The Role of Sphingosine Kinases and SKAM1 in Cutaneous Wound Healing

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Statement of Authorship

Chan H and Pitson SM (2013). Post-translational regulation of sphingosine kinases.

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Huasheng Chan: Wrote the article.

I hereby certify that the statement of contribution is accurate

Signed: *Date:*

Stuart M. Pitson: Prepared figures and edited contribution from the other author.

I hereby certify that the statement of contribution is accurate and I give permission for the inclusion of this paper in the thesis

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Abstract

Sphingosine kinases (SKs) phosphorylate sphingosine to generate the bioactive lipid sphingosine 1-phosphate (S1P). SKs and S1P regulate a diverse range of cellular processes, including cell proliferation, survival, differentiation, migration, smooth muscle cell contraction, inflammation, cytoskeleton reorganisation and angiogenesis, mainly via the engagement of S1P to a family of five S1P-specific G protein-coupled receptors (GPCRs). As such, the SKs and S1P are involved in regulating a plethora of cellular processes that are known to be fundamental to wound healing.

The role of SK and S1P in cancer and other diseases including asthma, hypertension, atherosclerosis and allergy are well established. Notably, however, the direct role of the SKs and S1P in wound healing has not been previously examined in any detail. My studies sought to fill this gap in knowledge. Using a well-established mouse model of incisional wound healing, I have shown that $SK1^{-/-}$, $SK2^{-/-}$, $SK1^{-/-}$ $SK2^{+/-}$ mice healed at a slower rate compared to wildtype mice. This may be attributed to a decrease in cellular proliferation in the early steps of wound repair. These studies highlight the importance of SKs in the very complex process of wound healing. My studies also examined the role of a relatively uncharacterised protein, fibroblast growth factor receptor-1 oncogenic partner 2 (FGFR1OP2), in wound healing. FGFR1OP2 is a protein that was identified from a yeast two-hybrid screen for SK1 interacting proteins. Unpublished work performed from the Pitson laboratory has shown that FGFR1OP2 can interact and activate SK1 in cells and *in vitro*. As such, we have more appropriately named this protein SKAM1 (Sphingosine Kinase Activating Molecule 1). SKAM1 has previously been reported to be upregulated following tooth extraction in rat oral mucosa. The Pitson laboratory has shown that

overexpression of SKAM1 induced collagen matrix contraction, an *in vitro* model of wound contraction, was mediated by SK1 and the S1P receptors, $S1P_{1/3}$.

Using a number of classical *in vitro* models of wound healing, I found that overexpression of SKAM1 in NIH3T3 fibroblasts did not affect cellular migration and proliferation. Notably, however, NIH3T3 fibroblasts overexpressing SKAM1 were resistant to serum deprivation-induced apoptosis. We also generated SKAM1 transgenic mice, where SKAM1 was ubiquitously expressed, to study the role of this protein in wound healing *in vivo*. We found no observable phenotypical difference between SKAM1 transgenic and wildtype mice at 12 and 48 weeks of age. Primary mouse embryonic fibroblasts (MEFs) isolated from SKAM1 transgenic embryos showed enhanced ability to contract collagen matrix compared with the wildtype. Somewhat surprisingly, the rate of wound healing following incisional wounding was similar between SKAM1A transgenic and wildtype mice. Notably, however, SKAM1A transgenic mice showed enhanced wound resolution compared with the wildtype following full-thickness excisional wounding. In addition, SKAM1 gene-trap mice with conditional potential have also been successfully generated and provide a tool for the study of the effect of SKAM1A knockout in wound healing *in vivo*.

The Pitson laboratory previously showed that a 35 amino acid peptide of SKAM1, $SKAM1^{71-105}$, can surprisingly still activate SK1 and enhance collagen matrix contraction. Further to this I have shown that a 30 amino acid cell-permeable peptide of SKAM1, TAT-$SKAM1^{76-105}$, was able to directly activate recombinant SK1 *in vitro* and when applied to cells. Notably, this effect was blocked by a mutant version of this peptide Tyr104→Phe mutation. Furthermore, NIH3T3 fibroblasts treated with TAT-$SKAM1^{76-105}$ showed enhanced collagen contraction, and more

importantly, intradermal injection of TAT-SKAM1A$^{76\text{-}105}$ into full-thickness excisional wounds resulted in enhanced wound resolution in mice. Neither of these effects was observed with the mutant peptide. Taken together, my findings suggest a potential therapeutic use of this peptide for the enhancement of wound repair.

In summary, my findings have demonstrated for the first time a novel role of SK and SKAM1 in wound healing. Knowledge gained from this study will be valuable for the development of potential new therapeutics for the improvement of wound healing.

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List of abbreviations

ABC	ATP binding cassette
ALX	Alloxan
AML	Acute myeloid leukaemia
Ang1	Angiopoietin 1
APN	Australian phenomics network
BrdU	5-bromo-2-deoxyuridine
BSA	Bovine serum albumin
BVDV	Bovine viral diarrhoea virus
CIB1	Calcium- and integrin-binding protein
CMV	Cytomegalovirus
CPP	Cell permeable peptides
CTGF	Connective tissue growth factor
DAPI	4', 6-diamidino-2-phenylindole
DMEM	Dulbecco's modified eagle medium
DMS	*N*, *N*-dimethylsphingosine
DTT	Dithiothreitol
EB	Extraction buffer
ECL	Enhanced chemiluminescence
ECM	Extracellular matrix
eEF1A	Eukaryotic elongation factor 1A
EGF	Epidermal growth factor
EGFP	Enhanced green fluorescent protein
ER	Endoplasmic reticulum
ERK	Extracellular regulated kinase

ES	Embryonic stem
FCS	Fetal calf serum
FDA	Food and drug administration
FGF	Fibroblast growth factor
FGFR	Fibroblast growth factor receptor
FGFR1OP2	Fibroblast growth factor receptor-1 oncogenic partner 2
FITC	Fluorescein isothiocyanate
GAPDH	Glyceraldehyde phosphate dehydrogenase
GPCR	G protein-coupled receptor
GST	Glutathione-S-transferase
H/E	Haematoxylin and eosin
HDAC	Histone deacetylase
HIV	Human immunodeficiency virus
HPLC	High performance liquid chromatography
IGF	Insulin-like growth factors
IGTC	International gene trap consortium
IL	Interleukin
IP	Intra-peritoneal
IRES	Internal ribosomal entry site
JNK	c-Jun N-terminal kinase
MEF	Mouse embryonic fibroblast
MMP	Matrix metalloproteinases
NOD	Non-obese diabetic
OPA	O-phthalaldehyde
PA	Phosphatidic acid

PBS	Phosphate buffered saline
PCNA	Proliferating cell nuclear antigen
PDGF	Platelet-derived growth factor
PHB	Prohibitin
PI3P	Phosphatidylinositol-3-phosphate
PKA	Protein kinase A
PKC	Protein kinase C
PLC	Phospholipase C
PP2A	Protein phosphatase 2A
PS	Phosphatidylserine
PTD	Protein transduction domain
PTEN	Phosphatase and tensin homolog deleted on chromosome 10
PTI-1	Prostate tumour inducer-1
PTK	Protein tyrosine kinase
rSK	Purified recombinant SK
S1P	Sphingosine 1-phosphate
$S1P_{1-5}$	Sphingosine 1-phosphate receptors 1-5
S1PP	Sphingosine 1-phosphate phosphatase
SDS	Sodium dodecyl sulphate
SIKE	Suppressor of IKappa kinase ε
SK	Sphingosine kinase
SKAM	SK activator molecule
SKi	2-(*p*-hydroxyanilino)-4-(*p*-chlorophenyl)thiazole
SKIP	SK interacting protein

SMA	Smooth muscle actin
SMP	Skim milk powder
Sph	Sphingosine
STZ	Streptozotocin
TASQ	Transgenic animal service of Queensland
TAT	Transactivator of transcription
TF	Tissue factor
TGF	Transforming growth factor
TIMP	Tissue inhibitor of metalloproteinases
TLC	Thin-layer chromatography
TNF	Tumour necrosis factor
TRAF	TNF receptor-associated factor
VCAM	Vascular cell adhesion molecule
VEGF	Vascular endothelial growth factor
vWF	Von Willebrand factor
Wit3.0	Wound inducible transcript of 3.0kb

CHAPTER 1

General Introduction

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See **Appendix 1** for author contributions

1. General introduction

1.1 Impaired wound healing: a significant health burden

Impairments in wound healing continue to pose a significant worldwide clinical burden and cause a high degree of morbidity and mortality (Sen, 2009; Sen et al., 2009). Aberrant wound healing response to cutaneous injury following disease, trauma and surgery often results in significant complications such as chronic infections, problematic scars and amputation (Ariyaratnam et al., 2010; Moxey et al., 2012). Problems that arise from these complications are not just limited to long-term physical and psychological trauma to the individual but also extend outwards and place a significant burden on families and communities for treatments (Van Loey and Van Son, 2003). In addition, burn injury is the most common household injury and results in millions of people worldwide acquiring burn scars each year, a large proportion of which occur in children (Peck, 2011). More importantly, the sharp rise in prevalence of diabetes and obesity have lead to increases in secondary complications including chronic, non-healing wounds [reviewed in (Tsourdi et al., 2013)]. Notably, as many as 15% of patients with both type-I or type-II diabetes can be expected to develop a non-healing wound, which in most cases results in amputation (Margolis et al., 2005). It is clear that wound healing complications can lead to long term economic impact, including loss of wages, constant health care cost and loss of skills as a result from scarring deformity and amputation (Margolis et al., 2005). Unfortunately, current wound healing treatments including garments, dressings, culture-based antibiotic treatment, topical growth factor/cytokine application or autologous skin transplantation are sub-optimal (Tsourdi et al., 2013). Therefore, the identification of new alternative therapies for the improvement of wound healing is of significant current need.

1.2 The process of cutaneous wound healing

Wound healing is a highly complex but coordinated process which restores the skin following injury. In normal skin, the epidermis and dermis act as a protective barrier against the external environment and in the event that this barrier is breached, the systematic physiological process begins to re-establish the integrity of the protective barrier [reviewed in (Velnar et al., 2009)]. The coordination of multiple cellular processes is a very delicate process, which ultimately determines the resultant scar formation from an unnoticeable hairline graze to keloid or hypertrophic scar contractures.

The restoration process is highly complex at the molecular level but can be divided into three distinct but interrelated phases consisting of: 1) inflammatory phase, 2) proliferative phase and 3) remodelling and resolution phase (Koopmann, 1995; Thomas et al., 1995; Yamaguchi and Yoshikawa, 2001). Each of these phases are discussed below.

1.2.1 Inflammatory phase

The inflammatory response occurs within minutes of the initial injury and can generally last up to several days [reviewed in (Artlett, 2013; Eming et al., 2007)]. Briefly, immediately after injury, the release of tissue factor (TF) results in the formation of the prothrombin activator complex which in turn cleaves inactive prothrombin to produce thrombin. Fibrinogen in the plasma is then cleaved by thrombin to produce fibrin. Fibrin monomers then polymerise with each other to form a scaffold which in turn binds directly to platelets to form a clot. The formation of the fibrin clot is integral in the early phase of wound healing as it serves as a provisional matrix which binds to a number of key inflammatory cell types including monocytes,

neutrophils and fibroblasts via their cell surface integrin receptors [reviewed in (Mosesson et al., 2001; Reinke and Sorg, 2012)]. Fibrin can also interact with several growth factors and cytokines including fibroblast growth factors (FGF) and insulin-like growth factors (IGF), which affects cellular migration and proliferation, as well as extracellular matrix (ECM) production. Concurrently with the formation of a clot, platelets are also activated by thrombin upon injury to undergo degranulation, releasing a range of growth factors vital in wound healing, including platelet-derived growth factor (PDGF), transforming growth factor (TGF) and epidermal growth factor (EGF) [reviewed in (Monaco and Lawrence, 2003)]. In addition, platelets can also stimulate vasodilation and increase vascular permeability to allow inflammatory cells to enter the wound (Singer and Clark, 1999).

Among all infiltrating leukocytes, neutrophils are the first population of inflammatory cells that arrive at the site of injury to remove cell debris and to combat invading pathogens (Eming et al., 2007). Upon arrival, neutrophils secrete a number of proinflammatory cytokines including interleukin (IL) -1α, IL-1β, IL-6 and tumour necrosis factor (TNF)-α. These factors help to recruit circulating monocytes into the wound site where they are activated and subsequently differentiate into macrophages (Koh and DiPietro, 2011). These macrophages then help to remove foreign materials via phagocytosis. Macrophages also produce a range of chemoattractants, cytokines, growth factors, fibronectin, elastin and complement factors to perpetuate the wound healing process [reviewed in (Eming et al., 2007)].

One of the most important cytokines released by the macrophages is TGF-β, which stimulates dermal fibroblasts to differentiate into myofibroblasts (Artlett, 2013). The myofibroblasts then produce collagen which forms part of the provisional

extracellular matrix (Gabbiani, 2003). The migration of fibroblasts into the wound space marks the beginning of the proliferative phase in wound healing.

1.2.2 Proliferative phase

The main objective in the proliferative phase is to restore tissue integrity. Fibroblasts are integral in this process for a number of reasons. Firstly, proliferation and migration of fibroblasts into the wound result in regeneration of new skin tissue (Reinke and Sorg, 2012). Secondly, wound-associated fibroblasts produce collagen based ECM that eventually replaces the fibrin matrix, thus regulating both growth and function of other cell types in the wound (Li and Wang, 2011). Thirdly, differentiated fibroblasts, known as myofibroblasts, are also responsible for the generation of contractile forces that bring the wound margins together via a process known as wound contraction (Darby et al., 2009).

Fibroblasts migrate into the wound by navigating along the provisional matrix. Thus, it is important to note that the arrangement of ECM in wound healing is crucial, with many problematic scars thought to be a consequence of disorganised ECM arrangement (Gabriel, 2011). The interactions between fibroblasts and the ECM are mediated by cell surface integrins that bind to several matrix components such as fibrin and fibronectin. IL-1 and TNF-α production by macrophages also induces the production of matrix metalloproteinases (MMP) that clear away damaged ECM and inflammatory debris, which then allows the migration of proliferative fibroblasts into the wound site. Initiation of permanent ECM production begins at the arrival of fibroblasts into the wound space. The production of new ECM by fibroblasts results in granulation tissue formation which then serves as a template for tissue growth and formation of myofibroblasts [reviewed in (Desmouliere et al., 2005)].

Upon TGF-β1 stimulation, fibroblasts differentiate into myofibroblasts which are the major contributor to extracellular matrix deposition. These specialised fibroblasts acquire some characteristics of smooth muscle (SM) cells, including the expression of alpha-smooth muscle actin (α-SMA) and the ability to generate mechanical forces which result in lamellipodia formation and subsequent wound contraction [reviewed in (Weber et al., 2012)].

One of the critical steps in wound healing is angiogenesis [reviewed in (Tonnesen et al., 2000)]. This is a process where new blood vessels are formed from neighbouring intact capillaries. Various growth factors and cytokines, including vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), angiopoietin and TGF-β are involved in this process. Interestingly, similar to the migration of fibroblasts into the wound, endothelial progenitor cells also use the ECM to migrate into the wound to initiate and assist in revascularisation (Tonnesen et al., 2000).

Re-epithelialisation is a process where epithelial cells migrate from the wound edge into the wound surface, which ultimately results in re-establishment of an intact epidermis [reviewed in (Reinke and Sorg, 2012)]. Keratinocytes are integral in this process. Keratinocytes migrate along the granulation tissue via ECM and this process continues until opposite ends of the migrating keratinocytes come in contact. Notably, rapid re-epithelialisation is desirable as it leads to restoration of the skin's function and prevention of further infection.

1.2.3 Remodeling and resolution phase

Successful repair after tissue injury requires resolution of the inflammatory response (Ortega-Gomez et al., 2013). While the molecular mechanisms underlying

the inflammatory response are well-defined, mechanisms that limit and down regulate this activity remained unclear. A number of early studies, however, have shown that resolution of inflammation appears to involve the upregulation of anti-inflammatory cytokines such as IL-10 and TGF-β1 (Sato et al., 1999; Werner and Grose, 2003), or upregulation of cytokine receptor antagonists (Eming et al., 2007).

Collagen remodelling also plays an important role at the final stages of wound healing [reviewed in (Ehrlich and Krummel, 1996)]. The remodelling phase involves the re-organisation of the tissue matrix, including the removal of fibronectin and hyaluric acid and the replacement by organised ECM framework composed of collagens. The degradation of initial matrix and the synthesis of new ECM are tightly regulated by MMPs (Zitka et al., 2010) which are in part regulated by various growth factors and cytokines, including PDGF, IL-1 and TGF-β (Werb et al., 1990). The regulation of new collagen matrix requires a fine balance between collagen production, breakdown and remodelling. Over time, the proportion of collagen I content in the granulation tissue increases and this corresponds to the decrease in collagen III until it returns to the basal collagen I to collagen III ratio of 90% : 10% in unwounded skin (Ehrlich and Krummel, 1996).

In human skin, collagen maturation occurs at approximately 7-10 days after injury where the deposited collagen fibres are re-organised into thick bundles parallel to the skin, correlating with increased tensile strength. Notably, scar formation is an inevitable outcome of adult wound healing. This is due to excessive production of collagen by myofibroblasts during the final remodelling phase (Li and Wang, 2011).

While the cells and cytokines involved in wound healing are now well defined, the molecular regulation of these complex processes remain poorly understood. As a consequence, the examination of the cell signalling pathways that

regulate wound healing and the identification of the molecular targets for therapeutic exploitation are of significant current need.

1.3 Sphingosine kinase and sphingosine 1-phosphate: new potential mediators of wound healing

1.3.1 SK and S1P in cellular signalling

The sphingosine kinases (SKs) and their lipid product sphingosine 1-phosphate (S1P) are involved in regulating a wide variety of cellular processes that are known to be fundamental in wound healing. Therefore, it raises the possibility that these enzymes may be important mediators of wound healing. This signalling pathway is a major focus of this thesis and is discussed in detail below.

The SKs are intracellular enzymes that catalyse the phosphorylation of sphingosine to generate the bioactive lipid S1P. S1P is involved in a diverse array of cellular processes including cell proliferation, apoptosis, calcium homeostasis, angiogenesis, vascular and neuronal maturation, cell migration and immune responses (Chan and Pitson, 2013; Hannun and Obeid, 2008; Maceyka et al., 2009; Pitson, 2011; Pyne and Pyne, 2010; Strub et al., 2010). Notably, many of these processes are critical aspects of wound healing, raising the hypothesis that the SK/S1P signalling axis may be important in wound healing. This hypothesis was examined in this thesis.

Two SKs exist, SK1 and SK2, that catalyse the same reaction but have at least some different roles and regulation. The roles and regulation of SK1 and SK2 are described in detail in section 1.4.

1.3.2 S1P signalling

S1P is generated in the cell and can either act as an intracellular second messenger (Strub et al., 2010) or signal extracellularly through a family of S1P-specific cell surface G protein-coupled receptors (Rosen et al., 2009). While the intracellular targets and roles of S1P are only now being elucidated, much more is known about the pathways downstream of the cell-surface S1P receptors.

1.3.2.1 Extracellular signalling of S1P

S1P can be exported from cells via ATP binding cassette (ABC) transporters (Strub et al., 2010) and spinster 2 (Spns2) (Mendelson et al., 2014). Extracellular S1P can regulate a number of cellular processes via the five cell surface S1P receptors, named $S1P_{1-5}$ (Rosen et al., 2009). These processes include cell proliferation, migration and survival (Sanchez and Hla, 2004). Since S1P receptors have differential tissue expression and are coupled to various G proteins, it is not surprising that these S1P receptors have the ability to differentially regulate a range of cell- or tissue-specific responses. The signalling function of each S1P receptor is discussed below.

$S1P_1$

In mammals, $S1P_1$ is expressed in all tissues (Aarthi et al., 2011). Interestingly, in addition to the plasma membrane, $S1P_1$ is also localised in various cellular compartments including the cytoplasmic vesicles, nucleus, and the perinuclear region (Rivera et al., 2008). $S1P_1$ couples only to G_i proteins to activate a number of signalling pathways, including ERK1/2 to enhance cell proliferation, Akt to enhance cell survival, and Rac to enhance migration (Fig. 1.1) (Kihara et al., 2007; Meyer zu Heringdorf and Jakobs, 2007). $S1P_1$ is required for lymphocyte egress from

the lymph nodes (Matloubian et al., 2004) since T-lymphocytes in mice lacking $S1P_1$ failed to egress from lymphoid organs (Allende et al., 2004a). In addition, $S1P_1$ is vital for vascular maturation and angiogenesis since $S1P_1$ knockout mice died *in utero* due to haemorrhage (Liu et al., 2000b). $S1P_1$ is also involved in transmission of nerve pulse (Meng and Lee, 2009), brain development (Dunlap et al., 2010) and mast cell responses [reviewed in (Aarthi et al., 2011)].

$S1P_2$

Like $S1P_1$, $S1P_2$ is also expressed in all tissues and couples to G_i (Aarthi et al., 2011) and therefore can stimulate ERK1/2 to induce cellular proliferation and cell survival (Blom et al., 2010). Apart from ERK1/2, $S1P_2$ has also been shown to be involved in other MAPK signalling pathways, including JNK and p38 MAPK, and therefore plays a role in cell stress, inflammation and apoptosis (Donati et al., 2005; Goparaju et al., 2005; Michaud et al., 2010). In addition to G_i, $S1P_2$ also couples to G_q and G_{12} proteins to activate Rho and inhibit Rac to promote stress fibre formation and inhibit cell migration (Meyer zu Heringdorf and Jakobs, 2007) (Fig. 1.1). Interestingly, $S1P_2$ has also been shown to inhibit proliferation in some cells, including hepatocytes (Ikeda et al., 2003) and keratinocytes (Schuppel et al., 2008). Previous studies have demonstrated that overexpression of $S1P_2$ impaired S1P-induced migration, wound healing and morphogenetic responses in senescent endothelial cells (Estrada et al., 2008). Furthermore, the same study has also shown that siRNA knockdown of $S1P_2$ or expression of dominant-negative PTEN (phosphatase and tensin homolog deleted on chromosome 10) restored functional impairments, suggesting that the impaired functions of senescent endothelial cells is

mediated through $S1P_2$ signalling and activation of the lipid phosphatase PTEN (Estrada et al., 2008).

$S1P_2$ knockout mice display a number of phenotypic abnormalities. They have high incidence of clonal B-cell lymphomas at old age and this appears to be due to the disruption of B-cell homeostasis in the germinal centre (Cattoretti et al., 2009). In addition, mice lacking $S1P_2$ also develop epileptic seizures and deafness, suggesting an important role of $S1P_2$ in the development of the auditory and vestibular systems (Herr et al., 2007). More importantly, $S1P_2$ knockout mice displayed impairment in hepatic wound healing following acute liver injury due to decreased myofibroblast proliferation and reduced hepatic matrix remodelling (Serriere-Lanneau et al., 2007).

$S1P_3$

$S1P_3$ is ubiquitously expressed and is predominantly localised in the plasma membrane (Dolezalova et al., 2003). Like $S1P_2$, $S1P_3$ also couples to G_i, G_q and G_{12} proteins (Davis and Kehrl, 2009) (Fig. 1.1). Although $S1P_3$ is coupled to the same classes of G proteins, it appears to regulate different signalling pathways to $S1P_2$ since it activates ERK1/2 , phospholipase C (PLC), Akt, Rho and Rac, and in some cases appear to have similar signalling effects to $S1P_1$ (Aarthi et al., 2011; Meyer zu Heringdorf and Jakobs, 2007; Pebay et al., 2007). Notably, $S1P_3$ couples most efficiently to G_q to stimulate PLC which results in the production of inositol 1,4,5-triphosphate and subsequent release of Ca^{2+} from internal stores and activation of protein kinase C [reviewed in (Aarthi et al., 2011)]. In addition, a number of studies have shown that $S1P_3$ signalling via the G_{12} and Rho pathway is pro-fibrotic and is involved in a number of fibrotic diseases, including cardiac and liver fibrosis (Keller et al., 2007; Li et al., 2009a; Li et al., 2009b). $S1P_3$ has been shown to be integral for

neurogenesis (Harada et al., 2004) and the expression of α-SMA after arterial injury (Grabski et al., 2009).

Knockout of $S1P_3$ alone does not result in any obvious phenotype (Kono et al., 2004). However, triple-knockout of $S1P_{1-3}$ leads to embryonic lethality due to severe vascular defects which appears worse than those resulting from $S1P_1$ knockout alone, suggesting that these three receptors have redundancy and cooperative functions for normal vascular development during embryogenesis (Kono et al., 2004).

$S1P_4$ and $S1P_5$

Unlike $S1P_{1-3}$, expression of $S1P_4$ and $S1P_5$ are more tissue specific [reviewed in (Aarthi et al., 2011)]. $S1P_4$ is expressed primarily in lymphoid tissues and leukocytes whereas $S1P_5$ is predominantly distributed in brain, skin and natural killer cells (Aarthi et al., 2011). Not much is known about the G proteins that couple to $S1P_4$ and $S1P_5$. A few studies, however, have shown that both $S1P_4$ and $S1P_5$ appear to be coupled to G_i and G_{12} proteins (Malek et al., 2001; Van Brocklyn et al., 2000; Yamazaki et al., 2000) (Fig. 1.1). Interestingly, although coupling to the same classes of G proteins, $S1P_4$ and $S1P_5$ appear to elicit different signalling function. Signalling via $S1P_4$ leads to the activation of ERK1/2, PLC, adenylyl cyclase, Rho and Rac to induce cell proliferation, cytoskeleton rearrangements and cell rounding (Anliker and Chun, 2004). $S1P_5$ signalling, however, elicits antagonistic effects to $S1P_4$ since it inhibits ERK1/2 to decrease cell proliferation (Novgorodov et al., 2007). Consistent with this, a later study has also demonstrated an anti-proliferative effect of $S1P_5$ using oesophageal cancer cells (Hu et al., 2010).

$S1P_4$ knockout mice have impaired T-cell function, dendritic cell migration and T_{17}-helper cell differentiation (Schulze et al., 2011), suggesting an integral role of

$S1P_4$ in the adaptive immune system. Mice lacking $S1P_5$ displayed defective natural killer cell egress from lymph nodes and chemotaxis to the sites of inflammation, highlighting the importance of $S1P_5$ in the innate immune response (Walzer et al., 2007).

1.3.2.2 Intracellular signalling of S1P

Apart from acting on receptors located on the plasma membrane, S1P can also function inside the cell to regulate a number of cellular responses including cellular proliferation, survival, epigenetic regulation and mitochondrial function (Maceyka et al., 2012). These effects of S1P are regulated via the interaction between S1P and a number of intracellular targets which have only been recently identified.

The role of S1P in epigenetic regulation was demonstrated in a study where intracellular S1P has been shown to interact directly with and inhibit histone deacetylases-1 and 2 (HDAC-1 and -2), enhancing histone acetylation thereby promoting transcription of the transcriptional regulator c-fos and cyclin-dependent kinase inhibitor p21 (Hait et al., 2009). In addition, S1P has also been identified as a cofactor for the ubiquitin ligase activity of TNF receptor-associated factor 2 (TRAF2) (Alvarez et al., 2010). Binding of S1P to TRAF2 increases its activity against IκB leading to subsequent activation of the NF-κB transcription factor to mediate pro-survival signalling. More recently, S1P has also been shown to interact with prohibitin-2 (PHB-2) (Strub et al., 2011), a highly conserved protein that regulates mitochondrial assembly and function (Artal-Sanz and Tavernarakis, 2009). The interaction between S1P and PHB-2 appears to be integral for mitochondria cytochrome-c oxidase assembly and mitochondrial function. Furthermore, S1P has also been shown to interact with the β-site amyloid precursor protein cleaving

enzyme-1 (BACE1), the rate-limiting enzyme for amyloid-β peptide (A_β) production, *in vitro* (Takasugi et al., 2011). Interestingly, the modulation of BACE1 proteolytic activity by S1P has been proposed to be linked to Alzheimer's disease (Maceyka et al., 2012; Takasugi et al., 2011). Recently, a study has shown that S1P can bind directly with the apoptosis inhibitor cIAP2 and mediates IL-1-induced expression of chemotactic factors CXCL10 and CCL5, which help to recruit mononuclear cells to the site of inflammation (Harikumar et al., 2014).

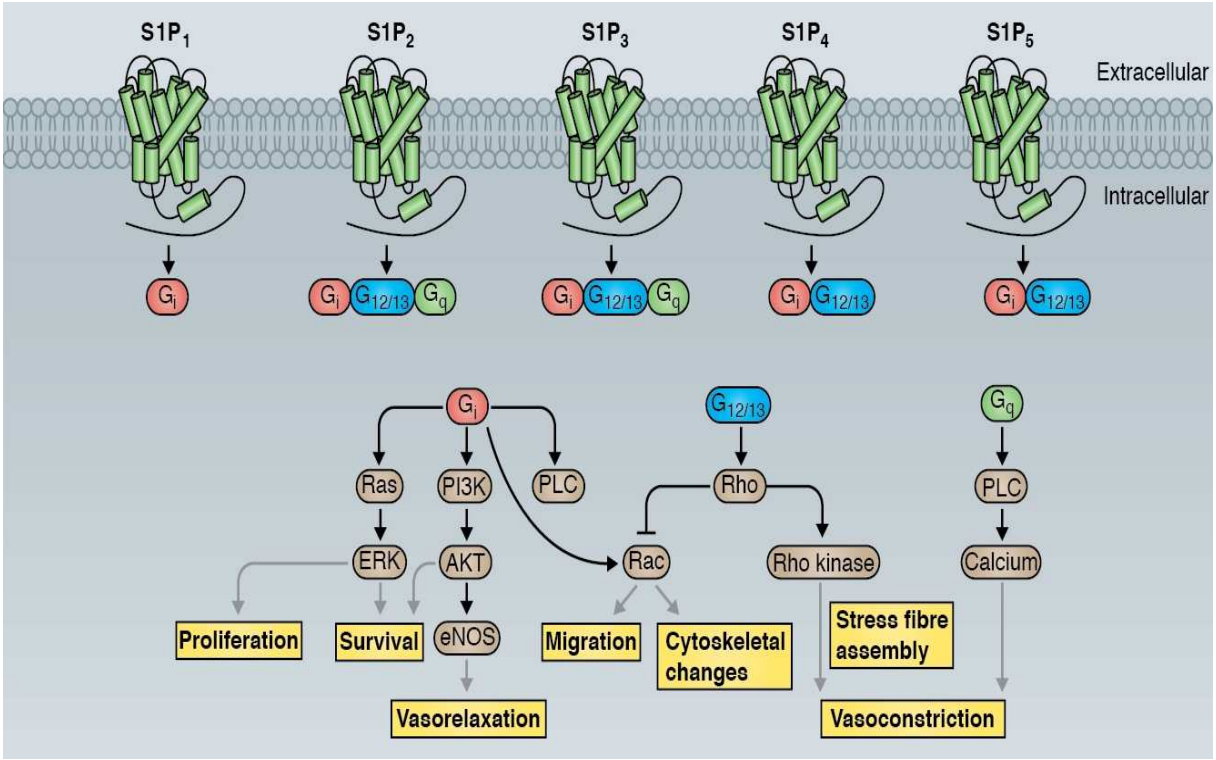

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Fig. 1.1 Signalling of $S1P_{1-5}$

A schematic representation of the signalling network of $S1P_{1-5}$.

1.3.3 SK1 and S1P in wound healing

SK1 and S1P have long been proposed to play a role in wound healing (Francis-Goforth et al., 2010; Watterson et al., 2007). Notably, however, strong experimental evidence to support this notion and details of the molecular mechanisms underlying the regulation of wound healing by SK and S1P remain lacking.

1.3.3.1 S1P in wound healing

Given its role in proliferation, survival, migration and differentiation, particularly in fibroblasts (Aarthi et al., 2011; Pitson et al., 2005), S1P appears to be a potential mediator of tissue repair and wound healing. Indeed, S1P is released by platelets at the site of injury (Lee et al., 2000) and is also enriched in acute wound fluid (Amano et al., 2004), suggesting a potential role of S1P in the process of cutaneous wound healing. Previous studies have shown that the application of S1P to diabetic wounds in mice significantly enhanced wound resolution, possibly due to increased angiogenesis (Kawanabe et al., 2007). Moreover, the wound healing effect of S1P appears to be greater when diabetic wounds were treated simultaneously with an $S1P_2$ antagonist (JTE-013) (Kawanabe et al., 2007), suggesting a negative role of $S1P_2$ in diabetic wound healing. More recently, a study has reported that S1P can enhance fibroblast-mediated collagen matrix contraction which appears to be mediated by the S1P receptors and through Rho and Rac pathways, although the specific S1P receptors involved were not examined in the study (Syuto et al., 2009). Clearly these studies are still in their infancy and further work is required to elucidate the molecular mechanisms underlying S1P-mediated wound contraction.

S1P has also been shown to be involved in the repair of the vasculature after injury. S1P stimulates proliferation and migration of human umbilical vein

endothelial cells and adult bovine aortic endothelial cells, possibly through G_i-mediated ERK1/2 phosphorylation and mobilisation of intracellular Ca^{2+} (Lee et al., 2000). More importantly, the same group has also shown that S1P treatment results in enhanced wound closure of endothelial cell monolayers *in vitro* (Lee et al., 2000). In addition, S1P has also been shown to induce endothelial cell invasion, a key process that is integral in the formation of new blood vessels and wound healing (Su et al., 2008). Notably, S1P-induced endothelial cell invasion appears to be mediated by the cell surface integrin α2β1 and via the activity of matrix metalloproteinases (MMPs) (Bayless and Davis, 2003). A more recent study has demonstrated that the activation of calpain and the subsequent translocation of membrane type-1 matrix metalloproteinase (MT1-MMP) to the plasma membrane are integral for S1P-induced cell invasion (Kang et al., 2011).

Surprisingly, in contrast to its pro-proliferative effect, S1P has been shown to inhibit proliferation of human keratinocytes *in vitr*o (Vogler et al., 2003). The anti-proliferative effect of S1P is not solely mediated by the cell surface G protein-coupled S1P receptors since a similar effect was observed when S1P was microinjected into cells (Vogler et al., 2003), suggesting an intracellular, anti-proliferative role of S1P. In addition, the inhibition of keratinocyte proliferation has been associated with inhibition of Akt/protein kinase B pathway and the activation of ERK (Kim et al., 2004). Interestingly, ERK activation is usually associated with cell proliferation (Torii et al., 2004). However, previous studies have demonstrated that ERK activation can also occur during keratinocyte differentiation (Marcinkowska et al., 1997). Indeed, S1P has also been shown to stimulate keratinocyte differentiation and migration, with both processes crucial for the re-epithelialisation of the cutaneous barrier (Vogler et al., 2003). Interestingly, S1P may help to prevent excessive proliferation of

keratinocytes during wound re-epithelialisation, which has been associated with skin diseases including psoriasis vulgaris (Manggau et al., 2001).

As mentioned above, S1P has also been associated with cellular differentiation. A number of studies have demonstrated that S1P can stimulate fibroblast differentiation into myofibroblasts, the key cell type that is responsible for wound contraction. Human fibroblasts treated with S1P differentiated into myofibroblasts in a dose-dependent manner (Keller et al., 2007). In addition, S1P treatment also substantially increased α-SMA expression, which is characteristic of myofibroblast differentiation. A similar effect was also observed when the cells were treated with FTY720, a therapeutic drug for forms of multiple sclerosis, which following SK2-mediated phosphorylation becomes a structural analogue of S1P (Keller et al., 2007). Notably, myofibroblast differentiation appears to be mediated by $S1P_3$. This notion is supported by the fact that myofibroblast differentiation is blocked by siRNA knockdown of $S1P_3$ and that fibroblasts isolated from $S1P_3$ knockout mice failed to differentiate to myofibroblasts in response to FTY720 treatment (Keller et al., 2007). Consistent with this, S1P can also stimulate human pulmonary myofibroblast differentiation via a Rho kinase-dependent manner (Urata et al., 2005). S1P has also been shown to be involved in synthesis and secretion of collagen. S1P stimulates collagen matrix production by fibroblasts in a comparable manner to TGF-β (Gellings Lowe et al., 2009; Youm et al., 2008). This is not surprising since it has been well established that there exists a ‘crosstalk’ between S1P and TGF-β signalling (Keller et al., 2007; Radeke et al., 2005; Sauer et al., 2004; Xin et al., 2004). Indeed, S1P has been shown to induce activation of the canonical TGF-β signalling pathway in numerous cell types, including dermal fibroblasts, and in some cases this can lead to excessive collagen production and development of tissue fibrosis. Notably, this

process appears to be independent of TGF-β itself, but dependent on S1P receptors. $S1P_{1-3}$ have all been implicated in TGF-β signalling pathway activation by S1P. However, the specific receptor involved appears to be cell type dependent [reviewed in (Shea and Tager, 2012)].

Taken together, it is evident that the SK1/S1P signalling pathway is involved in many of the cellular events that are fundamental to wound healing. Therefore, it is of significant interest to understand how this SK1/S1P axis can be manipulated to improve wound healing.

1.3.3.2 SK in wound healing

It is known that activation of SK1 leads to enhanced cell proliferation and survival, particularly in fibroblasts (Xia et al., 2000), through a diverse range of important pro-proliferative, pro-survival regulatory pathways including mobilisation of intracellular calcium (Mattie et al., 1994), activation of ERK1/2 (Pitson et al., 2000), PI3-K (Song et al., 2011) and NF-κB (Xia et al., 2002), and inhibition of JNK (Cuvillier et al., 1998) and caspases (Edsall et al., 2001). Interestingly, many of the growth factors and cytokines that are released during wound repair, including TNF-α, PDGF and EGF are also known to stimulate SK1 activity (Doll et al., 2005; Olivera et al., 1999a; Pitson et al., 2003). In addition, the involvement of SK1 in cell migration, a critical component of wound healing, has also been demonstrated by numerous studies (Spiegel et al., 2002; Spiegel and Milstien, 2011). Furthermore, SK1 has also been associated with vascular, gastric and airway smooth muscle cell constriction (Ammit et al., 2001; Bolz et al., 2003; Keller et al., 2006; Rosenfeldt et al., 2003; Watterson et al., 2005), a process which has some similarity to wound contraction since it is mediated by α-SMA (Grinnell, 1994). Very recently, a study has shown that

application of an expression plasmid encoding SK1 onto full-thickness excisional wounds significantly enhanced wound resolution in diabetic rats (Yu et al., 2014), suggesting a role of SK1 in wound healing.

1.3.4 SK/S1P in tissue fibrosis

The role of SK and S1P in fibrogenesis has been elucidated by a key study showing that SK1 transgenic mice with ubiquitous overexpression of SK1 (~20-fold higher than endogenous) developed progressive myocardial degeneration and cardiac fibrosis (Takuwa et al., 2010). SK1-induced cardiac fibrosis appears to be mediated through $S1P_3$, Rho/Rac and Smad3 signalling pathways since inhibition of the Rho signalling by pitavastatin or deletion of $S1P_3$ prevented SK1 transgenic mice from developing cardiac fibrosis (Takuwa et al., 2010). Notably, SK1 transgenic mice with cardiac fibrosis also have elevated reactive oxygen species (ROS) which appears to contribute to disease progression (Takuwa et al., 2010).

SK1 has also been shown to play a role in fibrogenesis in the lung. SK1 levels are elevated in lung tissues isolated from patients with idiopathic pulmonary fibrosis (IPF) and this correlates with disease severity and poor prognosis (Huang et al., 2013; Milara et al., 2012). Furthermore, SK1 knockout mice are resistant to bleomycin-induced pulmonary fibrosis (Huang et al., 2013). Similarly, administration of an SK inhibitor, SKi, into mice also attenuated pulmonary fibrosis (Huang et al., 2013), suggesting that SK1 plays a critical role in the pathology of this disease.

A number of studies have shown that S1P plays a role in liver fibrosis via $S1P_{1-3}$. Indeed, S1P levels are elevated in fibrotic liver (Li et al., 2011). Previous studies have shown that $S1P_2$ knockout mice are more resistant to carbon tetrachloride-induced liver injury compared to wildtype (Ikeda et al., 2009).

Moreover, $S1P_2$ deficient mice also showed enhanced liver regeneration following dimethylnitrosamine-induced acute liver injury, suggesting a pathological role of $S1P_2$ in liver fibrosis. Interestingly, S1P/$S1P_3$-mediated homing of bone marrow-derived mesenchymal stem cells to the liver has also been shown to contribute to liver fibrosis (Li et al., 2009b). Consistent with this, a subsequent study showed that transplanted bone marrow cells migrated to the liver of the recipient in response to liver injury (Li et al., 2009a). Notably, siRNA knockdown of $S1P_3$ blocked S1P-induced bone marrow cell migration *in vitro*, suggesting that $S1P_3$ is important for bone marrow cell homing to the liver (Li et al., 2009a). In addition, a more recent study has shown that $S1P_1$ and $S1P_3$ mediate S1P-induced human hepatic myofibroblast (hMF) migration to the liver since siRNA knockdown of both of these S1P receptors markedly reduced migration of hMFs *in vitro* (Li et al., 2011). In contrast, chemical inhibition of $S1P_2$ results in enhanced migration of hMFs *in vitro*, suggesting an anti-migratory role of $S1P_2$ in hMF migration (Li et al., 2011). A recent study has demonstrated that SK1 and S1P are associated with angiogenesis in liver fibrosis (Yang et al., 2013). Indeed, S1P levels in fibrotic liver correlates with the expression of angiogenic and fibrotic markers, including angiopoietin 1 (Ang1), CD-31, vascular cell adhesion molecule-1 (VCAM-1), von Willebrand factor (vWF), and collagen I and III (Yang et al., 2013). Notably, the pro-angiogenic effect of S1P appears to be mediated by $S1P_1$ and $S1P_3$ since simultaneous chemical inhibition of both receptors by an $S1P_{1/3}$ inhibitor, VPC23019, reduced the expression of angiogenic markers (Yang et al., 2013). More importantly, mice treated with VPC23019 and SKi, a SK1 inhibitor, are more resistant to carbon tetrachloride-induced liver fibrosis compared with wildtype controls (Yang et al., 2013). Taken together, these suggest that the SK1/$S1P_{1/3}$ axis plays an integral role in the development of liver fibrosis.

1.4 Post-translation modification of SKs

The regulation of the sphingosine kinases is important for the studies outlined in this thesis. The following published review details current knowledge of this complex and still evolving area.

Chan, H. & Pitson, S.M. (2013). Post-translational regulation of sphingosine kinases. *Biochimica et Biophysica Acta (BBA) – Molecular and Cell Biology of Lipids, v. 1831 (1), pp. 147-156*

1.5 Fibroblast growth factor receptor-1 oncogenic partner 2 (FGFR1OP2): A novel protein involved in wound healing

1.5.1 FGFR1OP2

FGFR1OP2 was first identified as a protein that was aberrantly fused to the FGF receptor-1 in a patient with T-cell lymphoblastic lymphoma (Grand et al., 2004). Chromosomal translocation results in the fusion of the cytoplasmic domain of FGFR1 to residues 1-132 of FGFR1OP2 (Grand et al., 2004). While no experimental evidence was provided by the study, this group proposed that the putative coil-coiled domains of FGFR1OP2 that are retained in the FGFR1-FGFR1OP2 fusion may promote dimerisation of the FGFR1 resulting in its subsequent ligand-independent activation of the receptor, leading to myeloproliferative syndrome (Grand et al., 2004). In a subsequent study, this FGFR1-FGFR1OP2 fusion was also identified in the KG-1 erythroleukaemic myeloid cell line (Gu et al., 2006). Furthermore, FGFR1OP2 has been identified as a component of striatin-interacting phosphatase and kinase (STRIPAK) complex which is involved in various cell signalling processes (Goudreault et al., 2009; Hwang and Pallas, 2014).

A more recent study has shown that the FGFR1-FGFR1OP2 fusion can lead to constitutive activation of FGFR1 (Chase et al., 2007). Furthermore, the same group also showed that the multi-targeted receptor tyrosine kinase inhibitor (TKI258) specifically inhibited proliferation and induced apoptosis of the FGFR1-FGFR1OP2 AML cell lines, KG1 and KG1A, in a dose-dependent manner (Chase et al., 2007). In addition, TKI258 treatment resulted in decreased activation and tyrosine phosphorylation of the FGFR1-FGFR1OP2 fusion protein as well as the downstream signalling molecules STAT5 and ERK (Chase et al., 2007).

While this fusion event involving FGFR1OP2 appears important in some leukemias, it does not appear relevant to the role of FGFR1OP2 in wound healing outlined below.

1.5.2 FGFR1OP2 in wound healing

In a screen for transcripts upregulated after wounding, Sukotjo et al. (2002) examined mRNAs in rat oral mucosal tissue following tooth extraction. A 3.0 kb mRNA was identified, which they termed wound inducible transcript of 3.0kb (wit3.0) (Sukotjo et al., 2002). Two alternative transcripts of wit3.0 were subsequently cloned (wit3.0α and wit3.0β) which have 99% and 100% amino acid sequence identity to alternative splice variants of the human FGFR1OP2 gene (Grand et al., 2004). This suggests that wit3.0 is the rat version of human FGFR1OP2. *In situ* hybridisation showed that wit3.0 mRNA is markedly upregulated in oral fibroblasts adjacent to the extraction site (Sukotjo et al., 2002). This was the first evidence showing that FGFR10P2 may be associated in wound healing in oral mucosa. In subsequent studies using quantitative RT-PCR, the same group showed that wit3.0 mRNA was elevated (up to 50-fold) seven days post-wounding (Sukotjo et al., 2003). Furthermore, overexpression of wit3.0α and wit3.0β in NIH3T3 fibroblasts and rat oral fibroblasts enhanced collagen matrix contraction in a classical *in vitro* model of wound healing (Sukotjo et al., 2003). More recently, Lin et al., (2010) demonstrated that lentiviral-mediated expression of FGFR1OP2 in excisional skin wound significantly facilitated wound closure *in vivo* (Lin et al., 2010). In addition, FGFR1OP2 expression in excisional wounds also results in decreased granulation tissue formation and reduced scarring (Lin et al., 2010). Notably, however, the molecular mechanism underlying FGFR1OP2-mediated wound closure was not

elucidated in the study and remains unknown. More recently, a study has identified a number of chemical compounds that can modulate FGFR1OP2 expression in fibroblasts (Cheng and Nishimura, 2012).

1.6 Biochemistry of FGFR1OP2

While searching for proteins that interact with SK1 by yeast two-hybrid technology (Zebol et al., 2009), a partial FGFR1OP2 cDNA encoding the C-terminal 90 amino acids (51-140) was isolated. Apart from a putative coiled-coil domain, FGFR1OP2 does not show any similarity to other known protein domains. Notably, however, FGFR1OP2 is related to a protein named SIKE (suppressor of IKapa kinase ε). While not much is known about SIKE, it has been shown to be a suppressor of the viral response by inhibiting IKapa kinase ε activity and type 1 interferon production (Huang et al., 2005b). In addition, like FGFR1OP2, SIKE has also been identified as a STRIPAK component (Goudreault et al., 2009; Hwang and Pallas, 2014).

Sequence analysis showed that there are four naturally occurring splice variants of FGFR1OP2 in humans, which we termed SKAM1A-1D (SK activator molecule 1A-1D). As mentioned above, FGFR1OP2 was initially identified as a protein fused with the FGF receptor-1 (FGFR1) as a result of a chromosomal translocation in a patient with T-cell lymphoblastic lymphoma. It was proposed, but never experimentally established, that this fusion may elicit constitutive signalling from the fused FGFR1 fragment that may generate oncogenic signalling. Thus, it has been known in the literature as FGFR1 oncogenic partner 2 (FGFR1OP2). Since this translocation appears rare, and does not represent the true physiological function of this gene product we felt that renaming FGFR1OP2 to SKAM1 (Sphingosine kinase activating molecule 1) was highly appropriate. These isoforms of SKAM1 come in

different sizes ranging from 140 to 253 amino acids (Fig. 1.2). Previous unpublished studies performed by the host laboratory using co-immunoprecipitation of overexpressed and endogenous proteins have confirmed that all isoforms of SKAM1 interacted with SK1 (Fig. 1.3). Notably, SKAM1 was shown to enhance the catalytic activity of SK1 in cells and *in vitro* (Fig. 1.4), thus resulting in the name SKAM1. These data suggest that SKAM1 contributes to the control of SK1 activity in cells.

