

## COLLLEGE OF MEDICINE, BIOLOGICAL SCIENCES AND PSYCHOLOGY

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# DEPARTMENT OF INFECTION, IMMUNITY AND INFLAMMATION

# THE ROLE OF K<sub>Ca</sub>3.1 IN IGA NEPHROPATHY

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# Abstracts

IgA nephropathy (IgAN) is the commonest pattern of primary glomerulonephritis worldwide, characterised by the deposition of IgA1 containing immune complexes in the mesangium leading to glomerular and tubulointerstitial fibrosis. A direct effect of IgA1 on mesangial cells (MC), podocytes and proximal tubular epithelial cells (PTEC) is believed to be crucial for the development of renal fibrosis in IgAN. The intermediate-conductance Ca<sup>2+</sup>- activated potassium channel, K<sub>Ca</sub>3.1, has emerged as an important regulator of fibroblast proliferation in renal and other diseases. The aim of this thesis was to investigate the expression of K<sub>Ca</sub>3.1 in cultured human MC, podocytes and PTEC and to determine the effect of blocking K<sub>Ca</sub>3.1 on the *in vitro* response of human MC and PTEC to IgA1, and of PTEC to monomeric IgA1 (mIgA) and polymeric IgA1 (pIgA).

Human MC, podocytes and PTEC were found to constitutively express K<sub>Ca</sub>3.1. IgA1 induced an up-regulation of K<sub>Ca</sub>3.1 synthesis by human MC and PTEC which could be inhibited by the K<sub>Ca</sub>3.1 selective blockers, TRAM-34 and ICA-17043, suggesting this effect was in part mediated by K<sub>Ca</sub>3.1 itself. Human MC exposed to IgA1 developed a pro-inflammatory phenotype, with secretion of IL-6, which was inhibited by TRAM-34. The ability of K<sub>Ca</sub>3.1 blockers to prevent IgA1 dependent changes in expression and synthesis of markers of fibrosis and epithelial mesenchymal transition (EMT) in PTECS suggests involvement of this potassium channel in tubulointerstitial damage in IgAN. In contrast with mIgA1, human serum plgA1 induces human PTECs to increase protein synthesis and gene expression of K<sub>Ca</sub>3.1, and markers of inflammation, EMT, and fibrosis (IL-6, TGF- $\beta$ , GDF-15, PDGF-AA,  $\alpha$ -SMA, FSP-1 and fibronectin). These results indicate the relationship between plgA1 and K<sub>Ca</sub>3.1 is pivotal to the mechanisms driving inflammation and fibrosis in the tubular interstitium. Controlling this relationship may prove critical in the search for a therapy to slow down renal damage in IgAN. The presence of K<sub>Ca</sub>3.1 in the exosome fraction of media from hTERT/PTECS incubated with IgA1 and urine from IgAN patients suggests the possibility of using K<sub>Ca</sub>3.1 as a marker for progression in IgAN.

These studies provide evidence that  $K_{Ca}3.1$  plays an important role in mesangial cell activation, EMT and ultimately extracellular matrix deposition and tubulointerstitial fibrosis in IgAN. The origin and role in IgAN pathogenesis of  $K_{Ca}3.1$  in urinary exosomes requires further investigation.

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# Contributions

Whole-patch clamping experiments using mesangial cells and HK2 cells were carried out by Dr. Mark Duffy.

The podocyte experiments were performed with Mr. Hamed Hohammed, an MSc student under my supervision.

The experiments studying  $K_{Ca}3.1$  in urine described in Chapter 6 were undertaken with Ms. Valeria Balan, a BSc student under my supervision. The experiment locating  $K_{Ca}3.1$  in urinary exosomes was performed by Mr. Christian Tang.

I confirm that unless otherwise stated, all of the work presented in this thesis is the candidate's own.

