Beta-adrenoceptor modulation of dermal endothelial cell function and angiogenesis

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Abstract

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Angiogenesis is an essential process in wound healing. An insufficient angiogenic response can result in chronic wounds, whilst an overzealous response can contribute to scarring and cancer metastasis. Beta-adrenoceptors (β-AR) are G protein-coupled receptors (GPCR) and have previously been shown to play a role in wound healing, however their role in angiogenesis, is currently unknown.

It was hypothesised that β-AR activation or blockade will reduce and promote human dermal microvascular endothelial cell (HDMEC) angiogenic functions and angiogenesis.

β-AR activation was both anti-motogenic and anti-mitogenic. In addition, protein kinase A and exchange proteins directly activated by cAMP (EPAC) played a role in modulating the β-AR-mediated decrease in migration rate. Meanwhile, immunoprecipitation studies revealed that both the β_1-AR and β_2-AR co-localised with EPAC. Finally, inhibiting cyclic adenosine monophosphate (cAMP) signalling pathways reduced proliferation. Perhaps a decrease in cAMP underpinned the β-AR-mediated decrease in proliferation rate.

In more complex environments, β-AR activation both promoted and delayed, whilst β-AR blockade promoted tubule formation. Meanwhile, β-AR activation and blockade both increased and reduced aortic outgrowth. In the chick chorio-allantoic membrane assay, β-AR activation and blockade both reduced and increase angiogenesis. Finally, enzyme linked-immunosorbent assays demonstrated that β-AR modulation altered vascular endothelial growth factor A secretion from human neonatal keratinocytes and fibroblast growth factor 2 secretion from HDMECs and human dermal fibroblasts.

In conclusion, activating or inhibiting β-ARs can modulate HDMEC function and angiogenesis *in vitro, ex vivo* and *in vivo*. Therefore, the use of β-AR agonists and antagonists could be promising modulators of angiogenesis.

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Publications

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Abbreviations

2D	two-dimensional
8-pCPT	8-CPT-2′-O-Me-cAMP
AC	adenylate cyclase
AGS	activator of G protein signalling
ANG	angiopoietin
aPKC	atypical protein kinase C
Arp	actin related protein
AR	adrenoceptor
AT	antithrombin
ATP	adenosine triphosphate
BCAEB	4-1-bis-4-methyl-phenyl-methyl-carbamoyl-3-2-ethoxy-benzyl-4-oxo-azetidine-2-yloxy-benzoic acid
BFA	brefeldin A
BM	basement membrane
BME	basement membrane extract
BSA	bovine serum albumin
β-AR	beta adrenoceptor
CAM	chick chorioallantoic membrane
cAMP	cyclic adenosine mono-phosphate
cGMP-PDE	cyclic guanosine monophosphate phosphodiesterase
CHO	Chinese hamster ovary
CT	connective tissue
CTGF	connective tissue growth factor
CTX	cholera toxin
DAG	diacylglycerol
DII4	delta-like 4
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethyl sulfoxide
DTT	dithiotheitol

EC	endothelial cell
EDTA	ethylenediaminetetraacetic acid
ECM	extracellular matrix
ECGM	endothelial cell growth medium
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
EPAC	exchange protein directly activated by cAMP
EPC	endothelial progenitor cell
EPH	ephrin
ELISA	enzyme linked immunosorbant assay
HB-EGF	heparin binding epidermal growth factor
EPAC	exchange protein directly activated by cAMP
ERK	extracellular signal-regulated kinase
FAK	focal adhesion kinase
FBS	foetal bovine serum
FA	focal adhesion junction
FGF	fibroblast growth factor
GDP	guanosine diphosphate
GM-CSF	granulocyte-macrophage colony-stimulating factor
GPCR	G protein-coupled receptor
GM	monosialotetrahexosylganglioside
Gna-1	glucosamine-phosphate N-acetyltransferase 1
GRB2	growth factor receptor bound protein 2
GRK	G protein-coupled receptor kinase
GTP	guanosine triphosphate
h	hour
HBSS	hepes buffered saline solution
HDF	human dermal fibroblast
HDMEC	human dermal microvascular endothelial cell
HGF	hepatocyte growth factor
HIF	hypoxia inducible factor
HNK	human neonatal keratinocyte

HUVEC	human umbilical vein endothelial cell
ICC	immunocytochemistry
IFN	interferon
IGF	insulin like growth factor
IP_3	inositol trisphosphate
IL	interleukin
iNOS	inducible nitric oxide synthase
JAM	junction adhesion molecule
kDa	kilodalton
KGF	keratinocyte growth factor
LMWH	low-molecular weight heparin
LPS	lipopolysaccharide
mAb	monoclonal antibody
mRNA	messenger ribonucleic acid
MAPK	mitogen activated protein kinase
MEK	mitogen activated protein kinase kinase
Min	minute
MMP	matrix metalloproteinase
MSP	macrophage stimulating protein
NK4	N-terminal hairpindomain and four kringle domains
NPY	neuropeptide Y
NO	nitric oxide
PAK	p21 activated kinase
PAI	plasminogen activator inhibitor
PAR	partitioning defective
PBMC	peripheral blood mononuclear cell
PBS	phosphate buffered saline
PDGF	placental derived growth factor
PDPK	phosphoinositide-dependent protein kinase
PF	platelet factor
PlGF	placental growth factor
PI3K	phosphoinositide 3-kinase

PIP	phosphatidylinositol 3-phosphate
PIP2	phosphatidylinositol 4,5-bisphosphate
PIP3	phosphatidylinositol 3,4,5-triphosphate
PKA	protein kinase A
PKB	protein kinase B
PKC	protein kinase C
PLC	phospholipase C
PLD	phospholipase D
PMA	phorbol-12-myristate 13-acetate
PMN	polymorphonuclear cell
PMSF	phenylmethanesulphonylfluoride
PMT	Pasteurella multocida
PTX	pertussis toxin
PP2A	protein phosphatase 2A
PVDF	polyvinylidene fluoride
RAP	ras-associated protein
RGD peptide	arginine, glycine and aspartic acid containing peptide
RGS	regulators of G protein signalling
RPMI	Roswell Park Memorial Institute Medium
ROS	reactive oxygen species
RT-PCR	real-time polymerase chain reaction
RTK	receptor tyrosine kinase
SCM	single cell migration
SDF	stromal cell-derived factor
SEM	standard error of the mean
SOS	son of sevenless
T47D	human ductal epithelial tumour cell line
TF	tissue factor
Th	T-helper
TFP	tri-functional protein
TGF	transforming growth factor
TIMP	tissue inhibitor of metalloproteinase

TM	transmembrane protein
TNF	tumour necrosis factor
tPA	tissue plasminogen activator
TP	thymidine phosphorylase
TPI	triose phosphate isomerase protein
TSP	thrombospondin
uPA	urokinase type plasminogen activator
uPAR	urokinase receptor
VEGF	vascular endothelial growth factor
VEGFR	vascular endothelial growth factor receptor
WASP	Wiskott Aldrich syndrome protein
WAVE	WASP family verprolin homologous protein
WASH	Wiskott–Aldrich syndrome protein and SCAR homologue

Chapter 1 Introduction

1.1 Mammalian skin

Mammalian skin is part of the integumentary system which is comprised of various appendages such as hair, nails and sweat glands. The main functions of the skin are to protect against microorganisms, help maintain haemostasis and to preserve fluid. The skin contains three distinct layers including: the epidermis; which is a superficial layer that acts as a barrier to water and pathogens, the dermis; which is a deeper layer that contains skin appendages and absorbs external stress, and the hypodermis or sub-cutaneous layer; which functions to attach the skin to the bone and muscle as well as providing the skin with vasculature and nerves (Mast, 2001, Wickett and Visscher, 2006, Proksch et al., 2008, Krieg and Aumailley, 2011, Prost-Squarcioni et al., 2008).

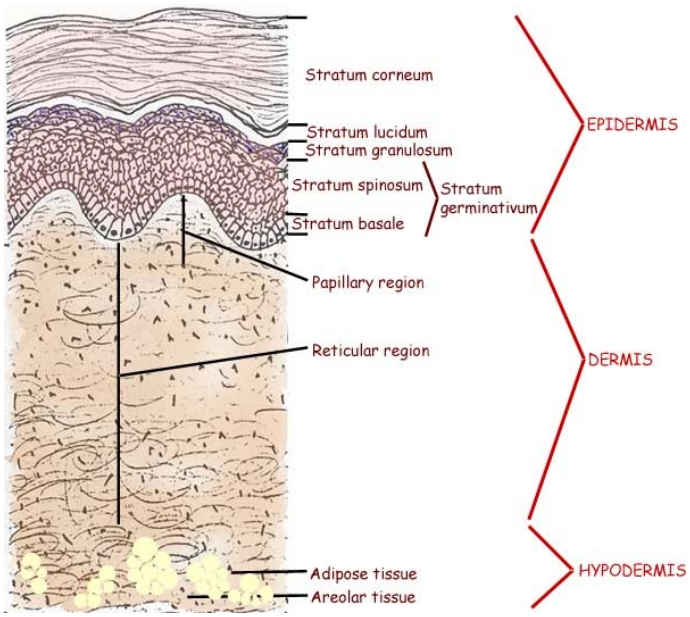


Figure 1.1: Mammalian skin. Image illustrating how mammalian skin is subdivided into three main layers: the epidermis, dermis and hypodermis. (Image taken from Patton, 2007)

The epidermis is the outermost layer of the skin and contains cells such as keratinocytes, mast cells, the pigment-containing melanocytes, the dendritic, antigen-processing langerhan cells and the pressure-sensing Merkel cells. The epidermal layer of the skin is subdivided into 5 different layers including the stratum corneum, stratum lucidum, stratum granulosum, stratum spinosum and the stratum basale, see figure 1.1 (Wickett and Visscher, 2006, Proksch et al., 2008). Keratinocytes are the main cell type of the epidermis and differ in their stage of differentiation depending on depth. In the deeper layers of the epidermis, they are undifferentiated and in the more superficial layers they are fully differentiated. Keratinocytes produce keratins which are the main structural component of the skin and play a major role in providing the barrier function of the skin. The stratum basale contains a single layer of keratinocytes which is anchored to the basement membrane via hemidesmosome junctions and contains the epidermal stem cells. Keratinocytes exist from the stratum basale to the stratum granulosum and undergo rapid mitosis in the stratum basale to provide keratinocytes to ascend and differentiate though the different layers of the epidermis. The key stages of keratinocyte differentiation occur in the stratum granulosum in which keratinocytes change in both shape and composition. Here, two types of granules are formed in keratinocytes including keratohyalin granules, which contain numerous proteins and lamella granules, which contain lipids. During the differentiation process, the nucleus is digested, the cytoplasm disappears and the plasma membrane becomes enveloped. In addition, keratin intermediate filaments aggregate to form microfibrils, aided by fillagrin and loricrin proteins found in keratohyalin granules. The lamella also secretes lipids into the intercellular space. The end result of these changes, are terminally differentiated keratinocytes termed,

corneocytes. Corneocytes are polyhedral shaped cells that lack a nucleus or any cytoplasmic organelles. Corneocytes in the stratum corneum are interlocked with one another and with keratinocytes in the stratum granulosum via desmosome junctions, which are specialised cell structures for cell-cell adhesion. Between these cells is a lipid-enriched extra-cellular matrix (ECM) which along with the corneocytes-lipid envelope, provides a hydrophobic water-proof barrier. The aggregated keratin microfibrils within the corneocytes provide mechanic strength, thus completing the "brick and mortar" model for the stratum corneum (Wickett and Visscher, 2006). As corneocytes ascend towards the surface of the skin, desmosomes disintegrate and corneocytes undergo shedding during the desquamation process in the stratum corneum (Fore, 2008, Wickett and Visscher, 2006, Proksch et al., 2008).

The basement membrane (BM) mechanically attaches the epidermis to the dermis. In addition, the BM regulates the passage of cells and molecules between the two layers and acts as a reservoir for growth factors and cytokines (Chan, 1997).

The dermis lies directly beneath the BM and is composed of connective tissue (CT) which protects the body by absorbing stress and strain. This region contains collagen, blood vessels, fibroblasts, mast cells, lymphatics and nerve endings responsible for the sense of touch and heat. The hair follicles, sebaceous glands and apocrine glands form the pilosebaceous units of the skin. The sebaceous glands produce a waxy/oily secretion that keeps the skin moisturised, while the aprocine and eccrine glands produce sweat and are responsible for the thermoregulation of the skin. The dermal layer of the skin can be divided into the papillary and reticular regions. The papillary region is directly below the epidermis, which is connected via the BM and contains a

thin arrangement of collagen fibres. The reticular region is below the papillary region and contains thicker collagen fibres that are arranged parallel to the surface of the skin. Both regions show different types of CT including loose CT and dense irregular CT. Dermal CT contains collagen, elastic and reticular fibres that are involved in mechanical stress absorption (Krieg and Aumailley, 2011, Prost-Squarcioni et al., 2008).

The hypodermis or sub-cutaneous layer is directly below the dermis and consists of adipose cells and loose connective tissue which contain large blood vessels and nerves (Fore, 2008).

1.2 Wound healing

1.2.1 Introduction

Wound healing is a highly evolved, organised and complex biological process involving the regeneration of the epidermal and dermal layers of the skin. Cutaneous wound healing can be divided into two types, full thickness and partial thickness cutaneous wound healing.

Full thickness, cutaneous wound healing occurs when the epidermis and dermis are compromised and involves multiple steps that occur in a highly orchestrated and temporal fashion (Stroncek and Reichert, 2008). A cascade of events takes place involving many different cell types and cellular processes. These events include coagulation, inflammation, re-epithelialisation, ECM deposition, angiogenesis, dermal remodelling and wound contraction, all of which are stimulated by an array of biological factors. The natural end point of full thickness, cutaneous wound healing is the formation of a scar (Falanga, 2005, Martin, 1997, Santoro and Gaudino, 2005, Stroncek and Reichert, 2008). On the other hand, partial thickness cutaneous wound healing occurs when the wound is superficial, usually caused by burns and abrasions, damaging mainly the epidermis. In partial thickness wounds, the dermis is left intact, leaving the basement membrane and the dermal appendages (hair follicles, sweat and sebaceous glands) undamaged. Therefore, coagulation and re-epithelialisation are the only processes required to regenerate the epidermis, thus resulting in minimal scar formation (Stroncek and Reichert, 2008).

1.2.2 Wound guidance cues

There are gradients of various motogenic guidance signals present that recruit cells into the wound site. As a result, a steering mechanism within a cell is activated along with the basic migration machinery to direct it to a desired location (Petrie et al., 2009). There are four main mechanisms that guide cells into the wound site including chemotaxis, haptotaxis, mechanotaxis and galvanotaxis. Chemotaxis allows for the directional migration of cells towards a gradient of chemoattractants such as growth factors, whereas haptotaxis is the directional migration in a gradient of adhesion molecules such as the ECM (Petrie et al., 2009). Mechanotaxis is the directional migration induced via mechanical forces such as shearing forces in blood vessels (Lamalice et al., 2007). The generation of local electric fields (galvanotaxis) within the epidermal and dermal layers of the skin are one of the earliest directional guidance cues for cell recruitment and migration (Fang et al., 1999, Pullar et al., 2006c, McCaig et al., 2005, Ojingwa and Isseroff, 2003). There is an asymmetric distribution of basolateral and apical Na^+ channels throughout the epithelium which creates a positive transepithelial potential. Upon tissue damage, this transepithelial potential collapses, creating a cathode (negative pole) at the centre of the wound site (Nuccitelli, 2003). Cells such as keratinocytes are able to sense applied electric fields and move directionally towards the cathode of an applied electric field *in vitro* (Pullar and Isseroff, 2005b, Nishimura et al., 1996). The exact process as to how cells sense and respond to these changes in electrical potential are still being investigated.

1.2.3 Stages of wound healing

Full thickness, cutaneous wound healing can be broken down into four distinct but overlapping stages: coagulation, inflammation, migration/proliferation and the contraction/remodelling phase.

The first stage is coagulation which occurs immediately upon tissue damage. Platelets are activated by the release of collagen from the surrounding tissue into the blood, resulting in the expression of various glycoproteins and integrins on their membranes. This enables platelets to adhere to various components of the ECM such as fibrin and fibronectin causing aggregation and the formation of a fibrin plug (Gurtner et al., 2008, Martin, 1997). The fibrin plug consists of a mass of platelets supported in a dense network of fibrin fibres cleaved by thombin from fibrinogen. There are also small amounts of other proteins present such as fibronecin, vitronectin and thrombospondin (Martin, 1997). The main purpose of the fibrin matrix is to provide a quick forming barrier that can protect the body from pathogens and prevent further trauma to the wound (Martin, 1997).

Activated platelets and ruptured cell membranes release various pro-inflammatory factors such as serotonin, bradykinin, prostaglandin, prostacyclins, thromboxane and histamine (Eming et al., 2007). These factors have a variety of functions which include increasing cell recruitment, migration and proliferation but are also involved with blood vessel vasoconstriction and vasodilation (Jeffcoate et al., 2004). Immediately after blood vessels haemorrhage, inflammatory factors such as thromboxanes and prostaglandins cause blood vessels to undergo vasoconstriction. This causes blood vessels to contract, therefore preventing further blood loss and allowing for the

collection of inflammatory cells and factors in the area. Vasoconstriction lasts for 5-10 minutes and is immediately followed by vasodilation which peaks at about 20 minutes post wounding. The main function of vasodilation is to increase blood flow in the body to tissues that need it the most. Histamine is the main factor involved in vasodilation and leads to blood vessels becoming larger and more porous. As a result, the surrounding tissues become more edematous as proteins and inflammatory cells such as neutrophils and monocytes leak into the wound site by a process known as extravasation (Martin, 1997).

Activated platelets also secrete various growth factors including platelet derived growth factor (PDGF) and transforming growth factor β1 (TGF-β1), see table 1.1. They both induce cell recruitment, migration, proliferation and cytokine release from cells such as macrophages, fibroblasts and smooth muscle cells. PDGF stimulates the chemotaxis of various cell types including neutrophils and macrophages and both the chemotaxis and proliferation of smooth muscle cells and fibroblasts, see table 1.1 (Arbabi et al., 2004). Cells adjacent to the wound use the fibrin plug as a scaffold to migrate into the wound bed (Falanga, 2005, Jeffcoate et al., 2004).

Growth factor	Source	Primary target cells and effect
Activin	Fibroblasts; keratinocytes	Keratinocytes, fibroblasts and stromal cells
CTGF	Fibroblasts; endothelial cells	Fibroblasts, downstream of TGF-β3
EGF	Platelets	Keratinocyte motogen and mitogen
FGFs 1, 2, and 4	Macrophages, fibroblasts and damaged endothelial cells	Angiogenic and fibroblast mitogen
FGF-7 (KGF)	Dermal fibroblasts	Keratinocyte motogen and mitogen
HB-EGF	Macrophages	Keratinocyte and fibroblast mitogen
IGF-1	Plasma; platelets	Endothelial cell and fibroblast mitogen
IL-1α and -β	Neutrophils	Early activators of growth factor expression in macrophages, keratinocytes and fibroblasts
PDGF	Platelets; macrophages; keratinocytes	Chemotactic for macrophages, fibroblasts and macrophage activation, fibroblast mitogen and ECM production
TGF-α	Macrophages; keratinocytes	Keratinocyte motogen and mitogen
TGF-β1 and -β2	Platelets; macrophages	Keratinocyte migration, chemotactic for macrophages and fibroblasts, fibroblast matrix synthesis and remodelling
TGF-β3	Macrophages	Anti-scarring
TNF-α	Neutrophils	Similar to the IL-1s
VEGF	Keratinocytes; macrophages	Angiogenesis

CTGF: connective tissue growth factor, EGF: epidermal growth factor, FGF: fibroblast growth factor, HB-EGF: heparin binding epidermal growth factor, IGF: insulin-like growth factor, IL: interleukin, KGF: keratinocyte growth factor, TNF: tumour necrosis factor and VEGF: vascular endothelial growth factor.

Table 1.1: Growth factor signals at the wound site. Table showing the various different growth factors present at the wound site, their source and their target cells. (Table adapted from Martin, 1997)

The inflammatory phase begins within minutes after injury and overlaps with the coagulation phase. Numerous immune T-helper (Th) cells and phagocytes are drawn to the wound site by various chemotactic signals (see table 1.1) from degranulating platelets, peptides cleaved from bacterial proteins, such as lipopolysaccarides and formyl-methionyl, and products of ECM proteolysis (Eming et al., 2005, Martin, 1997). The first phagocytes to arrive at the wound site are neutrophils which are polymorphonuclear cells (PMN) that transmigrate between the ECs in the blood vessel walls via the process of extravasation. Neutrophils begin the debridement of necrotic tissue and kill pathogens by phagocytosis. They can also kill pathogens though the release of reactive oxygen species (ROS), cationic peptides, eicosinoids and proteases (elastase, cathepsin G, proteinases 3 and urokinase-type plasminogen activator (uPA)), which also contribute to the clearing and cleansing of the wound, see table 1.2. In addition, neutrophils can release a cocktail of pro-inflammatory cytokines to amplify the inflammatory response, including TNF-α, IL-1β, IL-6, IL-8 and VEGF to stimulate the wound repair response, see table 1.2. Other PMNs that contribute to wound inflammation are basophils and eosinophils. Basophils and eosinophils are involved in the removal of pathogens from the wound site, however their precise function is still under investigation (Eming et al., 2005).

Mast cells also play a role during wound inflammation. They are an important source of pro-inflammatory mediators and cytokines (see table 1.2) promoting inflammation and vascular changes. Mast cells contribute to the characteristic signs of inflammation at the wound site including heat, pain, redness and swelling. After 2 days post wounding, monocytes extravasate from blood vessels and differentiate into macrophages to replace PMNs which either undergo apoptosis or are degraded by

other immune cells. Macrophages have a very similar role to PMNs. They are a major source of growth factors and cytokines (i.e. PDGF and TGF-β) which promote the recruitment of cells required for the proliferative stages of wound healing, therefore amplifying the chemical signals produced earlier by platelets and neutrophils, see table 1.2. The inflammatory phase lasts as long as there is debris or bacteria in the wound with a prolonged inflammatory phase leading to tissue damage and chronic wound healing (Eming et al., 2005, Martin, 1997, Martin and Leibovich, 2005). In addition, cells of the adaptive immune response are also present at the wound site including T-helper cell, Th1, Th2 and γδT. These cells can contribute to wound repair processes such as re-epithelialisation and tissue remodelling though the release of various mediators, see table 1.2. however their precise functions are still under investigation (Eming et al., 2005).

Cell type	Functions	Mediators
Macrophages	Phagocytosis of PMN and fragments of tissue degradation	
	Amplify inflammatory response	
	Anti-inflammatory function	TNF-α, IL-1β, and IL-6
	Stimulate repair response: angiogenesis and fibroplasia	IL-10 and TGF-β1, PDGF, VEGF, FGF-2, TGF-α, and TGF-β
	Fibrolysis	tPA, uPA, u-PAR, and PAI-1/-2
Mast cells	Control vascular permeability	Histamine
	Control influx of PMN	Chymase, tryptase
	Regulate tissue remodelling	
PMNs (Neutrophils)	Phagocytosis of infectious agents	ROS, cationic peptides, eicosanoids, proteases (elastase, cathepsin G, PR-3, and uPA)
	Macrophage activation though phagocytosis	
	Amplify inflammatory response	TNF-a, IL-1β, IL-6
	Stimulate repair response	VEGF, IL-8
T cell: Th1/Th2	Regulate tissue remodelling	CD40 ligand; IL-2, TNF-α/ IL-4, -5,-10
γδT cells	Keratinocyte proliferation, differentiation, hyaluronan synthesis in Kc	FGF-7, FGF-10, and IGF-1

CD: cluster of differentiation, PAI: plasminogen activator inhibitor, tPA: tissue plasminogen activator, uPAR: urokinase receptor.

Table 1.2: Wound inflammatory cells, their function and the mediators released.
(Table adapted from Eming, 2005)

The migration/proliferation stages of wound healing occur days after wounding and are characterised by various processes, some of which overlap including angiogenesis, fibroplasia, granulation tissue formation, collagen deposition and re-epithelialisation (Martin, 1997). The granulation tissue appears during this stage of wound healing and consists of a mesh of blood vessels, ECs, inflammatory cells, fibroblasts and provisional ECM components. Fibroblasts migrate and deposit ECM components which are required to fill the void left by the wound and to rebuild the ECM structural support network. Fibroblasts enter the wound from normal adjacent unwounded tissue. They migrate from the wound edge initially using the fibrin/fibronectin scab as a matrix until collagen and other ECM are deposited. Indeed, fibroblasts secrete various ECM molecules such as glycosaminoglycans, proteoglycans, hyaluranan, elastin, fibronectin and collagen. Fibroblasts initially lay down collagen III, which functions to increase the strength of the wound. Initially, collagen synthesis exceeds collagen degradation by collagenases which are secreted by fibroblasts. Eventually, synthesis and degradation equal out and there is no net gain in collagen (Arbabi et al., 2004, Falanga, 2005, Jeffcoate et al., 2004).

Re-epithelialisation is stimulated by various growth factors (see table 1.3). Wound edge and dermal appendage keratinocytes migrate over the provisional matrix, to seal the void left by the wound (Santoro and Gaudino, 2005). Keratinocytes are normally anchored to the BM by desmosomes and hemidesmosomes which attach to ECM components such as laminin-5, via α6β4 intergrins (Santoro and Gaudino, 2005). Therefore, to allow re-epithelialisation to begin, cell junction disassembly occurs as a result of “inside-out” signalling. The intracellular domain of the β4 integrin is phosphorylated, causing the detachment from laminin 5, thus releasing keratinocytes

from the BM and the ECM. Various integrins (see table 1.2) are up-regulated to serve as temporary linkages to ECM proteins present in the wound environment such as fibronectin and vitronectin (Martin, 1997). Keratinocyte migration is further enhanced by growth factors (see table 1.1 and 1.3) and via the expression of tissue plasminogen activator (tPA), urokinase type plasminogen activator (uPA) and various matrix metalloproteinases (MMP) (see table 1.3) which degrade the BM and aid in keratinocyte migration across the provisional wound matrix (Santoro and Gaudino, 2005). Keratinocytes migrate directionally and advance in sheets from the edge of the wound until migration is halted via contact inhibition (Fisher and Yeh, 1967, Guelstein et al., 1973, Pullar et al., 2006a).

	Reepithelialisation (migration)	Reepithelialisation (proliferation)
ECM	Laminin-5	Laminins and Collagens
Growth factors	MSP, TGF-β1	EGFs, TGF-α, KGF, GM-CSF
Integrins	α3β1, α6β4, αvβ6	αvβ3/β5, β4, β1
MMPs	MMP 1, 2, 9, 10	MMP 3

GM-CSF: granulocyte macrophage-colony stimulating factor, MSP: macrophage stimulating protein

Table 1.3: Signals involved in keratinocyte migration. Table showing the various pro-motogenic and pro-mitogenic signals that alter keratincoyte behaviour during re-epithelialisation. (Table adapted from Santaro, 2005)

Angiogenesis is the formation of new blood vessels from pre-existing ones. The formation of neo-vascular beds is essential for wound healing as a continuous supply of oxygen and nutrients is required for cells involved in regeneration. The process of angiogenesis occurs about days post wounding and occurs alongside fibroplasia (Bauer et al., 2005). Studies have shown that microvessel density increases 3 days post injury, then decreases during the vascular remodelling phase but can remain elevate for up to 2 years in scars (Brown et al., 2002, Kilarski et al., 2009, Kumar et al., 2009). ECs are guided to the wound site via the topographical fibrin/fibronectin scab, various growth factors and electrical guidance cues (Zhao et al., 2003). ECs from uninjured parts of blood vessels develop pseudopodia aiding their migration though the ECM and into the wound site. To allow ECs to migrate though the ECM, collagenases and plasmin degrade part of the ECM and the fibrin plug. MMPs aid EC migration by degrading the BM and parts of the neodermis. EC growth and proliferation is also stimulated by hypoxia, as a low oxygen environment induces platelets and macrophages to release pro-angiogenic factors which chemotactically attract ECs. All these factors aid in EC recruitment, proliferation and consequently angiogenesis (Eming et al., 2007, Lamalice et al., 2007).

In addition to angiogenesis, lymphangiogenesis also plays a role in wound healing. Oedema often occurs after tissue damage due to vasodilation, increased vascular permeability and damage to lymphatic vessels (Alitalo et al., 2005, Shaw and Martin, 2009). Therefore, lymphangiogenesis is required to restore lymph vessels and drain the tissue fluid and large proteins from the extravascular space. Lymph fluid is then filtered by the lymph nodes and returned to the blood circulation though the left and right

lymphatic ducts in the jugular area, where the jugular vein meets the subclavian vein (Alitalo et al., 2005).

In summary, the migration/proliferation phase of wound healing is characterised by ECM deposition, angiogenesis and re-epithelialisation. Fibroblasts produce a provisional ECM scaffold to allow for subsequent cell migration, proliferation and further ECM deposition. Keratinocytes then proliferate and migrate from the wound edges to reform the epithelial barrier. Angiogenesis drives these processes by providing a continuous supply of oxygen and nutrients.

The final phase of wound healing is the contraction and remodelling of local tissues, which can last up to 3 years depending on the depth of the wound. Wound contraction is achieved by the actions of myofibroblasts. TGF-β1 stimulates fibroblast differentiation to myofibroblasts with the aid of mechanical stress and cellular fibronectin (ED-A splice variant). Myofibroblasts specifically express smooth muscle α actin (SM-α actin), found on bundles of microfiliaments. These microfiliaments terminate at the cell surface in the fibronexus, which connect intracellular actin with transmembrane integrins and extracellular fibronectin domains. Therefore the fibronexus is able to transmit force from stress fibres to the ECM allowing for wound contraction (Desmouliere et al., 2005). Wound contraction diminishes when ECM synthesis and degradation come to equilibrium. The deposited collagen is remodelled and type III collagen is replaced with the stronger type I collagen, in an ordered fashion, along tension lines. As a result, the tensile strength of the wound increases from around 50% to 80% compared with normal tissue. Scarring is improved as blood

vessels undergo apoptosis when they are no longer needed (Falanga, 2005, Jeffcoate et al., 2004).

Scarring is the natural end result of wound healing and represents the final point of the healing process (Diegelmann and Evans, 2004). A scar is essentially fibrous tissue that has replaced normal tissue. It is less resilient to UV radiation and lacks sweat glands and hair follicles which do not grow back. The reasons behind scarring are not fully understood, although it appears that evolution has primed us to heal quickly but imperfectly, augmenting various processes such as inflammation and angiogenesis. Collagen is a major component of the connective tissue and comprises about 30% of the total protein in the human body. In the skin, collagen is regularly arranged in a basket weave-like pattern and is the main structural support network of the skin, providing strength, organisation and integrity (Diegelmann and Evans, 2004). However, during wound healing, collagen is deposited in disorganised bundles generating new tissue that exhibits differing tensile strength, texture and quality compared with normal tissue (Rhett et al., 2008).

The inflammatory cascade during wound healing plays a role in scarring although the exact mechanisms are unknown. Myofibroblasts are known to be hyper-stimulated. They reconstitute and remodel the collagen matrix poorly. Many animal models have been generated with inflammatory cell types such as macrophages, neutrophils or mast cells missing or genetically altered (knocked out/knockdown) in an attempt to understand their roles in wound healing and wound scarring see table 1.4. The PU.1 knockout mouse, lacking macrophages, neutrophils and mast cells, exhibits scarless

wound healing. However is its clear from table 1.4 that the role of inflammation in scarring is contentious and still under investigation (Martin et al., 2003).

Knockout/knockdown mouse	Missing cell types			Wound phenotype
	Macrophages	*Neutrophils*	*Mast cells*	
Macrophage antisera (Martin et al., 2003)	Missing			Poor debridement/retarded repair
Neutrophil antisera (Silvestri et al., 1999)		Missing		Normal healing
Platelet antisera (Szpaderska et al., 2003)				More macrophages but otherwise normal healing
PU.1 KO (Martin et al., 2003)	Missing	Missing	Missing	Speedier repair and reduced scarring
Neutrophil antisera (Dovi et al., 2003)		Missing		Speedier re-epithelialisation
Kit W KO (Egozi et al., 2003)			Missing	More neutrophils but otherwise normal healing

Table 1.4: Inflammatory cell transgenic studies. Table showing genetic alteration of macrophages, neutrophils and masts cells and the consequences for wound healing (Table adapted from Martin and Leibovich, 2005)

A number of cytokines have also been implicated in scarring which include the Th2 cytokines IL-4, IL5, IL-13 and IL-21. TGF-β1, TGF-β2 and TGF-β3 growth factors have been found to play critical roles in fibrosis (Rhett et al., 2008, Wynn, 2008). Adult wounds predominantly express TGF-β1 and TGF-β2. Antibodies to both TGF-β1 and TGF-β2 have reduced scarring and fibrosis (Shah et al., 1994). Topical applications of TGF-β3, down regulate TGF-β1 and TGF-β2 (Shah et al., 1995) and improve scarring therefore suggesting that the expression and balance of these growth factors are critical during wound healing (Berman et al., 2004, Ferguson et al., 2009, Martin, 1997, Martin and Leibovich, 2005).

Interestingly, wounds that are less than 0.56 mm in depth completely regenerate and do not scar (Martin and Leibovich, 2005). The reasons behind this are not completely understood, although it is known that different types of wound healing will occur depending on the depth of the wound. As previously described, partial thickness wounds in which the injury only penetrates the epidermis, just requires epithelialisation compared with full thickness dermal wound healing which requires, inflammation, dermal remodelling, angiogenesis, epithelialisation and wound contraction (Stroncek and Reichert, 2008). Another hypothesis, states that there are distinct phenotypes of fibroblasts that can colonise the wound site, stimulated by fibrogenic cytokines or thermal injury (Martin and Leibovich, 2005). Embryonic and oral wounds also heal without a scar, although the mechanisms behind this are still under investigation. It is known, however, that there are lower levels of inflammation, angiogenesis and growth factors such as VEGF-A, TGF-β1 and TGF-β2 in oral and embryonic wounds (Redd et al., 2004, Shah et al., 1994, Shah et al., 1995, Szpaderska et al., 2005, Wilgus et al., 2008). Lower levels of VEGF and consequently angiogenesis, result in less vascularity resulting in less fibrous tissue deposition and scarring (Redd et al., 2004, Szpaderska et al., 2005, Wilgus et al., 2008).

In summary, full thickness-dermal wound healing involves the coordination of numerous processes, cell types and biological factors to stimulate the regeneration of the epidermal and dermal layers of the skin. The processes of coagulation, inflammation, migration/proliferation of various cell types and wound contraction/ECM remodelling occur in a highly temporal and spatial manner, sometimes overlapping with one another. Immediately upon wounding, coagulation occurs, which is immediately followed by inflammation that happens within minutes

after wounding. The migration/proliferative stages of wound healing occur within hours, and encompass the processes of ECM deposition, angiogenesis and re-epithelialisation. Finally, the wound contraction and ECM remodelling phases, occurs within days after wounding, with the formation of a scar representing the end point of wound healing.

1.3 Impaired wound healing

1.3.1 Fibrosis

Excessive scar formation can lead to fibrosis. Normally, scarring is the end result of wound healing. However, during fibrosis, there is a reduction in the apoptosis of various cell types including myofibroblasts and vascular cells and an excess of ECM deposition (Gabbiani et al., 1972). Excessive collagen deposition results in a loss of structure and function, while too little will cause the wound to weaken and dehisce (Diegelmann and Evans, 2004). Aberrations in the inflammatory response, along with the interplay between various cells types (I.e. myofibroblasts), growth factors, cytokines and components of the ECM, may play a role in excessive scarring. The therapeutic treatment of fibrotic scars involves reducing the inflammatory response, altering collagen production and surgically altering the physical shape of the scar (Verhoeckx et al., 2006). Some examples of pathological scars include hypertrophic scars and keloids, see figure 1.2 (Arbabi et al., 2004).

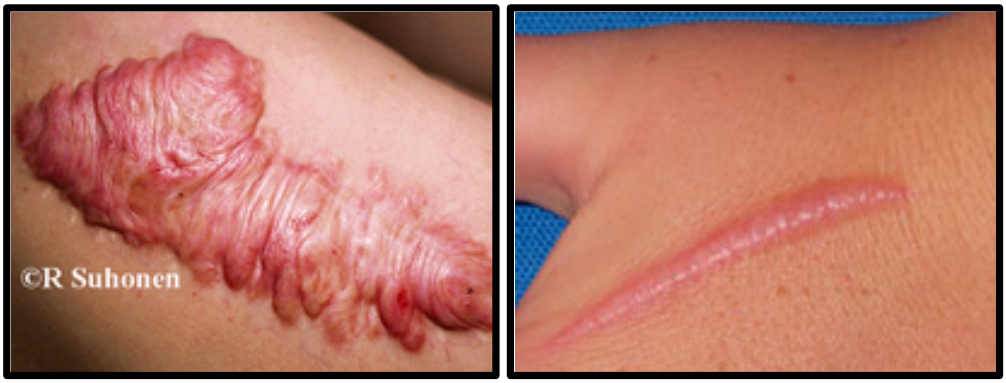


Figure 1.2: Excessive scarring. Image illustrating the two most common types of pathological scars: hypertrophic and keloids scars (Image adapted from Rothfield, 2010)

Keloids are scars that invade the surrounding tissue with a poor likelihood of regression and predominantly occur in dark-skinned people with a possible familial component. Hypertrophic scars, on the other hand, are non-invasive and may regress over time. They are red/purple coloured skin elevations and remain in the original site of the wound. Hypertrophic scars can occur several weeks after surgery, at which point they increase in mass, stabilise and then may regress. This entire process typically takes up to two years. Keloids however do not regress and can occur outside of the original wound area and may not necessarily appear after wounding (Arbabi et al., 2004).

1.3.2 Chronic wounds

Chronic wounds can be defined as wounds that undergo a delayed healing response by failing to progress though the normal stages of healing (Menke et al., 2007). In the United Kingdom alone, approximately 1 in 14 individuals will develop chronic wounds, which equates to about 650,000 people annually, with an estimated cost to the national health service of about £3 billion, in 2005/2006. Chronic wounding is a major source of morbidity and mortality leading to an array of psychological, sociological and

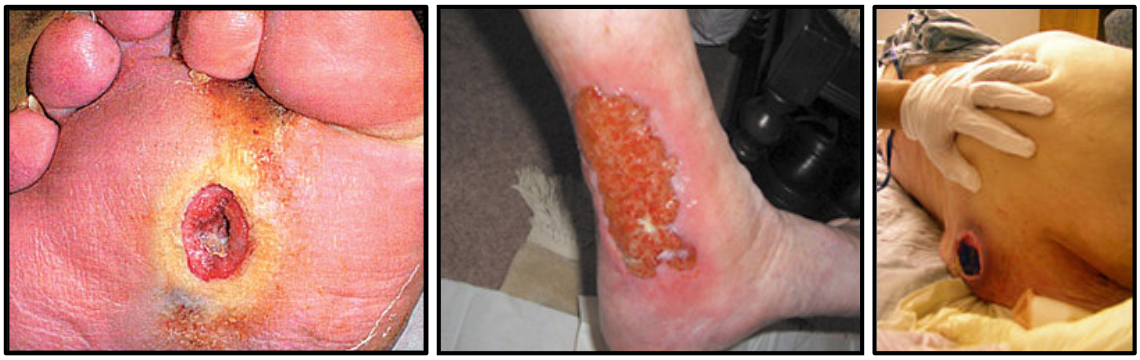
economic burdens to society. Therefore, it is important to understand the wound healing process, in order to develop therapeutic treatments to improve and accelerate the healing process (Thomas, 2006, Pullar et al., 2006a).

Chronic wounds almost always occur in an individual with a pre-existing condition such as a systemic illness like diabetes, peripheral vascular disease (i.e. atherosclerosis) psoriasis or atopic eczema. Also increased age, repeated trauma and various comorbid ailments can contribute such as vasculitis, immune suppression, pyoderma gangrenosum and other diseases that cause ischemia. Negative emotional states increase stress, which in turn, increase cortisol levels, which has a detrimental effect on immunity and wound healing (Diegelmann and Evans, 2004, Ojingwa and Isseroff, Menke et al., 2007). Chronic wounds are characterised by defective cell migration, delay in angiogenesis and re-epithelialisation, abnormal levels of biological factors such as growth factors and degradative enzymes and an “over-zealous” inflammatory response (Brem and Tomic-Canic, 2007).

Excessive or insufficient cell migration can contribute to chronic wounds. Excessive fibroblast migration in addition to abnormal proliferation, differentiation and excessive ECM production can lead to fibrosis, which can contribute to chronic wounding (Hinz, 2007, Lorena et al., 2002). The insufficient migration of ECs and keratinocytes delay angiogenesis and re-epithelialisation (Falanga, 2005, Stojadinovic et al., 2005). Chronic wounds also have high levels of degradative enzymes such as MMPs and have low levels of growth factors such as PDGF, EGF, KGF and VEGF (Diegelmann and Evans, 2004, Menke et al., 2007).

In acute wounds, inflammatory cells clear the wound of pathogens, debris and any necrotic tissue, subsequently preparing the wound site for cell migration, proliferation and ECM deposition. However, in chronic wounds, excessive inflammation causes additional injury which further promotes inflammation, leading to the persistence of neutrophils, monocytes and macrophages in the wound (Martin and Leibovich, 2005, Smith, 2006). Inflammatory cell persistence is thought to be a consequence of tissue trauma caused by pressure, leading to the recruitment and activation of neutrophils, ischemic reperfusion injury, high levels of pathogens, and inflammatory cell trapping in capillaries. This "over-zealous" inflammatory response leads to an abnormal inflammatory profile in chronic wounds. The persistence of neutrophils leads to high levels of secreted degradative enzymes such as collagenase (MMP8) and neutrophil-derived elastase. Due to the low levels of the degradative enzyme inhibitors such as tissue inhibitor of metalloproteinases (TIMP) and α2-macroglobulins in a chronic wound, the presence of these degradative enzymes leads to wound degradation (Menke et al., 2007). The persistence of inflammatory cells also results in high levels of pro-inflammatory cytokines such as TNF-α, and toxins such as ROS which causes cellular and ECM damage and a breakdown of the growth factors such as PDGF that promote cell migration, proliferation and ECM (Diegelmann and Evans, 2004, Menke et al., 2007).