Studies by the host laboratory have suggested the involvement of SK1 in SKAM1-mediated wound healing. Briefly, overexpression of SKAM1 or SK1 resulted in enhanced collagen contraction by NIH3T3 fibroblasts, an *in vitro* model of wound contraction. In addition, SKAM1-induced collagen contraction in NIH3T3 fibroblasts is mediated by SK1 and $S1P_{1/3}$ since targeting SK1 or $S1P_{1/3}$ via chemical inhibition or overexpression of a dominant negative SK1 resulted in significantly impaired collagen contraction by these cells. In addition, embryonic fibroblasts from $SK1^{-/-}$ mice showed significant impairment in collagen contraction compared with the wildtype. These preliminary data can be found in sections 3.3 and 4.3.

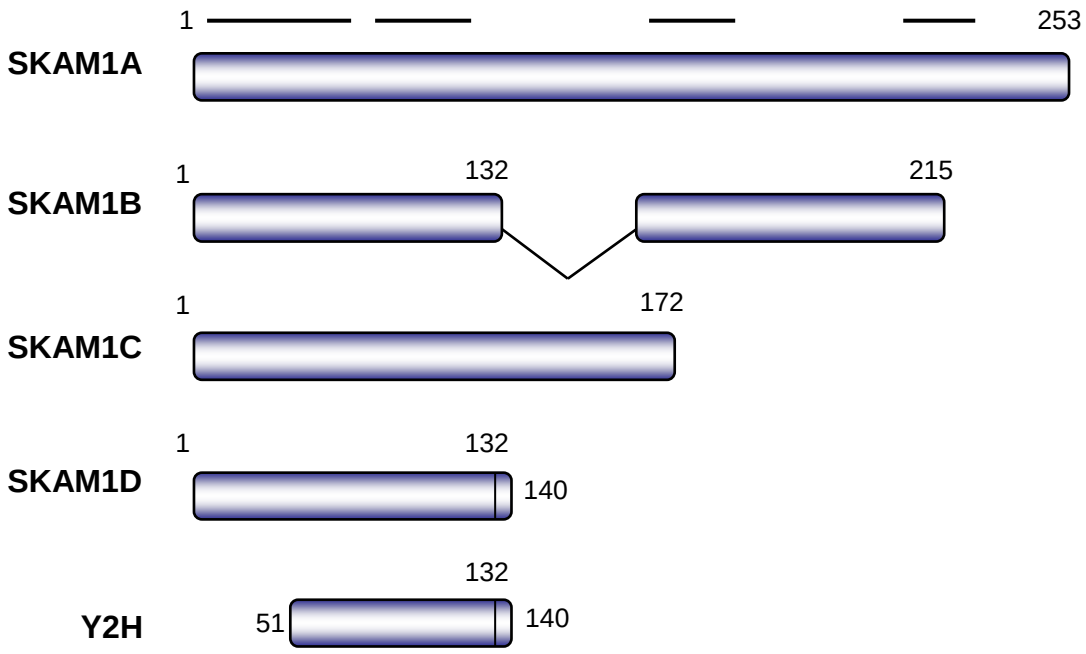
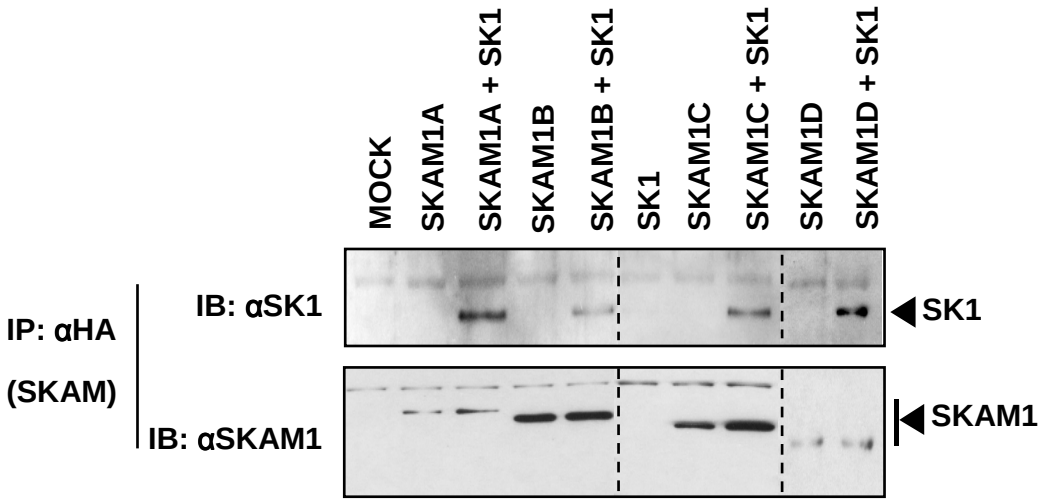


Fig. 1.2 SKAM1 isoforms

A schematic representation of the conserved regions of the SKAM1 isoforms. Residues 1-132 of SKAM1 is fused to the cytoplasmic region of FGFR1 in a patient with T-cell lymphoblastic lymphoma (Grand et al., 2004). Y2H is the fragment of SKAM1 that was isolated from the yeast two-hybrid screen for SK1-interacting proteins. Bars (top) represent the putative coiled-coil domains of SKAM1.

A


MOCK
SKAM1A
SKAM1A + SK1
SKAM1B
SKAM1B + SK1
SK1
SKAM1C
SKAM1C + SK1
SKAM1D
SKAM1D + SK1
IP: αHA
(SKAM)
IB: αSK1
IB: αSKAM1
SK1
SKAM1

B

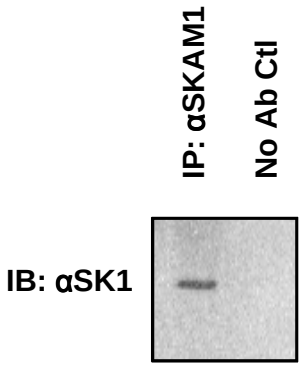

IP: αSKAM1
No Ab Ctl
IB: αSK1

Fig. 1.3 All naturally occurring SKAM1 isoforms interact with SK1

HEK 293T cells were mock transfected or transfected with vectors encoding SKAM1 isoforms alone, SK1 alone or SKAM1 isoforms and SK1. The next day lysates were prepared and HA-tagged SKAM1 isoforms immunoprecipitated with anti-HA antibodies and the immunocomplexes isolated with protein A-Sepharose. The anti-SK1 immunoblot (upper panel) shows that SK1 co-immunoprecipitates with all SKAM1 isoforms. The lower panel (immunoblot for SKAM1) confirms that all SKAM1 isoforms were present in the immunoprecipitates (A). Endogenous SK1 was co-immunoprecipitated from lysates of untransfected HEK 293T cells with anti-SKAM1 antibodies and the immunocomplexes isolated with protein A-Sepharose. Immunoblotting with endogenous SK1 antibodies confirmed that endogenous SK1 and endogenous SKAM1 interact. A 'no antibody control' (No Ab. Ctl.) was included to discount any non-specific protein interaction on the protein A-Sepharose (Pham & Pitson, unpublished) (B). All SKAM1 and SK1 proteins migrated at the expected molecular weights. The expected molecular weights for SKAM1A-D are approximately 28, 24, 18 and 14 kDa, respectively. The expected molecular weight of SK1 is approximately 42 kDa.

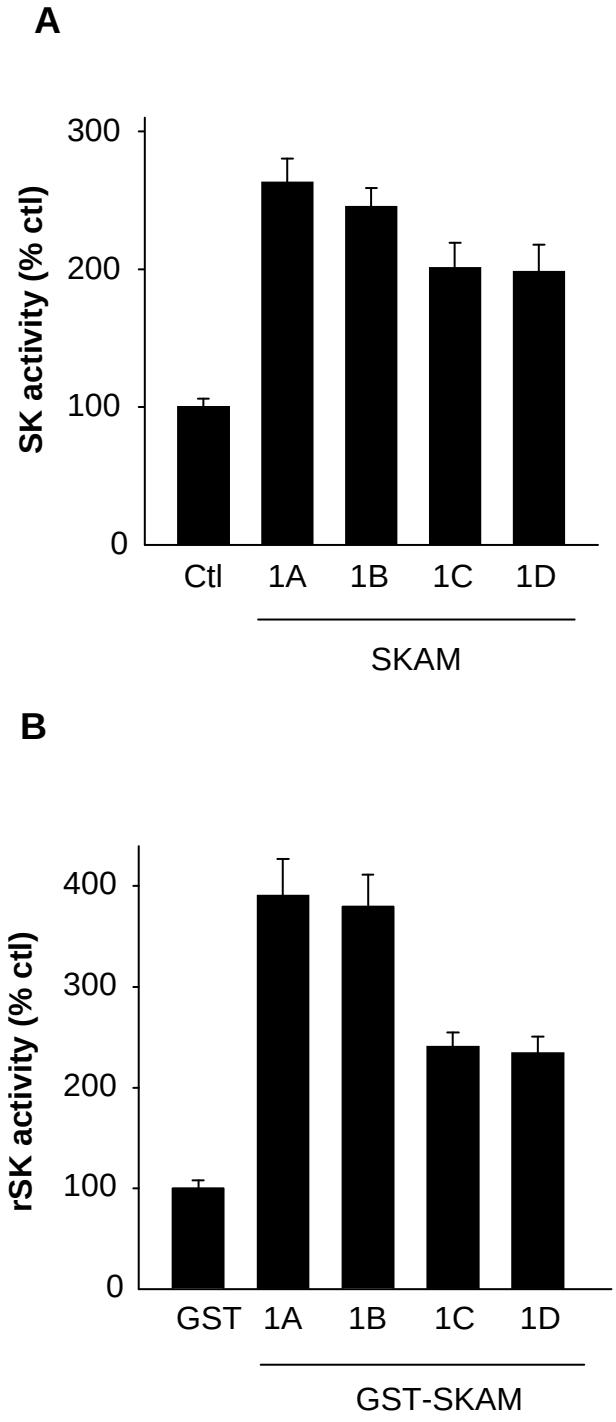

A
300
200
100
0
SK activity (% ctl)
Ctl
1A
1B
1C
1D
SKAM
B
400
300
200
100
0
rSK activity (% ctl)
GST
1A
1B
1C
1D
GST-SKAM

Fig. 1.4 All isoforms of SKAM1 activate SK1 in cells and *in vitro*

All the SKAM1 isoforms were over-expressed in HEK 293T cells, lysates prepared and the level of endogenous SK1 activity determined, using assay conditions largely specific for SK1, and not SK2, activity (Liu et al., 2000a). In comparison to the vector control all forms of SKAM1 were able to increase the activity of endogenous SK1 activity (A). Data are the mean (± S.D.) from five independent experiments, each analysed in duplicate. GST-SKAM1 fusion proteins (1A, 1B, 1C and 1D) were produced in *E.coli* and purified. The effect of these purified SKAM1 fusion proteins on the activity of purified recombinant SK1 was determined using an *in vitro* SK1 activity assay. All SKAM1 isoforms were able to increase SK1 activity when compared to the GST control (B) Data were obtained from four individual experiments, each performed in duplicate and expressed as mean ± S.D. (Pham & Pitson, unpublished).

1.7 Aims and hypothesis of the thesis

1.7.1 Hypothesis

Given that SK1 and S1P are involved in many cellular processes that are fundamental to wound healing, and that SK1 activity can be regulated by SKAM1, a protein that is induced following wounding, and previously shown to enhance wound healing, I hypothesised that SK1 and SKAM-mediated activation of SK1 is involved in critical aspects of wound healing; fibroblast migration and proliferation, and wound contraction.

1.7.2 Aims

The general aim of this project was to elucidate the role of SK and SKAM1 in wound repair. Previous studies have shown that SKAM1 is highly expressed in fibroblasts of wounded tissue and can enhance wound resolution. Although the underlying mechanism of SKAM1-mediated wound contraction is not known, further studies using an *in vitro* model of wound healing demonstrated that SKAM1 enhanced wound contraction. In addition, previous studies from the Pitson laboratory showed that SKAM1 interacts directly with SKs and enhanced SK catalytic activity. However, the role of the SK in wound healing has not been previously directly examined in any detail. The known roles of SK1 in enhancing cell proliferation, survival, migration and also contraction of smooth muscle cells, together with its activation by SKAM1 suggest that SK may mediate the effects of SKAM1 on enhancing wound healing. Thus, the specific aims of my studies were:

1) To characterise the role of SKs in wound healing using SK deficient mice.

2) To investigate the role of SKAM1 in wound healing using *in vitro* wound healing models and to generate mouse models to study the role of SKAM1 in wound healing *in vivo*.
3) To examine the effect of cell-permeable SKAM1 peptides on wound healing *in vitro* and *in vivo* models of wound healing.

CHAPTER 2

Materials and Methods

2. Materials and methods

2.1 Cell culture and transfection

NIH3T3 murine fibroblasts (ATTC CRL-1658) were maintained in Dulbecco's modified eagle medium (DMEM) containing 10% fetal calf serum (FCS) and 1% penicillin/streptomycin (both from Gibco, Life Technologies). Cell cultures were kept at 37˚C and 5% CO_2. Cells were transfected at ~70% confluence using the Lipofectamine 2000 transfection protocol (Invitrogen) and incubated for 48hrs before passaging. After 48hrs of incubation, cells were passaged 1:10 and selected with G418 at 0.8mg/ml. Media was changed every 3-4 days and cells were selected for approximately two weeks. Remaining cells after passaging were pelleted and resuspended in 300µL of extraction buffer (EB) containing 50mM Tris/HCl (pH 7.4), 150mM NaCl, 2mM Na_3VO_4, 10mM NaF, 1mM EDTA, 10% glycerol and 10mM β-glycerophosphate, 1mM dithiothreitol (DTT) and Complete^{TM} protease inhibitor cocktail. Whole cell lysates were made by sonication using the bath sonicator (3 x 10 sec cycles on ice) (Bioruptor) and were used in Western blot analysis.

2.2 Protein assays

Protein concentrations in whole cell lysates were determined by aliquoting 10µl of 1:10 diluted lysate into a 96-well plate in triplicate. Standards were bovine serum albumin (BSA) ranging from 0.05mg/ml to 0.5mg/ml. 200µl of the Bio-Rad Protein Assay reagent (Bio-Rad Laboratories, CA) was added to samples and standards and the protein concentration measured at 595nm using an ELISA plate reader.

2.3 *In vitro* scratch wound assay

Cells were plated into a 6-well plate (Falcon) and cultured at 37°C and 5% CO_2 in complete DMEM (Gibco, Life Technologies) containing 10% FCS and 1% penicillin/streptomycin until a confluent monolayer was formed. Once confluent, the cells were washed with serum-free DMEM and cultured overnight with DMEM containing 1% charcoal-stripped FCS (Invitrogen) and 1% penicillin/streptomycin. The next day, 'wounds' were created by scratching the surface of each well using a sterile P1000 pipette tip (Gilson). After scratching, the dislodged cells were removed by carefully replacing the media with fresh DMEM containing 1% charcoal-stripped FCS and 1% penicillin/streptomycin. Mitomycin C (2μg/ml) (Sigma) was added to block cell proliferation so that cell migration alone could be observed. Cells were then allowed to migrate into the wound gap over a period of 72hrs. Photos were taken regularly over a 30hr period. The rate of wound closure was quantified using Image J software.

2.4 *In vitro* fibroblast-populated collagen contraction assay

Sterile 22mm x 22mm glass coverslips were placed into 6-well dishes and incubated at 37˚C for 30min to warm. Cells were harvested, counted using a haemocytometer and resuspended in chilled collagen gel mix containing 70% of bovine collagen gel (BD Biosciences), 10% 0.1M NaOH, 10% 10x M199 media (Sigma) and 10% milli Q water. The cell/collagen gel mix (4×10^5 cells/400μl collagen gel) was then carefully pipetted onto the warmed glass coverslips and incubated at 37˚C and 5% CO_2 for 1hr to allow for the gel to set. Phenol red-free DMEM (Gibco) containing 5% FCS and 1% penicillin/streptomycin was then added and cells in collagen gel were incubated at 37˚C and 5% CO_2 overnight. Gels were

lifted off the coverslip the next day, returned to media and photos of gels taken at various time points to measure contraction. Gel contraction was quantified using Image Quant software and expressed as percentage of initial gel size.

2.5 Cell proliferation and apoptosis assays

BrdU (5-bromo-2-deoxyuridine) incorporation into nascent DNA was used as a measure of cell proliferation. Cells were seeded in triplicate in a 96-well flat bottom plate containing 10% FCS and 1% penicillin/streptomycin in DMEM. Cells were then incubated in 37˚C and 5% CO_2 for 3hr to allow cells to bed down. Cells were washed with serum-free media and then cultured in serum-free DMEM containing 1% penicillin/streptomycin for 24hr. Media was then replaced with DMEM containing 1% penicillin/streptomycin and 10μM BrdU (Roche) and incubated in 37˚C and 5% CO_2 for 3hr. BrdU incorporation was then detected according to manufacturer's protocol.

To assess apoptosis, NIH3T3 cells were seeded into an 8-well chamber slide (Labtek) in complete DMEM containing 10% FCS and 1% penicillin/streptomycin. The cells were incubated at 37°C and 5% CO_2 for 4hr to allow for cell adherence. The media was then removed and replaced with serum-free DMEM and incubated for 8hr at 37°C and 5% CO_2. The serum-free media was then removed and cells were stained with 1μg/ml 4',6-diamidino-2-phenylindole (DAPI) in methanol for 15min at room temperature. Cells were then washed with room temperature methanol, air dried and a coverslip applied with DAKO mounting medium (Dako). Apoptotic cells were identified by condensation and fragmentation of nuclei using fluorescence microscopy and expressed as percentage of total cells counted. A minimum of 300 cells were scored per point.

2.6 RNA preparation and cDNA synthesis

RNA was prepared from wounded and unwounded mouse skin. Skin tissue was homogenised in Trizol (Invitrogen) using a glass homogeniser. Chloroform was then added and mixed by shaking vigorously for 15sec. Samples were then left at room temperature for 5min and then centrifuged at 13,000g for 15min at 4˚C to separate the two phases. The top aqueous phase was collected and isopropanol was added to precipitate RNA. Samples were then centrifuged at 13,000g at 4˚C to pellet the RNA, supernatant removed, the pellet washed with RNase-free 75% ethanol and then dissolved in RNase-free water. Equal amount of RNA was then used as template to synthesise cDNA using the QuantiTect reverse transcription kit (Qiagen).

2.7 Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR)

qRT-PCR was performed using Taqman Gene Expression kit (Applied Biosystems). Each reaction contained 2µl of RNase-free H_2O, 5µl of Taqman Universal Master Mix (Applied Biosystems), 0.5µl of 20x Taqman target primer, 0.5µl of 20x Taqman control primer and 2µl of cDNA. qPCR cycle was conducted under the following conditions: Hold 1 at 50°C for 2min, hold 2 at 95°C for 15min, 40 cycles of denaturing at 95°C for 30 sec, annealing at 58°C for 21sec, and extension at 72°C for 15sec completing with hold 3 at 72°C for 30sec on the Rotor-Gene RealTime PCR machine (Corbett Life Sciences/QIAGEN). Relative levels of target genes (FGFR1OP2 Cat. No. Mm01604388_g1, Acta-2 Cat. No. Mm0156133_m1) were analysed using the Rotor-Gene software and normalised to the corresponding control (mGAPDH Cat. No. 4352339E).

2.8 Western blotting

Cells were harvested in cold phosphate buffered saline (PBS) and centrifuged at 1,500 rpm at 4°C for 5min. Cell pellets were then resuspended in EB and sonicated using the bath sonicator (Bioruptor). Cell lysates were clarified by centrifuging at 13,000g for 20 min, then boiled in sodium dodecyl sulphate (SDS) sample buffer for 5min, and briefly centrifuged. Proteins were separated using 12% polyacrylamide gels and transferred to nitrocellulose membranes. For FLAG immunoblots the membranes were placed in blocking buffer composed of PBS containing 5% skim milk powder (SMP) and 0.1% Triton X-100. The membrane was then placed on a rocker for 1hr at room temperature. The membranes were incubated with anti-FLAG primary antibody (1:5000, Sigma) in PBS containing 5% SMP and 0.1% Triton X-100 for 1hr. The membranes were washed 3 x 5min in PBS containing 5% SMP and 0.1% Triton X-100 and then incubated with anti-mouse HRP secondary antibody (1:10000, Pierce) in PBS containing 5% SMP and 0.1% Triton X-100 for 1hr. The membranes were washed 3 x 5min in PBS and then incubated with enhanced chemiluminescence (ECL) solution (Amershambiosciences) for 1min and exposed to a LAS4000 imaging system (Fujifilm). All other antibodies used in the Western blots were performed in the same manner unless otherwise described.

2.9 Protein extraction from mouse tissues

Frozen mouse tissue was homogenised using a glass homogeniser in protein lysis buffer containing 50mM Tris (pH 7.5), 1mM EDTA, 50mM NaCl, 0.1% Triton X-100 and 1 x complete protease inhibitor cocktail (Roche). Homogenised samples were then sonicated on ice using a bath sonicator (Bioruptor) for 3 x 10sec cycles. Lysates were then clarified at 13,000g for 20min at 4°C, and the supernatant

collected. Protein concentration was then determined using Bio-Rad Protein Assay reagent (Bio-Rad Laboratories, CA). Protein samples were snap frozen in liquid nitrogen and stored at -80°C.

2.10 Sphingosine kinase assays

Sphingosine kinase activity was routinely determined using D-*erythro*-sphingosine (Biomol) and [γ^{32}P]ATP (Perkin Elmer) as substrates. Cell lysates or purified proteins were incubated with 100μM sphingosine (prepared from a 2mM stock of solubilised in 5% Triton X-100), [γ^{32}P]ATP (1μM : 10μCi/μl), 0.5mM 4-deoxypyridoxine (in the case of cell lysates) in assay buffer (100mM Tris-HCl, pH 7.4, 10mM $MgCl_2$, 1mM Na_3VO_4 and 10mM NaF) to a total reaction volume of 100μl. The reaction was incubated at 37^{o}C for 30min. The enzyme reaction was then stopped by the addition of 700μl chloroform/methanol/conc. HCl (100/200/1, v/v), 70μl of chloroform and 20μl of 5M KCl. Phases were separated by vortexing (for purified protein assays) and by vortexing and centrifugation (13,000 × g, 5min, 37°C) when assaying cell lysates. Following phase separation, the aqueous top phase was removed and 50μl of the chloroform bottom phase was spotted onto aluminium backed silica thin-layer chromatography (TLC) plates (Sigma). S1P was then resolved using 1-butanol/ethanol/acetic acid/water (80:20:10:20, v/v) as mobile phase. Radiolabel incorporated into S1P was quantified by exposure of the plate to a storage phosphor screen read with a Molecular Dynamics Typhoon PhosphorImager. A unit (U) of SK activity is defined as the amount of enzyme required to produce 1pmol S1P / min.

2.11 Animal ethics

All animal studies were performed with the approval of both the University of Adelaide animal ethics committee and the Central Adelaide Local Health Network animal ethics committee. Animal ethics approval numbers are S-2013-096, S-2013-024, 89a/09 and 47/13.

2.12 Full-thickness incisional wound healing study in mice

Twelve week old C57BL/6 female mice were individually caged for at least one day prior to surgery for acclimatisation. Mice were anaesthetised by isofluorane inhalation. Two 1cm full-thickness incisions were then performed on the dorsal skin of mice. Photo of wounds were taken immediately after surgery, Betadine applied and Temgesic (buprenorphine) was intra-peritoneally (i.p.) injected at 0.1mg/kg for pain relief. Mice were then returned to an individual cage containing soaked food, water and plastic housing. Wounds on mice were then allowed to heal via secondary intention (wounds that are intentionally left open to heal). Wound photos were taken daily. Mice were observed daily and humanely culled at day 3, 4, 7, 14 or 21. Wounds were then excised from the back of the mice and cut in half. Half of the wound was placed in a tissue biopsy cassette and fixed in 10% formalin for wound measurement, and the other half was snapped frozen and used for biochemical analyses including Western blotting, RNA extraction and qRT-PCR. Wound margins were measured from haematoxylin/eosin stained sections and were quantified using the Research AnalySIS software.

2.13 Full-thickness excisional wound healing study in mice

Twelve week old female C57BL/6 mice were individually caged for at least one day prior to surgery for acclimatisation. Mice were anesthetised by isofluorane inhalation, and using a 6mm biopsy punch (Stiefel), two full-thickness excisional wounds were created on the dorsal skin of each mouse, one on each side of the midline. Photographs of wounds were taken immediately after surgery, Betadine was applied to wounds, and Temgesic (buprenorphine) was IP injected for pain relief. Mice were then returned to an individual cage containing soaked food, water and plastic housing. Wounds were allowed to heal by secondary intention. Photos of wounds were taken daily until day 14. Wound area was quantified using the ImageJ software. Mice were culled at day 21 and wounds were excised and cut into half. Half of the wound was placed in a tissue biopsy cassette and fixed in 10% formalin for wound analyses, and the other half was snapped frozen and used for biochemical analyses including Western blotting, RNA extraction and qRT-PCR.

2.14 Generation of SKAM1A gene-trap mice

Commercially available C57BL/6 mouse embryonic stem (ES) cells containing the SKAM1A gene-trapped 'knockout-first' allele (clone ID: EPD0680_1_E09) were purchased from the International Gene Trap Consortium (IGTC) and were used to generate SKAM1A gene-trap mice by the Australian Phenomics Networks (APN) at Monash University. ES cells containing the gene-trapped SKAM1A allele were microinjected into blastocysts of C57BL/6 mice to generate chimeras. The resulting chimeras were bred with wildtype C57BL/6 mice to generate heterozygous SKAM1A gene-trap mice. Heterozygous SKAM1A gene-trap

mice were identified by PCR using genomic DNA isolated from tail biopsies. The following primers were used:

Primer 1: 5'-CAGTTATGATGAATTTCTTGATACC-3', Primer 2: 5'-GAGAAACAGGATACACTGAGTCC-3' and Primer 3: 5'-CAACGGGTTCTTCTGTTAGTCC-3'. Primers P1 (located at the 3' end of the 5' homology arm) and P2 (located in exon ENSMUSE00001273520) amplify a product of 495bp from the wild type allele. Primers P1 and P3 (located in the en-2 intron) amplify a product of 269bp from the targeted allele.

2.15 Generation of transgenic mice

2.15.1 Generation of *pCX-FLAG/SKAM1A-IRES-EGFP* construct

The construct *pcDNA3-FLAG/SKAM1A-IRES-EGFP* [previously generated in the Pitson Laboratory for mammalian expression of FLAG-tagged SKAM1 and also enhanced green fluorescence protein (EGFP) via an internal ribosomal entry site (IRES)] was subject to Quickchange mutagenesis to remove the EcoRI site located at the 3' end of FLAG/SKAM1A. This procedure was performed so that the FLAG-SKAM1A-IRES-EGFP DNA fragment resulting from the EcoRI digest of *pcDNA3-FLAG/SKAM1A-IRES-EGFP* could be subcloned into the pCX vector (Okabe et al., 1997) as a single fragment. Oligos used for Quickchange mutagenesis: 5' - CTGAGGAAGAGCTGAATTAGTTAACCTCGAGCGG – 3' and 5' – GACTCCTTCTCGACTTAATCAATTGGAGCTCGCC – 3'. Methylated DNA templates were removed by digesting with DpnI. The DNA construct was then transformed into DH5α competent *E. coli* via heat shock at 42°C and then selected with 100 μg/L of ampicillin at 37°C for 24hr. Mutation of the EcoRI site was confirmed by sequencing. A new EcoRI restriction site was introduced at the 3' end

of EGFP cDNA via PCR using T7 and ECFP reverse primers (T7 primer 5' – TAATACGACTCACTATA -3' and ECFP primer 5' – TAGAATTCACTTGTACAGCTCGTCCATGC -3'). PCR clean up was performed using the QIAQUICK purification protocol (QIAGEN). The PCR product was then digested with EcoRI followed by gene cleaning and gel purification (Ultra Clean DNA purification, Mo Bio). The purified PCR product was then ligated into EcoRI/CiP-digested *pCX-EGFP* vector (provided by Okabe M., Genome Information Research Centre, Osaka University, Japan) (Okabe et al., 1997). The resulting DNA construct was denoted *pCX-FLAG/SKAM1A-IRES-EGFP*. Orientation of insert was verified by digesting the construct with HindIII and XhoI. Sequencing confirmed the integrity of the cDNA sequence.

2.15.2 Generation of SKAM1A transgenic mice

50μg of *pCX-FLAG/SKAM1A-IRES-EGFP* DNA construct was digested with SalI, BamHI and FspI for 24hr at 37°C. This procedure was performed to remove most of the backbone vector and generate a linear expression fragment containing the CMV enhancer, intron, FLAG-SKAM1A-IRES EGFP cDNA and poly-A stop signal. Gel electrophoresis confirmed successful digestion. Digested DNA was then sent to The Transgenic Animal Service of Queensland (TASQ) to generate SKAM1A transgenic mice by pronuclear injection. Briefly, microinjected embryos from C57BL/6 mice were transferred to the oviducts of pseudopregnant females. Tail biopsies from offspring resulting from the microinjected embryos were used for transgenic screening by PCR. Oligos used for transgenic mice screening were: FLAG 5' – GACTACAAGGACGACGATGA – 3' and SKAM 5' – TAGAATTCAGCTCTTCCTCAGACTC - 3'. Transgenic positive founders identified

by PCR were then sent from TASQ to the IMVS Animal Care Facility and were bred with wildtype C57BL/6 mice for colony establishment.

2.15.3 Screening for SKAM1A transgenic mice

Genomic DNA was prepared from tail biopsies using the DNeasy Blood and Tissue Kit (QIAGEN). Transgenic positive mice were identified by PCR as described above.

2.16 Focus formation assay

T75 flask containing confluent NIH3T3 cells were resuspended in 15ml of complete DMEM containing 10% FCS and 1% penicillin/streptomycin. 2ml of cell suspension were added to a 6-well plate in duplicate and cultured at 37°C and 5% CO_2 for approximately two weeks. Media was changed every 2-3 days. After approximately two weeks, when foci are macroscopically visible, the media was removed and cells washed, fixed and stained with 0.05% methylene blue.

2.17 Isolation of primary mouse embryonic fibroblasts (MEFs)

Day 13.5 embryos were isolated from the uterus of pregnant mice. Visible internal organs of the embryos were removed and the remainder of the embryo was transferred into a tissue culture plate and then minced using a surgical blade. Embryos were then digested with trypsin for 20min at 37°C and 10% CO_2, transferred into DMEM containing 10% FCS and 1% penicillin/streptomycin, and then centrifuged at 2000rpm for 5min. The pellet was resuspended in 10% FCS and 1% penicillin/streptomycin and transferred into a T25 flask and cultured at 37°C and 10% CO_2 for 24hr. Media was changed the next day to remove non-adherent cells.

Resultant primary MEFs were analysed before five passages. Genotyping was performed on primary MEFs to confirm their genotype.

2.18 Extraction of S1P from plasma for high performance liquid chromatography (HPLC)

Plasma was prepared from whole blood of mice collected via cardiac puncture. Determination of S1P levels in these samples was then performed by Ms Lorena Davies (Pitson Lab) using HPLC. Briefly, plasma (100µl) was added to a tube containing 270µl of chloroform: methanol: conc. HCl at a ratio of 100:200:1 and C_{17}-S1P (internal control) at a final concentration of 200pmol per sample. Samples were mixed by vortexing. 70µl of chloroform and 20µl of 2M KCl were added into each sample and then mixed by vortexing. Samples were centrifuged at 14,000g for 5min at 4°C for phase separation. The aqueous upper layer was removed by aspiration. The organic layer was then transferred into a new tube. Samples were dried down using a SpeedVac Concentrator (Thermo Scientific) for 15min at room temperature. Dried samples were then resuspended in a solution containing methanol: 70mM K_2HPO_4 (9:1) and 1mM EDTA (275µl). Samples were then sonicated on ice for 15sec using a bath sonicator (Bioruptor) for 4 cycles (25sec on and 25sec off). Samples were then incubated with 25µl o-phthalaldehyde (OPA) solution (5mg OPA, 100µl ethanol, 5µl β-mecaptoethanol and 5ml of 3% boric acid) for 15min at room temperature in dark to fluorescently label S1P. After 15min incubation, the samples were briefly centrifuged at 14,000g for 10min at 4°C, and the supernatant transferred into a HPLC vial (Phenomenex) and injected onto the column (Waters-Xbridge C_{18} 5µm). Solvents: K_2HPO_4/methanol at gradient 22% K_2HPO_4/78% methanol to 100% methanol over

20min with a flow rate of 1ml/min. S1P levels were detected at 455nm and quantified using a fluorescence detector and Waters Breeze 2 System software.

2.19 Histology, immunohistochemistry and image analysis.

Histological sections (4μm) of mouse skin wounds embedded in paraffin were cut and stained with haematoxylin and eosin or subjected to immunohistochemistry following antigen retrieval as previously described (Adams et al., 2009). Briefly, tissue was sectioned (4μm) onto 'snow-coat' slides, dried overnight and subject to dewaxing in xylene. Sections were brought through graduated ethanol baths to remove xylene, washed in water and rinsed in PBS. Sections were boiled in the target retrieval solution. Sections were then washed in PBS and treated with trypsin (Sigma). Following antigen retrieval, sections were blocked in 3% normal goat serum, and primary antibodies against SKAM1 (1: 200) (Protein Tech), PCNA (1: 200) (Sigma) or collagen I (Rockland) was applied and incubated overnight at 4°C. Species specific Alexa Fluor 488-conjugated secondary antibodies (1: 200) (Invitrogen) were used and incubated for 1hr at room temperature. Fluorescence intensity was determined using AnalySIS software package (Soft Imaging System GmbH, Munster, Germany), and the number of fluorescing cells in the dermis of the wounds was counted in a blinded manner. Negative controls included replacing primary antibodies with normal mouse or normal rabbit IgG. Primary or secondary antibodies were omitted to verify the staining and detect nonspecific binding. All control sections had negligible immunofluorescence.

2.20 Histological analysis of SKAM1A transgenic mice

Tissues and organs of SKAM1A transgenic mice were extracted, fixed in 4% paraformaldehyde, sectioned (4μm) and stained with haematoxylin and eosin. All histological samples were analysed by the Australian Phenomics Network at SA Pathology.

2.21 Effect of cell permeable SKAM peptides on SK1 activity *in vitro*

Cell permeable SKAM1 peptides were synthesised containing the protein transduction domain of the Human Immunodeficiency Virus transactivator of transcription (TAT) protein (YGRKKRRQRRR) (Becker-Hapak et al., 2001). TAT-SKAM1A$^{76\text{-}105}$ (YGRKKRRQRRRGIRELQQENKELRTSLEEHQSALELIMSKYR) and TAT-SKAM1A$^{76\text{-}105(Y104F)}$ (YGRKKRRQRRRGIRELQQENKELRTSLEEHQSALELIMSKFR) peptides were synthesised by China Peptides. Fluorescein isothiocyanate (FITC)-labelling of these peptides was also done by China Peptides. Purity of each peptide (>90%) was determined by China Peptides using mass spectrophotometry and HPLC. Peptides were dissolved in milli Q water at a concentration of 1mg/ml and were stored at -80°C. Purified recombinant His-tagged human SK1 was generated in insect cells as previously described (Pitson et al., 2002). For the *in vitro* SK assay, each peptide was incubated with recombinant SK1 at a 50:1 ratio (each reaction contained 50ng of peptide (9.5×10^{-3}nmol) and 1ng of recombinant SK1 (2.4×10^{-5}nmol)). The *in vitro* SK1 activity assay was performed as described in section 2.12.

2.22 Delivery of TAT fusion peptides into NIH3T3 cells

NIH3T3 cells were seeded into a 6cm plate and were cultured in DMEM containing 10% FCS and 1% penicillin/streptomycin until 80% confluent. NIH3T3 cells were then treated with 0.5μM of FITC, TAT-SKAM1A$^{76\text{-}105}$-FITC or TAT-SKAM1A$^{76\text{-}105(Y104F)}$-FITC for 1hr at 37°C and 5% CO_2. Cells were washed twice with PBS, harvested and seeded onto poly-L-lysine coated coverslips. Cells were then incubated with DMEM containing 10% FCS for 4hr at 37°C and 5% CO_2 for cell adherence. Cells were then fixed with 4% paraformaldehyde for 10min at room temperature. After fixing, cells were washed twice with PBS containing 0.1% Triton X-100. Cells were permeabilised with PBS containing 0.1% Triton X-100 for 10min and stained with DAPI. Coverslips were then mounted onto microscope slides using DAKO mounting medium. Samples were visualised on confocal microscope Zeis LSM700.

2.23 Effect of cell permeable SKAM peptides on NIH3T3 fibroblast-mediated collagen matrix contraction

NIH3T3 fibroblasts were seeded onto 6cm plates containing 10% FCS and 1% penicillin/streptomycin DMEM and cultured until 80% confluent. Cells were pre-treated with 0.5μM of peptide for 1hr at 37°C and 5% CO_2, then washed in PBS, harvested and seeded into the collagen gel as described in section 2.4. Cells were incubated with 0.5μM of TAT-SKAM1A$^{76\text{-}105}$ or TAT-SKAM1A$^{76\text{-}105(Y104F)}$ for 24hr.

2.24 Delivery of SKAM peptides into full-thickness excisional wounds

Mice were anaesthetised by isofluorane inhalation and photographs of wounds were taken before injections were performed. Peptides were freshly prepared and

were kept on ice during the course of administration. Using an insulin syringe, 200µl of TAT-SKAM1A$^{76\text{-}105}$ (0.5µM), TAT-SKAM1A$^{76\text{-}105(Y104F)}$ (0.5µM) or saline (0.9% NaCl) was injected intradermally into the adjacent skin tissue of each wound. Mice were then returned to individual cages after the procedure was performed.

CHAPTER 3

The Role of Sphingosine Kinases in Wound Healing

3. The role of sphingosine kinases in wound healing

3.1 Abstract

Sphingosine kinases (SKs) are intracellular signalling enzymes that generate the bioactive lipid sphingosine 1-phosphate (S1P). SKs and S1P regulate a diverse range of cellular processes, including cell proliferation, survival, differentiation, migration and angiogenesis, mainly via the action of S1P as a ligand for a family of five S1P-specific G protein-coupled receptors (GPCRs). As such, SKs and S1P are involved in regulating a wide array of cellular processes that are known to be critical in wound healing. These together with unpublished findings from the Pitson laboratory that show involvement of the SKs in fibroblast-mediated collagen matrix contraction, suggest that the SKs may be important mediators of wound healing. In light of this, I examined the role of SKs in wound healing *in vivo* using a well established mouse model of incisional wound healing using SK knockout mice. A significant delay was observed in $SK1^{-/-}$, $SK2^{-/-}$ and $SK1^{-/-}SK2^{+/-}$ mice, compared to that of wildtype mice. Furthermore, impaired wound healing observed in SK deficient mice appeared to correlate with decreased cellular proliferation during the wound repair process. Taken together, these results strongly suggest that SK1 and SK2 play an important positive role at least in the early stages of wound healing.

3.2 Introduction

SKs are intracellular enzymes that catalyse the generation of S1P, a bioactive signalling lipid that is involved in controlling many biological processes, including calcium mobilisation, cell proliferation, apoptosis, migration and cytoskeleton rearrangement, mainly via the cell surface S1P-specific G protein-coupled receptors, $S1P_{1-5}$ (Hait et al., 2006). Since these cellular processes are also known to be fundamental in wound healing, this raises the real potential for the SKs and S1P to be mediators of wound healing. Indeed, SK1 and S1P have long been proposed to play a role in wound healing (Francis-Goforth et al., 2010; Watterson et al., 2007), although evidence to support this hypothesis remains scant. Notably, S1P is released by platelets at the site of injury (Lee et al., 2000) and is also enriched in acute wound fluid (Amano et al., 2004). Furthermore, while no molecular details were examined, previous work has also shown that topical application of S1P to diabetic wounds and psoriasis lesions in mice significantly enhanced wound resolution (Kawanabe et al., 2007; Schaper et al., 2013). Very recently, a study has shown that application of an expression plasmid encoding SK1 onto full-thickness excisional wounds significantly enhanced wound resolution in diabetic rats (Yu et al., 2014). These, coupled with our considerable preliminary data that showed the involvement of the SKs and S1P receptors in fibroblasts-mediated collagen matrix contraction (detailed below), indicate that the SKs and S1P play a critical role in wound healing. To date, however, a direct role for SK/S1P in wound healing has not been demonstrated. Therefore, the focus of this chapter of my thesis was to examine the roles of SKs in wound healing. Here, I have shown for the first time a direct positive role for the SKs in wound healing of the skin.

3.3 Preliminary data leading to the project

3.3.1 SK1 overexpression enhances fibroblast-mediated collagen contraction

To examine the role of SK1 in wound healing, the host laboratory initially employed the fibroblast-populated collagen contraction assay, a well-established *in vitro* model of wound healing (Vernon and Gooden, 2002). Collagen contraction was significantly enhanced by NIH3T3 fibroblasts stably overexpressing SK1, suggesting a potential role of SK1 in wound repair (Fig. 3.1A).

3.3.2 Genetic ablation or chemical inhibition of SK1 results in reduced collagen contraction

The effect of SK1 inhibition on collagen contraction was then examined. Compared with untreated control cells, collagen contraction was significantly attenuated in NIH3T3 fibroblasts treated with SKi, a chemical inhibitor of SK1 (French et al., 2003) (Fig. 3.1B). In addition, MEFs from $SK1^{-/-}$ mice showed a significant reduction in collagen contraction compared with MEFs from wildtype mice (Fig. 3.1C). Together, these data suggest a role of SK1 in wound healing.

3.3.3 The $S1P_{1/3}$ antagonist VPC23019 blocks SK1-induced collagen contraction

Having established a role of SK1 in collagen contraction, examination of whether the S1P receptors are involved in this process was performed next. Using a number of commercially available S1P receptor antagonists, the host laboratory has shown that SK1-induced contraction was significantly reduced in NIH3T3 cells treated with VPC23019 (a $S1P_{1/3}$ antagonist) (Fig. 3.2). Notably, this effect was not observed in NIH3T3 cells treated with JTE013 (a $S1P_2$ antagonist) (Fig. 3.2). This

suggests that SK1-induced collagen contraction by NIH3T3 fibroblasts is mediated via the extracellular function of S1P on $S1P_1$ and/or $S1P_3$.

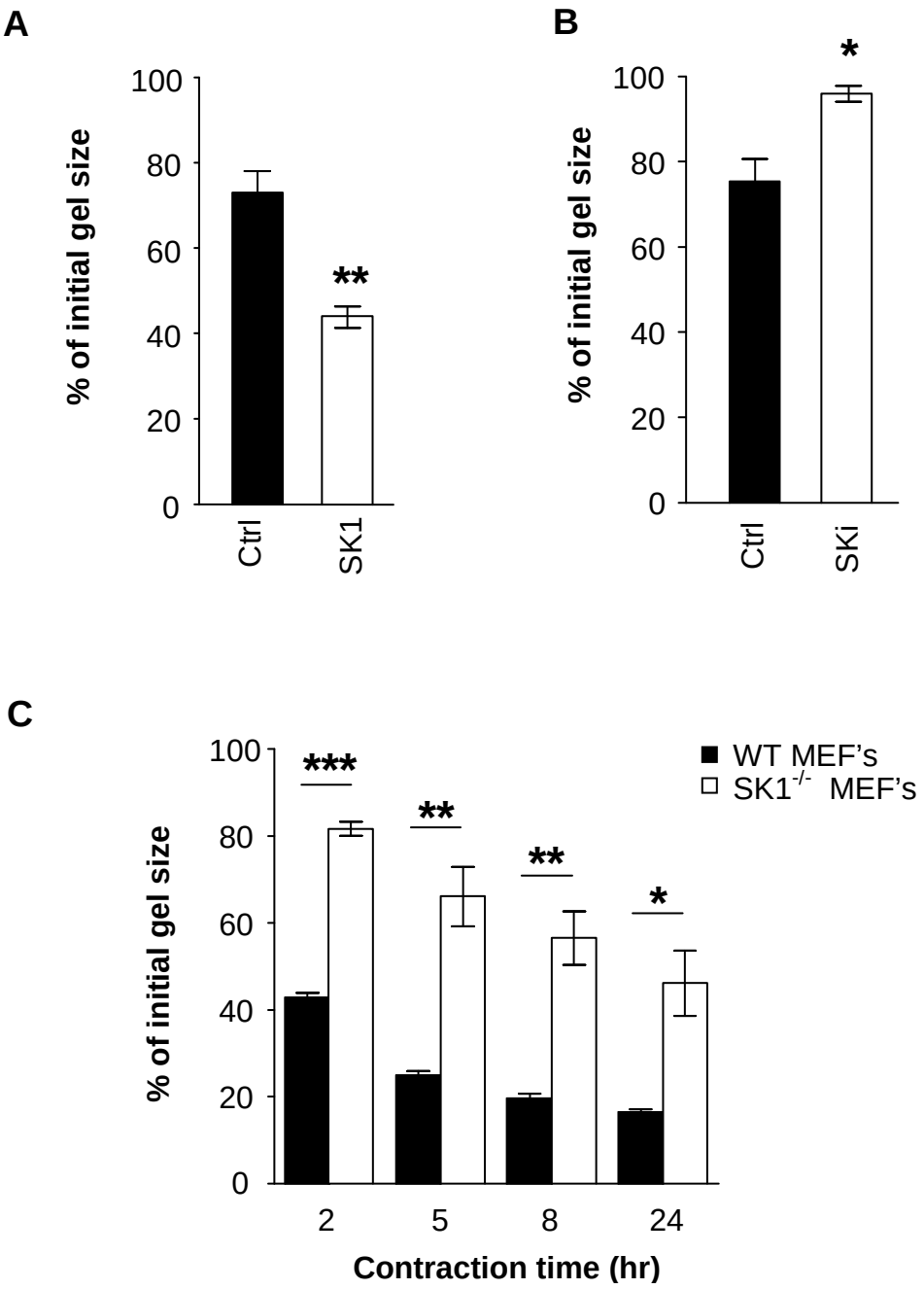

A
% of initial gel size
100
80
60
40
20
0
**
Ctrl
SK1
B
% of initial gel size
100
80
60
40
20
0
*
Ctrl
SKi
C
% of initial gel size
100
80
60
40
20
0
***
**
**
*
WT MEF's
SK1-/- MEF's
2
5
8
24
Contraction time (hr)

Fig. 3.1 SK1 enhances fibroblast-mediated collagen contraction.

NIH3T3 cells overexpressing SK1 or vector control cells were subject to collagen gel contraction assays, n=4, **p<0.005 for gel sizes of 28hr (A). Untransfected NIH3T3 cells were treated with SKi (5μM) and gel sizes assessed at 28hr, n=4,*p<0.05 (B). Primary MEFs were harvested from wildtype or $SK1^{-/-}$ embryos and subjected to collagen gel contraction assays and gel size measured at various times, n=3, *p<0.05, **p<0.005 and ***p<0.0005 (C) (Gliddon & Pitson, unpublished). The initial gel size immediately after release from the coverslip (time 0hr) was defined as 100%.

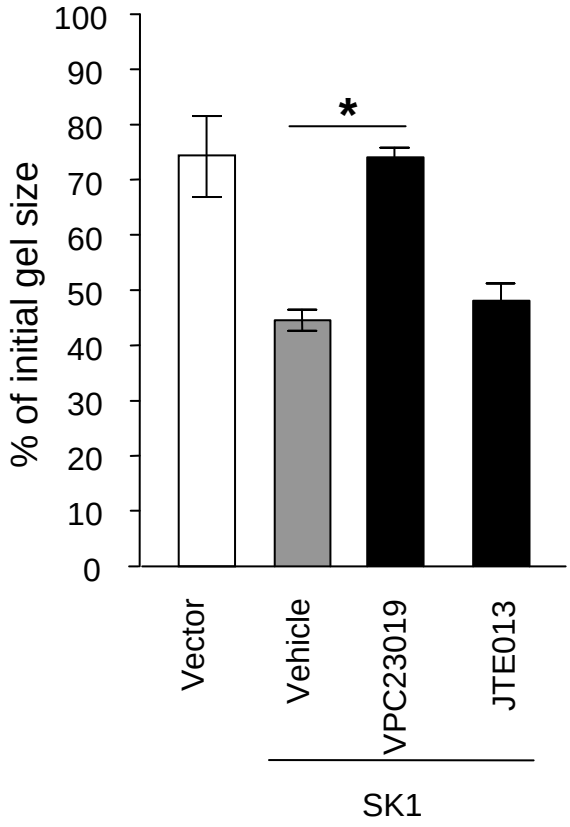


Fig. 3.2 SK1-induced contraction is mediated by $S1P_{1/3}$.