# **Presentations**

- Nilasari D, Molyneux K, Bradding P, Barratt J, K<sub>Ca</sub>3.1: A potential antifibrotic target in IgA Nephropathy, East Midlands Renal Showcase, Leicester, UK, 2015 (Oral) – Best basic science oral presentation prize
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AKI	Acute kidney injury
α-SMA	Alpha smooth muscle actin
ANOVA	Analysis of variance
bFGF	Basic fibroblast growth factor
BMP-2	Bone morphogenetic protein 2
BSA	Bovine serum albumin
Ca <sup>2+</sup>	Calcium
CD	Cluster of differentiation
CKD	Chronic kidney disease
dlgA	Dimeric IgA
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
ELISA	Enzyme-linked immunosorbent assay
EMT	Epithelial Mesenchymal Transition
ESRD	End stage renal disease
Fab	Fragment, antigen binding
Fc	Fragment, crystallisable
FcRγ	Fc receptor γ subunit
Fn	Fibronectin
FPLC	Fast protein liquid chromatography
FSP-1	Fibroblast specific protein-1
Gal	Galactose
GalNac	N-acetyl galactosamine
GDF-15	Growth differentiation factor 15
GdplgA1	Galactose deficient polymeric IgA1
GFR	Glomerular filtration rate
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GWAS	Genome wide association study
НАА	Helix Aspersa agglutinin
HCI	Hydrochloric acid
HK-2	Human kidney-2
HLA	Human leukocyte antigen
HMC	Human mesangial cell
HRP	Horseradish peroxidase

HSP	Henoch-Schönlein purpura
ICAM	Intercellular adhesion molecule
lg	Immunoglobulin
IgAN	Immunoglobulin A nephropathy
IL	Interleukin
J chain	Immunoglobulin joining chain
K <sub>Ca</sub> 3.1	Calcium activated potassium channel 3.1
kDa	Kilodaltons
MAC	Membrane attack complex
MCP	Monocyte chemoattractant protein
MHC	Major histocompatibility complex
MIF	Macrophage migration inhibitory factor
mlgA	Monomeric IgA
MMP	Matrix metallopeptidase
mRNA	Messenger RNA
PAI-1	Plasminogen activator inhibitor type <b>1</b>
PAS	Periodic acid-Schiff
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PCR	Protein:creatinine ratio
PDGF	Platelet-derived growth factor
pIgA	Polymeric IgA
plg	Polymeric immunoglobulin
plgR	Polymeric immunoglobulin receptor
PMP	Per million population
PTEC	Proximal tubular epithelial cells
RANTES	Regulated on activation, normal T cell expressed and
RNA	Ribonucleic acid
RRT	Renal replacement therapy
SC	Secretory component
sCD89	Soluble CD89
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel
	electrophoresis
SEM	Standard error of the mean
S-IgA	Secretory Immunoglobulin A

siRNA	Small interfering RNA
SNP	Single nucleotide polymorphism
TAE	Tris-acetic acid-EDTA
TBS	Tris-buffered saline
TfR1	Transferrin receptor
TGF-β	Transforming growth factor beta
Th	T helper
Thr	Threonine
TNF	Tumor necrosis factor
TRP	Transient receptor potential

# Chapter 1: Introduction

## 1.1 IgA Nephropathy

### 1.1.1 Immunoglobulin A

Immunoglobulin A (IgA) is the most abundant immunoglobulin produced in humans. The human body synthesizes 66 mg of IgA per kg body weight daily, compared with 34 and 7.9 mg of IgG and IgM respectively. In humans, IgA exists as two isotypes, IgA<sub>1</sub> and IgA<sub>2</sub> (Figure 1-1). The IgA in human serum is 90% IgA1 and 10% IgA2, while in mucous excretions, the proportion of IgA2 can be as high as 50%. This characteristic distribution may reflect the fact that plasma cells in the bone marrow are the source of serum IgA and plasma cells in lamina propria and other mucosal immune sites are the source of secreted IgA.

### 1.1.2 Structural Features of IgA

Like all immunoglobulins, IgA molecules are composed of two identical heavy chains ( $\alpha$ 1 or  $\alpha$ 2, ~55 kDa) and two identical light chains ( $\kappa$  or  $\lambda$ , ~25 kD). The Fab region binds to antigen while the Fc region mediates receptor and effector mechanisms (Figure 1-1). The structures of the two subclasses of IgA differ only in that IgA<sub>1</sub> has a hinge region located between regions C1 and C2 comprising 16 amino acids with 3-6 O-linked oligosaccharides attached. The sugar chains are composed of combinations of N-acetylgalactosamine, sialic acid, and galactose (Woof and Russell, 2011).

In humans, IgA exists in two forms, monomeric (~160 kD) and dimeric (polymeric). Dimeric IgA is made up of two monomers linked by a polypeptide called joining (J) chain. J chain is a small polypeptide consisting of 137 amino acids with a molecular weight of 15kD. Dimeric IgA is most frequently found in mucosal secretions, colostrum, milk, saliva, tears, nasal fluid, and respiratory, gastrointestinal and genitourinary mucus (Woof and Mestecky, 2005). It is synthesized by mucosal immune cells then crosses the mucosal barrier by binding to a specific receptor termed polymeric immunoglobulin receptor (pIgR).

