>Monoclonal purified IgG2a</td>
<td>200 µg/ml</td>
<td>1:100 WB</td>
<td>Santa Cruz, UK</td>
</tr>
<tr>
<td>NFκBp50</td>
<td>sc-7178</td>
<td>Rabbit</td>
<td>Monoclonal purified IgG2a</td>
<td>200 µg/ml</td>
<td>1:100 WB</td>
<td>Santa Cruz, UK</td>
</tr>
<tr>
<td>Beta actin</td>
<td>ab6276</td>
<td>Rabbit</td>
<td>Polyclonal IgG</td>
<td>100 µg/ml</td>
<td>1:100 EB</td>
<td>Abcam, UK</td>
</tr>
<tr>
<td>GAPDH</td>
<td>ab9485</td>
<td>Rabbit</td>
<td>Polyclonal IgG</td>
<td>100 µg/ml</td>
<td>1:100 WB</td>
<td>Abcam, UK</td>
</tr>
<tr>
<td>SB 203580,P38 inhibitor</td>
<td>S8307</td>
<td>-</td>
<td>-</td>
<td>50 mg/ml</td>
<td>1:10,000 WB</td>
<td>Sigma Aldrich, UK</td>
</tr>
<tr>
<td>Chelerythrine chloride, PKC inhibitor</td>
<td>C2932</td>
<td>-</td>
<td>-</td>
<td>50 mg/ml</td>
<td>1:10,000 WB</td>
<td>Sigms Aldrich, UK</td>
</tr>
</tbody>
</table>
Table 2.4: Secondary antibodies used in immunofluorescence (IF) and Western blotting (WB).

<table>
<thead>
<tr>
<th>Secondary Antibody</th>
<th>Clone/ Cat no.</th>
<th>Host</th>
<th>Isotype</th>
<th>Stock con.</th>
<th>Working dilution</th>
<th>Label</th>
<th>Use</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-mouse</td>
<td>A11004</td>
<td>Goat</td>
<td>IgG (H+L)</td>
<td>2 mg/ml</td>
<td>1:200</td>
<td>Alexa Fluor 568</td>
<td>IF</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>Anti-rabbit</td>
<td>A11008</td>
<td>Goat</td>
<td>IgG (H+L)</td>
<td>2 mg/ml</td>
<td>1:100</td>
<td>Alexa Fluor 488</td>
<td>IF</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>Anti-goat</td>
<td>A11055</td>
<td>Donkey</td>
<td>IgG (H+L)</td>
<td>2 mg/ml</td>
<td>1:100</td>
<td>Alexa Fluor 488</td>
<td>IF</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>Anti-goat</td>
<td>C5284</td>
<td>Rabbit</td>
<td>IgG</td>
<td>2 mg/ml</td>
<td>1:10,000</td>
<td>HRP</td>
<td>WB</td>
<td>Bio-Rad, UK</td>
</tr>
<tr>
<td>Anti-rabbit</td>
<td>170-6515</td>
<td>Goat</td>
<td>IgG</td>
<td>2 mg/ml</td>
<td>1:5000</td>
<td>HRP</td>
<td>WB</td>
<td>Bio-Rad, UK</td>
</tr>
</tbody>
</table>
2.3 Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis and Western Blotting

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) is used to separate molecules based on size. The gel is a cross-linked polymer matrix used to support and separate the molecules, and can be of constant density or a gradient (Anderson, 1998).

Electrophoresis involves applying an electric current to the gel and allowing the proteins to migrate through the matrix. In order for the proteins to migrate through the gel, they are negatively charged by exposure to the detergent SDS. The amount of bound SDS is relative to the size of the protein, and proteins have a similar charge to mass ratio. Bands in different lanes separate based on the individual component sizes, and a molecular weight (MW) marker that produces bands of known sizes is used to help identify proteins of interest (Anderson, 1998).

2.3.1 Preparation of Cell Lysates

Passages 4 and 11 from the different cell lines described in Section 2.1 were washed twice with PBS at RT. Cells were lysed in a solubilisation buffer with 20% sodium dodecyl sulphate (SB20; 0.1 M Tris at pH 6.8, 20% SDS, 10 mM EDTA) with about 10 μl for each cm² of cells. The viscous cell lysates were collected and transferred to a 1.5 ml microfuge tube. Samples were sonicated at high power for 10-15 seconds to reduce viscosity and to shear the DNA of the lysed cells, before storing at -20°C.

2.3.2 Determination of Protein Concentration and Preparation of Samples for SDS-PAGE

The protein concentration of each cell lysate was measured using the Micro BCA protein assay kit (Cat no. 23235, Thermo-Scientific, UK) according to the manufacturer’s instructions. The Micro BCA™ protein assay kit is a detergent-compatible bicinchoninic acid formulation for the
colorimetric detection and quantitation of total protein. Samples were diluted 1:50 in distilled water to obtain final compatible concentrations with the manufacturer’s recommendation from the Micro BCA protein assay kit. BSA (2 mg/ml) standards were diluted to obtain a range of concentrations from 0.025 to 1 mg/ml and the protein-free sample was used as the blank. A 50 µl volume of the diluted protein sample, together with the standard protein dilutions were mixed with equal amounts of the BCA working reagent, freshly prepared according to manufacturer’s instructions. The colorimetric reaction was placed on a 60°C heating block to develop for 60 minutes, and the absorbance of the standard/samples was read at 562 nm using a NanoDrop-300 spectrophotometer. The concentration of the protein sample was calculated from a standard curve produced from the measurement of the standard BSA serial dilutions (Figure 2.1).

Figure 2.1: Example of the standard curve obtained using the BCA protein assay.
The concentration of protein samples were determined by the division of the absorbance value obtained for the sample by the gradient of the straight line. This concentration value in µg/ml was then multiplied by the dilution factor to obtain the true protein concentration of the sample.
2.3.3 SDS-PAGE

Protein samples were separated according to their molecular weight by SDS-PAGE. Samples were mixed with the loading buffer at a 1:1 ratio. Loading buffer was composed of sample buffer (0.5 M Tris HCl pH6.8, 0.01 g bromophenol blue and glycerol), SB20 supplemented with 5% 2-mercaptoethanol and 0.05% bromophenol blue. All proteins bind to the negative charge of the detergent and form a rod-like micelle that allows them to be separated on a polyacrylamide gel according to size. The presence of 2-mercaptoethanol is required to break the disulphide bridges that form between the cysteine residues of peptides. Bromophenol blue is a dye that migrates through the gel at approximately the same speed as glycine, the smallest known amino acid, and thus allows the leading edge of migration to be visualised to prevent runoff of the proteins. For samples run together on the same gel it was ensured that each had a similar detergent concentration. Different detergent concentrations substantially affect migration of the samples and the width of the final bands. Subsequent quantification relied on all bands being of an equal width.

SDS polyacrylamide gels were prepared containing 12.5%, v/v (40% bis/acrylamide solution), SDS-PAGE separating gel buffer (25.3%, v/v), ammonium persulphate (1.49%, v/v), tetramethylethelinediamine (TEMED; 0.06%, v/v), and made up with distilled water to the appropriate volume. Reagents were mixed by gentle stirring to avoid the formation of bubbles and directly loaded into a gel caster (BioRad, UK). The resolving gel was overlaid with butanol to ensure a flat edge which was washed away when polymerisation was complete. The butanol on the surface of the gel was removed and the top of the gel was washed with distilled water. The stacking gel containing acrylamide: bis/acrylamide (4.5%, v/v), SDS-PAGE stacking gel buffer (25%, v/v), ammonium persulphate (1.57%, v/v), TEMED (0.28%, v/v) and distilled water up to the appropriate volume. The stacking gel was overlaid on the resolving gel in the gel caster and a sample-well comb was inserted. The stacking gel was allowed to polymerise and
after the removal of the comb, the wells were washed several times with distilled water to remove excess acrylamide.

A kaleidoscope pre-stained protein standard ranging from 200 kD – 12.5 kD in size (BioRad, UK) was also loaded onto each gel in order to estimate the molecular mass of the sample bands (Figure 2.2).

![Molecular weight standards](image)

**Figure 2.2: Protein molecular weight standards.**

Electrophoresis through the stacking gel was performed at a constant voltage of 120 V and through the separating gel at a voltage between 160 - 200 V. Proteins resolved by SDS-PAGE were transferred to a polyvinylidene difluoride (PVDF) membrane (Amersham, UK) by placing the membrane (pre-equilibrated with transfer buffer) in direct contact with the gel. The membrane and gel were sandwiched with filter paper pads previously soaked in transfer buffer on both sides (Figure 2.3) and then placed in a wet blotting transfer unit (BioRad, UK) and transferred at 100 V for 1hr or at 30 V overnight at 4°C. Following transfer the efficiency of transfer was assessed by staining the PVDF membrane with Ponceau S solution for 2
minutes at RT and then washing the membrane with distilled water to visualise the protein bands.

Figure 2.3: Wet transfer ‘sandwich’ set-up.
The membrane and gel were placed in the direction of the current flow which is indicated in the apparatus diagram.

Protein binding sites on the PVDF membrane were blocked with skimmed milk in PBS (5%, w/v) for 1hr at RT with gentle agitation. Following blocking, the solution was discarded and the membranes were then incubated with the fresh blocking buffer containing the appropriate primary antibodies (Table 2.2 and Table 2.3). The membranes were incubated for 1hr at RT by rolling and were then washed three times with PBS and 0.1% Tween for 10 minutes. The membranes were incubated for 1hr at RT with the secondary antibody conjugated to horse-radish peroxidase (Table 2.4) diluted in blocking buffer. The membranes were repeatedly washed following incubation.

The detection of immunolabelled proteins was carried out using enhancing chemiluminescence (Amersham, UK). A reaction mix of stable peroxide solution mixed with an equal amount of luminol buffer was prepared and the membrane was incubated with this solution at RT for 1 min. The solution was then discarded and the membrane placed in a
photographic cassette and exposed to X-ray film (Hyperfilm; Amersham, UK). The protein markers on the membranes allowed the estimation of the approximate MW of the protein signals on the film.

Semi-quantification of protein blots was carried out by scanning the developed films using a SynGene Bioimaging Densitometer controlled by a GeneSnap (Syngene, UK) and all data analyses were performed using GraphPad Prism (GraphPad Software Inc., version 4.02).

2.4 Peptides used as Gap Junction Blockers

The peptide $^{43}$GAP27 was synthesised by Activotec (UK) and provided at >80% purity as a dry powder. Details are provided in Table 2.5.

Table 2.5: Details of the peptide used in this study

<table>
<thead>
<tr>
<th>Peptide Name</th>
<th>Target</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>$^{43}$GAP27</td>
<td>EL 2 (Cx37 and Cx43)</td>
<td>NH2-SRPTEKTIFII-OH</td>
</tr>
</tbody>
</table>

A scrambled peptide was not utilised as a control since a number of studies have been carried out demonstrating the specificity of the GAP27 peptide in disrupting GJIC between cells (Chaytor et al., 1997; Leybaert et al., 2003; Evans and Leybaert, 2007).
2.5 Assessment of Functional Gap Junction Communication using Dye Transfer between Monocytes and HUVECs

This assay aims to quantify the level of gap junctional formation and communication between monocytes and HUVECs, which is achieved by loading either one of the cell types with a specific dye known to permeate principally through GJs.

2.5.1 Isolation of Peripheral Blood Mononuclear Cells

Peripheral blood mononuclear cells (PBMCs) from anticoagulant-treated venous blood were isolated by layering onto Histopaque-1077 (Cat no. 10771, Sigma-Aldrich, UK), a solution containing polysucrose and sodium diatrizoate, adjusted to a specific density of 1.077 g/ml (Björkqvist et al., 2008). During centrifugation, erythrocytes and granulocytes rapidly settle to the bottom of the tube, while lymphocytes and other mononuclear cells remain at the plasma-Histopaque interface (Figure 2.4). Most platelets are removed by low speed centrifugation during the washing steps.

Freshly collected human blood from healthy volunteers, (50 ml) was placed in Falcon 50 ml conical centrifuge tubes (Cat no. 14-432-22, fisher scientific, UK) and diluted in two volumes (1:2) of PBS supplemented with EDTA (2 mM) (Cat no. BE02-017F, Sigma-Aldrich, UK). Next, 35 ml of the diluted blood sample was carefully layered onto 15 ml of histopaque-1077 solution (Cat no. 10771, Sigma-Aldrich, UK) (1:3) and subjected to centrifugation (Cat no. LMC-4200R, Laboratory Refrigerated Centrifuge, BioSan, UK) at 1500 xg for 40 minutes at RT in a swinging bucket rotor without brakes (Figure 2.4).
Figure 2.4: PBMC isolation using Histopaque-1077.
The original tube shows the Histopaque-1077 solution at the bottom before layering the blood above it without disturbing the solution as shown in the before centrifugation tube. The after centrifugation tube shows how red blood cells are pushed down and the Histopaque-1077 solution is separated between red blood cells and the mononuclear cell layer and plasma.

Following centrifugation, the upper layer was aspirated leaving the mononuclear cell layer undisturbed at the interface. The interface of cells was then carefully transferred to a new 50 ml Falcon conical centrifuge tubes and diluted with 2 mM EDTA/PBS before being again subjected to centrifugation at 2500 ×g for 10 minutes at 4°C.

The supernatant was then discarded and the cell pellet was re-suspended in RPMI 1640 culture medium supplemented with 10% FBS and 5% pencillin/streptomycin. Finally, 2 µl of the cell suspension was mixed with 18 µl of a 1:1 (v:v) solution of 0.4% trypan blue (Cat no. T6146, Sigma Aldrich, UK) in PBS, resulting in a 1:10 dilution of the cell suspension and a haemocytometer was used to count the cells under a light microscope. Cell viability was determined by calculating the percentage of unstained cells in the total population.

2.5.2 Isolation of Monocytes
Monocytes were isolated by positive selection using a MACS® human CD14+ MicroBeads Cell Isolation Kit (Cat no. 130-050-201, Miltenyi Biotec, UK) according to the manufacturer’s instructions. Monocytes were magnetically labelled with CD14+ MicroBeads (CD14 + is the
only marker for monocytes) by re-suspending the PBMC pellet in 80 μl of buffer (supplied in the kit) with 20 μl of MicroBeads antibody per 10×10^6 total cells and was then incubated for 15 minutes at 4°C. The suspension was loaded into a MACS column and placed in the magnetic field of a MACS separator. The magnetically labelled CD14+ cells were retained within the column while the unlabelled cells ran through; thus this cell fraction is depleted of CD14+ cells. After removing the column from the magnetic field, the magnetically retained CD14+ cells were eluted as the positively selected cell fraction (Figure 2.5).

Figure 2.5: Isolation of monocytes by positive selection using a MACS® human CD14+ MicroBeads cell isolation kit.
2.5.3 Evaluation of Calcein Dye Transfer between Monocytes and HUVECs using Flow cytometry

Calcein AM (MW: 994.8, Cat no. C3099, Invitrogen, UK) is a polyanionic acetoxyethyl (AM) fluorescein ester derivative. It is a membrane permeable fluorescent indicator that is widely used when studying living cells. Modification of carboxylic acids with the AM ester group results in an uncharged molecule that can permeate cell membranes, and once inside a cell, lipophilic blocking groups are cleaved by nonspecific esterases, resulting in a fluorescent and negatively charged molecule (MW: 623.1) that is both active and cell impermeable (Figure 2.6). In the case of calcein AM, the AM ester is colourless and non-fluorescent until hydrolysed (Oviedo-Orta et al., 2000).

![Calcein AM and Calcein](image)

**Figure 2.6: Chemical structure of calcein AM before and after modification by intracellular esterases.**

HUVECs were dye-loaded with 5 μM calcein AM (the stock concentration is 1M and it was diluted to the working concentration, 5 μM, with EBM-2) for 30 minutes at 37°C in a 5% CO₂ humidified incubator. Next, the HUVEC monolayer was washed with PBS three times for 5 minutes to eliminate any dye excess. Isolated monocytes then were added to the loaded monolayer and incubated for 30 minutes, 1-hour, 2-hours, 3-hours, 4-hours and 5-hours at 37°C in a 5% CO₂ humidified incubator to allow the establishment of GJ communication between the HUVECs and monocytes. The monocytes were then collected, washed three times for 5 minutes, and re-suspended in 4% paraformaldehyde (Cat no. P6148, Sigma-Aldrich,
UK) for fixation for 10-15 minutes. Fixed monocytes were washed and subjected to centrifugation three times with PBS for 5 minutes at 1000 ×g at 4°C. The pellet was then re-suspended in 500 μl of FACS buffer, PBS and 0.1% sodium azide, and sorted by flow cytometry to examine for possible dye transfer from the HUVECs to the monocytes (Figure 2.7).

Figure 2.7: Evaluation of calcein AM dye transfer between monocytes and HUVECs using flow cytometry.

Samples were analysed by flow cytometry for calcein AM (excitation at 488nm and emission at 535nm) using a FACS Canto (Fluorescence-Activated Cell Sorting (FACS)) with FACS Diva software version 5.2 (BD Biosciences, UK) with the assistance of Dr Rachel Butler.

Flow cytometry is a laser-based, biophysical technology employed in cell counting, cell sorting, biomarker detection and protein engineering, by suspending cells in a stream of fluid and passing them by an electronic detection apparatus. It allows the simultaneous multi-
parametric analysis of the physical and chemical characteristics of up to thousands of particles per second.

Before each experiment both positive (monocytes loaded with calcein AM without any contact with the HUVEC monolayer) and negative controls (monocytes not loaded with calcein AM or contact with the HUVEC monolayer) were used to assess auto fluorescence, and separate cell samples were also labelled with isotype matched negative controls, at the same concentration of calcein AM. Data from 100,000 cells were acquired for each sample and the results were expressed as the mean green fluorescent intensity caused by dye transfer above the arbitrary value set using the negative cells. Two main conditions were used, activity in EGM and in EBM.

2.6 Trans-Endothelial Cell Migration

The TEM assay was performed using 6-well Transwell plates and polyester membranes with a 3.0 µm pore size to allow monocyte migration (Corning incorporated, Corning, USA). Each Transwell was coated with a fibronectin and gelatin mixture (fibronectin 1 mg/ml, Cat no. F1141, Sigma Aldrich, UK. And 5 µg/ml of gelatin, Cat no. G1393, Sigma Aldrich, UK) for 1 hour at 37 °C in a 5% CO₂ incubator. The coating mixture then removed and the inserts allowed to dry for 20-30 minutes at 37 °C in a 5% CO₂ incubator. HUVECs (4.0×10⁶ cells) were seeded and grown for 5 days to allow the formation of a contact tight monolayer.

In some experiments HUVEC monolayer activation occurred via incubation with 25 ng/ml of TNF-α for 6 hours to overnight at 37 °C in a 5% CO₂ incubator to allow complete stimulation of HUVECs, this is explained in much more detail in Chapter 5.

Monocytes or THP-1 cells were added to the upper compartment (3-5×10⁵ cells) and after 6 hours of incubation at 37 °C in a 5% CO₂ atmosphere, the cells in the upper compartment as well as those that had transmigrated to the lower compartment were collected separately.
(Figure 2.8), marked with a CD14+ antibody and then fixed with 4% paraformaldehyde in preparation for counting by flow cytometry. The number of migrated cells was calculated as:

\[
\text{Transmigration (\%)} = \frac{\text{Migrated cells in bottom well}}{\text{Total cells added into the upper well}} \times 100
\]

![Diagram of trans-endothelial migration assay](image)

Figure 2.8: The trans-endothelial migration assay.

(A) HUVECs were seeded and grown for 5 days to allow the formation of a tight monolayer. (B) Monocytes or THP-1 cells were added to the upper compartment. (C&D) After 6 hours, the cells in the upper compartment as well as those that had transmigrated to the lower compartment were harvested, marked with a CD14+ antibody and then fixed for counting by flow cytometry.