NIH3T3 cells stably overexpressing SK1 were subject to the collagen gel contraction assay with VPC23019 (10μM) or JTE013 (1μM) and gel size assessed at 28hr, n=3,*$p<0.05$ (Gliddon & Pitson, unpublished). The initial gel size immediately after release from the coverslip (time 0hr) was defined as 100%.

3.4 Results

3.4.1 SK knockout mice and relative plasma S1P levels

To examine the role of SK1 and SK2 we obtained $SK1^{-/-}$ and $SK2^{-/-}$ mice from R. Proia (NIH, Bethesda, USA) (Allende et al., 2004b; Mizugishi et al., 2005). The two SKs may have some compensatory roles since single knockout of either gene results in mice with no gross defects, but SK1/SK2 double knockout mice die *in utero* (Mizugishi et al., 2005). Thus, we also bred mice possessing only one functional SK allele; $SK1^{-/-}SK2^{+/-}$ mice (Mizugishi et al., 2005). Again, these mice had no gross defects in their appearance or behaviour, although $SK1^{-/-}SK2^{+/-}$ female mice are infertile (Mizugishi et al., 2007).

Examination of S1P in the plasma of the SK knockout mice showed that $SK1^{-/-}$ mice have reduced plasma S1P levels (Fig. 3.3), which is consistent with previous published work (Allende et al., 2004b). Furthermore, also in agreement with previous studies, the plasma S1P levels of the $SK2^{-/-}$ mice were higher compared to the wildtype (Fig. 3.3) (Kharel et al., 2012; Olivera et al., 2007; Sensken et al., 2010; Zemann et al., 2006). Notably, we have shown for the first time that plasma S1P levels in $SK1^{-/-}SK2^{+/-}$ mice is significantly lower compared to the wildtype (Fig. 3.3).

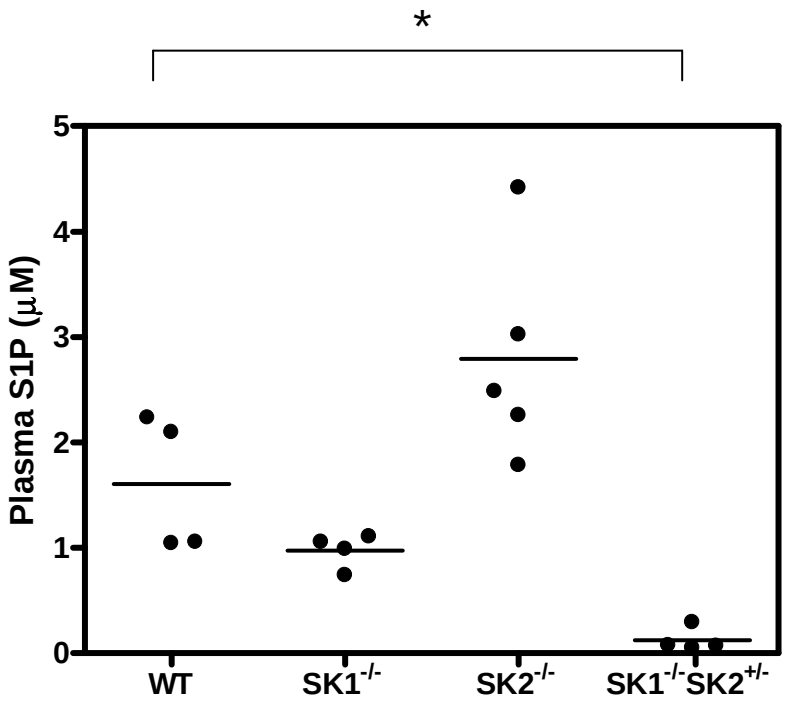


Fig. 3.3 Plasma S1P levels of wildtype, $SK1^{-/-}$, $SK2^{-/-}$ and $SK1^{-/-}SK2^{+/-}$ mice

Plasma was prepared from whole blood harvested from 12 weeks old female C57BL/6 mice and subject to HPLC analysis by Ms Lorena Davies. *p=0.004 compared with the WT control. Statistical significance was calculated using the student's t-test.

3.4.2 SK deficiency impairs wound healing

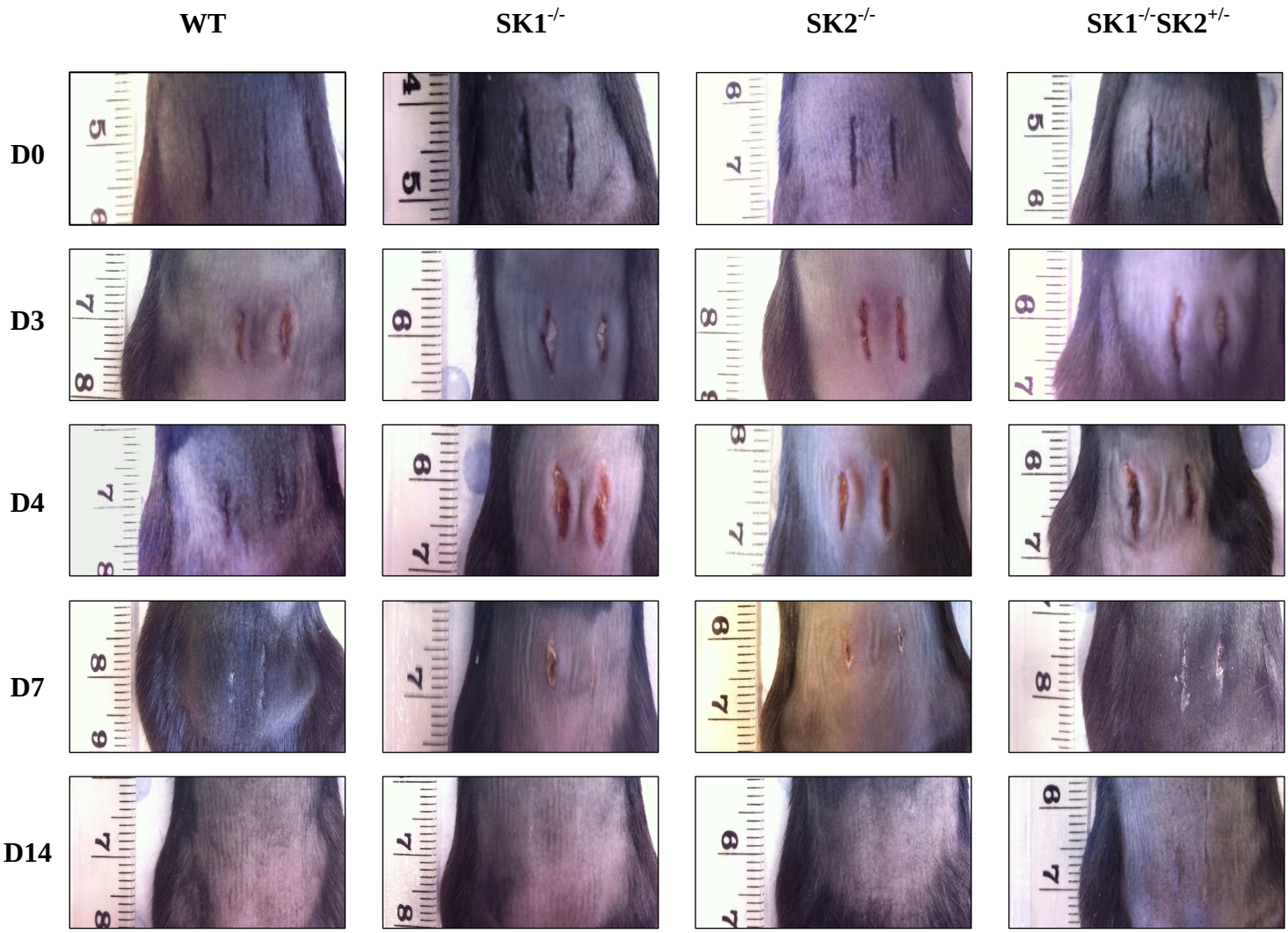
To investigate the role of SKs in wound healing *in vivo*, I initially employed a murine model of incisional wound healing. Briefly, two 1cm incisional wounds were created on the dorsal skin of mice at day 0 and wounds were left to heal via secondary intention (see Materials and Methods for details). Images of the wounds were captured at day 0, 3, 4, 7 and 14 post-wounding for visual inspection. Mice were humanely culled at day 3, 4, 7 and 14 post-wounding and wounds were harvested for histology, immunohistochemistry and qPCR analyses.

Incisional wounds created on the dorsal skin were identical throughout all genotypes at the start of the experiment. Visual inspection of the wounds at day 3, 4 and 7 post-wounding showed that the wound size of the $SK1^{-/-}$, $SK2^{-/-}$ and $SK1^{-/-}/SK2^{+/-}$ mice were macroscopically larger compared to that of the wildtype control (Fig. 3.4A). This was most evident on day 4 (Fig. 3.4A). Although all SK deficient mice displayed impaired wound healing at earlier time points (day 3, 4 and 7), the wounds of these mice, like that of the wildtype, were healed at day 14 (Fig. 3.4A). It is important to note, however, that although all mice healed at day 14, the wounds of the wildtype mice were healed around day 8 whereas the SK deficient mice healed around day 10, suggesting that SK deficiency impairs wound healing.

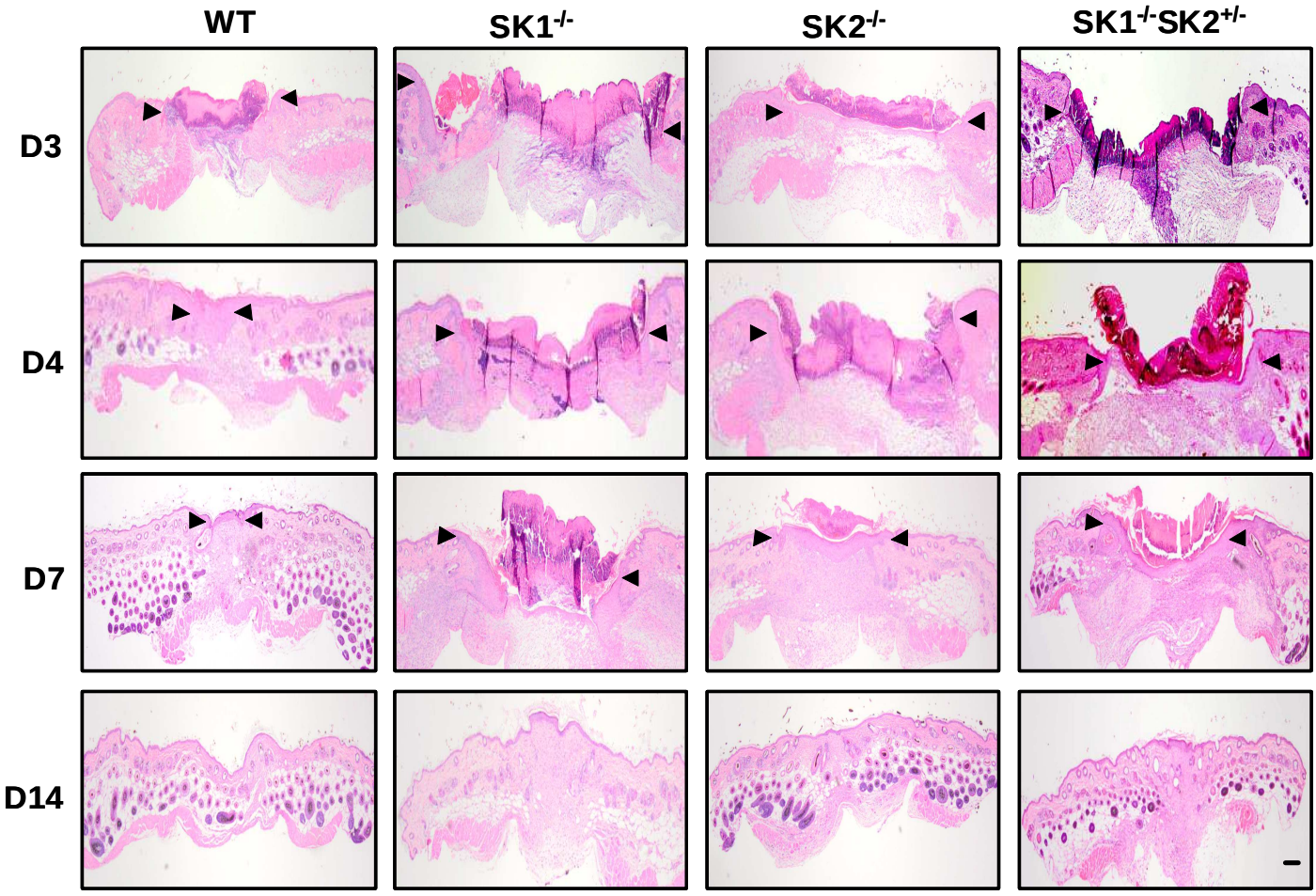
To measure the rate of wound resolution, wounds were harvested from mice of each genotype, cross-sectioned and subject to H/E staining (Fig. 3.4B). The rate of wound resolution was then determined by measuring the wound margin, which is defined as the distance between each migrating tongue of the dermis of a wound (represented by arrows in Fig. 3.4B) (Cowin et al., 2006). Histological analysis showed that the wound margins of $SK1^{-/-}$, $SK2^{-/-}$ and $SK1^{-/-}SK2^{+/-}$ wounds were significantly larger compared with the wildtype control at day 3, 4 and 7 post-

incisional wounding (Fig. 3.4C). Interestingly, the wound margin of the $SK2^{-/-}$ wounds appeared to be slightly smaller compared with $SK1^{-/-}$ and $SK1^{-/-}SK2^{+/-}$ mice but this was not significant. Complete closure of wounds was observed at day 14 in all genotypes (Fig. 3.4). Notably, no phenotypic abnormalities were observed in the skin of SK deficient mice after wound repair (Fig 3.4B). Taken together, these results strongly suggest that SK1 and SK2 play an important positive role in cutaneous wound healing.

A



B



C

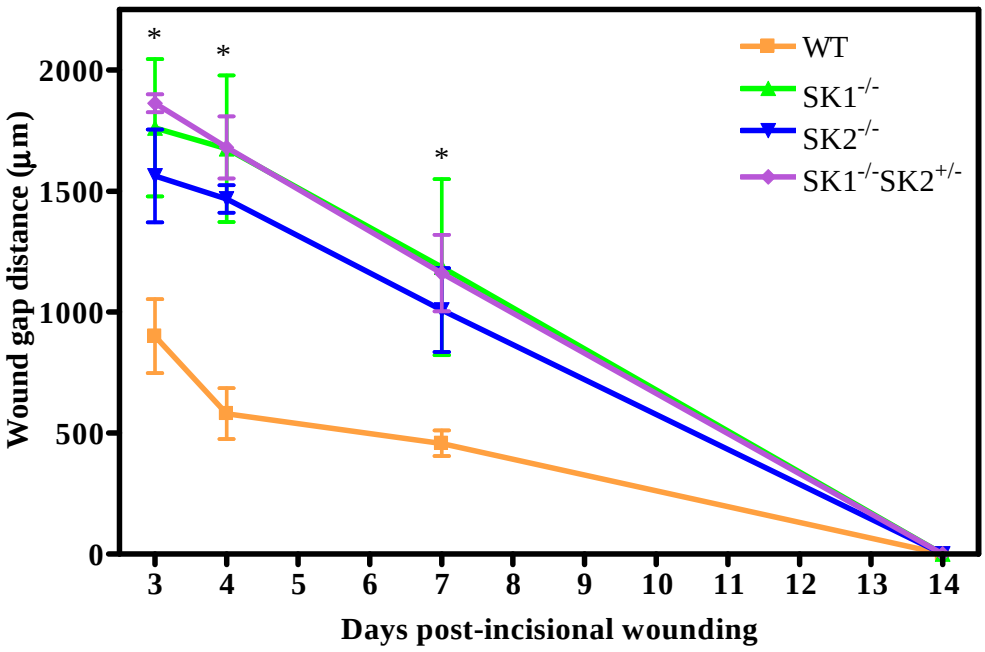


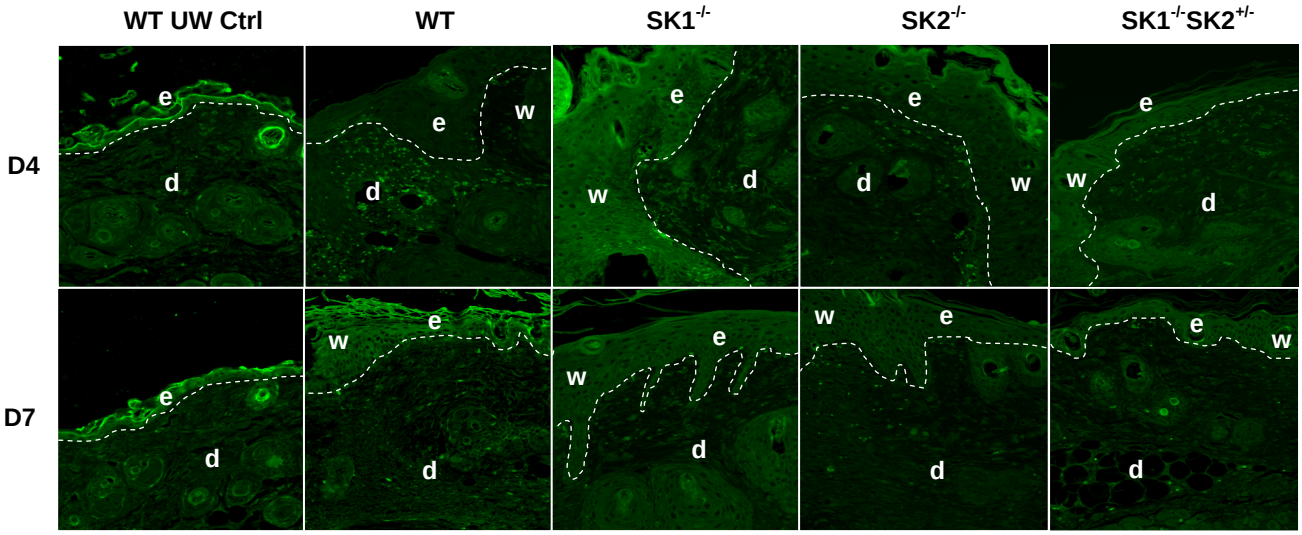
Fig. 3.4 SK deficient mice have impaired wound healing.

Two, full-thickness 1cm incisions were performed on the dorsal skin of twelve weeks old wildtype, $SK1^{-/-}$, $SK2^{-/-}$ and $SK1^{-/-}SK2^{+/-}$ female mice (A). Wounds were allowed to heal by secondary intention for 3, 4, 7 and 14 days. Images of wounds were taken at day 3, 4, 7 and 14 post-wounding. Wounds were harvested at day 3, 4, 7 and 14 post-wounding and fixed. Cross sections were prepared from the harvested wounds and were stained by H/E (B). Wound margins (represented by arrows) were measured from H/E stained sections (B) and were quantified using the Research AnalySIS software (C). Data represents mean ± SD (n=4), *$p<0.05$ (for all three SK knockout lines) compared with the corresponding WT control. Statistical significance was calculated using the student's t-test.

3.4.3 Proliferation is impaired in SK deficient wounds

Since proliferation is a critical aspect of wound repair (Yamaguchi and Yoshikawa, 2001), I next examined whether this was affected in SK deficient mice during wound repair. Histological sections prepared from wildtype, $SK1^{-/-}$, $SK2^{-/-}$ and $SK1^{-/-}SK2^{+/-}$ wounds were subject to immunohistochemistry for proliferating cell nuclear antigen (PCNA) as a marker for proliferating cells. PCNA positive cells were counted in wounds harvested from wildtype, $SK1^{-/-}$, $SK2^{-/-}$ and $SK1^{-/-}SK2^{+/-}$ mice within the migrating tongue of the dermis at the wound edge and the dermal wound bed where cell proliferation in wounds is most prevalent (Fig. 3.5)(Cowin et al., 2007). At day 4 post-incisional wounding, the number of proliferating cells in $SK1^{-/-}$, $SK2^{-/-}$ and $SK1^{-/-}SK2^{+/-}$ were substantially lower compared with wildtype (Fig. 3.5). A similar number of proliferating cells were observed in $SK1^{-/-}$, $SK2^{-/-}$ and $SK1^{-/-}SK2^{+/-}$ wounds. Notably, the number of PCNA positive cells in the wildtype wound was lower at day 7 compared to day 4 (Fig. 3.5) with a similar number of proliferating cells observed in wildtype, $SK1^{-/-}$, $SK2^{-/-}$ and $SK1^{-/-}SK2^{+/-}$ wounds at day 7 (Fig. 3.5). Interestingly, in contrast to my findings, other studies have shown that cellular proliferation was higher at day 7 compared to earlier time points, including day 3 and day 4 (Chen et al., 2014; Cowin et al., 2007). My findings suggest that impaired wound healing response in $SK1^{-/-}$, $SK2^{-/-}$ and $SK1^{-/-}SK2^{+/-}$ appears to be associated with decreased cellular proliferation early in the wound healing response.

A


WT UW Ctrl
WT
SK1-/-
SK2-/-
SK1-/-SK2+/-
D4
D7
e
d
w

B

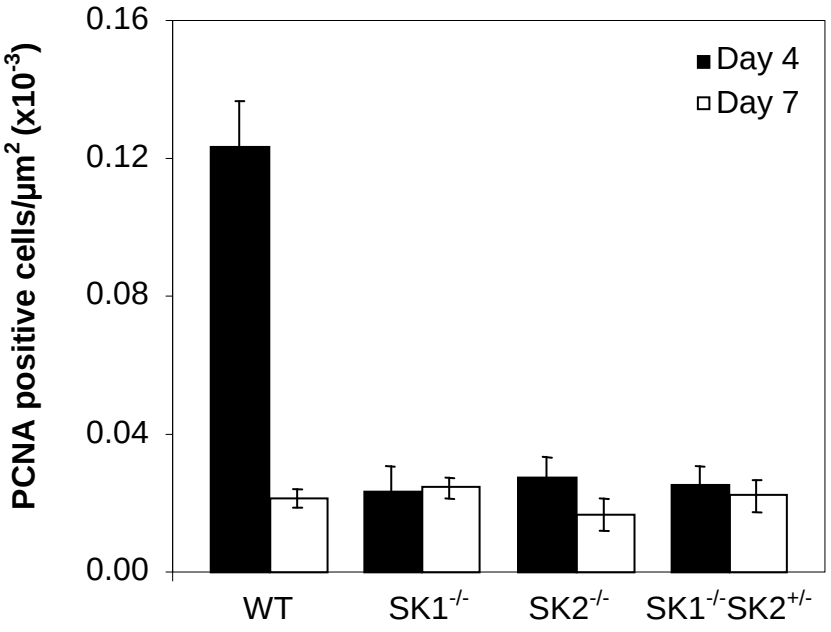

PCNA positive cells/µm2 (x10-3)
0.16
0.12
0.08
0.04
0.00
Day 4
Day 7
WT
SK1-/-
SK2-/-
SK1-/-SK2+/-

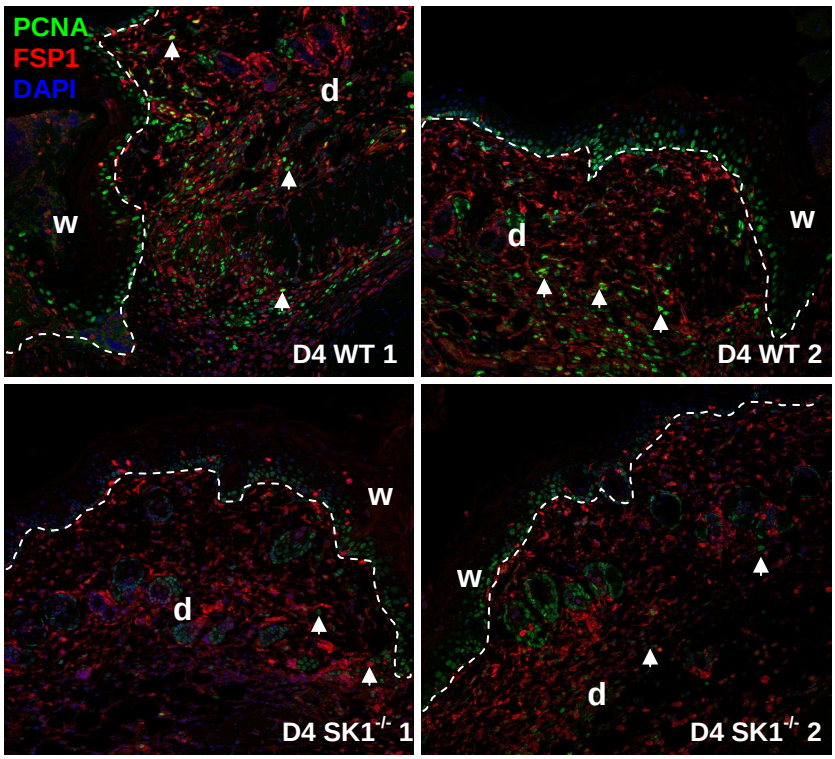
Fig. 3.5 Proliferation is impaired in SK deficient wounds

Two, full-thickness incisions were created on the dorsal skin of twelve weeks old wildtype, $SK1^{-/-}$, $SK2^{-/-}$ and $SK1^{-/-}SK2^{+/-}$ female mice. At day 4 and day 7 post-wounding, wounds were harvested, cross-sectioned and subject to immunohistochemistry for PCNA. Proliferating cells were counted within the migrating tongue of the dermis at the wound edge and the dermal wound bed using the Research AnalySIS software. Data are expressed as number of PCNA positive cells per area (μm^2). The experiment was performed using 2 mice per genotype. Data represent mean ± range. Epidermis, dermis and wound are denoted as e, d and w, respectively.

3.4.4 Number of proliferating fibroblasts is decreased in $SK1^{-/-}$ wounds

To determine whether fibroblast proliferation is impaired in $SK1^{-/-}$ wounds, histological sections prepared from day 4 wildtype and $SK1^{-/-}$ wounds were subject to immunohistochemistry for PCNA and fibroblast-specific protein 1 (FSP1). The number of proliferating fibroblasts (denoted by arrows) was counted as described above. Consistent with my findings above, the number of proliferating fibroblasts is dramatically reduced in $SK1^{-/-}$ wounds (Fig. 3.6). This suggests that the impaired wound healing observed in $SK1^{-/-}$ mice may be a consequence of decreased fibroblast proliferation. It is important to note that this data is preliminary, having been generated from the wounds of only two mice, and thus, needs to be repeated in future studies.

A


PCNA
FSP1
DAPI
d
w
D4 WT 1
d
w
D4 WT 2
w
d
D4 SK1-/- 1
w
d
D4 SK1-/- 2

B

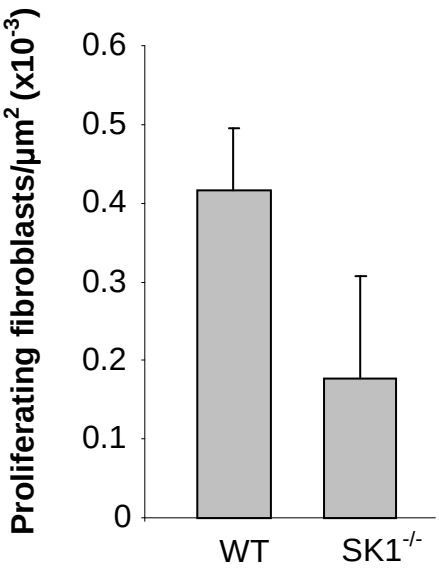

Proliferating fibroblasts/µm2 (x10-3)
0.6
0.5
0.4
0.3
0.2
0.1
0
WT
SK1-/-

Fig. 3.6 Number of proliferating fibroblast is reduced in SK1$^{-/-}$ wounds

Two, full-thickness incisions were created on the dorsal skin of twelve weeks old wildtype and SK1$^{-/-}$ female mice. At day 4 post-wounding, wounds were harvested, cross-sectioned and subject to immunohistochemistry for PCNA and FSP1. Proliferating fibroblasts were counted within the migrating tongue of the dermis at the wound edge and the dermal wound bed using the Research AnalySIS software. Data are expressed as number of proliferating fibroblasts per area (μm^2). The experiment was performed using 2 mice per genotype. Data represent mean ± range. Dermis and wound are denoted as d and w, respectively.

3.4.5 Collagen I expression is not affected in SK deficient wounds

Collagen I is an important matrix protein and its restitution after wounding contributes to overall wound strength and scar formation (Fletcher, 2000). Thus, I next examined whether the collagen I expression pattern was altered in SK deficient mice compared to wildtype mice in response to wounding. Histological sections prepared from day 14 wildtype, $SK1^{-/-}$, $SK2^{-/-}$ and $SK1^{-/-}SK2^{+/-}$ wounds were subject to immunohistochemistry for collagen I (Fig. 3.7). Notably, the increase in collagen I synthesis is a common feature of later stage wound healing in the skin [reviewed in (Baum and Arpey, 2005; Ehrlich and Krummel, 1996)]. Consistent with this, my immunofluorescence data revealed that collagen I expression was upregulated in wildtype mice on day 14 (Fig. 3.7). Similar levels of collagen I was also observed, however in response to wounding in $SK1^{-/-}$, $SK2^{-/-}$ and $SK1^{-/-}SK2^{+/-}$ mice at day 14 (Fig. 3.7). These results suggest that injury-induced collagen I production in mice is not affected by SK deficiency.

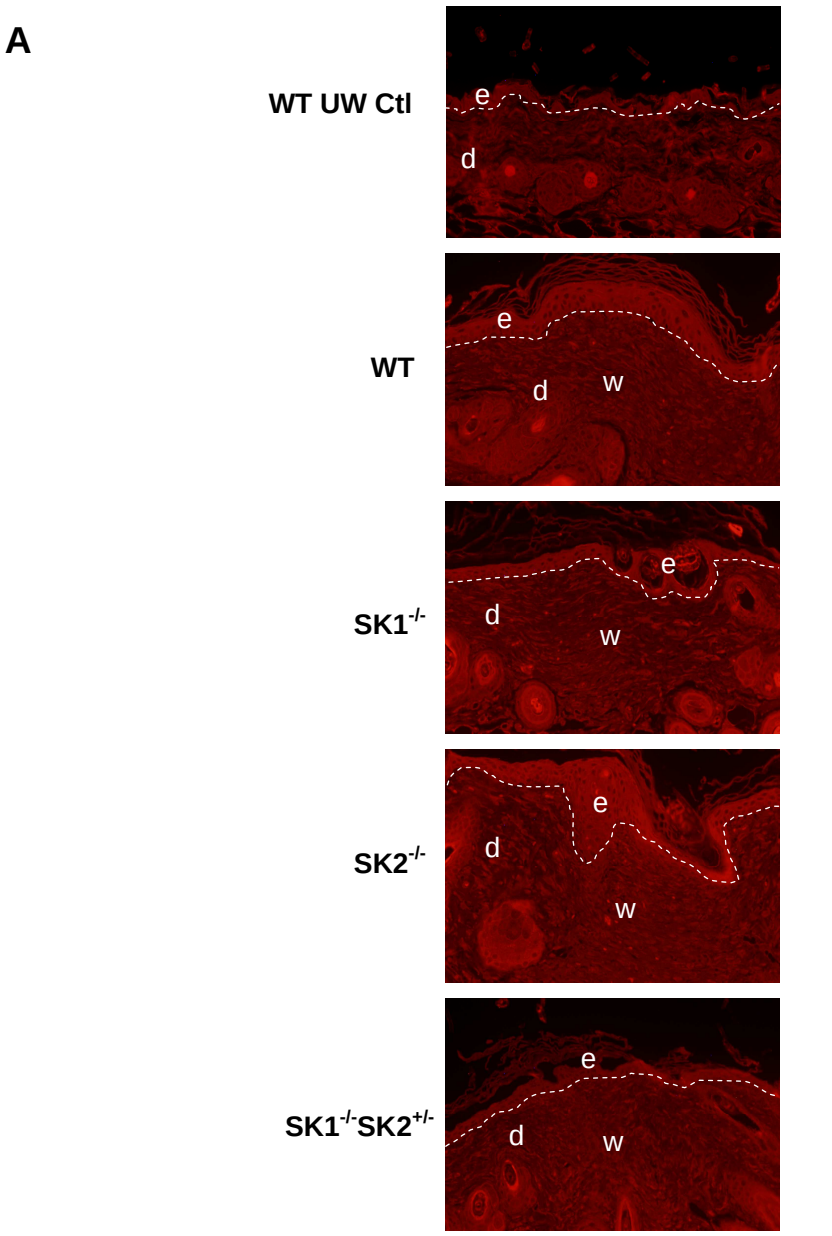

A
WT UW Ctl
e
d
WT
e
d
w
SK1-/-
e
d
w
SK2-/-
e
d
w
SK1-/-SK2+/-
e
d
w
B
Collagen I
(Fluorescence Intensity)
100
90
80
70
60
50
40
30
20
10
0
WT UW
WT
SK1-/-
SK2-/-
SK1-/-SK2+/-
Day 14 wounds

Fig. 3.7 Collagen I expression is not affected in SK deficient wounds

Two, full-thickenss incisions were created on the dorsal skin of twelve weeks old wildtype, $SK1^{-/-}$, $SK2^{-/-}$ and $SK1^{-/-}SK2^{+/-}$ female mice. At day 14 post-wounding, wounds were harvested, cross-sectioned and stained for collagen I. Expression levels of collagen I in the dermis were measured using the Research AnalySIS software and are expressed as fluorescence intensity (arbitrary units/μm^2). The experiment was performed using 2 mice per genotype. Data represent mean ± range. Epidermis, dermis and wound are denoted as e, d and w, respectively. UW denotes unwounded skin.

3.4.6 Myofibroblasts numbers are not affected in SK deficient wounds

Since myofibroblasts are the cell type responsible for ECM production, wound contraction and scar formation (Hinz, 2007; Hinz et al., 2007), I next examined whether the distribution and the number of myofibroblasts were altered in the SK deficient mice in response to wounding. The presence of myofibroblasts was determined by measuring the fluorescence intensity of α-SMA staining within the dermal wound bed where these cells are normally found (Fig. 3.8) (Baum and Arpey, 2005). Since myofibroblasts are usually found in the later stage of wound healing (Baum and Arpey, 2005), histological sections were prepared from day 14 wildtype, $SK1^{-/-}$, $SK2^{-/-}$ and $SK1^{-/-}SK2^{+/-}$ wounds, and subject to immunohistochemistry for α-SMA (Fig. 3.8). Immunofluorescence analysis showed that α-SMA expression was upregulated to a similar extent in response to wounding in all genotypes (Fig. 3.8). In addition, distribution of myofibroblasts within the dermal wound bed of $SK1^{-/-}$, $SK2^{-/-}$ and $SK1^{-/-}SK2^{+/-}$ appeared to be similar to that of the wildtype (Fig. 3.8). These data suggest that the number and distribution of myofibroblasts within the dermal wound bed in mice during later stages of wound healing are not affected by SK deficiency. It is important to note that this data is preliminary, having generated from the wound of only two mice, and thus, needs to be repeated in future studies.

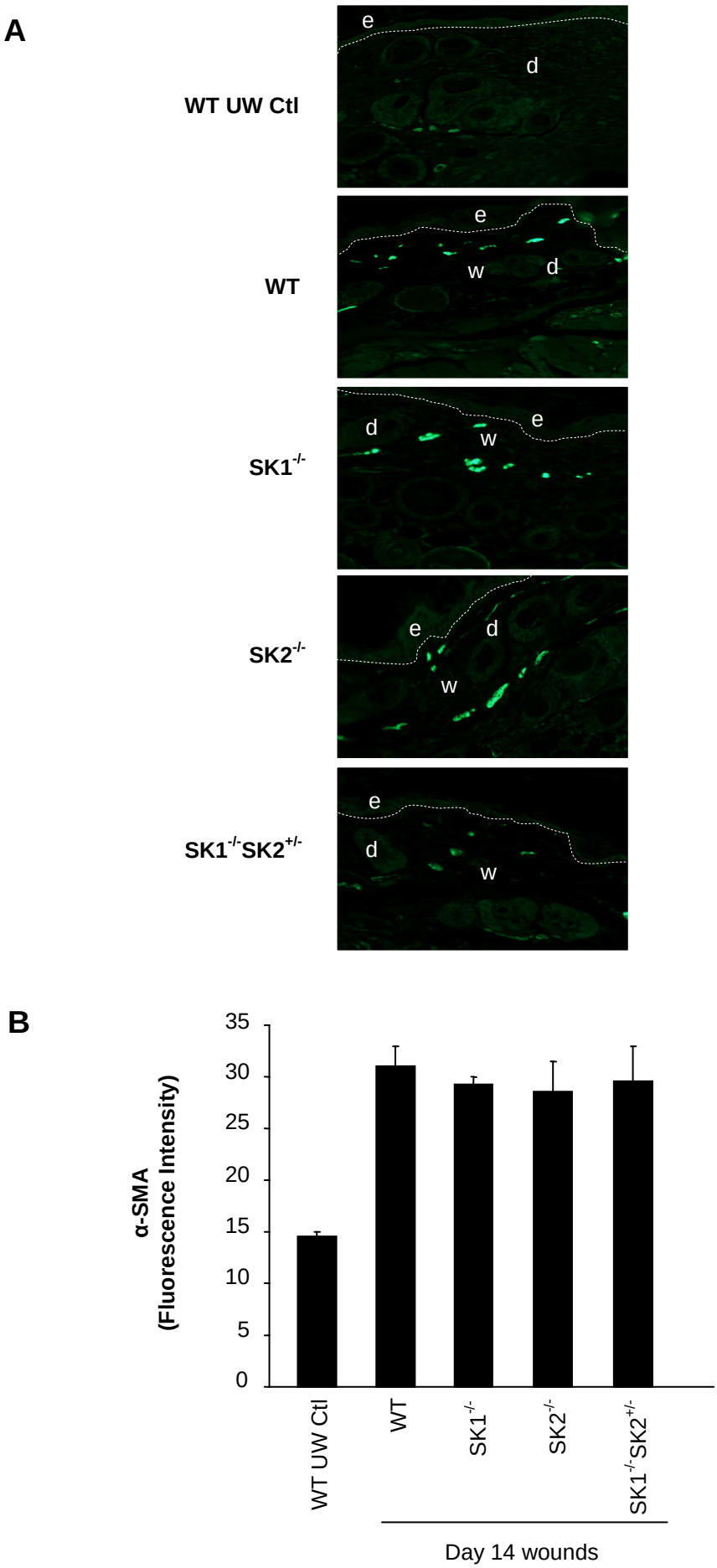

A
WT UW Ctl
WT
SK1-/-
SK2-/-
SK1-/-SK2+/-
e
d
w
B
α-SMA
(Fluorescence Intensity)
35
30
25
20
15
10
5
0
WT UW Ctl
WT
SK1-/-
SK2-/-
SK1-/-SK2+/-
Day 14 wounds

Fig. 3.8 Myofibroblasts expression is not affected in SK deficient wounds

Two, full-thickness incisions were created on the dorsal skin of twelve weeks old wildtype, $SK1^{-/-}$, $SK2^{-/-}$ and $SK1^{-/-}SK2^{+/-}$ female mice. At day 14 post-wounding, wounds were harvested, cross-sectioned and stained for α-SMA. Expression levels of α-SMA in the dermis were measured using the Research AnalySIS software and are expressed as fluorescence intensity (arbitrary units/μm^2). The experiment was performed using 2 mice per genotype. Data represent mean ± range. Epidermis, dermis and wound are denoted as e, d and w, respectively. UW denotes unwounded skin.

3.5 Discussion

While the roles of SKs in cancer and other disease conditions have been well established [reviewed in (Heffernan-Stroud and Obeid, 2013; Orr Gandy and Obeid, 2013)], their roles in wound healing are unknown. In this study, I have shown for the first time that the SKs play an important role in wound healing of the skin (Fig. 3.4). Furthermore, my studies have also shown that SK deficient mice have impaired cellular proliferation during the early stage of wound healing (Fig. 3.5). Collagen I expression levels in the wounds of SK deficient mice were similar to that of the wildtype mice at day 14 post-incisional wounding (Fig. 3.7). In addition, my studies also showed that the number of myofibroblasts in the SK deficient mice was similar to that of the wildtype at day 14-post-incisional wounding (Fig. 3.8).

Although the role of SK in wound healing has not been described, given that SK1 is involved in the regulation of a variety of fundamental cellular processes that are also known to be critical for wound healing, it appears reasonable to hypothesise that this enzyme may also be involved in wound healing. Indeed, my studies presented here showed that SK deficient mice have impaired wound healing compared with wildtype mice (Fig. 3.4). This strongly suggests that the SKs play a crucial role in wound healing *in vivo*.

Proliferation is one of the fundamental aspects during early stage of wound healing (Krieg and Heckmann, 1989). Therefore, it is not surprising that alteration in cellular proliferation can have significant impact on the outcome of wound healing. This notion is supported by previous studies showing that the deletion of pro-proliferative proteins, in most cases, resulted in impaired wound healing in mice (Liang et al., 2012; Rashel et al., 2014). Since SK1 and in some cases SK2, have been shown to promote cellular proliferation [reviewed in (Hannun and Obeid, 2008;

Neubauer and Pitson, 2013; Pyne and Pyne, 2010)], I hypothesised that SK knockout mice would have reduced cellular proliferation in the healing wound compared with the wildtype. As expected, my results showed that the wounds of $SK1^{-/-}$, $SK2^{-/-}$ and $SK1^{-/-}SK2^{+/-}$ mice at day 4 post-wounding have reduced number of proliferating cells compared with the wildtype (Fig. 3.5). While it is tempting to speculate that the impaired wound healing observed in the SK deficient mice were in part due to impaired cellular proliferation, further experiments involving additional mice will need to be performed to validate the result. Interestingly, the decrease in cellular proliferation in $SK2^{-/-}$ mice following wounding suggests that SK2 plays a pro-proliferative role, like SK1. This is in contrary to a number of studies that suggest that SK2 is pro-apoptotic (Igarashi et al., 2003; Maceyka et al., 2005; Okada et al., 2005). Indeed, although there is compelling evidence to suggest that SK2 can have a physiological role in mediating apoptosis, there are now many studies that also supporting a role for SK2 in promoting survival and proliferation, much like SK1 (Nemoto et al., 2009; Sankala et al., 2007; Schnitzer et al., 2009; Van Brocklyn et al., 2005). Thus, it is tempting to speculate that both SKs are essential for wound healing and a slight decrease in total SK expression is sufficient to impair wound healing.

While other studies have shown that the number of proliferating cells in mouse wounds peaked at approximately day 7 (Chen et al., 2014; Cowin et al., 2007), this was not observed in my study. This could be due to an issue with the preparation of day 7 tissue sections since all genotypes showed consistently low number of proliferating cells. The immunostaining of day 7 skin tissues for proliferating cells should be repeated in future studies. In addition, the total number of proliferating fibroblasts (Fig. 3.6) appeared to be higher than the total number of proliferating cells (Fig. 3.5). While the reasons for this discrepancy are unknown this may have been

caused by the immunohistochemistry experiments shown in Fig. 3.5 and 3.6 having been performed on different days using wounds from different mice. In addition, the staining background in Fig 3.5 is higher compared to Fig. 3.6 which may have caused PCNA positive cells to appear as non-proliferative cells in Fig. 3.5. Both experiments will need to be repeated with a larger cohort of animals to clarify this issue.

My data also showed that there was substantially less proliferating fibroblasts in the day 4 wounds of $SK1^{-/-}$ compared with the wildtype (Fig. 3.6). This suggests that the impaired wound healing observed at least in the $SK1^{-/-}$ mice is, in part due to decreased fibroblast proliferation. This may also be a reason for the impaired wound healing observed in $SK2^{-/-}$ and $SK1^{-/-}SK2^{+/-}$. It is important to note that since this study was performed using only 2 mice per genotype, this study will need to be repeated with additional mice per genotype to validate these findings.

Interestingly, while it was not statistically significant, the $SK2^{-/-}$ mice appeared to have less impaired healing compared with $SK1^{-/-}$ and $SK1^{-/-}SK2^{+/-}$ mice (Fig. 3.4C). Notably, consistent with previous studies (Kharel et al., 2012; Olivera et al., 2007; Sensken et al., 2010; Zemann et al., 2006), our data also showed that circulating S1P levels in $SK2^{-/-}$ mice were approximately 2-fold higher than the wildtype (Fig. 3.3), possibly due to the compensatory effect and the upregulation of SK1 expression (Liang et al., 2013). Thus, whether this increase in circulating S1P contributed to the slightly less impaired healing observed in $SK2^{-/-}$ mice awaits further investigation. My findings however, suggest that circulating S1P is unlikely to play a role in wound healing since significant defects in wound healing were also observed in $SK2^{-/-}$ mice (Fig. 3.4C). Since the deletion of either SK isoform resulted in impaired wound healing, it appears reasonable to speculate that rather than circulating S1P, wound-localised S1P may play an important role in wound healing. Indeed, this

notion is supported by the fact that S1P is enriched in wounds (Amano et al., 2004) and that application of S1P into diabetic wounds significantly enhanced wound resolution (Kawanabe et al., 2007). Furthermore, although no evidence showing a direct increase in wound-localised S1P levels, a recent study has shown that the application of an expression plasmid encoding SK1 into wounds of diabetic rats significantly improved wound healing (Yu et al., 2014). My findings together with the published data suggest that wound-localised S1P may play an integral role in the process of wound healing. Future studies should examine the S1P levels in the wounds of SK deficient mice. We speculate that the levels of S1P in the wounds of these mice would be reduced, thus resulting in impaired cutaneous wound healing.

Collagen production and wound contraction by wound-associated myofibroblasts are essential for later stage wound healing [reviewed in (Baum and Arpey, 2005; Ehrlich and Krummel, 1996)]. Although the exact origin of myofibroblasts in the healing wound is not clear, it is thought that the majority is recruited locally from the dermis and tissues around the wound site (Hinz et al., 2007). Notably, a key feature of myofibroblasts is to provide contractile forces that bring the wound edges together, which is observed in normal wound healing (Bainbridge, 2013). Previous studies have shown that S1P is important for myofibroblast differentiation of fibroblast from various tissues (Gellings Lowe et al., 2009; Sobel et al., 2013), including the skin (Keller et al., 2007), and the expression of ECM components including collagen I and III (Hamidi et al., 2014). Thus, the levels of S1P in a wound could impact on the outcome of wound healing. In my study, the impaired wound healing observed in the SK knockout mice could be a consequence of decreased local S1P levels. Therefore, I hypothesised that the wounds of SK deficient mice would contain less myofibroblasts and collagen I compared with

wildtype mice, therefore resulting in impaired wound healing. Interestingly, however, my findings showed that collagen I expression levels and the number of myofibroblasts were similar between the wounds of SK deficient mice and wildtype at day 14 post-wounding (Fig. 3.7 and 3.8). It is important to note, however, that my studies only focused on collagen I since it is the most abundant type of collagen of the ECM and it makes up about 80% of total collagen content in the granulation tissue (Ehrlich and Krummel, 1996). Notably, there are 16 different types of collagen, some of which have unknown function while others, including collagen III and VII, are essential for wound healing (van der Rest and Garrone, 1991). Although my study has shown that collagen I expression levels was unaffected in the wounds of SK deficient mice, further studies need to be performed to examine whether other types of collagen such as collagen III or ECM components are altered by SK deficiency.

In conclusion, my studies in this chapter have established for the first time a novel role of SK in wound healing. It is clear that further work remains to be done to understand the underlying cellular and molecular defects in wound healing of SK deficient mice. Notably, since a number of experiments performed in this chapter have only analysed a limited number of animals (n=2), these studies will need to be repeated with additional mice to validate these results. Nonetheless, knowledge gained from these studies will provide useful insights to the wound healing process and may be exploited as a therapeutic treatment for problematic wounds such as diabetic foot ulcers. For example, S1PR agonists can be used as potential treatment to stimulate healing in problematic wounds. Alternatively, given that SK is important for wound healing, future wound healing therapies should also exploit ways to increase SK activity in problematic wounds, such as the strategy examined in Chapter 5.