Upon crossing the mucous membrane, dimeric IgA acquires a part of the pIgR called the secretory component (Figure 1-1) this form of IgA is called secretory IgA (sIgA) (Woof and Kerr, 2006).



**Figure 1-1. Structure of immunoglobulin A. (a).** Monomeric IgA1 (mIgA1); (b). mIgA2; (c) dimeric IgA (dIgA) and (d).secretory IgA1 (S-IgA1). Heavy chains are shown in pink and light chains are shown in blue. J chain is shown in yellow and secretory component in purple. On the monomeric form of IgA, O-linked sugars (on the IgA1 hinge) are shown as green circles, while N-linked oligosaccharides are shown in red. Adapted from Woof and Kerr (2006)

#### 1.1.2 Functions of IgA

The function of IgA in the mucosal compartment is clear: it contributes to the body's defence mechanism by protecting mucosal membranes from bacterial invasion. However, the role of IgA in the blood is not so well understood: it is

thought to play a role as a second line of defence by eliminating the pathogens that have invaded the systemic compartment. (Favre *et al*, 2005).

### 1.1.3 Epidemiology

Chronic kidney disease is a global health problem and a significant economic burden. The prevalence of CKD is steadily rising. It is estimated that early CKD prevalence ranges from 2.9% to 7.0% of the population of the UK (MacGregor, 2007). Many patients with CKD ultimately develop end stage renal disease (ESRD) when the need of dialysis and renal transplantation is mandatory. Furthermore, people with CKD have a 20-fold increased risk of developing cardiovascular disease, a leading cause of death worldwide (Jha et al, 2013; Stel et al, 2017). Data from the Global Disease Burden 2015 study shows an average of 1.2 million deaths (32% increase since 2005) and 19 million disability cases from cardiovascular diseases were attributable to reduced kidney function (Couser et al, 2011; Eckardt et al, 2013). The impact of renal disease on populations is compounded by the lack of access to dialysis and kidney transplantation in some parts of the world. It is estimated that 2.3-7.1 million people with CKD died without access to dialysis in 2010. Worldwide, 2.62 million people received dialysis in 2010 and this number is estimated to double by 2030 (Liyanage et al, 2015)

IgA nephropathy (IgAN), also known as *Berger's Disease*, is the most prevalent pattern of primary glomerulonephritis in most Western and Asian countries. The disease was first identified in 1968 by Jean Berger using immunofluorescence. It is characterized by a diffuse mesangial deposition of IgA (Berger and Hinglais, 1968). Thirty to twenty percent of IgAN patients develop chronic kidney disease (CKD) within 20 years of first diagnosis.

The estimated incidence of IgAN is 2.5:100,000, although this varies in different ethnic groups, being higher in Asian populations and less frequent in African Americans (Figure 1-2) (Jha *et al*, 2017). The incidence of IgAN in Australia is 105 per million population per year (pmp/year), 39-45 pmp/year in Japan, and 25-31 pmp/year in France. A mass urine screening policy carried out in China, Japan and Korea has led to a higher detection frequency in children and high

school students (Schena and Nistor, 2018). IgA nephropathy is more common in males in European populations but occurs with equal frequency in Chinese ethnic groups.



**Figure 1-2. Geographical variations in the prevalence of IgAN.** Percentages represent the proportion of cases of IgAN compared to all native kidney biopsies performed. The numbers in brackets represent minority racial groups, African Americans in the United States of America, and Polynesians in New Zealand. Adapted from Feehally and Floege, 2010.

### 1.1.4 Diagnosis

### 1.1.4.1 Clinical Features and Prognosis

Patients diagnosed with IgAN face very variable clinical manifestations ranging from asymptomatic to rapidly progressive glomerulosclerosis (Zhang *et al*, 2015). The first step to being diagnosed with IgAN is frequently the presence of hematuria, proteinuria, flank pain, oedema or high blood pressure. The clinical manifestation of IgAN varies in frequency with age (Figure 1-3) and none of the pattern is pathognomonic of IgAN (Feehally and Floege, 2010).

On finding raised serum creatinine a renal biopsy is carried out. The presence of IgA in the glomeruli confirms an IgAN diagnosis.