2.7 Trans-Endothelial Electrical Resistance Monitoring

TEER monitoring in perfusion culture is considered to be a reliable indicator of monolayer integrity and barrier function. Therefore 6-well snapwell tissue culture inserts were coated with a fibronectin and gelatin mixture (fibronectin 1 mg/ml, Cat no. F1141, Sigma Aldrich, UK, and 5 μg/ml of gelatin, Cat no. G1393, Sigma Aldrich, UK) for 1 hour at 37 °C in a 5% CO₂ incubator on a polyester membrane with a 0.4 μm pore size (Corning Costar, Corning, NY). HUVECs were cultured at 8-10×10⁶ cells/well and grown to confluency. Both the upper
compartment (luminal side) and lower compartments (intimae side) were separately accessible for medium removal, exchange, and recovery of cells. Monolayer integrity was tested by assessing the TEER using an Epithelial Voltohmmeter (EVOM, World Precision Instruments, Sarasota, FL) (Figure 2.9).

As advised by the manufacturer, for short-term storage (less than 2 weeks) electrodes were submerged in an electrolyte solution (0.10–0.15 M KCl or NaCl). The electrode cable was connected to the electrode port on the EVOM volt-ohm meter so that the system was internally short-circuited and electrode symmetry maintained. For long-term storage the electrode was rinsed with distilled H₂O and stored in dry, dark conditions.

To sterilise the electrodes they were immersed in ethanol for 5 minutes and allowed to air dry for 1 minute. The electrodes were then rinsed in a sterile electrolyte solution in PBS, before each resistance measurement. With the volt-ohm meter set to the resistance setting, the electrodes were placed vertically in a the well containing the transwell insert, with the short electrode in the upper chamber and the long electrode in the lower chamber touching the bottom of the well. Once the EVOM reading had stabilised, the resistance value was recorded (given in ohms; Ω) for each well. The resistivity calculation (resistance normalised to area; Ω×cm²) of a sample was performed by subtracting the background resistance (TEER of transwell inserts without cells) from the total resistance value. Measurements were repeated every day until the TEER value had reached a maximum and measurements plateaued, indicating the formation of a permeability barrier, which typically took 3-7 days from the initial cell plating.
Figure 2.9: TEER measurements.
The EVOM instrument (upper left) and diagram of its components used for TEER measurements (upper right). The lower picture shows an actual TEER measurement, (EVOM, World Precision Instruments, USA, Ghaffarian and Muro, 2013).
2.8 Statistical Analysis

Data are presented as mean values ± standard error of the mean (SEM) for \( n \) separate measures. In general each experiment was repeated three times (\( n=3 \)) unless otherwise stated. Differences between two data sets were compared using unpaired Student’s \( t \)-tests. Differences between multiple data sets were tested by one-way analysis of variance (ANOVA) with Bonferroni post-hoc tests. In some experiments when there may be interaction between two independent variables on the dependent variable, two-way ANOVA was performed and indicated as appropriate in figure legends. The null hypothesis was rejected at \( p<0.05 \), and significance was indicated as *\( p<0.05 \), **\( p<0.01 \) and ***\( p<0.001 \), using the appropriate symbols as indicated. Statistical analyses were carried out using GraphPad Prism software (version 5.02, GraphPad Software Inc., USA)
Chapter 3 - Characterisation and Functional Assessment of Cx43 Expression in HUVECS and Human Peripheral Monocytes
3.1 Introduction

The endothelial layer is a one-cell thick layer which lines all vessels. It responds to several physical and chemical signals by the production of a wide range of factors that regulate vascular tone, cellular adhesion, thromboresistance (resistance by a blood vessel to thrombus formation), SMC proliferation, and vessel wall inflammation. The importance of the endothelium was first recognised by its effect on vascular tone, which is achieved by the production and release of several vasoactive molecules that relax or constrict the vessel, as well as by a response to and modification of circulating vasoactive mediators, such as bradykinin and thrombin. This vasomotion plays a direct role in the balance of tissue oxygen supply and metabolic demand through regulation of vessel tone and diameter, and is also involved in the remodelling of vascular structure and long-term organ perfusion (Morel, 2014; Brisset et al., 2009; Schechter and Gladwin, 2003).

Endothelial activation or endothelial dysfunction represents a switch from the normal phenotype towards one that involves the host defence response. Cardiovascular risk factors can activate molecular machinery in the endothelium that results in the expression of chemokines, IL-6 and IL-8, cytokines, TNF-α and IFN-γ, and adhesion molecules, including platelet endothelial cell adhesion molecule 1 (PECAM-1), intercellular adhesion molecule-1 (ICAM-1), and vascular cell adhesion molecule-1 (VCAM-1), designed to interact with leukocytes and platelets and to target inflammation to specific tissues in order to clear the cause of endothelial activation, for example oxLDL (Matsuzawa et al., 2015; Sena et al., 2013; Bafana et al., 2011).

ECs adhere to one another through junctional structures formed by transmembrane adhesive proteins that are responsible for homophilic cell-to-cell adhesion. In turn, the transmembrane proteins are linked to specific intracellular partners, which mediate anchorage to the actin
cytoskeleton, and as a consequence, stabilise junctions. Junctions in endothelial and epithelial cells share common features and in both cell types two major types of junctions have been described, AJs and TJs. However, epithelial cells also form desmosomes, which are absent in the endothelium (Bazzoni and Dejana, 2004). In addition to cell-to-cell adhesion, another type of junction, the GJ, mediates cell-to-cell communication.

GJs are specialised cell membrane domains consisting of clusters of protein channels that link the cytoplasmic compartments of neighbouring cells, forming pathways for the direct exchange of ions and small molecules (Yamasaki and Naus, 1996). The component proteins of GJ channels are connexins, a multigene family of conserved proteins, with different members expressed in different cell types, tissues, and species (Bruzzone et al., 1996; Kumar and Gilula, 1996). At least 21 different mammalian connexins have thus far been identified, with four present in healthy vascular walls, Cx37, Cx40, Cx43 and Cx45, although their expression varies according to the vascular territory and species. Usually, Cx37, Cx40 are Cx43 expressed in ECs, while Cx43 and Cx45 are mostly present in SMCs. Of note, Cx43 has also been described in ECs of large vessels in rabbits, hamsters and rats. Homomeric and heteromeric connexons and homocellular and heterocellular GJ channels are found in the vascular wall between ECs, between SMCs and in some cases between ECs and SMCs, allowing the passage of transverse and longitudinal signals along the vessel wall (Wit et al., 2006; Sandow et al., 2003; Looft-Wilson et al., 2004).

The importance of vascular connexin has been demonstrated by assessing their deletion on vascular function using knock-out mice. Cx45 knockout mice die in utero due to the interruption of vessel maturation (Kruger et al., 2000), whereas Cx43 knockout mice die shortly after birth due to cardiac malformations resulting in the obstruction of the outflow tract (Vink et al., 2004). Although Cx40 knockout mice are viable, they are hypertensive (Wit
et al., 2003) and display an increased sensitivity for cardiac arrhythmias (Simon et al., 1998), while the double deletion of Cx37 and Cx40 in mice induces embryonic death due to the excessive dilation of blood vessels (Simon and McWhorter, 2002).

Physiological control of vascular tone is in part regulated by GJ intercellular communication. Indeed GJs have been implicated in the radial transmission of hyperpolarisation from ECs to SMCs (the endothelium-derived hyperpolarising factor (EDHF) phenomenon) and in the longitudinal transmission of electrical signals (de Wit and Griffith, 2010). The role of connexin isoforms in vascular tone has been investigated in vitro by targeting gap junction communication and in vivo using transgenic animals. For example, α-glycyrrhetinic acid, which inhibits gap junctional communication, blocks the EDHF phenomenon (Chaytor et al., 2000). The use of specific peptides targeting Cx37, Cx40 or Cx43 in the rat hepatic artery has shown that the inhibition of the EDHF response depends on more than one connexin subtype (Chaytor et al., 2001), and that Cx37 and Cx40 are implicated in endothelium-dependent subintimal smooth muscle hyperpolarisation, whereas Cx43 is involved in the spread of subintimal hyperpolarisation through the media (Chaytor et al., 2005). Moreover, along the arterial wall connexin expression is influenced by blood flow, and oscillatory shear stress present at the branch points of arteries induces Cx43 expression in ECs (Gabriels and Paul, 1998), whilst simultaneously reducing expression of Cx37 (Pfenniger et al., 2012). The disturbed shear stress in these regions induces endothelial dysfunction and makes them a starting point for atherosclerosis plaque development (Davies and Civelek, 2011).

Monocytes are members of the mononuclear phagocyte system that originates in the bone marrow (Whitelaw and Bell, 1966) and monocytes travel through peripheral blood vessels after leaving the bone marrow. Compared to healthy conditions, in many disease states there is increased bone marrow production of monocytes that have a shorter circulation time
(Leder, 1967). At the site of inflammation, monocytes differentiate into macrophages or DCs by growing and increasing their lysosomal content, the amount of hydrolytic enzymes, the number and size of mitochondria, and the extent of their energy metabolism (Hume et al., 2002; Grage-Griebenow et al., 2001).

HUVECs have played a major role and are widely used as an EC model system, and have been used to study several aspects of EC function and modulation. For example, the regulation of EC function and the role of the endothelium in the response of the blood vessel wall to stretch, shear forces, and the development of atherosclerotic plaques and angiogenesis. The human umbilical cord has become one of the most important sources of vascular ECs, and the availability of these cells has played a major role in the development of the field of vascular biology (Yamada et al., 1992; Rice et al., 1988; Libby, 2000; Davies, 1997). Perfusion of the human umbilical cord vein with collagenase results in a pure preparation of the single layer of ECs that line this vessel (Gimbrone Jr and García-Cardeña, 2013). Initial passages of these cells, which are grown in the presence of heparin and pituitary extract, maintain nearly all of the features of native vascular ECs, including the expression of EC specific marker, such as von Willebrand factor (vWF) and an endothelial specific adhesion molecule, expression of receptors for growth factors, cytokines, vasoactive ligands, and specific signalling pathways for vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), transforming growth factor-β (TGF-β), TNF-α and angiotensin II (Goldberger et al., 1994; Namiki et al., 1995; Nozawa et al., 2000; Muscella et al., 1997).

HUVECs have provided a critical in vitro model for major breakthroughs in molecular medicine, including seminal insights into cellular and molecular events in the pathophysiology of atherosclerosis and plaque formation, and mechanisms for the control of angiogenesis or neovascularisation in response to hypoxia and inflammation in tumours, ischemic tissue,
in embryogenesis (Burns and DePaola, 2005; Kokura et al., 1999; Wei Zhang et al., 2003). Monolayers of HUVECs have been used for the study of the interaction of leukocytes and macrophages with the EC layer in vascular tissues, resulting in the discovery of adhesion molecules, chemokines and kinases that mediate the interaction of inflammatory cells with the endothelial surface and their migration into the media (Bevilacqua et al., 1989). Monolayers of HUVECs have been generated on deformable surfaces or in chambers, which have allowed the study of the effects of shear stress and pulsatile flow on cell signalling in order to reproduce the effects of blood flow on EC function in vivo. These monolayers have been used to identify transcription factors such as KLF2, which regulates the expression of adhesion molecules such as VCAM-1 and endothelial adhesion molecule E-selectin in response to stress and pro-inflammatory cytokines, such as TNF-α, that mediate changes in cell adhesion and migration, and play a role in the early changes of atherosclerosis (Parmar et al., 2006; Parmar et al., 2005; Dai et al., 2004).

Connexins are expressed in multiple cell types of the immune system. Levy et al. (1976) first reported the establishment of intercellular communication between macrophages based on the electrical coupling of adherent murine macrophages. Subsequently, GJs were morphologically described in the progeny of canine macrophages by freeze fracture electron microscopy (Porvaznik and MacVittie, 1979). Electron microscopy laid the foundation for describing GJ structures established between murine macrophages and an intestinal epithelial cell line (Martin et al., 1998), between hamster monocytes, as well as monocytes and neutrophils (Jara et al., 1995), and between rainbow trout macrophages and neutrophils (Köllner and Kotterba, 2002). Furthermore, the evidence related to GJIC between monocytes/macrophages and other cells has been supported by dye transfer assays (Martin et al., 1998).
To date, Cx43 has been found to be the most predominantly expressed connexin in human monocytes/macrophages, and Cx43 mRNA has been detected in macrophage foam cells of human atherosclerotic carotid arteries (Polacek et al., 1993). However, the contribution of Cx43 in monocytes TEM remains poorly identified. Interestingly, Cx37 was also found to be expressed in macrophages in early atheromas but not Cx43 (Kwak et al., 2002). The induced expression of other connexins in monocytes/macrophages was not detected after treatment with lipopolysaccharide (LPS), TNF-α or IFN-γ (Polacek et al., 1993; Eugenan et al., 2003).
3.2 Hypothesis

Cx43 expression in both monocytes and HUVECs is required for the formation of functional GJs which promote monocyte TEM.

3.2.1 Aims and Objectives

To characterise and functionally assess Cx43 expression in both HUVECs and human peripheral monocytes.

1. Characterisation of HUVECs and monocytes obtained from peripheral blood using immunoconfocal microscopy and a Western blotting techniques.

2. Characterisation of functional Cx43 GJ formation by the use of the dye transfer technique in the presence and absence of growth factors, GJ blockers and Cx43−/ in monocytes and THP-1 cells.

3. Examination of the role of Cx43 in the formation of functional GJs between monocytes and HUVECs using the dye transfer assay, TEM assay and TEER measurements.
3.3 Materials and Methods (see chapter 2 for more details)

3.3.1 Cell characterisation:

As described before in chapter 2, each cell type was maintain in its specific type of media. Human umbilical vein endothelial cells were maintained in endothelial growth medium (EGM-2). Human monocytes were obtained from volunteer’s peripheral blood which then characterised and isolated by positive selection using MACS human CD14+ MicroBeads cell isolation kit.

HeLa wild-type cells and HeLa cells transfected with Cx37, Cx40 or Cx43 were used in this study for control experiments (kindly donated by Dr Emmanuel Dupont). HeLa cells were maintained in complete Dulbecco’s modified Eagle’s medium.

The human monocyte-like cell line Tamm-Horsfall Protein-1 (THP-1) was purchased from the European Collection of Cell Culture (ECACC, UK); these cells were used as a negative control in some experiments as they lack for the expression of Cx37, Cx40, and Cx43. THP-1 cells were maintained in complete Roswell Park Memorial Institute media.

Unless stated, all experiments for western blot, confocal microscopy, Dye transfer assay, trans-e.

3.3.2 Dye Transfer:

Human umbilical vein endothelial cells monolayers were loaded with 5 mM calcein-AM dye. Suspensions of freshly isolated monocytes from peripheral blood were added to HUVEC monolayers that have been loaded with calcein-AM. Calcein transferred to monocytes was measured by flow cytometry and normalised to positive control values obtained from monocytes directly loaded with calcein and the values presented as percentage of calcin
transferred to monocytes. A negative control used, Cx43 deficient monocyte cell-line, THP-1 cells.

3.3.3 Trans-Endothelial Migration (TEM):
HUVECs were seeded and grown on 6-well transwell polyester plates with 3.0 µm pore size membranes. Monocytes were then added to the upper compartment (3-5×10⁵ cells). After six hours, the cells in the upper compartment as well as those that had transmigrated to the lower compartment were collected separately, marked with CD14+ antibody and counted by flow cytometry.

3.3.4 Trans-Endothelial Electrical Resistance (TEER):
HUVECs were seeded and grown on 6-well Transwell polyester plates with 0.8 µm pore size membranes. HUVEC monolayer functional integrity was assessed by measuring TEER using an Epithelial Voltomhmmeter (EVOM, World Precision Instruments, USA).
3.4 Results

3.4.1 Cell Characterisation

3.4.1.1 Detection of Endothelial Cell Markers and Connexin Expression in HUVECs

In all experiments HUVECs were grown to 85-90% confluency to achieve a monolayer of ECs with a cobblestone-like appearance, thereby mimicking the lining of the vasculature in the arterial intima (Baudin et al., 2007). HUVECs were cultured and maintained in EGM-2 and monitored by phase-contrast microscopy over time to obtain an optimal confluent monolayer for experiments. Figure 3.1 shows the effect of incubation time on HUVEC monolayer morphology. After one day of incubation, the HUVEC monolayer covered around 30-50% of the total surface area, and after two days of HUVECs covered around 85% of the total surface area which is the optimal cell surface for either splitting or for experiments. However, when HUVECs were incubated for more than three days, they covered the entire surface area and started to squeeze between each other and contact inhibit neighbouring cells causing some to detach.

Cultured HUVECs were stained for specific endothelial markers, such as vWF and PECAM-1 (Figure 3.2). vWF was mainly expressed by HUVECs within the cytoplasm, whereas PECAM-1 was mainly localised on the cellular membrane with low expression in the cytoplasm.

As ECs lie in very close proximity to SMCs within the vasculature HUVEC cultures were assessed for contamination with SMCs (Figure 3.2 and Figure 3.3). Although HUVEC purity was high, in a few purchased patches contamination with SMCs was observed. This was exhibited by prominent SMC-actin stress-fibre labelling and the clustering in groups of 2-3 cells, compared to pure batches of HUVECs.
In Figure 3.3, this particular batch of HUVECs is estimated by visual inspection to be <5% contaminated with SMCs. Every HUVEC batch bought from Lonza was assessed to check for specificity and purity, and only pure HUVECs were used in the subsequent experiments.
Figure 3.1: Effect of incubation time on cultured HUVECs confluency.
HUVECs were grown for different durations (1-4 days) to achieve a cobblestone-like appearance and monitored by phase-contrast microscopy; different magnifications are shown from left to right. (A) Day 1, early-confluency with a confluent monolayer of around 30-50%, (B) Day 2-3, optimal confluent monolayer at around 85%-90% (C) Day 4, post-confluency at around 90-100% or even over 100% were cells are in contact resulting in inhibition.
Figure 3.2: Immunofluorescence labelling of HUVECs for endothelial cell markers.
Cells were cultured for 48 hrs using primary antibodies against endothelial cell (EC) markers; von Willebrand Factor (vWF, green) and Platelet Endothelial Cell Adhesion Molecule 1 (PECAM-1, green) and smooth muscle (SMC actin, green). vWF (cytoplasm) and PECAM-1 (cell membrane) were expressed by ECs whereas immunofluorescence labelling illustrates no specific smooth muscle (SMC actin, green) contamination of the HUVEC population. Cells were counter stained with DRAQ 5, a nuclear dye.
Figure 3.3: Purity assessment of HUVECs by immunofluorescence.
Cells were cultured for 48 hrs using primary antibodies against von Willebrand Factor (vWF, green) and actin from smooth muscle cells (SMC actin, red). Double immunofluorescence labelling illustrates SMC contamination of the HUVEC population (right image), with distinct filamentous labelling distinguishable from the anti-vWF staining (green) of ECs. Cells were counter stained with DRAQ 5, a nuclear dye.
Expression levels of Cx37, Cx40, and Cx43 were assessed (Figure 3.4), with Cx43 found to be predominantly expressed by HUVECs, demonstrating the typical punctate labelling around EC borders (membrane). HUVECs also displayed some significant intracellular labelling, localised to one side and in close proximity to the nucleus, a localisation consistent with the Golgi apparatus. However, Cx37 and Cx40 labelling could not be observed by either immunofluorescence confocal microscopy or Western blotting, thus enabling the assessment of the role of Cx43 without interference from others connexins.

Figure 3.5 shows the level of protein expression for Cx37, Cx40, and Cx43 at different passages (4 and 11) next to the expression of transfected Cx37, Cx40 and Cx43 HeLa cells, which were used as a positive control. Equal amounts of total protein were loaded, and it can be seen that passage differences did not affect Cx43 expression in HUVECs (HUVECs Passage 4: 3.35 ± 0.14, HUVECs Passage 11: 3.28 ± 0.14) compared to HeLa-Cx43 cells (3.85 ± 0.14; P>0.05; two-way ANOVA). However, as mentioned earlier, Cx37 and Cx40 could not be detected by Western blotting.