There are many types of chronic wounds including venous leg ulcers, diabetic foot ulcers and pressure ulcers, see figure 1.3.



Diabetic foot ulcer ***Venous leg ulcer*** ***Pressure ulcer***

Figure 1.3: Chronic wounds. Image illustrating the three most common types of chronic wounds: diabetic foot ulcer, venous leg ulcer and a pressure ulcer. (Image taken from: AAFP, 2002)

Diabetic foot ulcers occur in 15% patients with diabetes, which affects 170 million people around the world. There are many causative factors leading to the development of chronic foot ulcers in patients with diabetes. Peripheral neuropathy for example, results in a loss of the protective sense to feel, mainly in the feet and legs, therefore increasing the chances of causing both physical and thermal injury. Other risk factors include atherosclerotic peripheral vascular disease, continuous trauma and increases in plantar fascia (thick CT that supports arch on bottom of the foot) pressure due to poor joint mobility and foot deformities (Lieschke and Currie, 2007). About 100 known deficiencies in wound healing have been identified in diabetic patients, some of these include decreased or impaired growth factor production, angiogenesis, re-epithelialisation, keratinocyte/fibroblast motogenesis and mitogenesis, collagen accumulation, quantity of the granulation tissue and high protease activity (Stojadinovic et al., 2005, Falanga, 2005). A number of pathological markers which correlate with diabetic delayed wound healing include overexpression of c-myc and

β-catenin, abnormal localisation of epidermal growth factor receptor (EGFR) and activation of the glucocorticoid pathway which can inhibit keratinocyte migration. Wound neovascularisation is also affected by a decreased secretion of stromal cell-derived factor-1α (SDS-1α) from epithelial cells and myofibroblasts, resulting in the lack of recruitment and mobilisation of bone marrow derived endothelial progenitor cells to the wound site (Brem and Tomic-Canic, 2007, Stojadinovic et al., 2005).

Venous ulcers are caused by the improper functioning of the blood valves in the veins causing the pooling of blood in the vessels and an increase in hydrostatic pressure. Subsequently gap junctions between the endothelial cells are weakened, leading to a leakage of blood exudate. The blood exudate is high in fibrinogen which polymerises to fibrin and is deposited around the vessel forming a fibrin cuff. The fibrin cuffs acts as an oxygen/nutrient diffusion barrier and sequesters cytokines, this leads to the surrounding tissue becoming necrotic, infected and ulcerated (Fonder et al., 2008).

Pressure ulcers occur at sites of bony prominence such as the hips, elbow, knees and plantar regions, whereby persistent pressure forces the bone against the dermis. As a result, blood flow from underlying capillaries is obstructed, causing local ischemia and tissue necrosis (Yarkony, 1994).

There are a number of treatments, currently available to treat chronic wounds. These include wound debridement (Lebrun et al., 2010), negative pressure therapy (Vuerstaek et al., 2006), wound care dressings (Fonder et al., 2008), bioengineered skin substitutes (Langer and Rogowski, 2009) and platelet gels containing growth factors (Anitua et al., 2008, Crovetti et al., 2004). Wound debridement involves the removal of foreign necrotic debris from the wound bed and edges, such as callus and eschar, and

has been used in the treatment protocols for diabetic foot ulcers. However, there is insufficient evidence, so far, to prove that wound debridement is an effective way of enhancing healing in a chronic wound (Lebrun et al., 2010). Some studies have shown that platelet gels may be useful in treating chronic wounds. Platelet gel is obtained from activated platelets and contains a plethora of growth factors which can promote cell migration, proliferation and differentiation (Anitua et al., 2008, Crovetti et al., 2004). However it is important to note that chronic wounds contain high levels of degradative enzymes which can degrade growth factors. Another treatment option is negative pressure therapy, which uses a vacuum to create a negative atmospheric pressure in the wound site to draw out oedema and promote angiogenesis (Vuerstaek et al., 2006). Further research is required to reveal the efficacy and cost effectiveness of these treatments.

1.4 Wound revascularisation

1.4.1 Introduction

Wound re-vascularisation is essential for successful wound healing and can occur by two main process; angiogenesis and vasculogenesis. Angiogenesis is most prevalent in embryogenesis, however it is down-regulated in adults, unless required in response to injury. Angiogenesis is the formation of new capillaries from pre-existing vasculature to distribute blood flow (Bauer et al., 2005, Eming et al., 2007). In addition, pre-existing vessels can also undergo growth by intussusception, in which blood vessels are split into two (Risau, 1997). If necessary, these new capillaries can then undergo arteriogenesis, whereby the luminal diameter of these capillaries can increase to form larger vessels such as arteries, to provide bulk flow. Arteriogenesis plays a major role in pathological conditions such as arteriosclerosis, whereby collateral remodelling and enlargement of arteriolar anastomoses, the reconnection of two vessels, is necessary to divert blood flow to reach the ischemic tissues (Carmeliet, 2000, van Oostrom et al., 2008). Vasculogenesis, on the other hand, is the *de-novo* formation of blood vessels utilising the specialist stem cell, the endothelial progenitor cell, which is derived from the bone marrow (Bauer et al., 2005, Risau, 1997).

1.4.2 Angiogenesis

Wound angiogenesis occurs from adjacent post capillary venules and utilises cell types such as microvascular ECs and pericytes. ECs line the lumen wall of the blood vessels and function as a barrier between the blood and the extravascular space. Angiogenesis occurs when ECs detach from adjacent vessels, proliferate and migrate directionally to the wound site to form tubule channels which connect to form loops. The newly formed tubules mature and are reinforced by the recruitment of periendothelial support cells such as smooth muscle cells and perictyes, as illustrated by figure 1.4 (Bauer et al., 2005, Eming et al., 2007).

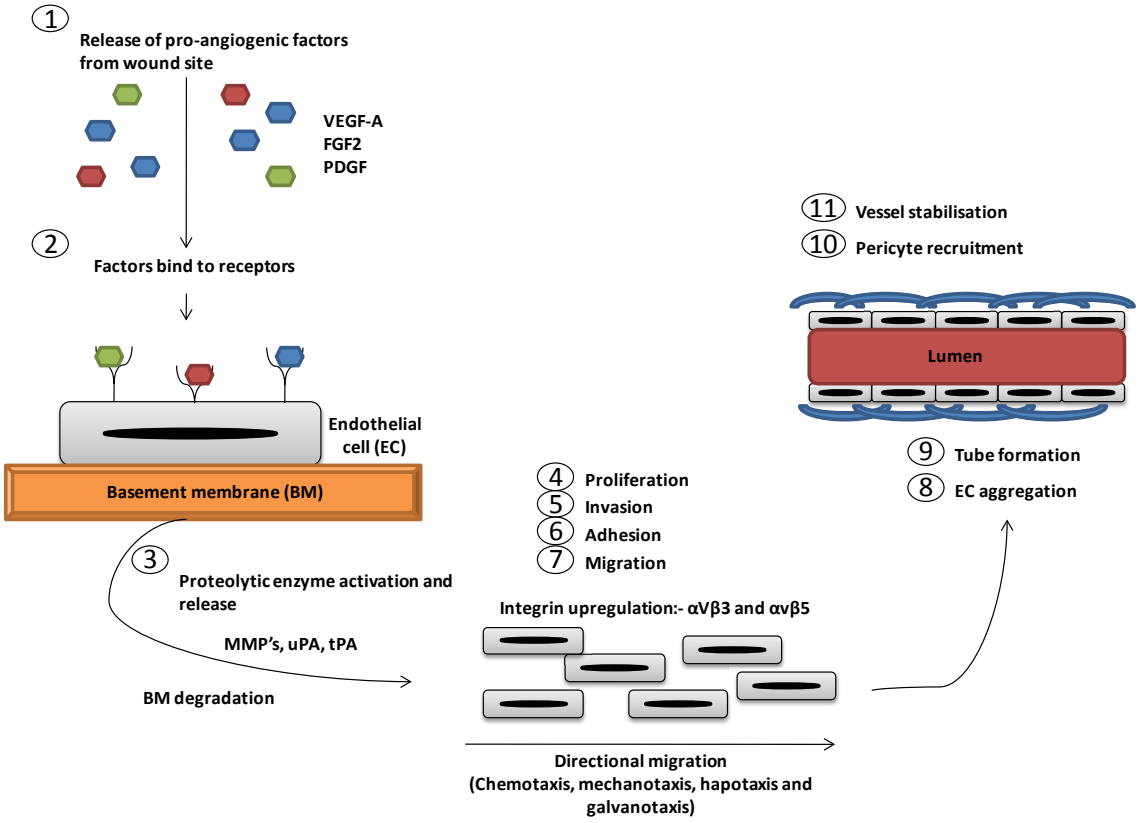


Figure 1.4: Wound angiogenesis: A cascade of events. Pro-angiogenic signals from the wound site stimulate ECs on adjacent, post capillary venules to detach (1, 2) and invade the surrounding ECM. EC invasion is stimulated by various proteolytic enzymes (3, 5). ECs proliferate (4) and migrate directionally into the wound site, guided by various cues (6, 7). ECs then aggregate to form leaky tubules which are then further enhanced and stabilised by pericytes (8, 9, 10 and 11). (Original figure, drawn by Andrew P O'Leary)

Angiogenesis occurs in three main stages: vessel destabilisation; vascular migration/proliferation and vessel stabilisation (Bouis et al., 2006). The destabilisation stage involves the detachment of ECs from adjacent blood vessels via the disruption of cell junctions. These include, focal adhesions (FA), claudins, occludins and junction adhesion molecule 1 (JAM-1) in tight junctions between ECs and periendothelial support cells. This in turn is stimulated by various cytokines, growth factors, cell-cell integrin mediated interactions and cell-ECM interactions, which can all cause EC activation and the subsequent destabilisation of the pre-existing vascular bed (Carmeliet, 2000, Polverni, 1995). Tissue injury leads to the release of pro-angiogenic factors from cells and from the ECM in which growth factors are sequestered. Upon wounding, platelets degranulate and release a cocktail of pro-angiogenic growth factors such as PDGF, VEGF, TGF-α, TGF-β, FGF-2, PD-EGF and angiopoietin-1 (Ang-1). These pro-angiogenic factors bind to their receptors on ECs to initiate various signalling pathways stimulating EC migration, proliferation and morphogenesis (Berrettini et al., 1987, Eming et al., 2007, Li et al., 2003b). During wound haemostasis, the presence of thombin potentiates the effects of VEGF by increasing the expressions and secretion of VEGF from cell types such as fibroblast (Huang et al., 2001). Thombin also stimulates ECs to release gelatinase A, which induces the local degradation of the BM see figure 1.6 (Berrettini et al., 1987, Eming et al., 2007, Li et al., 2003b).

The vascular proliferative/migration phase is characterised by the production of proteolytic enzymes by activated ECs and the release of growth factors and cytokines by smooth muscle cells, macrophages and fibroblasts, which promote EC migration into the surrounding ECM. Wound inflammation further potentiates wound angiogenesis by the release of growth factors and cytokines from monocytes and

macrophages such as PDGF, VEGF-A, IL-8, Ang-1, FGF-2, TGF-α and TNF-α. ECs degrade the ECM and BM by the upregulation of proteolytic enzymes such as MMPs, uPA and tPA, see table 1.4 and 1.5. Migration is directional and is guided by galvanotactic, chemotactic, mechanotactic and haptotactic cues which is further enhanced by the upregulation of integrins ($\alpha_v\beta_3$ and $\alpha_v\beta_5$) (Bauer et al., 2005, Eming et al., 2007).

Normally, luminal ECs exist in a resting (quiescent-phalanx) state, however, upon stimulation by pro-angiogenic factors, they can become remodelling ECs, termed "stalk" cells which maintain the structural and functional integrity of the vessel. The ECs that detach and lead the invasion process are termed "tip" cells, characterised by long filopodial protrusions that coordinate the invasion and migration process. EC differentiation is regulated by the NOTCH ligand Delta-like 4 (DII4), Jagged 1 ligand and Wnt signalling pathways, which determine the Stalk/Tip cell "decision" process. NOTCH is a single-pass transmembrane receptor and upon activation, DII4 ligand reduces VEGF receptor expression and signalling in adjacent stalk ECs reducing the tip cell phenotype. In contrast, Jagged 1 ligand antagonises DII4 NOTCH signalling and is believed to be a potent pro-angiogenic regulator of angiogenesis (Adams and Eichmann, Benedito et al., 2009). Wnts are secreted glycoproteins that signal primarily though the frizzled 7 pass transmembrane receptors and are believed to be essential in influencing EC behaviour and tubulogenesis (Franco et al., 2009). Tip cells respond to a variety of attractive and repulsive guidance cues such as ephrins, semaphorins, netrins and slits which are important ligands for directing the formation of new EC connections. Slits and netrins act as repulsive guidance cues whereas semaphorins and netrins function in vascular patterning (Adams and Eichmann, 2010).

Angiogenic growth factors play an important role in stimulating angiogenesis. Perhaps the most important group of pro-angiogenic factors belong to the VEGF family, which consists of VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E, VEGF-F and placental growth factor (PIGF) (Hoeben et al., 2004). The VEGF gene is located on chromosome 6 and is composed of 8 exons, therefore VEGF can also differentially divided based on the different splice variants with $VEGF_{121}$, $VEGF_{165}$, $VEGF_{189}$, $VEGF_{206}$ being the most common, whilst other less common variants include $VEGF_{145}$ and $VEGF_{183}$ (Otrock et al., 2007).

VEGF-A is a 34-46kDa secreted glycoprotein which can bind to the VEGF receptor tyrosine kinases (RTK) on ECs. In wound healing, VEGF-A is secreted by platelets, keratinocytes, neutrophils and macrophages (Polverini et al., 1977, Taichman et al., 1997, Weninger et al., 1996). VEGF-A can bind to VEGFR1 and VEGFR2. VEGFR1 has a high ligand binding affinity, but low signalling capacity, whereas VEGFR2 has a low binding affinity but high signalling capacity. In addition, neuropilins, expressed on arteries (neuropilin 1) and veins (neuropilin 2), can act as co-receptors for VEGF-A 165, PIGF-2, VEGF-B, VEGF-E (Bouis et al., 2006, Eming et al., 2007). Semaphorins can influence VEGF binding to neuropilin receptors and inhibit or augment angiogenesis. For example semaphore 3A is an endogenous inhibitor of angiogenesis and competes with VEGF for neuropilin receptors (Acevedo et al., 2008). On the other hand, class 4 semaphorins (Zhou et al., 2012) and semaphorin 5A (Sadanandam et al., 2010) cooperate with VEGF to promote angiogenesis. VEGF-A is the most pro-angiogenic growth factor and induces EC proliferation sprouting and tubulogenesis and is unregulated in wound healing (Bates and Jones, 2008). VEGF-A can also upregulate levels of endothelial nitric oxide synthase (eNOS), therefore increasing NO causing

vasodilation (Hattori et al., 2007). Alternately, a recently discovered isoform of VEGF, $VEGF_{165b}$ has been shown to be an endogenous inhibitor of VEGF (Bates et al., 2002).

VEGF-B binds to VEGFR1 and regulates EC migration and ECM degradation by increasing the activity of uPA and tPA (Bouis et al., 2006). VEGF-C and VEGF-D also promote blood vessel growth though VEGFR2 and VEGFR3 while VEGF-E can promote angiogenesis though VEGFR2. The functions of VEGF-F and its link to wound healing have not been investigated (Eming et al., 2007, Otrock et al., 2007). PIGF is upregulated during cutaneous wound repair and is localised to wound edge keratinocytes, ECs surrounding the wound and within the granulation tissue. PIGFs can form heterodimers with VEGF-A to bind to the VEGFR2 or to the VEGFR1/VEGFR2 heterodimer receptor complex and are believed to enhance VEGF-A activity, although its precise role in wound angiogenesis is not completely understood (Eming et al., 2007).

VEGF mRNA expression can be controlled by a wide range of factors. Hypoxia is the most well known of these and can stimulate transcription in ECs via the formation of a DNA transcription factor complex. This complex includes hypoxia inducing factor (HIF-1α), HIF2 and the adapter protein p600. The complex then initiates VEGF-A gene transcription consequently leading to VEGF production, promoting blood vessel formation (Hattori et al., 2007). Hypoxia can also lead to increased eNOS-NO activity in ECs, therefore increasing levels of NO, promoting vasodilation, angiogenesis and subsequently local blood flow (Berrettini et al., 1987).

Other important pro-angiogenic growth factors include FGF-1, FGF-2, PDGF and TGF-β1. FGF-1 and FGF-2 are growth factors that can bind heparin in the ECM and

exert their effects though the RTKs, FGFR1 and FGFR2 see table 1.5 and 1.6. FGF-1 and FGF-2 stimulate EC migration and proliferation as well as stimulating the production of degradative enzymes such as collagenase and plasminogen activator to promote invasion. PDGFs on the other hand can exist as heterodimers (PDGF-AB) or homodimers (PDGF-AA or PDGF-BB), composed of chains A and B. PDGF has a role in vessel remodelling and stability by recruiting pericytes, although their precise role is still under investigation. TGF-β1 is both pro and anti-angiogenic, whereby dose-dependent concentrations influence TFG-β1 functions. Low doses of TGF-β1 will upregulate other pro-angiogenic growth factors and proteinases whereas high doses can inhibit EC growth, promote BM reformation and stimulate differentiation of SMCs and recruitment (Otrock et al., 2007).

	Pro-angiogenic factors	Anti-angiogenic factors
Platelet-derived regulators	HGF	Angiostatin, HGF/NK4
	Thymidine phosphoylase	mAb, TPI
	Neuropeptide γ	PF4
RTK-Binding factors	VEGF	mAbs to VEGF or VEGFRs, soluble VEGFR, RNA aptamers, RTK inhibitors
	FGF-2	mAbs, Suramin, Suradista, polysaccharides, peptides, IFNα
	PDGF	RTK inhibitors (Gleevec)
	EGF	mAbs, RTK inhibitors (Iressa)
	Ang1, Ang2, TIE2-mab	Ang2, Gna1, soluble TIE2
	CDT6, ARP1, -2, PGAR	Angioarrestin
	Ephrins	Soluble EphA2

Eph: ephrin, Gna-1: glucosamine-phosphate N-acetyltransferase 1, HGF: hepatoctye growth factor mAb: monoclonal antibody, PF: platelet factor, RTK: receptor tyrosine kinase, TPI: triose phosphate isomerase protein.

Table 1.5: Pro and anti angiogenic factors affecting EC proliferation and migration. (Table adapted from Bouis, 2006)

	Pro-angiogenic factors	Anti-angiogenic factors
Coagulation	TF	TFPI, mAbs, LMWH
	Thombin	TFPI, TM, Hirudin, TSP-1, ABT-510, Heparin, LMWH
Endogenous inhibitors		Angiostatin, Endostatinm Arrestin, Canstatin, Tumtatin, Restin, Prolactin, Vasostatin, Kininostatin
Fibrinolysis	uPA	PAI-1, -2, uPAR-binding peptide, Maspin, Aprotinin
	tPA	PAI-1, -2, uPAR-binding peptide, Maspin, Aprotinin
	Plasmin	α2-antiplasmin, α2-macroglobulin
Integrins	αvβ3 and αvβ5	Mabs (Vitaxin) or RGD peptides (Anginex, Cilengitide)
Proteolysis	MMP1, 2 and 9	TIMPs, Endostatin, synthetic peptides (Batimastat, Marimastat), AG3340, Minocycline
	Chymases	BCEAB
	Heparanases	Suramin analogues, denatured collagen mAbs

BCEAB:4-1-bis-4-methyl-phenyl-methyl-carbamoyl-3-2-ethoxy-benzyl-4-oxo-azetidine-2-yloxy-benzoic acid, LMWH: low molecular weight heparin, TSP: thrombospondin, RGD: arginine–glycine–aspartic acid containing peptide, TFPI: tissue factor pathway inhibitor, TM: transmembrane.

Table 1.6: Pro and anti angiogenic factors affecting the BM and ECM. (Table adapted from Bouis, 2006)

Vascular stabilisation and remodelling is mediated by the Ang family of proteins and their receptor, Tie2. The two main proteins of this family are Ang1 and Ang2 which can both bind to the tyrosine kinase receptor Tie2/Tek. Ang1 binds to the Tie2 receptor, while Ang2 is an antagonist of Ang1 binding to the Tie2 receptor. Ang2 is present in high levels at sites of blood vessel remodelling and stabilisation in adults. Angiopoietin knockout mice have abnormal vasculature remodelling and a failure to recruit smooth muscle cells and perictyes, which encase and give strength to endothelial tubes, indicating an essential role for this family in the final stages of angiogenesis (Lamalice et al., 2007, Otrock et al., 2007). Angiopoietins also have a role in EC migration and proliferation. Expression of Ang2 antagonizes Ang1/Tie2 signalling, resulting in the loosening of the tight vasculature structure and exposing ECs to pro-angiogenic signals such as VEGF. In the presence of VEGF, Ang2-exposed ECs are activated and begin migrating and proliferating, allowing for the formation of new capillary sprouts and tubes. However, the absence of VEGF is associated with vascular regression and Ang2-induced apoptosis of ECs. This suggests that vascular regression is associated with high Ang2 levels and low pro-angiogenic factors (Lamalice et al., 2007). The binding of Ang-1 to Tie2 on ECs stimulates the production of PDGF which in turn leads to the recruitment of smooth muscle cells and perictyes to the neovasculature (Bauer et al., 2005, Eming et al., 2007). Recent research revealed that Ang1, Ang2 and Tie2 are expressed in fibroblasts, myofibroblasts and ECs suggesting a role in human dermal wound repair and scarring (Staton et al., 2010).

At the later stages of wound healing, angiogenesis is reduced, growth factor levels decline and wound oxygen levels return to normal. There are various endogenous

inhibitors of angiogenesis that are important in controlling levels of angiogenesis, see table 1.6. Vascular proliferation is inhibited by the release of the TGF-β1 from pericytes (Faler et al., 2006), interferon-β (INF-β), secreted from epidermal cells, vasostatin and endostatin, which is a cleavage product from collagen XVIII (Bauer et al., 2005, Eming et al., 2007).

1.4.3 Vasculogenesis

Vasculogenesis is the *de novo* formation of blood vessels from the differentiation of bone marrow derived endothelial progenitor cells (EPC). Vasculogenesis was only thought to play a role in the embryo, however evidence suggests that post-natal vasculogenesis may occur in pathologies such as wound healing. EPCs are mobilised from the bone marrow by cytokines such as VEGF-C and SDF-1α and enter the peripheral circulation (Bauer et al., 2005). The basal level of circulating EPCs at any one time is estimated be about 0.002%, however, when neovascularisation is required, EPCs are released into the circulation in larger numbers and differentiate into ECs (Katso et al., 2001). The extent to which vasculogenesis plays a role in wound healing is poorly understood, although it appears to contribute to neovascularisation during wound healing.

1.5 Beta-adrenoceptors

G protein-coupled receptors (GPCR) are a family of transmembrane receptors which contains about 1000-2000 members and comprises >1% of the genome in vertebrates (Bockaert and Pin, 1999). β-ARs are a member of this superfamily, and are responsible for mediating the effect of their natural catecholamine ligands, adrenaline and noradrenaline. β-ARs can be divided into β_1-AR, β_2-AR and β_3-AR which vary in their protein sequences (Hall, 2004, Wallukat, 2002). The β-AR subtypes are encoded by genes on chromosomes 10 for the β_1-AR (Frielle et al., 1987), 5 for the β_2-AR (Sheppard et al., 1983) and 8 for the β_3-AR (Emorine et al., 1989). The β_1-ARs are predominantly expressed in the heart and brain and have a function in heart muscle contraction (Frielle et al., 1987, Hall, 2004) whereas the β_2-ARs are widely expressed throughout the body and have a function in smooth muscle relaxation (Hall, 2004, Sheppard et al., 1983). The β_3-ARs are most prevalent in the adipose tissue and can enhance lipolysis (Emorine et al., 1989, Frielle et al., 1987, Hall, 2004). GPCRs consist of 7 transmembrane helical segments, an external ligand binding domain and an intracellular domain that is linked to a G protein (Bockaert and Pin, 1999, Neer, 1995). G proteins are trimeric in structure and are composed of α, β and γ subunits, which act as important signal transducers (Hamm, 1998). Agonist activation of a GPCR stimulates the exchange of GDP for GTP on the α subunit instigating the dissociation of the βγ subunits (Gilman, 1987, Hamm, 1998). The subunits interact with their corresponding membrane effector molecules which are able to generate secondary messenger molecules such as cyclic adenosine mono-phosphate (cAMP), inositol triphosphate (IP_3) and diacylglycerol (DAG) and control intracellular concentrations of ions such as Na^+, Ca^{2+} and K^+, see table 1.7 (Hamm, 1998). The intracellular change in concentration

of secondary messengers and/or alteration of ion concentration causes the activation or inhibition of signalling cascades resulting in a change in cell function (Vazquez-Prado et al., 2003).

Gα subunits contain intrinsic GTPase activity, and after several seconds, GTP is hydrolysed back to GDP, and the Gα subunit reassociates with the Gβγ subunit (Cabrera-Vera et al., 2003b). The GTPase activity of G proteins is highly regulated though the actions of regulators of G protein signalling (RGS) and activators of G-protein-mediated signalling (AGS). RGSs can regulate GPCRs by increasing the GTPase activity of Gα subunits, therefore increasing GTP/GDP hydrolysis, returning G proteins to their GDP bound state. RGS are also believed to influence signalling specificity and effector functions, although the exact function of RGSs is still under investigation (Wettschureck and Offermanns, 2005). AGSs can also affect G proteins, some by promoting GDP/GTP exchange on Gα subunits (e.g. AGS1) and some independently of GDT/GTP exchange (e.g. AGS3), the precise mechanisms are still under investigation (Blumer et al., 2005).

The most common G proteins include G stimulatory (G_s), G inhibitory (G_i) and G_q which in turn are linked to their corresponding membrane effector molecules such as adenylate cyclase (AC) and phospholipase (PL) C, see table 1.7 (Offermans, 2003).

Family	Subtype	Effector
Gs	$G\alpha_{a(S)}$[a]	↑ AC
	$G\alpha_{a(L)}$[a]	↑ GTPase of tubulin
		↑ src
	$G\alpha_{olf}$	↑ AC
Gi	$G\alpha_{i1}$	↓ AC
	$G\alpha_{i2}$	Rap 1 GAP
	$G\alpha_{i3}$	GRIN 1 and 2
	$G\alpha_{oA}$[a]	↑ GTPase of tubulin
	$G\alpha_{oB}$[a]	↑ src
	$G\alpha_{Z}$	Ca^{2+} and K^{+} channels
	$G\alpha_{t1}$	↑ cGMP-PDE
	$G\alpha_{t2}$	
	$G\alpha_{g}$	Unknown
Gq	$G\alpha_{q}$	↑ PLCβs
	$G\alpha_{11}$	↑ brutons tyrosine kinase ($G\alpha_q$)
	$G\alpha_{14}$	
	$G\alpha_{15\ or\ 16}$	
G_{12}	$G\alpha_{12}$	↑ NHE-1
		↑PLD
	$G\alpha_{13}$	↑p115RhoGEF
		↑iNOS

NHE: sodium/proton exchanger

Table 1.7: Classification of G-subtypes and their effectors. (Table adapted from Cabrera-Vera, 2003)

Figure 1.5 illustrates that agonist activation of G_s coupled receptors stimulates the exchange of GDP for GTP on the G_α subunit resulting in the dissociation of the $G_{\alpha s}$ subunit from the $G_{\beta\gamma}$ subunits. The $G_{\alpha s}$ subunit binds to and activates AC subsequently catalysing the conversion of adenine triphosphate (ATP) to cAMP. The intracellular increase in cAMP levels, activates cAMP dependent protein kinases, such as protein kinase A (PKA) and exchange protein directly activated by cAMP (EPAC) (figure 1.5)

(Hall, 2004, Rooij et al., 1998, Strosberg and Nahmias, 2007). EPAC is a guanine nucleotide exchange factor which activates its downstream effector molecule Rap1, a small ras like GTPase, though the exchange of GDP to GTP (Kopperud et al., 2003, Rooij et al., 1998). To regulate high concentrations of cAMP a family of enzymes known as phosphodiesterases degrade cAMP, returning concentrations to basal levels (Offermans, 2003). G_i coupled receptors exert the opposite effect of Gs and inhibit AC and consequently decreases the production of cAMP from ATP. This reduces the activity of cAMP-dependent molecules such as PKA and EPAC (Cabrera-vera et al., 2003a, Offermans, 2003).

G_q coupled receptors activate PLC resulting in the formation of IP_3 and DAG from phosphatidylinositol 4,5-bisphosphate (PIP_2) generating an increase in intracellular Ca^{2+} and activation of PKC (figure 1.6) (Offermans, 2003, Hall, 2004). Within the past decade there has been a growing body of evidence showing that $G_{\beta\gamma}$ subunits also act as important GPCR regulators and signal transducers. $G_{\beta\gamma}$ dimers are known to act on a number of ion channels for ions such as Ca^{2+}, K^+ and Na^+, bind PLC-β, AC, Akt/PKB, phosphoinositide-3-kinase (PI3K) and stimulate GRK activation (Hamm, 1998, Bommakanti et al., 2000, Camps et al., 1992).

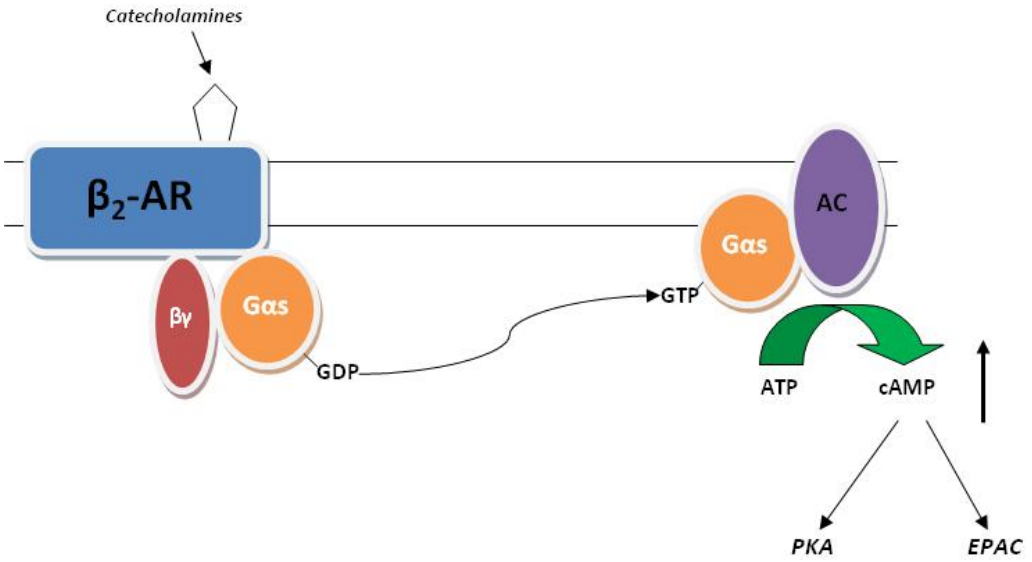


Figure 1.5: Illustration of a GPCR linked to a Gαs G protein. Upon catecholamine activation of the β_2-AR, a conformation changes stimulates the exchange of GDP for GTP on Gαs resulting in the dissociation of Gαs from Gβγ. Gαs binds and activate AC which catalyses the conversion of ATP to cAMP. cAMP can now activate cAMP dependent molecules such as PKA and EPAC. (Original figure, drawn by Andrew P O'Leary)

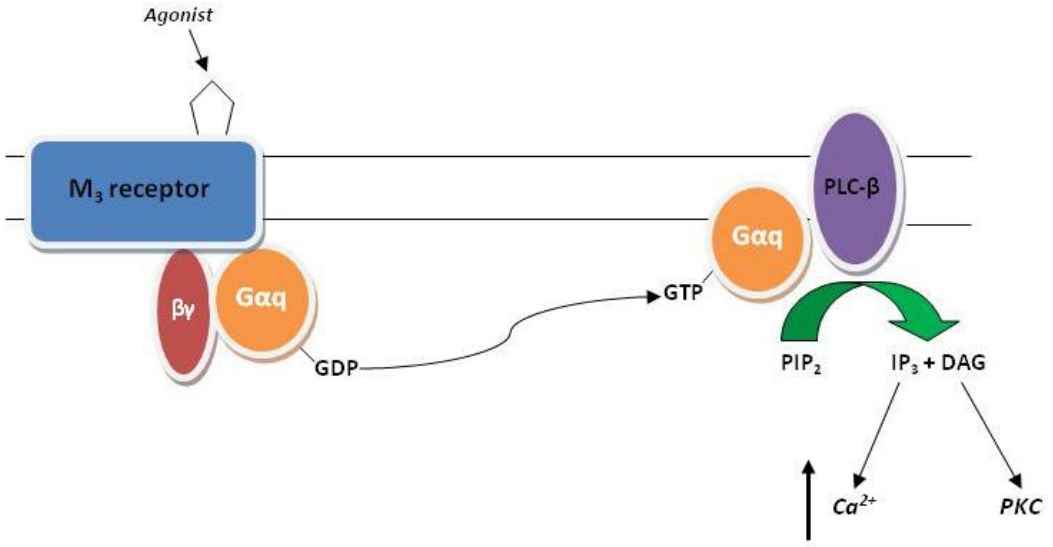


Figure 1.6: Illustration of a GPCR linked to a Gαq G protein. Agonist activation of M_3 receptor results in a conformation change causing an exchange of GDP for GPT on $G\alpha_q$. $G\alpha_q$ dissociates from Gβγ and activates PLC resulting in the formation of IP_3 and DAG from PIP_2, generating an increase in intracellular Ca^{2+} and activation of PKC. (Original figure, drawn by Andrew P O'Leary)

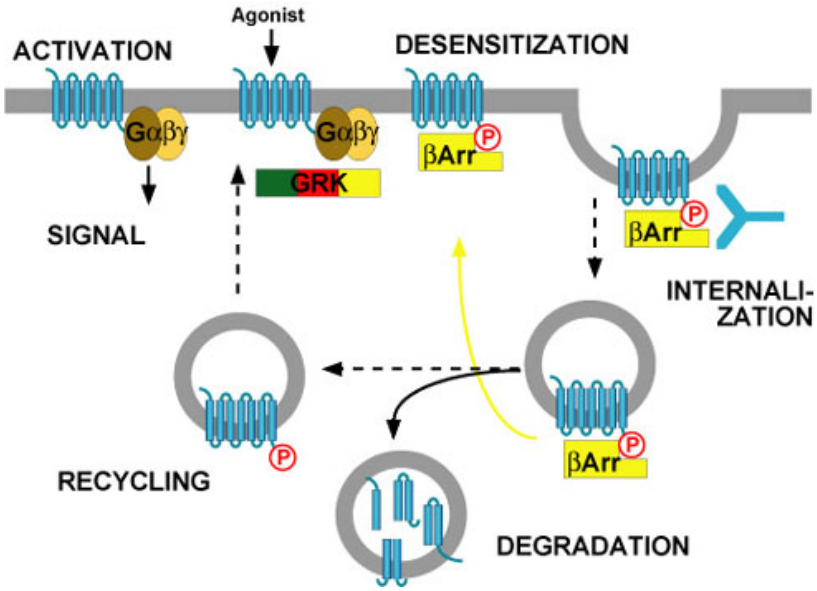


Figure 1.7: GPCR desensitisation mechanism. Upon agonist binding, GPCRs can undergo G protein uncoupling via GRK phosphorylation. Receptor phosphorylation allows for β-arrestin recruitment and subsequent clathrin mediated internalisation to an early endosomes. Receptor complex can either be degraded or recycled back to the cell surface (Image taken from Krasel, 2009)

GPCRs also have an important regulatory feedback mechanism which primarily occurs at the G protein level. One such mechanism is the process of desensitisation and resensitisation, which is a physiological response to protect the receptor from overstimulation, preventing further ligands from activating the receptor. Overstimulation of a GPCR with an agonist can result in a decrease in receptor responsiveness resulting in a reduced effect, this is termed receptor desensitisation (Ferguson, 2001). Desensitisation can occur through a variety of mechanisms which include: receptor phosphorylation; internalisation and downregulation of the receptor. The resulting effect can be reduced responsiveness to the ligand or a complete termination of the receptor-mediated effect. Arrestin-dependent desensitisation is initiated upon receptor phosphorylation by second messenger-dependent protein kinases such as PKA and PKC and GRKs. This allows for the binding of a family of

proteins knows as arrestins which are regulators of signal transduction (Balk et al., 2008, Ferguson, 2001). For example, β-arrestins can bind to the β_2-AR which serves to further block any G protein signalling via receptor uncoupling and to target the receptor for the next stage of desensitisation, internalization.

As a result of arrestin binding, the receptor is targeted for internalisation via endocytosis as illustrated in figure 1.7. Endocytosis may occur though clathrin-coated pits or via a clathrin independent mechanism such as caveolin or flotilin-mediated endocytosis. The type of endocytosis that occurs is dependent on tissue location and cell type (Doherty and McMahon, 2009). For clathrin-mediated endocytosis, the membrane undergoes invagination, which causes the receptor and agonist to be compartmentalised into clathrin-coated vesicles. The vesicles are then uncoated and form early endosomes. Vesicle trafficking and fusion to early endosomes is promoted by the GTPases, Rab5 and Rab4, which are reported to be important in the budding, fusion and recycling of the vesicle to the cell surface. The receptor can subsequently be dephosphorylated by G protein coupled receptor phosphatases and can be recycled back to the cell surface or be targeted to and degraded in the lysosomes (figure 1.7) (Ferguson, 2001). RGSs can also negatively regulate GPCRs. RGSs are GTPases that have the ability to return G proteins to their GDP bound state thus inactivating and reducing the responsiveness of the receptor. Therefore, RGSs dampen the signalling capability of the GPCR from any ligands that may have bound to the receptor (Ferguson, 2001, Vries et al., 2000).

β-ARs are believed to undergo receptor dimerisation to form homodimers or heterodimers with other receptor subtypes which may regulate receptor

internalisation and signalling between β_1-ARs and β_2-ARs (Hall, 2004). As well as targeting the receptor for internalisation, it is also believed that arrestin binding may initiate a second wave of signalling via c-src and the mitogen activated protein kinase (MAPK) cascade (Luttrell et al., 1999, Cao et al., 2000). An example of G protein independent signalling is the β_2-AR mediated activation of ERK via the transactivation of the EGFR. After GRK phosphorylation and subsequent G protein uncoupling, β-arrestins are recruited to the receptor. The β_2-AR/GRK/β-arrestin complex co-localises with a dimerised/cross-phosphorylated c-src/EGFR complex, whereby clathrin mediated endocytosis occurs allowing the ERK signalling to occur in the absence of a EGFR ligand (Maudsley et al., 2000).

Therefore, the β-AR family are capable of signalling though G protein dependent or independent mechanisms. The β_1-AR, β_2-AR and β_3-AR can couple to $G_{\alpha s}$ and $G_{\alpha i}$, although all three β-AR subtypes are capable of coupling to other types of G proteins depending on conditions and cell type (Hall, 2004, Xiao et al., 1999b, Soeder et al., 1999, Daaka et al., 1997, Kobilka, 2007).

1.6 The role of β-ARs in skin physiology

The β-ARs were first identified in cutaneous tissue over 30 years ago, however only in the last 10-15 years has research begun to unravel their multifarious and complex functional roles in regulating skin physiology. The β-ARs are widely distributed throughout the skin and are expressed on all cell types including keratinocytes (Steinkraus et al., 1991), fibroblasts (Berrettini et al., 1987, Furlan et al., 2005, Katanko et al., 1992), melanoctyes (Gillbro et al., 2004), endothelial cells (Howell, 1988, Steinberg et al., 1984), langerhans cells (Seiffert et al., 2002), neutrophils (de Coupade et al., 2004), mast cells (Barnes, 1993), eosinophils (Barnes, 1993) and macrophages (Verhoeckx et al., 2006).

The β-ARs bind their endogenous catecholamine ligands, adrenaline and nor-adrenaline. Catecholamines provide important biological functions, acting as both neurotransmitters and endocrine hormones and are part of the body's response to stress. Basal levels of circulating adrenaline are approximately 0.3-0.8nM in blood plasma (Schulz et al., 2004, Sedowofia et al., 1998) but can rise to about 3 to 12nM upon tissue injury or surgical stress (Crum et al., 1990, Matsui et al., 1991, Sedowofia et al., 1998). Catecholamines are synthesised by the adrenal medulla or by pre-synaptic neurons, comprising the neuro-endocrine system (Flierl et al., 2008, Slominski, 2005). Catecholamine biosynthesis involves the conversion of L-tyrosine to adrenaline and nor-adrenaline, catalysed by numerous enzymes, see figure 1.8 (Schulz et al., 2004, Blaschko, 1973).