CHAPTER 4

Characterisation of the Role of SKAM1 in Wound Healing

4. Characterisation of the role of SKAM1 in wound healing

4.1 Abstract

SKAM1 is a protein that was identified in a yeast two-hybrid screen as a SK1 interacting protein. Previous studies performed in the Pitson laboratory have confirmed the interaction between SKAM1 and SK1 in cells, and shown that SKAM1 can directly enhance SK1 enzymatic activity both in cells and *in vitro*. Interestingly, SKAM1 has also been shown previously to be involved in wound healing. However, the mechanism of SKAM1-mediated wound healing was unknown. In this Chapter I detail studies to elucidate the biological effects of SKAM1, and develop mouse systems to enable future *in vivo* work to understand SKAM1 biology. Given the ability of SKAM1 to activate SK1 and the known effects of SK1 in enhancing cell proliferation, survival and migration, here I examined the ability of SKAM1 to elicit similar effects in cultured cells. I have shown that while SKAM1 overexpression did not alter cell migration and proliferation, it did result in enhanced cell survival in response to serum deprivation. Notably, however, unlike SK1 overexpression, overexpression of SKAM1 in NIH3T3 fibroblasts did not result in neoplastic transformation. In studies to examine SKAM1 in cutaneous wounds, I found that SKAM1 mRNA was significantly upregulated in the wounded skin of mice. In addition, immunohistochemistry with anti-SKAM1 antibodies showed an increased number of SKAM1 positive cells in wounded skin compared to unwounded skin. I subsequently generated SKAM1A transgenic mice to study the role of SKAM1 *in vivo*. Notably, SKAM1A was overexpressed in all tissues examined of the transgenic mice. Extensive analysis of various mouse organs revealed no observable phenotypical differences between SKAM1A transgenic and wildtype mice when compared at 12 or 48 weeks of age. Primary mouse embryonic fibroblasts (MEFs)

isolated from SKAM1A transgenic embryos, however, showed enhanced ability to contract collagen matrix compared with the wildtype. This led me to examine the rate of wound healing following incisional and excisional wounding in these mice. I found no observable difference in wound healing between the SKAM1A transgenic and wildtype mice following incisional wounding. SKAM1A transgenic mice did, however, showed enhanced wound resolution compared with wildtype mice following full-thickness excisional wounding.

4.2 Introduction

SKAM1/FGFR1OP2/wit 3.0 is protein that was identified in a yeast two-hybrid screen as a sphingosine kinase 1 (SK1) interacting protein. Four naturally occurring splice variants of SKAM1 exist (A, B, C and D), which all interact with SK1 (Fig. 1.3). Notably, the interaction of all isoforms of SKAM1 can enhance the catalytic activity of SK1 in *vitro* and in cells (Fig. 1.4). Interestingly, SKAM1 expression was found to be upregulated in the oral mucosa following tooth extraction in rat (Sukotjo et al., 2002; Sukotjo et al., 2003). Studies have also shown that high levels of SKAM1 were expressed in the connective tissue adjacent to the tooth extraction socket (Sukotjo et al., 2002; Sukotjo et al., 2003). This was the first evidence suggesting that SKAM1 may be associated with the wound healing of the oral mucosa. In addition, overexpression of SKAM1 in NIH3T3 and rat oral fibroblasts was shown to result in enhanced collagen gel contraction by these cells, suggesting that SKAM1 may be involved in wound healing (Sukotjo et al., 2003). Notably, a more recent study has shown that lentiviral-mediated SKAM1 overexpression in excisional skin wounds significantly enhanced wound closure (Lin et al., 2010). Together, these findings suggest that SKAM1 may be an important mediator of wound healing. To date, however, the molecular mechanism underlying SKAM1-mediated wound closure is unknown. Therefore, the focus of my work is to fill in this gap with knowledge by 1) characterising the role of SKAM1 in fundamental aspects of wound healing, including proliferation, survival and migration, and 2) developing mouse models to study SKAM1 in wound healing *in vivo*.

4.3 Preliminary data: Targeting SK or $S1P_{1/3}$ blocks SKAM1-induced collagen contraction

Previous studies have shown that SKAM1 can enhance collagen contraction by fibroblasts and lentiviral-mediated delivery of SKAM1 to mouse excisional wounds enhanced wound healing (Lin et al., 2010). However, the underlying mechanism of SKAM1-mediated wound healing was unknown. Since SKAM1 interacts with SK1 and enhances its activity, the Pitson Laboratory examined whether SK1 is involved in this process. Consistent with previous studies (Lin et al., 2010; Sukotjo et al., 2003), SKAM1 overexpression significantly enhanced collagen contraction by NIH3T3 cells (Fig. 4.1A). Notably, this process was dependent on SK1 since the overexpression of a dominant-negative SK1 [SK1(G82D)] (Pitson et al., 2000), or the treatment of a SK1 inhibitor, SKi, blocked SKAM1-induced contraction by NIH3T3 cells (Fig. 4.1B). Furthermore, SKAM1-induced collagen contraction was shown to be mediated by $S1P_1$ and $S1P_3$ since antagonism of both $S1P_1$ and $S1P_3$ (with VPC23019) significantly blocked SKAM1-induced collagen contraction (Fig. 4.2). Antagonism of $S1P_2$ (with JTE013) had no effect (Fig. 4.2). Interestingly, inhibition of either $S1P_1$ (with W146) or $S1P_3$ (with CAY10444) alone had no significant effect on SKAM1-induced collagen contraction (Fig. 4.2). A combination of both antagonists, however, blocked the effects of SKAM1, suggesting that signalling via either $S1P_1$ or $S1P_3$ is sufficient (Fig. 4.2).

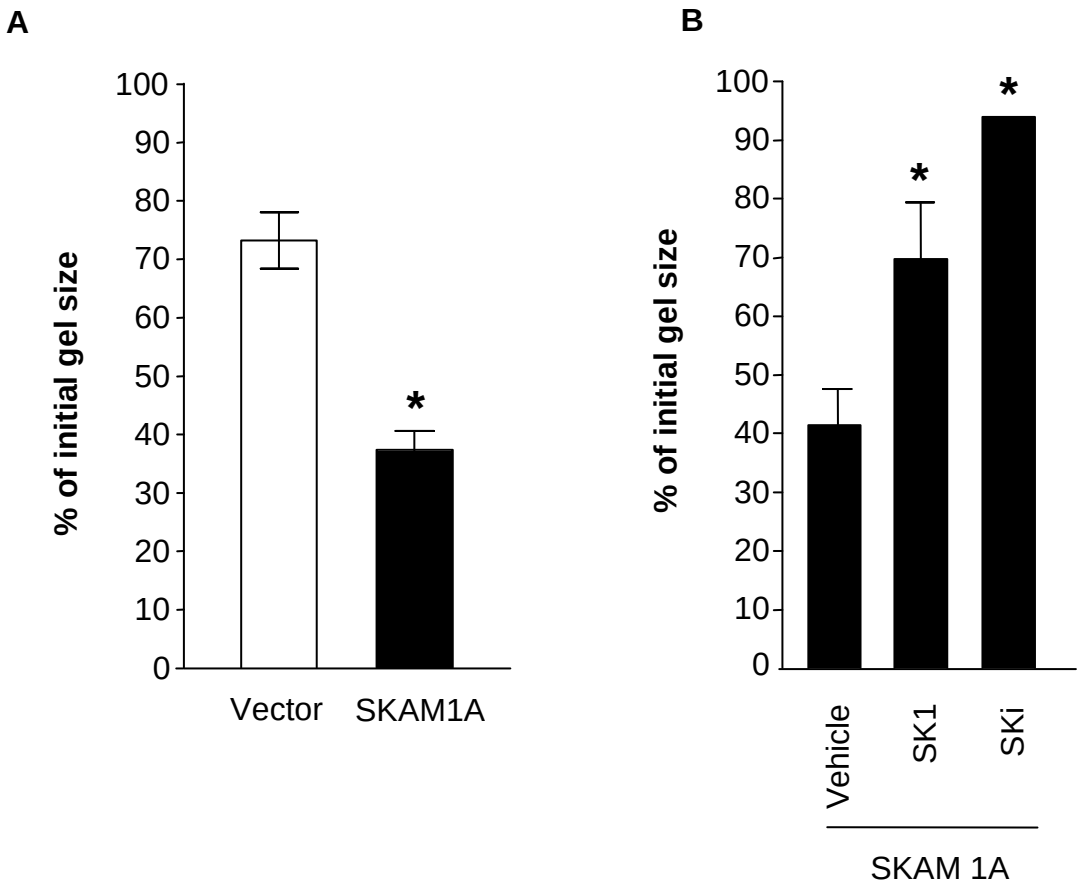


Fig. 4.1 SKAM1-induced collagen contraction is mediated by SK1

NIH3T3 cells transiently overexpressing SKAM1A or vector control cells were subject to collagen gel contraction assay and gel sizes assessed at 28hr after release, n=4,*p<0.0005 (A). NIH3T3 cells transiently overexpressing SKAM1A were treated with SKi (5μM) or co-transfected with SK1(G82D) and were subjected to collagen contraction assay with gel sized assessed 28hr after release, n=4,*p<0.05 (B). Statistical analysis was performed using student's t-test. (Gliddon & Pitson, unpublished).

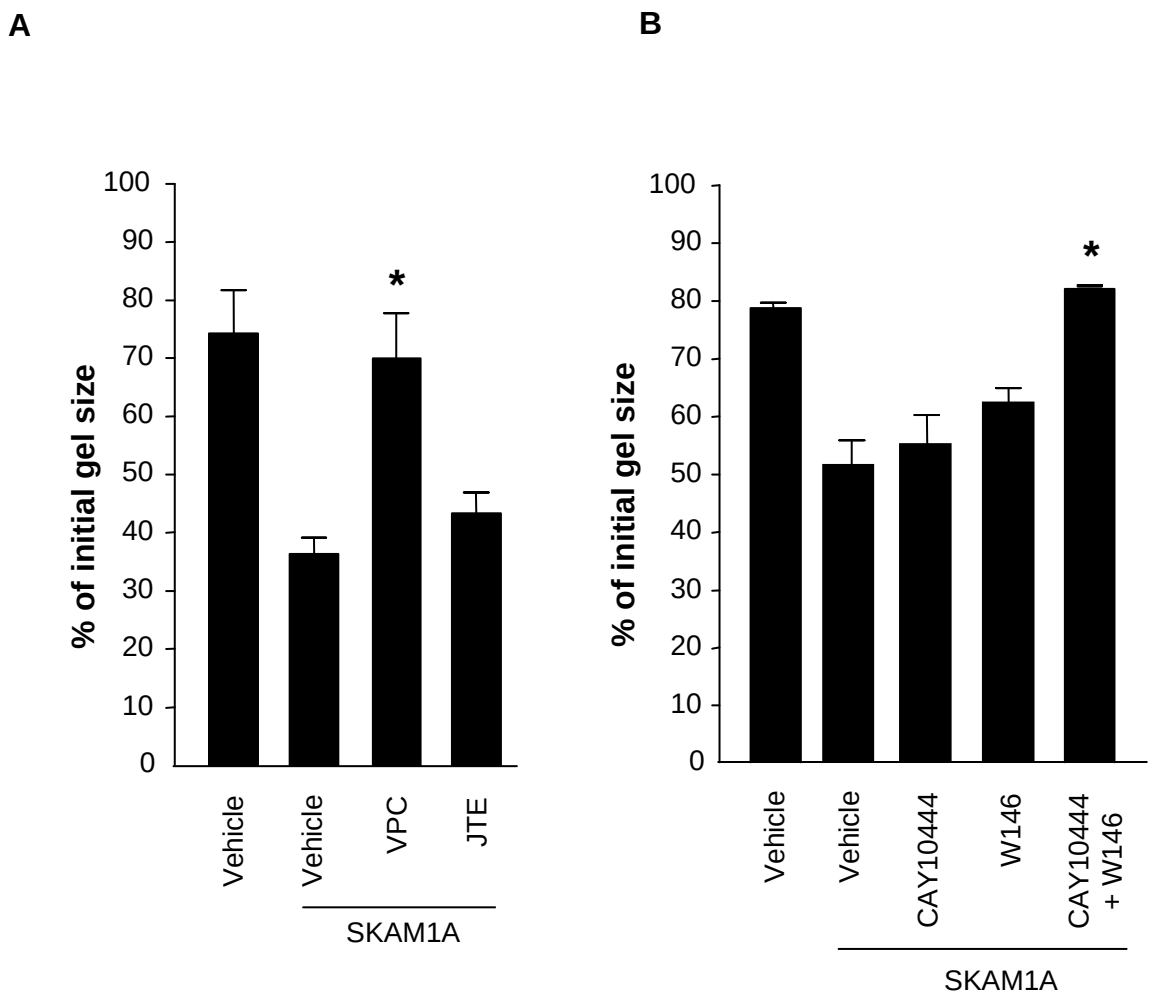


Fig. 4.2 Targeting $S1P_{1/3}$ blocked SKAM1-induced collagen contraction by NIH3T3 cells

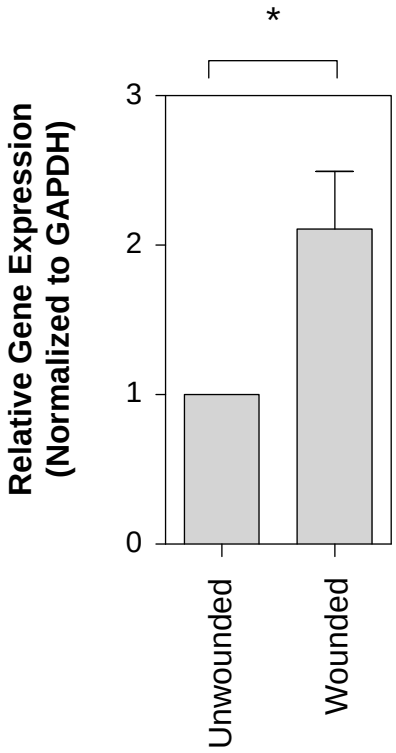
NIH3T3 cells transiently overexpressing SKAM1A were treated with VPC23019 ($S1P_{1/3}$ antagonist) or JTE013 ($S1P_2$ antagonist) and subjected to collagen contraction assays with gel sizes assessed at 28hr after release, n=4, *$p<0.05$ (A). NIH3T3 cells overexpressing SKAM1A were treated with CAY10444 ($S1P_3$ antagonist), W146 ($S1P_1$ antagonist) or both and subjected to collagen contraction assays with gel sizes assessed at 28hr after release, n=4,*$p<0.05$ (B). Statistical analysis was performed using student's t-test. (Gliddon & Pitson, unpublished).

4.4 Results

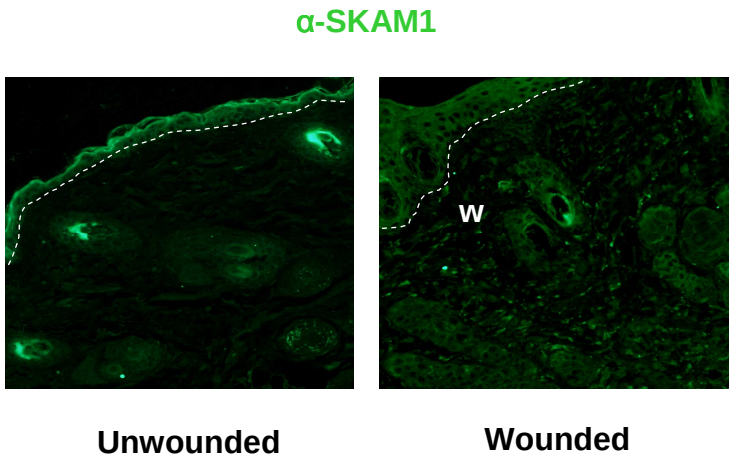
4.4.1 SKAM1 is upregulated during wound healing *in vivo*

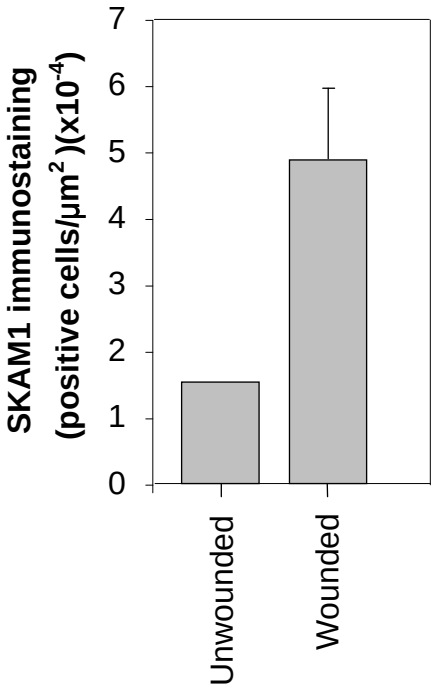
Previous studies have shown that SKAM1 expression was significantly upregulated following wounding in the rat oral mucosa by tooth extraction (Sukotjo et al., 2002). To examine whether SKAM1 was also upregulated in skin following injury, twelve week old C57BL/6 female wildtype mice were subject to incisional wounding via the formation of 1cm long full-thickness incisional wounds on their backs. Wounded and unwounded skin tissues were collected at day 7 post-wounding and were subjected to qPCR analysis for SKAM1 mRNA expression. QPCR results showed that, like in the wounded oral mucosa, SKAM1 mRNA levels were also significantly upregulated (2-fold) in the wounded skin tissue compared with the unwounded control (Fig. 4.3A). This upregulation of SKAM1 mRNA in skin wounds of mice, however was lower than that observed in the oral mucosa of rats. Furthermore, immunostaining of histological sections derived from wounded and unwounded skin tissues with an anti-SKAM1 antibody showed the wounded skin had higher numbers of SKAM1 positive cells (Fig. 4.3B) and higher overall levels of SKAM1 in the dermis (Fig. 4.3C) compared with the unwounded control. The commercially available rabbit SKAM1 polyclonal antibody (Protein Tech) was tested by the manufacturer against endogenous SKAM1 in mouse thymus tissue. Western blot shows the antibody to be specific against SKAM1A in mice. The antibody, however, has not been previously examined for use with immunofluorescence.

A


*
Relative Gene Expression
(Normalized to GAPDH)
3
2
1
0
Unwounded
Wounded

B


α-SKAM1
W
Unwounded
Wounded


SKAM1 immunostaining
(positive cells/μm²)(x10⁻⁴)
7
6
5
4
3
2
1
0
Unwounded
Wounded

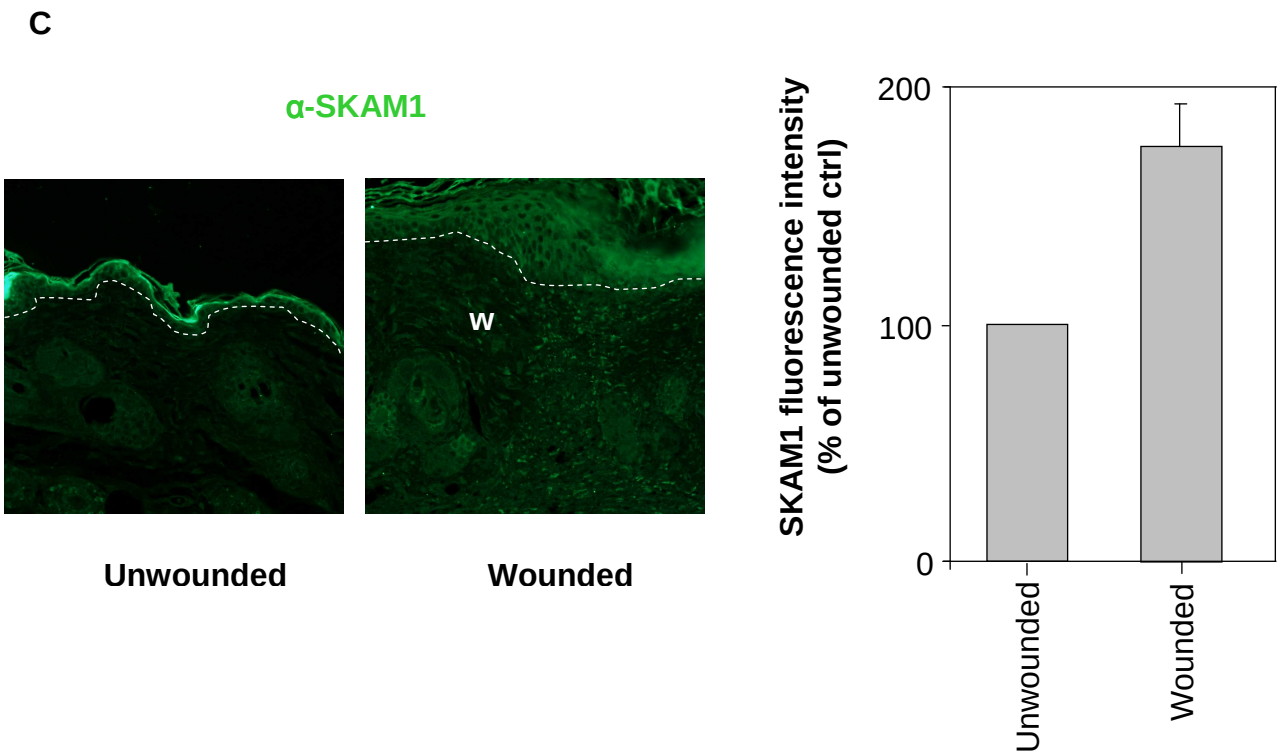


Fig. 4.3 SKAM1 was upregulated during wound healing *in vivo*

QPCR analysis of wounded and unwounded mouse skin tissues harvested at day 7 post-incisional wounding. The expression of mRNA was normalised against the housekeeping gene glyceraldehyde phosphate dehydrogenase (GAPDH) (A). Data represent the mean ± SD from three independent experiments, each analysed in triplicate. *p< 0.05 compared with the unwounded control. SKAM1 immunostaining of day 7 wildtype wounded and unwounded skin (B and C). Data represent number of SKAM1 positive cells per μm^2 (B) and percentage difference in total fluorescence intensity (C) in the dermis. Dotted line and 'w' indicate the epidermis and the wound, respectively. Skin sections were immunostained with primary rabbit anti-SKAM1 polyclonal antibody. Fluorescence was detected at 488nm. Data quantified using the AnalySIS software and represents the mean ± range from two independent experiments (C). Statistical significance was calculated using the student's t-test.

4.4.2 SKAM1 expression appears to be regulated by TGFβ1 and PDGF

Growth factors and cytokines are critical mediators of wound healing (Eming et al., 2007). Given that SKAM1 expression was induced by wounding, we sought to examine whether SKAM1 expression is regulated by growth factors commonly associated with the wound response. NIH3T3 fibroblasts were treated with a number of growth factors for 24hr and SKAM1 mRNA assessed by qPCR (Fig. 4.4). As shown in Fig. 4.4, SKAM1 mRNA expression appeared modestly upregulated following exposure to TGFβ1 or PDGF treatment, but was unaltered by the presence of VEGF and connective tissue growth factor (CTGF). It should be noted, however, that positive controls for the effectiveness of growth factor stimulations were not performed in these studies, so the results should be interpreted with some caution.

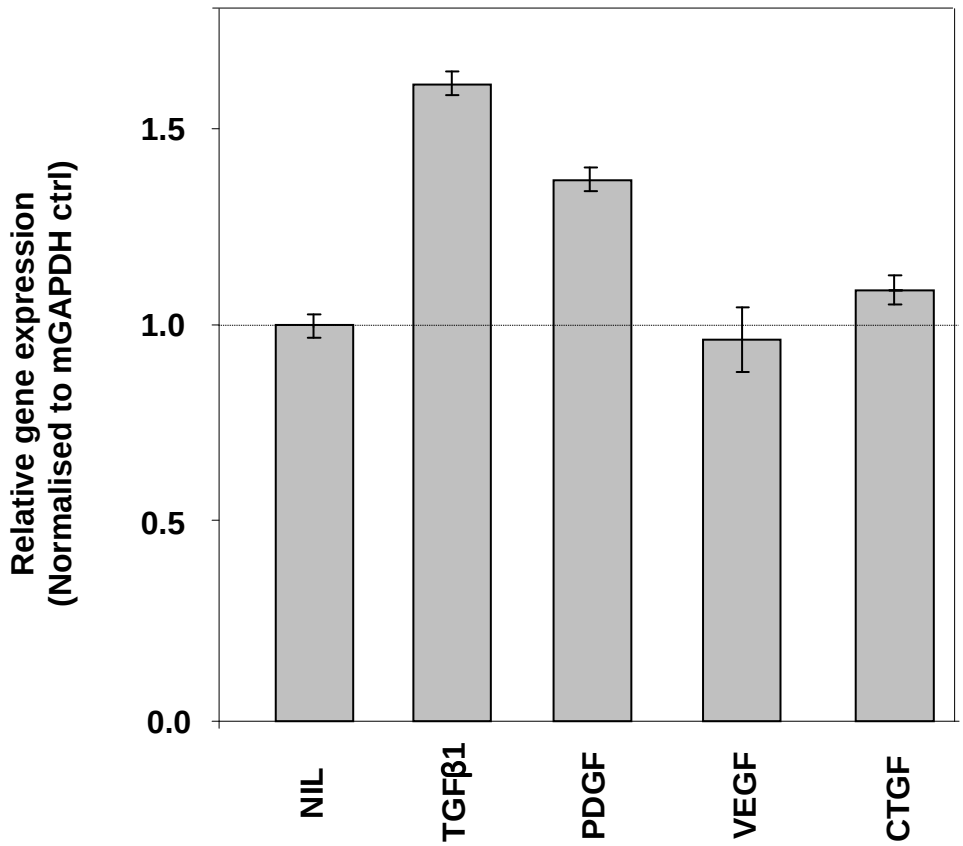


Fig. 4.4 SKAM1 expression appears to be regulated by TGFβ1 and PDGF

NIH3T3 fibroblasts were cultured in serum-free DMEM containing 1% penicillin/streptomycin for 24hr at 37°C and 5% CO_2. Cells were then treated with serum-free DMEM containing TGFβ1 (0.2ng/ml), PDGF (6ng/ml), VEGF (10ng/ml) or CTGF (1µg/ml) for 24hr. RNA was isolated from cells using Trizol and was subject to cDNA synthesis. qPCR was performed as described in section 2.7. Data represents the mean ± range from two independent experiments each performed in triplicate.

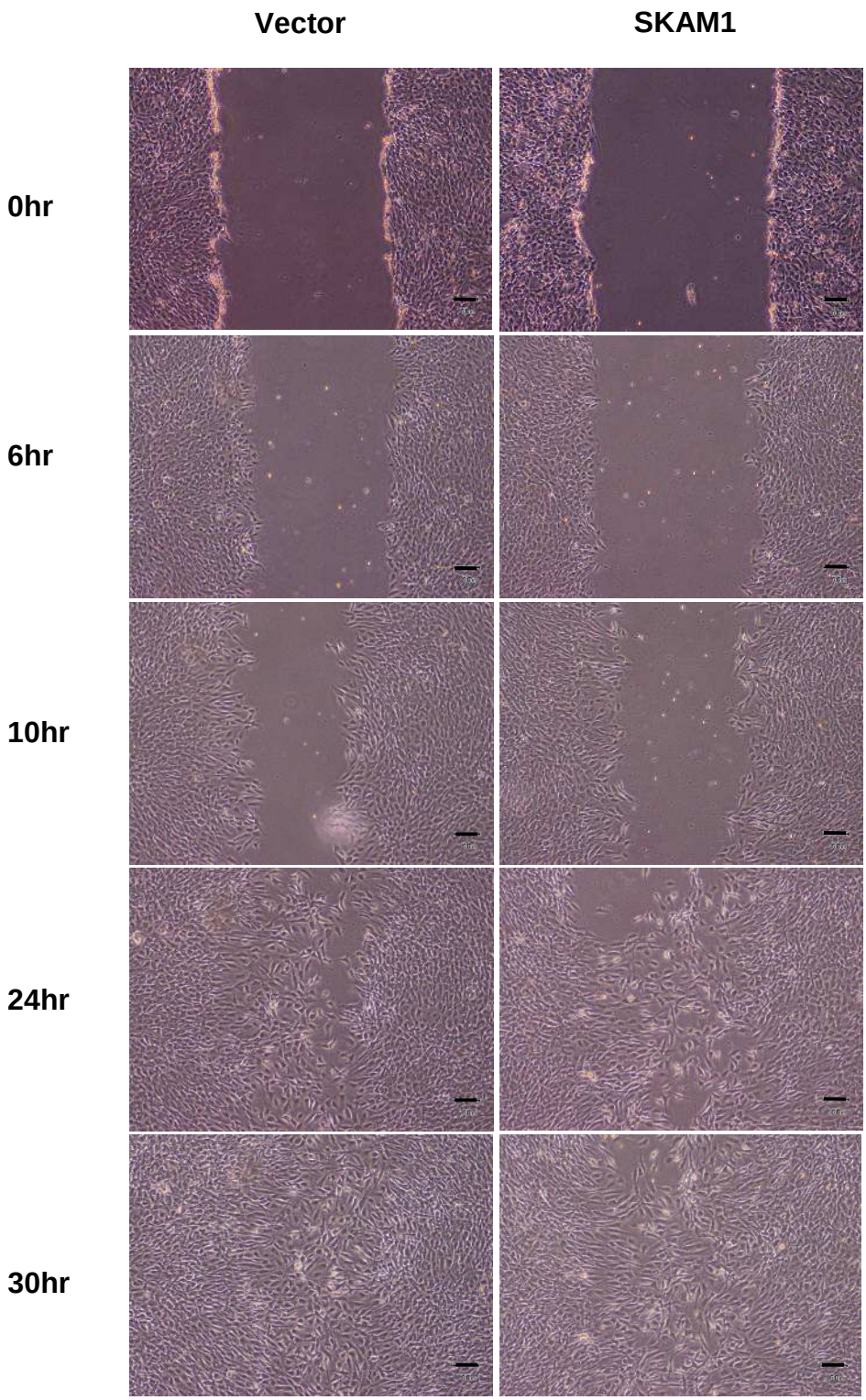
4.4.3 SKAM1 overexpression does not affect cell migration

It has long been known that cell migration plays a critical role in wound healing (Bainbridge, 2013). Since SK1 has been shown to be involved in cell migration by numerous studies (Bao et al., 2012; Li et al., 2012; Maceyka et al., 2008; Schwalm et al., 2010), I therefore sought to examine the role of SKAM1 in cell migration using the *in vitro* scratch wound assay. In this assay a 'wound' is made in a confluent monolayer of cells and the migration of cells into the scratch assessed over time in the presence of the proliferation inhibitor mitomycin C. Thus, migration of NIH3T3 cells stably expressing SKAM1A was analysed in this assay, and compared to vector control cells. Cell migration was not affected by SKAM1A overexpression in NIH3T3 cells, suggesting that SKAM1A may not play a role in cell migration in these cells (Fig. 4.5).

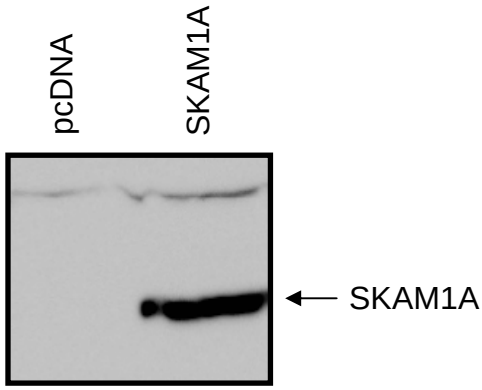
4.4.4 SKAM1 overexpression prevents serum-deprivation-induced apoptosis

Since apoptosis plays a crucial role in wound healing (Greenhalgh, 1998) and elevated SK1 activity, such as that observed following SKAM1 overexpression, is usually associated with enhanced cell survival [reviewed in (Van Brocklyn and Williams, 2012)], I examined the role of SKAM1 overexpression in cell survival. Consistent with previous studies, SK1 overexpression significantly decreased serum-deprivation induced apoptosis in NIH3T3 cells compared to the vector control cells (Fig 4.6A). Notably, SKAM1A overexpression also had a similar anti-apoptotic effect (Fig 4.6A). These results suggest that, like SK1, SKAM1A protects cells from apoptosis.

A



B


pcDNA
SKAM1A
SKAM1A

C

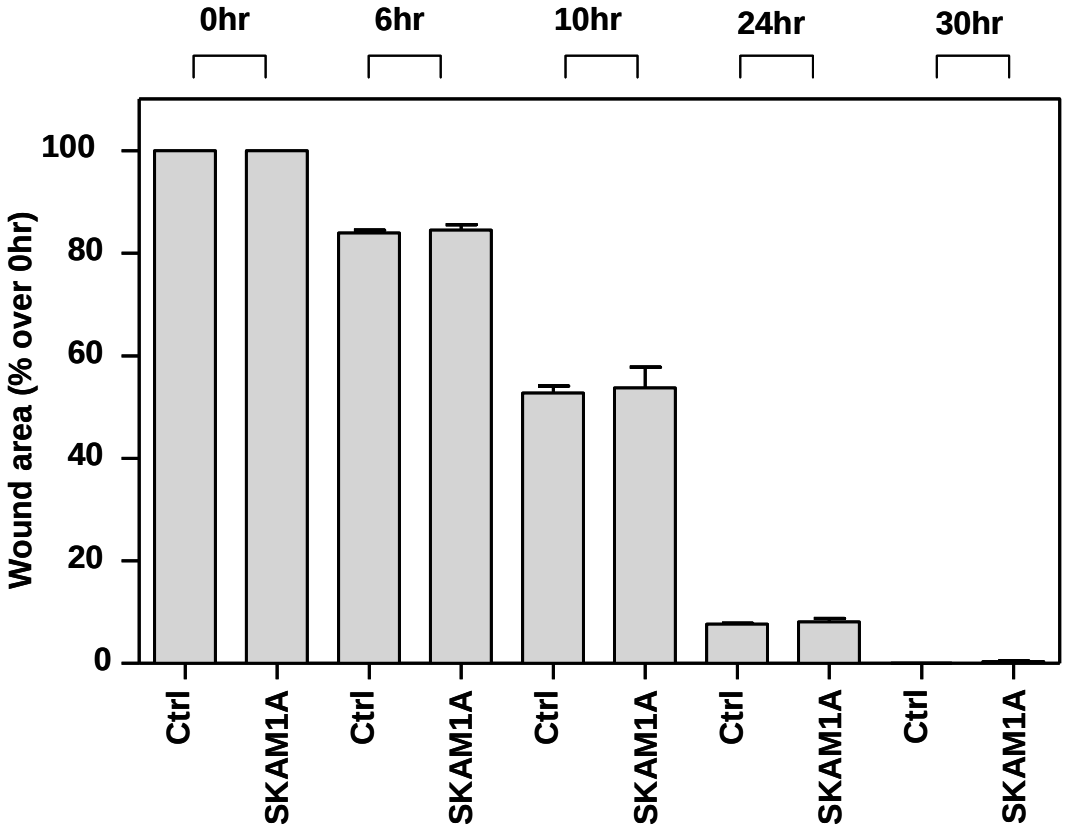

0hr
6hr
10hr
24hr
30hr
Wound area (% over 0hr)
100
80
60
40
20
0
Ctrl
SKAM1A
Ctrl
SKAM1A
Ctrl
SKAM1A
Ctrl
SKAM1A
Ctrl
SKAM1A

Fig. 4.5 SKAM1 overexpression does not affect cell migration.

NIH3T3s stably overexpressing SKAM1A (pcDNA3-FLAG/SKAM1A-IRES-EGFP) or the vector control cells transfected with pcDNA3-IRES-EGFP were seeded onto a 6-well plate containing 10% charcoal-stripped FCS and 1% penicillin/streptomycin and were allowed to grow until a confluent monolayer was formed. A scratch was formed by manually scraping the cells off the surface of the 6-well plate using a pipette tip. Cells were allowed to migrate into the 'wound' for 30hr in the presence of mitomycin C (2μg/ml) (A). Bars represent 50μm. SDS-PAGE using clarified lysates prepared from remaining cells after seeding were assessed for FLAG-tagged SKAM1A overexpression by anti-FLAG monoclonal antibody (B). Data was quantified using the ImageJ software and represents the mean ± range from two independent experiments, each performed in duplicate (C).

4.4.5 SKAM1 overexpression does not affect cellular proliferation

Given that SKAM1 can activate SK1 and that elevated SK1 activity has also been shown to stimulate cellular proliferation (Olivera et al., 1999b; Xia et al., 2000), I next examined the effect of SKAM1 overexpression on cellular proliferation using NIH3T3 cells stably overexpressing SKAM1A. Consistent with previous studies (Olivera et al., 1999b; Xia et al., 2000), SK1 overexpression resulted in increased cellular proliferation in NIH3T3 cells (Fig. 4.6B). Interestingly, however, SKAM1A overexpression did not affect proliferation of NIH3T3 cells (Fig. 4.6B).

4.4.6 SKAM1 overexpression does not result in neoplastic transformation

Previous studies have shown that SK1 overexpression in NIH3T3 cells induced neoplastic transformation (Pitson et al., 2005; Xia et al., 2000). Given that SKAM1 is an activator of SK1 and other SK1 activator proteins can induce neoplastic transformation (Leclercq et al., 2011), I next examined whether SKAM1 overexpression in NIH3T3 lead to neoplastic transformation. Focus formation assays were performed using NIH3T3 cells stably expressing the SKAM1A, SK1 or vector control cells. Consistent with previous studies, SK1 overexpression in NIH3T3 cells resulted in neoplastic transformation (Fig. 4.7). NIH3T3 cells stably overexpressing SKAM1A, however, did not acquire the neoplastic transformed phenotype.

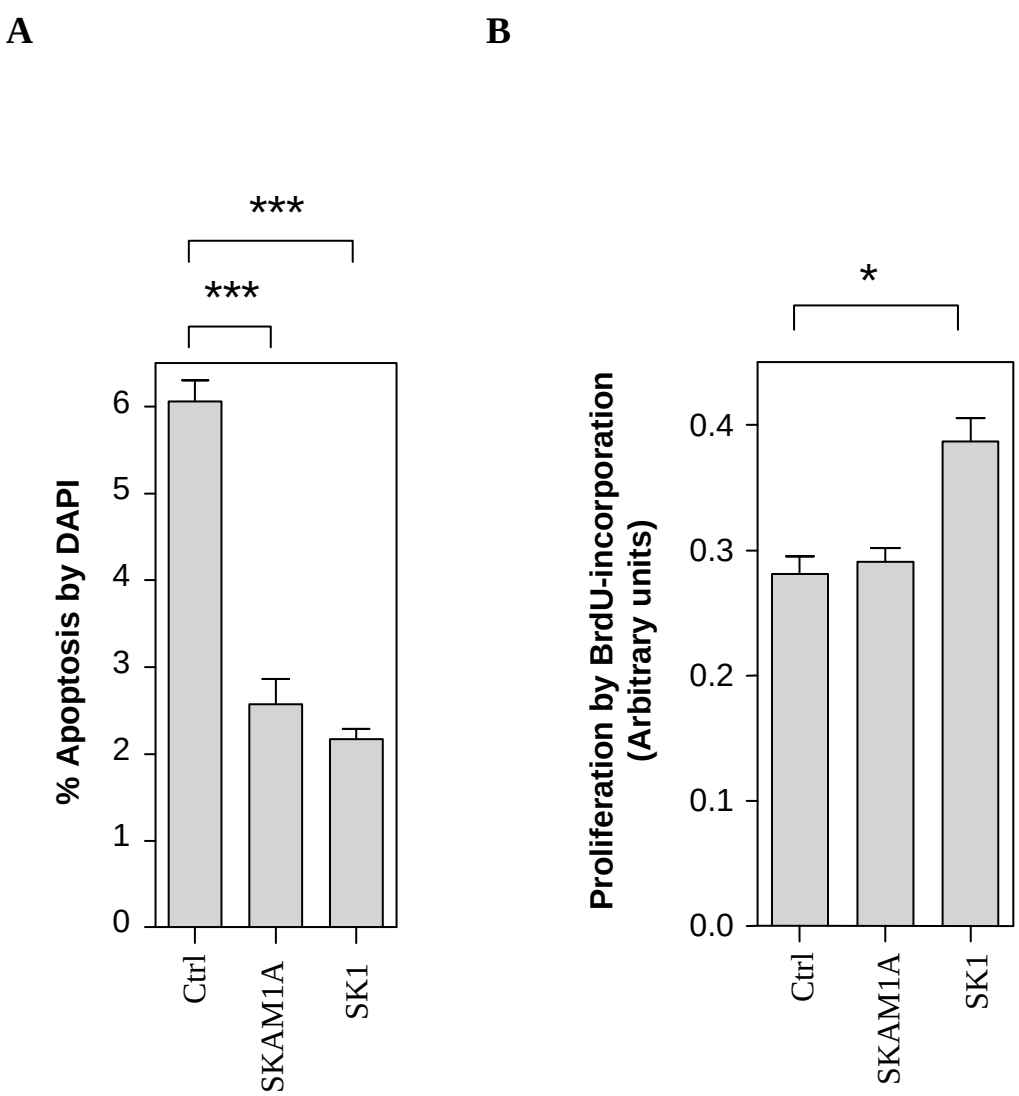


Fig. 4.6 SKAM1A overexpression protects NIH3T3 fibroblasts against serum-deprivation-induced apoptosis but does not affect cellular proliferation.

NIH3T3 cells stably overexpressing SKAM1A or SK1 and vector control cells were analysed for apoptosis and proliferation by DAPI staining (A) and BrdU labelling (B), respectively. Data represents the mean ± SD from three independent experiments, each analysed in duplicate. *$p<0.05$ and ***$P<0.001$. Statistical analysis was performed using the student's t-test.

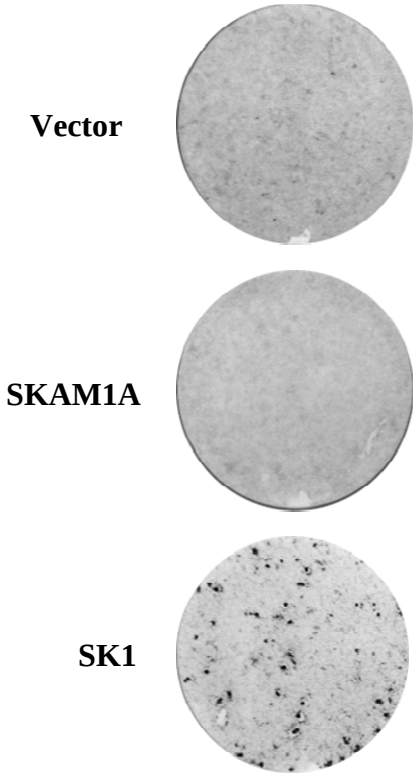


Fig. 4.7 NIH3T3 cells stably expressing SKAM1A did not acquire a neoplastic transformation phenotype.

The ability of SKAM1A to induce neoplastic transformation was examined using focus formation assays. Cells were cultured at confluency in 0.5% FCS, 1% penicillin/streptomycin for approximately three weeks with media replaced every 2-3 days. Data shown is representative of two independent experiments.

4.4.7 Generation of mouse models to study the role of SKAM1A in wound healing

To study the role of SKAM1A in wound healing *in vivo*, we sought to overexpress or knockout SKAM1A in mice.

4.4.7.1 Generation of SKAM1A gene-trap mice

To study the effects of SKAM1A knockout on wound healing *in vivo*, we employed the Australian Phenomics Networks at Monash University to generate SKAM1A gene-trap mice. Commercially available mouse embryonic stem (ES) cells containing the SKAM1A gene-trapped 'knockout-first' allele (EPD0680_1_E09) were purchased from the International Gene Trap Consortium and were used to generate SKAM1A gene-trap mice. The gene trap cassette (L1L2_Bact_P) is inserted between exon 2 and 3 of SKAM1 (Fig. 4.8A). The cassette is composed of an FRT site followed by lacZ sequence and a loxP site. This first loxP site is followed by a neomycin cassette under the control of the human beta-actin promoter, SV40 polyA, a second FRT site and a second loxP site. A third loxP site is inserted downstream of exon 3, resulting in exon 3 being flanked by loxP sites. Thus, a conditional allele can be generated by Flp recombinase expression in mice carrying this allele (Fig. 4.8B). Subsequent Cre expression results in a knockout mouse (Fig. 4.8B). Notably, although a protein fragment of SKAM1 (the first 59 amino acid) could still be expressed in the gene-trap mice, this does not contain the region of SKAM1 known to be involved in SK1 interaction and activation.

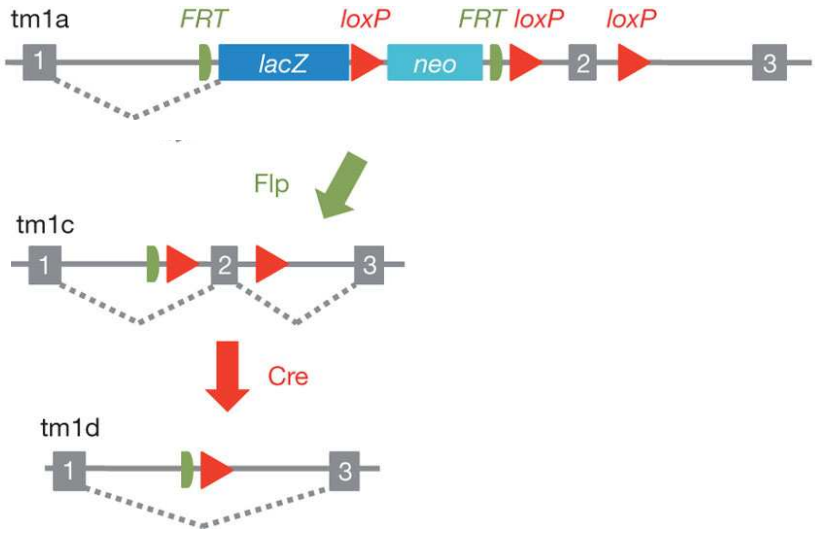
ES cells containing the SKAM1A mutant allele were microinjected into blastocysts to generate chimeras. The resulting chimeras were then bred with wildtype to confirm germline transmission of the mutation. Three heterozygous animals

(mouse numbers 57, 59 and 61), two female and one male mice, were identified (Fig. 4.9). These are currently being used to establish SKAM1A gene-trapped mouse lines and therefore were not yet available for use in the current studies. Notably, heterozygous SKAM1A gene-trapped mice appear phenotypically normal. Although we expect 25% of the offspring from heterozygous SKAM1A gene-trapped in breeding to be homozygous SKAM1A gene-trapped, current attempts to generate homozygous SKAM1A gene-trapped mice have failed (of 55 mice born from heterozygous SKAM1A gene-trapped breeding, 10 were wildtype and 45 were heterozygous SKAM1A gene-trapped), suggesting that SKAM1A may be essential for mouse development.

A

Adapted from International Knockout Mouse Consortium

B



Adapted from Skarnes et al. (2011)

Fig. 4.8 Schematic of the 'knockout-first' conditional allele.

Schematic representation of the 'knockout-first' conditional SKAM1 allele used to generate SKAM1 gene-trap mice (A) (Skarnes et al., 2011). The 'knockout-first' allele (tm1a) contains an IRES:*lacZ* trapping cassette and a floxed promoter-driven *neo* cassette inserted into the intron of a gene, disrupting gene function. Flp converts the 'knockout-first' allele to a conditional allele (tm1c), restoring gene activity. Cre deletes the floxed exon of the tm1c allele to generate a frameshift mutation (tm1d), triggering nonsense mediated decay of the deleted transcript (B).