![Figure 3.4: Cellular distribution of Cx37, Cx40, and Cx43 in HUVECs.](image)

Immunofluorescence labelling of HUVECs cultured for 48hrs using primary antibodies directed against Cx37, Cx40 and Cx43. Cx43 was the best expressed and outlined the cell borders (green) whereas Cx37 and Cx40 could not be detected. Draq 5 (blue) was used to counterstain the nucleus.
Figure 3.5: Level of Cx37, Cx40, and Cx43 protein expression in cultured HUVECs. Different passages (4 and 11) were compared to a positive transfected HeLa cell line with Cx37, Cx40, and Cx43. The level of Cx43 protein expression showed no significant difference due to passage in cultured HUVECs (HUVECs Passage 4: 3.35 ± 0.14, HUVECs Passage 11: 3.28 ± 0.14, HeLa-Cx43 cells 3.85 ± 0.14) whereas both Cx37 and Cx40 could not be detected in either passage of cultured HUVECs compared to the positive control of transfected HeLa cell line. Data are expressed as means ± SEM, P>0.05; Two-way ANOVA.

3.4.1.2 Monocyte Characterisation

3.4.1.2.1 Detection of Monocyte Markers and Connexin Expression in Human Monocytes

The monocyte CD14 marker was utilised to differentiate monocytes from other leukocytes, and levels of Cx43 expression were assessed as this enables the formation of GJs to occur between different cell types, with the expression of at least one connexin needing to be present. Figure 3.6 shows the expression of the CD14+ marker on monocytes as well as Cx43 using image confocal laser scanning microscopy, demonstrating the typical distribution of connexin expression which is localised to the plasma membrane, thereby in position for the establishment of GJs between cells.

The level of Cx43 protein expression in monocytes was also assessed by Western blotting in comparison to HeLa cells transfected with Cx43 (monocytes: 3.22 ± 0.15, HeLa-Cx43 cells 7.70 ± 0.29; P<0.001; unpaired t test) (Figure 3.7).
Figure 3.6: Immunofluorescence labelling of monocytes for CD14 and Cx43 expression.
The cellular distribution of CD14 monocyte marker and Cx43 in human monocytes. Cx43 expression was detected using a polyclonal antibody against human Cx43 and a secondary antibody labelled with Alexa Fluor 488. Cells were counter stained with DRAQ, a nuclear dye. CD14, is a cell plasma membrane marker characteristic for the differentiation of human monocytes.

Figure 3.7: Level of Cx43 protein expression in human monocytes.
Monocyte expression was compared to a positive HeLa cell line transfected with Cx43, (monocytes: 3.22 ± 0.15, HeLa-Cx43 cells 7.70 ± 0.29;*** P<0.001 HeLa cells compared to human peripheral monocytes. Unpaired t test. n=3, data are expressed as means ± SEM.
3.4.1.2.2 Characterisation of Peripheral Blood Mononuclear Cells and Isolated CD14+ Monocytes by Flow Cytometry

The effectiveness of the MACS® human CD14+ MicroBeads Cell Isolation Kit was checked. Monocytes from PBMCs can be used directly in experiments; however, positive selection of monocytes from PBMCs can be efficient and yield purified monocytes in contrast to using the whole buffy coat layer, which can lead to interference from other leukocytes. In the next set of experiments, monocytes obtained using the buffy coat layer directly and monocytes positively selected from the buffy coat using the MACS human CD14+ MicroBeads Cell Isolation Kit were assessed.

As shown in Figure 3.8, the CD14+ antibody showed specificity and efficiency for the selection of the monocyte population (P3, green population of CD14+) when compared to monocytes obtained directly from the buffy coat layer without positive selection (P3, green population of PBMC). In addition, platelets, red blood cells and/or debris (P1, red population) and lymphocytes (P2, blue population) are also shown in Figure 3.8. The calculated percentage of monocytes in PBMCs compared to CD14+ selected cells is also indicated. The percentage of monocytes was higher in CD14+ cells at around 58.46% ± 1.44 compared to 21.23% ± 0.71 in PBMCs (P<0.001; two-way ANOVA). According to the manufacturer’s protocol for the MACS® Human CD14+ MicroBeads Cell Isolation Kit, the monocyte percentage should be around 80-90%. The percentages were calculated by dividing the number of monocytes (PBMC or CD14+ derived) by the total number of cells counted by the flow cytometry as follows:

\[
\% \text{ of Monocytes} = \frac{\text{Number of monocytes}}{\text{Total number of cells counted by the flow cytometry}}
\]

In all future experiments, monocytes were positively selected and obtained using the MACS Human CD14+ MicroBeads Cell Isolation Kit.
Figure 3.8: Flow cytometric analysis of monocytes isolated from peripheral blood and positively selected using microbeads.

Panels A and B are representative data of cell density for PBMCs and CD14+ monocytes. Panel C shows the statistical analysis for PBMCs and CD14+ monocytes. P1 (Red) represents platelets, RBCs and/or debris; P2 (Green) represents lymphocytes, including B cells, T cells and NK cells; P3 (Blue) represents the monocyte population. The percentage of monocytes is higher after positive selection using CD14+ (B) compared to the non-selected population from PBMCs (A) 58.46% ± 1.44 compared to 21.23% ± 0.71, respectively. n=3, data are expressed as means ± SEM. *** P<0.001 CD14+ selected method compared to PBMCs, two-way ANOVA.
3.4.2 Evaluation of Dye Transfer between Monocytes and HUVECs

The aim of these experiments was to investigate whether CD14+ monocytes can establish GJ communication with HUVECs \textit{in vitro}. This was accomplished by loading HUVECs with calcein AM and two types of monocytes were used; human monocytes and THP-1 cells, which have the same characteristics as monocytes but lack expression of connexins.

3.4.2.1 Optimisation of Dye Transfer Conditions

Figure 3.9 shows effective calcein AM loading in HUVECs using epifluorescence microscopy. Panel A illustrates the HUVEC monolayer loaded with calcein AM, onto which non-loaded monocytes can be dropped to allow GJ formation between monocytes and HUVECs. For the monocytes (Panel B), flow cytometric analysis was used to detect calcein AM loaded cells in suspension, which was used as an indication of positive and negative loaded monocytes for comparison with monocytes that have taken up the dye from loaded HUVECs. Panel C shows calcein loaded and non-loaded THP-1 cells, as positive and negative controls, which were included in all experiments involving HUVECs and monocytes.
Figure 3.9: Optimisation of dye transfer - loading of calcein AM in HUVECs, monocytes and THP-1 cells.

(A) Left, phase contrast image of calcein AM loaded HUVEC monolayer; right, fluorescent image taken using an epifluorescent microscope. (B) Left, Flow cytometric analysis of non-loaded monocytes with used as a negative control; right, loaded monocytes used as a positive control. (C) Left, Flow cytometric analysis of non-loaded THP-1 cells used as a negative control; right, loaded THP-1 cells used as a positive control.

When the calcein AM is inside a cell it is generally unable to diffuse out (Oviedo-Orta et al., 2000); however, in ECs some of the dye may leak out via non-specific dye transfer routes. Therefore, multiple washing steps were applied, during which the dye transferred to
monocytes was reduced (Figure 3.10). After washing steps, as THP-1 cells lack expression of connexins, no dye transfer occurred when THP-1 cells were dropped onto the calcein AM loaded HUVECs. However, calcein AM did transfer to the monocytes after contact with the loaded HUVECs (Figure 3.10).

Figure 3.10: Effect of multiple washes on calcein AM loading in monocytes.
Multiple washing steps were employed, to ensure that dye transfer only occurred through GJs. Connexin expression in both monocytes and HUVECs is indicated; peak 1 indicates dye loading due to calcein leaking from ECs whilst peak 2 indicates dye transfer from actual contact between ECs and monocytes. No dye transfer occurred between THP-1/HUVECs which indicates the absence of GJ.
3.4.3 The Formation of Gap Junctions Mediates Intracellular Communication between Monocytes and HUVECs

3.4.3.1 Time course for Gap Junction Formation

Calcein AM is known to permeate only through functional GJs, and Figure 3.11 and Figure 3.12 show direct intercellular communication through GJ channels assessed using flow cytometry to measure the amount of fluorescent calcein AM transferred from loaded HUVECs to monocytes in EGM and EBM. EGM was used to determine the effect of growth factors on GJ formation and communication. Monocytes in both EGM and EBM showed dye transfer indicating GJ communication between monocytes and HUVECs. After 30 minutes of incubation in EBM, about 67.80% ± 1.35 (P<0.001, one-way ANOVA) of the total percentage of monocytes had taken up calcein AM (Figure 3.12) compared to 32.70% ± 1.42 (P<0.001, one-way ANOVA) when EGM was used. During the next 300 minutes, dye transfer slowly increased to a steady level to around 84.33% ± 0.88 in EBM and 61.43% ± 0.29 in EGM (P<0.001, one-way ANOVA).

![Figure 3.11: Effect of length of incubation on dye transfer from HUVECs to monocytes in EGM and EBM.](image)

EBM shows more efficient dye transfer, thus increased GJ formation and communication, with around 67.80% ± 1.35 of monocytes showing dye transfer within the first 30 mins compared to 32.70% ± 1.42 in EGM. n=3, data are expressed as means ± SEM.
Figure 3.12: Gap junction formation represented by dye transfer after 30 minutes from HUVECs to monocytes in EGM and EBM.
HUVECs were first loaded with calcein AM and co-cultured with unloaded monocytes. Direct intercellular communication through GJs was assessed using flow cytometric analysis by measuring the amount of fluorescent calcein AM transferred from HUVECs to monocytes, *** P<0.001 % of loaded monocytes in EBM 67.80% ± 1.35 compared to positivity loaded monocytes, δδδ P<0.001 % of loaded monocytes EGM 32.70% ± 1.42 compared to positivity loaded monocytes. n=3, data are expressed as means ± SEM, One-way ANOVA.

3.4.3.2 Cx43 Expression in HUVECs Mediates Gap Junction Formation between Monocytes and HUVECs.

GJ formation has been demonstrated to occur between HUVECs and monocytes through dye transfer, but the next step was to determine whether dye transfer only occurred through GJs. The GJ blocker ⁴³GAP27, a specific peptide blocker for Cx43, was used together with THP-1 cells which are deficient in Cx43.

The effect of ⁴³GAP27 on Cx43 was assessed by incubating HUVECs with ⁴³GAP27 for between 0 – 24 hours. Figure 3.13 shows that ⁴³GAP27 had no effect on the levels of total Cx43 expression over the 24 hours, consistent with its functional effect to block and close the gap junctions, rather than inhibiting connexin synthesis.
Figure 3.13: Effect of $^{43}$GAP27 on Cx43 expression in HUVECs.
(A) Expression of Cx43 in HUVECs over time; (B) Effect of GAP27 on the expression of Cx43 in HUVECs over time. (C) Analysis of total Cx43 protein expression in control HUVECs and HUVECs treated with $^{43}$GAP27; $^{43}$GAP27 has no effect on Cx43 expression level as it acts functionally by closing the channel and does not inhibit synthesis. n=3, data are expressed as means ± SEM, P>0.05 two-way ANOVA.
Figure 3.14 shows the effect of \(^{43}\text{GAP27}\) on dye transfer between HUVECs and monocytes in both EBM and EGM, which was reduced or almost inhibited compared to when it was absent (EBM: 67.80% ± 1.35, EGM: 32.70% ± 1.42, EBM with \(^{43}\text{GAP27}\): 18.65% ± 4.36, EGM with \(^{43}\text{GAP27}\): 12.45% ± 1.23). The amount of calcein AM that transferred in presence of \(^{43}\text{GAP27}\) was almost identical in both EGM and EBM, showing that growth factors had no effect, as observed when \(^{43}\text{GAP27}\) was absent.

![Flow cytometry analysis](image)

**Figure 3.14: Assessment of gap junction formation in the presence of \(^{43}\text{GAP27}\).**

Left - flow cytometric analysis of the effect of \(^{43}\text{GAP27}\) (300μM) on dye transfer between monocytes and HUVECs in different media. \(^{43}\text{GAP27}\) blocks GJs and inhibits GJ dye transfer between monocytes and HUVECs in both EGM and EBM. *** P<0.001 % of calcein AM loaded in monocytes in both EBM and EGM treated with \(^{43}\text{GAP27}\) (EBM with \(^{43}\text{GAP27}\): 18.65% ± 4.36, EGM with \(^{43}\text{GAP27}\): 12.45% ± 1.23) compared to its absence EBM and EGM (EBM: 67.80% ± 1.35, EGM: 32.70% ± 1.42 respectively). n=3, data are expressed as means ± SEM, one-way ANOVA.

GJ formation was next assessed in THP-1 cells (Figure 3.15), where almost no dye transfer occurred between THP-1 cells and HUVECs in both EGM and EBM compared to the normal controls (in EBM: THP-1 8.13% ± 0.18 vs. monocytes 66.33% ± 1.33, P<0.001, t-test; in EGM: THP-1 4.11% ± 0.8 vs. monocytes 30.32% ± 2.83, P<0.001, t-test). THP-1 cells lack connexin expression and consequently no GJs are formed.
Figure 3.15: Assessment of gap junction formation between THP-1 cells and HUVECs
Left - flow cytometric analysis of dye transfer between THP-1 cells and HUVECs; almost no dye transfer occurred in both EGM and EBM compared to the normal controls due to the lack of connexin expression by THP-1 cells. Right - statistical analysis of flow cytometry results; in EBM: THP-1 8.13% ± 0.18 vs. Monocytes 66.33% ± 1.33, *** P<0.001, t-test whereas in EGM: THP-1 4.11% ± 0.8 vs. Monocytes 30.32% ± 2.83, *** P<0.001, t-test respectively. n=3, data are expressed as means ± SEM.

3.4.4 Monocyte Adhesion and Trans-Endothelial Migration

Figure 3.16 shows the number of monocytes which migrated to the lower chamber when HUVECs were incubated in the presence or absence (control condition) of $^{43}\text{GAP27}$. The migration of monocytes from the upper to lower chamber was slow in the absence of $^{43}\text{GAP27}$ (0.13×$10^5$ ± 0.008×$10^5$ vs. total number of monocytes: 4.5×$10^5$ ± 0.05×$10^5$). However, the combined total number of monocytes in both the upper and lower chambers were less compared to the beginning of the experiment, which indicates that some may have adhered to the monolayer and/or were in the middle of the migration process (the total was (adhered control): 1.1×$10^5$ ± 0.01×$10^5$, adhered $^{43}\text{GAP27}$: 0.006×$10^5$ ± 0.0001×$10^5$, P<0.001, one-way ANOVA).

Moreover, the number of migrated monocytes was even less or no cells migrated when HUVECs were treated with $^{43}\text{GAP27}$ (0.006×$10^5$ ± 0.0003×$10^5$ vs. monocytes from the lower chamber under the control condition: 0.13×$10^5$ ± 0.008×$10^5$). However, almost no monocytes
adhered to the monolayer when HUVECs were incubated with \(^{43}\text{GAP27}\) compared to the control condition. These data indicates the role of Cx43 in TEM.

Figure 3.16: Effect of \(^{43}\text{GAP27}\) treatment of HUVECs on the number of migrated monocytes.
The number of migrated cells from the upper to lower chamber was slow in the control condition (HUVECs not incubated with \(^{43}\text{GAP27}\)) and even slower or not at all when HUVECs were treated with \(^{43}\text{GAP27}\); however, more cell adhesion occurred under the control condition compared to when HUVECs were treated with \(^{43}\text{GAP27}\). *** P<0.001 monocytes collected from the upper chamber when HUVECs treated with \(^{43}\text{GAP27}\) compared to number of monocytes in control upper chamber (4.0×10^5 ± 0.01×10^5 vs. 2.9×10^5 ± 0.06×10^5), ααα P<0.001 adhered monocytes calculated from the difference between the upper and lower chambers for HUVECs treated with \(^{43}\text{GAP27}\) compared to adhered monocytes calculated from the difference between the upper and lower chambers for the control condition (0.006×10^5 ± 0.0001×10^5 vs. 1.1×10^5 ± 0.01×10^5), and βββ P<0.001 monocytes collected from the lower chamber when HUVECs were treated with \(^{43}\text{GAP27}\) compared to the number of monocytes in lower chamber under the control condition (0.006×10^5 ± 0.0003×10^5 vs. 0.13×10^5 ± 0.008×10^5). n=3, data are expressed as means ± SEM, one-way ANOVA.
3.4.5 Human Umbilical Vein Endothelial Cells Monolayer Integrity

3.4.5.1 Optimisation of Trans-Endothelial Electrical Resistance on HUVECs

Monolayer integrity was tested by assessing the TEER using an EVOM. HUVECs showed different TEER measurements over the first 5 days and then reached a steady level at 6 days of about 30 – 40 Ω.cm² compared to the blank (Figure 3.17).

![Figure 3.17: Optimisation of TEER measurements for HUVECs over 14 days.](Image)

During the first 5 days the TEER measurements varied between 70 Ω.cm² to 40 Ω.cm² until they reached a steady level of resistance after day 6 between 35-45 Ω.cm². n=6, data are expressed as means ± SEM.

3.4.5.2 The Effect of Ca²⁺ on HUVEC Monolayer, Trans-Endothelial Electrical Resistance Integrity.

Ethylene glycol tetraacetic acid (EGTA) was used to chelate extracellular Ca²⁺ in these experiments (Flora and Pachauri, 2010) where it would be expected to alter the properties of both GJs and TJs. Addition of 5 mM EGTA to HUVEC culture (Figure 3.18) caused the TEER to change after 1 minute (30-10 Ω.cm²) and a steady state was reached after approximately 5 minutes (10 Ω.cm²). More ever, the effect of re-establishing [Ca²⁺] by adding excess DMEM, showed that the TEER increased within 2 minutes to the original measurement after 3-4 minutes.
Figure 3.18: Effect of EGTA on HUVEC monolayer integrity.
EGTA, a Ca\textsuperscript{2+} chelating agent caused the TEER measurements to drop after 1 minute to 10 Ω.cm\textsuperscript{2}; however, this effect was recovered by adding calcium via DMEM, and normal TEER measurements were regained after 5 mins. n=6, data are expressed as means ± SEM.

3.4.5.3 Trans-Endothelial Electrical Resistance Measurements under Treatment with a Gap Junction blocker in the Presence and Absence of EGTA

TEER measurements were taken every minute from HUVEC culture; control conditions and when treated with the GJ blocker \textsuperscript{43}GAP27, both in the presence and absence of 5 mM EGTA, as shown in Figure 3.19. Treating the HUVEC monolayer with \textsuperscript{43}GAP27 reduced the TEER to 50.0 ± 2.5 Ω.cm\textsuperscript{2}, which after adding EGTA was reduced further to a level similar to that observed for EGTA treatment alone, to 5.0 ± 1.3 Ω.cm\textsuperscript{2}. The effect of EGTA in the presence of \textsuperscript{43}GAP27 was also reversed by washing it out with excess DMEM solution.
Figure 3.19: Effect of $^{43}$GAP27 treatment of HUVECs on TEER measurements in the absence and presence of EGTA.

Treatment of the HUVEC monolayer with $^{43}$GAP27 resulted in a TEER measurement of around 50.0 ± 2.5 $\Omega$.cm$^2$ compared to 88.0 ± 1.2 $\Omega$.cm$^2$ in the control condition. However, adding EGTA caused the resistance to drop in both conditions to almost 5.0 ± 1.3 $\Omega$.cm$^2$. This effect was reversed when EGTA was cleared by adding DMEM. *** P<0.001 TEER measurements for the HUVEC monolayer treated with $^{43}$GAP27 compared to the normal HUVEC control monolayer for each time point. n=6, data are expressed as means ± SEM, Unpaired t tests.
3.5 Discussion

Several studies have reported significant changes in protein expression and the cellular distribution of connexin isoforms (Cx37, Cx40 and Cx43) in vascular endothelial GJs during the progression of atherosclerosis (Figueroa and Duling, 2009). Activation of ECs by a variety of pro-inflammatory stimuli, such as oxLDL, occurs during early atherosclerosis, triggering the expression of adhesion molecules and chemo-attractant proteins promoting adhesion to monocytes. Subsequently, blood monocytes transmigrate across the endothelium to penetrate the sub-intimal layer (Charo and Taubman, 2004; Hickey et al., 1997).