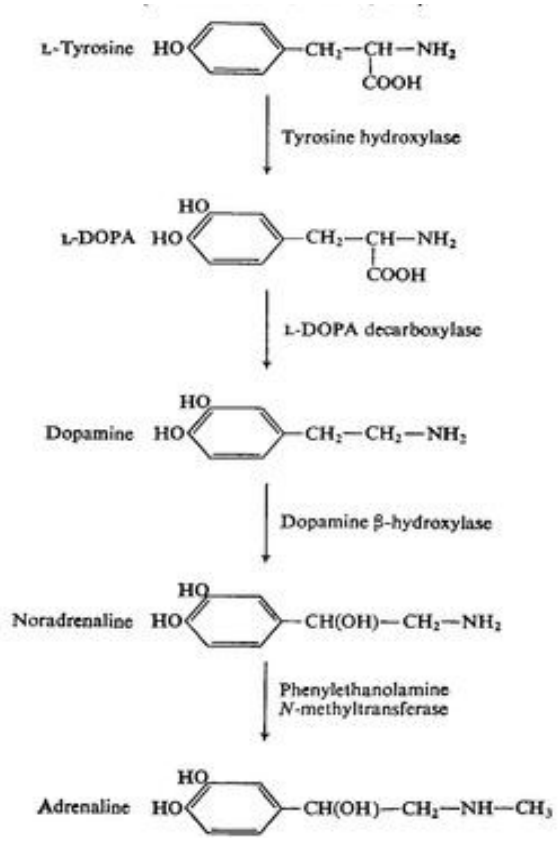


Figure 1.8: Catecholamine biosynthesis pathway. Catecholamine biosynthesis involves the conversion of L-tyrosine to L-dopa (L-3,4-dihydroxyphenylalanine) by tyrosine hydroxylase which is the rate-limiting step for catecholamine biosynthesis. L-DOPA is converted to dopamine by DOPA decarboxylase aromatic L-amino acid decarboxylase. Finally, dopamine β-hydroxylase convert's dopamine to nor-adrenaline after which, phenylethanolamine-N-methyl transferase (PNMT) can catalyze the synthesis of adrenaline from nor-adrenaline. (Image taken from Blaschko, 1972)

Keratinocytes express two of the enzymes required to synthesise catecholamines, tyrosine hydroxylase and phenylethanolamine-N-methyl transferase (Schallreuter et al., 1992, Pullar et al., 2006b). Catecholamines can also be synthesised and secreted by local cell types such as keratinocytes (Pullar et al., 2007), corneal epithelial cells (Pullar et al., 2007) and melanoctyes (Gillbro et al., 2004) to act in an autocrine or paracrine fashion in the skin. Therefore, the β-ARs form part of a sub-cutaneous β-AR network allowing cells to respond rapidly to catecholamines in their local environments.

Previous research has shown that catecholamines play a major role in the body's response to stress and can alter wound healing. Early work on the adrenoceptors (AR) found an anti-motogenic role for catecholamines on epidermal migration during wound closure in adult newts (Donaldson and Mahan, 1984). Chronically stressed mice also exhibited a delay in wound healing due to the increased levels of adrenaline, resulting in decreased re-epithelialisation, inflammation, angiogenesis and wound contraction (Romana-Souza et al., 2010a). However, a non-selective β-AR antagonist, propanolol, reversed the deleterious effects observed on re-epithelialisation and wound contraction, leading to improved wound repair (Romana-Souza et al., 2010a, Romana-Souza et al., 2010b). Contradictory data has revealed a beneficial role for catecholamines in which nor-adrenaline depleted mice showed a reduction in re-epithelialisation rates compared with control mice (Gosain et al., 2006, Pullar et al., 2008). Therefore, catecholamines can alter various processes of wound healing, implicating the ARs as a possible therapeutic target in the skin.

Keratinocytes are the most abundant cell type in the epidermis and express β_2-ARs, therefore particular interest has been shown to its functional significance in the skin

(Schallreuter, 1997, Orenberg et al., 1983, Steinkraus et al., 1992, Steinkraus et al., 1996). Research has linked the abnormalities β_2-AR density and function to the physiology of various skin disorders such as vitiligo, atopic eczema and psoriasis. In vitiligo, there are abnormally high levels of tetrahydrobiopterin, an essential cofactor involved in the catecholamine biosynthesis pathway producing high levels of adrenaline and noradrenaline which control a high β_2-AR density on undifferentiated/proliferating keratinocytes. Receptor numbers correlate with calcium uptake and release into the cell cytosol, therefore differentiating keratinocytes in patients suffering from vitiligo have an inability to uptake calcium (Schallreuter, 1997). In ectopic eczema, there is a low density of β_2-ARs as a result of a point mutation on the β_2-AR gene, altering its structure and function on keratinocytes and peripheral blood lymphocytes (Schallreuter, 1997). Interestingly, keratinocytes in psoriatic lesions, showed a low cAMP response upon β_2-AR activation (Eedy et al., 1990). This may contribute to the unusually high proliferation rate of keratinocytes in psoriasis as research has shown that the modulation of cAMP can alter keratinocyte proliferation (Takahashi et al., 2004).

Research during the last decade has found a critical role for the β-ARs in regulating keratinocyte and fibroblast physiology and wound healing using a variety of *in vitro, ex vivo* and *in vivo* models.

β-AR agonists have been shown to be anti-motogenic and anti-mitogenic in keratinocytes, decreasing migration (Pullar et al., 2003), proliferation and delaying re-epithelialisation both *ex vivo* and *in vivo* (Pullar et al., 2006a). The mechanisms behind the anti-motogenic effect observed was revealed to be via a PP2A mediated

mechanism preventing ERK phosphorylation (Pullar et al., 2003) and altered localisation to the lamellipodial edge (Pullar et al., 2006a, Pullar et al., 2003), see figure 1.9. Immunocytochemistry studies, using antibodies against actin and vinculin, an FA protein, revealed that β-AR activation can alter the cytoskeletal organisation in migrating keratinocytes. Further research has revealed a role for the β_2-AR in oral keratinocyte physiology and wound repair. A non-selective β-AR agonist, isoproterenol, reduced single cell migration and scratch wound healing *in vitro* though an ERK1/2 and p38 dependent mechanism. These effects were reversed by pre-incubating with the non-selective β-AR antagonist, timolol (Steenhuis et al., 2011).

On the other hand, non-selective and selective β-AR antagonists such as timolol and ICI 118,551 respectively were found to be pro-motogenic in the single cell migration (SCM) assay and scratch wound assay and ultimately promoted skin wound re-epithelialisation in an *ex vivo* chronic human skin wound healing model (Pullar et al., 2006b). The ICI 118,551-mediated increase in keratinocyte migration was possibly via the blockade of the endogenously synthesised catecholamine ligands (Pullar, 2006a) known to have a deleterious effect on keratinocyte migration (Pullar et al., 2006a, Pullar et al., 2007, Pullar et al., 2003, Pullar et al., 2008, Ghoghawala et al., 2008, Sivamani et al., 2009). Also, immunocytochemistry studies revealed that ICI 118,551 preserved a more migratory keratinocyte phenotype with localised, vinculin-stained FAs at the leading and trailing edges of the lamellipodium (Pullar et al., 2006a, Pullar et al., 2006b). In addition, the β-ARs also play a role for regulating corneal epidermal wound repair. Corneal epithelial cells can also synthesise catecholamines, therefore β-AR antagonists were shown to enhance corneal wound healing by blocking

autocrine/paracine catecholamine binding, whilst a β-AR agonist delayed wound healing (Pullar et al., 2007, Ghoghawala et al., 2008).

In contrast, β-AR activation in human dermal fibroblasts is both pro-mitogenic and pro-motogenic. Mechanistic studies revealed that a cAMP/PKA signalling pathway transduced the β-AR-mediated increase in fibroblast proliferation, whilst the β-AR mediated, src-dependent, transactivation of EGFR, and subsequent downstream phosphorylation of ERK, was essential for the β-AR agonist mediated increase in dermal fibroblast migration, see figure 1.10 (Pullar and Isseroff, 2006). In addition, β-AR activation delayed collagen gel contraction via a cAMP dependent mechanism (Pullar and Isseroff, 2006, Pullar and Isseroff, 2005a). This β-AR mediated delay in collagen gel contraction *in vitro* could translate to a decrease in wound contraction *in vivo* as fibroblasts are responsible for generating the mechanical forces required for wound contraction in the granulation tissue (Gabbiani et al., 1972).

Further work has also supported the therapeutic potential for using beta blockers to enhance wound healing. A study in 2003, found that β_2-AR antagonists increased epidermal barrier recovery, post tape stripping (Denda, 2003). In addition, β-AR blockade by propanolol, stimulated protein synthesis and protein deposition rates in a rabbit skin wound model, which might be beneficial to wound healing (Zhang et al., 2009). In contrast, studies investigating the effect of the non-selective β-AR antagonist, propanolol-mediated beta-blockade on rat excisional cutaneous wound healing found that it was impaired (Romana-Souza and Monte-Alto-Costa, 2009, Romana-Souza et al., 2006, Romana-Souza et al., 2009).

Studies have also shown a role for the β-ARs in burn wound healing. A cohort study found that hospital burns patients who had been administered propanolol, a non-selective β-AR antagonist, during their stay, healed faster compared with control groups (Arbabi et al., 2004). This was further supported by an efficacy study on beta blockade in adult burns patients (Mohammadi et al., 2009, Pereira et al., 2007). An *ex vivo* study, using a human burn skin model found that stress-induced adrenaline levels delayed keratinocyte migration and consequently decreased re-epithelialisation however treatment with β_2-AR antagonists (ICI 118,551) inhibited this effect and consequently enhanced the rate of burn wound re-epithelialisation (Sivamani et al., 2009).

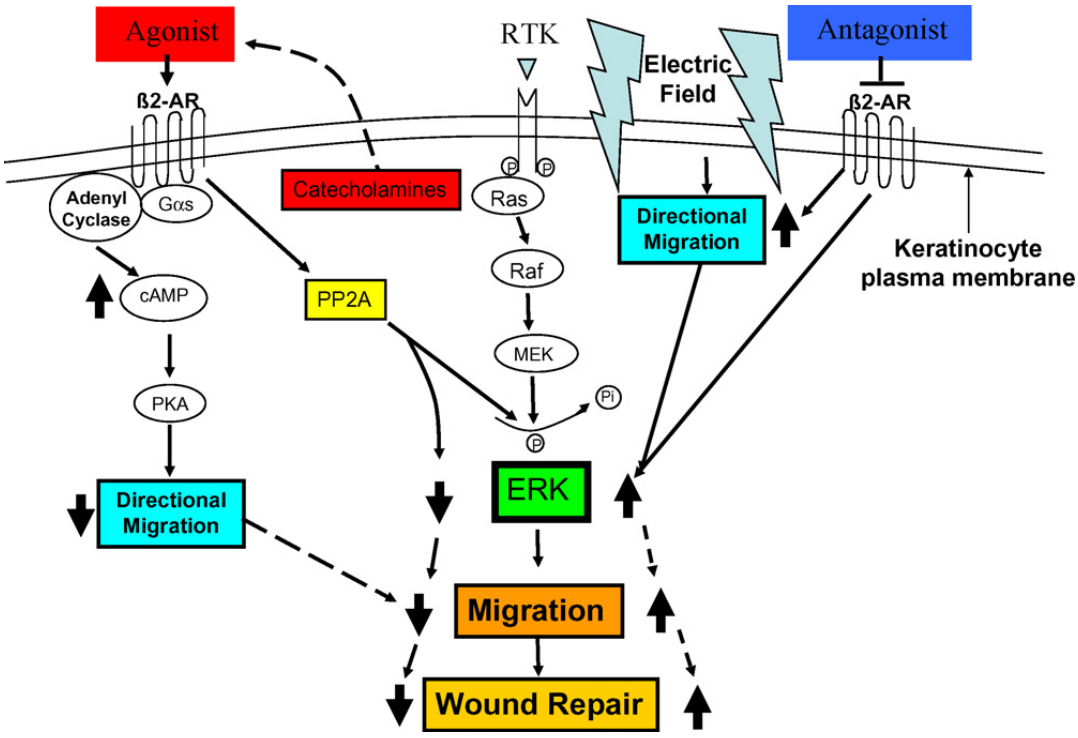


Figure 1.9: Illustration of β-AR signalling pathways for keratinocyte migration *in vitro.* Endogenously produced catecholamines, decrease migration though the dephosphorylation of ERK by a PP2A dependent mechanism. β-AR agonists also increase cAMP which decreases the directionality of migration. β_2-AR antagonists on the other hand prevent β-AR agonists from binding and increase directional migration. (Image taken from Pullar et al., 2008)

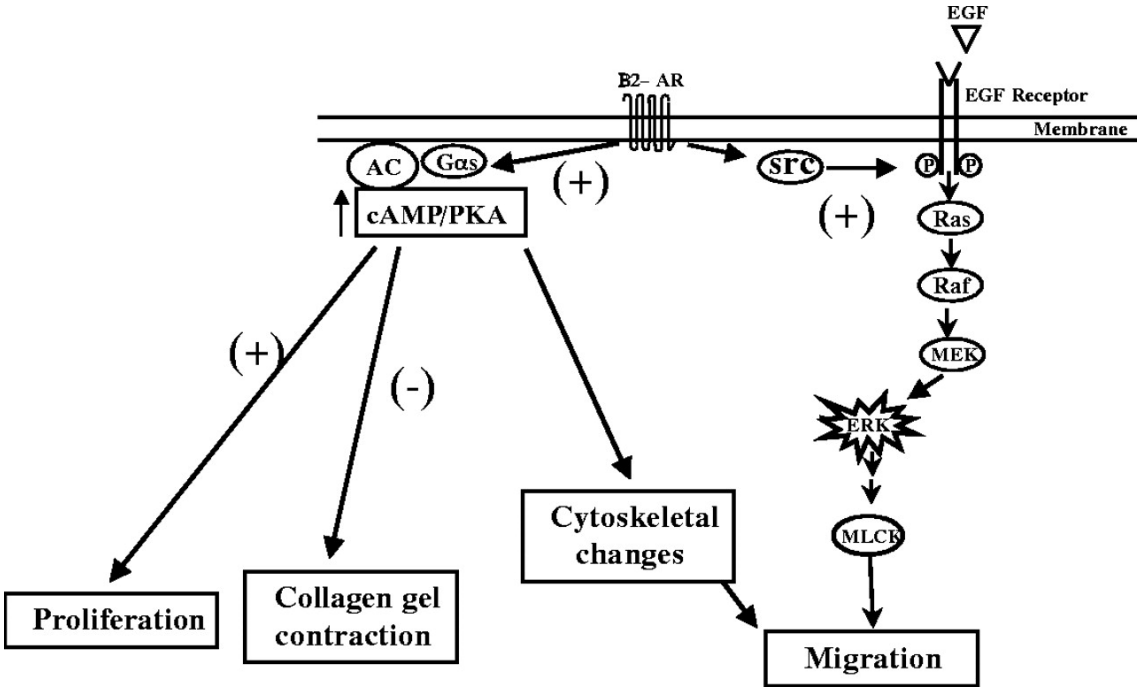


Figure 1.10: Illustration of β-AR signalling pathways for fibroblast function *in vitro*. β-ARs promoted dermal fibroblast cell migration though the modulation of ERK by the src-mediated transactivation of the EGFR. β-AR modulation of cAMP and PKA, meanwhile, were shown to alter proliferation, collagen gel contraction and cyctoskeletal changes. (Image taken from Pullar and Isseroff, 2006)

β-ARs can also play a role in modulating inflammation. β-AR agonists can regulate many aspects of mast cell, neutrophil, monocyte and macrophage physiology and have a detrimental effect on chemotaxis (Silvestri et al., 1999), migration (Sadowska et al., 2005), recruitment (Johnson, 2002, Li et al., 2003a), phagocytosis (Gosain et al., 2009), adhesion to the endothelium (Bowden et al., 1994), generation of ROS and production of pro-inflammatory mediators (Bowden et al., 1994, Gosain et al., 2009, Johnson, 2002, Li et al., 2003a, Maris et al., 2004, Muthu et al., 2005, Perkins et al., 2004, Sadowska et al., 2005, Silvestri et al., 1999). In addition, β_2-AR agonists can promote the release of growth factors from macrophages such as TNF-α and VEGF (Muthu et al., 2005, Verhoeckx et al., 2006). However, β-AR blockade can also be anti-inflammatory, reducing PMN leukocyte recruitment, migration, ROS release and cytokine production, see table 1.8 (Barker et al., 2005, Djanani et al., 2003, Dunzendorfer and Wiedermann, 2000, Jaboureck-Bouttier et al., 1999). Furthermore, β_2-AR blockade by ICI 118,551, attenuated the hyperinflammatory response induced by traumatic injury in mice (Rough et al., 2009). In conclusion, there is a large body of evidence suggesting that β-ARs can modulate inflammation. Indeed it appears that the role of the β-ARs in wound inflammation is complex.

Inflammatory cell type	β-AR agonist, modulation of inflammatory cytokine generation	Reference
Basophils	↓ histamine, ↓ IL-4 and ↓ IL-13	(Johnson, 2002)
Eosinophil	↓ peroxidase, ↓leukotriene , ↓ superoxide and eosinophil derived neurotoxin	(Johnson, 2002)
Macrophage	↑ IL-10, ↓ IL-8, ↓IL-1β, ↓IL-2, ↓ TNF-α, ↑GM-CSF, ↑ oncostatin M and ↑ VEGF	(Muthu et al., 2005, Verhoeckx et al., 2006)
Mast cell	↓ IL-5, ↓ TNF-α, ↓ GM-CSF, ↓MIP-1α, ↓leukotriene C, ↓leukotriene D and ↓ prostaglandin D_2	(Johnson, 2002)
Monocyte	↑ IL-8, ↓ IL-8, ↓ TNF-α, ↑ IL-10 and ↓MIP-1α	(Johnson, 2002, Li et al., 2003a, Maris et al., 2004)
Neutrophil	↓ IL-8, ↓macrophage inflammatory protein, ↓ cytokine-induced neutrophil chemo-attractant, ↓ TNF-α and ↓ bacterial permeability increasing protein	(Johnson, 2002, Maris et al., 2004)
T lymphoctytes	↓ TNF-α, ↓IL-2, ↓IL-3, ↓IL-4, ↓ IL-5 and ↓ GM-CSF	(Johnson, 2002)

Table 1.8: Table showing the β-AR agonist mediated release of inflammatory cytokines from numerous inflammatory cell types. (Original table, drawn by Andrew P O'Leary)

A number of studies within the last two decades have linked the β-ARs to the regulation of EC function and angiogenesis. Previous *in vitro* work has revealed that the β_2-AR can regulate the vasculature though the control of nitric oxide (NO) release (Ferro et al., 1999) and transendothelial permeability (Baluk and Mcdonald, 1994, Zink et al., 1995). Furthermore, the β-ARs have been demonstrated to regulate macrovascular EC function including proliferation of human coronary artery smooth muscle cells and human coronary ECs (Brehm et al., 2001), proliferation and eNOS activity in human umbilical vein endothelial cells (HUVEC) (Sexl et al., 1995, Seya et al., 2006), migration, proliferation and differentiation of HUVECs (Lamy et al., 2010), MMP9 secretion and differentiation of human brain microvascular endothelial cells

(Annabi et al., 2009) and the migration, proliferation, invasion and cell elongation of human retinal and choroidal ECs (Steinle et al., 2003, Steinle et al., 2005). Meanwhile *in vivo* studies have found that β-ARs blockade increased angiogenesis in a rat cutaneous wound healing model (Romana-Souza et al., 2010b, Romana-Souza et al., 2006, Romana-Souza et al., 2009). These will be discussed in more depth in section **3.1, introduction**.

Stem cells have been shown to play a role in wound repair. Different subtypes of stem cells are recruited to the wound site from the bone marrow and differentiate into specialised wound cell types such as leucocytes, epithelial like cells, fibroblasts and ECs (Borue et al., 2004, Fathke et al., 2004, Lau et al., 2009, Medina et al., 2007, Sivan-Loukianova et al., 2003). The less differentiated stem cell, $CD34^-$ is involved in wound healing (Badiavas et al., 2003, Borue et al., 2004, Fathke et al., 2004) and can give rise to the intermediate stem cell, the circulating blood fibrocyte (collagen I^+/$CD11B^+$/$CD13^+$/$CD34^+$/$CD45RO^+$/MHC class II^+/$CD86^+$) and the circulating bone marrow derived hematopoietic stem cell ($CD34^+$) (Abe et al., 2001, Medina et al., 2007, Sivan-Loukianova et al., 2003, Yang et al., 2002). Research has revealed a role for the β-ARs in the modulation of stem cells. Different subtypes of the β-ARs are expressed on bone marrow derived stem cells (bMSC) (Akavia et al., 2006, Bertani et al., 2005). Catecholamine activation of the β-ARs can regulate mobilisation and migration of $CD34^+$ cells (Spiegel et al., 2007). In addition, selective agonists for β_2-AR and β_3-AR cooperate to induce the mobilisation of bMSCs (Mendez-Ferrer et al., 2010). Further work has revealed that β-AR blockade post injury prevents bone marrow suppression of bMSCs and allows for their release into the blood, which could assist in wound healing (Beiermeister et al., 2010, Mohr et al., 2011).

In summary, β-ARs play a role in wound healing and my work will focus specifically on their role in angiogenesis, essential for wound repair.

1.7 Pharmacology of β-AR agonists and antagonists

An agonist is a substance that binds to a receptor, activates it and elicits a cellular response whereas an antagonist is a substance that prevents the response of an agonist (Kenakin, 2004). When experimenting with receptor agonists and antagonists *in vitro* and *in vivo*, it is important to take into account the pharmacodynamics, pharmacokinetics, (route of administration, absorption, distribution and elimination), bioavailability, dose, affinity, potency, efficacy and intrinsic efficacy (Feldman et al., 1997). Dose is the amount of agonist given to elicit a desired effect. Affinity on the other hand is the strength of binding of the drug to its biological target such as a receptor which is denoted, K_d which is actually the reciprocal of affinity and defined as the concentration of ligand required to occupy 50% of the receptors. The efficacy is the relationship between receptor occupancy and ability to initiate a maximal response. Similarly, potency refers to drug activity expressed in terms of the amount required to produce an effect of given intensity. For example, a highly potent drug such as morphine can elicit a high response at low concentrations. Subsequently, potency is proportional to affinity and efficacy. Finally, intrinsic efficacy is defined by the property of a drug that determines the amount of biological effect produced per unit of drug-receptor complex formed (Feldman et al., 1997, Kenakin, 2004).

Another important factor that needs to be taken into account when exposing receptors to drugs at high concentrations or for long durations is receptor desensitization and various side effects. For example, isoproterenol, a β-AR agonist used in the treatment of heart diseases, can cause receptor desensitisation and have various side effects such as tachycardia and arrhythmias (Lanzara, 2005).

Receptors can be constitutively active whilst some exist as quiescent receptor systems. Thus, some receptors such as rhodopsin require an agonist to become active (Palczewski, 2006), whereas some receptors such as the β_2-AR can spontaneously transition between inactive and active conformations in the absence of an agonist (Zhou et al., 2000, Varma, 1999, Chakir et al., 2003). There are many β-AR modulators available that change the basal activity of the receptor by either stimulating the receptor or by blocking the receptor from their endogenous ligands via classical antagonism (Vilardaga et al., 2005, Zhu, 2005). Timolol is a non-selective β-AR antagonist, but can exhibit inverse agonism. Inverse agonism can occur upon binding of an antagonist to a GPCR that is constitutively active which will stabilise the inactive conformation and consequently reduce the continuous downstream signalling of the receptor (Vilardaga et al., 2005). Therefore, the β_2-AR is an interesting receptor in that it can exist in multiple conformational states including active, resting, inactive and one of spontaneous activation (Zhou et al., 2000, Varma, 1999, Chakir et al., 2003).

Selective β_2-AR agonists, such as salbutamol and terbutaline are a widely prescribed subset of drugs used to treat asthma and chronic obstructive pulmonary disease due to their ability to cause bronchodilation. β-AR agonists, such as isoproterenol, are used to treat conditions such as bradycardia (Rang et al., 2003). Alternately, β-AR antagonists ("beta-blockers") are also a widely prescribed sub-set of drugs that are used to treat an array of conditions (Rang et al., 2003). A β-AR antagonist such as timolol can reduce intra-ocular pressure, a major risk factor in glaucoma, the leading cause of blindness in the world (Coleman and Brigatti, 2001, Feldman, 2004, Medeiros and Weinreb, 2002, Quigley, 1996, Sharif et al., 2001, Tan et al., 2002). Beta-blockers are often used to treat hypertension and cardiovascular dysfunctions. For example,

timolol and oxprenolol have been used to treat hypertension to alleviate the symptoms of angina pectoris and to prevent cardiac dysrhythmias. Other β-AR antagonists such as propanolol, alprenolol, atenolol and oxprenolol are used to treat benign essential tremor. However, despite the widespread use of β-AR antagonists, there have been few studies on the ability of these drugs to improve wound healing.

Both formoterol and salmeterol are long acting selective β_2-AR agonists; however they differ in their speed of action and intrinsic efficacy. Formoterol has a rapid onset of action, whereas salmeterol has a much slower onset of action. These differences can be attributed to their physiochemical and pharmacological properties, affecting their tissue diffusion rates and interactions with the β_2-AR. Both formoterol and salmeterol are lipophilic and therefore enter and are stored within the lipid bilayer. Salmeterol is highly lipophilic, while formoterol has only moderate lipophilicity and is more hydrophilic. Therefore salmeterol is partitioned in the lipid bilayer whereas formoterol rapidly diffuses though the tissues with some molecules partitioned in the lipid bilayer and some that remain in the extracellular space. The moderate lipophilicity and hydrophilicity of formoterol produces a fast onset of action at the β_2-AR (Lotvall, 2001). The lipophilic nature of these β_2-AR agonists may also explain their long duration of action. One theory states that ligands which partition themselves within the lipid bilayer (formotereol), are retained and act as a depot (Anderson, 1993, Lotvall, 2002). In contrast, salmeterol may elicit sustained β_2-AR effects by binding to an alternative anchored binding site within the lipid bilayer (Lotvall, 2002, Rong et al., 1999). No such binding site has been found for formoterol so the depot hypothesis seems more plausible (Anderson, 1993, Lotvall, 2002).

On the other hand, salbutamol is a short acting, selective β_2-AR agonist which is water soluble with low lipid solubility, restricting its partition into cell membranes. Salbutamol is generally a partial agonist (dependent on system), and therefore generally unable to elicit a response equivalent to a full agonist such as isoproterenol (Lotvall, 2001, Lotvall, 2002). The obvious advantage of using partial agonists would be to prevent adverse effects which may be more prominent upon the use of full agonists. Such effects include reducing overstimulation of the receptor to avoid desensitisation, adaption, tolerance and dependence issues (Zhu, 2005). Isoproterenol on the other hand in some systems is a non-selective, full β-AR agonist which binds to both β_1-AR, β_2-AR and β_3-AR, see table 6.1 (Berlan et al., 1993, Galitzky et al., 1993, Soeder et al., 1999).

In general, care must be taken when interpreting data from radioligand binding studies and functional assays. For example, a ligand may act as a full agonist on one cell type or in a particular environment but may behave as a partial agonist in a different cell type in a more physiological environment. For example, one study revealed that agonist potency at the β_3-AR was dependent on receptor expression and the nature of the assay. Indeed, a selective β_3-AR antagonist, SR 59230A was found to have agonist activity when there was a 10-fold increase in β_3-AR receptor expression in CHO cells (Wilson et al., 1996). It is therefore important that data regarding the affinity and efficacy of the ligand should be taken in context.

In conclusion, the pharmacology of β-AR agonists and antagonists is complex. The efficacy and potency of β-AR agonists and antagonist are system and response dependent. For example it is well known that β-AR agonists may be partial agonists on

one response or in one system but act as full agonists on other responses or in other systems. Furthermore, purported selectivity of particular β-AR agonists and antagonists may only be selective or non-selective is a particular system at a specific concentration. Therefore one can only assume a certain level of response or selectivity if the relevant pharmacological studies have been done in that particular system or appropriate pharmacology has been performed in the system of interest.

1.8 Thesis hypothesis and aims

It is hypothesised that β-AR activation will inhibit HDMEC function and angiogenesis whist β-AR blockade will promote HDMEC function and angiogenesis.

The aim of this investigation is to determine the role of the β-AR family in angiogenesis. Angiogenesis is an essential process in wound healing and aberrations have been associated with both chronic wound healing and scarring (Mohammad et al., 2008, Wynn, 2008). Wound angiogenesis occurs via the microvascular endothelium at post-capillary venules as opposed to the macrovascular endothelium (Staton et al., 2006). Therefore, human dermal microvascular endothelial cells (HDMEC) will be used to study the effects of the β-AR family in modulating HDMEC function. A range of β-AR agonists and antagonists will be used to elucidate the roles that β-ARs play in modulating processes known to be critical for angiogenesis.

Processes required for wound angiogenesis such as migration, proliferation, secretion of pro-angiogenic growth factors (VEGF-A and FGF-2), and formation of tubule-like structures will be explored in the presence or absence of β-AR agonists and antagonists. A range of *in vitro* techniques will be used including the single cell migration assay, proliferation assay, scratch wound assay, enzyme-linked immunosorbent assay (ELISA) and the two-dimensional (2D) tubule assay. In addition, cell signalling studies using pharmacological agents and techniques such as Western blotting and ICC will reveal the β-AR activated downstream signalling pathways that underpin the observed changes to HDMEC physiology *in vitro*. Finally, vessel outgrowth and angiogenesis will be investigated using the *ex vivo* rat aortic ring assay and the *in vivo* chick chorioallantoic membrane (CAM) assay.

Overall, this research hopes to reveal how β-ARs regulate angiogenesis. β-AR-mediated alterations in angiogenesis could improve healing in chronic wounds or reduce scarring in acute wounds (Gosain et al., 2006, Pullar et al., 2008). Modulation of angiogenesis could also help to treat other pathophysiologies such as cancer (Perez-Sayans et al., 2010).

Chapter 2 Materials/Methods

2.1 Materials

2.1.1 General lab reagents

2-mercaptoethanol, bovine serum albumin (BSA), ethylenediaminetetraacetic acid disodium salt dehydrate, forskolin, dimethyl sulfoxide (DMSO), DL-dithiotheitol (DTT), donkey serum, glycine, isopropyl alcohol, phenylmethanesulphonylfluoride (PMSF), phosphate buffered saline (PBS) tablets, sodium chloride, sodium dodecyl sulphate, sodium fluoride, sodium orthovanadate, sulphuric acid 2M, Tris, Triton X 100 and Tween 20 were obtained from Sigma Aldrich (Poole, U.K). Methanol, high performance liquid chomatography grade obtained from Fisher Scientific (Loughborough, U.K). Quantikine duo set enzyme-linked immunosorbant assay (ELISA) kits for human VEGF-A and FGF-2 were obtained from R&D Systems (Minneapolis, U.S).

2.1.2 Western blotting, immunocytochemistry and immunoprecipitation reagents

4X sample buffer, 20X DTT, Bradford reagent assay dye, criterion XT pre-cast 10% Bis-Tris gels and XT MOPs running buffer 20X were obtained from Bio-Rad laboratories (Hemel Hampstead, U.K). Non-fat dried milk was purchased from Morrisons supermarkets (Leicester, U.K). Protease inhibitor cocktail tablets were purchased from Roche diagnostics (Indianapolis, U.S). Chemiluminescence western blotting detection reagents were obtained from GE Healthcare (Buckinhamshire, U.K). PageRuler plus prestained protein ladder was obtained from Fermentas Life Sciences (St Leon Rot, Germany). ProLong Gold antifade reagent and Texas red-X phalloidin were purchased from Invitrogen (Paisely, U.K). Protein A-Sepharose beads were purchased from

BioVision (Bedfordshire, U.K.). Polyvinylidene flouride (PVDF) was obtained from Roche Diagnostics USA (Indianapolis, U.S).

2.1.3 Antibodies

Anti-alpha-tubulin antibody, monoclonal anti vinculin antibody and anti goat IgG secondary antibody were obtained from Sigma Aldrich. Cy-2 conjugated AffiniPure $F(ab')_2$ fragment donkey anti-mouse IgG (H+L) was purchased from Jackson ImmunoResearch laboratories INC. (Suffolk, U.K.). EPAC rabbit polyclonal was purchased from Santa Cruz Biotechnology (Middlesex, U.K.). Primary monoclonal phospho-PKA substrate (RRXS/T) (100G7E) rabbit mAB, monoclonal β-actin (13E5) rabbit mAb, polyclonal p44/42 MAPK (Erk1/2) and phospho-p44/42 MAPK (Erk1/2) (Th202/Tyr204) antibodies and secondary anit-rabbit HP and anti mouse HP-linked antibodies were obtained from New England Biolabs (Hitchen, U.K). Primary rabbit polyclonal antibody phenylethanolamine N-methltransferase, goat polyclonal β_1-AR antibody, rabbit polyclonal β_2-AR antibody and goat polyclonal β_3-AR antibody were purchased from Abcam (Cambridge, U.K.).

2.1.4 Bacterial toxins

Cholera toxin from Vibrio cholera was obtained from Sigma Aldrich. Pasteurella multocida toxin and pertussis toxin were purchased from Calbiochem (Nottingham, U.K.).

2.1.5 Cell culture reagents

Basement membrane extract, BME (Cultrex) was obtained from R&D Systems (Minneapolis, U.S). Foetal bovine serum (FBS), trypsin/ethylenediaminetetraacetic

acid, soybean trypsin inhibitor, HEPEs buffered saline solution (HBSS) and coating matrix (collagen I) were obtained from Cascade Biologics though Invitrogen.

2.1.6 PKA and EPAC modulators

Reagents

8-CPT-2ME-cAMP sodium salt was purchased from Tocris Biosciences (Bristol, U.K). Adenosine 3',5'cyclic monophosphorothioate- Rp-Isomer-triethylammonium salt, adenosine 3',5'cyclic monophosphorothioate- Sp-Isomer-triethylammonium salt and brefeldin A from Eupenicillium brefeldianum were purchased from Calbiochem (Nottingham, U.K.).

2.1.7 β-AR agonists and antagonists

Reagents

Salbutamol hemisulfate, atenolol, ZD7114 hydrochloride, formoterol hemifumarate, ICI 118-551 hydrochloride and SR 59230A hydrochloride were purchased from Tocris Biosciences. Isoproterenol hydrochloride was purchased from Calbiochem. Timolol maleate salt was obtained from Sigma Aldrich.

Key

β-AR non-selective, full agonist – isoproterenol, partial agonist – salbutamol, β_2-AR selective, full agonist – formoterol and β_3-AR selective, full agonist – ZD7114.

β-AR non-selective antagonist – timolol, β_1-AR selective antagonist – atenolol, β_2-AR selective antagonist – ICI 118,551 and β_3-AR selective antagonist – SR 59230A.

2.1.8 Cell lines and cell culture

Standard cell culture conditions

All cells were grown at 37°C with 5% CO_2/95% air in a humidified atmosphere. Cells were cultured in 35, 60 and 90 mm plastic cell culture dishes and 6, 12, 24, 48 and 96 well plastic plates were obtained from Fisher Scientific. Two to three donor cell strains were used and experiments were performed using cell passages between 3 and 7 for HDMECs and HNKs and between passages 3 to 15 for HDFs. All cells were grown to 70/80% confluence for routine culture, and also before experiments.

Culture of primary HDMECs

Primary HDMECs (4C1290 and 2C0670 donors), isolated from human neonatal dermis, were purchased from Cascade Biologics though Invitrogen and the CADMEC 1300 donor was purchased from Cell Applications (San Diego, U.S). Cells were grown in endothelial cell growth medium (ECGM) MV supplemented with microvascular growth supplement (MGS) (0.05ml/ml fetal calf serum, 0.004 ml/ml endothelial cell growth supplement, 10ng/ml EGF, FGF-2, IGF, VEGF-165, ascorbic acid, 90 µg/ml heparin and hydrocortisone) (PromoCell, Heidelberg, Germany) and antibiotics (100 U/ml penicillin and 100µg/ml streptomycin) (Sigma Aldrich). Cells were grown on attachment factor (AF) containing 0.1% gelatine from Cascade Biologics though Invitrogen.

Culture of primary HNKs

Primary HNKs (6C0336, 5C1254 and 6C341 donors), isolated from human neonatal foreskin, (were purchased from Cascade Biologics. Cells were grown in keratinocyte growth medium (Epilife, 0.06 mM Ca^{2+}) supplemented with human growth supplement

(0.2 ng/ml EGF, 5 µg/ml insulin, 5 µg/ml transferrin, 0.18 µg/ml hydrocortisone and 0.2% bovine serum pituitary extract) and antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin) from Cascade Biologics.

Culture of primary HDFs

Primary HDFs (6C193, 3C0657 and 789013 donors), isolated from human neonatal foreskin, were purchased from cascade biologics though Invitrogen. Cells were grown in Dulbeccos Modified Eagle Medium (DMEM) supplemented with 10% foetal bovine serum (FBS) (Cascade Biologics though Invitrogen) and antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin) from Cascade Biologics.

Human ductal epithelial tumour cell line (T47D)

The T47D cell line (a kind gift from Professor Maggie Manson) was sub-cultured in Roswell Park Memorial Institute medium (RPMI) (Invitrogen) supplemented with 10% FBS and 100 U/ml penicillin/100 µg/ml streptomycin (Invitrogen).

Isolation and activation of neutrophils and macrophages from peripheral human blood

Neutrophils and macrophage cell lines were a kind gift from Dr. Bernard Burke. Isolation and activation of neutrophils and macrophages were carried out by Dr. Gabrielle Le Provost.

Human blood (20 ml) was taken from healthy volunteers with the approval of the local Ethics Committee. The red blood cells were sedimented using 6% dextran. Neutrophils were purified by density gradient centrifugation with histopaque (Sigma Aldrich). The neutrophils obtained were typically 96% pure. The cells were activated with 50 ng/ml

GM-CSF (Invitrogen) for 30 min in RPMI (Invitrogen) supplemented with 2% fetal bovine serum (FBS) prior to plating for ELISA.

Peripheral blood mononuclear cells were isolated from heparinised (10U/ml) blood from healthy volunteers, with the approval of the local Ethics Committee, by centrifugation on Ficoll-Paque Plus (Amersham, Little Chalfont, UK). Cells were re-suspended at 5 x 10^6 cells/ml in Iscove's-modified Dulbecco's medium (Sigma Aldrich) supplemented with 2.5% human AB serum (Sigma Aldrich, Poole, U.K.), 2 mM L-glutamine (Invitrogen) 200 U/ml penicillin/200 μg/ml streptomycin (Life Technologies) and 100 ng/ml recombinant macrophage colony stimulating factor (a kind gift of the Genetics Institute, Cambridge, USA).

1x10^6 PBMC were plated into a 12-well plate and cells were incubated for 5 days, in complete Iscove's media (Sigma Aldrich), to obtain a mixed population of lymphocytes and macrophages. The lymphocytes were removed by 3 washes in HBSS, then the adherent macrophages were activated by 100ng/ml lipopolysaccharides (LPS) from *Salmonella abortus equii* (Sigma Aldrich) in Iscove's media supplemented with 0.5% FBS, for one h, prior to treatment for ELISA.

The U937 monocytic cell line was sub-cultured in RPMI (Invitrogen) supplemented with 10% FBS and 200 U/ml penicillin/200 μg/ml streptomycin (Invitrogen). Differentiation was induced by plating 1x10^6 cells/well into 6-well plates, in the presence of 250 nM phorbol-12-myristate 13-acetate (PMA) (Sigma Aldrich) for 2 days. Fresh media without PMA was added for one more day, prior to treatment for ELISA. Adherent cells were treated with media alone (RPMI + 2% FBS) or media containing β-AR agonist or antagonist for 2 h, then activated by LPS for a further 24 h for ELISA.

2.2 Methods

2.2.1 Single cell migration assay

HDMECs were plated onto collagen I (30 μg/ml; Cohesion Technologies, Invitrogen) coated 35mm plastic cell culture dishes, at a density of $3.5x10^3$ per cm^2 for 2 hours (h) at 37°C. In some experiments HDMECs were serum starved for 24 h. HDMECs were then the incubated with media alone or media containing β-AR agonists or antagonists at time 0. In some experiments, HDMECs were initially pre-incubated with β-AR agonists or antagonists for 60 minutes (min) prior to their addition at time 0 to determine if pre-incubation with the β-AR agonists or antagonists would have an additional effect on HDMEC SCM. The 35mm dishes were placed in a heating chamber, designed to maintain the medium at 37°C on an inverted Nikon phase contrast microscope. Time lapse images of the cells were digitally captured every 10 min over a 1 h period, in an automated manner using Improvision Open Labs 5.0 or Volocity 5.5.2 software (Perkin Elmer, Coventry, U.K.) on a Macintosh GS. The cells' migratory paths were manually tracked using the same software, the data were exported to FileMaker Pro 3.0, whereby the data were analysed and the speed and distance travelled by each cell were calculated using a cell tracking programme customised for use in the Pullar laboratory.

2.2.2 Scratch wound assay

The wells of a 48 well plate were demarcated with two vertical parallel lines and three crossing horizontal lines. HDMECs were seeded at a density of $3x10^4$ cells/well in triplicate on collagen I (30 μg/ml) coated-demarcated 48 well plates. A sterile pipette tip was used to scratch an approximately 1 mm wide gap re-creating the wound edge,

at the centre of the wells. Wells were then washed with HBSS and media was added alone or containing β-AR agonist or antagonist treatments. The areas between the vertical and horizontal demarcated areas of each well were then photographed at 0, 6, 12, 24 and 32 h using Improvision Velocity 5.5.2 software (Perkin Elmer) until the gaps/spaces had closed. The data were then analysed by using NIH image 1.6 (public domain image processing and analysis program for Windows developed at the National Institute of Health and available on the internet at http://rsb.info.nih.gov/nih-image), which determined the area of the wounds. The percentage of wound healing was then calculated by dividing the area of the wound at time X by the area of the wound at time 0 and multiplying by 100.

2.2.3 2D tubule assay

The wells of a 96 well plate were demarcated to produce a 3X3 grid. The centre of the top left, top right, bottom left, bottom right and the central square were demarcated with a cross. BME (Cultrex) was thawed on ice at 4°C. 50 µl of BME was added to the centre of each well in duplicate on the demarcated 96 well plate and left to set at 37°C for 30 min. HDMECs were grown to 70/80% confluence, trypsinised and 50 µl containing $20x10^4$ cells, was added to the top of the BME and left to incubate for 2 h at 37°C. 50 µl of either media alone or media containing 2X (two times concentration, 20 µM) β-AR agonist or antagonist was added to each well. The cross at the centre of the 5 demarcated square in the wells were then photographed at 0, 6, 12, and 24 h on an inverted Nikon eclipse phase contrast microscope using Improvision Open Labs 5.0 (Perkin Elmer) software on a Macintosh. Tubule formation was analysed in a single-blind fashion by counting the number of tubule-like structures in 5 images, taken at pre-determined positions in each well, in duplicate.