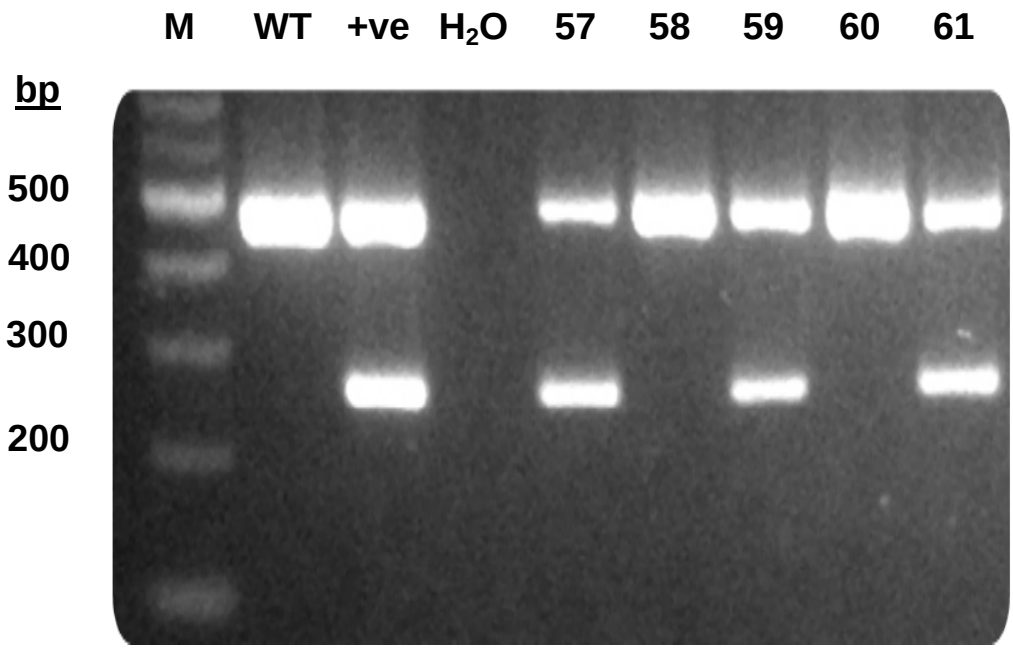


Fig. 4.9 Identification of three SKAM1A gene-trap mice by PCR

PCR was performed using the genomic DNA extracted from tail biopsies (see Materials and Methods for detail). Genomic DNA prepared from Fgfr1op2 targeted embryonic stem cells clone (EPD0680_1_B11) was used as the positive control (+ve). Numbers indicate the offsprings derived from pronuclear injection. WT represents genomic DNA prepared from wildtype C57BL/6 mice. M represents 1Kb Plus DNA ladder marker.

4.4.7.2 Generation of SKAM1A transgenic mice

4.4.7.2.1 Generation of SKAM1A expression construct

Initial attempts to generate SKAM1A transgenic mice used the pcDNA3 vector employing the CMV promoter. Unfortunately, these were unsuccessful due to lack of transgene expression (data not shown). In the second attempt, we employed the pCX vector with the chicken β-actin promoter, CMV enhancer and an intron (Okabe et al., 1997). To generate an expression construct driving expression of FLAG-tagged SKAM1A, as well as EGFP via an IRES, I subcloned the cDNA encoding for human SKAM1A into this vector downstream of the chicken β-actin promoter (Fig. 4.10). To confirm expression of the resulting construct pCX-FLAG/SKAM1A-IRES-EGFP, I performed transient transfection in NIH3T3 fibroblasts (Fig. 4.11A). As shown in Fig. 4.10A and B, high levels of FLAG/SKAM1A protein were expressed in the transfected NIH3T3 fibroblasts. Notably, the transfection efficiency and expression levels of the pCX construct in NIH3T3 fibroblasts were markedly higher compared with the pcDNA3 construct (Fig. 4.10A and B).

Only the ~4.5 kb DNA fragment resulting from the *SalI* and *BamHI* restriction enzyme digest containing the CMV enhancer/chicken β-actin promoter to the rabbit β-globin poly-A signal was to be used for pronuclear injection (Fig. 4.12A) to generate transgenic mice. Therefore, I next examined the ability of this DNA fragment to express SKAM1A and EGFP when transfected into in NIH3T3 fibroblasts. As shown in Fig. 4.12B and C, this DNA fragment alone was sufficient to drive SKAM1A transgene expression in NIH3T3 fibroblasts. DAPI staining should also be performed in future studies to identify the proportion of cells expressing this construct.

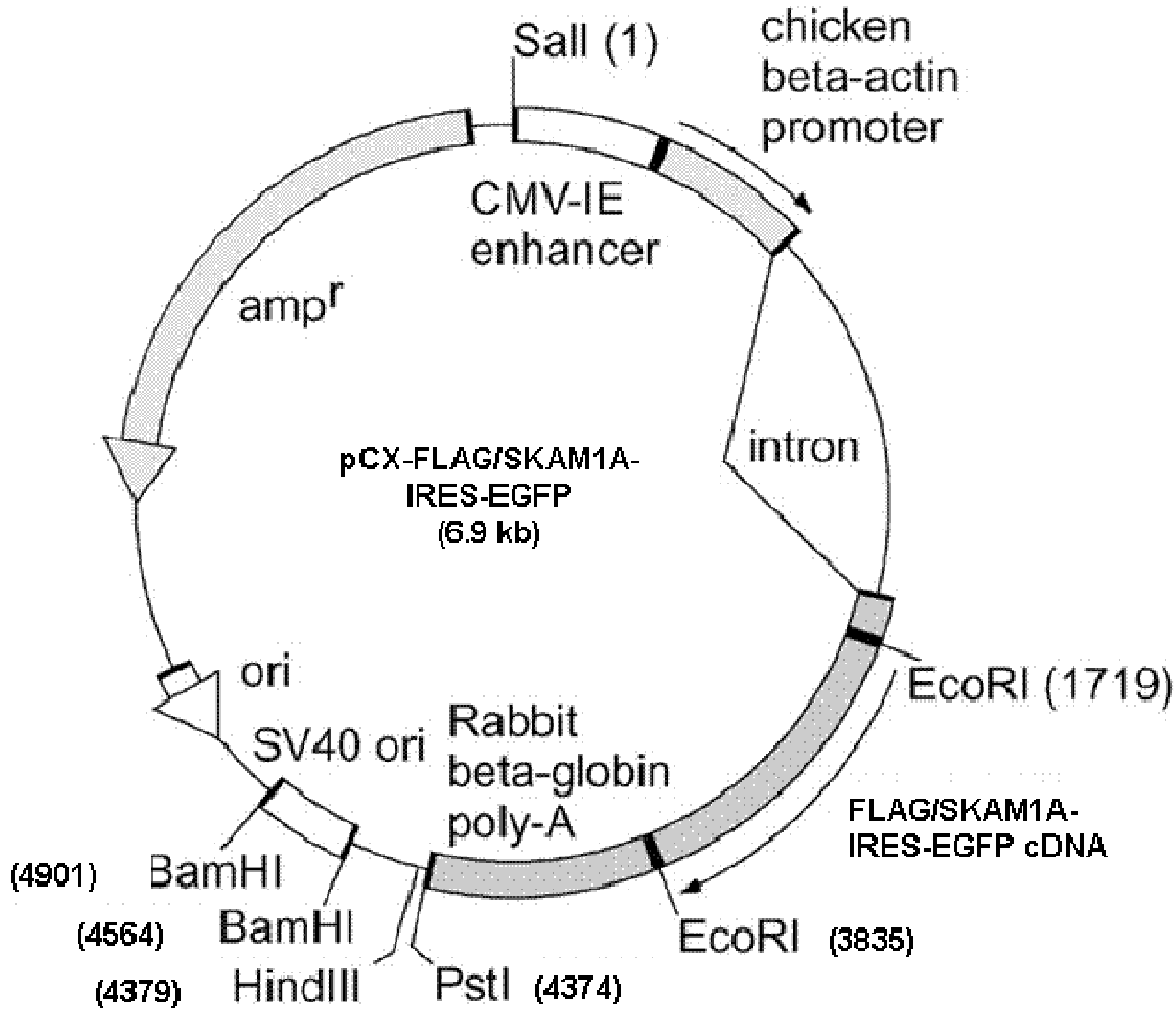
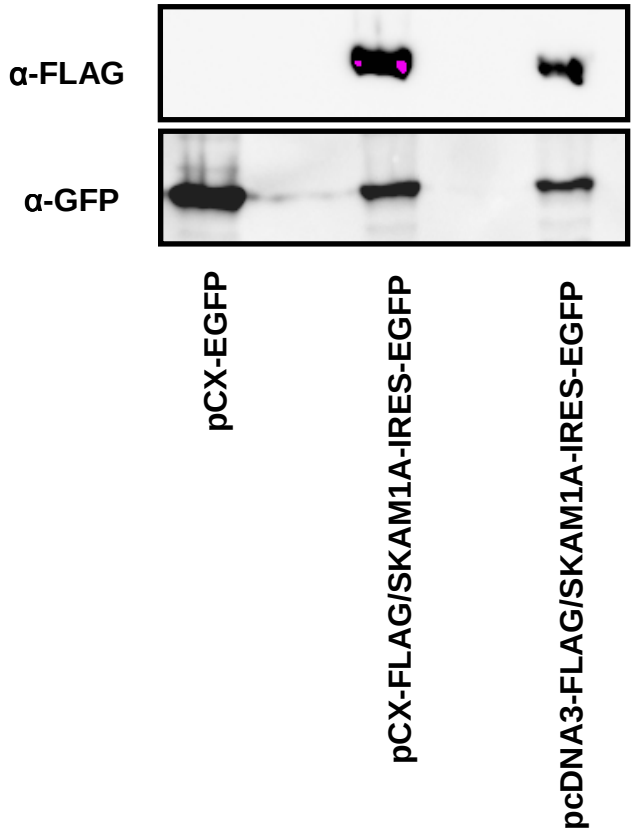


Fig. 4.10 Restriction map of pCX-FLAG/SKAM1A-IRES-EGFP

FLAG/SKAM1A-IRES-EGFP cDNA was subcloned downstream of the chicken β-actin promoter of the pCX-EGFP construct (see section 2.12 for detail).

A


α-FLAG
α-GFP
pCX-EGFP
pCX-FLAG/SKAM1A-IRES-EGFP
pcDNA3-FLAG/SKAM1A-IRES-EGFP

B

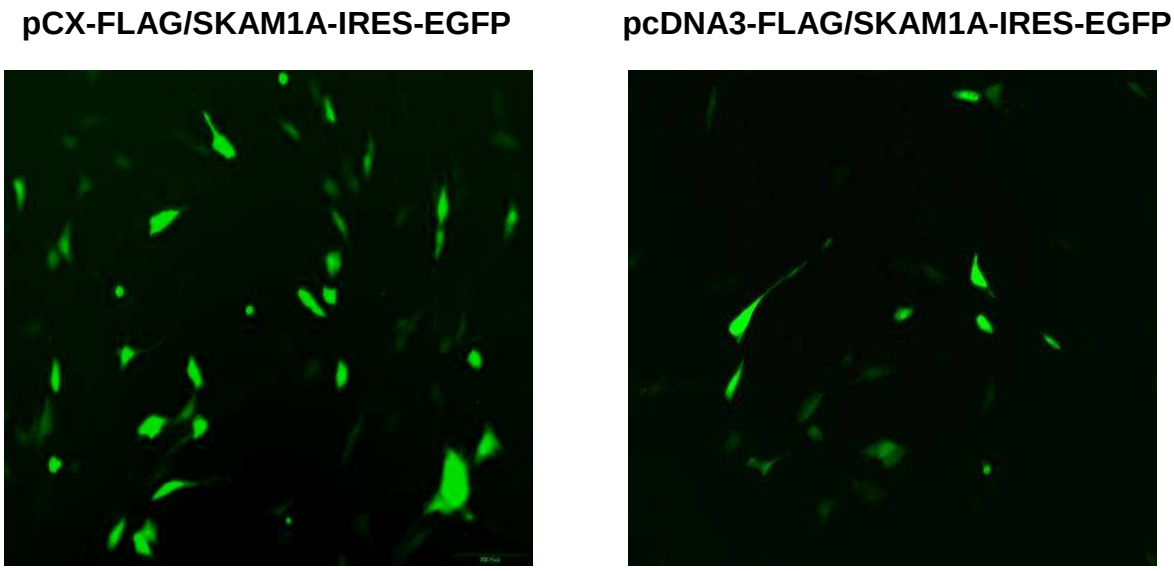
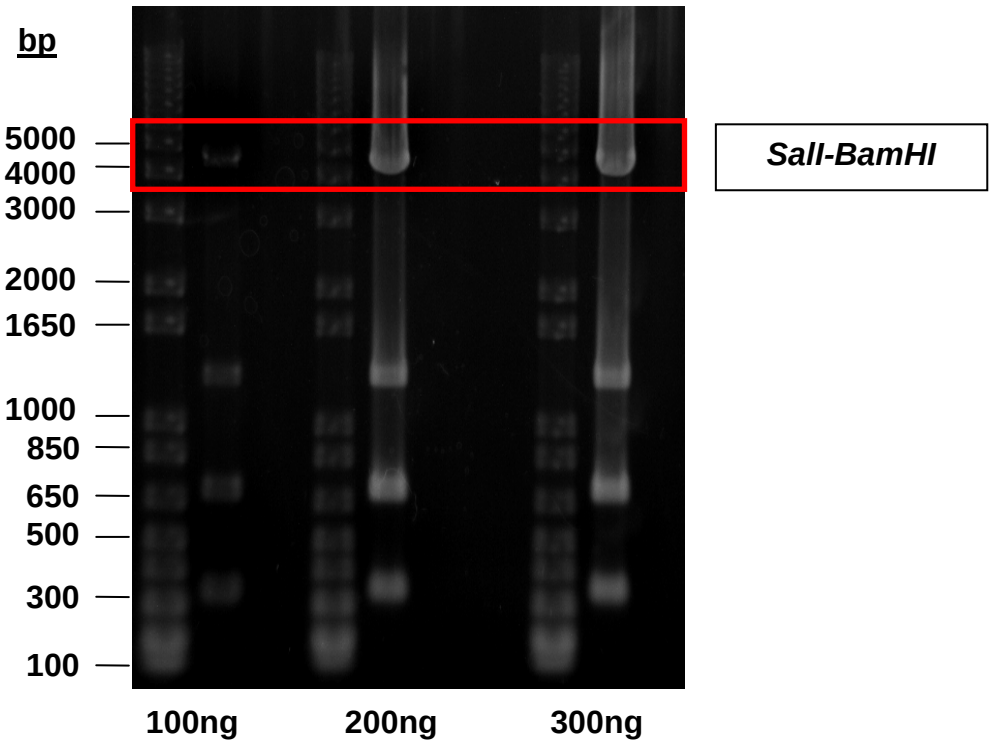


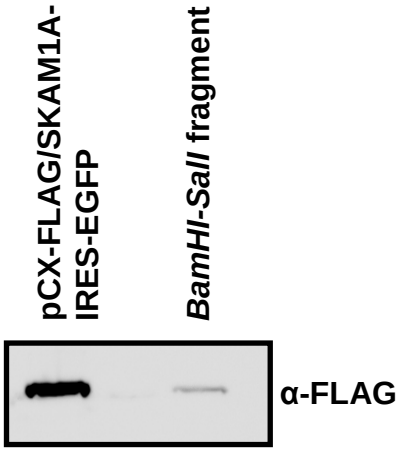
Fig. 4.11 pCX-FLAG/SKAM1A-IRES-EGFP is highly expressed in transiently transfected NIH3T3 fibroblasts

NIH3T3 fibroblasts were transiently transfected with pCX-FLAG/SKAM1A-IRES-EGFP or pcDNA3-FLAG/SKAM1A-IRES-EGFP using Lipofectamine 2000 transfection reagent. Western blot with anti-FLAG and anti-GFP antibodies were performed using clarified cell lysates (A). Cells were also visualised for GFP expression by fluorescence microscopy (B).

A


bp
5000
4000
3000
2000
1650
1000
850
650
500
300
100
100ng
200ng
300ng
SalI-BamHI

B


pCX-FLAG/SKAM1A-IRES-EGFP
BamHI-SalI fragment
α-FLAG

C

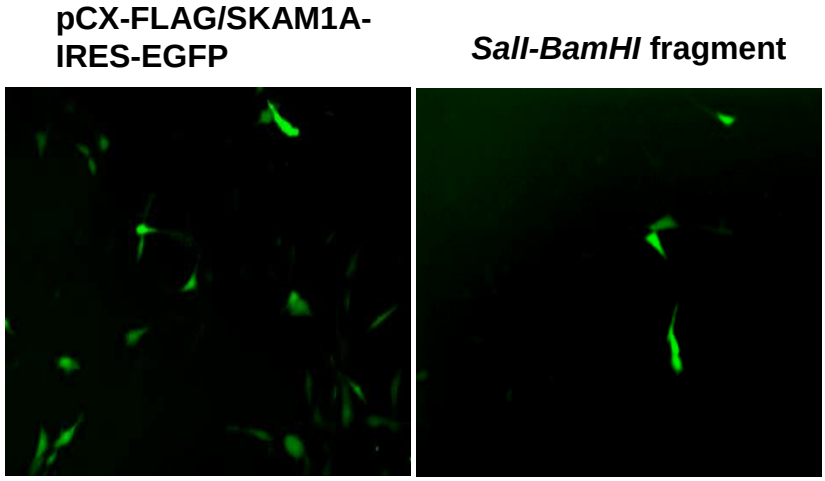

pCX-FLAG/SKAM1A-IRES-EGFP
SalI-BamHI fragment

Fig. 4.12 SalI-BamHI DNA fragment is expressed in NIH3T3 fibroblasts

pCX-FLAG/SKAM1A-IRES-EGFP was digested with restriction enzymes SalI, BamHI and FspI yielding a 4.5kp fragment (A). NIH3T3 fibroblasts were transiently transfected with pCX-FLAG/SKAM1A-IRES-EGFP or the SalI-BamHI digested DNA fragment using Lipofectamine 2000 transfection reagent. Western blot with anti-FLAG antibodies was performed using clarified lysates prepared from NIH3T3 fibroblasts transiently transfected with pCX-FLAG/SKAM1A-IRES-EGFP or the Sal-BamHI DNA fragment (B). Cells were also visualised for GFP expression by fluorescence microscopy (C).

4.4.7.2.2 Generation of SKAM1A transgenic mice by pronuclear injection

Since the pCX-FLAG/SKAM1A-IRES-EGFP construct was successfully expressed in mouse fibroblasts, this construct was provided to the Transgenic Animal Service of Queensland (TASQ) to generate SKAM1A transgenics via pronuclear injection. Three SKAM1A transgenic founder mice, denoted as B1(a), B2 and B4, were successfully generated from a total of 30 pronuclear injections (Fig. 4.13). These founder mice were identified by transgene-specific PCR as described in Chapter 2. All three founders were bred with wildtype C57BL/6 mice to establish the transgenic mouse lines (data not shown). To confirm germline transmission, PCR was performed using genomic DNA isolated from tail biopsies of the F1 generation (see Materials and Methods for details). Notably, germline transmission was successfully detected in the B2 and B4 founder lines (Fig. 4.14). Unfortunately, the B1(a) founder mouse experienced birthing difficulties and had to be humanely culled and excluded from the study.

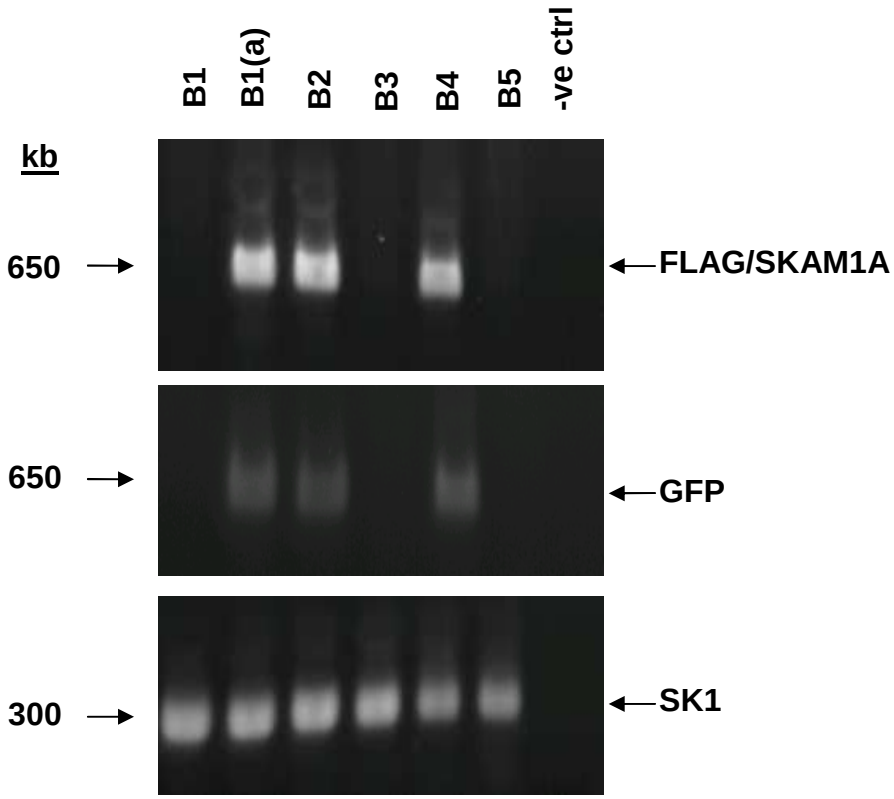


Fig. 4.13 Three transgenic positive founder lines were identified by PCR

Genomic DNA prepared from tail biopsies was subject to transgene specific PCR for the FLAG/SKAM1A transgene as described in section 2.15.

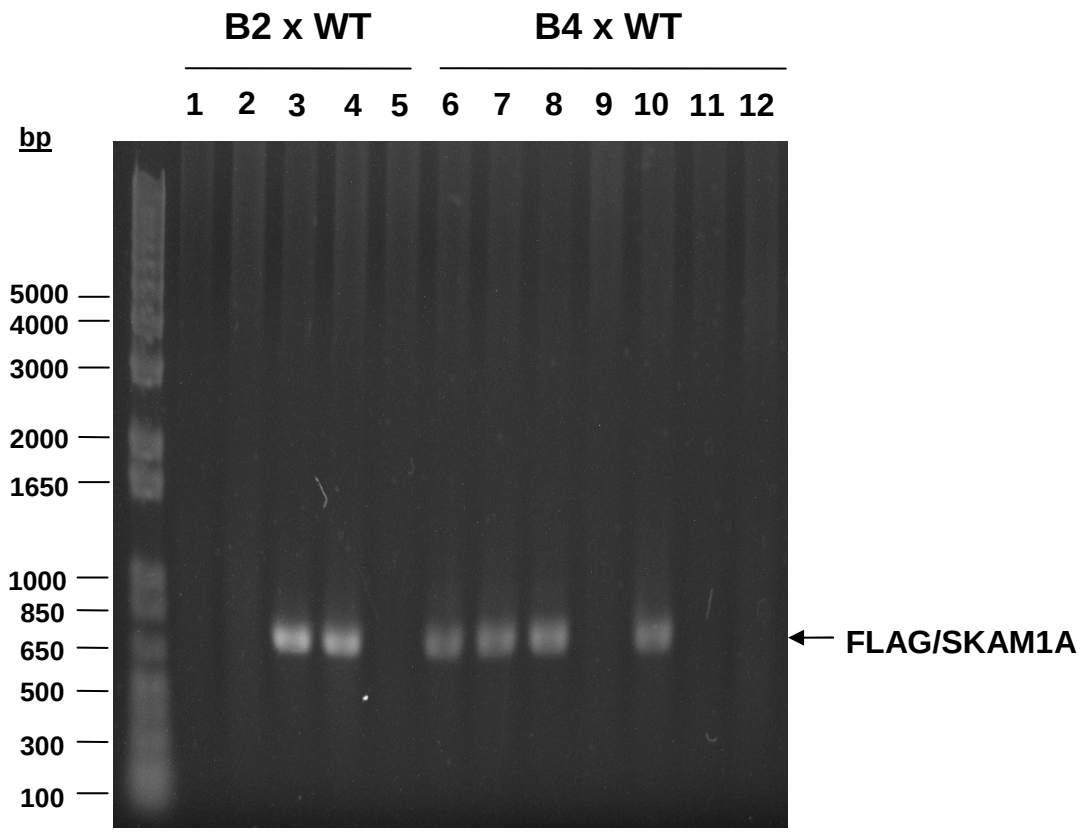


Fig. 4.14 Germline transmission was observed in the F1 progeny

Genomic DNA was isolated from tail biopsies of the F1 progeny. Transgene specific PCR for FLAG/SKAM1A transgene was performed as described in section 2.13.

4.4.8 Analysis of SKAM1A transgenic mice

4.4.8.1 SKAM1A transgene is expressed in a wide variety of tissues in SKAM1A transgenic mice

To confirm SKAM1A protein expression in transgenic mice, Western blot analysis was performed using lysates prepared from a variety of tissues of SKAM1A transgenic and wildtype mice. As shown in Fig. 4.15, FLAG-tagged SKAM1A expression was detected in the skin, as well as other tissues of the transgenic mice (derived from B2 line), including the heart, brain, lung, liver, kidney and spleen. Notably, SKAM1A expression levels were relatively higher in the lung and kidney compared with other organs (Fig. 4.15).

4.4.8.2 SKAM1A transgenic mice displayed normal phenotype

Previous studies have shown that SK1 transgenic mice displayed cardiac fibrosis (Takuwa et al., 2010). Given that SKAM1 can activate SK1, I next examined SKAM1A transgenic mice for phenotypic abnormalities, particularly for signs of tissue fibrosis, in a range of organs, including the eye, skin, heart, lung, brain, stomach, small intestine, pancreas, liver, kidney, muscle, intercostal muscle (Fig. 4.16A-D). Histological sections prepared from the organs of 12 week old female and male SKAM1A transgenic and wildtype littermates were subject to H/E staining (Fig. 4.16A-D). Both 12 week old male and female SKAM1A transgenic mice appeared to be normal and did not display any phenotypic abnormalities or signs of tissue fibrosis (Fig. 4.16A-D). Similarly, we did not observe any phenotypic abnormalities in aged (48 week old) SKAM1A transgenic male and female mice (Fig. 4.16E-H).

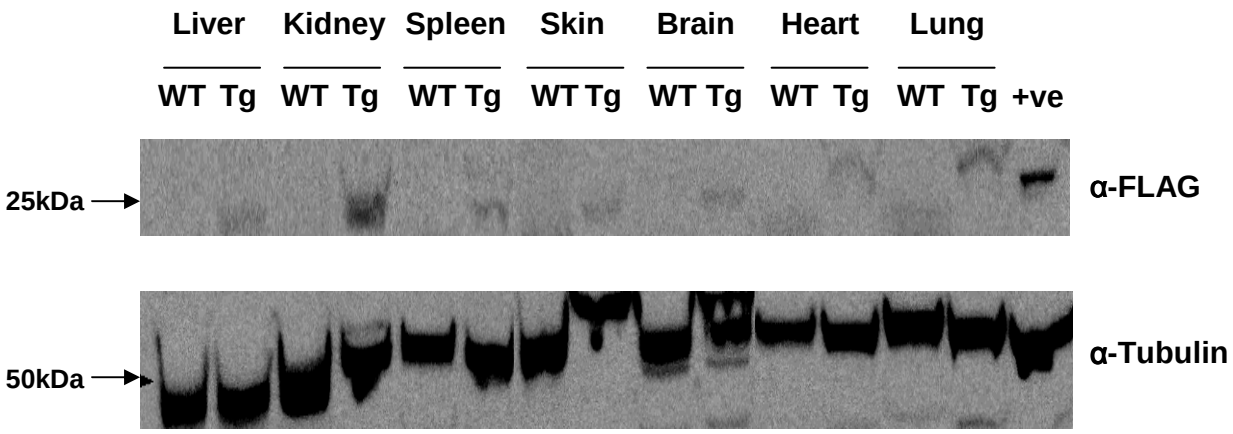
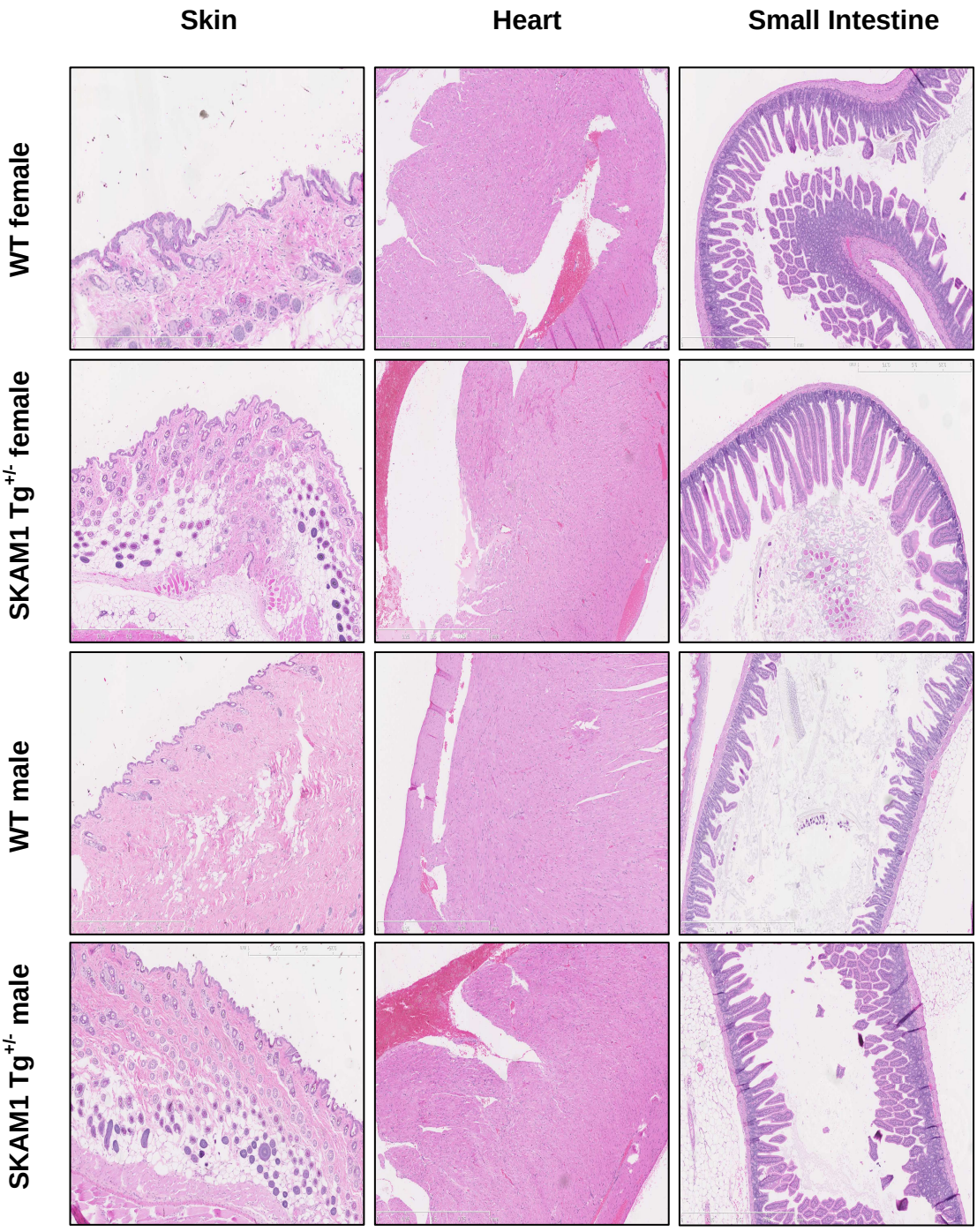


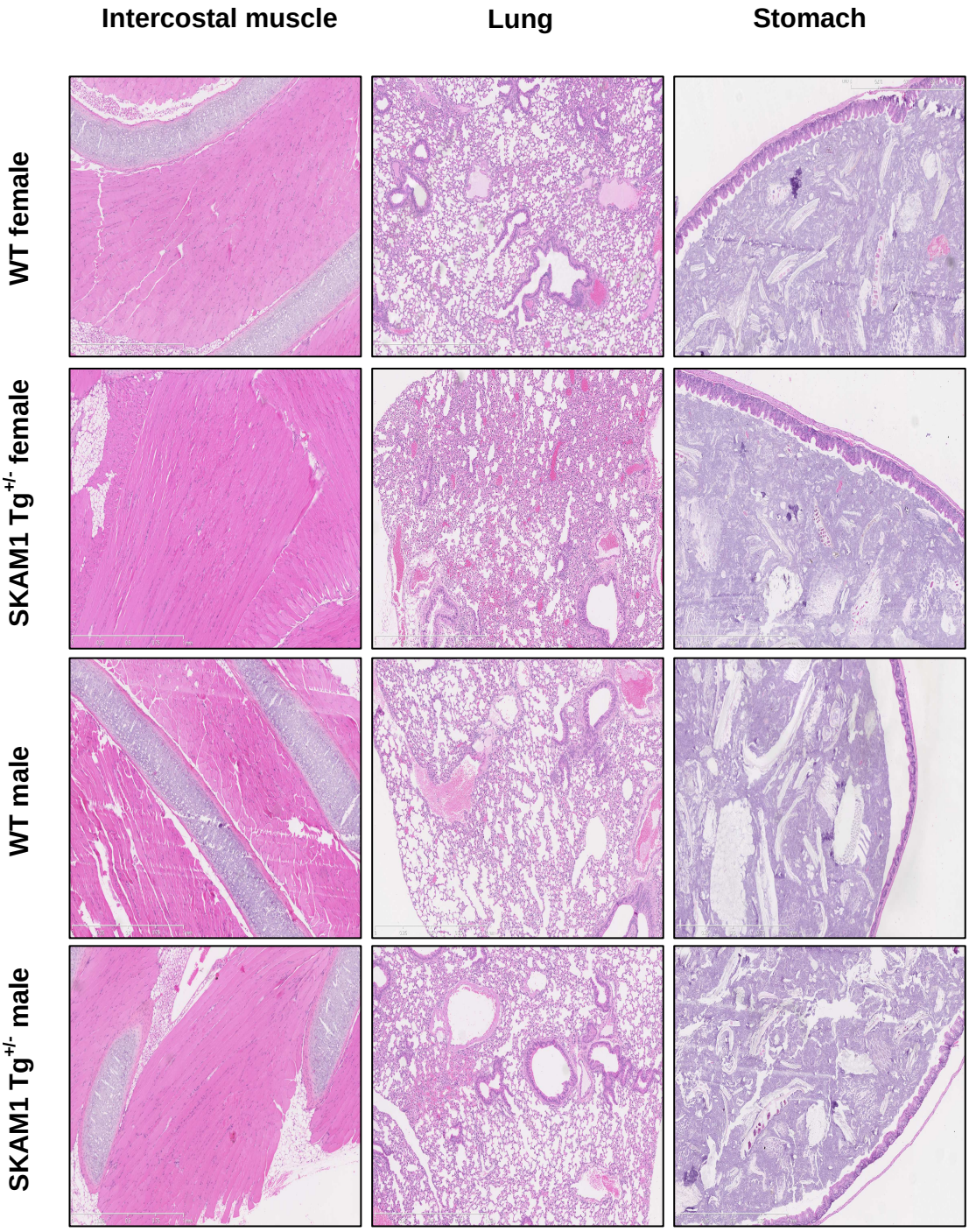
Fig. 4.15 SKAM1A is ubiquitously expressed in transgenic mice

Western blots were performed using lysates prepared from various tissues of wildtype (WT) and SKAM1A transgenic (Tg) mice. Expression of the SKAM1A transgene was detected with anti-FLAG antibodies. Anti-tubulin antibodies were used for loading control.

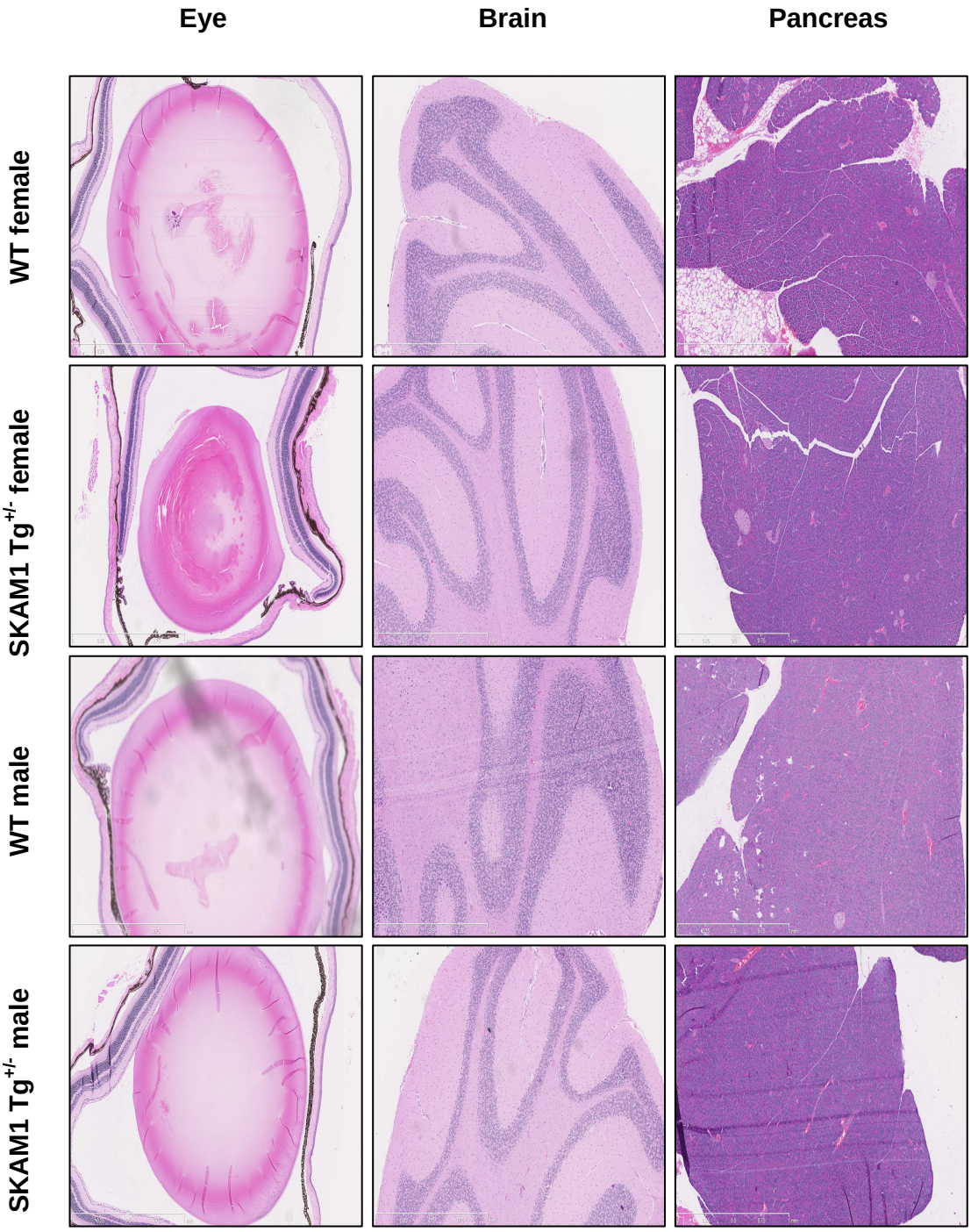
A


Skin
Heart
Small Intestine
WT female
SKAM1 Tg+/- female
WT male
SKAM1 Tg+/- male

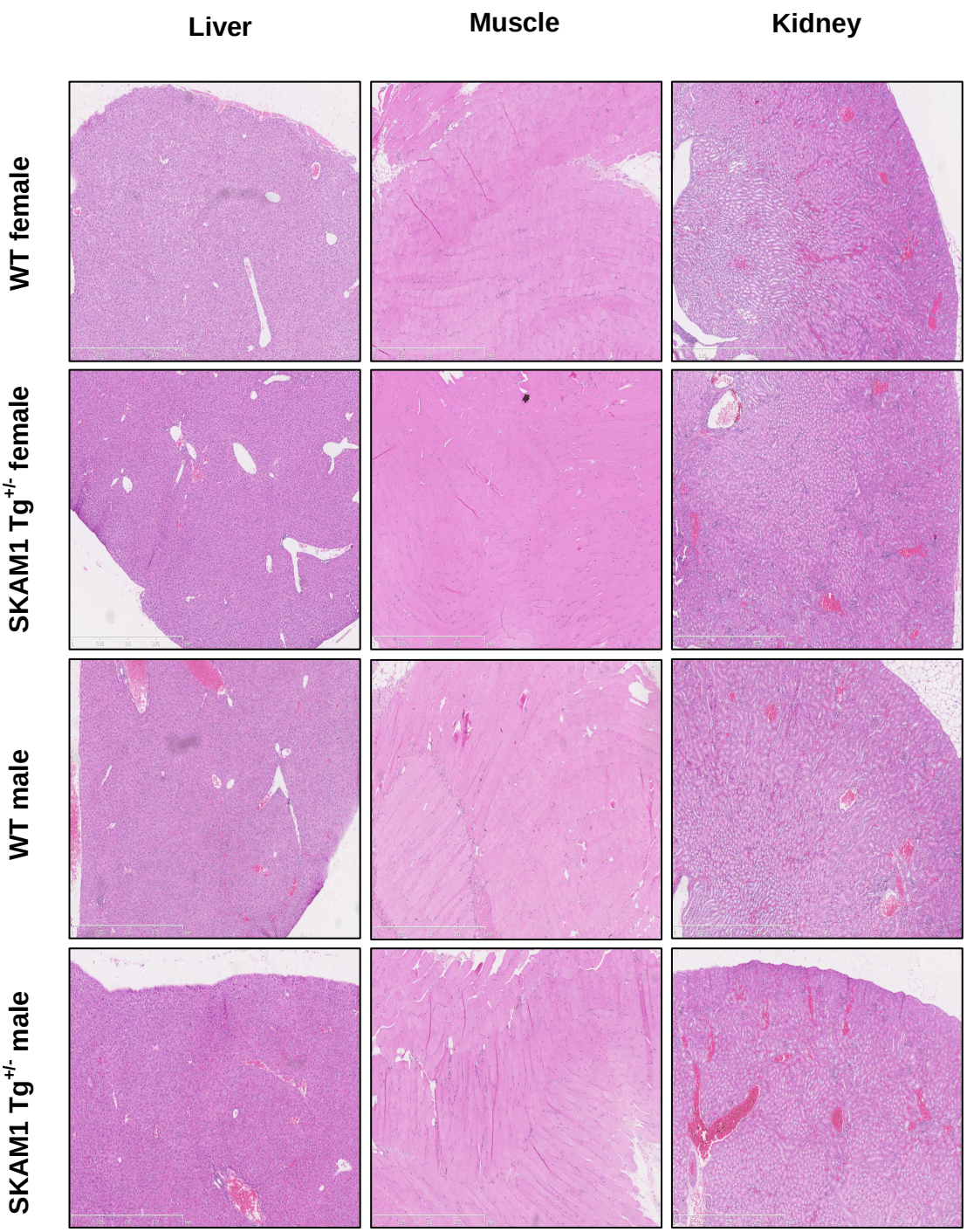
B


Intercostal muscle
Lung
Stomach
WT female
SKAM1 Tg+/- female
WT male
SKAM1 Tg+/- male