In this study, the characterisation of model ECs, namely HUVECs and monocytes was performed, and the purity of both HUVECS and monocytes was assessed together with the level of connexin expression. The formation of GJ communication between different cell types was also assessed, and communication via the identified channels was verified to be through GJs by using the blocker 43GAP27. Different conditions were tested by using EGM and EBM. In the TEM assay and TEER measurements, HUVEC monolayer integrity was assessed and measured with/without the addition of 43GAP27.

HUVECs were chosen as an in vitro model of ECs, as they are relatively easy to culture, and are morphologically, as well as functionally stable, throughout several cell culture passages (Figure 3.1). Indeed, HUVECs have been widely used as a cell-based model for endothelial activation, monocyte adhesion and recruitment assays (Singh et al., 2009). The culture of primary ECs was first reported by Jaffe in 1973, and although primary cultured cells provide an attractive in vitro model with very close representation of the physiological relevance in vivo, they are known to undergo senescence following a limited number of cell divisions (after passage 14) and also are not susceptible to atherosclerosis. However, they have been
extensively useful in studying endothelial dysfunction and possibly early stages of atherosclerosis (Kwak et al., 2009; Parmar et al., 2006; Parmar et al., 2005; Dai et al., 2004).

HUVECs in culture were clearly identified using EC markers, including vWF and PECAM-1. vWF was used as a differentiation marker, and is a multimeric glycoprotein which is synthesised exclusively in ECs as a carrier for coagulation factor VIII in the plasma. vWF is stored in the cytoplasm of ECs within rod-shaped cytoplasmic vesicles, known as Weibel-Palade bodies (Mannucci, 1995). In this study the HUVECs stained for vWF displayed clear granular staining, indicative of cellular distribution within Weibel-Palade bodies (Figure 3.2). PECAM-1 is a transmembrane glycoprotein surface molecule, which is constitutively expressed on the surface of ECs near intercellular junctions (Muller, 2009). It is involved in cellular processes associated with cell growth, adhesion and migration, and was also used as a differentiation marker in this study (Figure 3.2) (Muller, 1995).

In terms of the contamination of HUVECs with SMCs, it was demonstrated that some of the batches of purchased HUVECs contain SMCs, as shown by parallel labelling of SMC-actin stress fibres, typical of well-differentiated vascular SMCs (Figure 3.2 and Figure 3.3). Cx43 expression by SMCs was detected and notably, the formation of Cx43 GJs between ECs and SMCs (data not shown). This was indicative of hetero-cellular communication that may modulate EC responses, in particular, the modulation of vasomotor responses. However, such contamination is expected to interfere and to generate false results related to in vitro assessments of permeability and migratory characteristics of ECs, such as TEER measurements and trans-well migration assays (Dewi et al., 2004; Evans et al., 2002). Thus with contaminated cultures difficulties arise due to variations between resistance measurement values. Therefore, each HUVEC culture purchased was examined and only pure HUVECs were used throughout this study.
HUVECs demonstrated the predominant expression of Cx43 compared to expression of Cx37 and Cx40 (Figure 3.4), which could not be detected by either immunofluorescence microscopy or Western blotting. This suggests that in EC culture conditions, communication is achieved predominantly through Cx43 channel coupling. This finding is in contrast with other studies, which have demonstrated substantial Cx40 and moderate Cx37 junctional labelling of HUVECs (Kwak et al., 2009; Parmar et al., 2006; Rijen et al., 1998). This difference may be due to methodological variations, such as the use of a different EC growth medium (RPMI-1640 with glutamax-I with 10% FBS vs. EGM with 2% FBS). Furthermore, in intact preparations of murine aortas, endothelial GJs were found to be composed of combinations of Cx37, Cx40 and Cx43 (Inai and Shibata, 2009; Yeh et al., 1997). Therefore, it is possible that connexin expression in ECs depends on the culture medium employed. The model developed in this thesis has the advantage that the role of Cx43 in mediating TEM can be studied without interference from other connexin isoforms.

This study involved isolating monocytes from human peripheral blood (Figure 3.7). It has been shown that the percentage of monocytes accumulating dye from loaded HUVECs increased when growth factors were absent from the culture medium (Figure 3.11 and Figure 3.12). Growth factors act on the receptor tyrosine kinase family, which play important roles in the vasculature. These receptors include VEGF, epidermal growth factor (EGF), fibroblast growth factor (FGF) and insulin-like growth factor receptor (IGF), with each receptor named after the growth factor that it selectively binds to. All these growth factors are present in the culture medium used in this study and are important for cell growth and proliferation (Mukherjee et al., 2006). These factors are external stimuli which activate or deactivate multiple signal transduction pathways, and in turn different kinases and phosphatases, which may regulate phosphorylation or dephosphorylation of connexions (Solan and Lampe, 2009). Most of the available reports related to the effect of growth factors on connexin expression, trafficking
and life time come from studies on Cx43. For example, FGF-2 treatment of cardiac monocytes was shown to have no effect on total Cx43 expression or its cellular distribution but increased connexin phosphorylation at serine residues, resulting in decreased GJ intercellular coupling (Doble et al., 1996). In addition, in rabbit lens epithelial cells, IGF-1 regulates increased PKCγ activity resulting in the disassembly of GJs, and hence a reduction in cell membrane GJ density (Lin, 2003). In summary, the results from this thesis agree with those of others and demonstrate that growth factors can indeed affect the expression and functionality of Cx43.

Evidence for GJ-mediated intercellular communication between monocytes and macrophages, as well as other cells, has been supported by dye transfer assays (Martin et al., 1998). In primary cultures of murine microglia, low levels of dye coupling were observed under control conditions, and was dramatically increased by TNF-α and IFN-γ alone or in combination with LPS, and inhibited by a GJ blocker (Eugenan et al., 2001). To date, Cx43 has been found to be the most predominantly expressed connexin in human monocytes/macrophages. Moreover, Cx43 mRNA has been detected in macrophage foam cells of human atherosclerotic carotid arteries (Kwak et al., 2002; Polacek et al., 1993). However, these findings conflict with other reports that have demonstrated the lack of GJIC between monocytes/macrophages and other cells (Eugenan et al., 2003). These latter reports showed that dye transfer was not observed between untreated human or mouse monocytes/macrophages, human monocytes/macrophages and ECs or human monocytes/macrophages and SMCs (Eugenan et al., 2003; Alves et al., 1996; Polacek et al., 1993). While the results presented in this thesis are different, dye transfer was observed between untreated HUVECs and human monocytes.

The inhibition of dye transfer between the two cell types was observed when using the GJ blocker 43GAP27. 43GAP27 peptides have been shown to act in a connexin-specific manner and
have been widely applied to block GJs composed of Cx37, Cx40 and Cx43 (Martin et al., 2005; Chaytor et al., 1997; Isakson and Duling, 2005). These peptides inhibit GJ transfer of fluorescent dyes (Chaytor et al., 1999), electrical coupling (Dora et al., 1999) and synchronised Ca\(^{2+}\) oscillations in SMCs (Isakson and Duling, 2005) and monolayer cell cultures (Boitano and Evans, 2000; Isakson et al., 2001). Furthermore, connexin mimetic peptides have also been shown to inhibit GJ dependent artery oscillations (Chaytor et al., 1997), and the intercellular propagation of calcium waves across GJs (Boitano et al., 1998), as well as dye coupling in HeLa cells expressing various recombinant connexins (George et al., 1998).

Braet et al. (2003) were the first to report that connexin mimetic peptides also inhibit connexin hemichannels. This observation was not unexpected, given the fact that the extracellular loop sequences, with which the peptides are likely to interact, are freely available in the connexin hemichannel form. Further work by other groups has demonstrated that \(^{43}\)GAP27 blocks the uptake of the reporter dye propidium iodide and ATP release in Cx43-expressing cells, indicating selective inhibition of connexin hemichannels composed of Cx43 by \(^{43}\)GAP27 (Braet et al., 2003a; Leybaert et al., 2003). Patch clamp studies have shown that bound connexin mimetic peptides reduce within minutes the voltage-induced opening of Cx43 channels expressed in cardiomyocytes (Hawat et al., 2010) and inhibition of ATP release or dye uptake via connexin hemichannels was found to follow similar kinetics (Leybaert et al., 2003; De Vuyst et al., 2007).

Several possible mechanisms of GJ blocking by connexin mimetic peptides have been proposed, including the reduced docking of two pre-formed connexin hemichannels with peptides on their extracellular loops, the breaking apart of existing GJ channels, and interactions of the peptides with an accessible connexin target followed by conformational changes that lead to GJ channel closure (Berthoud et al., 2000; Evans et al., 2006). Further
possibilities include the diffusion of the peptides into the extracellular space surrounding the GJ channels and into the intercellular clefts, resulting in the separation of independent effects on GJ coupling from the effect on connexin hemichannels (Evans et al., 2006).

Normally, ECs and monocytes do not adhere, and thus TEM is low. However, physiological states, such hypercholesterolemia, insulin resistance, hypertension, smoking, mechanical damage, and infectious agents, are associated with the activation of the endothelium, which precedes the transmigration process of monocytes across the EC barrier. Both TNF-α and IFN-γ cytokines potentiate the production of adhesion molecules that can promote leukocyte adhesion to the endothelium (Mantovani et al., 1992). HUVECs treated with TNF-α along with IL-1 and LPS for 8 hours displayed a 60-70% increase in ICAM-1 expression (Myers et al., 1992). Similarly, human saphenous vein ECs treated with IFN-γ (with either TNF-α or IL-1α) showed higher VCAM-1 expression (Wang et al., 2007). With respect to these studies, TNF-α and IFN-γ could also be potentially relevant to connexin expression. One of the first studies that has explored this reported that TNF-α treatment of HUVECs led to the redistribution of Cx43, consequently impairing GJ functionality, while Cx37 and Cx40 expression was also decreased by TNF-α (Rijen et al., 1998).

As previously described, GJs (especially those composed of Cx43) and TJs are associated together (Nagasawa et al., 2006). GJIC, controlled by E-cadherin, involves post-translational regulation (assembly and/or function) of the GJ protein Cx43 (Jongen et al., 1991). TJ proteins (ZO-1) affect cadherin-based AJs assembly in terms of binding of ZO-1, E-cadherin and α-catenin (Contreras et al., 2002).

EGTA (5 mM) is a chelator of extracellular calcium ions (Ca^{2+}), and has lower affinity for magnesium ions (Mg^{2+}), making it more selective for Ca^{2+}. Treatment of HUVECs with EGTA reduced the TEER within one minute (Figure 3.18). This rapid reduction in TEER suggests an
extracellular action of EGTA to chelate Ca\(^{2+}\). The use of Ca\(^{2+}\) free solution in the TEER experiments was designed to show that what was being measured was due to the existence of a high resistance monolayer of cells, and not an artefact.

Connexons and hemichannel openings are increased by low extracellular Ca\(^{2+}\) (Zhao et al., 2006; Bennett et al., 2003), which enhances the release of small molecules, such as ATP, glutamate, and prostaglandins. The opening of hemichannels causes a drop in TEER, but the mechanisms remain unknown (Evans et al., 2006). With respect to TJs, low Ca\(^{2+}\) levels cause the reassembly of TJ integrated proteins, resulting in the inhibition of functional as well as the morphological reassembly of TJs, because of altered interactions between TJ proteins and the cytoskeleton. Reassembly of these proteins causes a leakage in the spaces between ECs, which leads to a reduction in TEER (Denker and Nigam, 1998).

In AJs, E-cadherin is a Ca\(^{2+}\) dependent protein, and low Ca\(^{2+}\) results in AJ dissociation mediated by PKA. This results in the redistribution of E-cadherin and ZO-1 (Nilsson et al., 1996), which disrupt AJS in ECs (Pokutta et al., 1994). This may be as a result of removing Ca\(^{2+}\) from binding sites on E-cadherin extracellular domains, causing a conformational change (Pokutta et al., 1994), thus interrupting cell-cell adhesion and reducing TEER. The recovery of normal Ca\(^{2+}\) by removing EGTA from the medium bathing ECs caused a rapid and complete recovery of TEER (Figure 3.18). Restoration of the extracellular Ca\(^{2+}\) closes hemichannels (especially Cx43) in ECs (Zhao et al., 2006; Bennett et al., 2003), and for TJs the restoration of extracellular Ca\(^{2+}\) causes the assembly of TJ proteins and restores TJ function. Similarly, in AJs, Ca\(^{2+}\) binding in the extracellular domain of E-cadherin allows them to regain their function (Contreras et al., 2002).

GJ blockers, such as 43GAP27, affected HUVECs GJIC as demonstrated by the reduced TEER to about 50% (Figure 3.19). GJ blockers disrupt hemichannels, especially Cx43 hemichannels,
(Dunlap et al., 1987) and are another indication that GJs (especially Cx43) play a role in the maintenance of ECs TEER (O’Donnell et al., 2014; Martin et al., 2005; Charles et al., 1992).
Chapter 4 - Effect of Suppression of Endogenous Connexin 43 in HUVECs on Gap Junction Formation between HUVECs and Human Peripheral Monocytes
4.1 Introduction

RNAi was employed to knockdown specifically the expression of connexins in order to establish their respective importance. RNAi is a naturally occurring post-transcriptional gene silencing mechanism employed by the cell to mediate gene regulation. The pathway is triggered by small RNA molecules that cause either a translational block or degradation of mRNA homologous in sequence to the RNA molecules. Post-transcriptional gene silencing was originally observed in plants, where it was termed ‘co-suppression’ (Napoli et al., 1990) and later correctly described by Fire et al. in 1998 in Caenorhabditis elegans as homology-dependent gene silencing. Since then similar findings have been described in several organisms including fruit flies (Kennerdell and Carthew, 1998), mice (Lewis et al., 2002) and human cells (Elbashir et al., 2001).

In animals and plants the two major classes of RNA molecules that regulate gene expression are: (1) siRNA, exogenously excised from long double-stranded RNA (dsRNA) derived from foreign nucleic acids, such as viruses, as part of the innate cellular defence mechanism (Carthew and Sontheimer, 2009); and (2) micro RNA (miRNA), which is non-coding RNA processed from stem-loop precursors transcribed in the nucleus to regulate endogenous gene expression (Filipowicz et al., 2008; Bartel, 2009). Despite these differences in origin, both processes require the cleavage of dsRNA by the ribonuclease III protein Dicer (Bernstein et al., 2001), producing RNA duplexes 21-23 nucleotides in length with dinucleotide overhangs at the 3’ end and monophosphate groups at the 5’ end (Zamore et al., 2000; Elbashir et al., 2001b). The 3’ overhang in the RNA duplex provides a recognition site for the nuclease-containing multi-protein structure called the RNA induced silencing complex (RISC) (Hammond et al., 2000; Martinez and Tuschl, 2004). The RNA duplex enters the RISC assembly pathway through the unwinding of the dsRNA by RNA helicase in an ATP-dependent step,
culminating in the stable association of one of the two strands with the Argonaute effector protein (Zamore et al., 2000; Nykanen et al., 2001). Strand selection is dictated by the relative thermodynamic stabilities of the two duplex ends (Carthew and Sontheimer, 2009). This guide strand directs mRNA target recognition by Watson-Crick base pairing, whereas the other strand of the original small RNA duplex is discarded. In the final step, the siRNA strand directs the RISC to where either: (1) it is perfectly complementary to RNA targets, which are then degraded by the RISC-associated endoribonuclease (Martinez et al., 2002); or (2) there is mismatch between the siRNA/target duplex leading to the suppression of mRNA translation or exonucleolytic degradation (Schwarz et al., 2004).

In mammalian cells, RNAi can be induced artificially by the introduction of synthetic double stranded siRNA molecules (21-23 nucleotides), the preferred approach for inducing RNAi in this study via long dsRNA (>30 nucleotides), or by transduction of plasmid or viral vector systems that express short hairpin RNA (shRNA), which are subsequently processed to siRNA by the cellular machinery. A schematic representation of the RNAi mechanism is illustrated in Figure 4.1.

The rapid development of the RNAi technique has been enhanced by a more complete knowledge of gene sequences. However, its application in mammalian cells has been hindered by an anti-viral interferon response triggered in the presence of long dsRNA molecules, resulting in the cessation of all protein synthesis (Clemens and Elia, 1997; Player and Torrence, 1998). However, the interferon response and the subsequent non-specific degradation of mRNA, has been demonstrated to only be triggered by dsRNA longer than 30 nucleotides, and dsRNA of 19-21 nucleotides in length were sufficient for RNAi activity (Bass, 2001; Elbashir et al., 2001; Caplen, 2002). Collectively, these findings have enabled the application of RNAi in mammalian cells and its potential use in therapeutic treatments, including malignant,
infectious and autoimmune diseases (McManus and Sharp, 2002; Lieberman et al., 2003; Sioud, 2004).

Figure 4.1: Schematic representation of the RNA interference pathway.
RNAi is a naturally occurring pathway that can be activated by: (1) siRNA produced in response to a virus; (2) miRNA to regulate gene expression; and (3) artificially, either by synthetic double-stranded siRNA molecules (19-21 nucleotides in length), long dsRNA molecules, or through shRNA expressed in plasmid or viral vector systems. miRNA and shRNA are produced in the nucleus as hairpin structures which are then exported and processed into long dsRNA molecules. dsRNA are cleaved into 21-23 nucleotide long RNA molecules by the enzyme Dicer in an ATP-dependent step. The processed siRNA is then incorporated into the RNA induced silencing complex (RISC) multi-protein complex, which contains a helicase that unwinds the duplex. The single strands of RNA in the duplex are then able to guide the RISC complex to the target mRNA. In the final step, selective gene silencing is achieved by either (1) the target mRNA being degraded by the RISC-associated endoribonuclease, or (2) repression of mRNA translation.
One of the main challenges in using RNAi as a tool to study gene function is the successful delivery of siRNA into cells. A variety of strategies have been developed for in vitro delivery of dsRNA, either by transfection (e.g. electroporation and lipid-mediated reagents) or through plasmid and viral vectors expressing shRNA (e.g. adenovirus and lentivirus). All of these techniques have benefits and drawbacks. Electroporation was the transfection method used in this study because it is a convenient and non-toxic delivery strategy for siRNA molecules into cells. It is also the method of choice for the in vitro delivery of a large variety of molecules, including ions, drugs, antibodies, cytokines and plasmid DNA, and also in vivo drug delivery to malignant tumours (Gehl, 2003; Mir, 2009). siRNA are small (21 nucleotides) and the molecules only need to be delivered into the cytoplasm in order to be active. Thus electroporation conditions can be less extreme, requiring lower voltages and shorter pulse durations.

An alternative strategy using lentiviral particles expressing shRNA is now commercially available as transduction-ready viruses for a target gene in any mammalian cell type, including primary and non-dividing cells. The benefits of using shRNA lentiviral particles include high transduction efficiency, and the possibility of developing stable expression of the shRNA while retaining high cell survival. Stable gene silencing for longer periods of time may also be advantageous if the target protein has a slow rate of turnover. One problem associated with this method is that integration of the transgene is random, so the silencing efficiency can vary considerably depending on the integration site. However, this can be overcome by selection of clones using an antibiotic resistance cassette.

Another important aspect for the induction of successful RNAi is the effectiveness of the siRNA sequence against the mRNA target. With the commercial availability of siRNA molecules, some empirical rules for the basic structure of effective siRNA sequences have emerged (Elbashir et
al., 2002; Reynolds et al., 2004; Luo and Chang, 2004), which have been taken into account in the development of siRNA design tools (i.e. algorithms) that are able to predict possible siRNA candidates for a target gene. These rules include: (1) avoiding target regions where the mRNA can form a hairpin structure affecting siRNA accessibility; (2) selecting a target site in a gene that is only specific to its sequence; (3) low guanine-cytosine content (less than 50%) to reduce thermal stability and facilitate the unwinding and incorporation of the strands into the RISC (Khvorova et al., 2003; Schwarz et al., 2004); and (4) keeping the length of dsRNA to 21-23 nucleotides to prevent an interferon response.