2.2.4 Proliferation assay

HDMECs were seeded at $3.5x10^3$ cells/well in 0.5 ml media for 2 h at 37°C. After 2 h, cells were treated with either media alone or β-AR agonists or antagonists at time 0. Treatments were replaced every 24 h and wells were washed, trypsinised and the cells counted using a haemocyometer. Cells were counted at 24, 48 and 72 h. In some experiments, HDMECs were initially pre-treated with a PKA inhibitor (rp cAMP; 50 μM), EPAC agonist (8-pCPT; 10 μM) or PTX 50 ng/ml for 60 min, 30 min and 16 h respectively before addition alone or with the β-AR agonist at time 0. Treatments were replaced every 24 h and wells were washed, trypsinised and the cells counted using a haemocyometer. Cells were counted at 72 h.

2.2.5 Detection of β-AR family on HDMECs

Cell treatments

HDMECs were seeded at a density of $2x10^5$ cells to collagen I (30 μg/ml) coated 90 mm dishes, while HNKs, HDFs and T47D were seeded at density of $1x10^6$ cells and incubated for 24 h. Cells were placed immediately upon ice and washed twice with 1 ml ice-cold phosphate buffered saline solution containing phosphatase inhibitors (50mM NaF and 1 mM Na_3VO_4) and scraped in 150 μl of lysis buffer (PBS containing 50 mM NaF, 1 mM Na_3VO_4,0.5% Triton X-100, 200 μg/ml PMSF, 1X protease inhibitor cocktail). The lysates were transferred to 1.5 ml microcentrifuge tubes and centrifuged at 14,000 x g for 10 min at 4°C. The lysates were then stored at -20°C and used within 1 to 2 weeks. The protein concentration of each sample was determined using the Bradford protein assay (Biorad).

Immunoblotting

55µg of HDMEC and HDF cell lysates were used, while for HNKs, 83µg of cell lysate was used. Cell lysates were added to an equal volume of 4X sample buffer and 20X DTT to make a final concentration of 1X sample buffer and 1X DTT. Samples were electrophoretically separated on 10% Bis-Tris gels using the Bio-Rad Criterean blotting system according to manufacturer's instructions. Proteins were transferred to PVDF membranes using the Criterean transfer system and immunoblotted with anti-β_1-AR, anti-β_2-AR, anti-β_3-AR or β-actin at 1:500, 1:500, 1:5000 and 1:1000 dilutions respectively in 5% non-fat, dried milk (5% milk, 0.1% Tween 20 in TBS buffer). Blots were developed using enhanced chemiluminescence (ECL) detection agents.

2.2.6 Detection of PNMT in HDMECs

Cell treatments

HDMECs were seeded at a density of $2x10^5$ cells on collagen I (30µg/ml) coated 90mm dish, while HNKs and HDFs were seeded at density of $1x10^6$ cells and incubated for 24 h. Cells were then lysed as previously described.

Immunoblotting

33µg of lysate was used for HDMEC, HNKs and HDFs. Cell lysates were added to an equal volume of 4X sample buffer and 20X DTT to make a final concentration of 1X sample buffer and 1X DTT. Protein samples were electrophoretically separated and transferred to PVDF membranes as previously described. PVDF membrane was then probed with anti-phenylethanolamine N-methltransferase or anti-β-actin at 1:50

dilution in 5% non-fat, dried milk (5% milk, 0.1% Tween 20 in TBS buffer). Blots were developed using enhanced chemiluminescence detection agents.

2.2.7 β-AR modulation of ERK1/2 phosphorylation

Cell treatments

HDMECs were seeded at a density of $8x10^4$ cells on collagen I (30 μg/ml) coated 35 mm dishes and incubated for 24 h. Cells were then washed with HBSS and serum starved for 24 h. Cells were preincubated with either media alone (control) or media containing 10 μM β-AR agonist or antagonist for 0 (no treatment), 1, 5, 10, 15 and 30 min at 37°C. Cells were then lysed as previously described.

Immunoblotting

10 μg of each protein sample was added to an equal volume of 4X sample buffer and 20X DTT to make a final concentration of 1X sample buffer and 1X DTT. Protein samples were electrophoretically separated and transferred to PVDF membranes as previously described. PVDF membrane was then probed with anti-ERK or anti-phospho-ERK at 1:1000 dilutions in 5% BSA (5% BSA, 0.1% Tween 20 in TBS buffer). Blots were developed using enhanced chemiluminescence detection agents.

Analysis

Densitometry analysis was performed on the scanned images using NIH image 1.6.

2.2.8 PKA inhibitor validation

Cell treatments

HDMECs were seeded at a density of 80,000 cells on collagen I (30 μg/ml) coated 35 mm dish and incubated for 24 h. Cells were then washed with HBSS and serum starved for 24 h. Cells were pre-incubated with either media, forskolin 50 μM or sp cAMP 50 μM for 60 min or pre-incubated with a PKA inhibitor (rp cAMP 50μM) alone for 120 min or for 60 min before addition of β-AR agonist and PKA inhibitor for a further 60 min. Cells were then lysed, as previously described.

Immunoblotting

10 μg of each protein sample was added to an equal volume of 4X sample buffer and 20X DTT to make a final concentration of 1X sample buffer and 1X DTT. Protein samples were electrophoretically separated and transferred to PVDF membranes as previously described. PVDF membrane was then probed with anti-phospho-PKA substrate mAB or anti-α-tubulin at 1:1000 dilutions in 5% BSA (5% BSA, 0.1% Tween 20 in TBS buffer). Blots were developed using enhanced chemiluminescence detection agents.

Analysis

As previously described.

2.2.9 Immunocytochemical analysis of actin and vinculin

Sterile, circular 13 mm glass coverslips were transferred to 12 well plates and coated with collagen I (30 µg/ml) for 30 min at 37°C. The coverslips were then washed with PBS, and 50 µl of $1x10^3$ cells were added to the coverslips and incubated for 3 h. Cells were treated with β-AR agonist or antagonists for 15 min. Coverslips were washed twice with PBS and fixed for 10 min in 4% paraformaldehyde. Cells were washed 3 times with PBS for 5 min between each step. Cells were then permeabilised with 0.1% Triton X-100 in PBS for 5 min and blocked with 5% donkey serum on PBS for 20 min. Primary monoclonal anti-vinculin antibody (A2547; 1:100; Sigma Aldrich) was added dropwise in 1X PBS and incubated for 1 h at 37^{o}C. A donkey anti mouse secondary Cy2 antibody (15-226-151; 1:50; Jackson Immunoresearch) was added in 1XPBS for 1 h at 37^{o}C. Phalloidin-Texas red (T7471; 1:25; Invitrogen) was added to the vinculin stained coverslips for 20 min. Standard controls were performed, whereby the primary or secondary antibody were incubated alone to ensure specificity. Finally, Prolong Gold antifade reagent was used in accordance with the manufacturer's instructions to mount coverslips onto glass microscope slides. The slides were left in the dark for 24 h. The slides were viewed on an inverted fluorescent Nikon eclipse (TE20000-E) phase contrast microscope using a 20X and 40X objective lens. The images were captured using a Hamamatsu digital CCD camera (model: C4742-80-12AG) and pseudo-coloured red for phalloidin-texas red (actin), green for Cy2 staining (vinculin) and blue for DAPI staining (nucleus). F-actin pixel density (intensity) was analysed using Improvision Volocity 5.5.2 software. The total number of FAs were analysed by counting the number of FAs in a single-blind fashion.

2.2.10 Immunoprecipitation

Anti-EPAC antibody (5 µg; 25632; Santa Cruz Biotechnology) was linked to 30 µl of pre-washed protein A sepharose beads (Biovision) overnight. HDMECs were seeded at a density of $3x10^5$ cells on collagen I (30 µg/ml) coated 90 mm dish and incubated for 24 h. Cells were either untreated or treated with 10µM β_2-AR/β-AR agonist and lysed, as previously described, in 500 µl lysis buffer. Lysates were then pre-cleared with 100 µl of pre-washed beads for 2 h at room temperature and then incubated with antibody bound beads overnight at 4°C on a rotary mixer. The beads were washed seven times with lysis buffer before adding 1x sample buffer. The samples were boiled at 95°C for 5 min and centrifuged to pellet the beads. The supernatants were loaded onto a 10% gel and electrophoretically separated and transferred to PVDF membranes as previously described. PVDF membrane was cut at the 70 kDa marker and probed with anti-EPAC (1:100) or anti-β_2-AR (1:100) antibody in 5% BSA (5% BSA, 0.1% tween 20 in TBS buffer). The membrane below the 70kDa marker was then striped for 30 min at 50°C in stripping buffer (62.5mM Tris.HCl ph6.8, 2% SDS and 100mM 2-mercaptoethanol) and re-probed for anti-β_1-AR 1:100 dilution in 5% BSA.

2.2.11 Rat aortic ring assay

Post schedule one euthanasia, the abdominal and aortic arcs were extracted from adult Whistar rats and placed into sterile PBS. The fibro-adipose tissue was then removed and aortas washed in warm, sterile PBS three times. Aortas were then cut into uniform, 1 mm sections and placed into basement membrane extract and incubated at 37°C for 30 min. Aortas were placed in media with 2% FBS and incubated until day 4 (72 h), whereby the media was either replaced with control media or media containing β-AR agonist or antagonist. Aortic endothelial outgrowth was then imaged

every 24 h using a Nikon eclipse Ti phase contrast microscope and Improvision Open Lab 5.0 software on a Macintosh GS. Images were analysed in a single-blind fashion using Improvision Volocity 5.5.2 software to investigate surface area covered by microvessel outgrowth from the aorta.

2.2.12 CAM assay

Fertilised eggs were obtained though the University of Leicester Biomedical Sciences Department from local hatcheries and incubated at 37°C in a humidified environment for 48 h. On day 3, approximately 5 ml of albumin was removed from the obtuse poles of the eggs using a 21G cannula. A square window (2 X 2 cm^2) was opened into the shell and shell membrane using a dremel cutter. The window was then sealed with parafilm and cello-tape and placed back in the incubator in a horizontal position. The eggs were then further incubated for 48 h. On day 5, β-AR agonist or antagonists treatments were dried onto the centre of sterile 13 mm circular coverslips for 2 h and then placed face down onto the CAM. Eggs were photographed at the centre of each coverslip every 24 h until day 10 using a Prior stereomicroscope (2.5X objective lens) and a premiere digital microscope eyepiece (Model MA88). Vascularisation was analysed in a single-blind fashion by counting the total number of branch points per field of view though the centre of the coverslip.

2.2.13 VEGF-A and FGF-2 ELISAs

HDMECs, HNKs or HDFs were grown to 70/80% confluence in 60 mm or 90 mm dishes and then seeded at a density of $80x10^4$ cells/well in 6 well plates. Cells were washed with HBSS and were serum starved for 24 h. Cells were incubated with either basal media alone or basal media containing β-AR agonists or antagonists for 6, 24 or 48 h

for HNKs and HDFs. For HDMECs, cells were serum-starved and treated in media plus 2% FBS. After each time point, the media was collected and stored at -80°C. Growth factor levels were determined using Quantikine R&D Systems duo set ELISA kits (Minneapolis, U.S), specific for human VEGF-A and human FGF-2.

2.2.14 Statistical analysis

Values are presented as mean ± SEM. Statistical analysis was performed using either a one-way or two-way ANOVA for one or two categorical variables, followed by the multiple comparison Dunnett's or Bonferroni post-test, using GraphPad Prism5 software. *P<0.05 was significant and **P<0.001 highly significant.

Chapter 3 The role of the β-AR family in dermal endothelial cell function *in vitro*

3.1 Introduction

3.1.1 Introduction

Cell migration, proliferation and tubule formation are essential physiological processes for angiogenesis, see section **1.4.2, angiogenesis** for further detail. The β-ARs are able regulate various wound healing processes and there is a growing body of evidence that β-ARs may regulate EC function and angiogenesis (Pullar et al., 2008).

3.1.2 Cell migration

The directional movement of cells to specific locations is essential for the maintenance and development of multicellular organisms. In particular, cell migration plays an important role in embryonic development, immune responses and wound healing (Horwitz and Webb, 2003). In order for cells to migrate, they undergo extensive actin remodelling allowing for various morphological changes to occur including extension, adhesion, translocation and de-adhesion (see figure 1.10). Three important structures containing actin filaments include filopodia, lamellipodia and stress fibres. Filopodia are a type of pseudopodia, a slim cytoplasmic projection from the cell, which contain cross-linked bundles of actin filaments. The main functions of these projections are to sense the external environment as well as adhering to the ECM (Lamalice et al., 2007). Lamellipodia are actin cytoskeletal projections found at the mobile/leading edge of the cell and are associated with actin polymerisation, allowing cells to undergo a "swimming like motility" as illustrated in figure 3.1 (Lamalice et al., 2007).

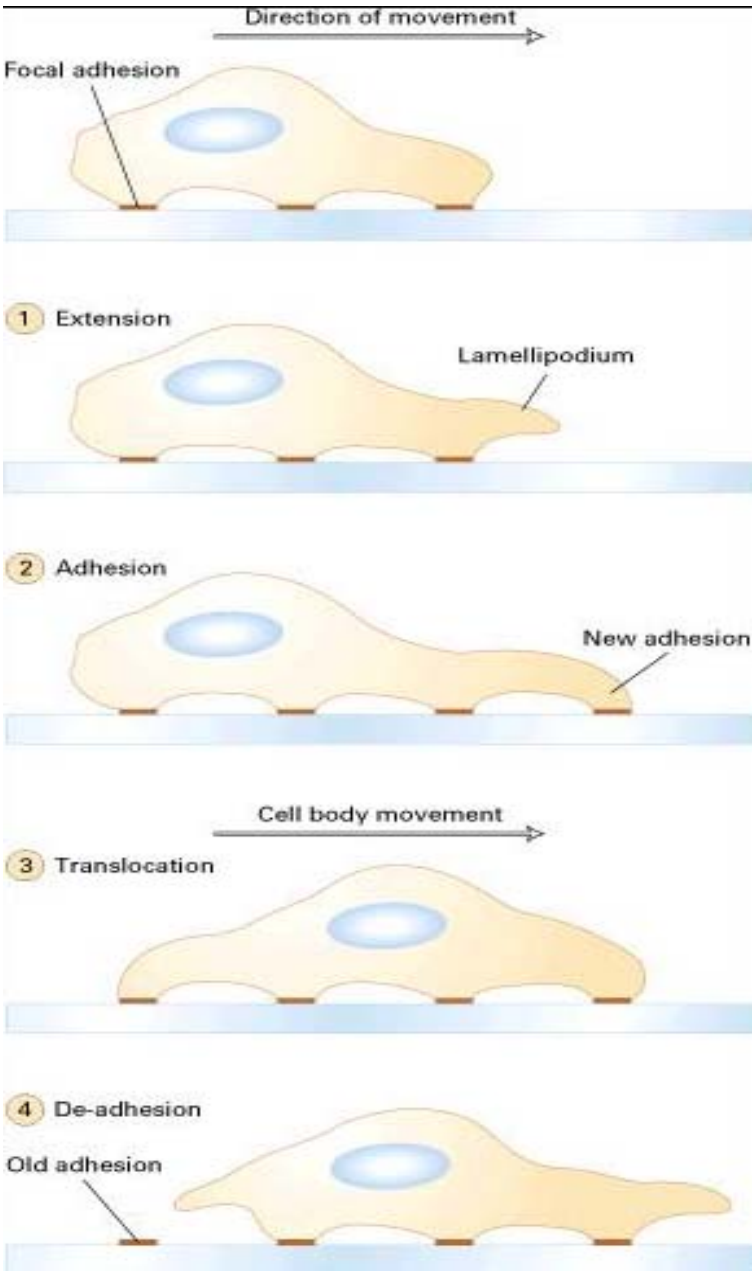


Figure 3.1: Image illustrating cell locomotion. To undergo migration, cell polarisation and formation of the lamellipodia occur. As the lamellipodia extends, old FAs at the rear of the cell are disassembled whilst new FAs are assembled at leading edge of the cell, allowing the cell to translocate forward. (Image taken from: Cell migration consortium, 2007)

Stress fibres also contain bundles of actin fibres. These bundles contain actin associated proteins such as myosin II, motor proteins and tropomyosins which act as accessory proteins (Lamalice et al., 2007). FA junctions serve as molecular attachments between the ECM and the cell cytoskeleton and act as signalling hubs, transmitting signals from the external environment to within the cell (Lamalice et al., 2007, Lo, 2006).

The migration of cells is essential in processes such as wound healing (Eming et al., 2007) and angiogenesis (Lamalice et al., 2007).

3.1.3 Cell proliferation

The cell cycle drives eukaryotic cell growth and proliferation which are essential during embryonic development and in processes such as wound healing. Cell proliferation is characterised by DNA replication and the segregation of chromosomes into two separate cells (Vermeulen et al., 2003). An important equilibrium exists between the addition and elimination of cells, which is highly regulated by cyclins, cyclin-dependent kinases and though a transcriptional regulatory network to prevent uncontrolled cell proliferation (Hipfner and Cohen, 2004, Vermeulen et al., 2003).

The proliferation of ECs is essential during wound healing and angiogenesis (Eming et al., 2007).

3.1.4 Tubule formation

Tubule formation is essential for neo-vascularisation *in vivo*. During sprouting angiogenesis, local ECs become activated, degrade the BM/ECM and differentiate into stalk and tip cells. EC tip cells develop filopodia to sense their environment and lead the invasion/migration process by growing from existing vessels and extend lumens

into newly formed branches. The cells at the trailing cord are stalk cells and eventually become lumenised. Two important factors believed to control tubule formation are the NOTCH signalling pathways and VEGF gradients, however the precise signalling mechanism are still under investigation. How ECs form lumen is still unclear although two proposed mechanisms include cord hollowing via cell rearrangement and vacuole fusion (Cleaver, 2011, Xu and Cleaver, 2011). *In vitro*, ECs do not form lumens, however they do undergo some of the processes that are indicative of in vivo EC tubulogenesis. When ECs are cultured on top of BME for example, ECs will differentiate into tubule-like networks. They will form discrete cellular aggregates, whereby ECs begin to align and elongate, forming cords. Eventually, these strings of aligned and elongated ECs connect to form tubule-like structures (Albini et al., 1987, Kleinman et al., 1986, Kubota et al., 1988).

3.1.5 The role of the β-ARs in EC function and angiogenesis

There is a growing body of evidence supporting a role for β-ARs in regulating EC physiology and angiogenesis. Previous work *in vitro* has demonstrated a role for the β-ARs in regulating the vasculature as research has shown that the β_2-AR can regulate the vasculature though the control of nitric oxide (NO) release (Ferro et al., 1999) and transendothelial permeability (Baluk and Mcdonald, 1994, Zink et al., 1995). A number of more recent studies have shown a role for the β-ARs in the modulation of macrovascular EC function. A selective β_1-AR antagonist, nebivolol inhibited proliferation of human coronary artery smooth muscle cells and human coronary ECs (Brehm et al., 2001), whilst nor-adrenaline promoted proliferation and eNOS activity in human umbilical vein endothelial cells (HUVEC) (Sexl et al., 1995, Seya et al., 2006). In contrast, β-AR blockade by propanolol, inhibited migration, proliferation and

differentiation of HUVECs (Lamy et al., 2010). In addition, propanolol inhibited MMP9 secretion and differentiation of human brain microvascular endothelial cells (Annabi et al., 2009). Activation of the β_3-AR is also pro-angiogenic. A β_3-AR agonist, BRL37344 promoted migration, proliferation, invasion and cell elongation in human retinal and choroidal ECs (Steinle et al., 2003, Steinle et al., 2005). Additional work by Steinle, Cappocia and Jiang in 2008, revealed that isoproterenol increased expression of pigment epithelial derived factor and Ang1, markers of a stable vasculature (Steinle et al., 2008). Apoptosis is also an essential process to halt angiogenesis, when it is no longer required, i.e. when a wound has healed. Nor-adrenaline induced apoptosis in rat/heart ECs via the β_1-AR and β_2-AR (Fu et al., 2004). Finally, the selective β_1-AR antagonist, nebivolol induced a moderate rate of apoptosis in human coronary artery smooth muscle cells and human ECs (Brehm et al., 2001).

The β-AR family can also regulate VEGF expression. Nor-adrenaline promoted VEGF expression in HUVECs (Sexl et al., 1995, Seya et al., 2006), while nor-adrenaline and a selective β_3-AR agonist, CL316,243 increased the expression of VEGF in murine adipocytes (Asano et al., 1997). The selective β_2-AR agonists, zilpaterol and clenbuterol stimulated VEGF secretion from macrophages via a cAMP dependent mechanism (Verhoeckx et al., 2006). The stress-induced activation of β_2-AR by nor-adrenaline in ovarian cancer cell lines, increased angiogenesis however, this was inhibited upon β_2-AR blockade (Thaker et al., 2006). Tumours in stressed animals had a β-AR mediated increase in the expression of VEGF, MMP2 and MMP5 (Thaker et al., 2006). Overall, the data suggests that the β-ARs may regulate VEGF mRNA expression, dependent on cell type.

In a study investigating post-ischemic angiogenesis in the hindlimb after femoral artery resection, there was a significant impairment in angiogenesis in β_2-AR knockout mice. However, restoration of the β_2-AR though gene therapy reinstated the angiogenic response, suggesting that β_2-AR activation promoted angiogenesis. Moreover, β_2-AR knockout mice exhibited an inhibition in the activation of nuclear factor kappa B (NF_KB), a transcription factor that can promote angiogenesis. NF_KB was unresponsive to both isoproterenol and TNF-α. Furthermore, the cAMP response element binding protein (CREB) which counter-regulates NF_KB was increased. Both NF_KB and CREB returned to normal levels and functioning states when the β_2-AR was restored by gene therapy (Ciccarelli et al., 2011). In addition, Iaccarino *et al* 2005 transfected rat femoral arteries with the human β_2-AR to study chronic ischeamia, resulting in enhanced angiogenesis, EC migration and proliferation (Iaccarino et al., 2005). In contrast, AR activation by nor-adrenaline resulted in a decrease in wound angiogenesis (Gosain et al., 2006), whilst the rotational stress-induced increase in adrenaline levels also impaired wound angiogenesis in mice (Romana-Souza et al., 2010a). Meanwhile, blockade of the β-ARs by propanolol led to an increase in angiogenesis in a rat cutaneous wound healing model (Romana-Souza et al., 2010b, Romana-Souza et al., 2006, Romana-Souza et al., 2009).

Therefore, these studies highlight the important role that the β-ARs have in regulating angiogenesis.

3.1.6 Chapter aims

This research will investigate the roles of β-ARs in angiogenesis. Here, the role of the β-AR family will be investigated in HDMEC migration, proliferation and tubule formation using single cell migration assays, scratch wound healing assays, proliferation assays and studying the formation of tubule-like structures on BME.

3.2 Results

3.2.1 HDMECs express β-ARs

ECs are known to express β-ARs. Adult human iliac vein, bovine fetal aortic (Howell, 1988), bovine aortic (Ahmad et al., 1990, Howell, 1988, Steinberg et al., 1984) and bovine pulmonary aortic (Ahmad et al., 1990) ECs express both β_1-AR and β_2-AR. Retinal ECs express both β_1-AR and β_3-AR (Steinle, 2003) while choroidal ECs express β_1-AR, β_2-AR and β_3-AR (Howell, 1988, Steinberg et al., 1984, Steinle et al., 2003, Steinle et al., 2005). However it is not known if HDMECs express β-ARs and if so, which subtypes they express. Western blotting studies revealed that HDMECs express all three subtypes of β-ARs, β_1-AR, β_2-AR and β_3-AR (figure 3.2).

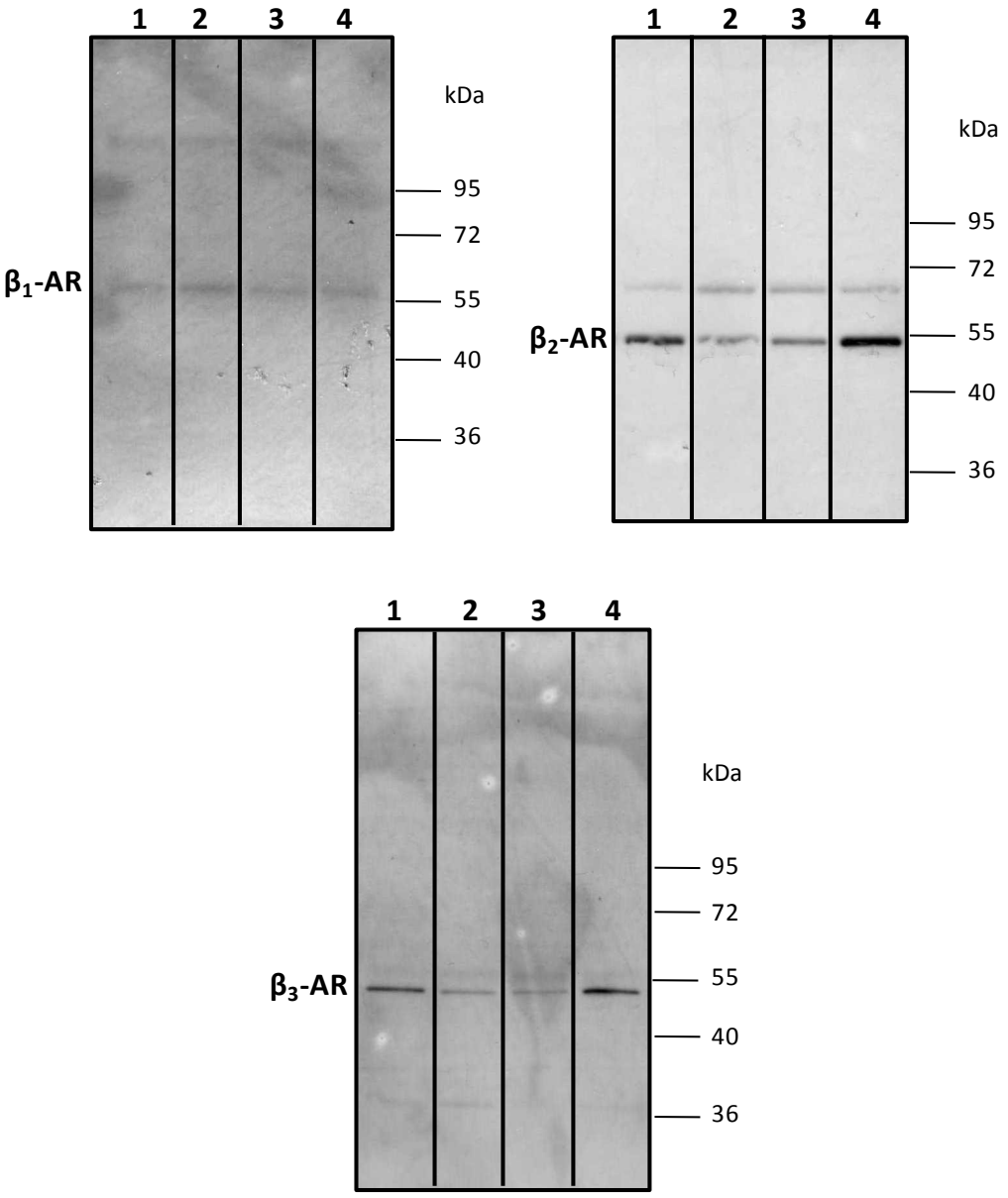


Figure 3.2: HDMEC β-AR expression studies

HDMEC strains 1-4 were lysed and separated electrophoretically, and membranes were immunoblotted with antibodies specific for β_1-AR (51 kDa), β_2-AR (47 kDa) and β_3-AR (55 kDa), as described in the methods.

3.2.2 β-AR activation decreases rate of HDMEC migration

EC migration is an important early step in skin wound angiogenesis (Eming et al., 2007, Lamalice et al., 2007). To investigate this, the single cell migration (SCM) assay was used, as described in the methods.

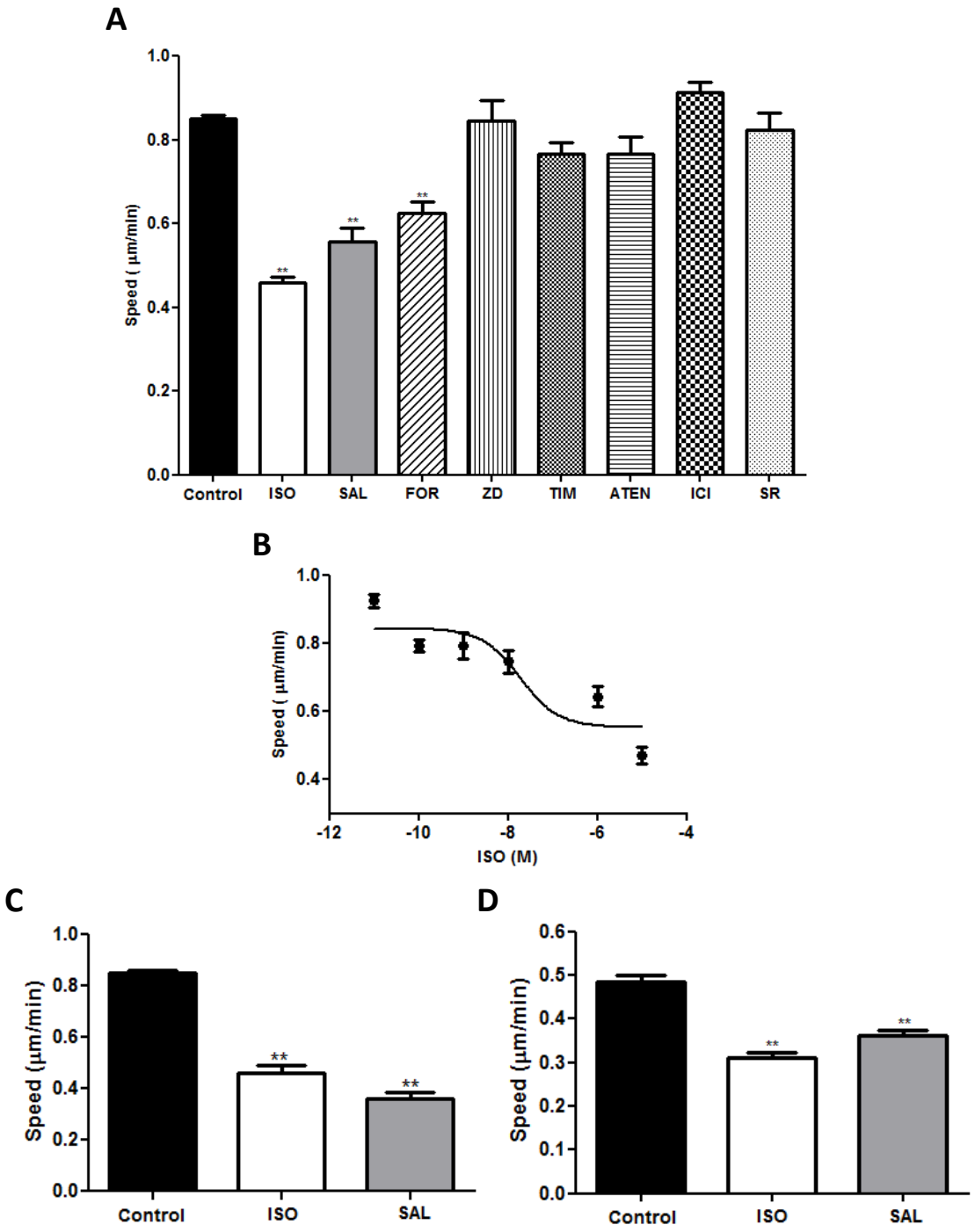
HDMECs express $\alpha_1\beta_1$, $\alpha_2\beta_1$ and $\alpha_3\beta_1$ integrins, which serve as receptors for collagen I (Furlan et al., 2005, Katanko et al., 1992). Collagen I is predominantly expressed during wound healing and is a natural substrate for cellular attachment, promoting growth, migration and proliferation. Therefore, HDMECs were plated onto a surface coated with collagen I matrix and SCM was performed in the presence or absence of a range of β-AR agonists and antagonists.

Initially, the β-AR agonists or antagonists were added at the start of the experiment at time 0. Isoproterenol reduced migration rate by 46% over the 1 h period, while salbutamol and formoterol significantly reduced migration rate by 34% and 27%, respectively. In contrast, ZD7114 and β-AR blockade by timolol, atenolol, ICI 118,551 and SR59230A had no significant effect on migration (figure 3.3A).

To investigate the parameters of the β-AR-mediated modulation of HDMEC SCM, experiments were performed in the presence of a range of β-AR agonist (isoproterenol) concentrations. β-AR activation generated a dose response effect on migration at concentrations ranging from 0.1 nM to 10 μM. The theshold dose, i.e. the lowest concentration at which an effect on migration was observed, was 0.1 nM and the half maximal effective concentration (EC_{50}) was calculated to be 19 nM (figure 3.3B).

To determine if pre-incubation with β-AR agonists or antagonists would have an additional effect on HDMEC SCM, HDMECs were pre-treated for 60 mins prior to the start of the experiments. Salbutamol had the most pronounced effect, reducing migration speed by 58% compared to a reduction of 34% when the salbutamol was added at time 0. In contrast, pre-incubating with isoproterenol had no additional effect on the rate of HDMEC migration which remained at 46% (figure 3.3C). Pre-incubation with ZD7114 and all the β-AR antagonists had no significant effect on migration speed (results not shown).

Exogenous growth factors or other serum factors could influence HDMEC SCM, therefore HDMECs were serum starved for 24 h. Indeed, in the absence of serum, HDMEC migration rate was reduced by 43%. Likewise, isoproterenol and salbutamol reduced migration speed by only 36% and 26% compared with 46% and 34% in serum-containing media, respectively (figure 3.3D).


A
Speed (µm/min)
1.0
0.8
0.6
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**
Control
ISO
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Speed (µm/min)
ISO (M)
-12
-10
-8
-6
-4
C
Speed (µm/min)
Control
ISO
SAL
D
Speed (µm/min)
0.6
0.5
0.4
0.3
0.2
0.1

Figure 3.2: β-AR activation decreases rate of HDMEC single cell migration

HDMEC SCMs were performed as described in the methods. Cells were treated with various β-AR agonists including, isoproterenol (ISO), salbutamol (SAL), formoterol (FOR) and ZD7114 (ZD). Cells were also treated with various β-AR antagonists including timolol (TIM), atenolol (ATEN), ICI 118,551 (ICI) and SR59230A (SR) at 10 μM, at time 0. The data shown were combined from 4-88 independent experiments, from 3 separate cell strains (control n=2219, ISO n=382, SAL n=122, FOR n=104, ZD n=648, TIM n=143, ATEN n=69, ICI n=82 and SR n=86) **(A)**. Cells were treated with the β-AR agonist (ISO) at a range of concentrations (0.1 nM-10 μM) at time 0. The data shown were combined from 3-88 independent experiments, from 3 separate cell strains (Control n= 2219, ISO 10 μM n= 382, ISO 1μM n=104, ISO 0.1 μM n=102, ISO 10 nM n=81, ISO 1nM n=206 and ISO 0.1 nM n=171) **(B).** Cells were pre-treated with β-AR agonists (SAL or ISO) at 10 μM for 60 mins and then added at time 0. The data shown were combined from 4-17 independent experiments, from 3 separate cell strains (control n= 443, ISO n=96 and SAL n=100) **(C)**. Cells were serum starved for 24 h and then treated with β-AR agonists (SAL or ISO) at 10 μM in serum free media, at time 0. The data shown were combined from 4-5 independent experiments from 3 separate cell strains, from 3 separate cell strains (control n= 181, ISO n=185 and SAL n=237) **(D)**. The data were averaged, statistically analysed using a one-way ANOVA followed by the Dunnetts test and graphically represented with the bars representing the means ±SEM. Only the differences versus the control are shown (** $p<0.001$).

3.2.3 β-AR activation delays HDMEC wound closure

β-AR activation by isoproterenol and salbutamol were anti-motogenic in HDMEC SCM. During angiogenesis, ECs migrate as a monolayer to re-vascularise the area, therefore, the scratch wound assay was used to investigate the role of the β-ARs in HDMEC migration from a monolayer wound edge. It is important to note that in this assay, the scratch will close by both proliferation and migration.

β-AR activation also had an anti-motogenic effect on migration from a monolayer wound edge. This is demonstrated in the images of an area of a representative scratch wound after 24 h, in the presence or absence of the β-AR agonists, isoproterenol or salbutamol (figure 3.3A). Isoproterenol and salbutamol reduced migration, decreasing the speed of wound closure as early as 12 h post wounding. After 32 h, control wounds were 92% healed while isoproterenol and salbutamol reduced wound closure by 25% and 26%, respectively (figure 3.3B). In contrast, ZD7114 and β-AR blockade had no effect on scratch wound healing (results not shown).

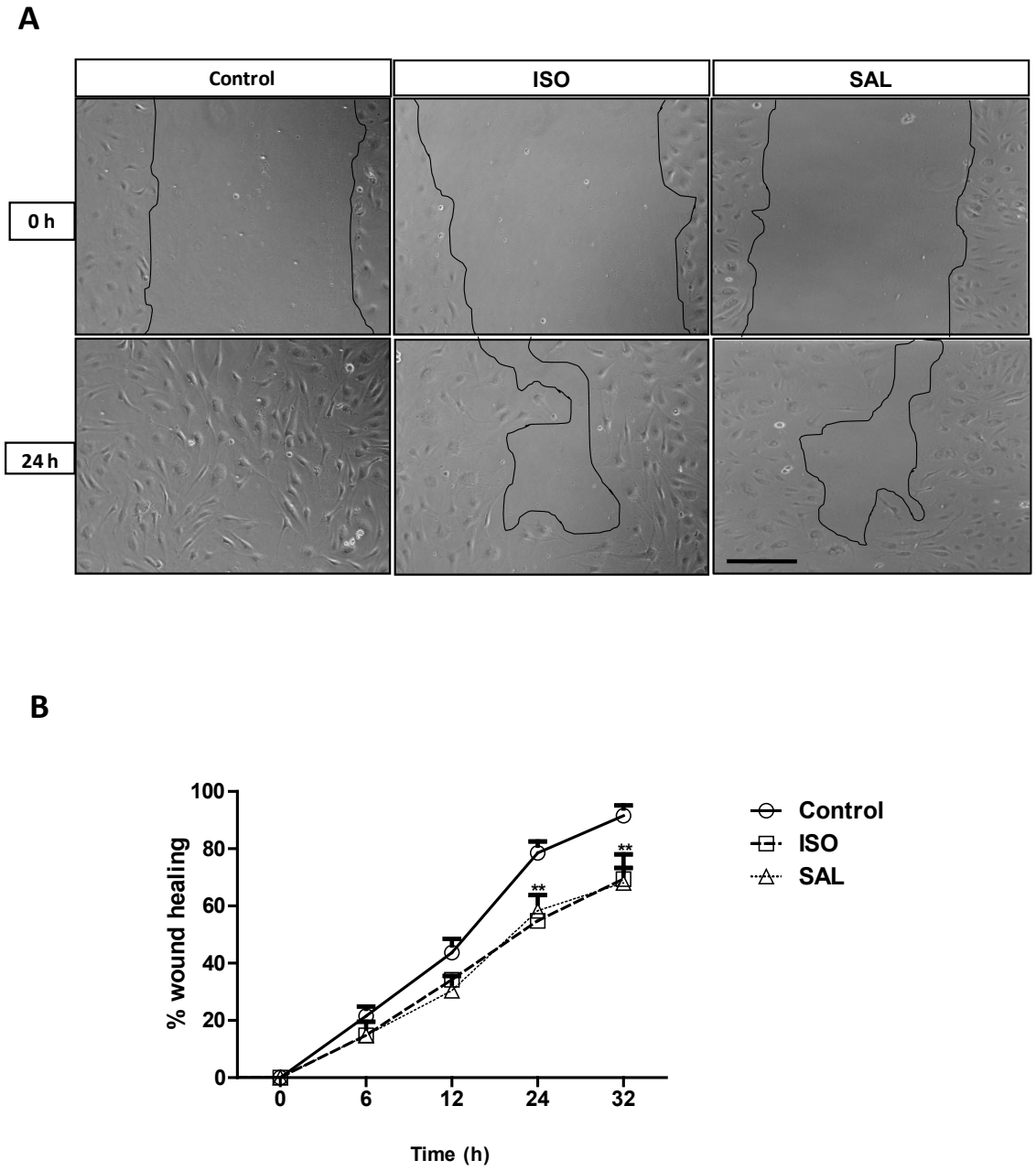


Figure 3.3: β-AR activation delays HDMEC scratch wound closure

Scratch wound assays were performed as described in the methods. Cells were treated with media alone or media containing β-AR agonists (isoproterenol; ISO or salbutamol; SAL) at 10 µM, at time 0. Demarcated areas of each well were photographed at 6, 12, 24 and 32 h. Images representative of control, ISO and SAL treated wounds at time 0 and 24 h are presented. The scale bar is 200 µM **(A)**. The data shown were the combined data from 3-6 independent experiments, from 2 separate cell strains. The data were averaged, statistically analysed using a two-way ANOVA followed by the Bonferroni test and graphically represented with the bars representing the means ±SEM. Only the differences versus the control are shown (control n= 6, ISO n=5 and SAL n=3). (** $p<0.001$) **(B)**.