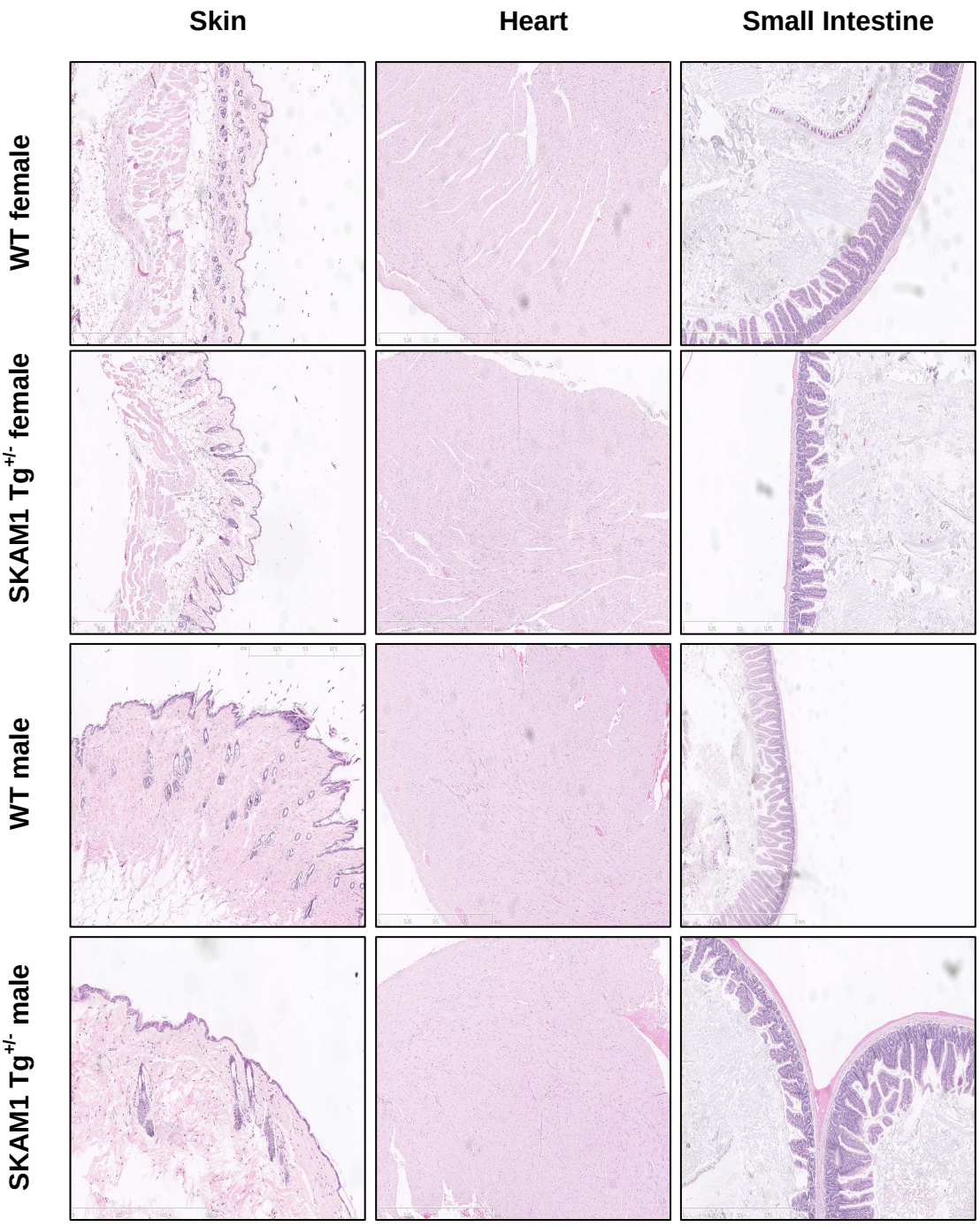
C


Eye
Brain
Pancreas
WT female
SKAM1 Tg+/- female
WT male
SKAM1 Tg+/- male

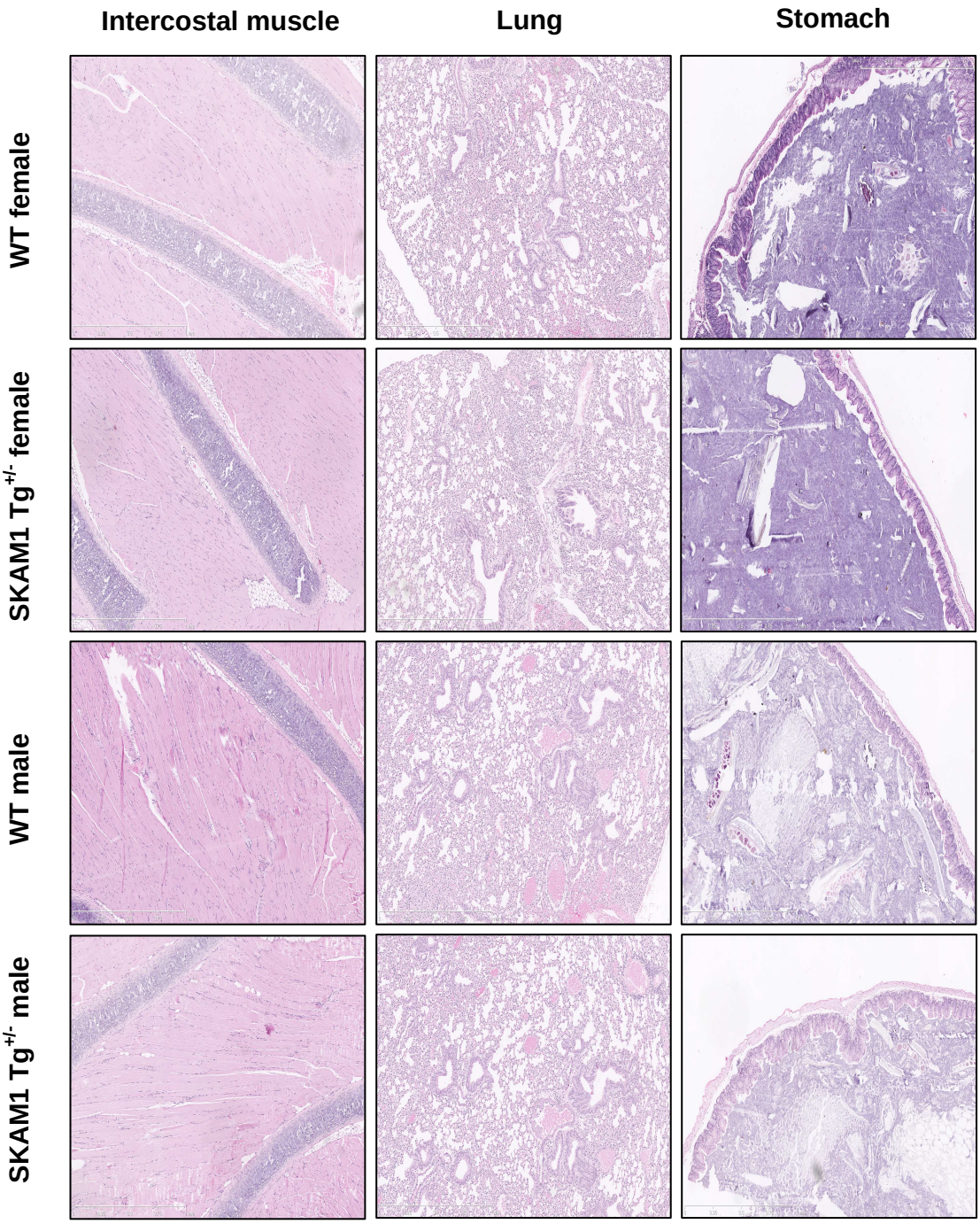
D


Liver
Muscle
Kidney
WT female
SKAM1 Tg+/- female
WT male
SKAM1 Tg+/- male

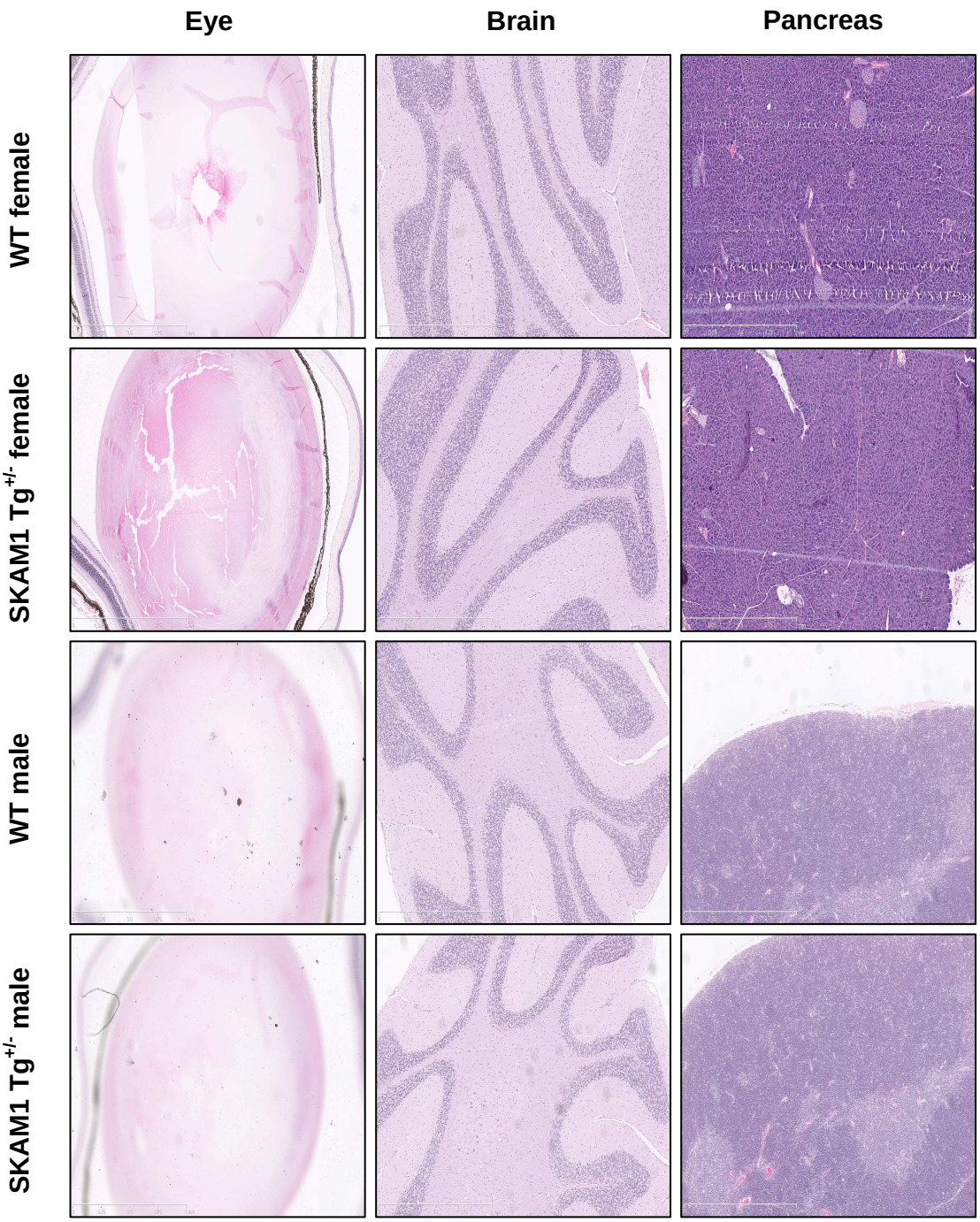
E


Skin
Heart
Small Intestine
WT female
SKAM1 Tg+/- female
WT male
SKAM1 Tg+/- male

F


Intercostal muscle
Lung
Stomach
WT female
SKAM1 Tg+/- female
WT male
SKAM1 Tg+/- male

G


Eye
Brain
Pancreas
WT female
SKAM1 Tg+/- female
WT male
SKAM1 Tg+/- male

H

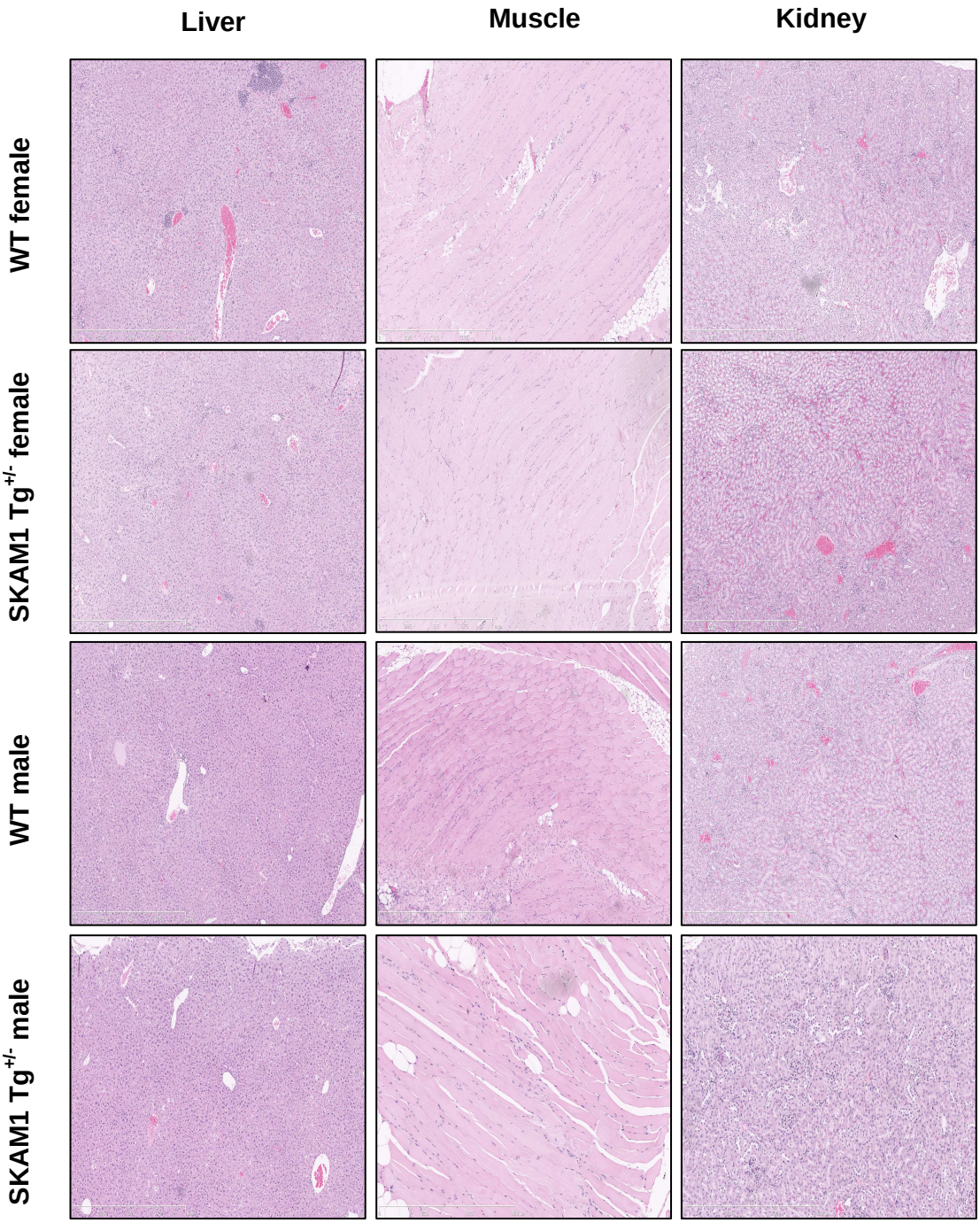

Liver
Muscle
Kidney
WT female
SKAM1 Tg+/- female
WT male
SKAM1 Tg+/- male

Fig. 4.16 SKAM1A transgenic mice display normal phenotype

Tissues and organs were harvested from 12 and 48 week old SKAM1A transgenic and wildtype mice. 4μm sections were prepared and stained with haematoxylin and eosin (H/E). Histological images of 12 week (A-D) and 48 week (E-H) old SKAM1A transgenic and wildtype mice. (A and E) H/E images of skin, heart and small intestine. (B and F) H/E images of intercostal muscle, lung and stomach. (C and G) H/E images of eye, brain and pancreas. (D and H) H/E images of liver, muscle and kidney. Images were taken using the NanoZoomer digital slide scanner XR-C12000 (Hamamatsu). Images are representative n=2 of 12 or 48 week old, male or female mice per genotype.

4.4.8.3 SKAM1A transgenic MEFs have enhanced collagen contraction

Preliminary data generated by the host laboratory have shown that overexpression of SKAM1A resulted in enhanced collagen contraction by NIH3T3 fibroblasts (Fig. 4.1). Consistent with these findings, primary embryonic fibroblasts isolated from SKAM1A transgenic mice showed enhanced ability to induce collagen contraction compared with wildtype MEFs (Fig. 4.17). These results suggest that SKAM1A may play a positive role in wound contraction.

4.4.8.4 SKAM1A transgenic mice appear to have slightly reduced levels of S1P in plasma compared with wildtype

To examine the S1P levels in the plasma of the SKAM1A transgenic mice, cardiac puncture was performed on 12 week old female SKAM1A transgenic mice. Sex and age matched wildtype mice were also included in this study as controls. Surprisingly, HPLC analysis showed that SKAM1A transgenic mice have reduced S1P levels in the plasma compared with wildtype mice (Fig. 4.18).

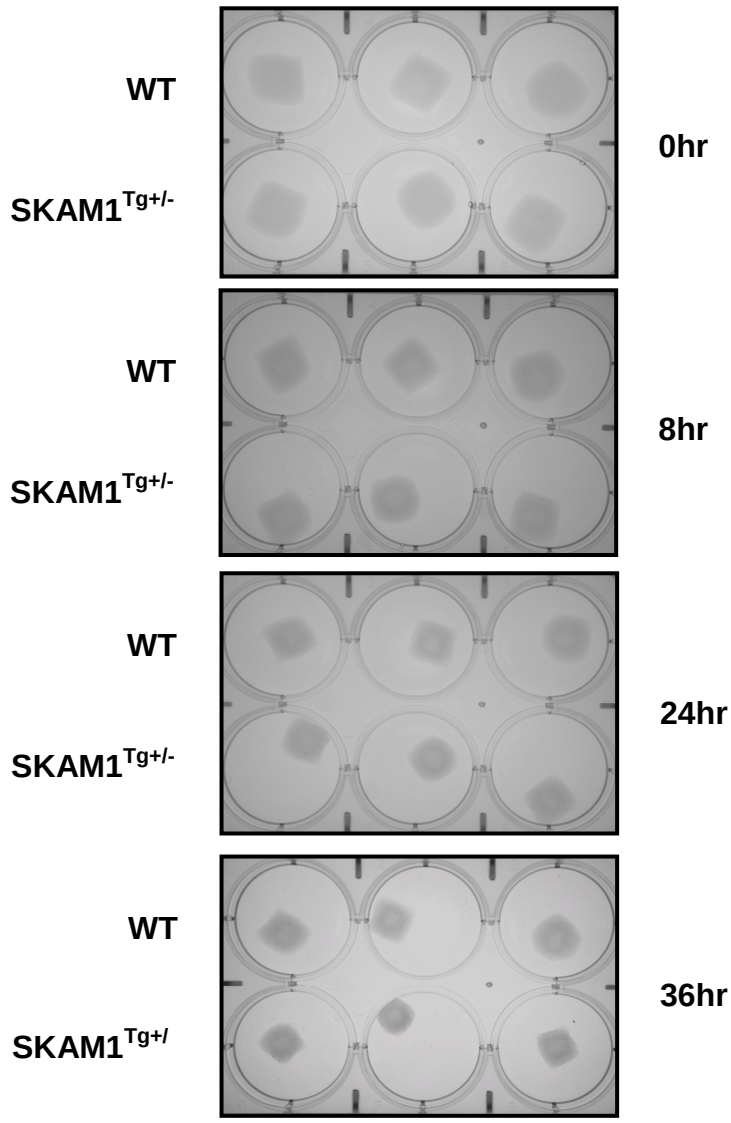
4.4.8.5 Wound healing of incisional wounds is similar between wildtype and SKAM1A transgenic mice

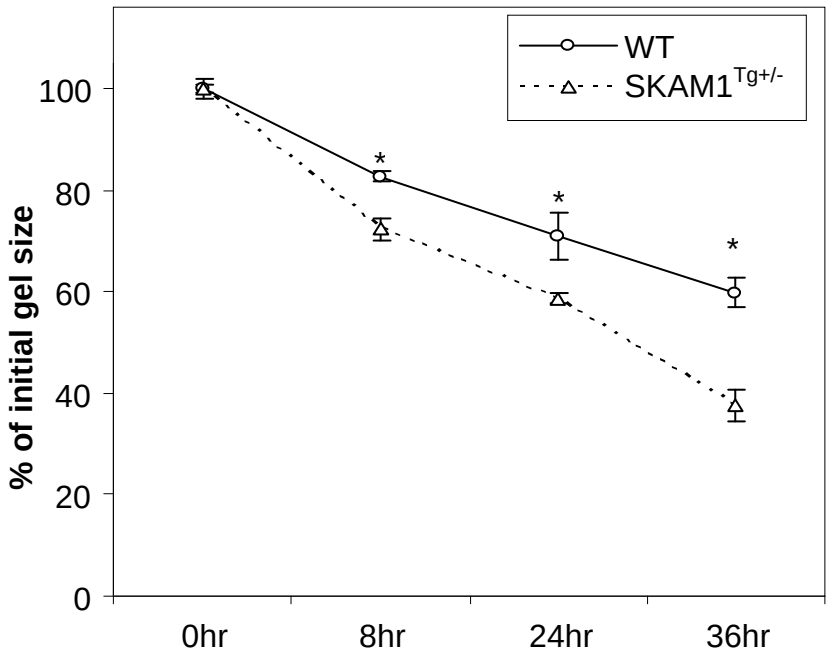
To study the role of SKAM1A in wound healing and to investigate whether SKAM1A overexpression enhances the rate of wound healing in mice, I performed incisions on the dorsal skin of 12 weeks old female SKAM1A transgenic and wildtype mice (Fig. 4.19). Somewhat surprisingly, visual inspection of wound images taken at day 4 post-wounding showed similar wound size between SKAM1A transgenic and wildtype mice (Fig. 4.19A and B). Furthermore, histological analysis

of wound tissue showed similar wound margins between SKAM1A transgenic and wildtype mice (Fig. 4.19C).

4.4.8.6 Wound healing of full-thickness excisional wounds is enhanced in SKAM1A transgenic mice

To further investigate the role of SKAM1A in wound healing *in vivo*, we employed the more severe full-thickness excisional wound healing model. Using a 6mm biopsy punch, two full-thickness excisional wounds were created on the dorsal skin of twelve week old C57BL/6 female mice, one on each side of the midline. Wounds were allowed to heal by secondary intention. Images of wounds were taken daily and size of the wounds was quantified. As shown in Figure 4.20, SKAM1A transgenic mice displayed a modest but significant enhancement in wound healing compared to wildtype mice, suggesting that SKAM1A plays a positive role in wound healing *in vivo*.


WT
SKAM1Tg+/-
0hr
WT
SKAM1Tg+/-
8hr
WT
SKAM1Tg+/-
24hr
WT
SKAM1Tg+/
36hr


WT
SKAM1Tg+/-
% of initial gel size
100
80
60
40
20
0
0hr
8hr
24hr
36hr
*
*
*

*p<0.01

Fig. 4.17 SKAM1A transgenic MEFs have enhanced collagen contraction

Individual embryos were isolated at embryonic day 13.5 and were subject to genotyping. Wildtype and SKAM1A transgenic mouse embryonic fibroblasts (MEFs) were subject to collagen gel contraction assay for 48hr (A). Area of each gel was measured and quantified using ImageJ software (B). Data represents mean ± SD derived from one experiment performed in triplicate. *$p<0.01$.

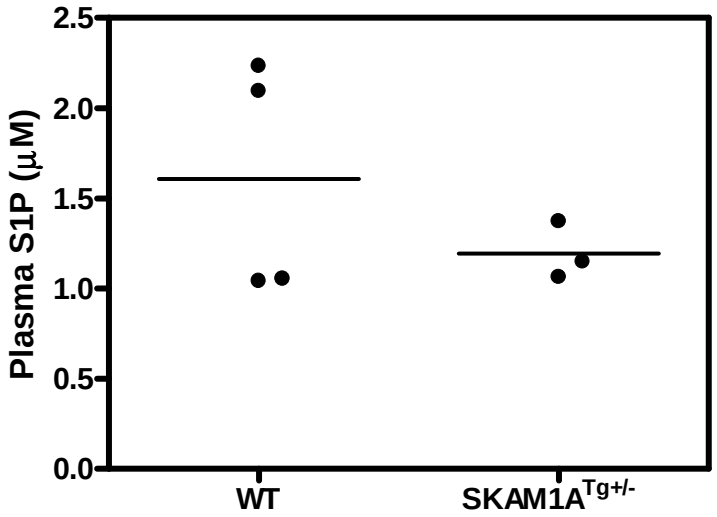


Fig. 4.18 SKAM1A transgenic mice showed slightly reduced S1P levels in plasma

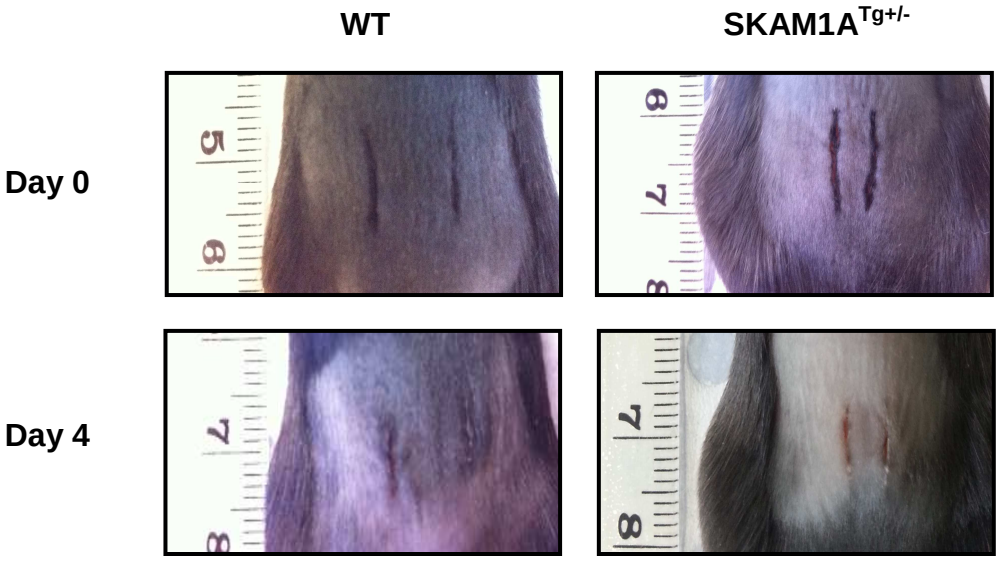
Cardiac puncture was performed on 12 week old female SKAM1A transgenic and wildtype mice. Plasma samples were prepared from whole blood and subject to HPLC analysis (refer to Chapter 2 Materials and Methods for details). Each dot represents the plasma S1P levels of an individual mouse.

	WT	SKAM1A$^{Tg+/-}$
WBC (K/µl)	8.2 ± 3.1	7.9 ± 0.3
NE (K/µl)	1.8 ± 1.2	1 ± 0.4
LY (K/µl)	6.2 ± 1.9	6.6 ± 0.2
MO (K/µl)	0.1 ± 0	0.3 ± 0
PLT (K/µl)	608 ± 74.8	727 ± 23.5
RBC (M/µl)	8.9 ± 0.4	9.1 ± 0.4

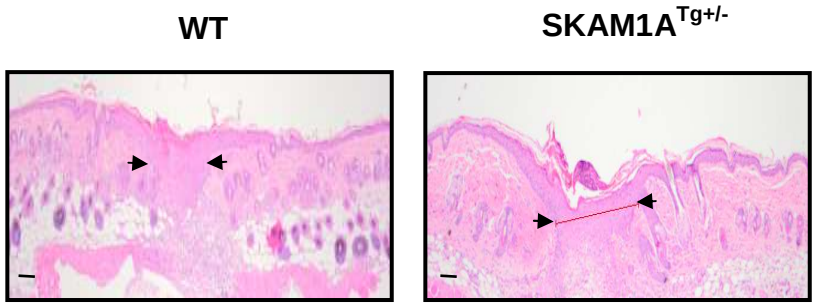
Table 4.1 Whole blood analysis of wildtype and SKAM1A transgenic mice.

Cardiac puncture was performed on wildtype (n=3) and SKAM1A transgenic (n=3) mice. Whole blood samples were analysed using Hemavet 950 and was performed by Dr Jason Powell. WBC: White blood cell. NE: Neutrophil. LY: Lymphocyte. MO: Monocyte. PLT: Platelet. RBC: Red blood cell.

A



B



C

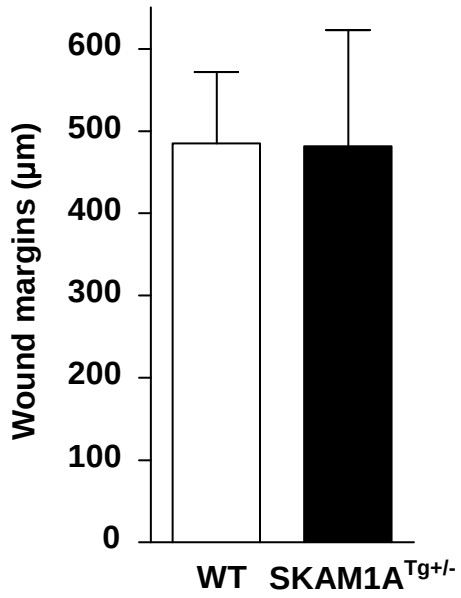
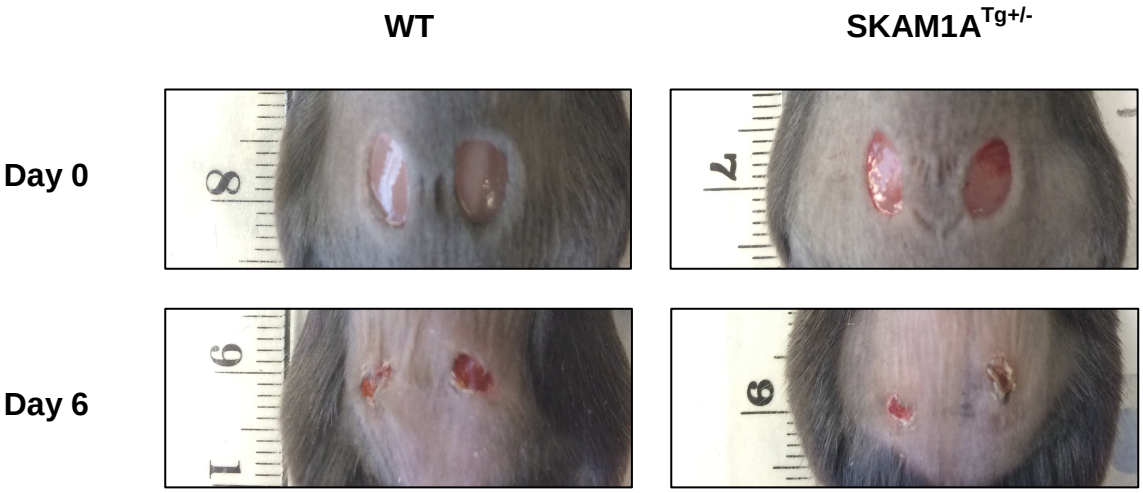


Fig. 4.19 Wildtype and SKAM1 transgenic mice healed at a similar rate following incisional wounding

Twelve weeks old WT and SKAM1 transgenic female C57BL/6 mice were subject to incisional wounding. Wound appearance at day 4 (A). Wounds were excised, cross-sectioned and stained with haematoxylin and eosin (H/E) (B). Wound margins were measured and quantified using the Research AnalySIS software. Wound gap distance was measured and quantified using the Research AnalySIS software (C). Data represents mean ± SD (n=3).

A


WT
SKAM1A Tg+/-
Day 0
Day 6

B

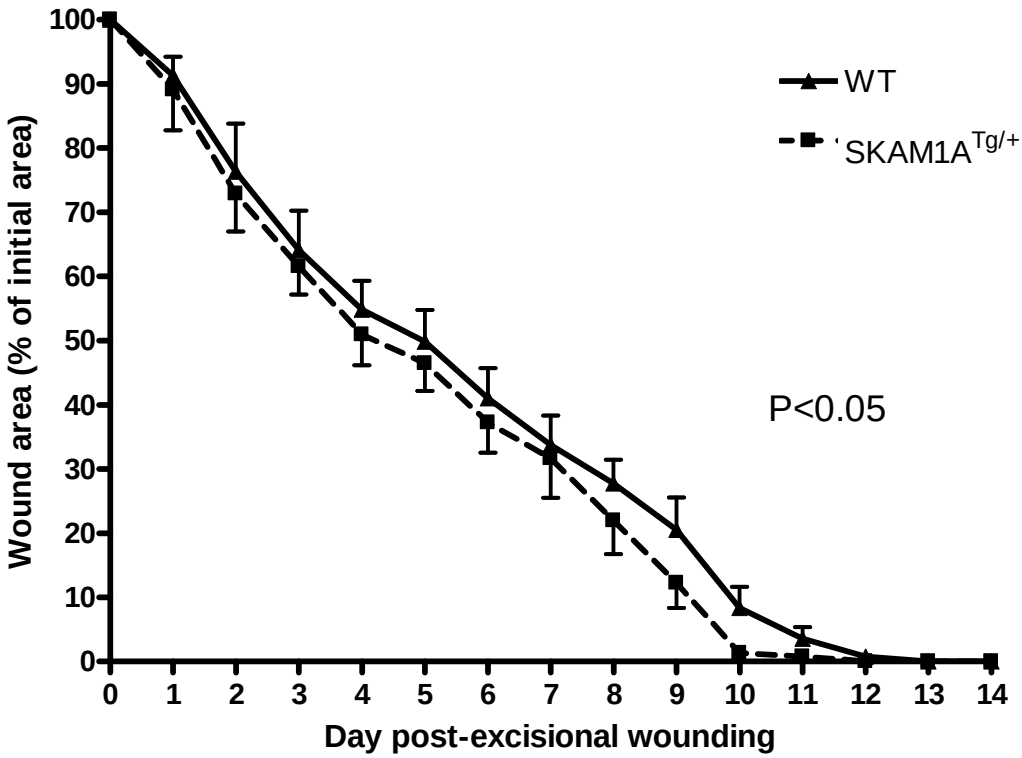

Wound area (% of initial area)
Day post-excisional wounding
WT
SKAM1A Tg/+
P<0.05

Fig. 4.20 Excisional wound healing is enhanced in SKAM1A transgenic mice

Twelve weeks old WT and SKAM1 transgenic female C57BL/6 mice were subject to full-thickness excisional wounding. Wounds were allowed to heal via secondary intention. Photos of wounds were taken daily from day 0 to day 14. Wound appearance at day 0 and 7 (A). Wound areas were measured and quantified using ImageJ software (B). Data is expressed as percentage of initial wound area (B). Statistical analysis was performed using 2Way ANOVA, $n=10$, $p<0.05$.

4.5 Discussion

The aim of this study was to characterise the roles of SKAM1 in fundamental aspects of wound healing, including cell migration, proliferation and survival, and to examine the roles of SKAM1 in wound healing *in vivo*. Data presented in this study have shown for the first time that SKAM1 expression was significantly upregulated in response to wounding in the skin (Fig. 4.3). Furthermore, this process appears to be regulated by growth factors including TGFβ1 and PDGF (Fig. 4.4). In addition, my studies have also shown that SKAM1 is involved in fundamental aspects of wound healing including cell survival (Fig. 4.6) and potentially wound contraction as assessed by fibroblast-mediated collagen gel contraction (Fig. 4.17). Notably, my studies also showed that SKAM1 plays a positive role in wound healing *in vivo* (Fig. 4.20).

SKAM1 was initially identified as a transcript that was upregulated following tooth extraction in rat (Sukotjo et al., 2002). Interestingly, the same group has shown that while SKAM1 was upregulated in the rat oral wound, this phenomenon was not observed in the wounded skin tissue of rat (Lin et al., 2010). This is somewhat surprising since my results showed that SKAM1A mRNA was significantly upregulated in response to wounding in the mouse skin (Fig. 4.3A). Furthermore, my immunostaining data have also shown that SKAM1 protein levels were substantially higher in the wounded skin compared with the unwounded skin in mice (Fig. 4.3B and C). The discrepancy in SKAM1 expression in the skin could be due to the different type of animals or wounding methods being used in these studies. Notably, while Lin and colleagues (2010) performed full-thickness excisional wounds in rats, we created full-thickness incisional wounds in mice. Thus, it is possible that the differences in these models may be responsible for the discrepant results. Our data

suggest that SKAM1 plays an important role in the wound repair of the skin. Indeed, this notion is supported by a study showing that lentiviral-mediated delivery of SKAM1 into excisional wounds in mice significantly enhanced wound resolution (Lin et al., 2010). This, combined with my findings strongly suggests that SKAM1 plays a positive role in cutaneous wound healing.

Interestingly, Lin et al., 2010 has shown that SKAM1 was expressed by fibroblasts in the granulation tissue juxtaposing the excisional wound site in mice. Consistent with this, my results also showed that SKAM1 was expressed by the cells located in close proximity to the incisional wound in mice (Fig. 4.3B). Therefore, given SKAM1 is enriched in the tissues surrounding the wound, it is tempting to speculate that SKAM1 may enhance wound healing possibly through modulating the function of wound-associated fibroblasts.

Cell survival has long been known to play an integral role in wound healing (Greenhalgh, 1998; Lam et al., 2013; Owen et al., 2011). My studies have shown for the first time that SKAM1 is involved in cell survival (Fig. 4.6). Although the mechanism underlying SKAM1-mediated cell survival is unknown, it appears reasonable to speculate that this process is most likely achieved by increased SK1 activity and the subsequent activation of pro-survival pathways downstream of SK1 (Mattie et al., 1994; Pitson et al., 2000; Shu et al., 2002; Song et al., 2011; Xia et al., 2002). Indeed, elevated SK1 activity has been shown by numerous studies to be associated with enhanced cell survival [reviewed in (Van Brocklyn and Williams, 2012). Furthermore, we (Fig. 4.3) and others (Sukotjo et al., 2002; Sukotjo et al., 2003) have shown that SKAM1 expression is upregulated in response to wounding, therefore it is tempting to speculate that SKAM1 may promote wound healing by protecting cells such as fibroblasts from stress-induced apoptosis during early stages

of wound healing. Interestingly, while SKAM1A overexpression enhances cell survival, it does not appear to affect cellular proliferation (Fig. 4.6). One possible explanation is that overexpression of SKAM1A decreases the overall ceramide levels in the cells, thereby promoting cell survival. This effect of SKAM1A, however may not be sufficient to stimulate cellular proliferation, at least in NIH3T3 cells. The role of SKAM1A in cellular proliferation requires further investigation and should also be examined using other cell lines, such as homozygous SKAM1A knockout MEFs.

Migration of cells into the wound bed is an important step in wound healing [reviewed in (Artlett, 2013; Baum and Arpey, 2005; Singer and Clark, 1999)]. During this process, cells undergo drastic changes in their cytoskeleton framework (Lees et al., 2013). Interestingly, previous studies have shown that SKAM1 was associated with the cytoskeletal network in wounded oral fibroblasts (Lin et al., 2010). This raises the possibility that SKAM1 may regulate cell migration through association with the cytoskeleton network. While no molecular detail was described, the same study has shown that SKAM1 was involved in ES cell-derived fibroblast migration (Lin et al., 2010). However, my studies showed that SKAM1 overexpression did not appear to affect cell migration in NIH3T3 fibroblasts, at least in 'two-dimensional' scratch wound assay (Fig. 4.5). It is important to note, however, that different cell types were used between the two studies. The effect of SKAM1 in cell migration may be cell type dependent. Clearly, the role of SKAM1 in cell migration needs further investigation using more advanced migration assays such as the Boyden chamber and microfluidic assays.

Experiments performed in Fig. 4.5 and 4.6 were preliminary studies to examine whether SKAM1A has any effect on cell migration, proliferation and apoptosis. The effect of SKAM1 on these cellular responses will need to be examined

in a thorough manner in future studies to confirm these observations. For example, clonal stable cells expressing low, medium and high of SKAM1 can be generated to examine the physiological role of SKAM1 in these processes.

Preliminary studies performed by the host laboratory have shown that the SKAM1 can directly activate SK1 *in vitro* and in cells (Fig. 1.4). This is the first report of a molecular function of this protein. Elevated SK1 activity and the resultant increase in its product S1P have been shown to be associated with oncogenesis [recently reviewed in (Heffernan-Stroud and Obeid, 2013)]. The ability of SKAM1 to directly activate SK1, together with its possible role in cell survival (Fig. 4.6), raises the possibility that SKAM1 may be involved in the development of an oncogenic phenotype. My studies however, have shown that SKAM1 does not cause neoplastic transformation of cells as assessed by focus formation assays in NIH3T3 fibroblast overexpressing SKAM1 (Fig. 4.7). NIH3T3 fibroblasts have been used extensively as a tool to study the oncogenic potential of a protein. This cell line was employed due to their preneoplastic characteristic which renders these cells to be sensitive to the one-hit transforming actions of many different oncoproteins (Clark et al., 1995). Indeed, not all proteins that can activate SK1 are associated with oncogenesis (Chan and Pitson, 2013). The phosphorylation at Ser225 of SK1 by ERK1/2 (Pitson et al., 2005) and the subsequent translocation of the enzyme to the plasma membrane by CIB1 (Jarman et al., 2010) are the prerequisites for SK1 oncogenic signalling. However, these are unlikely events in SKAM1-mediated SK1 activation since SKAM1 does not phosphorylate SK1. Notably, the Pitson Laboratory has shown that the eukaryotic elongation factor 1A (eEF1A), like SKAM1, can directly interact and activate SK1 *in vitro* (Leclercq et al., 2008). Furthermore, eEF1A binds SK1 at the same site as SKAM1 as shown by competitive pull-down assay (Pitson, unpublished) and can

induce neoplastic transformation via SK1 in NIH3T3 cells (Leclercq et al., 2011). Although it is an interesting observation that eEF1A, but not SKAM1, is able to induce neoplastic transformation in NIH3T3 cells, this could be due to the different binding affinities of the two proteins with SK1, the subcellular location at which SK1 is activated, or the sustainability of SK1 activation. This requires further examination.

Previous studies have shown that SK1 transgenic mice have 20-fold higher SK1 activity compared with wildtype (Takuwa et al., 2010) and they developed cardiac fibrosis (Takuwa et al., 2010). Given that SKAM1A is an activator of SK1, we expected a similar phenotype may present in the SKAM1A transgenic mice. Interestingly, however, while SKAM1A protein was ubiquitously overexpressed in SKAM1A transgenic mice, these mice appear phenotypically normal (Fig. 4.16). Furthermore, my studies also attempted to determine the levels of SK activity in the tissue and organs of the SKAM1A transgenic mice. However, the SK activity assay results were not shown due to inconsistent data, possibly due to the method used to extract SK1 from tissues/organs of SKAM1A transgenic mice. This method will need to be optimised in future studies. While the levels of SK1 activity in the SKAM1A transgenic mice have not yet been determined, HPLC analysis showed that these mice have surprisingly less S1P in the plasma compared with age and sex matched wildtype (Fig. 4.18). One possible reason is that SKAM1A overexpression in the tissues of SKAM1A transgenic mice could lead to higher tissue S1P and thus resulting in reduced sphingolipid availability for the synthesis of S1P in the blood. Interestingly, recent analysis of whole bleed of SKAM1A transgenic mice by the Pitson Laboratory showed these mice had similar levels of white blood cells, neutrophils, lymphocytes, monocytes, and red blood cells to that of the wildtype mice (Table 4.1). Notably, the number of platelets appears to be slightly elevated in the

SKAM1A transgenic mice compared to the wildtype (Table 4.1). Future studies should focus on the elucidation of the underlying cause for these observed phenomenons. Ideally, each study should be examined with additional mice per genotype to enable statistical analyses to be performed and data to be interpreted with confidence.

It can be speculated that the upregulation of SKAM1A following cutaneous wounding (Fig. 4.3) may lead to the activation of SK1 and increased levels of wound-localised S1P which can promote wound healing. Since SKAM1A transgenic mice have higher levels of SKAM1A in unwounded skin compared to wildtype mice, this could result in higher levels of wound-localised S1P following wounding and thus resulting in enhanced wound healing as observed in SKAM1A transgenic mice (Fig. 4.20). This hypothesis requires further investigation.

It is predicted that overexpression of SKAM1A, like SK1, would result in increased cellular proliferation. However, my data showed that SKAM1A is not involved in cellular proliferation at least in NIH3T3 cells. It is important to note that while SKAM1A does not appear to play a role in cellular proliferation *in vitro*, this may not be the case *in vivo*. Future studies should examine the role of SKAM1A in proliferation using other cell lines or skin fibroblasts isolated from SKAM1A transgenic and gene-trap mice. Alternatively, cellular proliferation can also be compared between wounds isolated from wildtype, SKAM1A transgenic and gene-trap mice.

While transgenic SKAM1A was expressed at very low levels, its expression, however, was detected using Western blot (Fig. 4.15). This suggests that the SKAM1A transgenic mice were expressing flag-tagged SKAM1A transgene. Indeed, low levels of SKAM1A expression could explain the lack of a phenotype in the

SKAM1A transgenic mice. It is important to note, however, that the SKAM1A transgenic mice generated in my study showed enhanced wound healing following excisional wounding. Furthermore, the transgenic mice analysed in this study is heterozygous for the transgene. Thus, this may explain the low levels of SKAM1A overexpression in these mice. Future studies will involve the generation of homozygous SKAM1A transgenic mice. We predict that the homozygous SKAM1A transgenic mice to have higher overall levels of SKAM1A overexpression compared to heterozygous SKAM1A transgenics. The level of SKAM1A overexpression in the transgenic mice can be detected using SKAM1 antibody. It is also of significant interest to examine the phenotype and wound healing response in these mice. Interestingly, while no phenotypical abnormalities were observed in the SKAM1A transgenic mice, primary mouse embryonic fibroblasts (MEFs) isolated from SKAM1A transgenic embryos showed enhanced collagen contraction compared with the wildtype MEFs (Fig. 4.17). This observation is consistent with the unpublished data performed by the host laboratory (Fig. 4.1). Given the enhanced ability of primary SKAM1A MEFs to contract collagen gels *in vitro*, and the pro-survival role of SKAM1A in fibroblasts, we speculate that the wound healing in SKAM1A transgenic mice would be more efficient compared with wildtype. Somewhat surprisingly, however, using the incisional model of wound healing, I initially found that the wildtype and SKAM1A transgenic mice healed at a similar rate following wounding (Fig. 4.19). It is important to note however, that the wound healing process in wildtype mice occurs efficiently in this model and therefore any enhancement in the rate of wound healing of the SKAM1A transgenic mice would be difficult to observe. Indeed, using a more severe model of wound healing, the full-thickness excisional model of wound healing, I have shown that the SKAM1A transgenic mice

had a small but significantly enhanced wound resolution compared with the wildtype (Fig. 4.20). Taken together, these data suggest that SKAM1A plays a positive role in wound healing *in vivo*. It should be noted that all my studies with SKAM1A transgenic mice used mice with only a single copy of the SKAM1A transgene. Future studies should also focus on the generation of homozygous SKAM1A transgenic mice. We expect the homozygous SKAM1A transgenic mice could have higher SKAM1A expression and to heal at a faster rate compared to heterozygous SKAM1A transgenic mice. This observation will strongly support the positive role of SKAM1A in wound healing.

The role of SKAM1A in wound healing will also need to be examined in other wound healing models where wound healing is impaired, such as a diabetic model. The sharp rise in prevalence of diabetes has lead to increases in secondary complications including chronic, non-healing wounds [reviewed in (Tsourdi et al., 2013)]. Non-healing wounds often result in other clinical complications such as infection and amputation (Margolis et al., 2005). To achieve this, diabetes could be induced in SKAM1A transgenic mice using streptozotocin (STZ) (Wu and Huan, 2008). Numerous studies have shown a clear advantage of this model of diabetes in wound healing experiments [reviewed in (Braiman-Wiksman et al., 2007)]. Alternatively, the SKAM1A transgenic mice can be crossed with db/db mice to generate diabetic mice overexpressing SKAM1A (Michaels et al., 2007). The effect of SKAM1A on wound healing may appear to be more significant in a diabetic model compared to a normal wound setting. These will be the focus of ongoing studies.

Since SKAM1A gene trap mice have also been successfully generated, future studies will involve the characterisation of the wound healing response of these mice using the wound healing models that have been established in the Pitson Laboratory.

It is of significant interest to study the wound healing response in homozygous SKAM1A gene-trapped mice. We expect these mice to heal at a slower rate compared to the wildtype or heterozygous littermates. This evidence will strongly support the notion that SKAM1A plays an integral role in cutaneous wound healing. It is important to note that SKAM1A may also have an unknown function that is important in development. Thus, deletion of both SKAM1A alleles could lead to embryonic lethality. Indeed, current attempts to generate homozygous SKAM1A gene-trapped mice have failed. This suggests that the loss of SKAM1A may block embryo survival. Notably, the 'knockout-first' system employed in my study to generate the SKAM1A gene-trapped mice enables SKAM1A expression to be rescued. This can be achieved by crossing SKAM1A gene-trapped mice with mice ubiquitously expressing Flp recombinase (see Fig. 4.8B). These mice can then be crossed with various Cre mice to generate conditional knockout mice in the tissue of interest, such as the skin. Using this system, future studies could generate a conditional knockout mouse line and specifically knockout SKAM1A in the skin of adult Cre mice, such as the K14-Cre mice (Jonkers et al., 2001) available commercially from the Jackson Laboratory, to investigate the effect of SKAM1A knockout in cutaneous wound healing using the wound healing models established in the Pitson Laboratory.

In conclusion, the animal models generated in my studies will greatly aid in the understanding of the physiological role of SKAM1A in wound healing. Therapeutic application of SKAM1A into problematic wounds such as diabetic foot ulcers will be the focus of future studies. Although some of the data presented in this Chapter remains preliminary, and requires further replication, these findings have made progress in the understanding of the role of SKAM1A in wound healing of skin.

CHAPTER 5

Effect of a Cell Permeable Peptide, TAT-SKAM1A$^{76\text{-}105}$, on Wound Healing

5. Effect of a cell permeable peptide, TAT-SKAM1A$^{76-105}$, on wound healing

5.1 Abstract

Sphingosine kinase activating molecule 1 (SKAM1) was initially identified as a protein that was significantly upregulated in the rat wounded oral mucosa. Subsequent studies have shown a positive role of SKAM1 in wound healing. Truncation studies performed by the Pitson laboratory have identified a 35 amino acid peptide of SKAM1A, SKAM$^{71-105}$, that can interact and activate SK1 in cells and *in vitro*. Furthermore, overexpression of this peptide in NIH3T3 fibroblasts significantly enhanced collagen gel contraction. Notably, this effect was lost by mutating the tyrosine residue at position 104 to a phenylalanine (Y104F). An even shorter version of this peptide, SKAM1A$^{76-105}$, can also still interact and activate SK1 *in vitro*. Thus, we sought to exploit this knowledge for the development of new agents to improve wound healing. To do this we generated cell-permeable versions of SKAM1A$^{76-105}$ peptide using the HIV-TAT protein transduction domain. TAT-SKAM1A$^{76-105}$, and its Y104F variant, TAT-SKAM1A$^{76-105(Y104F)}$ were synthesised and employed in cells and *in vivo* models of wound healing. I showed that TAT-SKAM1A$^{76-105}$ is cell-permeable, and that TAT-SKAM1A$^{76-105}$, but not TAT-SKAM1A$^{76-105(Y104F)}$, directly activated SK1 *in vitro* and in cells. Application of these peptides to *in vitro* models of wound contraction showed that treatment of NIH3T3 cells with TAT-SKAM1A$^{76-105}$, but not TAT-SKAM1A$^{76-105(Y104F)}$, dramatically enhanced the ability of these cells to contract collagen gels. Furthermore, extension of these studies into the full-thickness excisional wound healing model in mice showed that TAT-SKAM1A$^{76-105}$ treatment significantly enhanced wound resolution in mice. Together, these findings suggest the cell-permeable SKAM1 peptide may have utility in improving wound resolution and

provide an impetus to extend these findings into porcine models of wound healing where the wound healing process better represents that of humans.

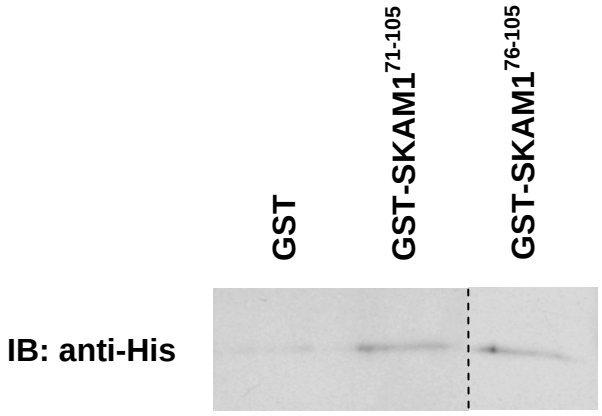
5.2 Introduction

Sphingosine kinase activating molecule 1 (SKAM1), also known as fibroblast growth factor receptor 1 oncogenic partner 2 (FGFR1OP2) or wit 3.0 was initially identified as a transcript that was highly expressed in the rat wounded oral mucosa (Sukotjo et al., 2003). This was the first evidence suggesting that SKAM1 may be important in wound healing. Subsequent studies have shown that overexpression of SKAM1 significantly enhanced collagen matrix contraction (Sukotjo et al., 2003), an *in vitro* model of wound contraction. Furthermore, lentiviral-mediated overexpression of SKAM1 in full-thickness excisional skin wounds significantly enhanced wound resolution in mice (Lin et al., 2010), suggesting that SKAM1 is potentially an important mediator of cutaneous wound healing. In addition, using an excisional wound healing model, my studies (Chapter 4) have shown that SKAM1A transgenic mice have enhanced wound resolution compared to wildtype mice.