In this chapter the optimisation and effect of Cx43 siRNA sequences is discussed. Several preliminary steps were required before RNAi could be used reliably to specifically knockdown connexin expression. The first stage of this study was to optimise: (1) the siRNA transfection efficiency; (2) the electroporation parameters to ensure high transfection efficiency with minimal cell death; (3) the concentration of siRNA required to maintain gene silencing without causing cell toxicity and off-target effects; and (4) the duration of the silencing effect.
4.2 Hypothesis

It was hypothesised that transfection of HUVECs with Cx43-siRNA will suppress total Cx43 protein expression levels leading to inhibition of functional GJ formation between HUVECs and human peripheral monocytes.

4.2.1 Aims and Objectives

1. To standardise and optimise Cx43-siRNA delivery regarding concentration and duration.
2. To assess the role of Cx43 in the formation of functional GJs between monocytes and HUVECs transfected with Cx43-siRNA using the dye transfer assay, TEM assay and TEER measurements.
4.3 Material and Methods

4.3.1 Connexin 43- Short interfering RNA (Cx43-siRNA)

4.3.1.1 Cx43-siRNA Sequences

A pre-designed siRNA sequence (Silencer® Select Pre-designed & Validated siRNA) was purchased from Ambion. The siRNA sequence was designed by applying an algorithm optimised to produce highly specific and efficient siRNA molecules that cause minimal off-target effects. The sequence used was chemically synthesised, high-performance liquid chromatography-purified (>95% purity) and in the form of annealed double-stranded molecules ready for transfection. The product was purchased as a 5 nmol quantity of lyophilised powder, and was reconstituted to 50μM in nuclease free water. The details of the siRNA duplex utilised in all the transfection protocols is shown in Table 4.1.

Table 4.1: Cx43-siRNA sequence.
The siRNA used in this study was pre-designed to be 21 nucleotides in length with dinucleotide 3’ overhangs that can be incorporated directly into the RISC complex by-passing the initial processing step involving the Dicer. Either of the two strands from the ds siRNA were able to guide the RISC to the target mRNA in a complementary driven fashion. Homology searches were performed with the basic local alignment search tool, BLAST (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi).

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
<th>Homology %</th>
</tr>
</thead>
</table>
| Mouse Cx43-siRNA      | Cx43mRNA 1579-1597
                        | 5'-GCUUAAAUUCUAGUAAGAGtt-3'   | 100        |
|                       | 3'-ctCGAAUUAAAGAUCAUUCUC-5'    |            |

4.3.1.2 Cx43-siRNA Delivery

Electroporation requires the application of a pulsed electrical field (Gene Pulser Transfection, model: 1652078, BioRad, UK) to induce the reversible breakdown of the lipid bilayer thereby creating temporary pores (40-120nm in diameter) in the plasma membrane that re-seal within seconds (Neumann et al., 1982). This process permeabilises the host cells to a wide variety of
molecules, such as siRNA. For small molecules, diffusion alone may be sufficient for intracellular uptake (Neumann et al., 1998). In this study a capacitance extender (Capacitance extender, model: 1652087, BioRad, UK) was used to increase the pulse duration and provide an electrophoretic drive for the negatively charged siRNA to enter the cells. Although electroporation is a convenient and efficient method of delivery, cell viability is a crucial aspect and is dependent upon the experimental conditions used (e.g. voltage parameter), with the parameters specific for each cell type. Thus a range of electroporation conditions, including the field strength (voltage) and pulse length (capacitance), were tested to ensure maximum transfection efficiency and cell recovery.

HUVECs were cultured to sub-confluency in a 75 cm² dishes (~8.5×10⁶ cells at confluency) and were no more than 4-5 days old. Cells were trypsinised, washed, collected by centrifugation and then re-suspended in EBM. Aliquots (0.4 ml) of 2×10⁶ cells in suspension together with 0.25 - 1 μM of siRNA (depending on the experiment) were placed in a sterile 0.4 cm electrode gap-cuvette and positioned for pulsing. Cells were subjected to ranges of single pulses of low voltage and high capacitance (e.g., 250 V at 960 μF) by using the capacitance extender (Figure 4.2). The time constant (τ) was recorded for each electroporation. The time constant is the length of time required for the voltage in the circuit to fall exponentially (1/e) by approximately 63% of its initial value, and is dependent on the volume in the electroporation cuvette and the conductivity of the medium. Although parameters for electroporation had been developed previously within our laboratory for SMCs, re-optimisation was necessary to maximise siRNA delivery to HUVECs and to minimise cell death.

Following transfection, cells were removed from the cuvette by gentle pipetting and seeded in a 6-well plate. Cells were seeded at confluency since these cells are mildly ‘contact-inhibited’, making them less likely to divide and prevent any dilution of the Cx43-siRNA. The
medium was changed 3-4 hours post-seeding to remove any dead cells and cellular debris caused by the electroporation, and incubated in an atmosphere of 5% CO₂ at 37°C for a minimum of 48 hours in order to recover from electroporation injury. The efficiency of Cx43-siRNA transfection was assessed by Western blotting of cells harvested 48-72 hours post-transfection.

Figure 4.2: Delivery protocol for Cx43-siRNA into HUVECs and the electroporater used.
4.4 Results

4.4.1 Optimisation of Electroporation Conditions for the Delivery of siRNA into HUVECs

For efficient down-regulation of connexins the electroporation conditions had to be optimised for minimal cell death and maximal siRNA suppression. In general, for each cell line, medium type and volume, an optimal time constant was experimentally determined which corresponded to fixed voltage and capacitance settings (Table 4.2).

Table 4.2: The corresponding voltages and capacitance setting for HUVECs

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Volume (µl)</th>
<th>Voltage (V)</th>
<th>Capacitance (µF)</th>
<th>Time Constance (ms)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HUVECs</td>
<td>400</td>
<td>200</td>
<td>960</td>
<td>31.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>220</td>
<td></td>
<td>26.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>240</td>
<td></td>
<td>25.6</td>
</tr>
</tbody>
</table>

Figure 4.3 shows the optimisation of the electroporation conditions for sufficient and efficient delivery of siRNA into HUVECs. Three samples were electroporated under different voltages and a 50-60% reduction in Cx43 levels can be observed after electroporation at 200 and 220 volts (0.57 ± 0.01, 0.58 ± 0.01 respectively vs. non-EP: 0.98 ± 0.03, P<0.001, one-way ANOVA), while at 240 volts the reduction is slightly lower (0.79 ± 0.01).
4.4.2 Determination of the Most Effective siRNA Concentration for Cx43 Downregulation in HUVECs

After establishment of the optimal electroporation conditions (220V), the next step was to electroporate HUVECs with varying amounts of Cx43-siRNA to determine the most effective siRNA concentration for maximal down-regulation of Cx43 levels. The concentrations used for the siRNA-titration curve ranged from 0.25 µM to 1.0 µM. As seen in Figure 4.4, there is little difference between the non-electroporated sample and the electroporated samples in the expression of Cx43; 1.0 µM of siRNA gave 50-65% of Cx43 expression (0.27 ± 0.03, P<0.001, one-way ANOVA) compared to 0.5 µM and 0.25 µM of siRNA (0.39 ± 0.1 and 0.56 ± 0.01, respectively, P<0.001, one-way ANOVA).
Figure 4.4: Effect of different Cx43-siRNA concentration on expression of Cx43.

HUVECs were transfected with a range of Cx43-siRNA concentrations and Cx43 expression levels were compared to cells electroporated without Cx43-siRNA. Maximal effects of siRNA knockdown for Cx43 were obtained at 1μM. *** γγγ, δδδ P<0.001 total Cx43 protein expression in HUVECs treated with 0.25 (0.56 ± 0.01), 0.5 (0.39 ± 0.1), and 1 μM (0.27 ± 0.03) Cx43-siRNA vs EP control. n=3, data are expressed as means ± SEM, one-way ANOVA.

To verify Cx43 suppression immunofluorescence experiments were performed using HUVECs cells treated with and without Cx43-siRNA and labelled with Cx43 antibodies. Figure 4.5 demonstrates that after treatment with siRNA most of the cells lacked Cx43 labelling. However, ~10-15% of the cells show normal to low levels of Cx43 present at the membrane, and in some cells, strong intracellular labelling could be observed, indicating that either Cx43 was not yet degraded or that some cells were not efficiently transfected. There was no difference in the labelling and distribution of Cx43 in the seeding and electroporation control (data not shown).
Figure 4.5: Immunofluorescence labelling of HUVECs treated with Cx43-siRNA.

Control and Cx43-siRNA treated HUVEC cells were labelled with the Cx43 antibody and incubated with Evans Blue in order to visualise the cells. In non-treated cells, Cx43 labelling is observed between cells (white arrows) and intracellularly. The overall labelling is significantly reduced in siRNA treated cells; however, residual labelling, intercellular and within GJs (white arrow), is still present. The cells were electroporated at 220V and 960μF.

A scrambled Cx43-siRNA was also applied to check the sensitivity and specificity of Cx43-siRNA for only Cx43 and the results are presented in Figure 4.6. Scrambled Cx43-siRNA has the same nucleotide composition but not the same sequence as Cx43-siRNA. This is achieved by either randomising (also known as scrambling) the nucleotides in the siRNA or reversing the sequence of the siRNA. When the sequences are confirmed to not target a known gene or have a miRNA seed region match with a known gene, then such siRNAs are appropriate as negative control siRNAs. However, it is not practical, and often impossible, to identify a scramble control for every gene-specific siRNA that is used, therefore a universal non-targeting control is more widely utilised.
Figure 4.6: Specificity of Cx43-siRNA in HUVECs for targeting Cx43 protein expression.
Scrambled Cx43-siRNA was used to test the specificity of the Cx43-siRNA used in this study. *** P<0.001 Total Cx43 protein expression in HUVECs treated with 1 μM of Cx43-siRNA at 220 volts vs cells treated with scrambled Cx43-siRNA (0.45 ± 0.01 vs. scrambled Cx43-siRNA; 1.35 ± 0.01, EP Control; 1.25 ± 0.20, Non EP; 1.12 ± 0.11). n=3, data are expressed as means ± SEM, one-way ANOVA.

4.4.3 Time course of Cx43 Suppression

The next step in planning the Cx43-loss-of-function experiments was to assess the duration of the siRNA-mediated Cx43 suppression. Cx43-siRNA was evaluated over the course of 16 days, and quantification of Cx43 siRNA indicated that the siRNA mediated effects were most prominent on day-2 (0.45 ± 0.01, P<0.001, one-way ANOVA) in contrast to day-4 (0.76 ± 0.01, P<0.001, one-way ANOVA) but connexin suppression was still maintained at over 50%. After day-4 the expression of Cx43 gradually increased until it reached the same as that of the control by day-12 to day-16 (1.36 ± 0.01) (Figure 4.7).
Figure 4.7: Length of effect of Cx43-siRNA in HUVECS on the suppression of Cx43 expression.

Cx43 suppression resulting from HUVECs treated with 1μM of Cx43-siRNA at 200V over the course of 16 days. Cx43 expression was downregulated for 2-4 days (0.45 ± 0.01, 0.76 ± 0.01, respectively) after transfection, however, this decrease in expression was lost over further days of incubation. ***, γγγ, δδδ P<0.001. n=3, data are expressed as means ± SEM, one-way ANOVA.

4.4.4 Role of Cx43 Expression on Gap Junction Formation between Monocytes and HUVECs in the Presence and Absence of Cx43-siRNA

4.4.4.1 Effect of Cx43-siRNA on Dye Transfer

Following the optimisation experiments, Cx43-siRNA functionality was assessed using different assays. The dye transfer assay was used to assess GJ formation when monocytes were in contact with HUVECs treated with Cx43-siRNA and loaded with calcein AM and compared to control samples. As shown in Figure 4.8, monocytes that were in contact with the HUVECs transfected with Cx43-siRNA showed a significant reduction in calcein AM dye
transfer of 9.93% ± 2.4 (P<0.001, one-way ANOVA) compared to 78.0% ± 2.8 for non-treated cells.

Figure 4.8: Effect of Cx43-siRNA on gap junction formation and communication between monocytes and transfected HUVECs.
Dye transfer after 30 mins incubation from Cx43-siRNA treated HUVECs to monocytes was reduced to 9.93% ± 2.4 compared to the non-treated control, while HUVECs treated with scrambled Cx43-siRNA showed similar level of dye transfer as the non-treated control, *** and γγγ P<0.001% vs scrambled Cx43-siRNA and control. n=3, data are expressed as means ± SEM, one-way ANOVA).

4.4.4.2 Effect of CX43-siRNA on Adhesion and Trans-Endothelial Migration
In the TEM assay monocytes were dropped and incubated on Cx43-siRNA treated HUVECs to assess whether monocytes needed to communicate via gap junction before migration. Figure 4.9 shows the number of monocytes that migrated to the lower chamber for HUVECs untreated or transfected with Cx43-siRNA. The migration of monocytes from the upper to lower chamber for non-transfected HUVECs, or for those treated with scrambled Cx43-siRNA were 0.14×10^5 ± 0.01×10^5, 0.14×10^5 ± 0.01×10^5, respectively. However, almost no monocytes migrated when HUVECs were transfected with Cx43-siRNA (0.05×10^5 ± 0.01×10^5, P>0.05, two-way ANOVA). In addition, the number of adhered monocytes were low compared to greater levels of cell adhesion for untreated HUVECs and those transfected with scrambled Cx43-siRNA (0.50×10^5 ± 0.01×10^5 vs. monocytes adhered on scrambled Cx43-siRNA HUVECs:
1.2×10⁵ ± 0.13×10⁵ and monocytes adhered on control HUVECs: 1.2×10⁵ ± 0.13×10⁵, P<0.001, two-way ANOVA).

**Figure 4.9: Effect of Cx43-siRNA transfected HUVECs on the migration of monocytes.**

The number of migrated cells was reduced in the presence of Cx43-siRNA compared to the controls and adherence was also lower. ***, γγγ P<0.001**, the number of monocytes collected from the upper chamber of HUVECs transfected with Cx43-siRNA (1 μM, 200V electroporation) compared to the number of monocytes in the upper chamber in the control and HUVECs transfected with scrambled Cx43-siRNA samples, respectively. βββ, δδδ P<0.001, the number of monocytes that had adhered was calculated from the difference between the upper and lower chambers of HUVECs transfected with Cx43-siRNA (1 μM, 200V electroporation) compared to number of monocytes adhered in the control and scrambled Cx43-siRNA samples, respectively. n=3, data are expressed as means ± SEM, two-way ANOVA.

**4.4.4.3 Trans-Endothelial Electrical Resistance Measurements of HUVECs Transfected with Cx43-siRNA in the Presence and Absence of EGTA**

TEER measurements were taken every minute for HUVECs transfected with Cx43-siRNA in the presence and absence of 5 mM EGTA, as shown in Figure 4.10. HUVEC monolayer that had been transfected with Cx43-siRNA had a reduction TEER to 38 ± 0.6 Ω.cm² and this was reduced by adding EGTA to a level similar to that following the addition of EGTA to non-transfected cells (2.0 ± 0.3 Ω.cm², see section 3.4.5.3). The washout of EGTA caused a recovery of TEER to the pre-intervention value.
Figure 4.10: Effect of Cx43-siRNA transfection of HUVECs on TEER measurements with or without EGTA.

Transfection of HUVEC monolayers with Cx43-siRNA resulted in TEER measurements below 40 Ω.cm² compared to 88 Ω.cm² for non-transfected cells. However, the addition of EGTA caused the resistance to drop in both samples to almost 2 Ω.cm², although this effect was abolished when EGTA was cleared out by adding DMEM, *** P<0.001 TEER measurements of HUVECs transfected with Cx43-siRNA compared to a control monolayer treated with 5 mM EGTA. n=6, data expressed as means ± SEM, unpaired t test.
4.5 Discussion

There are currently a range of techniques available to study connexin gene function, including the transfection of cell lines (Halliday et al., 2003), the use of mutant constructs through molecular cloning (Joshi-Mukherjee et al., 2008), and cells from animal knockout studies (Beauchamp et al., 2004). In this investigation the effect of Cx43-siRNA on Cx43 protein expression in HUVECs was assessed and hence functional GJ formation between HUVECs and monocytes.

Cultured ECs are renowned for being difficult to transfect, whether for the purpose of exogenous over-expression of plasmid DNA or for genetic knockdown via silencing RNA. Therefore, optimal conditions are absolutely necessary for achieving relatively high transfection efficiency coupled with low cellular toxicity. In this study, an optimised protocol was used that knocked-down gene expression using siRNA in primary cultures of HUVECs (Table 4.2 and Figure 4.3). Previous studies have shown that siRNA delivery by chemical transfection caused a wide range of side effects depending on the cell type (Charolidi, 2008), and this led the physical delivery method of electroporation being explored. A key advantage of electroporation over other methods is that electropermeabilisation is independent of the cell-cycle stage, making it possible to deliver siRNA to non-dividing or slowly dividing cells (Ovcharenko et al., 2005). It also requires fewer pipetting steps, thereby reducing the variability of procedures and is more cost-effective when compared with chemical transfection.

The optimum electroporation parameters are generally specific for each cell type, but the key parameters that influence electroporation efficiency are the voltage and the pulse-length of the applied electric field. From the range of voltages and capacitances tested, the optimum electroporation parameters for siRNA delivery in HUVECs were at a lower voltage and higher
capacitance compared to those required for other cells. The voltage, which presumably
determines the pore size and number of pores formed in the plasma membrane, was the more
important parameter in determining the effectiveness of transfection. However, an increase
in voltage caused a significant decrease in the total level of CX43 protein expression
(Figure 4.3). This negative effect on cell viability was minimised by decreasing the pulse length
(capacitance) of the electrical field. Both the size of the electrical field and the pulse length
are also critical in ensuring cell recovery and membrane pore re-sealing. When the pores are
open for a prolonged period, cellular toxicity may be increased by the influx of external
medium (Bonnafous et al., 1999). In extreme conditions, intracellular content may leak out
due to osmotic swelling, leading to cell death (Gehl, 2003). It is also important to ensure that
the parameters are not too extreme on the cellular structure, since the intact function of the
cytoskeleton is important for pore closure (Teissie and Rols, 1994).

siRNA is highly specific, but its use at high concentrations may cause non-specific target effects
(e.g. interferon response) or off-target effects that include any alterations of non-target genes
(Shrey et al., 2009). It was also important to check for siRNA-mediated toxicity, since a
potential disadvantage of electroporation is that it requires higher amounts of siRNA for
successful gene silencing in comparison to chemical transfection (Ovcharenko et al., 2005). At
all the siRNA concentrations tested (0.25-1μM) knockdown of Cx43 did not affect cell survival.

The effectiveness of siRNA is transient and is also dependent on the half-life of the target
protein (Kurreck, 2009). A pronounced inhibition of protein expression was observed within
48 hours post-transfection, and although the level of inhibition decreased at day 3, a
significant level of down-regulation was still maintained. This may be expected since the half-
life of connexins is short, ranging from 1-5 hours (Laird, 2006). A reduction in the effectiveness
of siRNA may also be caused by cell division. It was also important that the duration of gene
silencing was sustained over days-2 and -3 for the monocyte TEM assays, which was the primary technique used to study connexin function.

In conclusion, siRNA parameters were optimised in terms of: (1) electroporation parameters to maximise siRNA transfection efficiency and minimise cell death (Figure 4.3); (2) siRNA concentration for best knockdown without causing any off-target effects (Figure 4.4); and (3) for how long the siRNA is stable (Figure 4.7).