3.2.4 β-ARs alter tubule formation on BME

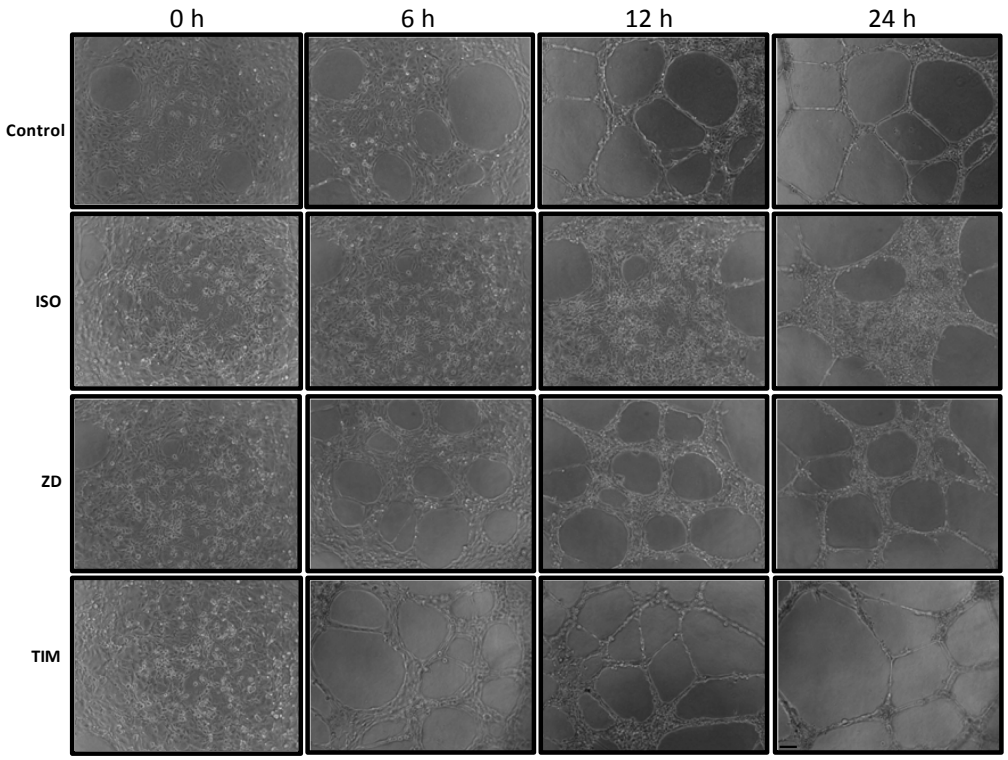
ECs undergo various physiological processes that contribute to angiogenesis including migration, proliferation, invasion, alignment, elongation and apoptosis. Interestingly, when ECs are cultured on top of basement membrane extract (BME) they organise into tubule-like structures. After a few h, ECs start to form discrete cellular aggregates, whereby ECs begin to align and elongate, forming cords. At about 6-12 h, these strings of aligned and elongated ECs connect to form tubule-like structures. At 24 h, ECs undergo apoptosis and the tubule-like structures break apart (Albini et al., 1987, Kleinman et al., 1986, Kubota et al., 1988). BME is a gelatinous mixture of proteins secreted by mouse tumour cells. The mixture consists of laminins, collagen type IV, fibrin, heparin sulphate, entactin and an array of growth factors including EGF, TGF-β and PDGF, therefore closely resembling the composition of the ECM (Kleinman et al., 1986, Ohashi et al., 2006). It is known that laminins and collagen I, and collagen IV to some extent, induce human endothelial cells to form capillary like structures (Kubota et al., 1988).

To explore HDMEC physiological processes in a more complex environment, HDMECs were cultured on top of BME for 24 h. HDMECS formed tubule-like structures at 0, 6 and 12 h, which eventually underwent apoptosis towards 24 h (figure 3.4A).

To quantitate HDMEC tubule development on BME, the number of tubule-like structures was counted per field of view. At 6 h ZD7114 and timolol promoted the development of tubule-like structures by 39% and 27%, respectively. On the other hand, isoproterenol appears to delay the formation of these tubule-like structures by 14% (figure 3.4B and 3.4C). Over time, at 12 and 24 h, the effects observed by

isoproterenol, ZD7114 and timolol appear to rescind and no longer alter tubule formation especially at 24 h, where there is a large decrease in control tubule-like structures by 51% compared with 6 h, demonstrating a degradation of the networks, see figures 3.4C.

A



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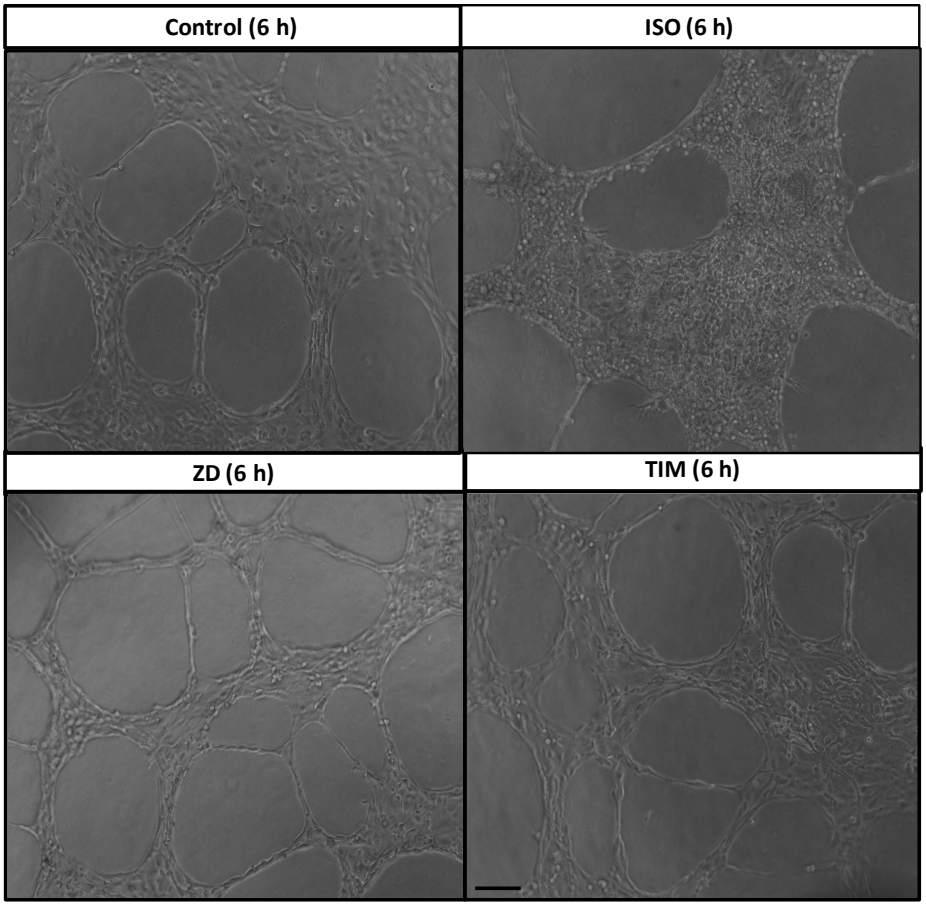

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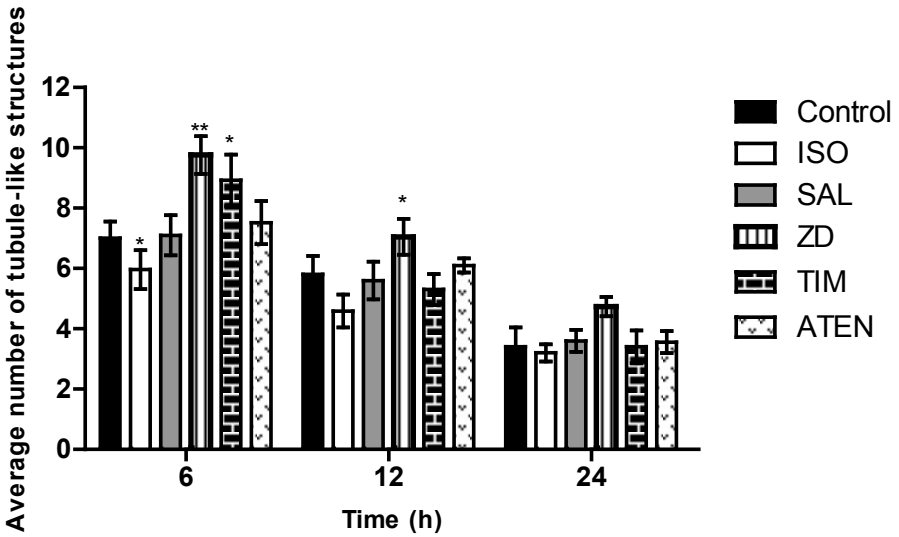


Figure 3.4: β-AR activation delays whilst β-AR blockade promotes formation of tubule like structures

2D tubule assays were performed as described in the methods. 50 µl of media alone or media containing 20 µM β-AR agonists (isoproterenol; ISO, salbutamol; SAL or ZD7114; ZD) or β-AR antagonists (timolol; TIM or atenolol; ATEN) was added at time 0 to each well containing 50 µl BME to give a final concentration of 10 µM. The wells were imaged at 0, 6, 12, and 24 h. Images representative of control, ISO, SAL and TIM treated cells at time 0, 6, 12 and 24 h are presented. The scale bar is 100 µM **(A)**. Images representative of control, ISO, ZD and TIM treated cells at 6 h are presented. The scale bar is 100 µM **(B)**. Tubule formation was analysed by counting the number of tubule-like structures in 5 images, taken at pre-determined positions in each well, in duplicate. The data shown were combined from 5 independent experiments, from 2 separate cell strains. Data were averaged, statistically analysed using the two-way ANOVA followed by the Bonferroni test and graphically represented with the bars representing the means ±SEM. Only the differences versus the control are shown (* $p<0.05$, ** $p<0.001$) **(C)**.

3.2.5 β_1-AR alters HDMEC proliferation

During angiogenesis, ECs proliferate and sprout outwards though the basement membrane (Eming et al., 2007). Therefore, the proliferation rate of HDMECs in the presence or absence of β-AR agonists or antagonists was determined every 24 h over a 72 h period. At 72 h, isoproterenol, significantly reduced proliferation by 44% (figure 3.5). However, salbutamol and ZD7114 and β-AR blockade had no effect on HDMEC proliferation rate.

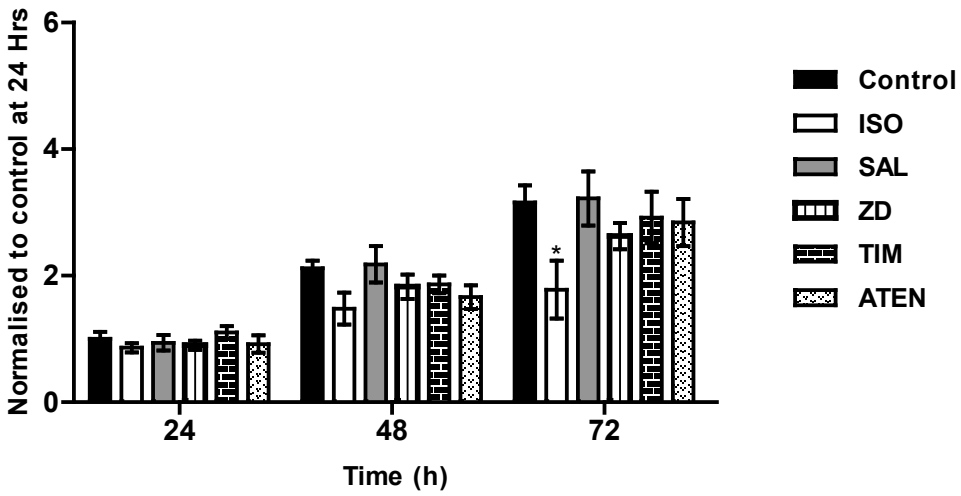


Figure 3.5: β-AR activation decrease cell proliferation at 72 h

Proliferation experiments were performed as described in the methods. HDMECs were treated with media alone or media containing β-AR agonists (isoproterenol; ISO, salbutamol; SAL or ZD7114; ZD) or β-AR antagonists (timolol; TIM or atenolol; ATEN) 10 µM, at time 0. The data shown were combined from 5 independent experiments, from 2 separate cell strains (control n= 5, ISO n=5, SAL n=5, ZD n=5, TIM n=5, ATEN n=5). The data were averaged, normalised to the control 24 h, statistically analysed using two-way ANOVA followed by the Bonferroni test and graphically represented with the bars representing the means ±SEM. Only the differences versus the control are shown. (* $p<0.05$).

3.3 Discussion

β-ARs can alter wound angiogenesis *in vivo* (Pullar et al., 2012, Romana-Souza and Monte-Alto-Costa, 2009, Romana-Souza et al., 2010a, Romana-Souza et al., 2010b, Romana-Souza et al., 2006, Romana-Souza et al., 2009). Physiological processes such as migration, proliferation and tubule formation play essential roles in a wound angiogenesis (Eming et al., 2007, Lamalice et al., 2007). Here, a range of experiments were performed to determine if β-AR activation or antagonism would alter EC physiological processes *in vitro*. Isoproterenol, salbutamol and formoterol were anti-motogenic whilst ZD7114 and β-AR blockade with timolol, atenolol and ICI 118,551 had no effect on HDMEC migration. Meanwhile, isoproterenol was anti-mitogenic whilst salbutamol and ZD7114 and β-AR blockade with timolol and atenolol had no effect on HDMEC proliferation. In the tubule assay, isoproterenol reduced tubule formation, whereas ZD7114 and timolol promoted tubule formation. In contrast, salbutamol and atenolol had no effect on tubule formation.

Various EC types express β-AR subtypes (Howell, 1988, Steinberg et al., 1984, Steinle et al., 2003, Steinle et al., 2005) however it was not known whether HDMECs express β-ARs. Western blotting studies using antibodies specific for the β-AR subtypes revealed bands at the predicted molecular weights for the β-ARs. However Western blotting also revealed various other bands raising the question of antibody specificity. In addition, GPCRs undergo post-translational modifications (Law, 2011) such as the β_2-AR which can be glycosylated (McGraw and Liggett, 2005). Consequently, receptors rarely appear at their predicted molecular weights. Therefore, further studies will be

required to confirm the expression of β-ARs in HDMECs such as real-time polymerase chain reaction (RT-PCR) to detect β-AR messenger ribonucleic acid (mRNA) expression.

A range of β-AR agonists (isoproterenol, salbutamol, formotereol and ZD7114) and antagonists (timolol, atenolol, ICI 118,551 and SR59230A) were used to provide evidence for the involvement of the β-ARs in regulating various functions of HDMECs *in vitro*. The various β-AR compounds were chosen to ensure that all three β-AR subtypes could be modulated. It is clear from various pharmacological studies (Baker, 2005b, Baker, 2010b, Hoffmann et al., 2004, Schnabel et al., 2000, Smith and Teitler, 1999) that some β-AR agonists and antagonists are selective whilst others are non-selective. Furthermore, even though some β-AR agonists such as ZD7114 are marketed as a selective β_3-AR agonist (Savontaus et al., 1998), recent pharmacological studies would dispute this (Baker, 2010b).

The expressions levels of the β-AR subtypes are unknown. According to recent β-AR radioligand binding studies (Baker, 2005b, Baker, 2010b), the concentrations of the β-AR agonists and antagonists used in this thesis, see section **2.1.7 β-AR agonists and antagonists**, only showed marginal selectivity. For example, salbutamol was used at 10μM and has K_d values of 21μM, 1μM and 105μM at the β_1-AR, β_2-AR and β_3-AR respectively (Baker, 2010b). Although these data show that salbutamol is selective for the β_2-AR, there will still be some occupancy at the β_1-AR and β_3-AR at the concentration used. Furthermore, research has shown that receptor numbers will dictate potency (Sato et al., 2007, Urban et al., 2007). Therefore it is possible for salbutamol to evoke responses through the β_1-AR and β_3-AR. Indeed, studies in CHO cells has shown that salbutamol evoked responses for β_1-AR with an EC_{50} of 0.3μM and

to 86% of the isoproterenol response (Baker, 2005c) while for the β_3-AR, the EC_{50} was 1.7μM which was 89% of the isoproterenol response (Baker, 2005a). Therefore, given the concentrations used, it is difficult to define selectivity of the compounds and determine the β-AR subtypes involved.

Although there is a likely lack of selectivity of the β-AR compounds used, there were clear differences in responses for migration, proliferation and tubule formation.

Isoproterenol, salbutamol and formoterol decreased HDMEC SCM while ZD7114 and-β-AR blockade with timolol, atenolol and ICI 118,551 had no effect on altering EC migration speed. These data are in contrast to research investigating macrovascular EC cell types. For example, the non-selective β-AR antagonist, propanolol inhibited HUVEC migration (Lamy et al., 2010). In addition, the selective β_3-AR agonist, BRL37344 in human retinal and choroidal ECs was found to be pro-motogenic (Steinle et al., 2003, Steinle et al., 2005). Other cell types also display similar anti-motogenic effects when exposed to β-AR agonists *in vitro*. In HNKs, isoproterenol decreased migration speed (Pullar et al., 2006a, Pullar et al., 2007, Pullar et al., 2003) whilst ICI 118,551 increased HNK migration (Pullar et al., 2007, Pullar et al., 2006b). On the other hand, isoproterenol in HDFs was found to be pro-motogenic (Pullar and Isseroff, 2006). Therefore, subtle differences exist between different types of cells and β-AR mediated effects are highly cell type specific.

Only isoproterenol modulated HDMEC proliferation *in vitro*. Previously, research has shown a role for β_2-AR in HUVEC proliferation (Lamy et al., 2010, Sexl et al., 1995, Seya et al., 2006) and a role for the β_3-AR in regulating human choroidal endothelial cells retinal EC proliferation (Steinle et al., 2003, Steinle et al., 2005). The β_3-AR agonist,

BRL37344 promoted proliferation in human retinal and choroidal ECs (Steinle et al., 2003, Steinle et al., 2005). On the other hand, the selective β_1-AR antagonist, nebivolol inhibited proliferation of human coronary ECs (Brehm et al., 2001) whilst the non-selective β-AR antagonist, propanolol also inhibited migration of HUVECs (Lamy et al., 2010). In addition, nor-adrenaline promoted the mitogenic activity of HUVECs (Sexl et al., 1995, Seya et al., 2006).

It appears that β-AR-mediated effects on HDMEC functions are specific to particular β-AR agonists. It is well known from numerous reviews that β-AR ligands exhibit "ligand-directed signalling" and can activate different signalling pathways at a particular receptor (Audet and Bouvier, 2008, Galandrin et al., 2007, Hoffmann et al., 2008, Kenakin, 2007, Perez and Karnik, 2005, Seifert and Dove, 2009) including salbutamol (Swaminath et al., 2005) and SR59230A (Sato et al., 2007). In the present study, isoproterenol reduced motility to a greater extent than salbutamol and formoterol, whereas ZD7114 had no effect on migration. Studies in other cell types and systems have shown isoproterenol and formoterol to be full agonists and salbutamol to be a partial agonist (Baker, 2010b), however, both formoterol and salbutamol had similar effects on HDMEC migration. Partial or full agonism is system dependent. For example, agonists may be partial agonists on one response or in one system or be full agonists on other responses and in other systems (Evans et al., 2010, Galandrin et al., 2007). Furthermore, structural differences in β-AR agonists may play a role in "ligand-directed signalling". Indeed, one study revealed that the catecholamine fragment, catechol and the β-AR agonist, salbutamol induced distinct conformational states to activate the β_2-AR. In addition, endogenous β-AR ligands, adrenaline and nor-adrenaline induced conformational changes that led to receptor internalisation.

Molecular modelling found that the aromatic ring of salbutamol did not interact with same receptor residues as the catechol moiety found in catecholamines (Swaminath et al., 2005). For example, it is possible that the conformations of the β-ARs brought about by isoproterenol and salbutamol differ in their ability to induce receptor phosphorylation and internalisation. Consequently, the differences in potency between salbutamol and isoproterenol could be due to the ability of β-AR ligands to induce internalisation and initiate a second wave of receptor signalling. Indeed, research has shown that distinct conformational changes induced by ligands at GPCRs can in turn influence effector protein association and subsequently differential signalling pathway activation (Hoffmann et al., 2008). Therefore, ligand-specific conformational changes by isoproterenol, salbutamol, formoterol and ZD7114 maybe influencing ligand potency and the activation of particular signalling pathways that modulate HDMEC function.

β-AR agonists can also influence β-AR G-protein coupling and can therefore regulate the activation of particular signalling pathways. For example, the β_2-AR can signal through Gαs and Gαi proteins in murine cardiac myocytes (Xiao et al., 1999a, Xiao et al., 1995, Zhu et al., 2001). Perhaps, ZD7114 is unable to induce an active conformation state at the β-ARs and influence specific G-protein coupling to regulate either HMDEC migration or proliferation. Furthermore, isoproterenol appears to be able to regulate both migration and proliferation of HDMECs. Therefore, it is reasonable to assume that either the same or different β-AR subtypes expressed in HDMECs may have differential coupling to different G proteins.

Another explanation for the functional differences observed by the β-AR agonists on HDMEC function could be due to the heterogeneous nature of the β-ARs. As mentioned in section **1.7, pharmacology of β-AR agonists and antagonist**, the genetics of β-ARs are complex with data showing that single nucleotide polymorphisms, also termed "haplotype", could alter the β-ARs responsiveness to an agonist. In fact, several single nucleotide polymorphisms have been identified for the β_2-AR (Green et al., 1995). This might explain why there were some variations in magnitude between the different agonists and antagonists between experiments. Therefore, a number of different EC strains were used in all experiments to compensate for any possible genetic variations of the β-ARs that might exist in the cell strain donors.

HDMECs demonstrated complex cellular behaviour when cultured on top of BME and form tubule-like structures *in vitro*. This behaviour highlights the importance of a "niche" environment, which is critical for influencing cell behaviour as BME is more similar to the wound environment. β-AR blockade by timolol enhanced the development of the tubule-like structures on BME, although no effect was found in influencing HDMEC migration, proliferation and scratch wound healing. This could be due to the complex environment that BME provides. It is possible that proteins and growth factors in BME could be influencing β-AR expression levels. For example, in cells with high β_3-AR expression, SR59230A behaved as a weak partial agonist for cAMP accumulation. However in cells expressing endogenous or low levels of β_3-ARs, SR59230A had no effect on cAMP accumulation (Sato et al., 2007).

The β-AR antagonist, timolol may be blocking the effect of an endogenous ligand found within the BME. This could explain how β-AR blockade enhanced tubule formation by

blocking the decrease in tubule formation induced by endogenously synthesised and secreted catecholamines. However, this was disregarded as a possible explanation as the manufacturer confirmed the absence of adrenaline and/or nor-adrenaline in their BME.

It was also not known if ECs are capable of synthesising and secreting endogenous catecholamine ligands, providing an autocrine AR network that could be modulated by β-AR activation or blockade. Therefore further work described in this thesis will determine if HDMECs express a key enzyme for catecholamine synthesis, phenylethanolamine-N-methyl transferase (Schallreuter et al., 1992, Pullar et al., 2006b) see chapter 4. Another possible mechanism to which β-AR antagonists may modulate tubule formation could be though the modulation of pro-angiogenic factors such as VEGF-A and FGF-2. Therefore ELISA studies will determine if β-ARs have any role in the generation or inhibition of VEGF-A and FGF-2 in HDMEC lysates, see chapter 5.

In conclusion, these data demonstrate a role for the β-ARs in modulating HDMEC function *in vitro*. Further work will investigate more complex models of angiogenesis *ex vivo* and *in vivo* and reveal the mechanism of how isoproterenol, salbutamol and formoterol modulate cell migration and isoproterenol modulates cell proliferation. In addition, how isoproterenol and ZD7114 and timolol alter tubule formation will be investigated.

Chapter 4 Investigating the mechanisms that underpin the β-AR-mediated modulation of endothelial migration and proliferation

4.1 Introduction

4.1.1 Introduction

Previously, this investigation has revealed a role for the β-ARs in regulating both EC migration and proliferation *in vitro*, both essential processes in angiogenesis. However, the mechanisms underpinning the β-AR-mediated modulation of migration and proliferation are unknown.

4.1.2 Mechanisms of cell migration

Cell migration is a complex process requiring numerous signalling proteins and extensive actin/cyctoskeletal remodelling.

Focal adhesions (FA) have an important role in cell migration acting as important transducers of external signals as well as securing the cell to the underlying matrix. One of the main components of FAs are integrins. Integrins form heterodimers with alpha and beta subunits that bind to ECM components such as collagen, fibronectin, vitronectin and laminins (Huttenlocher and Horwitz, 2011). The intracellular side of FAs contain proteins including vinculin, α-actinin, filamin, talin and parvin/actopaxin which link to the cytoskeleton. Signalling proteins such as focal adhesion kinase (FAK), src and paxillin are also present which are involved in transmitting signals from the external environment, see figure 4.1 (Lo, 2006, Petit and Thiery, 2000, Zamir and Geiger, 2001).

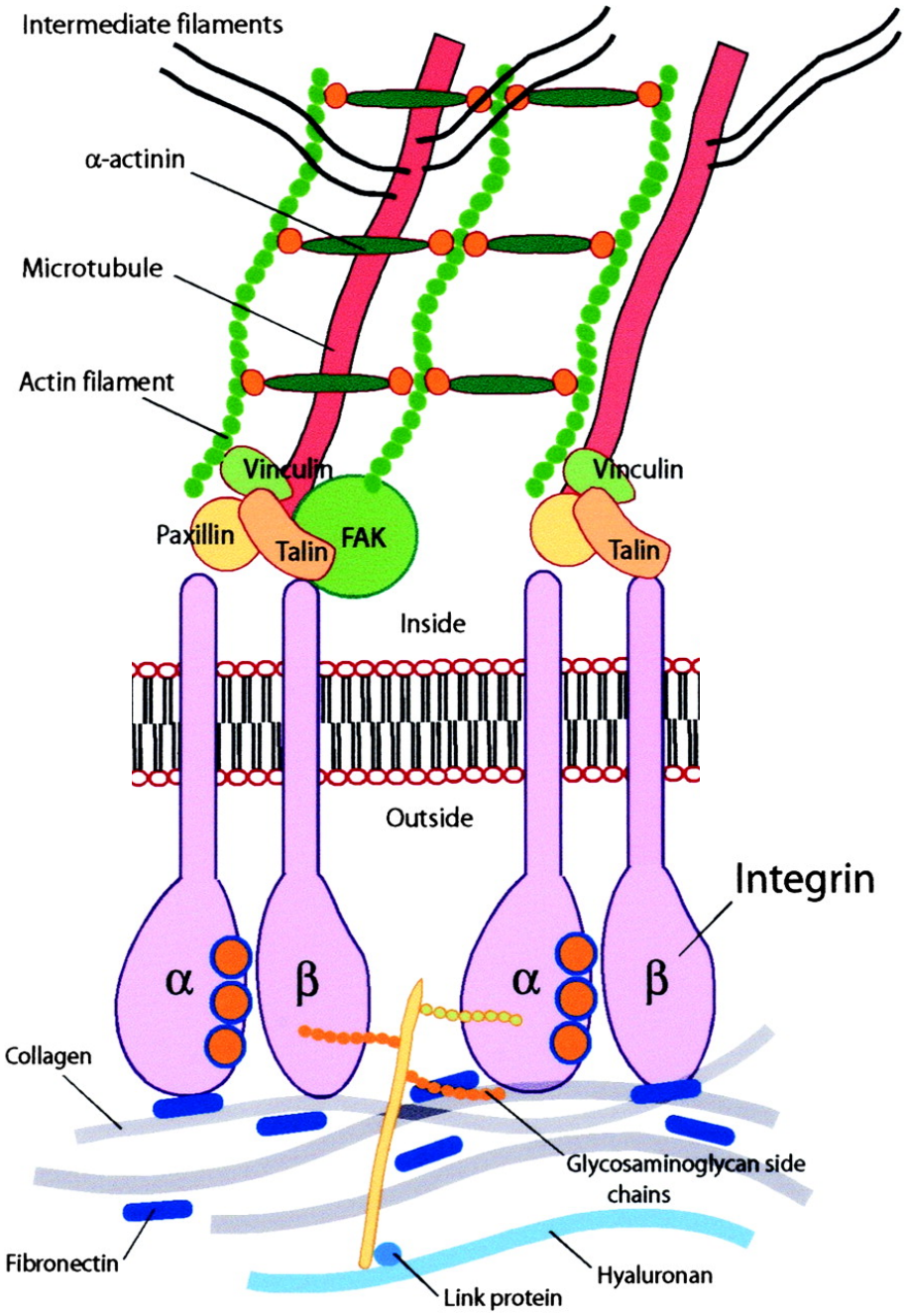


Figure 4.1: Focal adhesion schematic. Transmembrane integrin subunits connect to ECM components such as collagen, fibronectin, hyaluronan and glycosaminoglycans. Integrin subunits also bind to intracellular adapter proteins, such as paxillin, talin, FAK, α-actinin and vinculin. Adapter proteins connect integrin subunits to the cytoskeletal components, such as actin, microtubules and intermediate filaments. (Image taken and adapted from Rodriguez-Pinon et al., 2005)

There are a variety of intracellular signalling events that take place in a highly organised and spatial manner to stimulate actin assembly/disassembly and attachment and de-attachment of FA junctions. The Rho-GTPases play a major role in cell motility, regulating the actin cytoskeletal and cell polarity. The three most important members of this family are RhoA, Rac1 and Cdc42. The Rho-GTPases switch between a guanine triphosphate (GTP)-bound active state and a guanine diphosphate (GDP)-bound inactive state. They are regulated by GTPase activating proteins (GAP), guanine nucleotide dissociation inhibitors (GDI) and guanine nucleotide exchange factors (GEF). GAPs promote the GDP bound state, while GEFs promote the GTP bound state and GDIs sequester Rho proteins (Bryan and D'Amore, 2007). The main effector of RhoA is the serine/theonine Rho associated kinase (ROCK), which is phosphorylated and in turn, phosphorylates and activates myosin light chain (MLC) and prevents myosin phosphatase from binding. MLC regulates the motor protein myosin which in turn leads to stress fibre formation and FA assembly. Another effector of RhoA is mammalian diaphanous (mDia) which triggers nucleation and polymerisation of unbranched actin filaments and microtubule capping. Rac1 and Cdc42 on the other hand, can modulate the effector p21-activated kinase (PAK). Activation of PAK by Rac1 and Cdc42 leads to the membrane localisation of PAK and the subsequent phosphorylation of LIM-kinase and its phosphorylation of cofilin, the negative regulator of actin polymerisation. PAK and LIM-kinase can also negatively regulate myosin light chain kinase (MLCK) and myosin, thus decreasing stress fibre formation. It is important to note that LIM-kinase is also an effector of ROCK (Bryan and D'Amore, 2007, Etienne-Manneville and Hall, 2002, Iden and Collard, 2008). The alternative downstream effectors of Rac1 and Cdc42 are N-Wiskott Aldrich syndrome protein

(N-WASP), WASP family verprolin homologous protein (WAVE) and Wiskott–Aldrich syndrome protein and SCAR homologue (WASH) which orchestrate the spatial and temporal activation of the actin related protein (Arp) 2/3 complex. The Arp2/3 complex contains 7 subsunits which can bind to monomeric subunits of actin, stimulating polymerisation, see figure 4.2 (Bryan and D'Amore, 2007, Pantaloni et al., 2001).

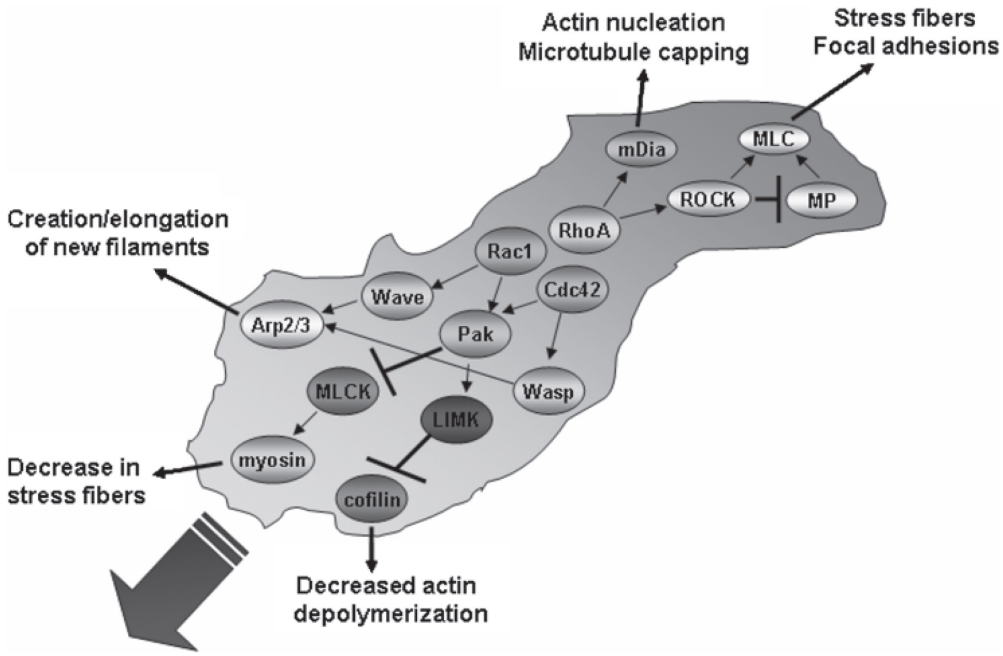


Figure 4.2: Rho-small GTPase control of cell migration. Image illustrating the spatial and functional roles of the main Rho-small GTPases involved in cell migration (Image taken from Bryan and D'Amore, 2007)

An important complex known as partitioning defective (PAR) is a major regulator of cell polarity consisting of PAR3, PAR6 and atypical protein kinase C (aPKC). The PAR complex is involved in the formation of the front and rear axis of migrating cells, asymmetric cell division, basal-apical polarity in epithelial cells and signalling via cdc42. Cdc42 is a member of the Rho family of GTPases and can be activated via integrin/ECM interactions at the cell membrane as previously described. Activated cdc42 can recruit and signal via the PAR complex whereby aPKC is activated. aPKC is an important regulator of cell polarity although its phosphorylation targets are not fully elucidated. To summarise, at the front and leading edge of the cell, signalling components involved with actin polymerisation are found including N-WASP, Arp 2/3 complex and GTPases such as Rac and Cdc42. In contrast, Rho is located at the trailing edge of the cell. Cdc42 and Rac1 can modulate RhoA activity in both the leading and rear edges but can also regulate Rac1 at a cell's leading edge (see figure 4.2) via PAR complex interactions (Etienne-Manneville, 2008, Petrie et al., 2009, Ridley et al., 2003, Sakumura et al., 2005). Therefore, cross talk between Rho, Rac and Cdc42, with Cdc42 controlling cell elongation, Rac mediating lamellipodia expansion and Rho inducing actin assembly and disassembly is essential for cell polarity and migration (Nobes and Hall, 1995, Sakumura et al., 2005).

Numerous downstream intracellular signalling molecules are also involved in cell migration including PI3K, Akt/protein kinase B (Akt/PKB), cAMP, PKA and EPAC (Ridley et al., 2003). PI3K regulates a diverse number of cellular functions including cell adhesion, cell survival, differentiation and cell migration. PI3K consists of a regulatory subunit (p85) containing src homology domains SH2 and SH3 and a catalytic subunit (p110) (Leevers et al., 1999, Merlot and Firtel, 2003). PI3K can be activated by src

which binds to the p85 subunit and mediates docking to receptor tyrosine kinases (RTK), allowing the p110 subunit to interact with phosphorylated tyrosine adaptor proteins and phosphorylate lipid substrates (Katso et al., 2001). PI3K can also be activated by βγ dimers liberated from subunits of G_i coupled GPCRs which bind to the p110 subunit (Brock et al., 2002, Stephens et al., 1994). PI3Ks can phosphorylate phosphatidylinositols which can generate phosphatidylinositol 3-phosphate (PIP), PIP_2 and phosphatidylinositol 3,4,5-triphosphate (PIP_3) which are important signal transducers (Leevers et al., 1999, Merlot and Firtel, 2003). PIP_3 generation can result in the activation and localisation of Akt/PKB to the plasma membrane at the leading edge of a migrating cell, where it is activated by protein dependent phosphoinositide kinase 1 (PDPK1) (Merlot and Firtel, 2003). Upon activation, Akt/PKB plays a role in myosin II assembly and actin remodelling induced by growth factors, integrins and GPCRs (Merlot and Firtel, 2003, Qian et al., 2003). Indeed, Akt/PKB can directly modulate the actin remodelling protein, girdin, enhancing fibroblast migration (Enomoto et al., 2005). Moreover, Akt/PKA can also augment mammary epithelial invasion by upregulating MMP2 activity (Chin and Toker, 2009).

In summary, cell polarisation and migration are controlled by an array of proteins, whereby external signals stimulate spatial and temporal signalling pathways involving small GPTases such as RhoA, Cdc42 and Rac1, via FA components such as integrins, to downstream signalling proteins. This induces extensive actin cytoskeletal remodelling therefore allowing the cell to polarise into a front and a rear axis. ATP and motor proteins such as myosin provide the force to propel the cell forward in a “swimming” like motility while FAs are continuously assembled and dissembled (Ridley et al., 2003).

4.1.3 Bacterial toxins as a means to study β-AR G protein coupling

GPCRs are known to influence EC function and angiogenesis (Richard et al., 2001). GPCRs can be coupled to a variety of G-proteins belonging to the Gαs, Gαi, Gαq and Gα12/13 families, see section **1.5, β-ARs**. The specific coupling of β-ARs relevant to the present study is, however, unclear. In order to study the G protein coupling of a particular GPCR, bacterial toxins can be used such as cholera toxin (CTX), pertussis toxin (PTX) and pasteurella multocida toxin (PMT) that modulate specific G proteins.

CTX is released from *Vibrio cholera* in an inactive form and consists of 6 subunits making up one copy of the enzymatic subunit (CTA) and 5 copies of the receptor binding subunit (CTB). It is believed that CTX binds to monosialotetrahexosylganglioside (GM) 1 receptor which is localised in lipid rafts and becomes internalised and taken up into early endosomes. CTX then undergoes retrograde transport to the tans/Golgi and ER. At some point, the CTA subunit dissociates from the CTB subunit. It is this CTA subunit that catalyses the ADP ribosylation at Gαs, locking Gαs in an GTP active form, thus allowing for continual activation of AC (Sánchez and Holmgren, 2008, Broecka et al., 2007, Cassel and Pfeuffer, 1978).

Conversely, PTX is released in an inactive form from *Bordetella pertussis*. PTX consists of 6 subunits making up one copy of the enzymatic subunit (PTA) and 5 copies of the receptor binding subunit (PTB). Upon binding to GM 1 receptor, it becomes internalised to the endosome and is transported in a retrograde fashion to the trans/Golgi and ER. At some point along this route, the PTA subunit becomes active, possibly though the action of glutathione and ATP. The PTA subunit catalyses the ADP

ribosylation at Gαi, preventing interaction with GTP, locking Gαi in a GDP inactive form, therefore preventing Gαi from inhibiting AC, leading to increased levels of cAMP. PTX can also affect Gαt and Gαo proteins (Fields and Casey, 1997, Aktories, 1997). PMT on the other hand is released from *Pasteurella multocida* and stimulates Gαq, Gαi and Gα12/13 activity. PMT binds to GM receptors and is internalised by endocytosis. It stimulates G proteins though the deamidation on the glutamine 205 residue (Orth et al., 2008, Ortha et al., 2009, Wilson and Ho, 2004).

4.1.4 The role of PKA and EPAC in cell migration and proliferation

Upon Gαs coupled receptor stimulation, AC catalyses the conversion of ATP to cAMP. Alternately, Gαi inhibits AC and consequently decreases the production of cAMP from ATP (Cabrera-vera et al., 2003a, Offermans, 2003). PKA and EPAC are two well-known downstream targets of cAMP, see section **1.5, β-ARs**.

PKA is a holoenzyme, consisting of two catalytic and regulatory subunits. Initial phosphorylation of the catalytic subunits by phosphoinositide-dependent protein kinase is an essential pre-requisite for optimal PKA activity. The regulatory subunit acts as high affinity cAMP receptors that sequester cAMP. This leads to the dissociation of the regulatory subunits from the catalytic subunits. Consequently, the liberated catalytic subunits can now phosphorylate and activate a variety of downstream targets including many cytoplasmic and nuclear proteins, enzymes and transcription factors (Cheng et al., 2008).

EPAC on the other hand, belongs to the GEF family of proteins and upon binding of cAMP, promotes the exchange of GDP to GTP on the ras-associated protein (Rap) 1 and Rap2 small GTPases. EPAC consists of two isoforms, EPAC1 and EPAC2 which are

both abundantly expressed, although EPAC1 is more abundant in the myocardium. EPAC consists of an N-terminal regulatory domain and a C-terminal catalytic domain. The catalytic domain consists of an exchange motif domain, which stabilises the GEF domain, a Ras association domain and a cdc25-homology domain that promotes the GDP/GTP exchange on Rap1 and Rap2. The regulatory domain consists of dishevelled, Eg1-10 and a pleckstrin homology domain which are responsible for membrane association and for binding cAMP with high affinity (Cheng et al., 2008, Roscioni et al., 2008).

Both PKA and EPAC are well known modulators of cell migration. PKA can directly or indirectly regulate many cell migration proteins such as RhoA, ROCK, MLCK (Howe, 2004), Cdc42 (Chahdi et al., 2005, Howe, 2004), Rac1 (Howe, 2004), p21 protein (Cdc42/Rac)-activated kinase 1 (Howe, 2004), adducin (Matsuoka et al., 1996), fodrin (Howe, 2004), vasodilator stimulated phosphoprotein (Howe, 2004, Lebrand et al., 2004), LIM and SH3 domain protein 1 (Howe, 2004, Keicher et al., 2004), paxillin (Han et al., 2003, Howe, 2004) and PLC-β_3 (Howe, 2004). These proteins are important regulators of the actin cytoskeleton and FA turnover which both play important roles in cell migration, as previously mentioned (Burridge and Fath, 1989, Pantaloni et al., 2001). EPAC on the other hand has been shown to act as either a positive (Baljinnyam et al., 2011, Yokoyama et al., 2008) or negative regulator of cell migration (Grandoch et al., 2009, Lyle et al., 2008, Yokoyama et al., 2008) dependent on cell type and system. EPAC can alter migration by directly regulating the downstream effector, Rap 1 (de Rooij et al., 1998, Kawasaki et al., 1998). Though Rap1, EPAC can alter integrin-mediated cell adhesion though the regulation of various integrins such as lymphocyte function-associated antigen 1 (de Bruyn et al., 2002, Ghandour et al., 2007, Katagiri et

al., 2004, Liu et al., 2002a, Reedquist et al., 2000, Sebzda et al., 2002, Shimonaka et al., 2003), very late antigen-4 (Arai et al., 2001, de Bruyn et al., 2002, Liu et al., 2002a, Reedquist et al., 2000, Sebzda et al., 2002, Shimonaka et al., 2003), macrophage integrin Mac-1 (Caron et al., 2000), the laminin receptor α5β1 (Sebzda et al., 2002), platelet integrin αIIbβ3 (Bertoni et al., 2002, Han et al., 2006) and the vitronectin and CD23 receptor αvβ3 (Gao et al., 2006). In addition, EPAC has also been shown to associate with microtubules in which light chain (LC) of microtubule associated protein (MAP) 1A, LC1, MAP 1B and tubulin regulate the guanine exchange factor activity of EPAC (Mei and Cheng, 2005, Borland et al., 2006, Gupta and Yarwood, 2005). It is hypothesised that such interactions could promote microtubule formation and establishment of cell polarity.