At present, there is no way of knowing, at diagnosis, how the disease will progress. Increasing age, the development of proteinuria and hypertension, have been identified as poor prognostic indicators. Some novel tests have been proposed, including clinical risk score proposed by Xie et al. (2012) (incorporating GFR, haemoglobin, albumin, and systolic BP at presentation), genetic risk score which requires genotyping of 7 GWAS SNPs, and glomerular density measurement on biopsy. However, there is no sufficient recommendation for clinical usage. Some emerging tests such as levels of galactose-deficient IgA1, antiglycan autoantibodies or markers of oxidative stress in serum, immunohistochemistry for fibroblast markers (e.g., FSP1, α-SMA) in biopsy material, urine and serum proteomics or microRNA profiling need to be standardised and validated.



**Figure 1-3 Age on clinical presentation of IgA nephropathy and IgA vasculitis.** IgA vasculitis is most common in childhood but may affect any age. Macrohematuria mostly occurs from childhood until age of 40. Patients are presenting with CKD ranging from age 20 to 80. Adapted from Feehally and Floege, 2010.

#### 1.1.4.2 Histology

The diagnosis of IgAN requires a renal biopsy and immunofluorescence as light microscopical changes vary widely. Diagnosis is confirmed by the finding of dominant or codominant IgA, with IgG or IgM, and variably coincide with C3, deposition in mesangial cells (Figure 1-4B). Other immune-complex GN may share the same features, and therefore must be excluded. Electronic microscopic shows mesangial dense deposits (Figure 1-4C).

The most common histologic finding seen in IgA biopsies is mesangial proliferation (Figure 1-4A). However, light microscopic histological features range from normal, crescentic to advanced glomerular sclerosis and tubular atrophy (Tumlin and Hennigar, 2004).

The Oxford classification of IgA nephropathy is an evidence-based histologic classification, which includes four variables (MEST) that have been tested and found to be independently linked to clinical outcome. The MEST-C score comprises mesangial hypercellularity (M); endocapillary hypercellularity (E); segmental glomerulosclerosis (S); tubular atrophy/interstitial fibrosis (T), Cresentic (C) (Figure 1-5) (Working Group of the International IgA Nephropathy Network and the Renal Pathology Society, Roberts *et al*, 2009). MEST score prognostic value has been tested and validated in adults and children by several studies in variable ethnicity (Working Group of the International IgA Nephropathy Network and the Renal Pathology Society, Coppo *et al*, 2010; Herzenberg *et al*, 2011; Edstrom *et al*, 2012; Alamartine *et al*, 2011; Lv *et al*, 2013; Lee *et al*, 2012; Zeng *et al*, 2012; Shima *et al*, 2012; Kang, S. H. *et al*, 2012; Katafuchi *et al*, 2011; Shi *et al*, 2011).



**Figure 1-4 Histologic variation in IgAN. A**.Hematoxylin-eosin staining shows expansion of mesangial matrix and hypercellularity. **B.** Immunoflourescent mircoscopy for IgA deposition within glomerular mesangium. **C.** Electron microscopy indicates mesangial matrix and presence of mesangial dense deposits (arrow). Adapted from Tumlin *et al.* (2007).



**Figure 1-5 Glomerular lesion in IgAN. A.** Mesangial hypercellularity with more than 4 nuclei. **B.** Endocapillary hypercellularity with occlusion of capillary lumina by cells. **C.** A fibrocellular crescent. **D.** Segmental sclerosis with tuft adhesions (Soares and Roberts, 2018)

#### 1.1.5 Pathogenesis, Origin and Disease Mechanism of IgA Nephropathy

#### 1.1.5.1 IgA Glycosylation

The majority of proteins in the serum are glycoproteins, with sugar chains Olinked or more commonly N-linked to the protein backbone. (Arnold *et al*, 2007). O-linked glycans are mostly found attached to membrane proteins. O-Linked glycans are usually attached to the peptide chain through a serine or threonine residue. IgA1 is one of the few serum glycoproteins which has O-linked glycans as well as N-linked glycans. (Arnold *et al*, 2007; Barratt *et al*, 1999)

The O-linked sugars are attached to serine or threonine residues in the hinge region of IgA1 (Figure 1-6). The synthesis of O-glycans is initiated by attachment of N-acetyl-galactosamine (GalNac) using the enzyme N-acetyl-galactosamyl-transferase 2 (GalNAc-T2). This sugar chain is elongated by the addition of a galactose moiety and or a sialic acid. Core 1  $\beta$ 1,3 galactosyltransferase (C1GalT1) attaches D-galactose in a  $\beta$ 1,3-linkage to the GalNac. C1GalT1 requires a chaperone protein called Cosmc (core 1  $\beta$ 1,3 galactosyltransferase molecular chaperone) to ensure its stability (Takahashi *et al*, 2012; Boyd *et al*, 2012). Sialic acids are attached using  $\alpha$ 2,6 or  $\alpha$ 2,3 sialyltransferases.