Previous truncation studies performed by the Pitson laboratory have shown that, somewhat surprisingly, a 35 amino acid peptide of SKAM1, SKAM1A$^{71\text{-}105}$, was able to interact and activate SK1 *in vitro* (Fig. 5.1). Notably, this 35 amino acid sequence is highly conserved between SKAM1 isoforms and a related protein known as suppressor of IKKε (SIKE) (Huang et al., 2005a), which we have termed SKAM2 as it also interacts with and activates SK (Fig. 5.2). Moreover, unpublished data from the Pitson laboratory has also shown that expression of the SKAM1A$^{71\text{-}105}$ peptide as a fusion protein with EGFP in NIH3T3 fibroblasts significantly enhanced collagen matrix contraction (Fig. 5.3). Interestingly, subsequent truncation studies performed by the Pitson laboratory have shown that a shorter version of this peptide, SKAM1A$^{76\text{-}105}$, was sufficient to interact with and activate SK1 *in vitro* (Fig. 5.1). These findings combined with the known positive roles of SK1 and SKAM1 in

wound healing raises the exciting possibility of generating a cell-permeable SKAM1-based peptide activator of SK1 that may be of potential use in enhancing wound healing. Thus, this chapter focuses on the development of a cell-permeable version of the SKAM1A$^{76\text{-}105}$ peptide and application of this peptide to wound healing in mice.

A


GST
GST-SKAM1 71-105
GST-SKAM1 76-105
IB: anti-His

B

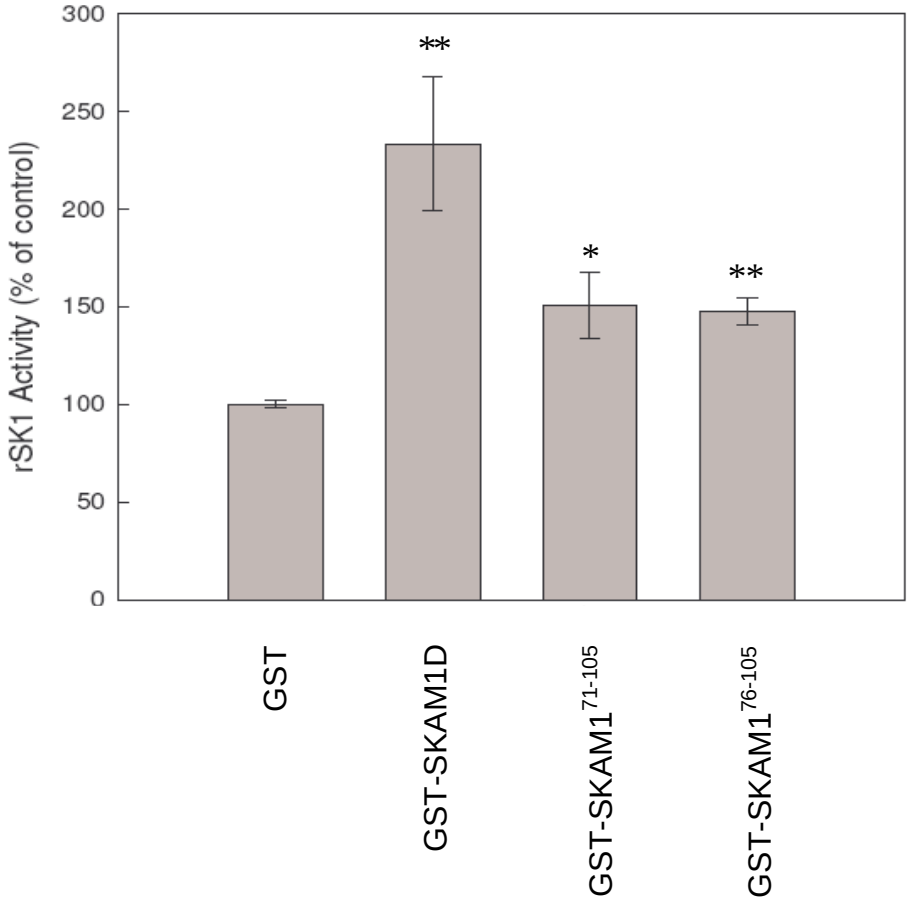

rSK1 Activity (% of control)
300
250
200
150
100
50
0
**
*
**
GST
GST-SKAM1D
GST-SKAM1 71-105
GST-SKAM1 76-105

Fig. 5.1 SKAM1 peptides retain the ability to interact with and enhance SK1 activity *in vitro*.

GST, GST-SKAM1A$^{71\text{-}105}$ and GST-SKAM1A$^{76\text{-}105}$ bound to GSH-sepharose were incubated with His-tagged purified recombinant SK1. SK1 associated with the SKAM1 fusion peptides was detected by immunoblotting with anti-His antibodies (A). GST, GST-SKAM1D, GST-SKAM1$^{71\text{-}105}$ and GST-SKAM1$^{76\text{-}105}$ were incubated with purified recombinant SK1 and examined for their ability to activate SK1 (B). Data are mean ± SEM of three experiments each performed in duplicate. $p < 0.05$, ** $p < 0.001$NB: SKAM1D used (B). (Leclercq & Pitson, unpublished).

```
SKAM1A    1 MSCTIEKALADAKALVERLRDHDDAAESLIEQTTALNKRVEAMK......   44
SKAM2     1 MSCTIEKILTDAKTLLERLREHDAAAESLVDQSAALHRRVAAMREAGTAL   50

SKAM1A   45 ..QYQEEIQELNEVARHRPRSTLVMGIQQENRQIRELQQENKELRTSLEE   92
SKAM2    51 PDQYQEDASDMKDMSKYKPHILLS....QENTQIRDLQQENRELWISLEE   96

SKAM1A   93 HQSALELIMSKYREQMFRLLMASKKDDPGIIMKLKEQHSKIDMVHRNKSE  142
SKAM2    97 HQDALELIMSKYRKQMLQLMVAKKAVDAEPVLKAHQSHSA.........  136

SKAM1A  143 GFFLDASRHILEAPQHGLERRHLEANQNELQAHVDQITEMAAVMRKAIEI  192
SKAM2   151 ............................EIESQIDRICEMGEVMRKAVQV  158

SKAM1A  193 DEQQGCKEQERIFQLEQENKGLREILQITRESFLNLRKDDASESTSLSAL  242
SKAM2   159 DDDQFCKIQEKLAQLELENKELRELLSISSES.LQARKENSMDTASQAIK  207

SKAM1A  243 VTNSDLSLRKS 253
```

Fig. 5.2 Sequence alignment of SKAM1A and SKAM2

Comparison of the SKAM1A and SKAM2 amino acid sequences reveals a stretch of 35 amino acids (underlined) that has high sequence identity between the two proteins. Amino acid changes in the SKAM2 sequence are in bold.

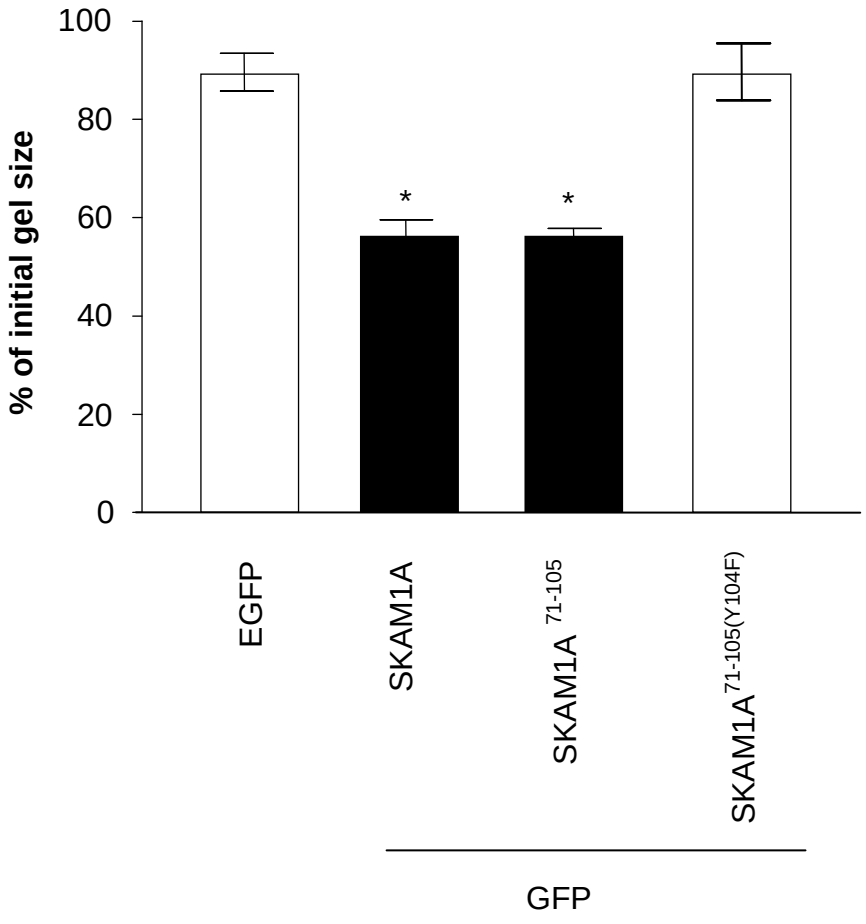


Fig. 5.3 NIH3T3 cells overexpressing EGFP-SKAM1A$^{71-105}$, but not EGFP-SKAM1A$^{71-105(Y104F)}$, enhance collagen contraction.

NIH3T3 cells overexpressing EGFP-SKAM1A, EGFP-SKAM1A$^{71-105}$ or EGFP-SKAM1A$^{71-105(Y104F)}$ were subject to collagen contraction assay with gel sizes measured 28hr after release, n=4, *$p<0.05$. Statistical calculation was performed using student's t-test. (Gliddon & Pitson, unpublished).

5.3 Results

5.3.1 Generation of a cell-permeable version of the SKAM1A$^{76\text{-}105}$ peptide

To generate a cell-permeable version of the peptide we had synthesised the SKAM1A$^{76\text{-}105}$ peptide fused, via a Gly linker, to the 11 amino acid minimal HIV-TAT cellular transduction peptide (YGRKKRRQRRR) (Becker-Hapak et al., 2001) at the *N*-terminus (Table 5.1). Previous studies in the Pitson Laboratory to examine the role of Tyr phosphorylation in the regulation of SKAM1 had generated a variant of SKAM1 containing a mutation of Tyr104→Phe that was unable to enhance collagen contraction (Fig. 5.3). Thus, a comparable cell-permeable version of the SKAM1A$^{76\text{-}105(Y104F)}$ peptide was also synthesised as a potential control.

To confirm cell permeability of these peptides, FITC-labelled TAT-SKAM1A$^{76\text{-}105}$ peptides (wildtype and Y104F) were also synthesised and applied at 500nM to NIH3T3 fibroblasts for 1hr. Cells were then fixed and stained with DAPI to visualise nuclei and assessed for peptide uptake via their FITC-label via confocal microscopy (Fig. 5.4). Both FITC-labelled TAT-SKAM1A$^{76\text{-}105}$ peptides (wildtype and Y104F) were localised in the cell cytoplasm as punctate spots, likely to be macropinosomes. Notably, this was not observed in the FITC control (Fig. 5.4). This type of staining is consistent with the uptake of other TAT-fused peptides (Shoji-Kawata et al., 2013; Yu et al., 2012) and suggests that the TAT-SKAM1A$^{76\text{-}105}$ and TAT-SKAM1A$^{76\text{-}105(Y104F)}$ peptides are cell-permeable. This study is preliminary and needs to be repeated to confirm the data.

5.3.2 TAT-SKAM1A$^{76\text{-}105}$ directly increases SK1 activity *in vitro*

To examine whether the TAT-SKAM1A$^{76\text{-}105}$ peptides retained their ability to activate SK1, I next performed an *in vitro* SK assay with purified recombinant SK1 in

the presence or absence of these peptides. Consistent with previous studies performed by the Pitson laboratory using GST-tagged peptides, incubation of recombinant SK1 with TAT-SKAM1A$^{76\text{-}105}$, but not TAT-SKAM1A$^{76\text{-}105(Y104F)}$, resulted in an approximately 2.5-fold increase in SK1 activity (Fig. 5.5). These results suggest that TAT-SKAM1A$^{76\text{-}105}$ is able to directly activate SK1 *in vitro* and that Tyr104 is important for the ability of the SKAM1 peptide to enhance the activity of SK1.

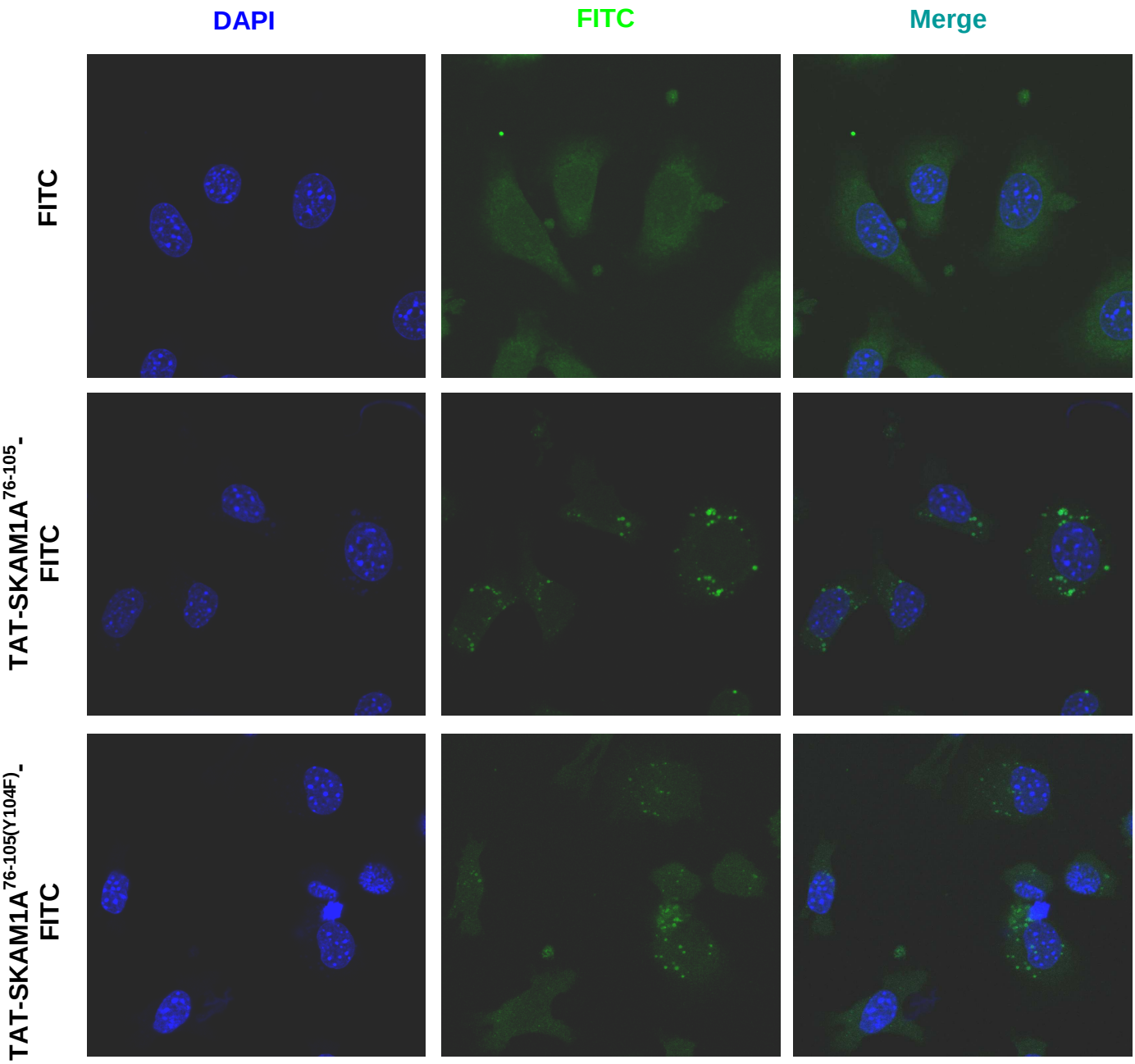

DAPI
FITC
Merge
FITC
TAT-SKAM1A76-105-FITC
TAT-SKAM1A76-105(Y104F)-FITC

Fig. 5.4 TAT-SKAM1A$^{76\text{-}105}$ and TAT-SKAM1A$^{76\text{-}105(Y104F)}$ are cell permeable. NIH3T3 fibroblasts were treated with 500nM of FITC control, FITC-labelled TAT-SKAM1A$^{76\text{-}105}$ or TAT-SKAM1A$^{76\text{-}105(Y104F)}$ for 1hr. Cells were washed with DMEM containing 10% FCS and 1% penicillin/streptomycin, harvested and then seeded onto poly-L-lysine coated coverslips and incubated for 4hr at 37°C and 5% CO_2. Cells were fixed, permeabilised and stained with DAPI. Peptides were visualised using a confocal microscope. Images are representative of one experiment performed in duplicate.

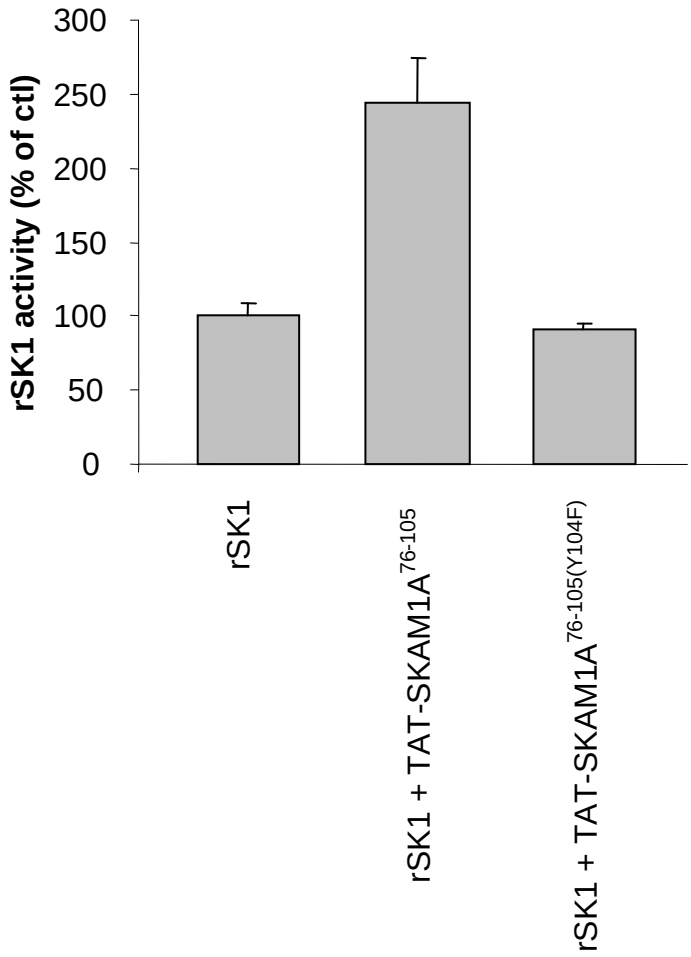


Fig. 5.5 TAT-SKAM1A$^{76\text{-}105}$, but not TAT-SKAM1A$^{76\text{-}105(Y104F)}$, directly activates recombinant SK1 *in vitro*.

TAT-SKAM1A$^{76\text{-}105}$ (50ng) or TAT-SKAM1A$^{76\text{-}105}$ (50ng) was incubated with purified recombinant SK1 (rSK1) (1ng). Each reaction contained 50ng of peptide (9.5 pmol) and 1ng of recombinant SK1 (0.024 pmol). The ability of the peptides to activate rSK1 was analysed in an *in vitro* SK1 activity assay. Data is mean ± range and is representative of two independent experiments performed in triplicate.

5.3.3 TAT-SKAM1A$^{76-105}$ treatment enhances cellular SK1 activity

To examine whether TAT-SKAM1A$^{76-105}$ retained its ability to activate endogenous SK1 in cells, NIH3T3 fibroblasts and primary MEFs were transduced with TAT-SKAM1A$^{76-105}$ and the SK activity subsequently analysed. As shown in Figure 5.6, cells that were treated with TAT-SKAM1A$^{76-105}$ had enhanced SK activity compared with the vehicle control. Interestingly, the effect of TAT-SKAM1A$^{76-105}$ on SK activity appeared to be greater in NIH3T3 fibroblasts compared with MEFs (Fig. 5.6), which could be due to lower expression levels of endogenous SKAM1 in NIH3T3 fibroblasts compared with MEFs as shown by qPCR studies (Fig. 5.7). These results suggest that TAT-SKAM1A$^{76-105}$ can enter the cells and subsequently enhance the activity of endogenous SK1. This study needs to be repeated to enable statistical analysis to be performed.

5.3.4 TAT-SKAM1A$^{76-105}$ treatment enhances collagen contraction by NIH3T3 fibroblasts

Studies performed by the Pitson Laboratory have shown that overexpression of EGFP-SKAM1A$^{71-105}$ in NIH3T3 fibroblasts resulted in enhanced collagen contraction by NIH3T3 fibroblasts (Fig. 5.3). Since TAT-SKAM1A$^{76-105}$ also directly increases SK1 activity *in vitro* (Fig. 5.4) and in cells (Fig. 5.6), I next examined the effect of this cell-permeable peptide on NIH3T3 fibroblast-mediated collagen contraction. Strikingly, NIH3T3 fibroblasts treated with TAT-SKAM1A$^{76-105}$ showed enhanced collagen contraction compared with the no treatment control (Fig. 5.8). In addition, this effect was not seen in the peptide with the Tyr104→Phe mutation (Fig. 5.8). Together, these results suggest that TAT-SKAM1A$^{76-105}$ can induce collagen

matrix contraction by NIH3T3 fibroblasts. This study needs to be repeated to enable statistical analysis to be performed.

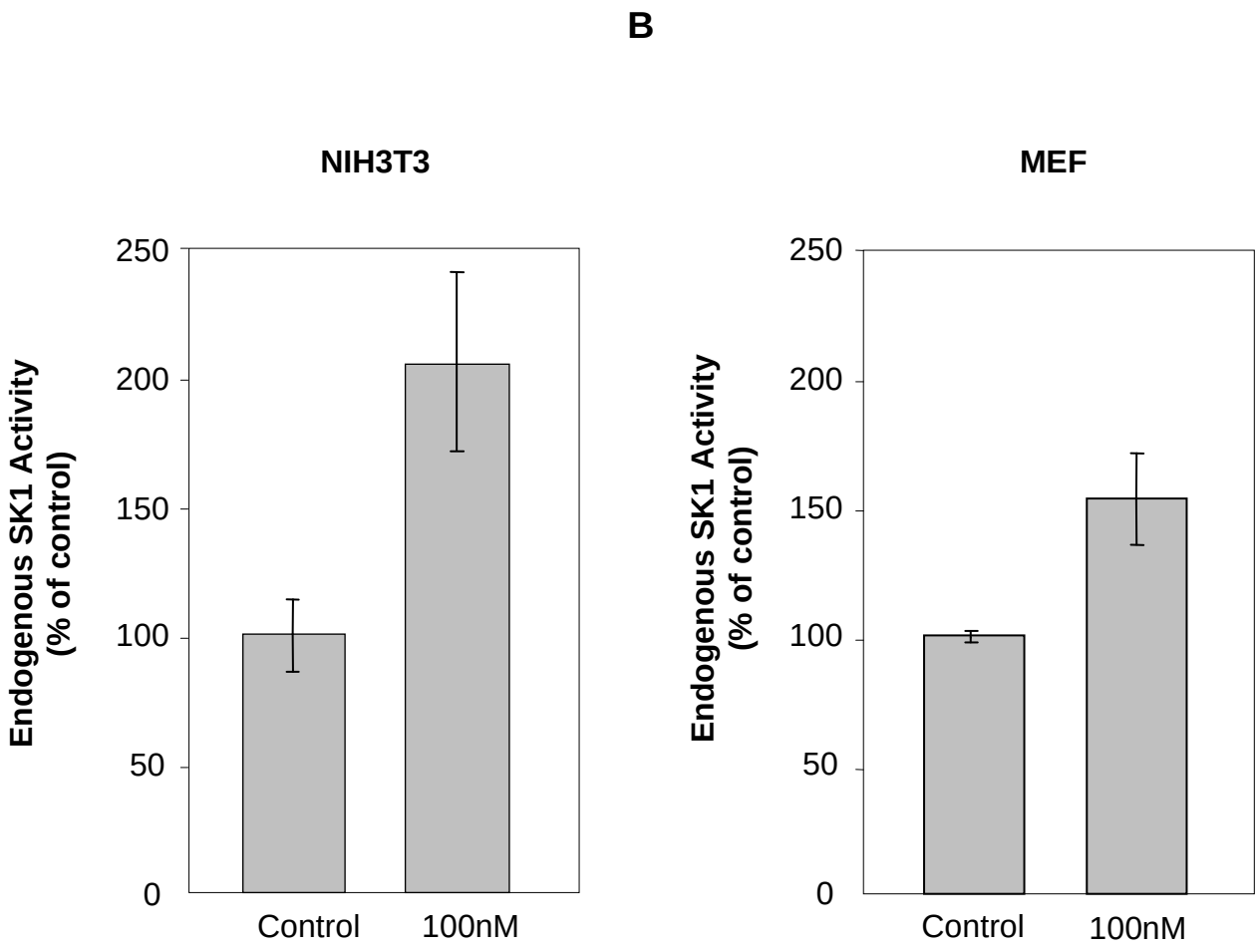


Fig. 5.6 TAT-SKAM1A$^{76\text{-}105}$ enhances endogenous SK1 activity in NIH3T3s and MEFs.

NIH3T3 fibroblasts were incubated with 100nM of TAT-SKAM1A$^{76\text{-}105}$ for 1hr (A). Primary MEFs were incubated with 100nM of TAT-SKAM1A$^{76\text{-}105}$ for 1hr (B). Endogenous SK1 activity was determined by an *in vitro* SK activity assay. Data is mean ± range of two independent experiments, each performed in duplicate.

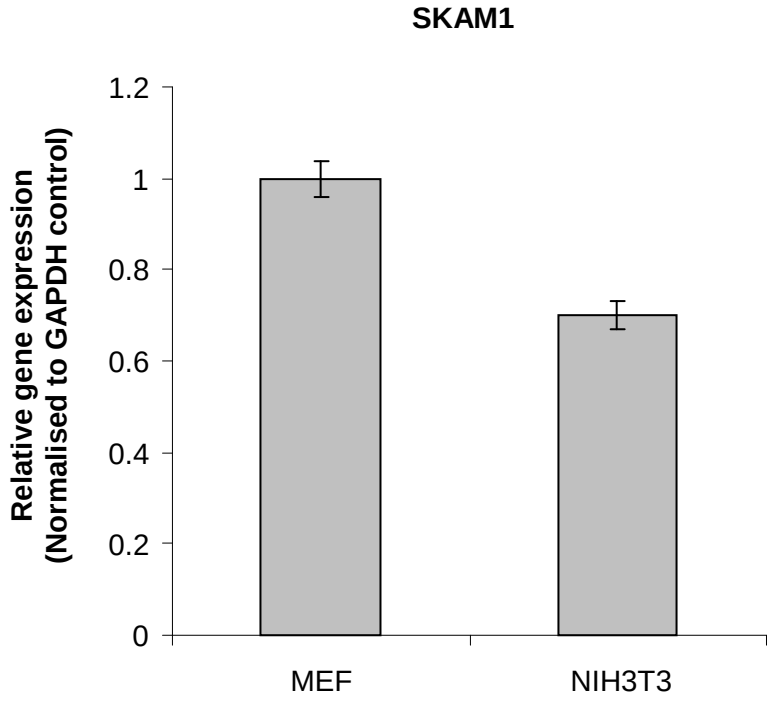
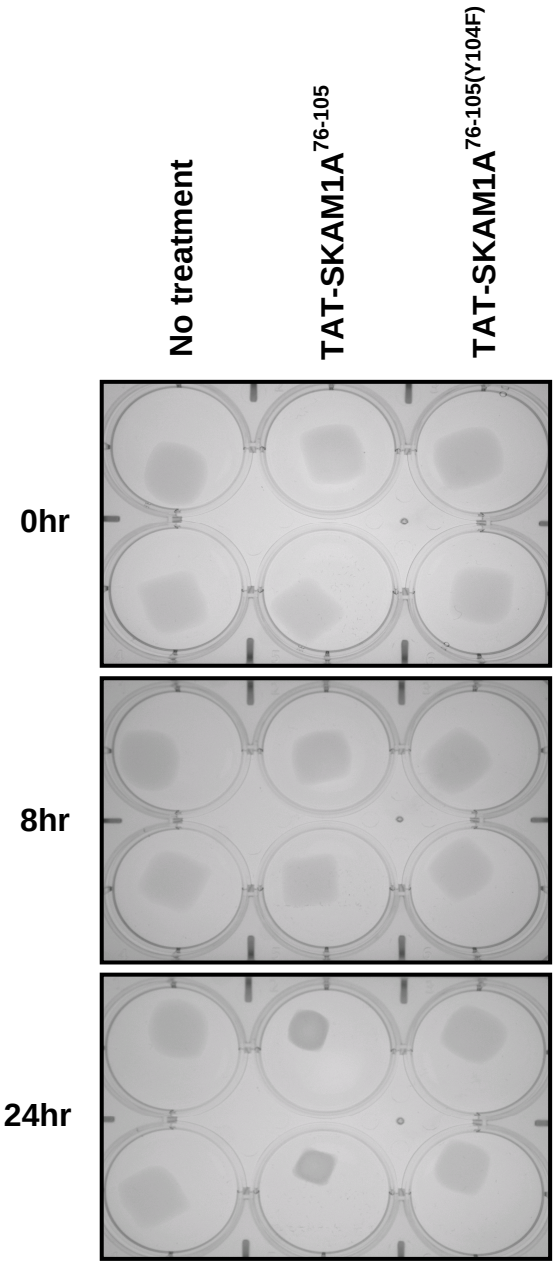


Fig. 5.7 SKAM1A mRNA expression appears to be lower in NIH3T3 fibroblasts compared with MEFs

RNA was isolated from cells using Trizol and was subject to cDNA synthesis. qPCR was performed as described in section 2.7. Data represents the mean ± range from two independent experiments, each performed in triplicate.

A


No treatment
TAT-SKAM1A76-105
TAT-SKAM1A76-105(Y104F)
0hr
8hr
24hr

B

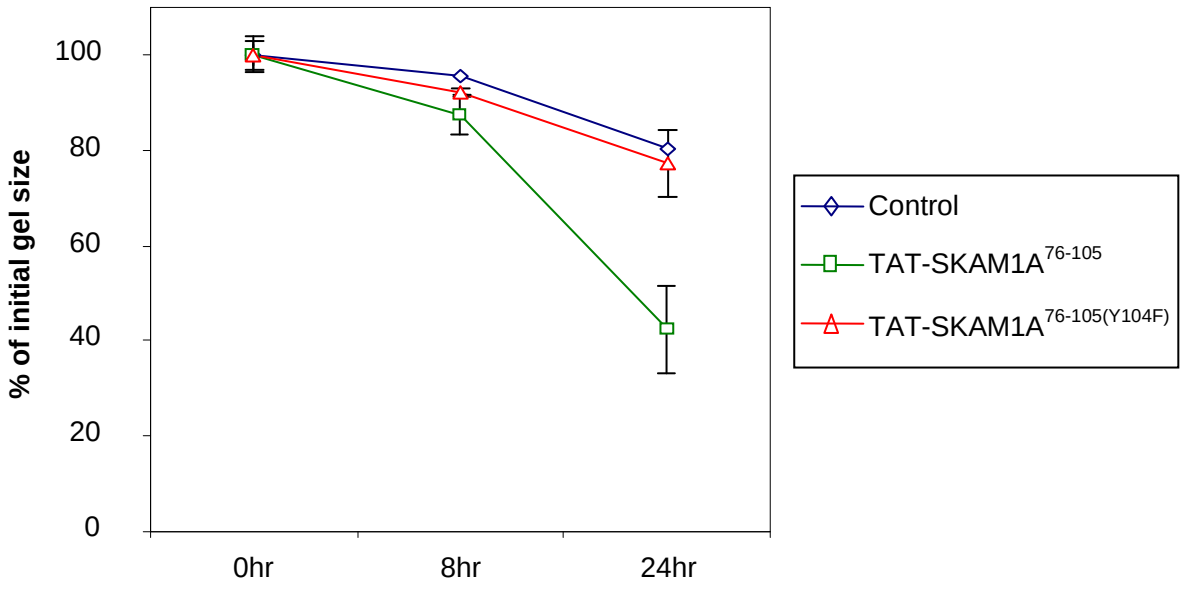

% of initial gel size
100
80
60
40
20
0
0hr
8hr
24hr
Control
TAT-SKAM1A76-105
TAT-SKAM1A76-105(Y104F)

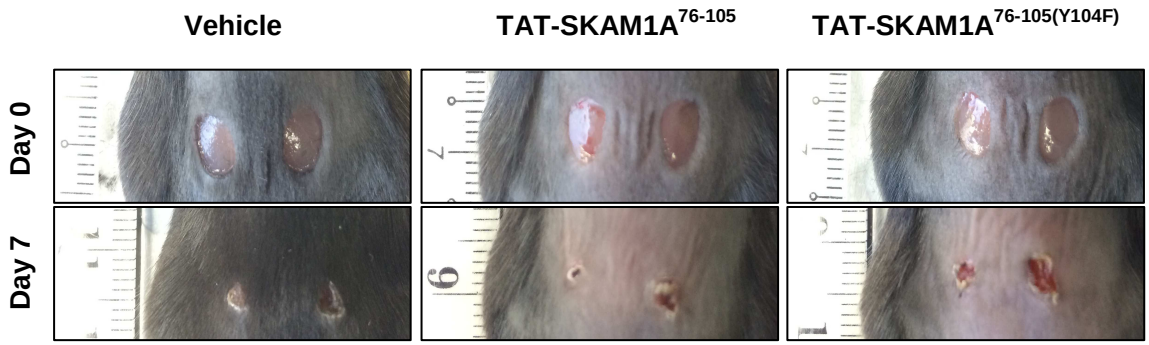
Fig. 5.8 TAT-SKAM1A$^{76\text{-}105}$, but not TAT-SKAM1A$^{76\text{-}105(Y104F)}$, enhances NIH3T3 fibroblast-mediated collagen contraction *in vitro*.

NIH3T3 fibroblasts were pre-treated with 500nM of TAT-SKAM1A$^{76\text{-}105}$ or TAT-SKAM1A$^{76\text{-}105(Y104F)}$ for 1hr before seeding into collagen gel. NIH3T3 cells were then incubated with 0.5μM of peptides (wildtype or mutant) for 24hr (A). Area of each gel was measured and quantified using ImageJ software (B). Data represent mean ± range derived from two experiments performed in duplicate.

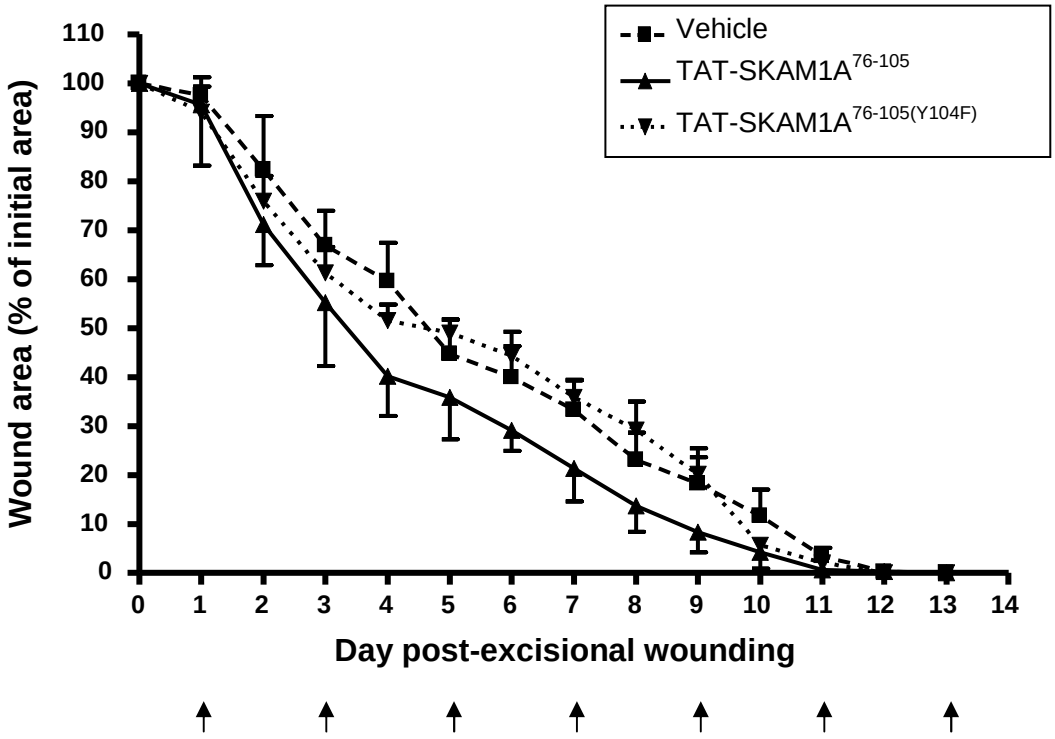
5.3.5 TAT-SKAM1A$^{76-105}$ treatment enhances wound resolution in mice

Since TAT-SKAM1A$^{76-105}$ can directly activate recombinant SK1 and also significantly enhance collagen contraction *in vitro*, this lead to the exciting possibility that this peptide may enhance wound healing *in vivo*. Thus, I examined the effect of TAT-SKAM1A$^{76-105}$ in wound healing using the full-thickness excisional wound healing model. Two full-thickness 6mm diameter excisions were created on the dorsal skin of twelve week old C57BL/6 female mice, one on each side of the midline (Fig. 5.9A) (see Chapter 2 for detail). A total of 21 mice (3 experimental groups, n=7) were used in the experiment. Group 1 received TAT-SKAM1A$^{76-105}$ (500nM) treatment, Group 2 received TAT-SKAM1A$^{76-105(Y104F)}$ (500nM) treatment and Group 3 (control) received saline vehicle control. Injections were performed intradermally adjacent to the skin tissue of each wound on day 1, 3, 5, 7, 9, 11 and 13. Images of wounds were taken daily and wound area was quantified using Image J software. As shown in Fig. 5.9B, TAT-SKAM1A$^{76-105}$ treated mice showed significantly smaller wound surface area compared with the vehicle control. Notably, this effect was not observed in mice treated with the Y104F mutant peptide, TAT-SKAM1A$^{76-105(Y104F)}$ (Fig. 5.9). The time to complete wound closure in, however was largely unaltered between the two treatments (Fig. 5.9).

A



B



Vehicle vs TAT-SKAM1A$^{76-105}$, P=0.0019

Vehicle vs TAT-SKAM1A$^{76-105(Y104F)}$, P=0.6517

SKAM1A$^{76-105}$ vs TAT-SKAM1A$^{76-105(Y104F)}$, P=0.0016

Fig. 5.9 Intradermal injection of TAT-SKAM1A$^{76-105}$, but not TAT-SKAM1A$^{76-105(Y104F)}$ into mouse excisional wounds enhanced wound resolution.

Using a 6mm biopsy punch, two full-thickness excisional wounds were created on the dorsal skin of 12 weeks old wildtype C57BL/6 female mice, one on each side of the midline. Wounds were allowed to heal by secondary intention. A total of 21 mice (3 experimental groups, n=7) were used in the experiment. Each group was treated with TAT-SKAM1A$^{76-105}$ (0.5μM), TAT-SKAM1A$^{76-105(Y104F)}$ (0.5μM) or saline. Injections were performed intradermally adjacent to the skin tissue of each wound on day 1, 3, 5, 7, 9, 11 and 13 (represented by arrows). Images of wounds were taken daily. Wound area was quantified using Image J software. Data is expressed as percentage fold-change compared with day 0. Statistical analysis was performed using 2Way ANOVA, n=7.

TAT-SKAM1A$^{76-105}$	YGRKKRRQRRRGIRELQQENKELRTSLEEH QSALELIMSKYR
TAT-SKAM1A$^{76-105(Y104F)}$	YGRKKRRQRRRGIRELQQENKELRTSLEEH QSALELIMSKFR
TAT-SKAM1A$^{76-105}$-FITC	YGRKKRRQRRRGIRELQQENKELRTSLEEH QSALELIMSKYR-FITC
TAT-SKAM1A$^{76-105(Y104F)}$-FITC	YGRKKRRQRRRGIRELQQENKELRTSLEEH QSALELIMSKFR-FITC

Table 5.1 Cell-permeable peptides of SKAM1.

5.4 Discussion

The aim of these studies was to examine the effect of a cell-permeable peptide, derived from SKAM1A, on wound healing. Data presented in this chapter has shown that the peptide, TAT-SKAM1A$^{76\text{-}105}$, can enhance SK1 activity *in vitro* and in cells (Fig. 5.5 and 5.6) and enhanced collagen gel contraction by NIH3T3 fibroblasts (Fig 5.8). More importantly, intradermal injection of TAT-SKAM1A$^{76\text{-}105}$ into full-thickness excisional wounds in mice significantly enhanced wound resolution (Fig. 5.9). In all cases, the TAT-SKAM1A$^{76\text{-}105(Y104F)}$ variant that does not activate SK1 had no effect, supporting the notion that effects were dependent on SK1.

In the last decade, considerable amount of effort has been made to develop new strategies to enhance wound healing of chronic wounds [reviewed in (Fonder et al., 2008; Tecilazich et al., 2013)]. Notably, however, the efficacy of the majority of therapeutic products that are applied topically to wounds is often limited by their poor penetration through the skin (Nasrollahi et al., 2012). This is not surprising since the skin, or more specifically the stratum corneum layer, which is highly rich in lipid, acts as a tight barrier to prevent the invasion of foreign particles from the external environment (van Smeden et al., 2014). However, in a wound, this protective barrier of the skin is breached, therefore presenting an opportunity for topical drug delivery into the wound. Notably, however, in addition to the skin barrier, the impermeability of the plasma membrane of the cell also has to be overcome for successful drug delivery into the cell (Foged and Nielsen, 2008). The use of cell-penetrating peptides (CPPs) to deliver molecules across the plasma membrane has been studied extensively in recent years [reviewed in (Bechara and Sagan, 2013)]. To date, several CPPs have been identified [reviewed in (Koren and Torchilin, 2012)]. These CPPs can be separated into three categories based on their overall charge and

hydrophobicity; amphipathic, cationic and hydrophobic (Koren and Torchilin, 2012). CPPs are typically short 10-30 amino acid sequences that generally consist of arginine, lysine and histidine. These positively charged residues are thought to facilitate the electrostatic interaction between the peptide and the negatively charged plasma membrane to promote cellular uptake (Yeung et al., 2008). Moreover, studies have shown that the positively charged components of the cell surface, such as proteoglycans, help to promote the cellular uptake of arginine-rich cell-penetrating peptides, like TAT (Amand et al., 2012; Naik et al., 2013). Notably, a number of wound healing studies have shown that the use of CPPs not only enhanced transdermal and transmembrane protein delivery, but also improved the therapeutic effects of the drugs on wound healing *in vivo* (Lee et al., 2013; Zhang et al., 2014). In my study, we utilised the cell-penetrating property of the HIV-1 TAT protein transduction domain (PTD) to deliver a 30 amino acid peptide of SKAM1A, denoted as SKAM1A$^{76\text{-}105}$, into cells. This method of delivery has been widely employed to deliver a variety of 'cargos' into cells, including nucleic acids, enzymes, nanoparticles and cytotoxic drugs [reviewed in (Zhang et al., 2012)]. Furthermore, in order to visualise the localisation of the peptide in cells, we conjugated FITC at the C-terminus of the SKAM1 peptides. Consistent with other studies (Kwon et al., 2005), we observed fluorescent puncta in the cells treated with TAT-labelled peptides likely to be macropinosomes, suggesting that the TAT PTD retained its cell-penetrating properties and was capable of delivering the SKAM1A peptides into cells (Fig. 5.4). Interestingly, the cellular uptake mechanism of TAT as well as other CPPs is not fully understood. However, it seems clear that two major types of cellular uptake mechanisms coexist; direct transduction and macropinocytosis [reviewed in (Koren and Torchilin, 2012). Hence, we speculate that the SKAM peptides traverse through

the plasma membrane via one or both of these mechanisms, most likely via the macropinocytosis since the peptides appeared as punctate spots in the cells.

Endocytic pathways provide highly efficient routes that enable macromolecular entity to be delivered across the plasma membrane (Lim and Gleeson, 2011). It is important to note, however, that it is possible that a fraction or perhaps most of the delivered molecules will be recycled or degraded in lysosomes (Lim and Gleeson, 2011). In my study, the presence of these fluorescent puncta in cells treated with SKAM1 peptides suggests that these peptides are likely to enter the cells via endocytic pathways. Although the uptake mechanism of cell-permeable peptides remains largely unclear, many studies suggest that the intracellular uptake of cell permeable peptides is via macropinocytosis [reviewed in (Lim and Gleeson, 2011)]. Macropinocytosis is an actin-dependent process initiated from surface membrane ruffles that give rise to large endocytic vacuoles called macropinosomes (Lim and Gleeson, 2011). Notably, these macropinosomes can be identified through the use of fluid phase markers, such as Lucifer Yellow, horseradish peroxidise and dextran (Lim and Gleeson, 2011). While the fate of these SKAM peptides located in macropinosomes was not examined in my study, the observed effect of SKAM1 peptides *in vitro* and *in vivo* suggests that SKAM1 peptides retained their ability to activate SK1. One possibility is that these SKAM1 peptides escaped from early stages of the endocytic pathway into the cytoplasm. Indeed, several studies have shown that cell-permeable peptides can be released from endosomes into the cytoplasm via a process called endosomal escape [reviewed in (Bechara and Sagan, 2013)]. While the molecular mechanism underlying this process is largely unknown, it has been proposed that the interaction between the cell-permeable peptides and the endosomal membrane can lead to destabilisation the endosomal membrane, thus resulting in the

release of the peptides into the cytoplasm [reviewed in (Bechara and Sagan, 2013)]. It is important to note that, in addition to macropinocytosis, a small proportion of the cell-permeable peptides can also enter cells via direct translocation. Thus, the observed effect of SKAM1 peptides could also be due to the activity of the peptides that have entered the cell independent of the endocytic pathway.

The Pitson laboratory showed that overexpression of EGFP-fused SKAM1A$^{71-105}$ in NIH3T3 fibroblasts resulted in enhanced collagen contraction (Fig. 5.3). Here, my findings have shown that exogenous TAT-SKAM1A$^{76-105}$ treatment also resulted in enhanced collagen contraction by NIH3T3 fibroblasts (Fig. 5.8), suggesting that this 30 amino acid peptide of SKAM1A is sufficient to stimulate collagen contraction by NIH3T3 cells. Notably, this effect was not observed in cells treated with TAT-SKAM1A$^{76-105(Y104F)}$ (Fig. 5.8) suggesting that Tyr104 is important for SKAM1A$^{76-105}$-induced collagen contraction. Previous studies performed by the Pitson Laboratory have shown that SKAM1-induced collagen contraction was dependent on the SK1 activity (Fig. 4.1). Therefore, given the fact that only TAT-SKAM1A$^{76-105}$, but not TAT-SKAM1A$^{76-105(Y104F)}$, stimulated collagen contraction by NIH3T3 fibroblasts (Fig. 5.8), it is likely that this was due to the inability of the mutant peptide to activate SK1. Indeed, despite both peptides being cell-permeable (Fig. 5.4), my *in vitro* studies have shown that only TAT-SKAM1A$^{76-105}$, but not TAT-SKAM1A$^{76-105(Y104F)}$, can activate recombinant SK1 (Fig. 5.5). This suggests that Tyr104 plays an important role in SKAM-mediated SK1 activation and collagen contraction.