PKA and EPAC can also effect cell migration and proliferation by modulating ERK1/2 activation though the regulation of the small GPTase, proto-oncogene serine/theonine-protein kinase 1. ERK1/2 alters cell migration, by phosphorylating and enhancing the activity of MLCK. MLCK in turn phosphorylates myosin light chains, which are wrapped around the head portion of myosins (Klemke et al., 1997). Myosins are ATP “powered” motor proteins that allow for translational movement along actin cables, thus allowing for the swimming-like motility observed during cell migration (Ridley et al., 2003). ERK1/2 can also regulate FAs and catecholamine biosynthesis, therefore highlighting ERK1/2 as an important possible downstream target of the β-ARs in the regulation of HDMEC cell migration (Dunkley et al., 2004, Liu et al., 2002b). ERK1/2 has also been demonstrated to promote proliferation, possibly though the up-regulation of the anti-mitogenic protein, p27Kip1 (Borland et al., 2009). However the precise signalling mechanisms and the roles of PKA and EPAC in both cell

migration and proliferation remains controversial and further investigations are required.

4.1.5 Chapter aims

The main aims of this chapter are to elucidate the mechanisms though which the β-ARs modulate HDMEC migration and proliferation. To determine if HDMECs synthesise and secrete catecholamine's providing an autocrine β-AR activation route, as previously described for keratinocytes (Pullar et al., 2006b) and melanocytes (Gillbro et al., 2004), western blotting will be performed to detect catecholamine synthesis enzymes. To investigate downstream signalling pathways that play a role in the β-AR-mediated modulation of HDMEC migration and proliferation, western blotting (ERK 1/2), pharmacological studies (Gαx, cAMP, PKA and EPAC) and ICC (actin and vinculin, an FA associated protein) will be performed.

4.2 Results

4.2.1 How β-ARs modulate dermal endothelial cell migration

4.2.1.1 Is an autocrine activation loop involved in the β-AR-mediated modulation of HDMEC physiological processes?

HNKs have been shown to generate and secrete catecholamines (Pullar et al., 2006b) as well as melanoctyes (Gillbro et al., 2004), whereas HDFs do not (Pullar et al., 2007, Schallreuter et al., 1992). It is not currently known whether ECs generate and secrete catecholamines. PNMT catalyzes the synthesis of adrenaline from nor-adrenaline (Schulz et al., 2004), **see section 1.7, the role of β-ARs in wound repair** for further detail. Therefore Western blotting was performed to detect PNMT expression in HDMECs. HNKs and human ductal breast epithelial tumor cell line T47Ds were used as positive controls while HDF was used as a negative control. Western blotting could not reveal the PNMT band in the HDMEC strains investigated (figure 4.3).

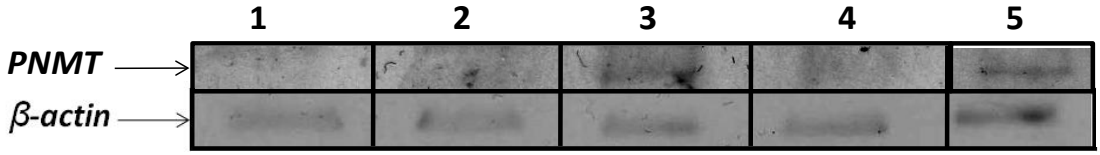


Figure 4.3: Phenylethanolamine N-methyltransferase (PNMT) was not detected in HDMEC cell lysates.

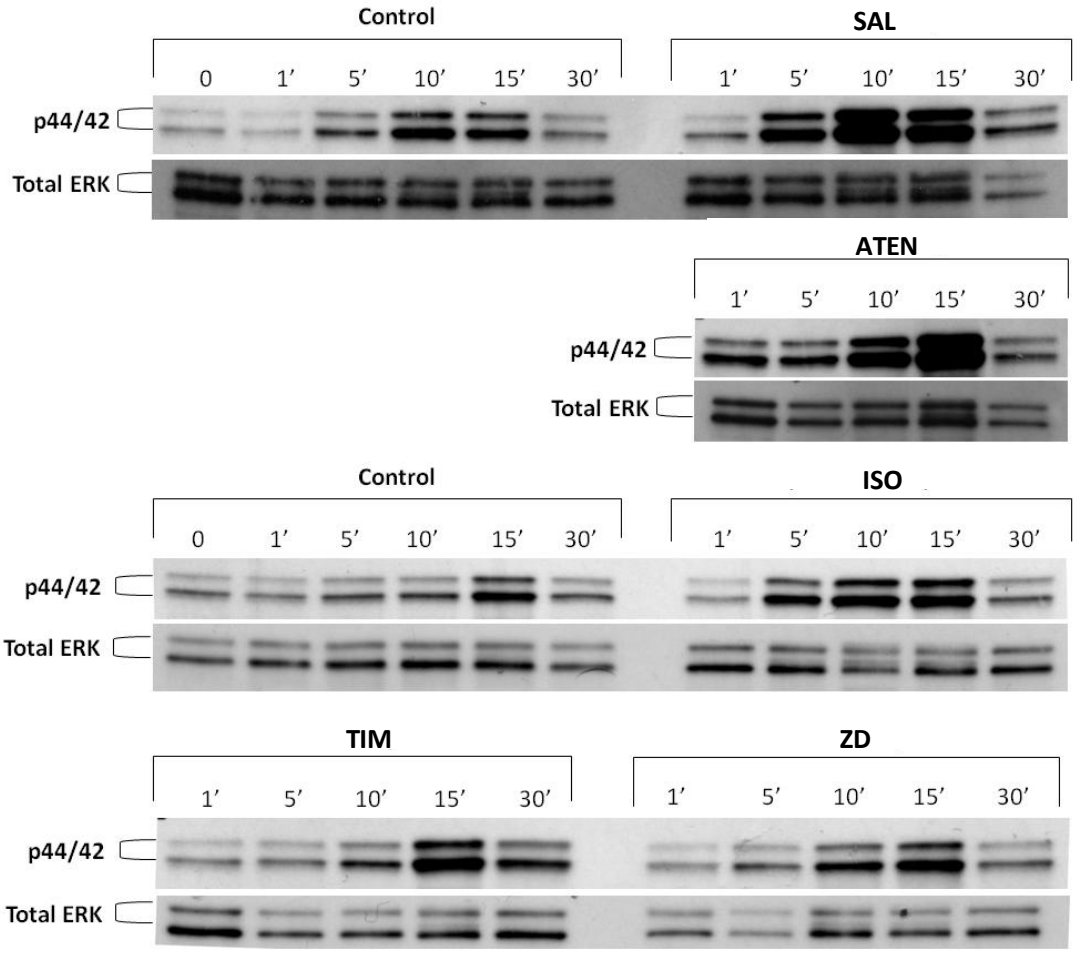
Cell lysates (HDMECs (1-2), HNK (3), HDF (4) and TD47D (5)) were lysed and separated electrophoretically, and membranes were immunoblotted with antibodies specific for anti-PNMT (~28 kDa) or anti-β-actin (~42 kDa) as described in the methods. The immunoblot shown is representative of one independent experiment.

4.2.1.2 Do β-ARs alter ERK 1/2 phosphorylation in HDMECs?

Previously, β-AR activation was found to be anti-motogenic. β-AR agonists, isoproterenol and salbutamol decreased HDMEC migration by 46% and 34% respectively, see section **3.2.2, β-AR activation decreases rate of HDMEC migration**. ERK1/2 is well known to influence EC motility (Klemke et al., 1997). β-ARs can modulate the ERK 1/2 signalling pathway though G_i and G_s coupled mechanisms, involving proteins such as PI3K, Akt and Src (Xiao et al., 1999a, Klemke et al., 1997, Gerber et al., 1998, Thakker et al., 1999, Munshi et al., 2000). Therefore, western blotting studies were used to investigate whether β-ARs alter HDMEC migration by signalling downstream to ERK1/2, consequently altering ERK1/2 phosphorylation.

Initially, we observed an increase in phospho-ERK1/2 over time in all treatments but also in the control (figure 4.2A). ERK1/2 can be activated mechanically in both the micro and macro vasculature (Birukov et al., 1997, Eskildsen-Helmond and Mulvany, 2003). Indeed, the addition of media alone to the cells was sufficient to activate phospho-ERK1/2 (figure 4.4A). Therefore, to reduce mechanical ERK1/2 activation, after serum starving the cells, fresh serum-free media alone containing 2X β-AR agonists/antagonist treatments, was carefully added to the top of the serum starved media at the side of the cell culture dish. This significantly lowered mechanical background activation of ERK1/2 and allowed for an improved ability to determine whether β-ARs affect ERK1/2 phosphorylation. However, β-AR modulation did not alter ERK phosphorylation (figure 4.4B).

A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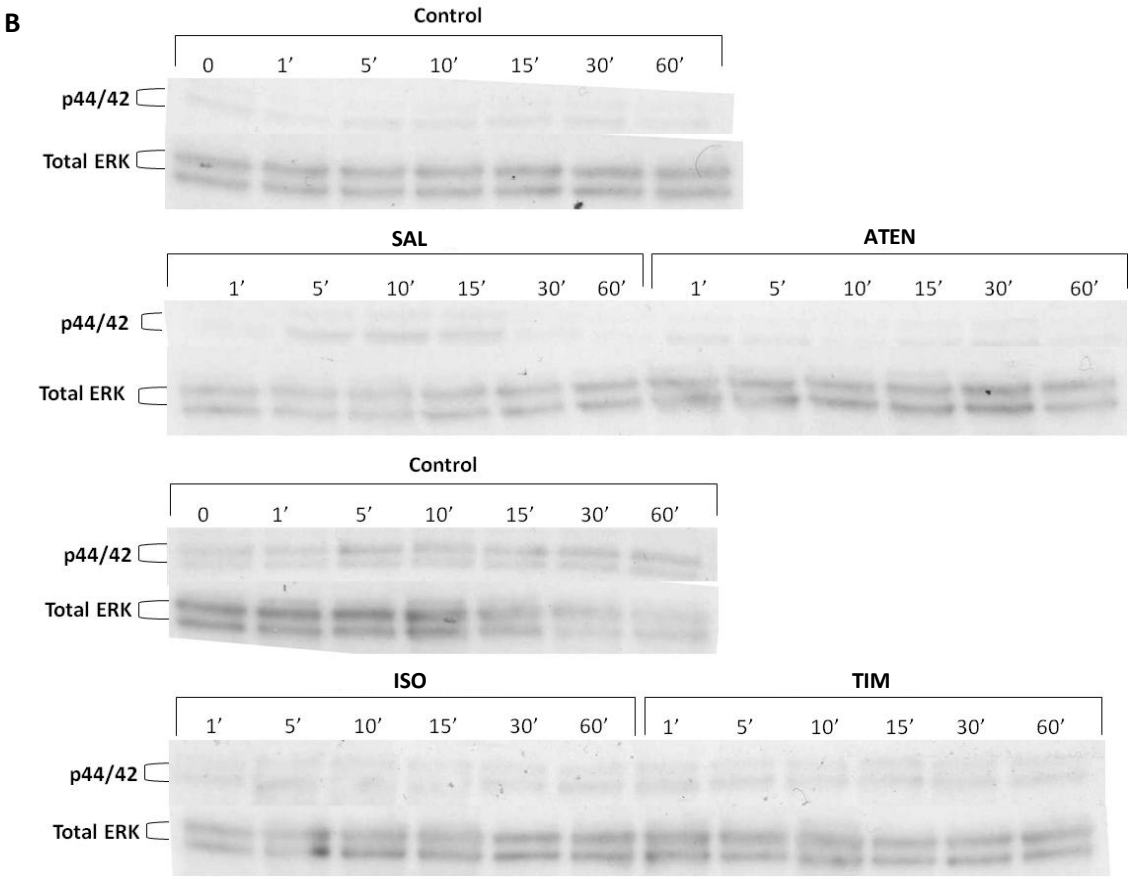


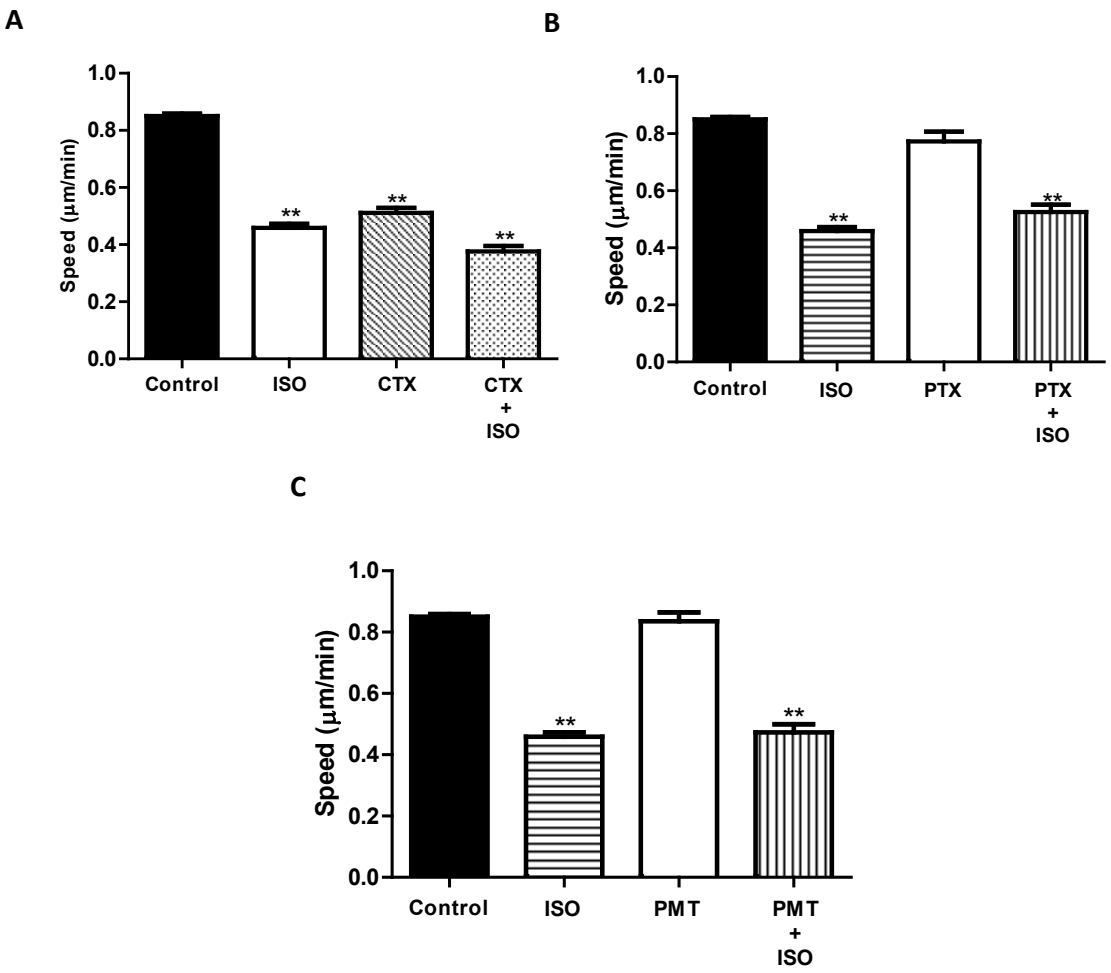
Figure 4.4: ERK1/2 phosphorylation is not altered by β-AR agonists and antagonists

HDMECS were serum starved and treated with either basal media alone or basal media containing either β-AR agonists (isoproterenol; ISO, salbutamol; SAL or ZD7114; ZD) or β-AR antagonists (timolol; TIM or atenolol; ATEN) at 10 µM for 0-30 min time points. Cells were then lysed and separated electrophoretically, and membranes were immunoblotted with antibodies specific for anti-ERK1/2 (44/42 kDa) and anti-total ERK1/2 (44/42 kDa). The data shown were representative of 3 independent experiments from 2 separate cell strains **(A)**. HDMECs were serum starved in 1 ml of basal media for 24 h and then treated with either 1 ml basal media alone or basal media containing 20 µM of β-AR agonists (ISO, SAL or ZD) or β-AR antagonists (TIM or ATEN) to each dish to give a final concentration of 10 µM for 0-60 min time points. Cells were then lysed and separated electrophoretically, and membranes were immunoblotted with antibodies specific for anti-ERK and anti-total ERK. The data shown were representative of 3 independent experiments from 2 separate cell strains **(B)**.

4.2.1.3 How do G proteins influence β_1-AR and β_2-AR-mediated HDMEC cell migration?

The β_1-AR and β_2-AR in the human heart cells (Brodde et al., 2006, Brodde and Michel, 1999, Steinberg, 1999, Xiao et al., 1999a, Xiao et al., 1999b, Xiao and Lakatta, 1993) and rat ventricular cardiomyocytes (Ponicke et al., 2006) couple to Gαs. Alternately, the β_1-AR in rat adipocytes (Chaudhry et al., 1994) and CHO cells (Martin et al., 2004) can couple to Gαi. In addition, Gαq coupled GPCRs activate PLC which is known to regulate angiogenesis (Bhattacharya et al., 2009, Husain et al., 2005). Therefore it is possible that in HDMECs, β_1-AR or β_2-AR could couple Gαq.

To determine if Gαs, Gαi or Gαq play a role in β-AR modulation of HDMEC migration, CTX, PTX and PMT were used to activate or inhibit Gαs, Gαi and Gαq/Gαi/$G\alpha_{12/13}$ respectively. CTX decreased SCM by 40%. Pre-incubation with both CTX and isoproterenol produced an additive effect, reducing SCM by 56% compared to 46% for isoproterenol alone (figure 4.5A). Both PTX and PMT alone did not alter HDMEC SCM. Pre-incubation with both PTX or PMT and isoproterenol did not alter isoproterenol mediated decrease in HDMEC SCM (figure 4.5B and 4.5C).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ISO
PMT
PMT + ISO

Figure 4.5: PTX and PMT had no effect on the β-AR agonist mediated decrease in HDMEC migration, therefore the β-AR mediated signalling mechanism is independent of Gi and Gq

HDMEC SCM experiments were performed as described in the methods. HDMECs were treated with either media alone or media containing a β-AR agonist (isoproterenol; ISO) at 10 µM at time 0 pre-treated with CTX (0.2 µg/ml) for 1 h before addition of CTX alone or CTX and ISO at time 0. The data shown were combined from 3-88 independent experiments, from 2 separate cell strains (control cell n=2219, ISO cell n=382, CTX n=233 and CTX + ISO n=118) **(A)**. HDMECs were pre-treated with PTX (100 ng/ml) for 16 h. The media was then replaced with media containing PTX alone or media containing both PTX and ISO at time 0. The data shown were combined from 3-88 independent experiments, from 2 separate cell strains (control cell n=2219, PTX cell n=111, ISO cell n=382, PTX + ISO cell n=155) **(B).** HDMECs were treated with ISO 10 µM at time 0. HDMECs were pre-incubated with PMT (100 ng/ml) for 2 h. The media was replaced with media containing PMT alone or media containing PMT and ISO. The data shown were combined from 6-88 independent experiments, from 2 separate cell strains (control cell n=2219, PMT cell n=294, ISO cell n=382, PMT + ISO cell n=199) **(C)**. The data were averaged, statistically analysed using one-way ANOVA followed by the Dunnetts test and graphically represented with the bars representing the means ±SEM. Only the differences versus the control are shown (** $p<0.001$).

4.2.1.4 Role of cAMP, PKA and EPAC in β-AR mediated cell migration

β_1-AR and β_2-AR in the human heart cells (Brodde et al., 2006, Brodde and Michel, 1999, Steinberg, 1999, Xiao et al., 1999a, Xiao et al., 1999b, Xiao and Lakatta, 1993) and rat ventricular cardiomyocytes (Ponicke et al., 2006) couple to Gαs, thus leading to the activation of AC and consequently an increase in cAMP (Ponicke et al., 2006, Xiao et al., 1999b).

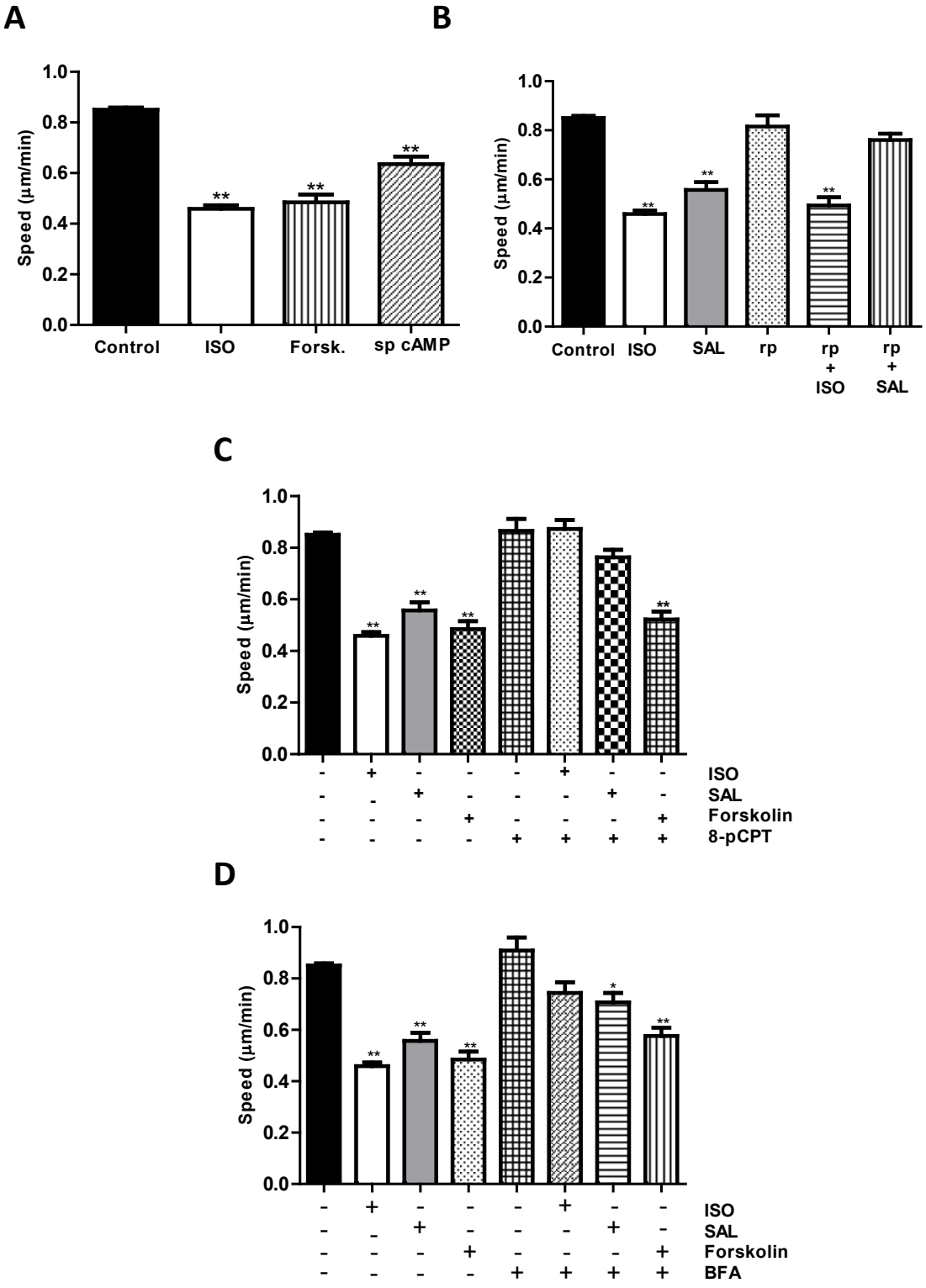
To confirm that cAMP plays a role in the β-AR-mediated HDMEC anti-motogenicity, a number of treatments were used to increase cAMP. Increasing cAMP via forskolin (AC activator) or sp cAMP (active cAMP analogue) decreased SCM by 43% and 25% respectively suggesting the involvement of cAMP in the β-AR-mediated decrease in HDMEC migration (figure 4.6A).

cAMP is able to modulate cell migration though the activation of cAMP dependent molecules, such as PKA and EPAC (Hanoune and Defer, 2001, Xiao et al., 1999b). To investigate the role of downstream targets of cAMP in β-AR-mediated HDMEC anti-motogenicity, PKA and EPAC modulators were used. The PKA inhibitor rp cAMP (rp) had no effect on cell migration alone (figure 4.6B) but prevented the β-AR agonist (salbutamol) mediated decrease in migration (figures 4.6B).

The EPAC agonist 8-CPT-2′-O-Me-cAMP (8-pCPT) and the EPAC inhibitor, brefeldin A (BFA) alone had no effect on HMDEC migration (figure 4.6C and 4.6D) but completely prevented the isoproterenol-mediated decrease in HDMEC SCM (figures 4C and 4D). However, while the EPAC agonist also prevented the salbutamol-mediated decrease in HDMEC SCM, the EPAC inhibitor only partially prevented the salbutamol-mediated

decrease in HDMEC SCM (figure 4.6D). In contrast, both the EPAC agonist and inhibitor did not prevent the forskolin mediated decrease in migration (figures 4.6C and 4.6D).

Efficient signal transduction involves scaffolding and the subsequent assembly of intracellular signalling complexes to the receptor. This brings into proximity of various signalling proteins, providing specific interactions and rapid signal relay. A number of these macromolecular signalling complexes have been discovered including the β_2-AR and PKA (Davare et al., 2001, Joiner et al., 2010, Luttrell et al., 1999). Therefore, it was hypothesised that the β_1-AR and β_2-AR in HDMECs were co-localised with EPAC. Consequently, EPAC was immunoprecipitated from HDMEC lysates and membranes probed for co-associated proteins (figures 4.7A, 4.7B and 4.7C). Immunoblotting the EPAC immunoprecipitates with the β_1-AR or the β_2-AR antibodies revealed that EPAC was associated with these receptors in HDMEC lysates (Figures 4.7B and 4.7C). Immunoblotting was also performed on β-AR and β_2-AR agonist treated HDMEC EPAC precipitates, however, no alteration in relative association were detected (results not shown).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rp + ISO
rp + SAL
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SAL
Forskolin
8-pCPT
BFA
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Figure 4.6: The role of cAMP, PKA and EPAC in the β-AR modulation of HDMEC single cell migration

HDMEC SCM experiments were performed as described in the methods. Cells were treated with a β-AR agonist (isoproterenol; ISO) 10 μM or cAMP elevators, forskolin (forsk) 50 μM and sp cAMP (sp) 50 μM at time 0 (control n=2219, ISO n=382, forsk n=118, sp cAMP n=132) **(A)**. Cells were treated with β-AR agonists (ISO or salbutamol; SAL) at time 0 or pre-incubated with a PKA inhibitor, rp cAMP (rp) 50 μM for 1 h before addition of both rp and SAL or ISO or rp alone at time 0 (control n=2219, ISO n=382, SAL n=122, rp cAMP n=70, rp cAMP + ISO n=81 and rp cAMP + SAL n=106) **(B)**. Cells were treated with either ISO or SAL at 10 μM or the cAMP modulator, Forskolin 50 μM at time 0 or pre-incubated with an EPAC agonist, 8-pCPT (8-pCPT-2'-O-Me-cAMP) for 1 h at 10 μM before addition of both 8-pCPT and SAL or ISO or 8-pCPT alone at time 0 (control n=2219, ISO n=382, SAL n=122, forskolin 50 μM n=118, 8-pCPT 10 μM n=105, 8-pCPT + ISO n=103, 8-pCPT + SAL n=86 and 8pCPT + forskolin n=84) **(C)**. Cells were treated with either ISO or SAL at 10 μM or the cAMP modulator, forskolin 50 μM at time 0 or pre-incubated with EPAC inhibitor, Brefeldin A (BFA) for 1 h at 10 μM before addition of both BFA and ISO or SAL or forskolin or BFA alone at time 0 (control n=2219, ISO n=382, SAL n=122, forskolin n=118, BFA n=76, BFA + ISO n=83, BFA + SAL n=74 and BFA + forskolin n=74) **(D)**. The data were combined from 3-88 independent experiments, from 3 separate cell strains. The data were averaged, statistically analysed using a one-way ANOVA followed by the Dunnetts test and graphically represented with the bars representing the means ±SEM. Only the differences versus the control are shown. (* $p<0.05$, ** $p<0.001$).

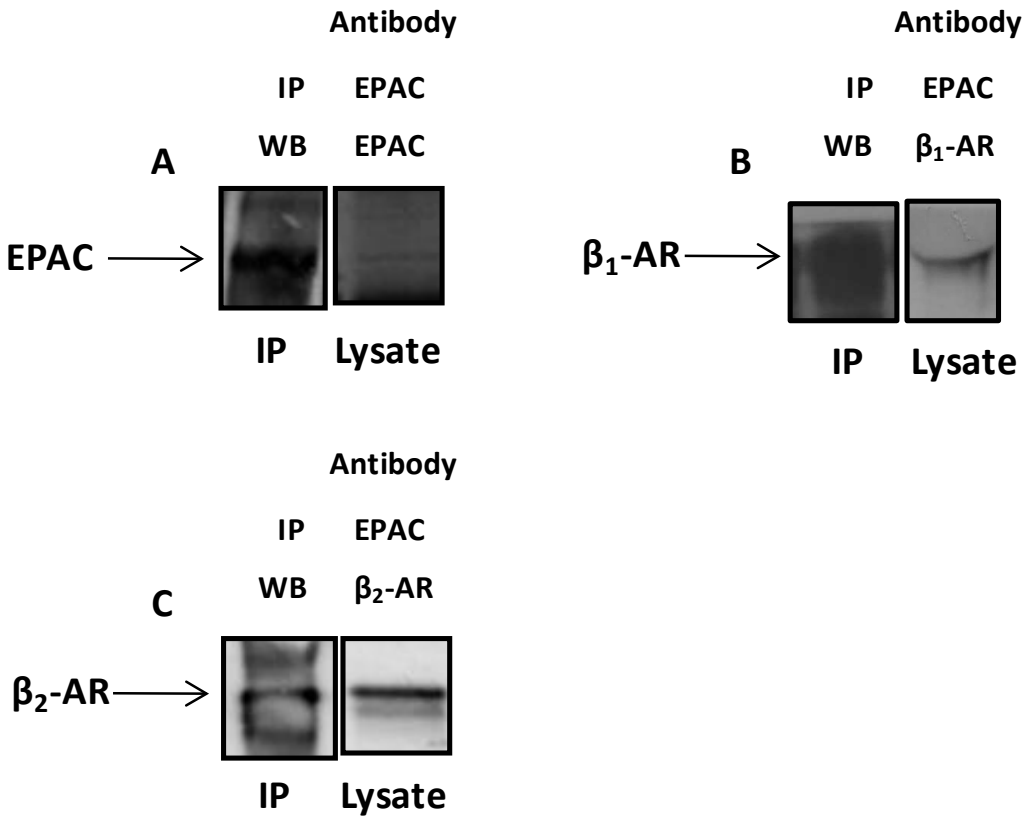


Figure 4.7: β_1-AR and β_2-AR co-localises with EPAC in HDMECs

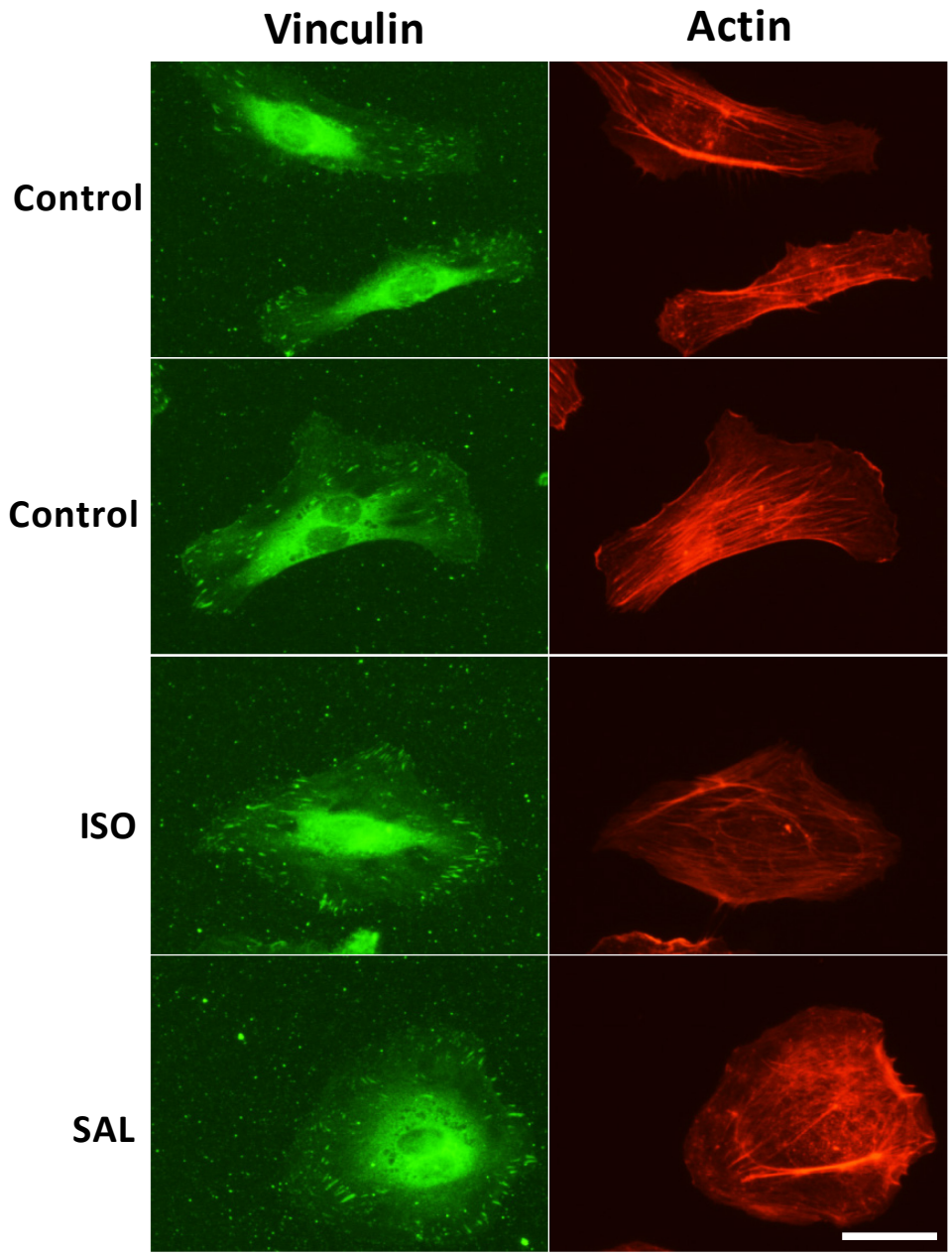
HDMECs were lysed and Immunoprecipitation was performed as described in the methods. Samples were separated electrophoretically, and membranes were immunoblotted with either an anti-EPAC 90 kDa **(A)**, anti-β_1-AR 47 kDa **(B)** or an anti-β_2-AR 51 kDa **(C)**. The data shown are representative of 3 independent experiments from 2 separate cell strains.

4.2.1.5 Immunocytochemical analysis of actin and vinculin

Actin filaments form part of the cell cytoskeleton and play a role in cell migration (Pantaloni et al., 2001). They terminate in focal adhesions (FA), which serve as mechanical attachments, connecting the ECM to the cytoskeleton. FAs also serve as signalling hubs, whereby numerous proteins including vinculin, allow for trans-cellular communication (Lo, 2006, Pantaloni et al., 2001, Petit and Thiery, 2000, Zamir and Geiger, 2001).

To visualise the effect of isoproterenol and salbutamol on the cytoskeleton, F-actin intensity and the number of FAs, using vinculin as a marker, were investigated. In the absence of β-AR agonists, HDMECs were polarised and crescent shaped with a broad lamellipodium. An abundance of actin stress fibres were observed at the cell periphery. In addition, multiple vinculin-stained FAs were present, located at both the leading and trailing edges of the cells (figure 4.8A). Isoproterenol and salbutamol had no significant effect on the intensity of actin staining or the number and spatial distribution of FAs. FAs were more symmetrically distributed around the cells plasma membrane (figure 4.8A). Quantitation showed that the total number of FAs was not significantly different (figure 4.8B).

A



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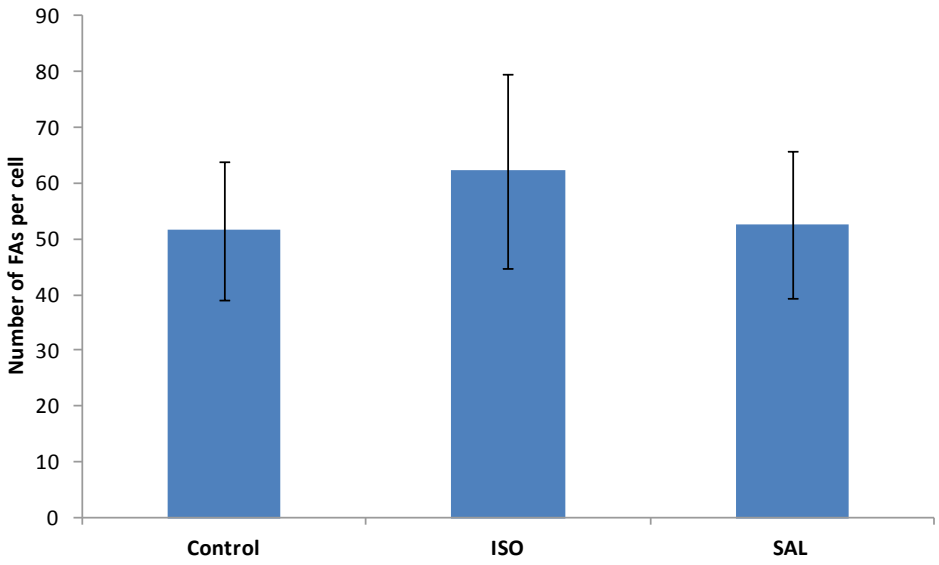

Figure 4.8: β-AR agonists alter the cytoskeletal conformation of HDMECs

Immunocytochemistry was performed as described in the methods. Cells were treated with either media alone or media containing β-AR agonists (isoproterenol; ISO or salbutamol; SAL) at 10 µM for 15 min. Cells were fixed and immunostained for actin (green) and vinculin (red) as described in the methods. Representative images of control, ISO and partial SAL treated cells are presented. The scale bar is 20 µm **(A)**. The numbers of FAs were quantitated as described. The data shown were the combined data from 3 independent experiments, from 2 separate cell strains. The data were averaged, statistically analysed using the one-way ANOVA and graphically represented with the bars representing the means ±SEM (control n=3, ISO n=3 and SAL n=3) **(B).**

4.2.2 How β-ARs modulate dermal endothelial cell proliferation

Previously, the β-AR agonist, isoproterenol decreased migration by 44% however, the mechanisms behind the β-AR mediated decrease in HDMEC proliferation are unknown.

Elevations in cAMP can increase the proliferation of vascular ECs (Torella et al., 2009), vascular smooth muscle cells (Favot et al., 2003, Chaudhry et al., 1994) and mouse embryonic stem cells (Kim et al., 2012). On the other hand, decreases in cAMP have reduced the proliferation of fischer rat thyroid cell line 5 (Ariga et al., 2000), thyrocytes (Kimura et al., 2001) and Swiss 3T3 fibroblasts (Lee et al., 1998).

Therefore, to determine if cAMP plays a role in the β-AR-mediated HDMEC anti-mitogenicity, treatments were used to modulate cAMP levels. An active cAMP analogue (sp cAMP) increased proliferation by 37% whereas pertussis toxin (PTX), a Gαi inhibitor, decreased proliferation by 77%. Pre-incubation with both PTX and isoproterenol had an additive affect and reduced proliferation by 59% compared to 38% for isoproterenol alone. Pre-incubation with both sp cAMP and isoproterenol completely prevented the isoproterenol-mediated decrease in proliferation and actually increased proliferation by 35% (figure 4.9A).

To investigate the role of cAMP-dependent molecules in the isoproterenol-mediated HDMEC anti-mitogenicity, PKA and EPAC modulators were used prior to the addition of isoproterenol. 48 h incubation with the EPAC inhibitor (BFA) induced cell death (data not shown). However, a PKA inhibitor (rp cAMP) and the EPAC agonist (8-pCPT) alone reduced HDMEC proliferation by 48% and 66% respectively. Pre-incubation with the PKA inhibitor or the EPAC agonist prior to the addition of isoproterenol, had an additive effect and further reduced cellular proliferation by 76% and 72% respectively.

In addition, pre-incubation with 8-pCPT prior to the addition of isoproterenol did not significantly reduce HDMEC proliferation compared with 8-pCPT alone (figure 4.9B).

Therefore, inhibiting PKA and EPAC reduced HDMEC proliferation suggesting that the β-AR mediated decrease in proliferation was by decreasing cAMP, suggesting a β-AR Gαi mechanism. However, PTX reduced proliferation therefore compromising its ability to be used as tool to investigate the β-AR Gαi mechanism.