Changes in GJ organisation and connexin expression can result in disturbances of normal cell-cell communication between monocytes and ECs. In this study the effects of changing Cx43 expression levels in HUVECs using RNAi to knockdown Cx43 expression was assessed. The major findings were: (1) knockdown of Cx43 produced a decrease in loading monocytes using the dye transfer assay by approximately 20% compared to 80% when Cx43 protein expression was normal; (2) knockdown of Cx43 produced almost no migration of monocytes in the TEM assay; and (3) TEER measurement with knockdown of Cx43 produced a decreased resistance of approximately 40 Ω.cm² compared to 85 Ω.cm² under normal conditions. All these findings will be discussed in detail in the next chapter.
Chapter 5 - Effect of Tumour Necrosis Factor Alpha on Connexin 43 Expression and its Function in Gap Junction Formation and Communication between HUVECs and Human Peripheral Monocytes
5.1 Introduction

TNF-α is a cytokine that contributes to a variety of inflammatory responses. The human TNF-α gene is located on chromosome 6 p21, and the most important source of this cytokine is monocytes/macrophages. When cells are stimulated with appropriate agonists, mRNA for TNF-α is induced and a membrane-bound precursor protein with a relative molecular mass of 26 kDa is produced. This precursor protein is processed by a membrane-bound metalloproteinase, TNF-α-converting enzyme, to generate secreted 17 kDa mature TNF-α (Nedwin et al., 1985).

TNF-α was thought to be produced primarily by macrophages, however, it is produced also by a broad variety of cell types including lymphoid cells, mast cells, ECs, cardiac myocytes, adipose tissue, fibroblasts, and neurons (Locksley et al., 2001).

TNF-α is a potent agonist activator of vascular EC, along with bacterial LPS and IL-1, and vascular ECs play an important role in the process of inflammatory responses (Brynskov et al., 2002). When ECs are stimulated with TNF-α, LPS, or IL-1, blood cell tethering molecules, such as E-selectin, ICAM-1, and VCAM-1 are expressed, together with signalling factors such as IL-8 and epithelial neutrophil activating peptide 78 (Ikuta et al., 1991; Imaizumi et al., 1997; Xia et al., 1998).

TNF-α binds to several receptors, all of which are cell type specific; TNF-receptor type 1 (TNFR1, 55 kDa) and type 2 (TNFR2, 64 kDa) (Theiss et al., 2005). TNFR1 is expressed in most tissues, and can be fully activated by both the membrane-bound and soluble trimeric forms of TNF-α, whereas TNFR2 is found only in cells of the immune system, and responds only to the membrane-bound form of the TNF-α homotrimer. Most information regarding TNF-α cell signalling pathways is based on the functional properties of TNFR1; however, the role of TNFR2 is likely to be underestimated (Theiss et al., 2005).
Binding of TNF-α to its receptor causes a conformational change to occur to the receptor, leading to the dissociation of the inhibitory protein silencer of death domains (SODD) from the intracellular death domain. This dissociation enables the adaptor protein, TNFRSF1A-associated via death domain (TRADD), to bind to the death domain, serving as a platform for subsequent protein binding. Following TRADD binding, three pathways can be initiated as demonstrated in Figure 5.1 (Wajant et al., 2002; Chen and Goeddel, 2002). The first and well-described pathway is initiated by activation of NF-κB, a heterodimeric transcription factor. Under physiological condition, NF-κB is located in the cytoplasm bound to an inhibitory protein, IκBα. Upon activation of TNFR1, IκB kinase (IKK) phosphorylates the inhibitory IκBα protein and it is subsequently degraded. This phosphorylation results in the dissociation of IκBα from NF-κB, which trans-locates to the nucleus and mediates the transcription of a vast array of genes involved in cell survival and proliferation, the inflammatory response, and anti-apoptotic factors. TNF-α also affects MAPK cell signalling pathways, and of the three major MAPK cascades, TNF-α induces a strong activation of the stress-related c-Jun N-terminal kinase (JNK) group, evokes the moderate response of the p38-MAPK, and is responsible for minimal activation of the classical extracellular signal–regulated kinases (ERKs).

Induction of death signalling is also affected by TNF-α, and like all death-domain-containing members of the TNFR superfamily, TNFR1 is involved in death signalling. However, TNF-induced cell death plays only a minor role compared to its overwhelming functions in the inflammatory process, and its death-inducing capability is weak compared to other family members (such as Fas), and often masked by the anti-apoptotic effects of NF-κB.

Another pathway through which TNF-α mediates the intracellular signalling pathways is through the activation of PKC. Several studies have shown that the PKC family of serine threonine protein kinases may be implicated in various aspects of TNF-α signalling. The PKC
family is composed of at least 11 isoforms, which are categorised into three groups (conventional PKCs α, βI, βII, and γ; novel PKCs δ, ε, η, and θ; and atypical PKCs ζ and λ/ι) according to their structure and mechanisms of activation (Azzi et al., 1992; Haller et al., 1994). Phosphorylation of serine threonine protein kinases residues is associated with the activation of PKCδ, PKCβII, and PKCζ, and in addition, it is currently believed that tyrosine phosphorylation may be important in the determination of substrate specificity of the enzyme (Gschwendt, 1999). Activation and inhibition of PKC by TNF-α in a variety of cell types has been reported (Schütze et al., 1990; Prasanna et al., 1998).
Figure 5.1: A general TNF-α signal transduction pathway.

Engagement of TNF-α with its cognate receptor TNFR1 results in the release of silencer of death domains (SODD) and formation of a receptor-proximal complex containing the important adaptor proteins TNFRSF1A-Associated Via Death Domain (TRADD), TRAF2, RIP, and FADD. These adaptor proteins in turn recruit additional key pathway-specific enzymes (for example, caspase-8 and IKKβ) to the TNFR1 complex, where they become activated and initiate downstream events leading to apoptosis, NF-κB activation, and JNK activation (adapted from QIAGEN, Gene gkop pathway).

TNF-α has been reported to trigger several cellular events to occur, including increased vascular permeability, up-regulation of cell surface adhesion molecules, and increased
migration of monocytes (Kleinbongard et al., 2010). Therefore, targeting the inflammatory pathway induced by TNF-α might be a promising therapeutic strategy to prevent atherosclerosis.

The integrity of the endothelial barrier is tightly regulated by intercellular junctional proteins to control para-cellular permeability. It is well established that ECs respond to acute-acting mediators, such as thrombin, by stimulating myosin light chain phosphorylation and stress fibre formation. As a result, cell retraction occurs which subsequently impairs barrier integrity (Birukova et al., 2004). In contrast, TNF-α-induced increased permeability is independent of the acto-myosin contractile mechanism (Petrache et al., 2001) and instead relies on the reorganisation of the junctional proteins (McKenzie and Ridley, 2007).

Recent studies have shown that the actions of TNF-α on ECs can occur through two specific transcription factors, NF-κB and activator protein-1 (AP-1), as well as induction of TEM (Collins et al., 1995). Both the NF-κB and the AP-1 pathways are activated by MAPKs pathways. Experiments in gene-knockout embryonic fibroblasts implicate MAPK-1 in the AP-1 pathway (Xia et al., 2000) and MAPK-3 in the NF-κB pathway (Yang et al., 2001) activated by TNF-α.

In response to TNF-α, IκB proteins are phosphorylated by IKK at critical serine residues, and once phosphorylated are rapidly ubiquitinated and then degraded by the cytosolic proteasome. In HUVECs, TNF-α causes the degradation of IκB-α, IκB-β, and IκB-ε (Johnson et al., 1996), and once an IκB protein is degraded, the associated NF-κB is free to move from the cytosol to the nucleus and activate transcription by binding to specific DNA sequences in the enhancers of target genes. TNF-α activated NF-κB consists of homodimers or heterodimers involving three different members of the Rel family, namely p50 (also called NF-κB1), p65 (also called Rel-A), and c-Rel. In experiments using the electrophoretic-mobility-shift assay, the
three E-selectin elements preferentially bind p50/p65 heterodimers (Lewis et al., 1994; Whitley et al., 1994).

AP-1 activation occurs when an MAP3K phosphorylates and activates several MAP2Ks, which in turn phosphorylate and activate several MAPKs, such as JNK-1 and JNK-2, and p38 MAPK (Baud and Karin, 2001). JNK-1 and JNK-2 phosphorylate the trans-activating domain of c-Jun, a component of AP-1, and thereby enable AP-1 to activate gene transcription. Both ICAM-1 and VCAM-1 also contain AP-1 binding sites (commonly called tetrahydrophorbol response elements) that bind AP-1 in TNF-α treated HUVECs (Cybulsky et al., 1993; Stade et al., 1990), which may explain the overexpression of ICAM-1 and VCAM-1 when ECs are treated with TNF-α (Ahmad et al., 1998).

However, other studies suggest that PKC has a role in mediating TNF-α in HUVECs. The inhibition of endothelial PKC modulates TNF-α-mediated effects on cell adhesion molecule (CAM) expression. Some studies have shown that PKC is translocated from the cytosol to the membrane of TNF-α activated HUVECs, while others have been unable to demonstrate this effect (Schütze et al., 1990; Deisher et al., 1993). It appears that PKC synthesis and by-products of PKC activity are increased in cytokine-stimulated HUVECs, yet multiple investigators have shown that PKC inhibitors decrease cell migration to and CAM expression in TNF-α stimulated HUVECs (Lane et al., 1990; Herbert, 1993).

Alterations in GJ expression have been noted in a number of different disease conditions with an underlying inflammatory process. In the liver, a rapid loss of Cx32 mRNA and protein was observed after the induction of an acute inflammatory state by the injection of endotoxin (Theodorakis and de Maio, 1999; Temme et al., 2000). Ischemia, which is known to up-regulate the expression of cytokines such as IL-1β and TNF-α in many tissues, has also been associated with the down-regulation of GJs composed of Cx32 in the liver (Gingalewski and De
Maio, 1997) and Cx43 in the heart (Gingalewski and De Maio, 1997). A more direct role for TNF-α in mediating the down-regulation of Cx43 in the heart after administration of bacterial endotoxin has been demonstrated by analysis of the response of the Cx43 promoter to this cytokine (Fernandez-Cobo et al., 1999). However, the injection of endotoxin into the kidneys or lungs resulted in increased expression of Cx43 at these sites (Fernandez-Cobo et al., 1997).

Although these data appear to be contradictory, it is possible that the influx of inflammatory cells could contribute to this response, since Cx43 has been detected in activated leukocytes (Jara et al., 1995). More recently, studies have examined the expression of Cx43 in reactive astrocytes associated with multiple sclerosis lesions. These lesions are known to contain high levels of IL-1β, a cytokine that down-regulates GJ connectivity, as well as Cx43 mRNA and protein in human astrocytes in vitro (John et al., 1999). Through immunohistochemistry, a marked loss of Cx43 immunoreactivity was demonstrated within the centre of active and chronic-active multiple sclerosis lesions, with normal expression of Cx43 immunoreactivity in the adjacent normal-appearing white matter (Brosnan et al., 2001). Taken together, these data support the conclusion that GJ expression may indeed be altered at sites of inflammation in vivo and could thus contribute to the pathogenic state.
5.2 Hypothesis

Because monocyte TEM requires functional GJs, it was hypothesised that TNF-α enhances this process, mediated by MAPKs or PKC.

5.2.1 Aims and Objectives

The aim of this study was to investigate the intracellular signalling pathway(s) mediating the effects of TNF-α on Cx43 expression.

1. Standardisation and optimisation of TNF-α in a time and dose dependent manner.
2. Assessment of the role of Cx43 in the formation of functional GJs between monocytes and HUVECs treated with TNF-α in the presence and absence of Cx43, using the dye transfer assay, TEM assay and TEER measurements.
5.3 Materials and Methods

5.3.1 Standardisation of TNF-α Concentration and Incubation Time

Different concentrations of TNF-α were used (6.25, 12.5, 25, and 50 ng/ml) to assess the most efficient concentration for Cx43 protein expression in HUVECs. After establishing an optimal concentration of TNF-α (25 ng/ml, see Figure 5.2), HUVECs were incubated for 1, 3, 6, 12, 24, 48, and 72 hours to assess the effectiveness of the length of TNF-α exposure on Cx43 expression. Subsequently, HUVECs were cultured in the presence or absence of 25 ng/ml TNF-α and the treated cells incubated overnight with TNF-α.

5.3.2 HUVEC Treatment with Chelerythrine Chloride

Chelerythrine chloride (CHE) affects the translocation of PKC from the cytosol to the plasma membrane; it is a potent and specific inhibitor of PKC and inhibits most of its isoforms, including α, β1, βII, γ, δ, ε, θ, η. CHE is supplied as powder, and for a 1 mM stock, 0.5 mg was reconstituted in 1.30 ml DMSO. The working concentration and the length of treatment was 5 μM overnight in the presence or absence of TNF-α.

5.3.3 Dye Transfer Assay

Normal HUVECs and HUVECs transfected with Cx43-siRNA monolayers were loaded with 5 mM calcein-AM dye in the presence or absence of 25 ng/ml TNF-α and/or 5 μM CHE. Suspensions of freshly isolated monocytes from peripheral blood were added to both monolayers and dye transfer to monocytes was measured by flow cytometry and normalised to positive control values obtained from monocytes directly loaded with calcein AM. The values are presented as the percentage of calcein AM transferred to monocytes.

5.3.4 Trans-Endothelial Migration Assay

Normal HUVECs and HUVECs transfected with Cx43-siRNA were seeded and grown on 6-wells Transwell polyester plates with 3.0 μm pore size membranes in the presence or absence of 25
ng/ml TNF-α and/or 5 μM CHE. Monocytes were then added to the upper compartment (3-5×10⁵ cells), and after six hours the cells in the upper compartment, as well as those that had transmigrated to the lower compartment, were collected separately, marked with the CD14+ antibody and counted by flow cytometry.

5.3.5 Trans-Endothelial Electrical Resistance Measurements

Normal HUVECs and HUVECs transfected with Cx43-siRNA were seeded and grown on 6-wells Transwell polyester plates with 0.8 μm pore size membranes in the presence or absence of 25 ng/ml TNF-α and/or 5 μM CHE. HUVEC monolayer functional integrity was assessed by measuring the TEER.
5.4 Results

5.4.1 Optimisation of the Effect of TNF-α in a Dose and Time Dependant Manner

Before investigating and assessing the effects of TNF-α on the role of Cx43 and the underlying mechanism, the appropriate concentration of TNF-α needed to be established and optimised in terms of how long ECs can be incubated with it without causing any lethal effects.

A range of TNF-α concentrations (6.25, 12.5, 25, and 50 ng/ml) were applied to HUVECs, and were chosen based on reports in the literature. The level of Cx43 protein expression in the treated HUVECs was examined by Western blotting (Figure 5.2). At higher concentrations of TNF-α there was decreased Cx43 protein expression, especially when the concentration of TNF-α reached 50 ng/ml (0.01 ± 0.00 vs. control: 1.4 ± 0.04, P<0.001, one-way ANOVA), where Cx43 levels were significantly very low and could barely be detected compared to the when 25 ng/ml TNF-α was applied (0.25 ± 0.01 vs. control: 1.4 ± 0.04, P<0.001, one-way ANOVA).
Figure 5.2: Effect of different concentrations of TNF-α on Cx43 expression in HUVECs.

A range of TNF-α concentrations (6.25, 12.5, 25, and 50 ng/ml) was applied to HUVECs. 50 ng/ml (1.4 ± 0.04) was the highest concentration used and it significantly reduced Cx43 expression to almost nothing, whereas 25 ng/ml (0.25 ± 0.01) was the optimal concentration of choice. ***P<0.001 levels of Cx43 protein expression in HUVECs treated with different concentrations of TNF-α (6.25, 12.5, 25, and 50 ng/ml compared to control. n=3, data are expressed as means ± SEM, one-way ANOVA.

After selecting the optimal concentration of TNF-α (25 ng/ml), the effect of the duration of TNF-α incubation on Cx43 protein expression was determined. Figure 5.3 shows Cx43 protein expression after incubation periods of 0, 1, 3, 6, 12, 24, 48, and 72 hours. Levels of Cx43 decreased significantly after an overnight of incubation (12 hours, 1.03 ± 0.2 vs. control: 1.19 ± 0.03, P<0.05, two-way ANOVA) and then a gradual decrease in expression was noted at 24 (0.8 ± 0.03, P<0.01), 48 (0.5 ± 0.01, P<0.001), and 72 (0.06 ± 0.01, P<0.001) hours, two-way ANOVA.
Figure 5.3: Effect of length of incubation with TNF-α on Cx43 expression in HUVECs.

Levels of Cx43 started to decrease significantly after 12 hours of incubation and decreased more with greater times. *P<0.05, **P<0.01, ***P<0.001 levels of Cx43 protein expression in HUVECs treated with TNF-α (25ng/ml) and incubated for 12, 24, 48, and 72 hours (1.03 ± 0.2, 0.8 ± 0.03, 0.5 ± 0.01, 0.06 ± 0.01, respectively) compared to control cells (1.19 ± 0.03). n=3, data expressed as means ± SEM, two-way ANOVA.

Interestingly, the morphology of the treated HUVECs also changed after 24 hours of incubation with 25 ng/ml of TNF-α (Figure 5.4). The normal morphology of HUVECs, as discussed in Chapter 3, is a cobblestone-shape and this morphology remained unchanged with the length of incubation after reaching confluence. However, when HUVECs were treated with TNF-α (25 ng/ml), the cell morphology started to change and become spindle-shaped after 24 hours of incubation, and even more spindle-shaped with increased cell detachment after 48 hours. Although, these data do not provide a functional assessment of the integrity of the HUVECs, it provides structural confirmation of HUVECs integrity.
Figure 5.4: Effect of length of incubation with TNF-α on the morphology of HUVECs.
(A) Cobblestone-shape morphology of HUVECs cultured under control conditions and in the presence of TNF-α (25 ng/ml) after 0.00 hours. (B) Cobblestone-shape morphology of HUVECs cultured under control conditions and the spindle-shaped morphology of HUVECs after 24 hours of exposure to TNF-α. (C) Morphology of both treated and untreated HUVECs after 48 hours.

5.4.2 Intracellular Signalling Mediates the TNF-α Effect on Trans-Endothelial Migration

5.4.2.1 Role of MAPK Pathways

Figure 5.5 shows the protein expression levels of both total and phosphorylated MAPKs that may mediate the effects of TNF-α; c-Jun N-terminal kinases (JNK, 2.05 ± 0.02, P>0.05), phosphorylated JNK (pJNK, 2.05 ± 0.02, P>0.05), ERK (1.32 ± 0.08, P>0.05), phosphorylated
ERK (pERK, 1.08 ± 0.05, P>0.05), P38 mitogen-activated protein kinases (1.08 ± 0.08, P>0.05) and phosphorylated P38 (pP38, 1.05 ± 0.08, P>0.05) under control conditions and when compared to a HUVEC monolayer activated by TNF-α (25 ng/ml, incubated overnight). The mean total expression of all MAPKs and their phosphorylated forms were not different when compared between inactivated HUVEC monolayer and those activated by TNF-α. This indicates that these proteins in this set of experiments are not mediating any observed effects of TNF-α.
Figure 5.5: Assessment of MAPKs expression in HUVECs treated TNF-α.

Western blots and mean protein expression levels for Cx43 and the MAPKs; c-Jun N-terminal kinases (JNK, 2.05 ± 0.02, P>0.05), phosphorylated JNK (pJNK, 2.05 ± 0.02, P>0.05), extracellular signal-regulated kinases (ERK, 1.32 ± 0.08, P>0.05), phosphorylated ERK (pERK, 1.08 ± 0.05, P>0.05), P38 mitogen-activated protein kinases (P38, 1.08 ± 0.08, P>0.05) and phosphorylated P38 (pP38, 1.05 ± 0.08, P>0.05). Cx43 protein expression is less than 50% (1.2 ± 0.1 vs. control: 2.9 ± 0.2) when the HUVEC monolayer was treated with TNF-α compared to untreated HUVECs. However, MAPK protein levels showed no significant difference in expression between untreated and treated HUVECs, ***P<0.001 levels of Cx43 protein expression in HUVECs treated with TNF-α (25 ng/ml) compared to control. n=3, data are expressed as means ± SEM, two-way ANOVA.
5.4.2.2 Role of Protein Kinase C

PKC is activated by TNF-α in vascular ECs. Therefore, the contribution of PKC pathway inhibition in ECs as a downstream target of TNF-α was investigated.