The effect of the mutant peptide was not tested on NIH3T3 and MEFs because it was not available at the time the study was performed. Future studies should examine the effect of the mutant peptide on NIH3T3s and MEFs, although we expect this peptide to have no effect on endogenous SK activity. Furthermore, future studies

should also include a concentration curve to obtain the optimal concentration for each peptide to be used in subsequent experiments.

Interestingly, two tyrosine residues (Tyr46 and Tyr104) are present in the full-length SKAM1A protein (Fig. 5.2). Previous mass spectrometry analysis of the FGFR1-SKAM1 fusion protein (FGFR1OP2) isolated from a patient with T-cell lymphoblastic lymphoma revealed that SKAM1A was phosphorylated at Tyr46 (Gu et al., 2006). Furthermore, a range of other phosphorylation sites in SKAM1 have also been observed in global phosphoproteomics studies, including Ser63, Thr64, Thr88, Ser95, Ser102, Ser141, Ser246 and Ser249 (Beres et al., 2010; Hoffert et al., 2006; Shiromizu et al., 2013; Trinidad et al., 2012; Zhou et al., 2013). Tyr104, however, has not been reported to be phosphorylated in any of these studies. It is tempting to speculate that the biological effects mediated by TAT-SKAM$^{76\text{-}105}$, including its ability to activate SK1 and stimulate collagen contraction, may be regulated by Tyr104 phosphorylation. However, my findings that the addition of purified wildtype peptide, but not the mutant peptide, to recombinant SK1 *in vitro* directly resulted in increased SK1 activity suggests that phosphorylation does not appear to play a role in the biological effects mediated by TAT-SKAM1A$^{76\text{-}105}$. Instead it appears that the biological effects of TAT-SKAM1A$^{76\text{-}105}$, including the ability to activate SK1 and stimulate collagen contraction, require the presence of a hydroxyl group of tyrosine 104, which is absent in phenylalanine. The hydroxyl group of tyrosine may allow the formation of hydrogen bond between TAT-SKAM1A$^{76\text{-}105}$ and SK1, enabling the peptide to stably interact with SK1 and activate this enzyme. Although these two amino acids have similar structure and chemical properties, the subtle difference in structure that exists between these two molecules may be a crucial determinant of its ability to interact and activate SK1. This hypothesis requires further investigation.

Nevertheless, the finding that the Y104F variant does not enhance SK1 activity or collagen contraction provided a valuable control peptide for my studies. Future experiments should also involve the use of either phosphorylated or phosphomimetic versions of the SKAM1 peptide to confirm that phosphorylation does not play a role in the activation of SK.

It was somewhat surprising that a 30 amino acid peptide of SKAM1 was sufficient to activate SK1 and stimulate collagen contraction *in vitro*. Previous unpublished NMR studies performed by the Pitson Laboratory have suggested that SKAM1A$^{76\text{-}105}$ peptide is unstructured in solution by itself. Thus, this peptide may require interaction with SK1 to adopt a stable conformation. Notably, it has been proposed that the conformational versatility and the size of CPPs play an important role in their cellular uptake (Eiriksdottir et al., 2010). Thus, given that TAT-SKAM1A$^{76\text{-}105}$ is small and does not spontaneously adopt a specific secondary structure may render it more able to be efficiently taken up by cells.

In my study, mice treated with SKAM1 peptide healed at approximately day 7 post-excisional wounding (Fig. 5.9). While it is difficult to compare the efficacy of wound healing agents with different mechanisms of action, the efficacy of SKAM1 peptide on wound healing in my study is comparable to other wound healing studies that have involved topical application of small peptides, including an antimicrobial peptide LL37 or Tiger17, on full-thickness excisional wounds in mice (Ramos et al., 2011; Tang et al., 2014). Both studies have shown that wounds treated with the respective peptides healed at approximately day 7 (Ramos et al., 2011; Tang et al., 2014), which is comparable to SKAM1 peptide treatment.

It is important to note, however, the total time taken for healing appears to be similar between the wildtype and the mutant peptide treatment (Fig. 5.9). This is an

interesting observation that requires further investigation using additional mice. A possible explanation for this phenomenon is that the efficacy of the wildtype peptide may have decreased over time as the wound heals. This may be due to the fact that at later stage of wound healing, the skin integrity has been re-established and is rich in collagens which could hinder the ability of the wildtype SKAM1 peptide to activate SK1 in fibroblasts. This notion requires further investigation.

My finding that full-thickness excisional wounds treated with TAT-SKAM1A$^{76\text{-}105}$ showed enhanced wound resolution in mice suggests that TAT-SKAM1A$^{76\text{-}105}$ shows promise as a potential future therapeutic treatment for problematic wounds. Although currently untested, this may be particularly applicable to slow-healing wounds such as diabetic foot ulcers in human. It is important to note, however, that major differences in wound healing exist between mice and human (Sullivan et al., 2001). Thus, future studies should examine the efficacy of the SKAM1 peptides in other animal models that can better replicate clinical human wound healing. One such model is the porcine wound healing model (Sullivan et al., 2001). Although no animal models can completely replicate human wound healing, porcine models of wound healing are excellent tools for the evaluation of therapeutic agents destined for used in human wounds since the wound healing in pig closely resembles that of human (Sullivan et al., 2001). Indeed, numerous studies have employed the porcine model to study the effects of topical agents on wound healing, many of which showed similar effects in subsequent human clinical trials [reviewed in (Sullivan et al., 2001)]. Thus, further studies using the cell-permeable SKAM peptide should focus on this porcine system. Successful outcomes in such a model may then provide impetus for human trials.

These data combined with my findings suggest that the TAT-mediated delivery system is an effective way to deliver peptides into cells and this may have therapeutic implications in future wound healing treatments. In my study, the intradermal application of SKAM1 peptides into full-thickness excisional wounds showed a substantial 3-fold improvement in wound resolution (determined by mean wound area at day 7) compared with previous studies using lentiviral-mediated overexpression system (Lin et al., 2010). This suggests that intradermal injection is not only an effective way to deliver SKAM1 into wounds, this peptide also appears to be more potent in enhancing wound healing compared with the full-length SKAM1A protein. Furthermore, the use of TAT to deliver SKAM1 peptides into wounds would appear to be a more likely therapy than previous studies using lentiviral-mediated delivery of SKAM1 into wounds (Lin et al., 2010) since the topical application of SKAM1 peptides can avoid systemic side-effects and is more likely to enhance patient's compliance and satisfaction in future clinical trials. Future work should focus on topical application of the TAT-SKAM1A$^{76\text{-}105}$ peptide to wounds, such as via a cream-based vehicle as mentioned in Chapter 6.

CHAPTER 6

General Discussion

Chapter 6: General discussion

S1P produced by the SKs is known to regulate a diverse range of cellular processes, including cell proliferation, survival, differentiation, migration, and angiogenesis, mainly via the engagement of S1P to a family of five S1P-specific G protein-coupled receptors (GPCRs) [reviewed in (Pitson et al., 2011)]. These S1P GPCRs regulate a wide array of cellular processes that are known to be critical in wound healing. To date however, little is known of the role of SK and S1P in wound healing. This was the focus of this thesis.

6.1 SK/S1P: New players in wound healing

Previous evidence has suggested a role for S1P in wound repair (Francis-Goforth et al., 2010; Watterson et al., 2007). Most notably, previous work has shown that S1P is enriched in acute wound fluid (Amano et al., 2004), and that application of S1P to diabetic wounds in mice significantly enhanced wound resolution (Kawanabe et al., 2007). Consistent with these findings, using the incisional wound model we observed a significant impairment in wound closure of $SK1^{-/-}$, $SK2^{-/-}$, and $SK1^{-/-}SK2^{+/-}$ mice (Fig. 3.4). Our further analysis suggested this impairment in wound healing may be due to decreased fibroblast proliferation (Fig. 3.5 and 3.6). It is not surprising that impaired fibroblast proliferation can be detrimental to wound healing since fibroblasts play a critical role in wound healing, particularly in the skin [reviewed in (Bainbridge, 2013)]. Fibroblasts are present in normal healing wounds, from late inflammatory phase until full re-epithelialisation of the skin has occurred (Forrest, 1983). During late inflammatory phase, growth factors and cytokines, including PDGF, IL-1β and TNF-α, released from platelets and macrophages located in the provisional matrix facilitate the recruitment of fibroblasts into the wound (Kim et al., 1999). Upon arrival

to the wound, fibroblasts proliferate and secrete new ECM components, including collagens, proteoglycans, fibronectin, tenascin, laminin and fibronectin, which are essential to the restoration of the skin integrity and function (McAnulty, 2007; Schultz et al., 2011). Notably, decreased fibroblast proliferation is a common feature found in many cases of impaired wound healing, particularly in diabetic wounds (Desta et al., 2010; Falanga, 2005; Loots et al., 1999). In addition, this is also one of the underlying causes of delayed wound healing observed in non-healing wounds of the elderly (Gosain and DiPietro, 2004). Thus, it is feasible that these non-healing wounds may have reduced SK activity, thereby resulting in decreased fibroblast proliferation and impaired wound repair. Future studies should examine S1P in these non-healing wounds and subsequently focus on how to enhance SK activity to stimulate wound healing in these wounds, such as the strategy examined in Chapter 5.

Plasma S1P levels are markedly reduced in $SK1^{-/-}$ and $SK1^{-/-}SK2^{+/-}$ mice of which the latter showed very low levels of plasma S1P (Fig. 3.3), which raised the possibility that systemic decreases in S1P in circulation may contribute to impaired wound healing. Interestingly, however, although $SK2^{-/-}$ mice also displayed impaired cutaneous wound healing, circulating S1P levels of $SK2^{-/-}$ mice are higher compared to wildtype mice [Fig. 3.3 and (Kharel et al., 2012; Olivera et al., 2007; Sensken et al., 2010; Zemann et al., 2006)]. These data suggest that the levels of circulating S1P do not have a defining role in the cutaneous wound healing response of SK deficient mice. Instead, I speculate that the S1P levels in the skin tissue, and not the circulation, may play a major role in the regulation of cutaneous wound healing. Notably, although their expression has not been examined in skin, both SK1 and SK2 are likely to be present in this tissue based on their broad expression in other tissues (Allende et al., 2004b; Fukuda et al., 2003; Liu et al., 2000a).

My findings that genetic ablation of either SK1 or SK2 impairs wound healing in mice is interesting. Given that SK1 and SK2 have different development expression, adult tissue distribution and subcellular localisation patterns, these suggest that distinct physiological roles may exist between these two enzymes (Chan and Pitson, 2013; Taha et al., 2006). Previous studies suggest that SK1 and SK2 can have opposing functions, with SK1 in almost all cases promoting cell survival and proliferation (Hannun and Obeid, 2008; Pyne and Pyne, 2010), while SK2 can be pro-apoptotic in some cases (Hofmann et al., 2008; Maceyka et al., 2005; Okada et al., 2005). Notably, however, a number of studies have shown that SK2 can be anti-apoptotic since the depletion of SK2 resulted in enhanced apoptosis and increased chemotherapeutic sensitivity in many cancer cells [reviewed in (Neubauer and Pitson, 2013)]. In addition, it has also been shown that SK2 appears to be pro-proliferative and is involved in cancer progression (Gao and Smith, 2011). My findings that the knockout of either SK1 or SK2 resulted in an impaired wound healing phenotype suggest that both enzymes contribute to wound healing, at least in the skin. Furthermore, my findings also suggest that both SK1 and SK2 appear to play a similar role in the wound healing process. This is not surprising since previous studies have shown that both enzymes appear to have at least some overlapping roles since mice lacking either SK1 or SK2 were normal, fertile and display no obvious abnormalities (Allende et al., 2004b; Kharel et al., 2005; Mizugishi et al., 2005), while SK1 and SK2 double knockout is embryonic lethal due to severe defects in neurogenesis and angiogenesis (Mizugishi et al., 2005). Thus, taken together, these suggest that stimulating both SK1 and SK2 activity in wounds may be a potential therapeutic treatment for aberrant wound healing. This hypothesis requires further investigation. Since my studies have shown that mice lacking either SK1 or SK2 had impaired

wound healing response, it can be speculated that a decrease in total SK in the skin is all that is required to cause impairment in wound healing in mice. Thus, it is important to examine the total SK activity and expression levels in the wounded skin tissues of the SK knockout mice. In addition, future studies should also examine the S1P levels in the wounded skin tissue to determine whether there is a difference between wildtype and SK deficient mice. The levels of S1P in the wounded skin tissue of $SK2^{-/-}$ mice would be of significant interest since the plasma S1P levels in $SK2^{-/-}$ mice is increased.

While decreased S1P in wounds appears a likely reason for the impaired wound healing observed in SK knockout mice, it should also be noted that these findings could also be due to the accumulation of the pro-apoptotic sphingolipids, ceramide or sphingosine, in the wound. Indeed, these sphingolipids are widely associated with growth arrest and apoptosis (Hannun and Obeid, 2008), which may provide an explanation for the decreased cell proliferation observed in the wounds of SK knockout mice. A number of direct targets of ceramide that appear to facilitate its pro-apoptotic effects have been identified, including the serine-threonine (Ser-Thr) protein phosphatases PP1 and PP2A, the Ser-Thr kinases protein kinase C zeta (PKCζ) and c-Jun N-terminal kinase (JNK), the kinase suppressor of Ras (KSR) and the protease cathepsin D (Hannun and Obeid, 2008; Modrak et al., 2006). The direct target that mediates the pro-apoptotic effects of sphingosine *in vivo* is, however, less clear. Nonetheless, a study has shown that sphingosine may exert its pro-apoptotic effects by modulating the function of the pro-survival adaptor protein 14-3-3 (Woodcock et al., 2010). Sphingosine interacts directly with 14-3-3 which leads to the phosphorylation and deactivation of the adaptor protein by protein kinases, such as PKA and PKCζ, and thus resulting in the inhibition of 14-3-3-mediated pro-survival

pathway (Woodcock et al., 2010). Notably, previous studies have shown that ceramide levels are slightly elevated in tissues of diabetic patients and this has been associated with many complications, including wound healing (Holland and Summers, 2008; Summers and Nelson, 2005). Interestingly, a study has shown that topical application of sphingosine or ceramide to full-thickness excisional wounds had no overall effect in diabetic mice (Wakita et al., 1998). Thus, it is tempting to speculate that this lack of effect could be due to increased levels of sphingosine and ceramide that are already present in the wound. Hence, further addition of these sphingolipids would have no effect on the healing of a diabetic wound. Future studies should examine the levels of ceramide and sphingosine in chronic non-healing wounds, such as the diabetic ulcers, as well as in the wounds of SK knockout mice. Elevated levels of these sphingolipids in these wounds would suggest a defect in the sphingolipid pathway, possibly due to decreased SK activity.

6.1.1 SK1 and SKAM1 in wound healing

As mentioned earlier, upregulation of SKAM1 (wit 3.0) was detected in rat oral mucosa following tooth extraction (Sukotjo et al., 2002). Since then a number of studies have demonstrated the role of SKAM1A in wound healing. The potential role for SKAM1 in wound healing and the findings that all naturally occurring SKAM1 isoforms are able to activate SK1 suggests that there may be a role for the activation of SK1 in SKAM1-induced wound healing. Indeed, SK and S1P have been previously associated in would healing (Francis-Goforth et al., 2010; Watterson et al., 2007). Two of the five S1P receptors, SIP_2 and $S1P_3$, were upregulated in response to acute liver injury (Serriere-Lanneau et al., 2007). This was accompanied with an increase in SK1 and SK2 mRNA and increased cellular SK activity. Notably, $S1P_1$ levels were

not altered (Serriere-Lanneau et al., 2007). Furthermore, $S1P_2$ knockout mice displayed reduced accumulation of hepatic myofibroblasts at the site of liver injury, as shown by lower induction of smooth muscle α-actin mRNA and lower TIMP-1 (an inhibitor of matrix metalloproteinases), TGF-β1 and PDGF-BB mRNA, indicating an overall reduction in tissue remodelling following injury (Serriere-Lanneau et al., 2007). In contrast to the observations of Serriere-Lanneau et al. (2007) in which $S1P_2$ knockout mice showed impaired healing in the liver following carbon tetrachloride-induced liver injury, Kawanabe et al., (2007) demonstrated that optimal cutaneous wound healing of diabetic mice was achieved using a combination of exogenous S1P and the $S1P_2$ antagonist JTE-013. This suggests that $S1P_2$, in this case, plays a negative role in S1P-induced cutaneous wound repair. Although these two findings are confounding, it is important to note that one study was performed in liver tissue (Serriere-Lanneau et al., 2007) while the other was performed using full-thickness excisional skin wounds (Kawanabe et al., 2007). Thus, it is reasonable to speculate that the opposing findings of these studies may be due to their biologically different environments and/or the differential expression and availability of the S1P receptors. Interestingly, however, $S1P_{1-3}$ are expressed in the skin and in the liver (Bu et al., 2010; Liu et al., 2011). This suggests that $S1P_2$ may have divergent roles in different organs. Notably, exogenous S1P treatment has been shown to stimulate activation of hepatic stellate cells *in vitro* (Liu et al., 2011). Activation of hepatic stellate cells is an important process in wound healing of the liver and is typical of all forms of liver injury (Khimji and Rockey, 2011). Furthermore, the study performed by Liu et al (2001) has also shown that $S1P_1$ and $S1P_3$, but not $S1P_2$, are important for the activation of hepatic stellate cells in response to S1P. Clearly the involvement of the S1P receptors in wound healing of the liver requires further investigation.

Kawanabe et al. (2007) showed that treatment of wounds with exogenous S1P in normal mice did not improve wound healing compared to vehicle treated wounds. However, intradermal injection of S1P into the wounds of diabetic db/db mice significantly enhanced wound resolution (Kawanabe et al., 2007). The fact that exogenous S1P treatment had no effect on normal wound healing, but significantly enhanced wound resolution of diabetic wounds, may suggest that S1P levels are higher in the normal wound compared to the diabetic wound. Thus, further addition of S1P into normal wound where S1P is enriched would have no additional effect whereas in the diabetic wounds, where S1P levels are present at low levels, additional S1P may help to promote wound healing via activation of the S1P receptors. This hypothesis requires further investigation.

Although the modulation of the S1P receptors has been a major focus of previous studies with the aim to improve wound repair and regeneration (Das et al., 2013; Kawanabe et al., 2007; Watterson et al., 2007), very little is known about the involvement of the specific S1P receptors in the wound healing process. These receptors are excellent targets for therapeutic purposes because they reside on the cell surface, signal through a number of well-characterised G-protein-coupled signalling pathways, and are ubiquitously expressed (Rosen et al., 2009). To date, a number S1P receptor agonists and antagonists are available, including SEW2871 (a selective agonist of $S1P_1$), CS-0777 (a selective agonist of $S1P_1$), FTY720 (an agonist of all S1PRs except $S1P_2$), VPC23019 (an antagonist of $S1P_1$ and $S1P_3$), JTE013 (an antagonist of $S1P_2$), W146 (a selective antagonist of $S1P_1$) and CAY1044 (a selective antagonist of $S1P_3$). These agents not only provide important tools to study of the involvement of the S1P receptors in wound healing, it also raises the exciting potential for the employment of these chemical agents to enhance wound healing by

targeting the SK/S1P axis. Notably, FTY720 has been established in the clinic as a therapy for multiple sclerosis (Chiba and Adachi, 2012). Unpublished work performed by the Pitson laboratory has shown that $S1P_1$ and $S1P_3$ are involved in the wound healing process *in vitro*. Thus, to examine the involvement of these S1P receptors in wound healing *in vivo*, future studies should examine the effect of the S1P receptor antagonists VPC23019 and JTE013 on wound healing in mice, using the mouse wound models that have been established; incisional and excisional wound models. In addition, if $S1P_1$ and/or $S1P_3$ are found to be involved in wound healing, subsequent studies using $S1P_1$ agonists (SEW2871 and CS-0777) should also be performed in diabetic mice, where wound healing is impaired, to examine if stimulation of the $S1P_1$ can enhance diabetic wound healing. Notably, no $S1P_3$-selective agonists are available, but since the main signalling pathways activated by $S1P_1$ and $S1P_3$ are similar, it is expected that $S1P_1$ signalling will be sufficient to enhance wound healing. Furthermore, the same experiment could also be performed using SK knockout mice to examine whether stimulation of $S1P_1$ by these agonists can rescue the impaired wound healing phenotype observed in SK knockout mice. Successful outcomes from these studies will raise the exciting potential for the topical application of these drugs to enhance cutaneous wound healing.

Findings from my studies suggest an alternative therapy to improve wound healing. This involves the stimulation of SK1 activity in cells using a cell-permeable SKAM peptide, TAT-SKAM1A$^{76\text{-}105}$. Elevated cellular SK1 activity may stimulate wound healing by both increasing S1P in the wound tissue which would result in the subsequent activation of the S1P receptors and also decreasing ceramide and sphingosine which appear elevated in diabetic tissue and may have a negative effect on wound healing (Summers and Nelson, 2005). Indeed, a recent study has shown that

topical application of a plasmid encoding SK1 cDNA to the wound surface of diabetic rats resulted in enhanced wound resolution (Yu et al., 2014). It is important to note, however, that the approach employed in the study to deliver SK1 into wounds is not relevant in the clinical settings since it has the risk that the SK1 cDNA-containing plasmid could integrate into the host genome and may elicit oncogenic signalling.

Interestingly, a SK1-interacting protein named FHL2/SLIM3 has been previously shown to be involved in wound healing (Kirfel et al., 2008; Wixler et al., 2007). S1P has been previously shown to trigger a signalling cascade that results in the nuclear translocation of FHL2 in response to the activation of the RhoA GTPase (Muller et al., 2000; Muller et al., 2002). In the nucleus, FHL2 acts as a coactivator of the androgen receptor (AR), a transcription factor which is involved in the regulation of a number of genes that are associated with cellular processes important in wound healing, including actin cytoskeleton remodelling, cell motility and cell adhesion (Muller et al., 2002). Notably, collagen contraction and cell migration were severely impaired in FHL2 deficient cells (Wixler et al., 2007). The authors concluded that FHL2 functions as a lipid-triggered signalling molecule in mesenchymal cells regulating their migration and contraction during cutaneous wound healing (Wixler et al., 2007). Subsequent studies have also shown that FHL2 deficient mice have impaired intestinal wound healing due to reduced collagen III expression in anastomotic segments after surgery (Kirfel et al., 2008). Taken together, it can be speculated that the impaired wound healing observed in the SK knockout mice may be a consequence of reduced translocation of FHL2 into the nucleus, due to reduced levels of SK in the wounded skin tissue, thus resulting in decreased expression of genes that are involved in the wound healing process. This hypothesis requires further examination.

6.2 FGFR1OP2/SKAM in wound healing

SKAM1 (known as FGFR1OP2 or wit 3.0 in the literature) is a protein recently identified by the Pitson laboratory to interact with and directly activate SK1. Although SKAM1 has been previously shown to be involved in wound healing, the molecular mechanism underlying SKAM1-mediated wound healing was not known.

My findings that overexpression of SKAM1 protects fibroblasts from apoptosis induced by serum deprivation suggests that SKAM1 plays a pro-survival role in cells (Fig. 4.6A). This is not surprising since SKAM1 is able to activate SK1, an enzyme that is known for its pro-survival effect in cells, particularly in fibroblasts [reviewed in (Van Brocklyn and Williams, 2012)]. Decreased cell survival has been previously shown to be associated with impaired wound healing (Ramaesh et al., 2006). In fact, this is an underlying cause of impaired wound healing in diabetic patients (Rai et al., 2005). Recent studies have shown that healing of full-thickness excisional wounds in mice can be improved by stimulating the survival of exogenous stromal stem cells using a non-immunogenic ECM patch material derived from porcine small-intestine submucosa (Lam et al., 2013). Taken together, these studies suggest that cell survival plays an important role in wound healing. The fact that SKAM1 overexpression resulted in enhanced cell survival of fibroblasts *in vitro* combined with my findings that SKAM1 is upregulated in the cells that are in close proximity to the wound raise the possibility that SKAM1 may mediate cutaneous wound healing by increasing the survival of fibroblasts in the wound. Future studies are required to determine the physiological relevance of the pro-survival role of SKAM1A in wound healing, with particular reference to its role in fibroblasts, since they play key roles in cutaneous wound healing. Fibroblasts are involved in key processes such as breaking down the fibrin clot, secreting new ECM components to

support other cells associated with effective healing, and they are also the precursor of myofibroblasts which are involved in wound contraction (Bainbridge, 2013). Although we proposed that the pro-survival effect of SKAM1 is mediated via the SK/S1P axis, further understanding of the molecular mechanism of SKAM1-mediated cell survival in fibroblast would be important for the development of therapeutic strategies to improve wound healing in the skin.

6.3 SKAM1A transgenic and gene-trap mice

In this study, two mouse lines were generated to investigate the role of SKAM1A in wound healing *in vivo*. Previous studies have shown that lentiviral-mediated delivery of SKAM1A into full-thickness excisional wounds accelerated wound resolution in mice (Lin et al., 2010). Consistent with these findings, my studies have shown that mice overexpressing SKAM1A have a small but statistically significant enhancement in wound healing compared with wildtype mice following full-thickness excisional wounding (Fig. 4.20). Notably, the enhanced wound healing observed in the SKAM1A transgenic mice may be a consequence of enhanced wound contraction. This is supported by the fact that the rate of wound closure in the SKAM1A transgenic mice was increased after day 7 (Fig. 4.20), at which the wound contraction phase is likely to occur, and that SKAM1A can enhanced fibroblast-mediated collagen contraction *in vitro* (Fig. 4.1 and 4.17). Future work should examine the role of SKAM1A in an impaired wound healing model, such as a diabetic model. Several mouse models of diabetes for wound healing studies are available, such as the non-obese diabetic (NOD) mice, ob/ob mice and db/db mice (Rees and Alcolado, 2005). In addition, diabetes can also be induced in mice to impair wound healing using chemicals such as streptozotocin (STZ) or alloxan (ALX) (Junod et al.,

1969; Lenzen and Panten, 1988). By far, the most commonly used mouse models of diabetes in wound healing studies are the db/db and the STZ model of diabetes (Jetten et al., 2014; Park et al., 2014; Ruzehaji et al., 2014; Ruzehaji et al., 2013). STZ is a toxic agent that depletes pancreatic beta islet cells, rendering the mouse diabetic due to the inability to produce sufficient insulin (Wu and Huan, 2008). It is important to note, however, it has been previously reported that the STZ model of diabetes may not be a suitable model of impaired wound healing for open wounds in mice. This notion is supported by a study showing that full-thickness excisional wounds in STZ-induced diabetic mice healed at a similar rate compared to the wildtype control (Fang et al., 2010). Recent studies, however, have shown that the STZ-induced diabetes can be an appropriate model of impaired wound healing in mice (Ruzehaji et al., 2014; Ruzehaji et al., 2013). Thus, the role of SKAM1A in wound healing can be examined using this impaired wound healing model. Alternatively, SKAM1A transgenic mice with impaired wound healing can be generated by crossing these mice with the db/db mice. This is a mouse model of diabetes that was first described by Hummel et al (1966) who identified random mutations in mice associated with obesity and excessive hunger (Hummel et al., 1966). It was shown by subsequent studies that the db/db mice have mutations in the leptin receptor, thus rendering these mice insensitive to leptin and resulting in a diabetic and obese phenotype (Coleman, 2010). This model of impaired wound healing has been widely employed in numerous wound healing studies (Immonen et al., 2014; Jetten et al., 2014; Park et al., 2014). Unlike the STZ model in which wound healing impairment was not always observed in excisional wounds (Fang et al., 2010), the db/db mice exhibit severe delay in excisional wound closure due to a significant decrease in granulation tissue formation, angiogenesis, proliferation (Michaels et al., 2007). Thus, this suggests that the db/db model may be

the most appropriate model of impaired wound healing for future studies examining the role of SKAM1A in wound healing.

The role of SKAM1A in wound healing should also be investigated using other well characterised wound healing models, such as a burn wound model and bleomycin-induced scar formation model, each of which focuses on different aspects of the wound healing process [reviewed in (Wong et al., 2011)]. Burn wounds differ significantly from surgical wounds, such as incisional wounds and excisional wounds, in both the direction and cellular mechanism of injury (Linares, 1996; Shakespeare, 2001). These wounds are characterised by heat-induced tissue coagulation and horizontal, not vertical injury as seen in surgical wounds (Linares, 1996). Surgical wounds heal rapidly via blood clot formation, re-epithelialisation and cellular proliferation. Although burn wounds heal via a similar process, these wounds generally heal more slowly, with oedema, extensive necrosis and hypoxia (Linares, 1996). Thus, it would be interesting to examine the role of SKAM1A in a burn wound model, such as the partial-thickness burn model, in which necrosis is involved in the wound healing process since my *in vitro* findings have shown that SKAM1A has a pro-survival role in fibroblasts. Furthermore, the involvement of SKAM1 in scar formation should also be examined in future studies. The bleomycin-induced scar formation model appears to be an ideal model to study the process of scar formation (Cameron et al., 2014). Bleomycin is an antibiotic and is widely used as an anti-cancer treatment (Umezawa, 1967). Bleomycin hydrolase inactivates bleomycin by hydrolysing the amide bond in the β-aminoalanineamide moiety. However, the lack of this enzyme in the lungs and the skin enables bleomycin to induce scarring in these organs in a dose-dependent manner (Muggia et al., 1983). This model will allow examination of whether SKAM1A is involved in the process of scar formation in the

skin, including changes in the composition of ECM components and the ratio of collagen I and III deposition in the scars. These studies will provide a greater understanding of the role of SKAM1A in different wound scenarios, which will be useful for future studies that involve the use of SKAM1A as a therapeutic treatment for aberrant wound healing.

While no work has been done on the SKAM1A gene trap mice due to issues with generating homozygous gene-trapped mice, the heterozygous SKAM1A gene-trap mice displayed an apparently normal phenotype without any observable gross abnormalities (data not shown). These mice should be further characterised in future studies. Since the SKAM1A gene trap mice have only one allele of the gene, we predict that these mice to have impaired wound healing response compared with wildtype mice. Our attempt to generate homozygous SKAM1A gene-trapped mice has failed (of 55 mice born from heterozygous SKAM1A gene-trapped breeding, 10 were wildtype and 45 were heterozygous SKAM1A gene-trapped), suggesting that SKAM1A may be essential for mouse development. Interestingly, homozygous SKAM1A gene-trapped embryos are viable at embryonic day 13.5 (E13.5) (data not shown). This suggests that embryonic lethality must have occurred after this stage of embryonic development. Current investigations are aimed at determining at which stage and why embryonic lethality occur as this will provide valuable information about the involvement of SKAM1A in embryonic development. Previous studies have shown that SK1 and SK2 double knockout embryos died *in utero* at E11.5 due to severe defects in angiogenesis and neurogenesis (Mizugishi et al., 2005). Since the death of SKAM1A gene-trapped embryos occurred later than E11.5, this suggests that embryonic lethality is not due to its role with SK. Notably, previous studies have shown that SKAM1 is a component of the STRIPAK complex (Goudreault et al.,

2009), which regulates a wide variety of cellular processes, including cell cycle control, apoptosis, Golgi assembly, neural and vascular development [reviewed in (Hwang and Pallas, 2014)]. Thus, the deletion of SKAM1 may have detrimental effects on embryonic development via altered signalling function of the STRIPAK complex. Notably, the versatile system employed in my study to generate gene-trapped mice enables embryonic lethality to be overcome by generating conditional SKAM1A homozygous gene-trapped mice. Briefly, SKAM1A wildtype mice can be obtained by crossing the heterozygous SKAM1A gene-trapped mice ubiquitously expressing Flp recombinase to remove the gene-trap cassette, whilst leaving loxP sites within the SKAM1 gene. These SKAM1A floxed mice can then be crossed with tissue-specific Cre recombinase expressing mice to achieve tissue specific SKAM1A knockout (Fig. 4.8). For example, skin specific SKAM1A knockout can be achieved by crossing the floxed SKAM1A mice with keratin 14 (K14)-Cre transgenic mice. The K14-Cre transgenic mice have been widely employed to study the effects of gene knockout in the skin (Scharfenberger et al., 2014). Notably, the murine K14 promoter is only expressed in the basal epidermal keratinocytes (Scharfenberger et al., 2014). However, since all epidermal cells of the skin are derived from the basal keratinocytes (Clayton et al., 2007; Qiu et al., 2011), the K14 promoter is often used to drive Cre recombinase expression in the whole skin epidermis. Thus, by crossing the floxed SKAM1A mice with K14-Cre transgenic mice, the effect of SKAM1A knockout in the skin in response to wounding can be studied. This should be the focus of future studies to enable the characterisation of the wound healing response in these mice. In addition, these mice can also be crossed with other Cre expressing mice to study the role of SKAM1A in mouse development, which is of significant interest.

6.4 Topical application of a cell-permeable SKAM peptide as a treatment for aberrant wound healing

Impairments in wound healing remain a major clinical challenge (Sen et al., 2009). Unfortunately, current wound healing treatments including debridement, garments, dressings, culture-based antibiotic treatment, or autologous skin transplantation are sub-optimal (Tsourdi et al., 2013). Although the use of growth factors as a treatment of impaired wound healing, particularly in patients suffering from diabetic wounds, has been extensively studied in the past (Erickson, 1991; Harding et al., 2002), the outcomes of the majority of human clinical trials, regarding the efficacy of growth factors in promoting wound healing, are generally disappointing (Bennett et al., 2003; Falanga et al., 1992; Richard et al., 1995). To date, the only growth factor that has passed the clinical trials and approved by the United States Food and Drug Administration (FDA) as a therapeutic treatment to promote healing of full-thickness neuropathic diabetic foot ulcers is recombinant human PDGF-BB (also known as becaplermin or Regranex), which is a variant of PDGF (Harding et al., 2002; Rennert et al., 2013). Regranex relies on the local delivery of PDGF-BB to accelerate wound healing via promotion of fibroblast migration and wound re-epithelialisation (Li et al., 2004; Pierce et al., 1991; Saba et al., 2002). It is worth noting, however, that this growth factor based product may not be effective in all wound types, as a previous clinical trial has shown that the application of Regranex gel was not superior over hydrogel dressings for the healing of hypertensive leg ulcers (Senet et al., 2011). Furthermore, the efficacy of Regranex has been questioned since a wide variation in complete wound recovery in treated patients was observed in previous clinical trials (Bennett et al., 2003). Notably, in one of the four clinical trials, conducted on 250 patients, Regranex treatment did not

achieve statically significant improvement in wound closure when compared to placebo control (36% improvement versus 32% improvement for placebo control) (Smiell et al., 1999). Taken together, these observations suggest that the efficacy of Regranex may be wound type-dependent and it may only be effective in a small subset of diabetic wounds with specific pre-existing pathophysiological conditions. Thus, given the modest efficacy of Regranex, its expensive cost and limited use, the identification and development of new alternative therapeutics for cutaneous wound repair is of significant current need.

My studies have shown that SKAM1 is an important positive mediator of wound healing. In addition, our findings that a 30 amino acid peptide of SKAM1 is sufficient to activate SK1 *in vitro* raised the exciting possibility of generating a cell-permeable SKAM1-based peptide activator of SK1 that may be of potential use in enhancing wound healing. Indeed, application of such a cell-permeable peptide of SKAM1, TAT-SKAM1A$^{76\text{-}105}$, into full-thickness excisional wounds in mice significantly enhanced wound resolution (Fig. 5.9). Notably, this finding is consistent with previous studies showing that lentiviral-mediated overexpression of full-length SKAM1 protein in full-thickness excisional wounds resulted in enhanced wound healing in mice (Lin et al., 2010). Taken together, my study suggests the therapeutic potential of this peptide for the improvement of wound healing.

While the cell-permeable SKAM1 peptide approach showed exciting findings, the persistence of SK1 activation following transduction of cells with the TAT-SKAM1A$^{76\text{-}105}$ peptide needs to be examined in future studies. If SK1 activation is only short-lived this would suggest proteolytic degradation of the peptides in cells or wounds. If this is the case, the use of retro-inverso peptides, composed of D-amino acids assembled in the reverse order of their native L-sequences may be beneficial.

These peptides appear to be more stable because they are not recognised by proteases and they generally retain their protein binding properties (Fischer, 2003; Taylor et al., 2010). It is important to note, however, that not all retro-inverso peptides retain the same binding affinity to proteins as their parent peptides, especially those that take up an alpha-helical conformation (Li et al., 2010; Pazgier et al., 2009; Worthylake et al., 1999). Notably, although prolonged SK1 activation by stable peptides could lead to oncogenic signalling, this is unlikely to occur since NIH3T3 cells stably overexpressing SKAM1 did not undergo neoplastic transformation (Fig. 4.7).

In my study, intradermal application of SKAM1 peptides onto full-thickness excisional wounds did not result in any observable skin irritation or allergic responses in mice. This suggests that the SKAM1 peptides, including the mutant control, were non-immunogenic and were well-tolerated in mice. Furthermore, the predicted short half-life of these peptides may also reduce the likelihood of an immune reaction when applied onto wounds since they are sensitive to proteolytic degradation. Further studies are required to examine the highest dosage of SKAM peptides that can be applied onto full-thickness excisional wounds without triggering aberrant immune response in mice.

Future studies should also examine the method of delivery of the cell-permeable peptide to wounds. In my study, peptides were delivered into mouse wounds via intradermal injections. Clearly, this method of delivery is neither an ideal nor a desirable treatment for problematic wounds in humans. An ideal method to deliver the SKAM1 peptide onto wounds would be via topical application. However, since the SKAM1 peptide is small, it is likely to be susceptible to degradation and inactivation when applied onto wounds. Thus, a suitable vehicle or formulation is necessary to preserve the biological activity of this peptide and to facilitate its release

when applied topically on wounds. In human clinical trials, recombinant human PDGF (Regranex) was delivered onto the lower extremity diabetic neuropathic ulcers in an aqueous gel which is mainly composed of carboxymethyl cellulose and water (Papanas and Maltezos, 2007). Notably, a study has shown that a similar vehicle, hydroxyethyl cellulose, combined with paraben preservatives appear to be a suitable vehicle for the delivery of peptides onto full-thickness excisional wounds in mice (Rodgers et al., 2011). Furthermore, the study also showed that this vehicle was well-tolerated in mice and no adverse effects were observed when topically applied on mouse wounds (Rodgers et al., 2011). More importantly, this vehicle in which the bioactive peptide of interest is resuspended, has passed Phase II clinical trials and is currently in Phase III clinical studies (Rodgers et al., 2011), suggesting that the vehicle or the peptide, so far has not caused any observable adverse effects in human. In addition, other studies have shown that a lipogel emulsion, mainly consisting of liquid paraffin and dimethicone (silicone), can also be used as a vehicle for peptides (Santiago et al., 2005). It is worth noting, however, that in this study the peptide was applied onto intact fibrotic skin tissue, not open wounds (Santiago et al., 2005). Thus, the effect of this vehicle on open wounds remains unknown. A more recent study has described the use of a cream-based vehicle composed of Sorbolene to topically deliver antibodies to pig wounds (Kopecki et al., 2013). Taken together, the vehicles employed in these studies will be useful references for future development of a biocompatible vehicle for SKAM1A peptide.

6.5 Murine models of cutaneous wound healing: Incisional and excisional models of wound healing

While all animal models of wound repair are imperfect reflections of human wound healing and its clinical challenges, they continue to be valuable biological tools that provide insights to basic processes of wound healing and for the development and validation of new therapeutic strategies for wound treatment (Wong et al., 2011). Two murine models of cutaneous wound healing were employed in my study; incisional and excisional models of wound healing. Both are acute wound models and involve the disruption of the skin integrity via surgical means.

In the incisional model, a clean incision into the skin with a sharp blade results in rapid disruption of the skin integrity with minimal collateral damage [reviewed in (Greenhalgh, 2005)]. This is followed by rapid extravasation of immune and red blood cells into the wound gap, and ultimately leading to the formation of a fibrin clot that bridges the wound margins. An incisional wound can heal in two ways; primary or secondary intention. Primary intention involves the use of mechanical means to rapidly heal the wound, including sutures, staples or clips, while wounds that are intentionally left open to heal is known as secondary intention. The latter was employed in my study to examine the early stages of wound healing, including inflammation, cell proliferation, re-epithelialisation and to a lesser extent wound contraction and scarring, which occur in the later phase of healing (Velnar et al., 2009). Although this model is advantageous to study some aspects of wound healing, it is, however, less adequate for physical evaluation of healing because of the limited area of wound healing activity. Thus, any modest enhancement or impairment of healing would be difficult to observe using this model. Indeed, this notion is supported by previous studies showing that smaller wounds, like incisional wounds, have smaller window for determining differences in wound closure than larger wounds (Greenhalgh et al., 1990). My initial studies showed that the rate of wound

healing appears to be similar between wildtype and SKAM1A transgenic mice following incisional wounding (Fig. 4.19). However, given the limitations of this model, I sought to further examine the wound healing of SKAM1A transgenic mice using the full-thickness excisional wound healing model, which is a more severe wound model that involves the complete removal of epidermis and dermis to the depth of fascial planes. This model offers the advantage of significant wound area and the involvement of all aspects of wound healing, in particular wound re-epithelialisation, wound contraction and scar formation (Greenhalgh, 2005). In addition, this model also allows visual inspection of the healing wound for wound assessment and comparison. Notably, while there was no observable difference in the rate of wound healing between wildtype and SKAM1A transgenic mice using the incisional model, this excisional model showed a small, but significant enhancement in wound healing in SKAM1A transgenic mice compared to wildtype mice (Fig. 4.20). This suggests that SKAM1A plays a role in wound healing, possibly in the later phase of wound healing, including wound contraction. This is consistent to *in vitro* data showing that SKAM1A can enhance fibroblast-mediated collagen gel contraction (Fig. 4.17).

6.6 Differences between murine and human wound healing

My initial studies have employed a number of *in vitro* wound healing models to elucidate the role of SKAM1 in wound healing. It is important to note however, that *in vitro* models have their own limitations and are designed to answer specific questions that involve a simple biological system, such as the response of one or two cell types to one or a few stimuli (Greenhalgh, 2005). Wound healing of the skin, however, is far more complex since it is a multistep process that requires multiple cell

types to function in a highly coordinated manner (Velnar et al., 2009). In addition, there are other factors that may influence the outcome of wound healing, such as temperature, oxygenation, nutritional status and overall health (Greenhalgh, 2005). Thus, the use of animal models is a more suitable tool that allows the study of wound healing. In my studies, I employed the murine model of incisional and excisional wounding to study the role of SKAM1 in wound healing. Although these murine models are commonly used to study wound healing, it is important to note that major differences in cutaneous wound healing exist between mice and humans [reviewed in (Dunn et al., 2013; Greenhalgh, 2005; Sullivan et al., 2001)]. In mice, wounds heal primarily due to the process of wound contraction [reviewed in (Wong et al., 2011)]. This is in part, due to mice having an extensive subcutaneous striated muscle layer called the panniculus carnosus that is largely absent in humans (Dunn et al., 2013). This muscle layer allows the skin to move independently of the deeper muscles thus contributing to the rapid contraction of the skin following wounding (Dunn et al., 2013). On the other hand, human wounds heal largely through granulation tissue formation and reepithelialisation [reviewed in (Greenhalgh, 2005)]. While my results showed that a cell-permeable peptide of SKAM1, TAT-SKAM1A$^{76\text{-}105}$, can enhance wound resolution of full-thickness excisional wounds in mice (Fig. 5.9), further studies are required to examine the efficacy of this peptide in animal models that more closely resemble wound healing in humans. One such system is the porcine model which is an excellent tool for the evaluation of therapeutic agents destined for used in human wounds for a number of reasons (Sullivan et al., 2001). Firstly, there are many similarities between human and pig, both anatomically and physiologically. More importantly, porcine skin resembles that of human skin. Both species have almost identical dermal-epidermal thickness ratio (Meyer et al., 1978) and both heal

primarily via granulation tissue formation and reepithelialisation (Sullivan et al., 2001). Thus, further studies using the cell-permeable SKAM1 peptide should focus on this porcine system, such as the well characterised partial or full-thickness excisional wound model and the burn wound model (Sullivan et al., 2001). Successful outcomes in such a model may then provide impetus for human trials.

6.7 Wound healing versus cancer: SK1 and SKAM1

Although a positive role of SK1 in would healing and then potentially a problematic role in tumourigenesis seems to be somewhat of a paradox, it is not surprising for a protein to be involved in these two biological responses. As both wound healing and cancer development involve cell proliferation, cell survival and migration, many studies suggest that cancers are analogous to unhealed wounds (Schafer and Werner, 2008). In fact, early studies have proposed that tumours have many similarities to wounds that do not heal [reviewed in (Dvorak, 1986)]. Thus, it is reasonable that a protein like SKAM1 that is able to induce activation of an enzyme heavily implicated in oncogenesis, like SK1, may regulate its functions through fine control of its effects on target proteins.

My data have shown that persistent SK1 activation by SKAM1, unlike eEF1A, did not result in neoplastic transformation in NIH3T3 fibroblasts as assessed by *in vitro* focus formation assays (Fig. 4.7). This suggests that the activation of SK1 by SKAM1 may be enough to stimulate wound healing, but not sufficient to induce oncogenic signalling. Since eEF1A and SKAM1 appear to bind SK1 in the same region (Leclercq, 2010) and have similar enhancing effects on SK1 activity, the reasons for this difference between oncogenic signalling by these proteins on SK1 remain unclear. SKAM1 and eEF1A binding to SK1 may differentially alter the

subcellular localisation of the enzyme. Previous studies have shown that the translocation of SK1 from the cytosol to the plasma membrane is integral for the oncogenic signalling of the enzyme (Pitson et al., 2005). This translocation process may occur when eEF1A binds to SK1, thereby rendering SK1 able to elicit its oncogenic signalling effects in cells. However, this may not be the case when SKAM1 binds to SK1. In this scenario, the activation of SK1 by SKAM1 in the cytoplasm may result in decrease ceramide level, and thus promoting cell survival. Such hypotheses concerning the role for SKAM1 in wound healing and cancer requires further investigation.

While there is still a significant amount of work to do to completely define how SKAM1 and SK1 interact, the data generated so far demonstrates the important role of SKAM1/SK1 interaction and subsequent activation of SK1 in cutaneous wound healing.

6.8 Conclusion, significance and future directions

In conclusion, data presented in this thesis has begun to elucidate the molecular mechanisms of involvement of SKAM1 and SK1 in wound healing. Knowledge gained from this study has provided greater understanding of molecular aspects of wound healing. It is evident that enhancing the SK/S1P axis can stimulate wound healing. This can be achieved by the application of a cell-permeable peptide of SKAM1, which appears to be promising new therapeutic for the improvement of wound healing. Therapeutic application of this peptide to wounds in porcine models and subsequently in human clinical trials should be the focus of ongoing studies. The generation of SKAM1 transgenic and knockout lines from my study will be invaluable tools for future studies to further investigate and characterise the function

of SKAM1 in wound healing. Future studies will involve the generation of SKAM1 homozygous transgenic and knockout lines to examine the effect of these on wound healing in mice. Furthermore, the role of SKAM1 in wound healing should also be examined in other impaired wound healing models, such as the burn wound model, bleomycin-induced excessive scar formation model, and in particular diabetes mellitus models. There is significant need to develop measures to improve wound healing in diabetic individuals, with 15% of diabetic patients expected to develop a non-healing wound (Brem and Tomic-Canic, 2007). Thus, significant health benefits would flow from the development of therapeutics to enhance wound healing, particularly with the incidence of diabetes on the dramatic increase. Since the crystal structure of human SK1 has recently been resolved (Wang et al., 2013), it is of significant interest to identify how SKAM1 and SK1 interact at the molecular level. One possible method is to generate SK1-SKAM1 peptide co-crystals and analyse this complex by X-ray crystallography. The knowledge gain from these studies will not only aid in the development of a chemical agonists or antagonists to manipulate the activity of SK1 to treat diseases such as cancer, but will also be valuable for the optimisation of the SKAM peptide to be used in future studies or the development of small molecule mimetics of the SKAM1 interaction.

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CHAPTER 1

Post-translational regulation of sphingosine kinases

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