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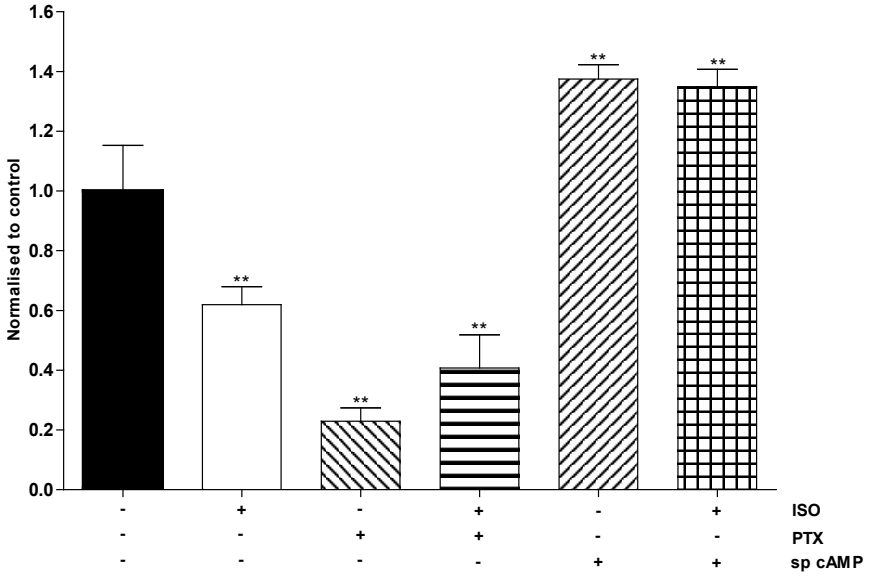
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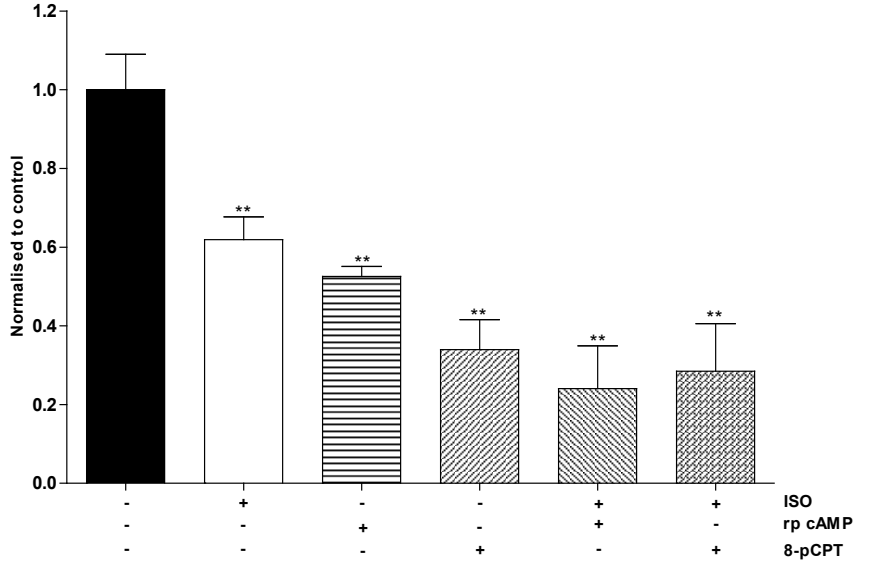
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Figure 4.9: β-AR activation decrease cell proliferation at 72 h

Proliferation experiments were performed as described in the methods. HDMECs were treated with a β-AR agonist (isoproterenol; ISO) 10 μM or sp cAMP 50 μM alone at time 0 or pre-treated with PTX 50 ng/ml for 16 h before addition of both ISO 10 μM and PTX or PTX alone at time 0. The media alone, ISO, PTX and sp cAMP were replaced every 24 h. Cells were counted at 72 h. The data shown were representative of 2 independent experiments from 2 separate cell strains (Control n=9, ISO n=9, PTX n=6, sp cAMP n=6 and sp cAMP and ISO n=6) **(A)**. HDMECs were treated with ISO 10 μM at time 0 or were pre-treated with the PKA inhibitors, rp cAMP 50 μM for 60 min or for 30 min with the EPAC agonist, 8-pCPT-2'-O-Me-cAMP (8-pCPT) before addition of both ISO and rp cAMP or 8-pCPT or ISO and rp cAMP or 8-pCPT alone at time 0. The media alone, ISO, rp cAMP and 8-pCPT were replaced every 24 h. Cells were counted at 72 h. The data shown were representative of 3 independent experiments from 3 separate cell strains (Control n=3, ISO n=3, rp cAMP n=3, 8-pCPT n=3, rp cAMP + ISO n=3 and 8-pCPT + ISO n=3) **(B)**. The data were normalised to control 72h, averaged, statistically analysed and graphically represented using one-way ANOVA followed by the dunnetts test and graphically represented with the bars representing the means ±SEM (** $p<0.001$).

4.3 Discussion

EC migration and proliferation are essential processes for not only wound angiogenesis but for cancer metastasis and numerous physiologic processes, including embryogenesis. This investigation has shown a role for the β-ARs in regulating HDMEC migration and proliferation. Although both PKA and EPAC played a role in the β-AR-mediated decrease in HDMEC migration by salbutamol, the β-AR-mediated anti-motogenicity by isoproterenol was mediated by EPAC alone (figure 4.10). In summary, β-AR activation by both isoproterenol and salbutamol decreased EC migration over a one hour period via cAMP-dependent mechanisms involving EPAC alone and PKA and EPAC, respectively. Both isoproterenol and salbutamol altered HDMEC morphology, FA localisation and distribution. Finally, HDMEC proliferation studies identified isoproterenol-mediated activation of the β-ARs as an anti-mitogenic mediator. Mechanistic studies demonstrated that sp cAMP prevented the isoproterenol-mediated reduction in HDMEC proliferation, while the PKA inhibitor and EPAC modulators decreased HDMEC proliferation alone. Perhaps the isoproterenol-mediated reduction in HDMEC proliferation occurs via a β-AR-mediated modulation/suppression of cAMP at 72 hours.

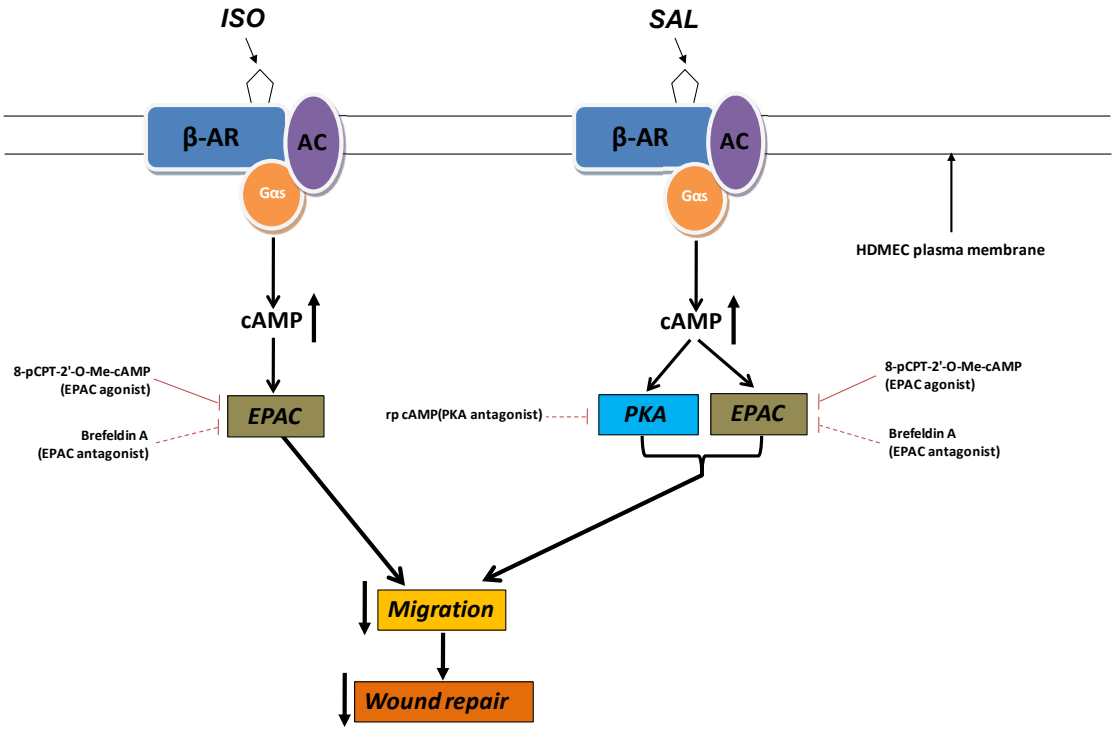


Figure 4.10: A diagrammatic representation of the differing signalling mechanisms in response to β-AR activation by isoproterenol (ISO) and salbutamol (SAL) in the regulation of HDMEC migration. β-AR activation by ISO leads to an increase in cAMP and subsequent activation of EPAC causing a decrease in migration. On the other hand, β-AR activation by salbutamol leads to an increase in cAMP and subsequent activation of both PKA and EPAC, causing a decrease in migration. (Original figure, drawn by Andrew P O'Leary)

β-AR antagonists did not alter HDMEC migration or proliferation *in vitro.* This would seem to suggest that HDMECs do not synthesise and secrete their own catecholamine ligands. None the less, western blotting studies attempted to detect PNMT, a key enzyme which converts nor-adrenaline to adrenaline. However, these studies were inconclusive as the antibody had poor specificity and a low PNMT detection rate. Even though the manufacture's positive control, T47D was used, and PNMT was detected, the expressions levels may vary between cell types. Consequently, PNMT expression in HDMECs may be below the level of detection. Furthermore, tyrosine hydroxylase converts L-tyrosine to L-dopa and is the rate limiting step in catecholamine biosynthesis (Nagatsu et al., 1964a, Nagatsu et al., 1964b) whereas PNMT catalyses the

conversion of adrenaline to nor-adrenaline (Schulz et al., 2004). Therefore further studies will be required to conclude that HDMECs do not express the necessary enzyme to synthesis adrenaline and nor-adrenaline. RT-PCR could be employed to detect PNMT and other enzymes involved in catecholamine biosynthesis. Furthermore, an adrenaline immune assay could be used to detect the secretion of adrenaline from HDMECs.

In keratinocytes, HUVECs, retinal and choroidal ECs, ERK1/2 plays a role in β-AR mediated signal transduction and the alteration of migration. Previous research has shown the ability of β-ARs to regulate cell migration through an ERK1/2 dependent mechanism in macrovascular ECs, keratinocytes and fibroblasts. β_3-AR activation by BRL37344 in human retinal and choroidal ECs, promoted migration though src, PI3K, MEK and ERK 1/2 mediated signalling pathways (Steinle et al., 2003, Steinle et al., 2005). Furthermore, β-AR activation by isoproterenol and salbutamol decreased keratinocyte migration via a PP2A-mediated mechanism reducing ERK phosphorylation and localisation to the lamellipodial edge (Pullar et al., 2006a, Pullar et al., 2003). In oral keratinocytes, β-AR activation by isoproterenol decreased SCM and scratch wound healing *in vitro* though an ERK1/2 and p38 dependent mechanism (Steenhuis et al., 2011). In contrast, the activation of the β-ARs by isoproterenol in human dermal fibroblasts promoted migration (Pullar and Isseroff, 2006). Mechanistic studies revealed a src-dependent, β-AR mediated transactivation of EGFR and subsequent downstream phosphorylation of ERK, was essential for the isoproterenol-mediated increase in dermal fibroblast migration (Pullar and Isseroff, 2006). However, work described in this thesis has found that β-AR modulation did not alter ERK1/2 phosphorylation in HDMECs, highlighting differences between cell types.

Subsequently, the role of upstream signalling proteins such as G proteins, cAMP, PKA and EPAC were investigated in the modulation of HDMEC migration.

Pharmacological studies revealed an anti-motogenic role for cAMP, PKA and EPAC in the isoproterenol/salbutamol mediated modulation of HDMEC migration. To investigate cAMP, a variety of cAMP modulating agents (forskolin, sp cAMP and CTX) were used to increase levels of cAMP. Forskolin is a well known AC activator which results in increased intracellular cAMP (Insel and Ostrom, 2003) whereas sp cAMP is an active, cell permeable cAMP analogue (Van Haastert et al., 1984). CTX on the other hand is a stimulator of Gαs proteins (Jacquemin et al., 1986). Furthermore, to investigate the downstream cAMP-dependent molecules, PKA and EPAC, rp cAMP, 8-pCPT and BFA were used. rp cAMP is an inactive cAMP analogue and was used to block downstream cAMP signaling and inhibit PKA (Van Haastert et al., 1984). BFA on the other hand is a lactone antibiotic and inhibits ADP-ribosylation factors (ARF), which are responsible for activating small GTPases (Tsai et al., 1994, Moringha et al., 1996). ARFs actions on EPAC are unknown, although ARF is structurally related to EPAC. Although there is no inhibitor for EPAC, BFA has been shown to be an inhibitor of EPAC function (Zhong and Zucker, 2005), but there is no evidence that it directly interacts with EPAC. Therefore, based on the available literature, BFA was used to inhibit EPAC (Zhong and Zucker, 2005, Gloerich and Bos, 2010). Finally, 8-pCPT was used as a selective agonist for EPAC1 (Enserink et al., 2002). For further details and concentration justifications of all cAMP, PKA and EPAC modulators used, see section **2.1.6, PKA and EPAC modulators**.

In various cell types, the β-AR-mediated alteration of migration is through cAMP, PKA and EPAC dependent pathways. In corneal ECs, β-AR activation by isoproterenol, led to AC activation, a subsequent increase in cAMP and activation of PKA (Grueb et al., 2008). In the present study, EPAC was shown to play a role in the isoproterenol mediated modulation of HDMEC migration. Other studies have shown that EPAC activation stimulates endothelial progenitor cell migration over fibronectin (Pelham, 1991) and inhibits the migration of human prostate carcinoma cells (Grandoch et al., 2009). Furthermore, cAMP, PKA and EPAC were demonstrated to regulate HUVEC migration and EC barrier permeability. More importantly, this regulation was via parallel and independent pathways, PKA was required for integrin mediated adhesion to control EC barrier permeability, whereas both PKA and EPAC were required for integrin mediated adhesion to control cell migration (Lorenowicz et al., 2008). EPAC also plays an important role in cell polarisation. In U937 cells, treatment with 8-pCPT increased the number of polarised cells on fibronectin, spatially distributed EPAC to the rear of the cell and stimulated chemotaxis (Klausner et al., 1992). In addition, previous research in Chinese hamster ovary (CHO) cells found that Rac inhibition involving cAMP and PKA could inhibit reorganisation of the actin cytoskeleton (Nagasawa et al., 2005). Furthermore, stimulation of smooth muscle cells by isoproterenol, forskolin or sp cAMP-induced actin depolymerisation, demonstrating a β-AR/cAMP/PKA dependent pathway (Hirshman et al., 2001). Finally, in Ovar3 cells, β-AR activation by isoproterenol induced integrin-mediated adhesion to fibronectin via a cAMP, EPAC and RAP1 dependent pathway (Rangarajan et al., 2003). Therefore, it is clear from the literature, that there is evidence supporting the finding of the present

study that β-AR activation by isoproterenol and salbutamol in HDMECs, reduces migration though cAMP, PKA and EPAC dependent signalling pathways.

Interestingly, the forskolin-mediated decrease in HDMEC migration by pre-treating with the PKA inhibitor or the EPAC agonist/inhibitor could not be restored. This could provide insights as to how the β-ARs signal to PKA and EPAC. Efficient signal transduction involves scaffolding and the subsequent assembly of intracellular signalling complexes to the receptor (Burack and Shaw, 2000). It is possible that isoproterenol/salbutamol stimulation allows for the formation of β-AR/EPAC or PKA signalling complexes, and it is this proximity that allows for efficient signal transduction. Indeed, IP data revealed that β_1-AR and β_2-AR associated with EPAC, an important downstream cAMP dependent modulator.

In the absence of isoproterenol/salbutamol stimulation, both the EPAC agonist and inhibitor had no effect on HDMEC migration. However, when HDMECs were pre-treated with the EPAC agonist or inhibitor, the isoproterenol/salbutamol mediated decrease in migration was prevented. It was curious that both the EPAC agonist and inhibitor behaved in the same way. The EPAC agonist, 8-pCPT is an agonist for EPAC1 at 10μM but not PKA (Vliem et al., 2008). The EPAC antagonist, BFA on the other hand is a fungal metabolite that inhibits ADP-ribosylation factors (Arf) which are responsible for activating small GTPases, therefore preventing GDP-GPT exchange. Arfs have important roles in intracellular function such as vesicle trafficking. Therefore, one of the main functions of BFA is the interference of anterograde transport from the ER to the Golgi. Although, Arfs are structurally related to EPAC1, BFAs action on EPAC1 and the pharmacology is currently unknown apart from the available literature that

demonstrates BFAs inhibitory actions on EPAC (Pelham, 1991, Klausner et al., 1992, Jackson and Casanova, 2000, Cox et al., 2004). Perhaps the modulation of EPAC by 8-pCPT or BFA prevented the binding of cAMP to the regulatory subunits of EPAC therefore preventing the isoproterenol/salbutamol mediated decrease in migration.

It is important to note that data from our laboratory has shown that a cAMP assay did not detect any global changes in cAMP in response to a β-AR agonist, isoproterenol in HDMECs (data not shown), suggesting that any β-AR mediated changes in cAMP levels may be a more localised phenomena. Past research has highlighted the importance of the formation of intracellular signalling complexes for the β-ARs, to facilitate functional and selective signal transduction. This has been observed in hippocampal neurons whereby the β_2-AR complexes with L-type calcium channels, AC and PKA (Davare *et al.*, 2001). One study found that the juxtaposition of EPAC with scaffolding proteins such as A-kinase anchor proteins and PDEs and also PKA, is important for exerting the biologic functions of EPAC (Breckler et al., 2011). The research described here indicates that EPAC signalling is regulated by cAMP in a spatial manner. Other findings have found that cAMP activation of EPAC leads to the translocation of EPAC to the plasma membrane where it can proximally activate the downstream effector, Rap1 (Ohba et al., 2003, Ponsioen et al., 2009, Wang et al., 2006). Finally, a study using fluorescent resonance energy transfer found that upon β-AR activation, cAMP was found in much higher concentrations at the plasma membrane (Dipilato et al., 2004). Indeed, evidence from the literature would seem to support that pools of cAMP become available in a spatial manner upon β-AR activation. Therefore, the functional differences that these data have revealed in the isoproterenol/salbutamol β-AR-mediated signalling pathways to PKA and EPAC could be due to the spatial localisation

and compartmentalisation of the β-ARs. In other words, the different β-AR agonists may be recruiting different molecules providing spatial differences in signalling.

ICC studies revealed that β-AR activation by isoproterenol and salbutamol promoted the anti-migratory phenotype of HDMECs compared with the crescent shaped morphology of control cells. Isoproterenol/salbutamol agonist-treated cells were not polarised, and had FAs throughout the cytoplasm, indicative of a stationary cell (Beningo et al., 2001, Ridley et al., 2003). Despite these observations, the methods used in the present study failed to show significant effects on actin and FAs with vinculin as a marker. This suggests that either such changes were not relevant or that the methodology was insufficiently sensitive. Pixel intensity was used to investigate actin expression. However no significant differences between β-AR agonist treatments were found. The actin filaments appeared to be more intense around the leading edge and rear of the cell. Therefore, a more spatial analysis of actin pixel intensity could yield some useful information about actin polymerisation. For example, it is well known that actin is extensively remodelled at the rear and leading edge of the ECs (Lamalice et al., 2007). Counting the total number of FAs yielded no significant differences between the control and isoproterenol/salbutamol agonist-treated cells. However, differences in size for the FAs were observed. Isoproterenol/salbutamol agonist-treated cells had a higher proportion of large FAs as opposed to the smaller, nascent FAs observed in migrating cells. Both of these characteristics observed in β-AR agonist treated cells are indicative of stationary/non-motile cells (Beningo et al., 2001). Therefore Improvision Volocity software could be used to investigate the size of the FAs during HDMEC migration.

HDMEC proliferation studies revealed a role for isoproterenol activation of the β-ARs in which cAMP, PKA and EPAC appear to play a role in the decrease in HDMEC proliferation. This is in contrast to previous research in macrovascular ECs. For example, β_1-AR blockade by nebivolol inhibited proliferation of human coronary ECs (Brehm et al., 2001). Nor-adrenaline promoted the proliferation of HUVECs though an ERK dependent pathway (Sexl et al., 1995, Seya et al., 2006). In addition, in human retinal and choroidal ECs, β_3-AR activation by BRL37344 increased their proliferation rate via src, PI3K, mitogen-activated protein kinase kinase (MEK) and ERK 1/2 signalling pathways (Steinle et al., 2003, Steinle et al., 2005).

In this thesis, the cAMP analogue, sp cAMP increased HDMEC proliferation whereas a combination of both isoproterenol and sp cAMP prevented the sp cAMP mediated increase in proliferation. Indeed, evidence from the literature revealed that elevations in cAMP also increased the proliferation of cells such as vascular ECs (Torella et al., 2009), vascular smooth muscle cells (Favot et al., 2003) and mouse embryonic stem cells (Kim et al., 2012). On the other hand, the anti-mitogenic effect of the PKA inhibitor and EPAC agonist alone suggests that the suppression of cAMP signalling prevents HDMEC proliferation. It was surprising that both the PKA inhibitor and EPAC agonists decreased proliferation. The PKA inhibitor and EPAC agonist mediated decrease in proliferation at 72 hours which would seem to indicate a basal tone of cAMP. As mentioned earlier, perhaps the PKA inhibitor and the EPAC agonist are preventing cAMP from binding to PKA and EPAC. In addition, the EPAC antagonist, BFA, also behaved unexpectedly and induced cell death. Indeed, one study found that in human cancer cells such as leukaemia and colon carcinoma cells, BFA can in fact induce apoptosis (Shao et al., 1996). Perhaps this is due to BFAs actions on intracellular

functions such as vesicle trafficking, as mentioned earlier (Pelham, 1991, Klausner et al., 1992, Jackson and Casanova, 2000, Cox et al., 2004). Consequently, the data appears to show that the modulation of cAMP can effect HDMEC proliferation. Therefore it was hypothesised that the isoproterenol-β-AR mediated decrease in HDMEC proliferation was via the suppression of cAMP, known to occur for the β_1-AR in CHO cells (Martin et al., 2004). To test this hypothesis, the Gαi inhibitor, PTX was used to suppress Gαi activity. It was believed that by pre-treating with PTX before addition of isoproterenol would prevent the isoproterenol-mediated decrease in HDMEC proliferation. However this was not the case, and in fact PTX was shown to inhibit proliferation therefore compromising itself as a tool to study the role of Gαi in proliferation. As a result, it is difficult to conclude the precise mechanism as to how isoproterenol decreases HDMEC proliferation. What is clear though is that the modulation of cAMP can increase or decrease HDMEC proliferation.

In conclusion, this chapter has provided a mechanistic insight into how the isoproterenol/salbutamol activation of the β-ARs modulates HDMEC migration and proliferation. Further work will investigate the roles of the β-ARs in angiogenesis in more complex *in vivo* models and how β-ARs may be able to alter angiogenesis though the actions of other wound cell types.

Chapter 5 Studying the role of the β-AR family in angiogenesis using *ex vivo* and *in vivo* models

5.1 Introduction

5.1.1 Introduction

The wound environment is complex and contains numerous cell types which secrete a multitude of growth factors and cytokines, many of which can modulate EC function and angiogenesis (Martin, 1997, Schultz and Wysocki, 2009). Therefore it is important to determine whether β-AR modulation can alter angiogenesis in more complex environments similar to an *in vivo* skin wound.

5.1.2 Angiogenic growth factors

There are numerous pro-angiogenic growth factors including VEGF-A, VEGF-B, VEGF-C, VEGF-D, PIGF, FGF-1, FGF-2, FGF-4, PDGF and TGF-β1 secreted from various cutaneous cells including keratinocytes, fibroblasts, ECs and macrophages, see **table 1.1: Growth factor signals at the wound site**. However, two of the most important pro-angiogenic growth factors are VEGF-A and FGF-2. VEGF-A is a pro-angiogenic growth factor that induces EC proliferation, sprouting and tubulogenesis and is upregulated during wound healing. VEGF-A can also upregulate levels of eNOS, increasing NO and inducing vasodilation (Hattori et al., 2007). FGF-2 is also an important family of pro-angiogenic growth factors, stimulating both EC migration and proliferation as well as stimulating the production of degradative enzymes such as collagenase and plasminogen activator to promote EC invasion (Otrock et al., 2007), see section **1.5, wound revascularisation** for further detail about VEGF-A and FGF-2. Many wound cell types secrete pro-

angiogenic growth factors such as VEGF-A and FGF-2. For example, keratinocytes are a major source of VEGF in the wound (Brown et al., 1992) while FGF-2 is secreted by dermal fibroblasts and ECs (Kandel et al., 1991, Schweigerer et al., 1987). Therefore it is possible that β-AR agonists or antagonists modulate growth factor secretion from cutaneous cells to alter EC function and angiogenesis.

5.1.3 *Ex vivo* and *in vivo* angiogenesis models

Determining the effect of β-AR modulation on EC function *in vitro* is necessary to explore the mechanisms which underpin functional changes. However, more complex models are required to assess how β-AR agonists and antagonists alter angiogenesis.

The aortic ring assay is an *ex vivo* organ culture assay developed by Nicosia, whereby rings of a rat aorta are placed in BME which stimulates the outgrowth of ECs and other cell types such as fibroblasts, SMCs and perictyes. This leads to the formation of 3-dimensional networks of cellular cords with occasional microvessels with lumina (Nicosia and Ottinetti, 1990). The microvessels that grow outwards from the aorta are similar to that *in vivo* in the sense that other cell types, as mentioned above are recruited to form the budding EC tube. Therefore, tests substances can be applied to the aortic rings to assess their effect on EC outgrowth and angiogenic activity. Perhaps another advantage of using the aortic ring assay is that ECs are representative of real life ECs as they have not been pre-selected by passaging and therefore are not in a proliferative state when used. The surface area of cell outgrowth can be quantitated or the number of tubule like structures or branch points can be counted (Auerbach et al., 2003).

The CAM assay allows the analysis of the effects of test substances on embryonic angiogenesis. Embryologists first experimented with CAMs 50 years ago avoiding the use of animals and the relevant training required for experimentation (Auerbach et al., 2003). The CAM assay utilises the chorioallantoic membrane which is a double layer mesoderm adjacent to the surface of the egg shell, where a dense vasculature network can develop (Cimpean et al., 2008, Auerbach et al., 2003). Fertilised hen eggs are incubated at 37°C with humidity for 5 days. The top of the shell is then excised and test substances can be placed on a glass coverslip, desiccated and added to the CAM, which is then incubated for a further 5 days. Stereomicroscopy is used to image angiogenic activity every 24 h until day ten of development. At day 10, the eggs are discarded. Blood vessel density is quantitatively evaluated using morphometric methods or by counting the number of branch points per image (Ribatti et al., 1996a, Auerbach et al., 2003).

In summary, both the rat aortic ring assay and the CAM assay are cost effective models and in line with replacement, reduction and refinement principles of reducing the use of animal models such as: subcutaneous implantation in animals; corneal rabbit model; zebra fish and rodent models. Consequently, both the rat aortic ring assay and CAM models were utilised to investigate the effect of β-AR agonists or antagonists on angiogenesis in more complex environments that more closely the *in vivo* environments of a wound.

5.1.4 Chapter aims

The main aims of this chapter are to determine the effect of β-AR agonists and antagonists on angiogenesis in more complex, physiological environments such as the rat aortic ring assay and CAM assay. In addition, ELISA studies will determine whether β-AR agonists and antagonists can alter growth factor secretion from a range of cutaneous cells including HNKS, HDFs, neutrophils and macrophages.

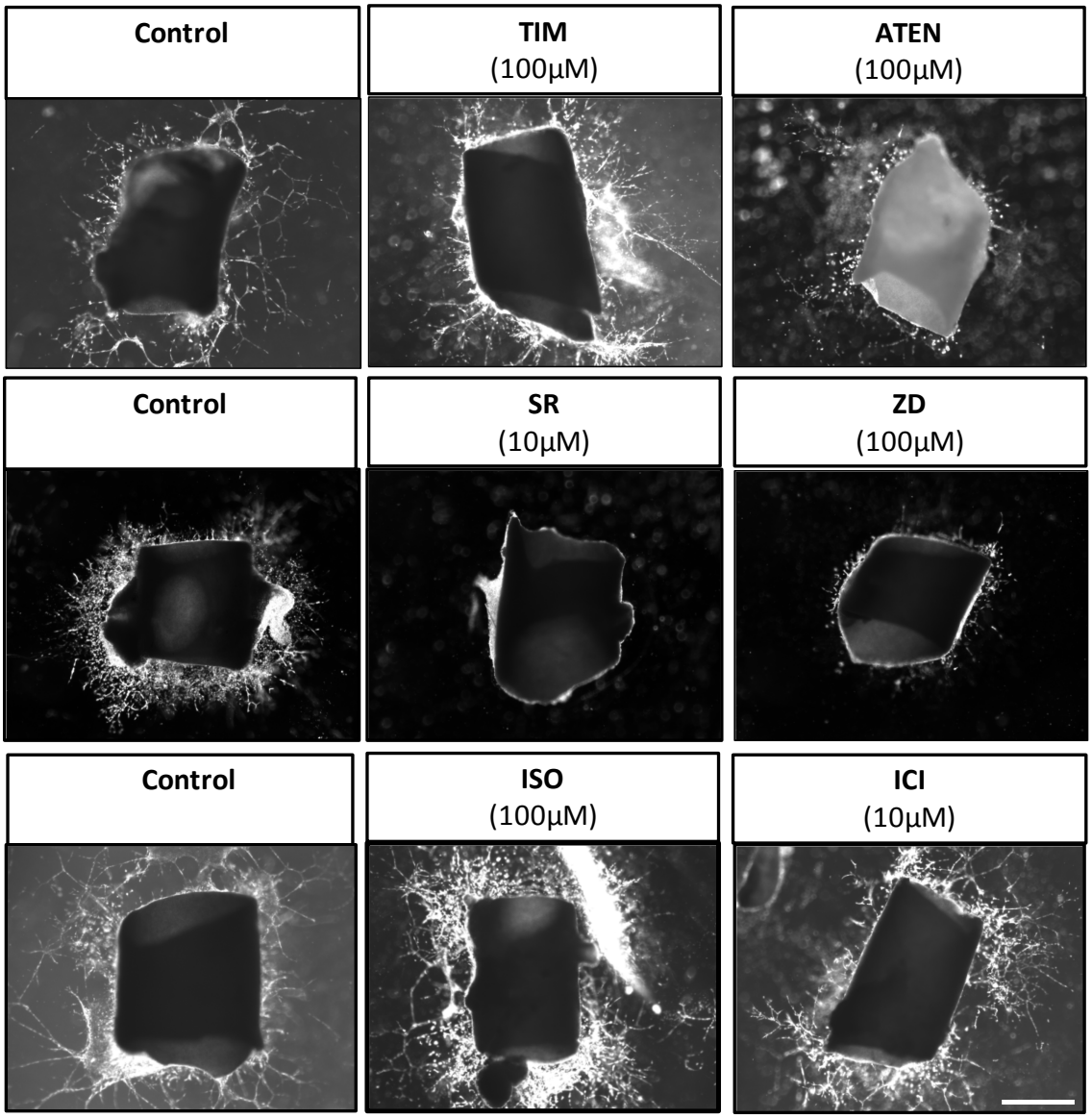
5.2 Results

5.2.1 β-AR modulation of microvessel outgrowth in the rat aortic ring assay

It important to understand how the β-AR family may affect EC function and consequently angiogenesis in more complex environments. Here, microvessel outgrowth from the rat aorta was observed in the presence and absence of β-AR agonists and antagonists.

Figure 5.1A illustrate aortic microvessel outgrowth after 8 days of development. The data show that isoproterenol increased microvessel outgrowth by 55%, whereas atenolol decreased microvessel outgrowth by 55%. In contrast, timolol and ICI 118,551 enhanced microvessel outgrowth by 23% and 35% respectively. Interestingly, ZD7114 and SR59230A significantly decreased microvessel outgrowth by 89% and 92% respectively (figure 5.1B).

A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TIM
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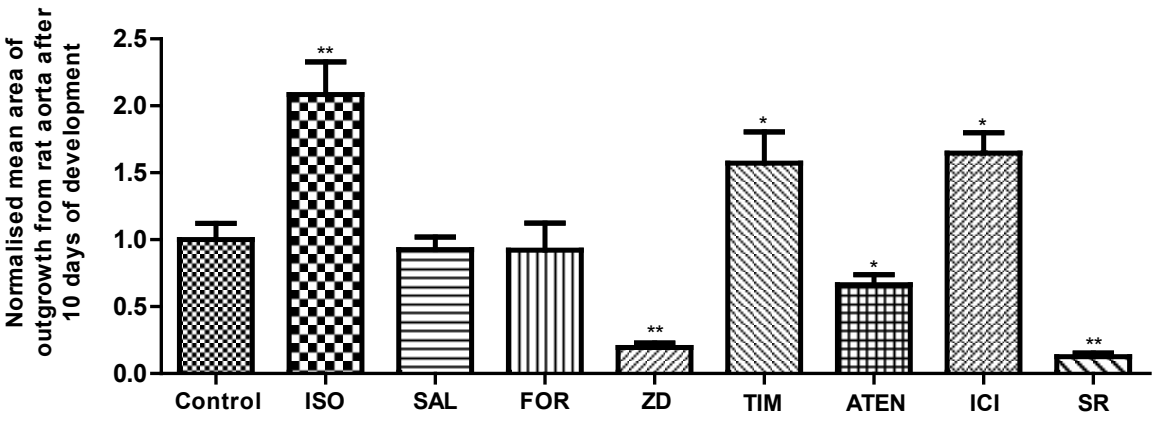


Figure 5.1: β-AR modulation of microvessel outgrowth

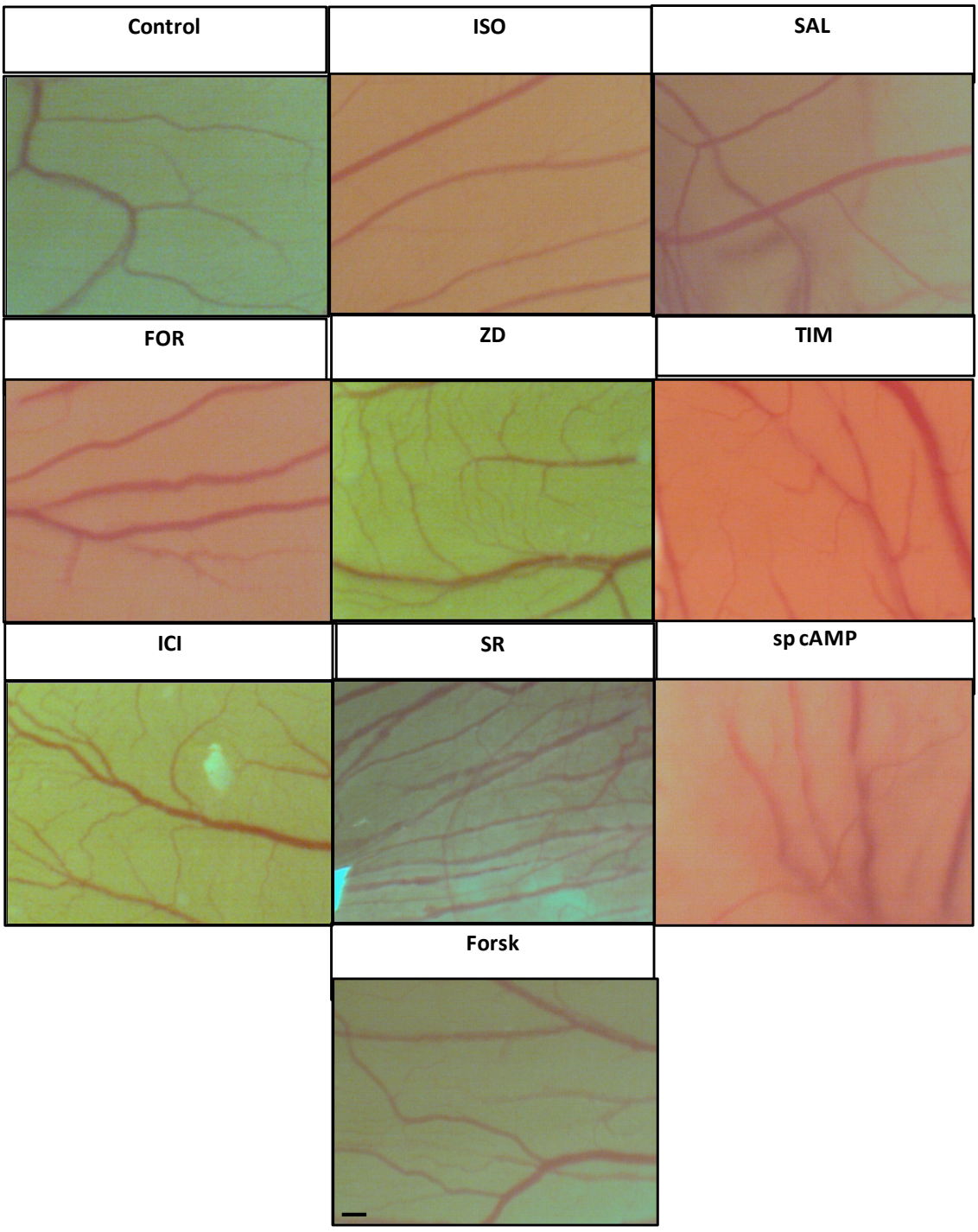
Rat aortic ring assays were performed as previously described in the methods. Aortas were treated with various β-AR agonists including isoproterenol (ISO; 100 µM), salbutamol (SAL; 100 µM), formoterol (FOR; 100 µM) and ZD7114 (ZD; 100 µM) at day 5. Aortas were also treated with various β-AR antagonists including timolol (TIM; 100 µM), atenolol (ATEN; 100 µM), ICI 118,551 (ICI; 10 µM) and SR 59230A (SR; 100 µM) at day 5. Aortic endothelial outgrowth was then imaged every 24 h until day 10 of development. Images representing aortic outgrowth after 10 days of culture when treated with various β-AR agonists and antagonists are presented. The scale bar is 1mm **(A)**. Aorta outgrowth surface area (mm^2) was divided by the aorta surface area (mm^2) for each treatment and then normalised to the average aorta outgrowth surface area of the control. The data were representative of 3-5 independent experiments (control n=13, ISO 100 µM n=3, SAL 100 µM n=4, FOR 100 µM n=6, ZD 10 0µM n=4, TIM 100 µM n=7, ATEN 100 µM n=4, ICI 10 µM n=4 and SR 100 µM n=5). There data were averaged, statistically analysed using the one-way ANOVA followed by the dunnetts test and graphically represented with the bars representing the means ±SEM. Only the differences versus the control are shown (* $p<0.05$, ** $p<0.001$) **(B)**.

5.2.2 β-AR modulation of embryonic vascularisation in the CAM

To investigate the roles of the β-ARs in embryonic vascularisation, the CAM assay was performed. The CAM is a copiously vascularised area which mediates gas exchange with the outer environment. The CAM is created by the merging of the allantois, an extra-embryonic membrane derived from the mesoderm, and the chorion-epithelium, derived from the ectoderm. Vascularisation encompasses the processes of vasculogenesis, angiogenesis and vessel growth by intussusception, which all contribute to chick-embryonic development (Ribatti et al., 1996b).

Images of CAMs, 9 days post-fertilisation are presented (figure 5.2A. Isoproterenol and formoterol decreased vascularisation by 45% and 29% respectively (figure 5.2B). Salbutamol did not alter vascularisation. ZD7114 enhanced vascularisation by 42%. Interestingly, when ZD7114 was used at 10 0μM, it had no effect. SR59230A at both 10 μM and 100 μM enhanced vascularisation by 46% and 42% respectively. In addition, ICI 118,551 also enhanced vascularisation by 48%. An active cAMP analogue, sp cAMP decreased vascularisation by 51%, whereas forskolin, an AC activator, had no effect on angiogenesis (figure 5.2B).

A



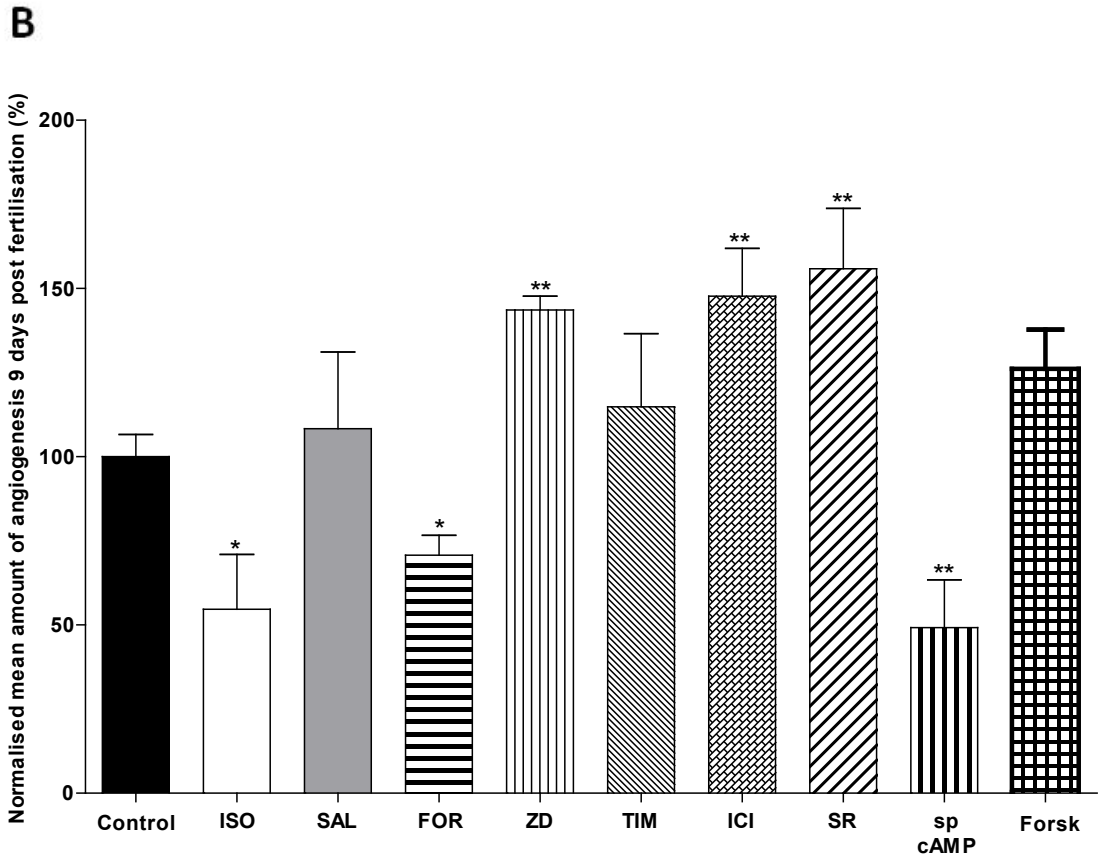


Figure 5.2: β-AR modulation of embryonic angiogenesis

CAM assays were performed as previously described in the methods. The CAMs were treated with various β-AR agonists including isoproterenol (ISO; 10 µM), salbutamol (SAL; 100 µM), formoterol (FOR; 100 µM) and ZD7114 (ZD; 10 µM) at day 5. The CAMs were also treated with various β-AR antagonists including ICI 118,551 (ICI; 100 µM) and SR 59230A (SR; 100 µM) at day 5. The CAMs were also treated with cAMP analogues, sp cAMP 50 µM or forskolin (forsk) 10 µM at day 5. Eggs were imaged every 24 h until day 10. Images representing angiogenesis 9 days post fertilisation are presented. The scale bar is 1 mm **(A)**. The data shown are combined from 3-16 independent experiments using a total of 51 eggs (control n=16, n=6, ISO 10 µM n=9, SAL 100 µM n=3, FOR 100 µM n=3, ZD 10 µM n=3, ICI 100 µM n=3, SR 100 µM n=3, sp cAMP 50 µM n=3 and forskolin n=4). Data taken from day 10 was averaged, statistically analysed using one-way ANOVA followed by the dunnetts test and graphically represented with the bars representing the means ±SEM. Only the differences versus the control are shown. (* $p<0.05$, ** $p<0.001$) **(B)**.