Figure 5.6 illustrates the expression of total PKC protein levels when a HUVEC monolayer is stimulated with TNF-α, and there was a significant increase in total PKC protein expression to almost two folds above that seen in the absence of TNF-α (1.72 ± 0.02 vs. control 0.96 ± 0.05, P<0.001, one-way ANOVA). The effect of the PKC inhibitor, CHE was assessed and it was found that the expression of PKC showed no significant difference in the absence of TNF-α with or without CHE. However, when the monolayer was activated with TNF-α, PKC protein expression was altered and increased one-fold in the absence of CHE, but when the PKC inhibitor was present this increase was negated (0.96 ± 0.02, P<0.001, one-way ANOVA).

The level of Cx43 expression in HUVECs was also measured when HUVECs were activated with TNF-α in the presence and absence of CHE. Cx43 expression decreased significantly under TNF-α stimulation (1.17 ± 0.09 vs. control 2.82 ± 0.07, P<0.001); however, this reduction was not apparent when CHE was applied to the activated cells (2.68 ± 0.10 vs. treated HUVECs with TNF-α: 1.17 ± 0.09, P<0.001). Therefore CHE stopped the mediating effect of TNF-α through inhibiting the action of PKC (Figure 5.7), one-way ANOVA.
Figure 5.6: Levels of PKC expression in HUVECs treated with a PKC inhibitor in the presence and absence of TNF-α.

Western blots and mean protein expression levels of PKC in HUVECs treated with/without both TNF-α and CHE. PKC expression was significantly increased when TNF-α was applied, however, the effect was abolished when CHE was used. *** P < 0.001 levels of total PKC expression in HUVECs treated with TNF-α (25 ng/ml) compared to control cells (1.72 ± 0.02 vs. control 0.96 ± 0.05), δδδ P < 0.001 levels of total PKC expression in HUVECs treated with TNF-α (25 ng/ml) compared to levels of total PKC expression in HUVECs treated with TNF-α (25 ng/ml, (0.96 ± 0.02 and 1.72 ± 0.02, respectively). n=3, data are expressed as means ± SEM, one-way ANOVA.
5.4.3 Role of TNF-α on Gap Junction Formation between HUVECs and Monocytes

5.4.3.1 Dye Transfer between Monocytes and HUVECs

After optimisation and the investigation of possible pathways that may mediate the effect of TNF-α, a set of functional experiments were undertaken to assess the role of TNF-α in different cell–cell communications and its role in the initiation of TEM, which leads to atherosclerosis.

Dye transfer experiments (Figure 5.8) showed that TNF-α significantly increased dye transfer between monocytes and HUVECs, to around 89.63% ± 2.35 compared to the control cells,
around 74.10% ± 1.43 (P<0.001, one-way ANOVA). However, when CHE was applied, this was abolished and dye transfer returned to that of the non-activated cells (74.10 % ± 1.43).

![Graph showing the effect of TNF-α on dye transfer between monocytes and HUVECs.](image)

**Figure 5.8: Effect of TNF-α on dye transfer between monocytes and HUVECs.**

TNF-α increased the number of dye-loaded monocytes (89.63% ± 2.35 vs. control 74.10% ± 1.43); however, this increasing was reduced when CHE was applied to almost the level seen for the non-activated cells (74.10 % ± 1.43). *** P <0.001 % of calcein AM loaded monocytes that transferred from HUVECs treated with TNF-α (25 ng/ml) compared to loaded monocytes from non-treated HUVECs control. δδδ P <0.001 % of calcein AM loaded monocytes that transferred from HUVECs treated with TNF-α (25 ng/ml) and CHE compared to loaded monocytes from HUVECs treated with TNF-α. n=3, data are expressed as means ± SEM, one-Way ANOVA.

### 5.4.3.2 Effect of TNF-α on Monocytes Trans-Endothelial Migration

The effect of TNF-α on GJ formation and communication was demonstrated in the dye transfer assay, and the next step was to examine adhesion and monocytes transmigration. HUVECs were seeded into 6-well Transwell plates and stimulated by adding TNF-α to the lower chamber of the insert. Monocytes were added to the upper compartment and allowed to adhere and migrate to the lower chamber. After 3 hours, the cells in the upper and lower chambers were collected and labelled with the CD14+ antibody to assess the percentage of monocytes in the upper and lower chambers. Figure 5.9 shows the number of monocytes present in the upper chamber after 3 hours, the number of monocytes that did not migrate because they had adhered (calculated as the difference between the monocytes in both the upper and lower chambers), and number of monocytes that had migrated into the lower
chamber. More monocytes migrated to the lower chamber when HUVECs were activated with TNF-α, at around $1.1 \times 10^5 \pm 0.04 \times 10^5$ compared to $0.1 \times 10^5 \pm 0.01 \times 10^5$ (P<0.001) for the non-activated monolayer, which is a 10-fold increase. However, this effect was abolished when a PKC inhibitor was applied (CHE: $0.1 \times 10^5 \pm 0.01 \times 10^5$, P<0.001), two-way ANOVA.

Figure 5.9: Effect of TNF-α on monocytes trans-endothelial migration.
An increased number of monocytes migrated from the upper to the lower chamber when HUVECs were activated with TNF-α, but this effect was negated when CHE was applied ($0.1 \times 10^5 \pm 0.01 \times 10^5$ vs. HUVECs stimulated with TNF-α: $1.1 \times 10^5 \pm 0.04 \times 10^5$). Similarly, there was an increase in the number of adhered monocytes (1.2$ \times 10^5 \pm 0.73 \times 10^5$ vs. adhered HUVECs stimulated with TNF-α: $1.5 \times 10^5 \pm 0.14 \times 10^5$, ** P<0.01 number of adhered monocytes calculated as the difference between the upper and lower chambers for HUVECs treated with TNF-α compared to the adhered control, ααα P<0.001 number of migrated monocytes in the lower chamber that crossed the HUVEC monolayer treated with TNF-α compared to the control, βββ P<0.001 number of monocytes collected from upper chamber for HUVECs with treated TNF-α and CHE compared to HUVECs treated with TNF-α alone, γγγ P<0.001 number of adhered monocytes calculated from the difference between monocytes in the upper and lower chambers for HUVECs treated with TNF-α and CHE compared to adhered HUVECs monolayer treated with TNF-α alone, δδδ P<0.001 number of migrated monocytes in the lower chamber that had crossed the HUVECs monolayer treated with TNF-α and CHE compared to HUVECs treated with TNF-α alone. n=3, data are expressed as means ± SEM, two-way ANOVA.

5.4.3.3 Effect of TNF-α on Trans-Endothelial Electrical Resistance Measurements
PKC has been shown to be activated by TNF-α in vascular ECs; therefore, the effect of TNF-α via PKC pathway inhibition on EC permeability and integrity was investigated. HUVECs were cultured in 6-well snapwell tissue culture inserts and grown to confluency. Both the upper
compartment (blood side) and lower compartment (intimae side) were separately accessible for medium removal, exchange, and the recovery of cells. Monolayer integrity was tested by assessing the TEER.

Figure 5.10 shows the TEER measurements for HUVECs under control conditions, HUVECs stimulated with TNF-α and incubated for 48 hours, and HUVECs treated with both TNF-α and the PKC inhibitor, CHE. From the reading measurements, the monolayer treated with both TNF-α and CHE managed to maintain its permeability and its integrity at around 62.28 ± 0.12 Ω.cm², whereas the monolayer that was treated only with TNF-α demonstrated a decrease in permeability and integrity as it was reduced to 32.15 ± 3.82 Ω.cm² (P<0.001, unpaired t test). The drop in TEER measurements suggests HUVEC cell-cell uncoupling.

![Figure 5.10: Effect of TNF-α on HUVEC monolayer integrity.](image)

TEER measurements showed a reduction in endothelial resistance when the monolayer was activated with TNF-α (32.15 ± 3.82 Ω.cm²) which led to a loss of integrity and increased cell-cell uncoupling; however, this effect was abolished when CHE was applied (62.28 ± 0.12 Ω.cm² vs. control: 62.28 ± 0.12 Ω.cm²). *** and ααα P<0.001 TEER measurements for treated HUVEC monolayer compared to normal control HUVEC monolayer, and HUVEC monolayer treated with both TNF-α and CHE, respectively for each time point. n=6, data expressed as means ± SEM, unpaired t test.
5.4.4 Role of TNF-α in Gap Junction Formation between HUVECs Transfected with Cx43-siRNA and Monocytes

5.4.4.1 Dye Transfer between Monocytes and HUVECs Transfected with Cx43-siRNA

The data in Chapter 4 showed that knocking-down expression of Cx43 significantly reduced the dye transfer process. Therefore, dye transfer between monocytes and HUVECs activated with TNF-α in the presence and absence of Cx43-siRNA with/without CHE was assessed. The TNF-α activated HUVEC monolayer treated with Cx43-siRNA showed dye transfer to occur in around 13.07% ± 1.72 (P<0.001, one-way ANOVA) of monocytes compared to 85.70% ± 2.85 for HUVECs transfected with scrambled Cx43-siRNA (Figure 5.11). Figure 5.12 illustrates the effect of the PKC inhibitor CHE when incubated with HUVECs transfected with Cx43-siRNA and stimulated with TNF-α, and it can be seen that the addition of CHE has no effect. TNF-α increases the number of dye-loaded monocytes for HUVECs transfected with scrambled Cx43-siRNA. However, this increase is reduced in TNF-α stimulated endothelial cells in the presence of CHE (85.70% ± 2.85, 74.77% ± 1.20, respectively), (P<0.001, one-way ANOVA).
Figure 5.11: Effect of TNF-α on dye transfer between monocytes and HUVECs transfected with Cx43-siRNA.

TNF-α increases the number of dye-loaded monocytes that dropped onto HUVECs transfected with scrambled Cx43-siRNA. However, this increase is reduced when HUVECs transfected with Cx43-siRNA are activated with TNF-α (85.70% ± 2.85, 13.07% ± 1.72, respectively). δδδ P <0.001 % of calcein AM loaded monocytes for HUVECs transfected with Cx43-siRNA compared to loaded monocytes for HUVECs transfected with scrambled Cx43-siRNA, γγγ P <0.001 % of calcein AM loaded monocytes for HUVECs transfected with Cx43-siRNA and treated with TNF-α compared to loaded monocytes for HUVECs transfected with scrambled Cx43-siRNA and stimulated with TNF-α. n=3, data are expressed as means ± SEM, one-way ANOVA.
Figure 5.12: Effect of TNF-α on dye transfer between monocytes and HUVECs transfected with Cx43-siRNA in the presence and absence of a PKC inhibitor.

TNF-α increases the number of dye-loaded monocytes for HUVECs transfected with scrambled Cx43-siRNA. However, this increase is reduced in TNF-α stimulated endothelial cells in the presence of CHE (85.70% ± 2.85, 74.77% ± 1.20, respectively), ααα P < 0.001 % of calcein AM loaded monocytes for HUVECs transfected with Cx43-siRNA compared to loaded monocytes for HUVECs transfected with scrambled Cx43-siRNA, δδδ P < 0.001 % of calcein AM loaded monocytes for HUVECs transfected with Cx43-siRNA and treated with TNF-α compared to loaded monocytes for HUVECs transfected with scrambled Cx43-siRNA and stimulated with TNF-α, and γγγ P < 0.001 % of calcein AM loaded monocytes for HUVECs transfected with Cx43-siRNA and treated with TNF-α and CHE compared to loaded monocytes for HUVECs transfected with scrambled Cx43-siRNA and stimulated with TNF-α and CHE.

n=3, data are expressed as means ± SEM, one-way ANOVA.

5.4.4.2 Effect of Endothelial Cx43 Expression on Monocytes Trans-Endothelial Migration

Figure 5.13 illustrates the effect of TNF-α on monocyte migration by knocking-down Cx43 expression in HUVECs through treatment with Cx43-siRNA. Activation by TNF-α resulted in increased monocyte migration for non-transfected HUVECs; however, for HUVECs transfected with Cx43-siRNA the effect of TNF-α was reduced both in terms of adhesion and migration to the lower chamber (0.36×10^5 ± 0.06×10^5, 0.12×10^5 ± 0.04×10^5, respectively, P<0.001, two-way ANOVA). The effect of the PKC inhibitor CHE was investigated and showed that when CHE was applied to HUVECs transfected with Cx43-siRNA, the number of cells in the upper chamber, number of adhered cells, and number of cells in the lower chamber were all affected (4.35×10^5 ± 0.16×10^5, 0.39×10^5 ± 0.06×10^5, 0.12×10^5 ± 0.04×10^5 respectively), as shown in Figure 5.14.
Figure 5.13: Effect of TNF-α on monocytes trans-endothelial migration for HUVECs transfected with Cx43-siRNA.

The number of migrated monocytes from the upper to the lower chamber was reduced for HUVECs transfected with Cx43-siRNA (0.36×10⁵ ± 0.06×10⁵) and even the increased number of migrated monocytes from the upper to the lower chamber following TNF-α activation was reduced (0.12×10⁵ ± 0.04×10⁵), *** P <0.001 number of monocytes collected from the upper chamber for HUVECs transfected with Cx43-siRNA compared to non-transfected HUVECs, ααα P <0.001 number of adhered monocytes calculated as the difference between monocytes in the upper and lower chambers for HUVECs transfected with Cx43-siRNA compared to non-transfected HUVECs, βββ P <0.001 number of migrated monocytes in the lower chamber that crossed HUVECs transfected with Cx43-siRNA compared to non-transfected HUVECs, γγγ P <0.001 number of adhered monocytes calculated as the difference between monocytes in the upper and lower chambers for HUVECs transfected with Cx43-siRNA and treated with TNF-α compared to non-transfected HUVECs treated with TNF-α, and δδδ P <0.001 number of migrated monocytes in the lower chamber that had crossed HUVECs transfected with Cx43-siRNA and treated with TNF-α compared to non-transfected HUVECs treated with TNF-α. n=3, data are expressed as means ± SEM, two-way ANOVA.
Figure 5.14: Effect of TNF-α on monocytes trans-endothelial migration for HUVECs transfected with Cx43-siRNA in the presence or absence of a PKC inhibitor.

The number of migrated monocytes from the upper to the lower chamber was reduced for HUVECs transfected with Cx43-siRNA (0.36×10^5±0.06×10^5) and even the increased number of migrated monocytes following TNF-α activation was reduced for HUVECs transfected with Cx43-siRNA in the presence and absence of CHE (0.12×10^5±0.04×10^5, 0.12×10^5±0.04×10^5, respectively), *** P <0.001 number of monocytes collected from the upper chamber for HUVECs transfected with Cx43-siRNA and treated with both TNF-α and CHE compared to non-transfected HUVECs treated with TNF-α and CHE, γγγ P <0.001 number of adhered monocytes calculated as the difference in monocytes between the upper and lower chambers for HUVECs transfected with Cx43-siRNA and treated with both TNF-α and CHE compared to non-transfected HUVECs treated with TNF-α and CHE, and δδδ P <0.001 number of migrated monocytes in the lower chamber that has crossed HUVECs transfected with Cx43-siRNA and treated with TNF-α and CHE compared to non-transfected HUVECs treated with TNF-α and CHE. n=3, data are expressed as means ± SEM, two-way ANOVA.
5.4.5 TNF-α Enhances IKKα and NFκBp50 Protein Expression in Endothelial Cells through Activation of Protein Kinase C

Prior to studying the effects of TNF-α on Cx43 phosphorylation, the effects of TNF-α on the level of protein expression of intracellular protein kinases leading to the activation of NFκB intracellular signalling pathways was investigated. The effect of TNF-α on PKC, as one of the main TNF-α mediators in this study, and on IKKα, IκBα and NFκBp50 protein expression in ECs was analysed by Western blotting.

The effect of TNF-α on IKKα, IκBα and NFκBp50 protein expression was detected and analysed by Western blotting in HUVECs with and without activation by TNF-α and in the presence or absence of the PKC inhibitor CHE (Figure 5.15). The effect of TNF-α on IKKα protein expression (1.44 ± 0.03) was reversed when CHE was applied (0.45± 0.02). In addition, CHE significantly reduced the increased expression of IKKα in HUVECs activated by TNF-α and maintained expression as in the control sample (0.45 ± 0.02, 0.45 ± 0.02, respectively) (P<0.001, one-way ANOVA). In contrast, the opposite was observed for IκBα where CHE abolished the reduction of IκBα expression caused by TNF-α activation and to that seen in the control sample (IκBα: 1.44 ± 0.03, with TNF-α: 0.53 ± 0.03, with TNF-α and CHE: 1.44 ± 0.03, P<00.1, one-way ANOVA). Expression of NFκBp50 was increased when HUVECs were activated with TNF-α; however, the effect was negated when CHE was applied (NFκBp50: 0.50 ± 0.03, with TNF-α: 2.11 ± 0.11, with TNF-α and CHE: 0.53 ± 0.03, P<00.1, one-way ANOVA).
Figure 5.15: Effect of TNF-α on IKKα, IκBα and NFκBp50 expression in HUVECs in the presence and absence of a PKC inhibitor.

(A) Effect of CHE on IKKα expression in non-activated and activated HUVECs, TNF-α increased the expression of IKKα, however, the effect was reduced when CHE was applied (IKKα: 0.45 ± 0.02, with TNF-α: 1.44 ± 0.03, with TNF-α and CHE: 0.45 ± 0.02).

(B) Effect of CHE on IκBα expression in non-activated and activated HUVECs, TNF-α decreased the expression of IκBα, however, the effect was negated when CHE was applied (IκBα: 1.44 ± 0.03, with TNF-α: 0.53 ± 0.03, with TNF-α and CHE: 1.44 ± 0.03).

(C) Effect of CHE on NFκBp50 expression in non-activated and activated HUVECs, TNF-α increased the expression of NFκBp50, however, the effect was reduced when CHE was applied (NFκBp50: 0.50 ± 0.03, with TNF-α: 2.11 ± 0.11, with TNF-α and CHE: 0.53 ± 0.03), *** P <0.001 levels of IKKα, IκBα and NFκBp50 protein expression in HUVECs activated with TNF-α compared to control HUVECs, γγγ P <0.001 levels of IKKα, IκBα and NFκBp50 protein expression in HUVECs treated with TNF-α and CHE compared to HUVECs treated with TNF-α alone. n=3, data are expressed as means ± SEM, one-way ANOVA.
5.4.6 TNF-α Induces Protein Kinase C Mediated Phosphorylation of Cx43 and Enhances Phosphorylated Cx43 Expression

The above section has shown that the expression of intracellular proteins linked to the NFκB pathway is altered by TNF-α. The next step involved investigating the mechanism of Cx43 phosphorylation by PKC and the effect of TNF-α on PKC-mediated phosphorylation of Cx43.

Figure 5.16 shows the effect of TNF-α on Cx43 expression, as well as the phosphorylated form of Cx43, pS368-Cx43. As observed previously, TNF-α reduced the expression of Cx43 in HUVECs; however, this reduction was abolished when the PKC inhibitor CHE was applied. In contrast, the expression of pS368-Cx43 was significantly increased under TNF-α stimulation (0.95 ± 0.02) compared to non-activated HUVECs (0.05 ± 0.01), although the effect of TNF-α was negated when CHE was applied (0.08 ± 0.02) (P<0.001, one-way ANOVA).
Fig. 5.16: Effect TNF-α on phosphorylated Cx43 expression in HUVECs activated with TNF-α in the presence and absence of a PKC inhibitor.

TNF-α reduced the expression of Cx43; however, this reduction was abolished when CHE was applied. Expression of pS368-Cx43 showed a significant increase under TNF-α stimulation (0.95 ± 0.02) compared to non-activated cells (0.05 ± 0.01), but again the effect was negated when CHE was applied (0.08 ± 0.02). *** P <0.001 levels of total Cx43 and pS368-Cx43 protein expression in HUVECs activated with TNF-α compared to non-activated HUVECs, respectively, γγγ P <0.001 levels of total Cx43 and pS368-Cx43 protein expression in HUVECs treated with TNF-α and CHE compared to HUVECs treated with TNF-α alone, respectively. n=3, data are expressed as means ± SEM, one-way ANOVA.
5.5 Discussion

GJ intercellular channels are constructed from a family of multispanning proteins known as connexins (White and Bruzzone, 1996). Among all the connexin isoforms, most is known about the biochemical properties and the post-translational modifications of Cx43. This connexin is the most widely expressed and predominant isoform endogenously expressed in most cell types. In fact, even cell lines derived from tissues that do not normally express Cx43 often express it extensively, and in some cases exclusively (Lampe and Lau, 2004b, Ek-Vitorin et al., 2006). To date, the only role of Cx43 is to form GJ channels between cells, and disruption of these channels by any means decreases intercellular communication. For these reasons, Cx43 was used as the primary framework for this study.