Many studies have demonstrated increased levels of galactose-deficient IgA1 in the serum from IgAN patients compared with healthy controls. This finding has been reproduced in populations from a variety of different ethnicities and geographic origins (Novak *et al*, 2018). Our group has found raised levels of undergalactosylated IgA1 in serum from IgAN patients with the more severe form of the disease compared with those with a more benign prognosis (ref) Furthermore, two studies observed mesangial galactose deficient IgA1 deposition (Allen *et al*, 2001; Hiki *et al*, 2001). These findings strongly support the hypothesis that galactose deficient IgA1 is important in the pathogenesis of IgAN.



**Figure 1-1. IgA1 structure and O-glycosylation pathway.** A IgA with hinge region that contains 5 O-glycans attached to serine and threonine residue. B IgA glycosylation is mediated by stepwise translational modification; 1). N-acetylgalactosamine (GalNAc) is added to serine/threonine residue by activity of N-acetylgalactosaminyl-transferase (GalNAcT2); 2). Galactose is added to GalNAc by core 1 beta 1,3-galactosyltransferase (C1GalT1) and core 1  $\beta$ 3GalT-specific molecular chaperone (Cosmc); 3). Sialic acid is added to galactose by  $\alpha$ 2,3 sialytransferase (ST2,3). 4). Sialic acid is added to GalNAc by ST2,6. C. Combination of different O-glycoforms of galactosylation and sialylation. Adapted from Yeo *et al*, 2018.

#### 1.1.5.2 The origin of pathogenic IgA in IgA nephropathy

Despite much research the pathogenesis of IgAN is still poorly understood. Along with the observation that patients with IgAN have raised levels of poorly galactosylated IgA1, we also know that this aberrant IgA has a tendency to form complexes with other antibodies in serum (Figure 1-7). These complexes have a tendency to be deposited in the glomerular mesangium and cause inflammation and fibrosis leading to a breakdown of the glomerular filtration barrier (Kim *et al*, 2015; Boyd *et al*, 2012).



**Figure 1-2.** Pathogenesis of IgA nephropathy. (1) Mucosal infection. (2) Mis-home of some IgA<sup>+</sup> ASC the systemic circulation. (3) Displaced IgA<sup>+</sup> ASCs secreted poorly galactosylated and polymeric) IgA1 into the systemic circulation. (4) IgA1 secretion is augmented by TLR ligation from mucosal-derived pathogen-associated molecular patterns (5) IgA1 immune complexes formation Poorly galactosylated polymeric IgA1 combine with: (a) IgG and IgA autoantibodies reactive to exposed neoepitopes in the poorly galactosylated IgA1 hinge region; (b) antimicrobial antibodies specific for carbohydrate components of the microbial cell wall (c) Shedding soluble CD89 from myeloid cells in response to polymeric IgA1 binding. (6) IgA1 immune complex deposition is leading to glomerular injury and tubulointerstitial scarring. Adapted from Boyd et al. (2012).

#### 1.1.5.3 Genetics of Immunoglobulin A Nephropathy

Urine abnormalities in healthy relatives of patients with IgAN have been observed in many studies, adding to accumulating evidence which suggests genetic components are important in the pathogenesis of IgAN. Several gene loci have been consistently linked with the IgAN phenotype, these include major histocompatibility loci (HLA-DR, -DQ, -DP and HLA-B), inflammatory mediators (TNF and  $\alpha$ -defensin), complement factor H, cytokines, and markers of innate immunity and mucosal integrity (Kiryluk *et al*, 2014). Some overlap between loci linked to IgAN and other immune-mediated diseases have been observed; IgAN and systemic lupus erythematosus (ITGAM-ITGAX and CFHR3,1) and IgAN and inflammatory bowel disease (CARD9, OSM). Single nucleotide polymorphisms (SNPs) in the genes coding for E-selectin (SELE) and L-selectin (SELL) have been found to be associated with rapid progression to ESRD in IgAN (Neugut and Kiryluk, 2018).

Serum galactose-defficient IgA1 (Gd-IgA1) has been identified as a risk factor in IgAN and shown to have a strong genetic determination (Gale *et al*, 2017. Gd-IgA1 heritability estimates range from 39-80% in Europeans and African Americans. Serum Gd-IgA level has been found to be higher in first degree relatives of IgAN patients and parents of paediatric patients with Henoch-Schönlein Purpura (HSP) or IgAN compared to unrelated controls (Neugut and Kiryluk, 2018).