5.2.3 β-AR modulation of angiogenic growth factors from wound cell types

5.2.3.1 VEGF-A ELISA

Cutaneous cells within the wound are capable of secreting a complex cocktail of biological factors that are capable of altering the recruitment of ECs to the wound and inducing angiogenesis. EC recruitment into the wound is mediated by VEGF which aids EC migration, proliferation, elongation and alignment (Bouis et al., 2006, Nissen et al., 1998). Keratinocytes are a major source of VEGF in the wound (Brown et al., 1992). Therefore VEGF-A ELISA studies will determine the ability of wound HNKs to secrete VEGF-A and the ability of β-AR agonists and antagonists to alter VEGF-A secretion from HNKs over 6, 24 and 48 h.

The data show that HNKs secrete VEGF-A at 6 h, and that this secretion increased at 24 and 48 h. Salbutamol reduced VEGF-A secretion at 6 and 24 h by 20% and 31% respectively. Isoproterenol also significantly reduced VEGF-A secretion at 6 and 24 h by 25% and 31% respectively. On the other hand, at 24 h, timolol significantly increased VEGF-A secretion by 23% (figure 5.3).

Finally, VEGF-A was also detected in HDMEC, HDF, U937 and neutrophil supernatants at <10 pg/ml, >10 pg/ml, >4000 pg/ml, <50 pg/ml and >10 pg/ml respectively, however β-AR activation or blockade had no effect on VEGF secretion (data not down).

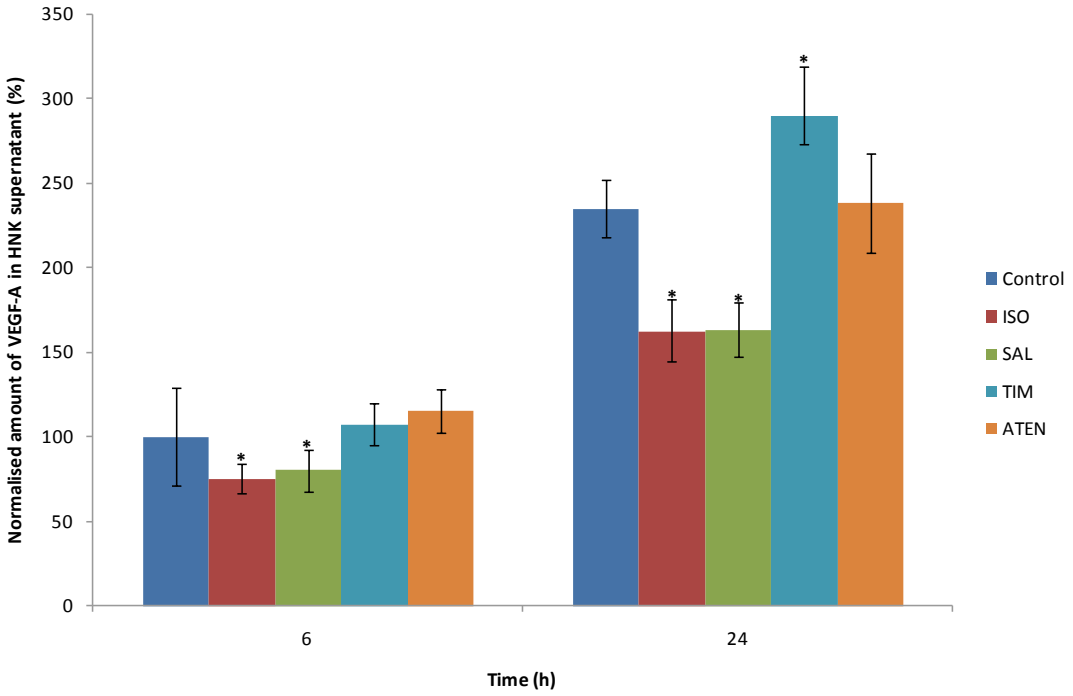


Figure 5.3: β-AR modulation of Human VEGF from HNK supernatants

HNKs were treated with either basal media alone or media containing various β-AR agonists including isoproterenol (ISO) and salbutamol (SAL) at time 0 for 6 and 24 h. HNKs were also treated with various β-AR antagonists including timolol (TIM) and atenolol (ATEN) at time 0 for 6 and 24 h. Growth factor levels were determined using Duo set ELISA kit specific for human VEGF-A as described in the methods. The data were combined from 5 independent experiments, from 2 separate cell strains (Control n=5, ISO n=5, SAL n=5, TIM n=5 and ATEN n=5). The data were averaged, statistically analysed using two-way ANOVA followed by the Bonferroni test and graphically represented with the bars representing the means ±SEM. Only the differences versus the control are shown. (* $p<0.05$).

5.2.3.2 FGF-2 ELISA

FGF-2 is also a potent inducer of angiogenesis and is synthesised and secreted by a number of wound cells such as dermal fibroblasts and ECs (Kandel et al., 1991, Schweigerer et al., 1987). Therefore FGF-2 ELISA studies will determine the ability of HDMECs and HDFs to secrete FGF-2 and the ability of β-AR agonists and antagonists to alter FGF-2 secretion from HDMECs and HDFs over 6, 24 and 48 h.

HDMECs secreted FGF-2 at 6 h which increased exponentially at 48 h (figure 5.5). At 6 h, isoproterenol and salbutamol significantly reduced FGF-2 secretion by 49% and 57% respectively. However at 48 h, salbutamol increased FGF-2 secretion 74% (figure 5.4).

HDFs secreted FGF-2 at 6 h which only slightly increases at 24 and 48 h (figure 5.5). Salbutamol increased FGF-2 secretion by 328% at 6 h whilst timolol decreased FGF-2 secretion by 33% at 24 h (figure 5.5).

Finally, FGF-2 was detected in HNK, U937 and neutrophil supernatants at <40 pg/ml, <1 pg/ml and <1 pg/ml respectively, however β-AR activation or blockade had no effect on FGF-2 secretion (data not shown).

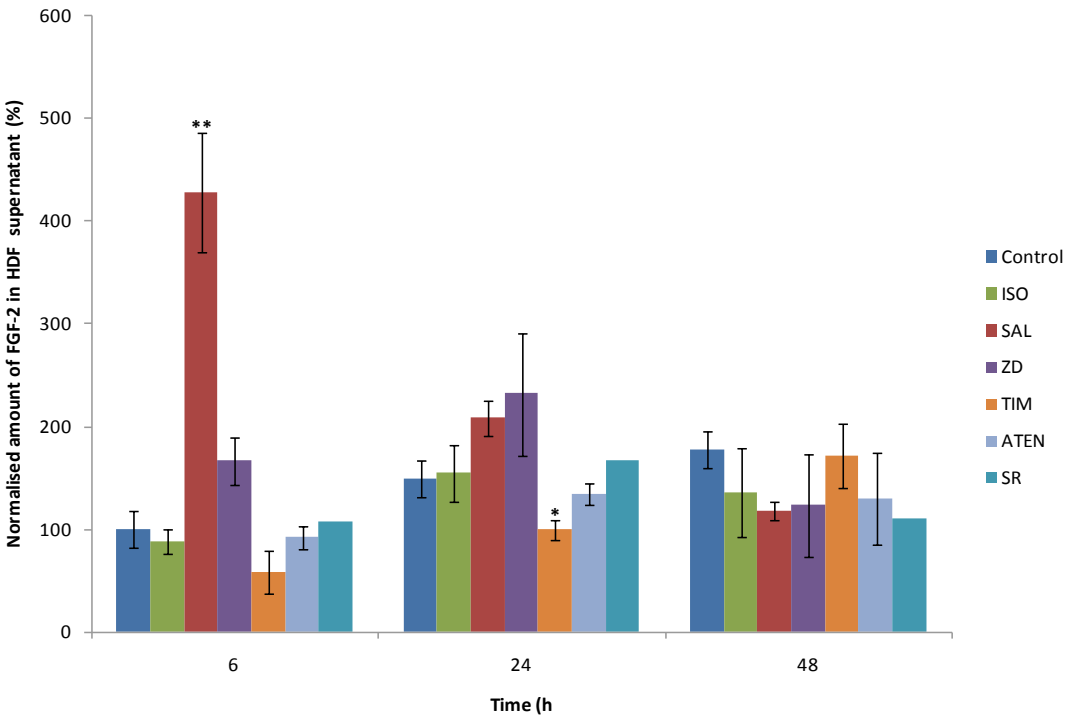


Figure 5.4: β-AR modulation of Human FGF-2 from HDF supernatants

HDFs were treated with either basal media alone or media containing various β-AR agonists at 10μM including isoproterenol (ISO), salbutamol (SAL) and ZD7114 (ZD) at time 0 for 6, 24 and 48 h. HNKs were also treated with various β-AR antagonists at 10 μM including timolol (TIM) and atenolol (ATEN) at time 0 for 6, 24 and 48 h. Growth factor levels were determined using Duo set ELISA kit specific for human FGF-2 as described in the methods. The data were representative of 4 independent experiments from 2 separate cell strains (Control n=4, ISO n=4, SAL n=4, ZD n=4, TIM n=4, ATEN n=4 and SR n=4). The data were averaged, statistically analysed using two-way ANOVA followed by the Bonferroni test and graphically represented with the bars representing the means ±SEM. Only the differences versus the control are shown. (* $p<0.05$, ** $p<0.001$).

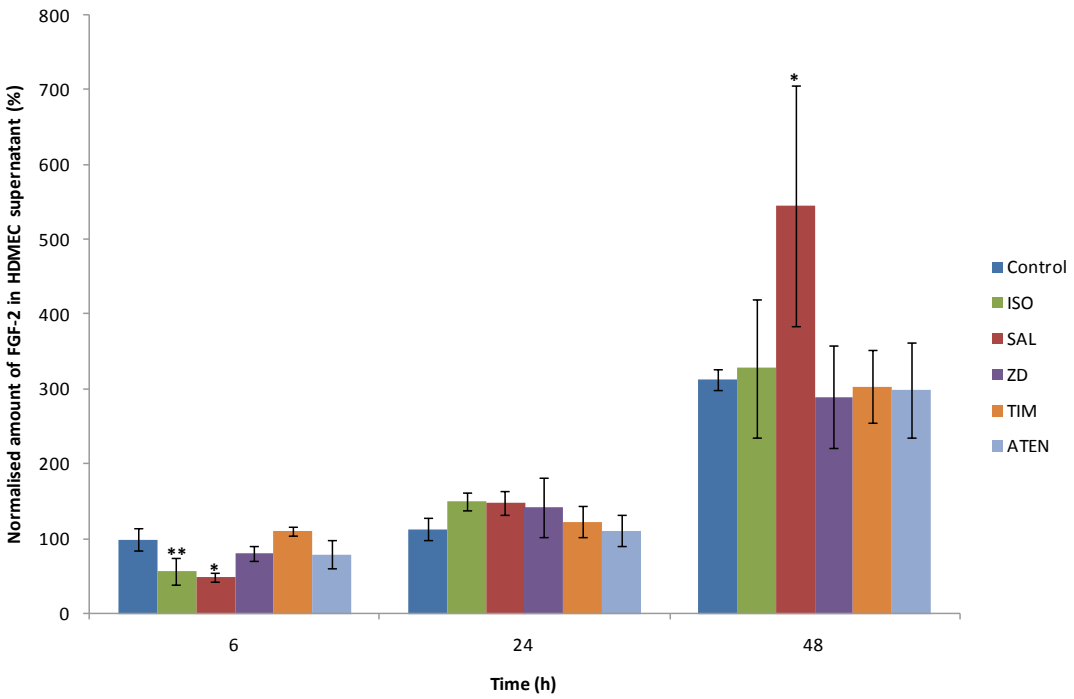


Figure 5.5: β-AR modulation of Human FGF-2 from HDMEC supernatants

HDMECs were treated with either basal media alone or media containing various β-AR agonists at 10µM including isoproterenol (ISO), salbutamol (SAL) and ZD7114 (ZD) at time 0 for 6, 24 and 48 h. HNKs were also treated with various β-AR antagonists at 10µM including timolol (TIM) and atenolol (ATEN) at time 0 for 6, 24 and 48 h. Growth factor levels were determined using Duo set ELISA kit specific for human FGF-2 as described in the methods. The data were representative of 4 independent experiments from 2 separate cell strains (Control n=4, ISO n=4, SAL n=4, ZD n=4, TIM n=4 and ATEN n=4). The data were averaged, statistically analysed using two-way ANOVA followed by the Bonferroni test and graphically represented with the bars representing the means ±SEM. Only the differences versus the control are shown. (* $p<0.05$, ** $p<0.001$).

5.3 Discussion

Angiogenesis is a multistage process requiring many cell types and biological factors to complete the process. This investigation has revealed a role for the β-ARs in regulating EC migration and proliferation and highlighted aspects of the signalling pathways involved. Here, two more complex angiogenesis assays, the *ex vivo* aortic ring assay and the *in vivo* CAM assay have demonstrated a role for the β-ARs in modulating angiogenesis. Isoproterenol and ICI 118,551 increased aortic outgrowth whilst timolol, SR59230A and ZD7114 reduced aortic outgrowth. Meanwhile, isoproterenol and formoterol reduced vascularisation in the CAM assay, whilst ICI 118,551, SR59230A and ZD7114 increased vascularisation in the CAM assay. In addition, ELISA studies revealed that β-ARs can alter the secretion of VEGF-A and FGF-2 from HDFs, HNKs and HDMECs. Moreover, salbutamol reduced whilst timolol increased the secretion of VEGF-A from HNKs. In contrast, salbutamol increased at 6 h whilst timolol reduced FGF-2 secretion at 24 h from HDFs. Furthermore, salbutamol decreased FGF-2 secretion at 6 h and increased FGF-2 secretion at 48 h from HDMECs. Finally, β-ARs did not alter the secretion of VEGF-A and FGF-2 from inflammatory cells including neutrophils and macrophages.

To summarise, as expected, in some situations, the agonists and antagonists had the opposite effect. However, bizarrely, in some instances, some agonists and antagonists had the same effect. β-AR activation by isoproterenol increased whilst β-AR blockade by timolol and SR59230A reduced aortic outgrowth. Interestingly, β-AR blockade by ICI 118,551 also increased aortic outgrowth whereas β-AR activation by ZD7114 reduced aortic outgrowth. Meanwhile, β-AR activation by isoproterenol and formoterol

reduced vascularisation in the CAM assay, whilst β-AR blockade by ICI 118,551, SR59230A increased vascularisation in the CAM assay. Interestingly, β-AR activation by ZD7114 also increased vascularisation in the CAM assay. Finally, ELISA studies revealed that agonists and antagonists had the opposite effect on growth factor secretion as one would expect. Indeed, β-AR activation by salbutamol reduced whilst β-AR blockade by timolol increased the secretion of VEGF-A from HNKs. In contrast, β-AR activation by salbutamol increased at 6 h whilst β-AR blockade by timolol reduced FGF-2 secretion at 24 h from HDFs. Interestingly, β-AR activation by salbutamol decreased FGF-2 secretion at 6 h and increased FGF-2 secretion at 48 h from HDMECs.

The rat aortic ring assay provided fascinating insights into how the β-AR agonists and antagonists may behave in a more complex environment with other cell types surrounding an ECM-like matrix. Although some of the β-AR agonists and antagonists behaved in a similar way to *in vitro*, numerous differences were observed. For example, isoproterenol, enhanced EC microvessel outgrowth, whereas atenolol, decreased angiogenesis. Alternately, ICI 118,551, enhanced EC microvessel outgrowth. Therefore one can postulate from these data that indeed, ICI 118,551 is pro-angiogenic whereas isoproterenol and atenolol are anti-angiogenic in more complex environments. These differences observed were not surprising as rat aortic ECs are macrovascular ECs and not microvascular ECs. Indeed, previous studies investigating the influence of β-ARs on marovascular EC function *in vitro* have already demonstrated the differences compared with the findings of this thesis for HDMECs, see section **3.1.5, the role of β-ARs in EC function and angiogenesis**. In addition, as previously mentioned, various cell types are present in the rat aorta, including SMCs and fibroblasts. Therefore it is reasonable to assume that β-AR agents may be affecting

SMCs and fibroblasts resulting in altered aortic cell outgrowth. Indeed previous research has already shown the role of the β_2-AR in modulating HDF (Pullar, 2006b) and SMC (Pullar and Isseroff, 2006, Gerthoffer, 2008) activity *in vitro*.

Interestingly, the β_3-AR has previously been shown to be pro-angiogenic (Steinle et al., 2003, Steinle et al., 2005). However, the aortic ring assay data has revealed a potent anti-angiogenic function for β_3-AR. Both activation and blockade of this receptor hugely reduced microvessel outgrowth, and in some cases completely prevented any out-growth from occurring. It was curious that both the β-AR agonist (ZD7114) and antagonist (SR59230A) behaved in the same way. Due to the complex pharmacology of the β-ARs, any number of explanations could be possible. There are a number of β-blockers that are known to act as partial agonists such as propanolol (Baker et al., 2003b), pindolol, alprenolol, acebutolol, labetolol (Baker et al., 2003a, Chidiac et al., 1994) and bucindolol (Maack et al., 2003).

The CAM assay provided the first opportunity for this study to investigate the effects that β-AR modulating agents may have in an *in vivo* environment. Also, the kind of doses that will be required to elicit a response in the CAM may be similar to those in more complex models. Here, the issue of bioavailability played a role as the doses required to elicit a response would be different to those used *in vitro*. It was found that some β-AR agents elicited a response at 10 μM whilst others were used at higher concentrations (100 μM) to take into count the diffusion gradient from the coverslip to the CAM though the tissue. Also, blood circulation may affect whether a β-AR agent reached their receptor targets as it is well known that drugs that bind to a whole range of substances in the blood such as plasma proteins. Interestingly, ZD7114 only elicited

a response at 10 μM and not at 100 μM. This could be due to receptor desensitisation, as result of the receptor being over stimulated.

As discovered *in vitro*, only some β-AR agonists and antagonists altered CAM vascularisation. Therefore ligand-directed signalling may be involved due to differences in ligand structure and their ability to alter receptor conformations and G protein coupling (Audet and Bouvier, 2008, Galandrin et al., 2007, Hoffmann et al., 2008, Kenakin, 2007, Perez and Karnik, 2005, Seifert and Dove, 2009), see section **3.3, discussion**. Furthermore, an alternative explanation as to why only some agonist/antagonist concentrations affected CAM vascularisation could be due to concentration-dependent signal pathway switching. Studies have shown *in vitro* that different concentrations of β-AR agonists can activate alternate signalling pathways. One study investigating β_2-AR activation in mouse embryonic fibroblasts found that alteration in agonist concentrations switched the GPCR between G protein-dependent signalling and G protein-independent signalling (Sun et al., 2007).

It was also curious to note that sp cAMP and not forskolin decreased vascularisation in the CAM, whereas the *in vitro* migration studies revealed that both sp cAMP and forskolin decreased migration. Forskolin had no effect on angiogenesis even though previous work revealed that forskolin promoted angiogenesis in the CAM (Namkoong et al., 2009). Perhaps species variation could explain these differences. Given that sp cAMP reduces angiogenesis to a similar extent to isoproterenol, it's reasonable to assume that the mechanism behind the isoproterenol mediated decrease may be cAMP dependent.

In addition to angiogenesis, CAM vascularisation encompasses the processes of vasculogenesis and vessel growth by intussusception (Ribatti et al., 1996b). Lymphangiogensis also plays a role during CAM development (Oh et al., 1997). Previous studies which inject mercox, a rapidly hardening polymer, to induce vascular corrosion casting, identified the presence of lymph vessels in the CAM, however, they are much smaller than normal blood vessels and are unidentifiable at the magnification used in this study (Oh et al., 1997). Therefore, although these data does not shed light on the role of β-ARs in lymphangiogenesis, it is possible that β-ARs may modulate vascularisation by vasculogenesis and vessel growth by intussusception in the CAM.

The rat aortic ring assay and the CAM assay are great models for investigating angiogenesis in more complex environments. However there are important limitations to each model that need to be taken into account. For example, with both assays, there is intra-species variation, subsequently requiring a large number of rats/chick embryos per study. Furthermore, rat aortas are large and difficult to handle making them difficult to cut into uniform sections for consistency and experimental reproducibility. The same can be said when preparing the CAMs, see section, **2.2, methods**. In addition, non-specific inflammatory reactions may develop from placing the coverslip on the CAM and in turn induce a secondary vasoproliferative response. This can therefore make the primary response difficult to quantify. To help reduce the severity of a secondary vasoproliferative response, the coverslip can be placed on the CAM at a very early stage of development when the host's immune system is relatively immature. In addition, neovascularisation and re-arrangement of vessels can be hard

to distinguish from each other. Therefore utilising sequential photography to image new vessel formation is a necessity (Staton et al., 2006).

A number of pro-angiogenic growth factors are known to stimulate angiogenesis including VEGF-A and FGF-2 (Bouis et al., 2006, Kandel et al., 1991, Nissen et al., 1998). Activating or inhibiting the β-ARs altered the amount of VEGF-A and FGF-2 secreted from HDFs, NHKs and HDMECs respectively. Past research has shown a role for the β-ARs in upregulating VEGF-A in HUVECs (Lamy et al., 2010), human choroidal endothelial cells (Steinle et al., 2008) and other cell types such as human melanoma cell lines (Yang et al., 2006). No VEGF-A was detected from HDMECs, however large amounts of VEGF-A was secreted from HNKs, whereby salbutamol, decreased secretion at 24 h by 31%. Interestingly, a huge amount of VEGF-A secretion was detected from U937 cells, however, β-AR modulation did not alter VEGF-A secretion. This is in contradiction to a study by Verhoeckx et al (2006), which found that the selective β_2-AR agonists, zilpaterol and clenbuterol increased the secretion of VEGF-A from U937 cells (Verhoeckx et al., 2006). ECs on the other hand are known to produce FGF-2 (Schweigerer et al., 1987). Interestingly for HDMECs, isoproterenol and salbutamol decreased FGF-2 secretion at 6 h and salbutamol increased FGF-2 secretion at 48 h. Data has also shown that timolol increased VEGF-A secretion from HNKs at 24 h. These data therefore show a novel role for the β-ARs in modulating VEGF-A secretion from HNKs and FGF-2 secretion from HDMECs.

In conclusion, this chapter has revealed that β-ARs can regulate angiogenesis in more complex environments. The β-ARs modulated pro-angiogenic growth factor secretion from HDMECs, HNKs and HDFs. The ability of β-ARs to modulate growth factor levels

from a variety of wound cell types might provide an explanation as to why different effects are observed *in vitro*, *ex vivo*, *in vivo*.

Chapter 6 Final discussion

6.1 Summary of research

Angiogenesis plays an important role in growth and development as well as in pathologies such as wound healing and cancer metastasis. HDMECs are the main cell type that contribute to angiogenesis during dermal wound healing. Compromised HDMEC function and blood vessel formation contribute to the delayed healing observed in chronic wounds. Indeed, the majority of chronic wounds have impaired angiogenesis (Falanga, 2005, Martin et al., 2002, Menke et al., 2007, Strodtbeck, 2001). On the other hand, an over-zealous angiogenic response is associated with both normal and pathological wound scarring (Diegelmann and Evans, 2004, Gabbiani et al., 1972). Meanwhile, abnormal angiogenesis is associated with tumour metastasis in all types of cancer including melanoma (Zetter, 1998).

This investigation has revealed a role for the β-ARs in the regulation of HDMEC function and angiogenesis. Isoproterenol and salbutamol were anti-motogenic whilst isoproterenol was anti-mitogenic. In addition, EPAC played a role in modulating the anti-motogenic effects of both the isoproterenol and salbutamol β-AR-mediated decrease in migration rate, while only PKA played a role in the salbutamol β-AR-mediated decrease in migration rate. Furthermore, immunoprecipitation studies revealed that both the β_1-AR and β_2-AR directly associate with EPAC. Finally, inhibiting cAMP signalling pathways reduced proliferation.

In more complex environments, the β-AR agonist, isoproterenol delayed, whilst both the β-AR agonist, ZD7114 and the β-AR antagonist, timolol promoted tubule formation. In contrast, isoproterenol and the β-AR antagonist ICI 118,551 increased aortic outgrowth whilst β-AR blockade with atenolol, SR59230A or β-AR activation by ZD7114 reduced aortic outgrowth. In the CAM assay, isoproterenol and the β-AR agonist, formoterol reduced angiogenesis whilst ICI 118,551, SR59230A and ZD7114 increased angiogenesis. Finally, ELISA studies demonstrated that isoproterenol and the β-AR agonist, salbutamol reduced while timolol increased VEGF-A secretion from keratinocytes. On the other hand, isoproterenol and salbutamol reduced FGF-2 secretion from HDMECs whereas salbutamol increased whilst timolol reduced FGF-2 secretion from HDFs. Overall, β-AR activation reduced the levels of pro-angiogenic growth factor secretion from HDMECs and HNKs while β-AR blockade increased pro-angiogenic growth factor secretion from HNKs.

It was important to determine whether the β-ARs could regulate HDMEC function and angiogenesis in more complex environments. *Ex vivo* and *in vivo* angiogenic models such as the 2D tubule assay, the rat aortic ring assay and the CAM assay, more closely resemble the kind of environment that exists within the body in which many cell types and biological factors are present that can also regulate angiogenesis. Although, *in vitro* migration and proliferation studies found no roles for β-AR antagonists, *ex vivo* and *in vivo* angiogenesis models revealed anti- or pro-angiogenic roles for β-AR antagonists. One hypothesis for this was that β-ARs could be altering angiogenesis by modulating the paracrine secretion of pro-angiogenic growth factors from other cell types which, in turn, act on HDMECs to regulate angiogenesis.

VEGF-A ELISA studies revealed that β-AR blockade by timolol increased VEGF-A secretion from HNKs. Therefore, perhaps the β-ARs mediate their pro-angiogenic mechanism *in vivo* by increasing the paracrine secretion of VEGF-A from other cell types such as keratinocytes. Furthermore, β-AR antagonism may be preventing the autocrine/paracrine action of noradrenaline/adrenaline. On the other hand, timolol could be activating specific receptor signalling pathways through inverse agonism or agonism perhaps unrelated to the more classical β-AR signalling pathways such as cAMP. Alternately, the isoproterenol/salbutamol β-AR agonist-mediated reduction in HDMEC migration rate and the isoproterenol β-AR-mediated reduction in HDMEC proliferation rate could play a role in the β-AR-mediated decrease in tubule formation and embryonic angiogenesis *in vivo*. Interestingly, isoproterenol/salbutamol also decreased FGF-2 secretion from HDMECs and VEGF-A secretion from HNKs. Perhaps β-ARs mediate their anti-angiogenic mechanism in more complex environments by modulating the autocrine or paracrine secretion of pro-angiogenic growth factors from HDMECs or other cell types to regulate angiogenesis.

The CAM assay provided the first opportunity to investigate the effects that β-AR agonists and antagonists may have in an *in vivo* environment. As a consequence, further pharmacological considerations have to be taken into account when investigating the effects of β-AR agonists and antagonists in more complex environments. For example, it is more difficult to determine exactly what the ligand concentration is at the site of action, making it difficult to extrapolate such considerations between *in vitro* and *in vivo* studies. Indeed, in whole-animal situations, pharmacokinetic and pharmacodynamic effects become extremely important in regulating concentrations of ligands at their site(s) of action.

It is possible to hypothesise about which β-AR agonist or antagonist would be the best to alter wound angiogenesis in chronic wounding and scarring. However, it is important to note that the pharmacology regarding the selectivity and the potency of the β-AR agonists and antagonists were not carried out for HDMECs. Therefore, β-AR selectivity was not assumed nor potency as this is system/response dependent, see section **6.2, limitations of experimental design**. Therefore, based on the pro or anti-angiogenic functions of the β-AR agonists and antagonists in HDMEC function and angiogenesis from this investigation and from data in the literature, two agents were suggested. Furthermore, based on the complex pharmacology and differences in signalling pathways that different β-AR subtypes may elicit, only selective β-AR agonists or antagonists should be used. The use of selective β-AR agents should reduce un-intentional side effects caused by the activation or inhibition of other β-AR subtype signalling pathways. Partial β-AR agonists should also help in reducing any un-intentional side effects as partial agonist should merely dampen down a particular process and not cause an almost or complete inhibition of a particular process (assuming that agonism is associated with inhibition). However, as mentioned previously in this thesis, partial agonism is system-dependent, therefore one would need to assess whether any selected compounds are partial in the target system. Furthermore, one would also need to assess the extent of agonism on specific signalling pathways and these would have to be clinically/therapeutically relevant ones.

Salbutamol, which is generally considered a partial β-AR agonist with some β_2-AR selectivity (Baker, 2010b) could be used to dampen angiogenesis and decrease scarring. Salbutamol is licensed for use in the USA (Food and Drug Administration

approved agent) and in Europe and is a commonly used anti-asthmatic with a fairly extensive history of use, potentially reducing the cost of development. However it is important to note that some of the clinical/therapeutic selectivity of salbutamol arises from its route of delivery. For example, the lung mainly has β_2-ARs, however, there have been reported side effects including adverse cardiac events even with aerosol administration. Therefore, salbutamol has a long history of use, its side effects are known and there is a lot of safety data available. Taking this in to account, this agent would most likely be best applied topically or though sub-cutaneous injection to limit dissipation of the agent away from the wound site. Although it is important to mention that these methods of drug delivery can also lead to an increase in circulating levels of drug.

On the other hand, ICI 118, 551 is a selective β_2-AR antagonist (Baker, 2005b) and could be used to increase angiogenesis in chronic wounds. This compound is not licensed for use in the USA or Europe and its limited solubility in aqueous solution would be problematic. Therefore, the only other antagonist used in the present study that may have clinical potential in wound healing and that is currently licensed for use is timolol, although the non-selective nature of its β-AR antagonism maybe problematic (Baker, 2005b).

In conclusion, this research strengthens our understanding of the role that the β-ARs play in HDMEC function and angiogenesis, subsequently supporting the development of therapeutic agents to regulate pathological angiogenesis. β-AR agonists could be used to regulate HDMEC-mediated angiogenesis in wound healing. For example, in acute wound healing, decreasing angiogenesis could help reduce scarring (Szpaderska

et al., 2005, van der Veer et al., 2011, Wilgus et al., 2008) whereas enhancing angiogenesis could improve healing in a chronic wound (Falanga, 2005). β-ARs could also be used in the treatment of other pathologies where HDMEC-mediated angiogenesis plays a central role for some types of cancer such as melanoma. Indeed, increasing or decreasing angiogenesis has been shown to alter tumour metastasis (Kerbel, 2008).

6.2 Limitations of experimental design

The focus of this thesis was to determine whether modulation of the β-ARs could alter HDMEC function and angiogenesis, however, an alternative experimental design could have addressed the pharmacology. Although the concentrations of the β-AR compounds used could modulate the β-ARs subtypes, selectivity could not be implied. Therefore, the β-AR mediated effects on HDMEC function and angiogenesis could be through the β_1-AR, β_2-AR or the β_3-AR or any combination of these, see section **3.3 discussion**.

The concentrations of the various β-AR agonists and antagonists used in this investigation to modulate β-ARs were estimated based on published *in vitro* studies. Recent publications on other cell types were used as a guide to understand potency and selectivity. There have been numerous β-AR agonist and antagonist binding studies (Smith and Teitler, 1999, Schnabel et al., 2000, Hoffmann et al., 2004), however the concentration required to elicit receptor responses was estimated from the most recent studies (Baker, 2005b, Baker, 2010b) and based on *in vitro* studies on HNKs (Pullar et al., 2006a, Pullar et al., 2003, Pullar et al., 2006b) and HDFs (Pullar and Isseroff, 2006, Pullar and Isseroff, 2005a).

Isoproterenol demonstrated potency across all β-AR subtypes whereas both salbutamol and formotereol demonstrated selectivity toward β_2-AR (table 6.1). Interestingly, ZD7114 demonstrated no selectivity across the β-AR subtypes on either binding or potency, suggesting similar levels of intrinsic efficacy at each β-AR subtype, see table 6.1 (Baker, 2010b, Baker, 2010a). Timolol showed selectivity for binding to the β_1-AR and β_2-AR (table 6.1). In contrast, ICI 118,551 is highly selective for the

β_2-AR, see table 6.1 (Baker, 2005b, Bilski et al., 1983). Finally, previous work has shown SR59230A to be selective for the β_3-AR (Manara et al., 1996) whilst other research has shown SR59230A to be a potent non-selective antagonist (Candelore et al., 1999). Furthermore, at high β_3-AR expression levels, SR59230A can be a partial agonist (Sato et al., 2007).

β-AR agonist or antagonist	β_1-AR K_D from binding (μM)	β_2-AR K_D from binding (μM)	β_3-AR K_D from binding (μM)	β_1-AR EC_{50} from cAMP (μM)	β_2-AR EC_{50} from cAMP (μM)	β_3-AR EC_{50} from cAMP (μM)	Reference
Isoproterenol	0.870	0.229	3.019	0.002	0.006	0.038	(Baker, 2010b)
Salbutamol	20.892	0.977	104.712	0.615	0.019	0.977	(Baker, 2010b)
Formoterol	0.776	0.002	1.513	0.005	0.00008	0.015	(Baker, 2010b)
ZD7114	0.026	0.048	0.165	0.008	0.022	Site 1: 0.028 Site 2: 2.290	(Baker, 2010b)
Timolol	0.005	0.0002	0.158	N/A	N/A	N/A	(Baker, 2005b)
Atenolol	0.218	1.023	0.158	N/A	N/A	N/A	(Baker, 2005b)
ICI 118,551	0.301	0.0005	0.363	N/A	N/A	N/A	(Baker, 2005b)

Table 6.1: K_D and EC_{50} values in μM of β-AR agonists and antagonists at human β_1-AR, β_2-AR or β_3-AR. K_D and EC_{50} values obtained by using clonal CHO-K1 cell lines transfected either with the human β_1-AR, β_2-AR or β_3-AR. Whole cell [^{3}H]-CGP 12177 radioligand binding and [^{3}H]-cAMP accumulation were measured in response to various β-AR agonists. Alternately, K_D for β-AR antagonist selectivity was obtained using ^{3}H-CGP 12177 in whole cell-binding studies. (Table adapted from both Baker, 2005 and Baker, 2010)

Given the relatively high concentrations of ligands used in the current study it is likely that for the majority, no selectivity can be implied and therefore the data cannot be used to interpret specific β-AR receptor subtypes involved, see section, **3.3 discussion**. Therefore without doing the appropriate pharmacology, see section **6.3, future directions,** non-selectivity was assumed for all β-AR agonists and antagonists at the concentrations used in this investigation. In addition, without direct measurement of

the relevant responses it is not possible to determine whether the agonists used are partial or full agonists at the concentrations used.

6.3 Future directions

This investigation looked at the roles of the β-ARs on regulating various functions of EC physiology *in vitro*. The four main functions of HDMECs that were focussed on were migration, proliferation, tubule formation and growth factor secretion. There are of course many more functions of ECs that contribute to angiogenesis *in vivo* such as alignment, elongation and apoptosis. Future studies could build up a more complete picture of the role of β-ARs in EC function and angiogenesis by investigating additional EC functions. EC alignment is commonly investigated by analysis of proteins in adheren junctions such as VE-cadherin. It would be interesting to see what effect β-AR manipulation would have on VE-cadherin expression and localisation at cell junctions, which could therefore impact on EC junctional integrity. Recent research has highlight the importance that apoptosis plays in angiogenesis, particularly in tubule formation (Segura et al., 2002). Therefore it would be curious to see how β-AR modulation would alter caspase activity, particularly that of caspase 3 which has been linked to microvessel density (Volm et al., 1999).

Further pharmacological studies in HDMECs could be designed to enable more precise identification of the β-AR subtypes involved in the various responses. For example, radioligand assays could be used which utilises a radioactively labelled ligand that can associate with a site of interest, such as a receptor allowing for the measurement of the amount and speed of binding. This in turns gives useful information such as the number of binding sites, ligand affinity and ligand accessibility to the binding sites (Bylund and Toews, 1993, Kenakin, 2004). This information could explain why activation of certain subtypes of the β-ARs may have more or less of an effect due to

differing populations of β-AR subtypes in HDMECs. Furthermore, the use of a variety of ligand concentrations and agonist/antagonist combinations in the experimental protocols could potentially help to tease out the receptor sub-types involved. Finally, genetic knock-out techniques could aid in determining which β-AR subtypes and signalling proteins are involved in the observed changes in HDMEC migration and proliferation. The knock-out of β_1-AR, β_2-AR, G proteins and EPAC could be used to further confirm the signalling pathways behind the β-AR mediated anti-motogenicity and mitogenicity. Indeed, there are already β_1-AR$^{-/-}$ mice, β_2-AR$^{-/-}$ mice and β_1-AR$^{-/-}$, β_2-AR$^{-/-}$ and β_3-AR$^{-/-}$ mice (Chruscinski et al., 1999, Jimenez et al., 2002, Mizobe, 2000), that could be exploited in such studies.

This investigation revealed that β-AR agonist and antagonist treatment altered the secretion of VEGF-A and FGF-2 from HDMECs, HNKs and HDFs. However, it is likely that other cell types and pro-angiogenic growth factors in the wound contribute to the β-AR agonist or antagonist-mediated promotion or inhibition of angiogenesis. One way to test this would be to use conditioned media, prepared from control cells and β-AR agonist or antagonist treated HDMECs, HNKs, HDFs, neutrophils and macrophages. Their relative contributions to the β-AR agonist or antagonist-mediated increase or inhibition in angiogenesis could be tested using various angiogenic models such as 2D tubule assay, aortic ring assay and the CAM assay. Any conditioned media that promotes or inhibits angiogenesis could then be tested by ELISAs for all the known angiogenesis promoting growth factors including VEGF-A, FGF-2, PDGF and the TGF-β family, using Duo set ELISA kits (R & D systems). In addition, a TransSignal angiogenesis antibody array (Panomics (http://www.panomics.com)) could be used to detect an even larger range of pro and anti-angiogenic growth factors from HDMECs, HNKs and

HDFs. This would enable the detection of pro-angiogenic factors such as VEGF, PIGF, G-CSF, HGF, FGF-1, FGF-2, TNF-α, TGF-α, Leptin, Ang, IL-1α, IL-1β, IL-6 and IL-8. Various anti-angiogenic factors could also be detected such as IFN-γ, IL-12, interferon gamma-induced protein-10, TIMP-1 and TIMP-2.

The diabetic mouse model (db/db) could be used to study the effect of β-AR agonists and antagonists on angiogenesis in a chronic wound healing model *in vivo*. Previous studies have only looked at β-AR modulation of angiogenesis in an acute wound healing model *in vivo* (Romana-Souza and Monte-Alto-Costa, 2009, Romana-Souza et al., 2010a, Romana-Souza et al., 2010b, Romana-Souza et al., 2006, Romana-Souza et al., 2009). Indeed, our laboratory has recently found a role for the β_2-AR in regulating angiogenesis in an acute mouse-wound model. β_2-AR antagonism increased angiogenesis, five days post-wounding, in an excisional mouse wound model *in vivo* (Pullar et al., 2012). In contrast, salbutamol reduced angiogenesis in the mouse excisional wound model, five days post-wounding (Pullar, personal communication). The db/db mouse has an autosomal recessive mutation resulting in abnormal leptin receptors causing hyperglycaemia and obesity, resulting in symptoms which resemble that of type II diabetes mellitus (Chen et al., 1996, Hummel et al., 1966, Sullivan et al., 2004). Therefore due to the physiology of the db/db mice, wounding is chronic and comparable to that of humans. Studies could involve creating a dorsal neck incision, then applying daily topical applications of hydrogel alone or hydrogel containing β-AR agonist or antagonist treatments. After a given number of days, the wounds could be biopsied and studied histologically using specific angiogenic markers such as platelet EC adhesion molecule-1 (Sullivan et al., 2004). Therefore the db/db mouse may

represent a novel model for investigating angiogenesis in a chronic wound environment *in vivo*.

In conclusion, future work could utilise genetic and pharmacological studies to add depth to the isoproterenol/salbutamol β-AR-mediated signalling pathways that regulate HDMEC migration and proliferation. In addition, the role of the β-AR family on wound angiogenesis could be explored in chronic mouse wound models. These data would provide novel insights into how the β-ARs could be used to alter angiogenesis in wound healing model and disease states, such as a chronic wound. Finally porcine models would close the gap between the experimental stages and clinical trials in human diseases such as chronic wounds, fibrosis and cancers such as melanoma.

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Images

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