The modulation of EC morphology from epithelial to fibroblastoid was consistently found in this study. This phenomenon has also been noted by others in HUVECs (Sato et al., 1986; Stolpen et al., 1986) and was accompanied by a change in cytoskeletal actin filament rearrangement, from dense peripheral bands with faint centrally located filaments, to parallel arrays of longitudinal stress fibres. Moreover, there is a loss of fibronectin, the major matrix constituent of cultured HUVECs (Jaffe and Mosher, 1978) after exposure to TNF-α (Stolpen et al., 1986).

After exposure to TNF-α, a 40% decrease of Cx43 protein expression in HUVECs was recorded. Levels of Cx40 and Cx37 were not measured due to their lack of expression in non-activated cells. This reduction of Cx43 may be caused by altered protein composition, since after exposure to TNF-α, GJs between HUVECs consist mainly of Cx43. Another mechanism responsible for the observed reduction may be connexin phosphorylation via PKC. It is known that the action of TNF-α is associated with the activation of PKC (Zhang et al., 2002; Javaid et al., 2003). It has been shown in this study that after activation of PKC, Cx43 expression in
HUVECs was reduced by 40%, and activation of PKC has also been shown to decrease the dye permeability of Cx43 GJ channels (Larson et al., 1990; Kwak and Jongsma, 1996), the channel type that is exclusively found in HUVECs after exposure to TNF-α. A third mechanism, by which Cx43 expression in HUVECs can be modified by TNF-α, is by changing the morphology of HUVECs. The transition from the typical cobblestone shape to the spindle-shaped appearance, thereby changing the contact area between adjacent cells (as depicted in Figure 5.4), may also lead to differences in Cx43 expression properties. Taken together, these three mechanisms lead to a substantial decrease in Cx43 expression in endothelial monolayers after exposure to TNF-α. A similar effect was found for SMCs from human umbilical arteries (Mensink et al., 1995).

In the discipline of GJ biology, PKC has received considerable attention because PKC activators which promote tumourigenesis, both increase Cx43 phosphorylation and decrease GJ communication in a number of different cell types (Brissette et al., 1991; Reynhout et al., 1992; Lampe, 1994). Moreover, several PKC isoforms are implicated in Cx43 regulation (Lampe and Lau, 2004; Bowling et al., 2001; Cruciani et al., 2001). The introduction of several inhibitors and activators of PKC specific for different isotypes has helped identify PKC isoforms responsible for Cx43 regulation (Bowling et al., 2001; Lampe and Lau, 2004; Cruciani et al., 2001). For example, PKCγ can phosphorylate Cx43 and reduce Cx43 GJ formation in lens epithelial cells, and experiments with specific inhibitors also indicate that PKCα, PKCβ and PKCδ can disrupt coupling between fibroblasts (Lampe and Lau, 2004b). Furthermore, PKCα and PKCε can phosphorylate Cx43 in cardiomyocytes (Bowling et al., 2001).

Phosphorylation of connexins has been implicated in the regulation of GJ communication at several stages during the connexin life cycle, including hemichannel oligomerisation, export of the protein to the plasma membrane, hemichannel activity, GJ assembly, GJ channel gating,
and connexin degradation (Lampe and Lau, 2004; Solan and Lampe, 2009; Solan and Lampe, 2005). Recent investigations have shown that activation of PKC can lead to the phosphorylation of serine residues in the cytoplasmic carboxyl-terminal domain of Cx43 (Bao et al., 2004; Lampe et al., 2000). On treatment with the PKC activator, phorbol 12-myristate 13-acetate, Cx43 phosphorylation is increased and GJ communication is decreased in cardiomyocytes, madin-darby canine kidney cells, rat liver epithelial cells and bovine lens cells (Lampe, 1994; Brissette et al., 1991; Reynhout et al., 1992).

The introduction of several inhibitors and activators of PKC that are specific for certain isoforms has led to the investigation of its role in the downregulation of GJIC. Recent studies have shown that overexpression of PKCγ or phorbol ester treatment of lens epithelial cells caused a reduction in the cell surface expression of Cx43 and allowed co-immunoprecipitation of PKCγ and Cx43 (Wagner et al., 2002). However, in various fibroblast systems, inhibition of GJ communication was found to be dependent on PKCα, PKCβ and PKCδ to different extents (Cruciani et al., 2001). PKCα and PKCε associate with Cx43 expressed in cardiomyocytes (Bowling et al., 2001) and FGF-2, which decreases cardiomyocyte GJ permeability and increases Cx43 phosphorylation, increased colocalisation of PKCε with Cx43 (Doble et al., 2000). In addition, our studies have shown that phosphorylated Cx43 expression is reduced in ECs treated with a PKC specific inhibitor. Similar effects were also found in TNF-α stimulated ECs treated with a PKC inhibitor indicating that Cx43 phosphorylation in ECs is PKC-dependent.

Thus, it several members of the conventional and novel PKC families influence GJ communication. One confounding factor is that different cells express distinct isotypes and consequently respond differently to these agents; therefore, discretion should be exercised when referring to the spectrum of effects of these inhibitors on this group of proteins. Understanding the actual sites of phosphorylation and the consequences of these events will
probably be necessary before the importance of different PKC isoforms in the regulation of GJ communication is fully understood.

Known inducers of NF-κB activity are highly variable and include reactive oxygen species, TNF-α, IL-1β, LPS, isoproterenol, cocaine, and ionising radiation. The principal signalling pathway following TNF-α release is protein tyrosine phosphorylation. The tyrosine phosphorylation cascade ultimately activates several transcription factors, such as NFκB (Hermiston et al., 2002). The common signalling element downstream of EC co-stimulation is phosphatidylinositol 3-kinase (PI3K). Co-stimulatory signalling via PI3K results in the recruitment of PKCθ, which is a Ca²⁺ independent serine/threonine kinase, an essential member of the NFκB activation cascade, and of central importance in the mediation of EC differentiation (Marsland and Kopf, 2008).

It is well documented that the effects of TNF-α are mediated through signalling pathways, especially via the activation of transcription factors, which in turn stimulate the expression of genes involved in the inflammatory and oxidative stress response or in cell cycle regulation. NFκB has often been referred to as a central mediator of the immune response because of its regulation of the expression of inflammatory cytokines, chemokines, immune receptors, and cell surface adhesion molecules (Li and Stark, 2002). NFκB is thought to be involved in multiple steps in the progression of atherosclerosis, including the initiation of monocyte adhesion, foam cell formation, and inflammation (de Winther et al., 2005). In macrophages, it has been reported that oxLDL activates NFκB via PKC and/or Ca²⁺-dependent pathways, and that this phenomenon does not involve the endocytic processing of oxLDL (Han et al., 2000). Of interest, the results in this study showed that in vitro stimulation of ECs with TNF-α enhanced PKC, IKKα, IKKβ and NFκBp50 protein expression. This could indicate that TNF-α activates NFκB in ECs through a PKC-dependent phosphorylation mechanism, and it should also be noted that
TNF-α might activate different signalling pathways, which interact with each other. These interactions may reflect the complicated cross-talk between intracellular signalling pathways induced by TNF-α and other pro-atherogenic signals.
Chapter 6 - General Discussion and Future Work
6.1 General Discussion

Atherosclerosis is a progressive multifactorial disease that affects medium size and large arterial vessels. From the initial phases of monocyte recruitment, to the eventual rupture of a vulnerable atherosclerotic plaque, inflammatory mediators appear to play a key role in the pathogenesis of atherosclerosis (Libby et al., 2011).

Recent data have shown evidence that GJs not only play a role in monocytes transmigration, but also contribute to the development of atherosclerosis (Wong et al., 2004). Three major connexins (Cx37, Cx40 and Cx43) are differentially expressed within atherosclerotic plaques at different stages of its development (Kwak et al., 2002; Burnier et al., 2009). Based on these findings, it was hypothesised that GJIC, specifically Cx43, plays a crucial role in the initial steps leading to monocyte activation and transmigration in response to atherosclerosis.

The first step was to characterise HUVECs, which were used as the in vitro cell model of choice. HUVECs are relatively easy to culture, and are morphologically as well as functionally stable throughout several cell culture passages. HUVECs in culture were clearly identifiable using EC markers, such as vWF and PECAM-1. However, there were some cell batches that showed contamination with SMCs, and these were excluded from this study. The expression profile of connexins in HUVECs and human monocytes was assessed. In this study, HUVECs showed an abundant expression of Cx43 in comparison to Cx37 and Cx40. This suggested that in EC culture conditions, communication is achieved predominantly through Cx43 channel coupling. This is in contrasts to other studies, which have demonstrated substantial Cx40 and moderate Cx37 junctional labelling of HUVECs (Rijen et al., 1998). This difference may be due to methodological variations, such as the use of a different EC growth medium. Furthermore, in intact preparations of mouse aortas, endothelial GJs composed of combinations of Cx37, Cx40 and Cx43 (Inai and Shibata, 2009; Yeh et al., 1997). Therefore, it is possible that expression of
particular connexions in ECs depends on the culture medium used. The precise composition and compatibility of GJs in this system would be of interest to investigate, ideally through the passage of low molecular weight dyes, such as Lucifer Yellow (Elfgang, 1995). Therefore, the model developed in this thesis has the advantage that the role of Cx43 in mediating TEM can be studied without interference from other connexin subtypes due to the low endogenous expression of other subtypes.

In this study the establishment of GJIC between monocytes and HUVECs was shown using the dye transfer assay and this communication was blocked and inhibited by $^{43}\text{GAP27}$. Evidence for GJ-mediated intercellular communication between monocytes and macrophages, as well as other cells, has been supported by the use of dye transfer assays (Martin et al., 1998; Eugenan et al., 2001). It was shown that the percentage of monocytes accumulating the dye from loaded HUVECs increased when growth factors were absent from the culture medium.

Under normal conditions, ECs and monocyte do not adhere and thus TEM is low. However, pathological conditions, such hypercholesterolemia, are associated with the activation of the endothelium, which precedes the transmigration process of monocytes across the EC barrier. Both TNF-$\alpha$ and IFN-$\gamma$ cytokines potentiate the production of adhesion molecules that can promote leukocyte adhesion to the endothelium (Mantovani et al., 1992). However, this process is not controlled by GJs alone, and TJs are involved which allow two adjacent cells to disassemble and the migration of monocytes to occur. GJIC, controlled by E-cadherin, involves post-translational regulation (assembly and/or function) of the GJ protein Cx43 (Jongen et al., 1991). TJ proteins (ZO-1) affect cadherin-based AJ assembly in terms of the binding of ZO-1, E-cadherin and $\alpha$-catenin (Contreras et al., 2002).

HUVEC monolayer integrity is more TJ-dependant compared to GJs and this was shown when TJs were disrupted by removing Ca$^{2+}$ via the use of EGTA; however, the presence of Cx43 was
required to maintain a complete integrated monolayer as the TEER measurements showed a reduction in monolayer resistance about 50% when the Cx43 mimic peptide was used. These finding shows that GJs (especially Cx43) play a role in the maintenance of ECs TEER (O’Donnell et al., 2014; Martin et al., 2005; Charles et al., 1992).

Using the 43GAP27 mimic peptide in this study was useful to establish and optimise GJIC between monocytes and HUVECs. However, as its function is to block the functional effect of Cx43 without affecting the level of Cx43 protein expression, a blocker or inhibitor at the gene level was required, which can be achieved either by knocking out or knocking down Cx43. siRNA was used in this study to assess the functional formation of GJs between monocytes and HUVECs. Different techniques are reported in the literature about the transfection of HUVECs and the most efficient and effective method identified was electroporation transfection. Previous studies have shown that siRNA delivery by chemical transfection caused a wide range of side effects depending on the cell type (Charolidi, 2008). A key advantage of electroporation over other methods is that electropermeabilisation is independent of the cell-cycle stage, making it possible to deliver siRNA to non-dividing or slowly-dividing cells (Ovcharenko et al., 2005). Changes in GJ organisation and connexin expression can result in disturbances to normal cell-cell communication between monocytes and ECs. In this study the effects of changing Cx43 expression levels in HUVECs using siRNA to knockdown connexin 43 expression was investigated. The major findings were that knockdown of Cx43 produced a decrease of monocytes loading using the dye transfer assay by approximately 20% compared to 80% when Cx43 protein expression was normal. However, knockdown of Cx43 produced almost no migration of monocytes in the TEM assay, and TEER measurements following the knockdown of Cx43 produced decreased resistance.
An examination of the involvement of the cytokine TNF-α was important to mimic the pathological condition, as TNF-α is one of the main cytokines released during the atherosclerosis process, especially during the initiation stage. In this study, TNF-α changed the morphology of HUVECs and reduced the level of Cx43 protein expression. The modulation of EC morphology from epithelial to fibroblastoid was consistently found, and this phenomenon has also observed by others in these cells (Sato et al., 1986; Stolpen et al., 1986). Three major pathways that mediate the effect of TNF-α were investigated; activation of MAPK cascades, activation of PKC, and activation of NFκB. In this study all possible pathways mediated by TNF-α were assessed and it was found that TNF-α mainly effected the PKC pathway, as none of the MAPKs was altered by TNF-α activation. In the discipline of GJ research, PKC has received considerable attention because PKC activators, which promote tumourigenesis, both increase Cx43 phosphorylation and decrease GJ communication in several cell types (Brissette et al., 1991; Reynhout et al., 1992; Lampe, 1994). The introduction of several inhibitors and activators of PKC specific for different isotypes has helped to identify PKC isoforms responsible for Cx43 regulation (Bowling et al., 2001; Lampe and Lau, 2004b, Cruciani et al., 2001). In this study, TNF-α mediation through PKC was assessed by inhibiting PKC using chelerythrine, and this furthermore abolished the effect of TNF-α. Thus, phosphorylated Cx43 expression was reduced in ECs treated with a specific PKC inhibitor and similar effects were also found in TNF-α stimulated ECs treated with a PKC inhibitor, indicating that Cx43 phosphorylation in ECs is PKC-dependent. Thus, it appears that several members of the conventional and novel PKC families influence GJ communication.

NFκB may be involved in multiple steps in the progression of atherosclerosis (de Winther et al., 2005). Cominacini et al. (2005) demonstrated that oxLDL dose-dependently increased the activation of NFκB in monocytes derived from human peripheral blood and that oxLDL had no effect in lymphocytes. Interestingly, this study showed that in vitro stimulation of HUVECs with
TNF-α not only increased NFκBp50 protein expression but also the expression of the key intermediate intracellular proteins IKKα and IKKβ, which are functionally involved in the activation of NFκBp50 through a series of phosphorylation events. This could be an indication that TNF-α, through HUVEC stimulation activates PKC, in turn exerting its activating effects on NFκB upon triggering of IKK activity. This study showed that CHE suppressed the expression of PKC and NFκBp50 in HUVECs cells stimulated with TNF-α. This is an indication that CHE inhibits NFκB activation in HUVECs exposed to inflammatory stimuli. Furthermore, the mechanism by which the CHE prevents NFκB activation is based on its effect on stabilising IκBα by inhibiting kinases, in turn masking the nuclear localisation signals of NFκB, keeping it sequestered in an inactive state.

Recent studies have shown that activation of PKC can lead to phosphorylation of serine residues in the cytoplasmic carboxyl-terminal domain of Cx43 (Lampe et al., 2000; Bao et al., 2004) and that treatment with a PKC activator phorbol 12-myristate 13-acetate, increases Cx43 phosphorylation, in turn decreasing GJ communication (Brissette et al., 1991; Reynhout et al., 1992; Lampe, 1994). This study showed that treatment with TNF-α increased Cx43 phosphorylation in HUVECs. The expression levels of phosphorylated Cx43 protein in HUVECs stimulated with TNF-α was significantly higher when compared to resting conditions, indicating that TNF-α exhibits a maximal effect on PKC phosphorylation of Cx43.
6.2 Key Findings in the Study

It was shown for the first time that two different cell types, HUVECs and monocytes, which express Cx43 form functional GJs and communicate through them. In addition, blockade of functional GJ channels could be established. It was also shown that Cx43 plays a role in the establishment of monocytes TEM and furthermore this process is enhanced by cytokine stimulation by TNF-α. This was assessed by Cx43 knocked-down in HUVECs transfected with Cx43-siRNA.

PKC has a role in mediating the effect of TNF-α in these processes; the results of the dye transfer assay, TEM assay and TEER measurement revealed that TNF-α had no affect when the PKC inhibitor CHE was applied. This study has shown the important role of GJ communication as an underlying mechanism during the early stages of atherosclerosis development, enhanced by the action of TNF-α. In summary, these results underline Cx43 as a cardiovascular risk marker and potential therapeutic target in the treatment of atherosclerosis.

In addition, this study has emphasised the involvement of TNF-α and intercellular communication between HUVECs and monocytes. A summary of the proposed mechanisms by which TNF-α could modulate GJIC leading to monocyte TEM is shown in Figure 6.1.
Figure 6.1: Proposed mechanism underlying the effects of TNF-α on Cx43 expression and gap junction mediated monocyte migration.

TNF-α uptake by TNFR1 triggers the activation of PKC and the subsequent phosphorylation of Cx43. The TNF-α induced PKC phosphorylation of Cx43 results in its direct interaction with other tyrosine kinases that could directly trigger the activation of NFκB through a series of phosphorylation events that include the phosphorylation of the IκBα protein by the IKK complex. Activated NFκB translocates into the nucleus and upregulates genes, which could reduce the expression of Cx43 or increase Cx43-pS368. In this type of cell TNF-α does not mediate the MAPK pathway.
6.3 Limitations of the Study and Recommended Future Work

This study has provided novel evidence for the importance of the role of TNF-α and importance of GJIC, especially Cx43 in monocytes TEM; however, this has given rise to many questions in need of further investigation.

The effect of TNF-α on Cx43 and its role during the initiation stage of developing atherosclerosis was examined. However, there are other cytokines involved in this process, such as IL-2, IL-4, IL-6, and IFN-γ, which may also have an effect on Cx43 expression and/or phosphorylation. In further studies a more accurate indication of cytokine secretion could be obtained by the intracellular detection of cytokines by flow cytometry. This method would have the added advantage that the cells could be double-labelled for the cytokine of interest and phenotypic surface markers. This would not only allow the identification of the cell type secreting the cytokine, but also provide additional information about the dynamic interaction between monocytes and HUVECs.

The reverse transcription polymerase chain reaction (RT-PCR) approach could also be employed to ascertain changes in mRNA and resolve doubts regarding the interpretations proposed in this study. Co-expression and immuno-precipitation studies could be employed to identify and characterise heterotypic coupling between ECs. In this manner an informative insight would allow the characterisation of different mechanisms for the regulation of connexin expression.

In addition, it was demonstrated through this study that PKC phosphorylates Cx43 and activates NFκB. However, the question relating to the mechanism of PKC specifically phosphorylating Cx43 without the intervention of other PKC isoforms needs to be further elucidated. Furthermore, channels’ permeability or stringency to different solutes may be decreased or increased by phosphorylation of Cx43 and phosphorylation in controlling the
gating system of the hemichannels may be residue-specific. It was not possible to discern a specific function associated with the phosphorylation of Cx43 during HUVEC activation, as there are multiple phosphorylation sites. This could be achieved through immunoprecipitation and immunoblotting analysis of PKCα, PKCβ, PKCγ, PKCδ, and PKCε mediated phosphorylation of Cx43 at serine residues under resting and TNF-α induced stimulatory conditions.
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