1.1.5.4 Mesangial deposition and the mechanism of kidney fibrosis in IgAN1.1.5.4.1 Mesangial deposition, cytokine release and cell activation

The pathogenesis of IgAN from the early stage of kidney injury to the formation of fibrotic tissue can be divided into 2 main processes. Firstly, a specific pathogenesis for IgAN which is widely accepted as being driven by poorly O-galactosylated IgA1. Secondly, a generic process of kidney fibrosis common to other causes of CKD.

In more detail, the deposition of IgA1-immune complexes which consist of poorly O-galactosylated IgA1 and O-glycan-specific antibodies results in

mesangial cell activation, leading to increased production of pro-inflammatory and pro-fibrotic cytokines, such as IL-6 and TNF- $\alpha$ , which promotes mesangial cell proliferation and extracellular matrix formation. At this point the glomerulus loses its permeability causing podocyte damage (glomerulopodocytic crosstalk), resulting in protein and high molecular weight compounds including IgAimmune complexes, coming into contact with epithelial cells lining the proximal tubules, triggering inflammation (glomerulotubular crosstalk), and ultimately driving tubulointerstitial fibrosis (Yeo *et al*, 2018). Surprisingly, the severity of IgAN progression has not been shown to be dependent on the amount of IgA deposition but on the speed of tubulointerstial fibrosis.

### 1.1.5.4.2 Kidney fibrosis

Kidney fibrosis is defined as scarring in the tubulointerstitial space after kidney insults of any cause. This is believed to be an important process underlying the progression of chronic kidney disease (CKD) to end stage renal failure (ESRD). Kidney scarring consists of glomerulosclerosis, tubular atrophy and interstitial fibrosis (Couser and Johnson, 1994; Kang *et al*, 2002).

Fibroblasts and myofibroblasts have been studied extensively and are known to be key factors in the pathogenesis of fibrosis in kidney and other solid organs. Over the latest few decades, several studies have attempted to understand the origin of those cells (Acloque *et al*, 2009; Acloque *et al*, 2008). The traditional concept which explains the pathogenesis of fibrosis in CKD suggests that myofibroblasts originate as resident fibroblasts and perivascular fibroblasts. Emerging evidence introduced two concepts known as epithelial mesenchymal transition (EMT) and endothelial mesenchymal transition (EndMT) (Acloque *et al*, 2008).

Fibroblasts are mesenchymal cells with spindle-shaped morphology. They are the source of components of extracellular matrix (ECM) which are essential to maintain tissue structures. In health, only a small number of fibroblasts are seen in the renal cortex, located at peritubular and perivascular sites. A number of markers have been proposed for renal fibroblasts, including vimentin, platelet-derived growth factor receptors  $\alpha$  and  $\beta$ , nerve growth factor, CD73, CD90 and

fibroblast-specific protein-1 (FSP-1) (Rodemann and Muller, 1991; Muller and Rodemann, 1991; Grupp and Muller, 1999).

In fibrogenesis, fibroblasts convert to myofibroblasts, a process which is marked by *de novo* expression of the myofibroblast marker,  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA). The presence of myofibroblasts has been thought to be a prognostic indicator for fibrosis progression, leading to ESRD. Several attempts have been made to attenuate the activity of myofibroblasts and inhibit their accumulation. Differentiation of fibroblasts into myofibroblasts occurs under the influence of a variety of cytokines and growth factors including TGF- $\beta$ , fibroblast growth factor (FGF), interleukin-1 (IL-1), connective tissue growth factor, platelet-derived growth factor (PDGF), tumour necrosis factor-  $\alpha$  (TNF-  $\alpha$ ), angiotensin II and aldosterone (Hinz *et al*, 2001; Hinz *et al*, 2003).

#### 1.1.5.4.3 Epithelial Mesenchymal Transition

Over the past two decades many researchers have described an alternative mechanism to explain the accumulation of myofibroblasts in the renal cortex. This mechanism involves the transformation of epithelial cells into a mesenchymal phenotype (epithelial mesenchymal transition-EMT). The transformed cells then pass through the basement membrane and migrate into the interstitium (Lovisa *et al*, 2016). Smad-3 is seen as the major regulator of EMT (Masszi *et al*, 2004). Liu (2004) has proposed 4 foundations of TGF- $\beta$ 1 driven EMT in fibrogenesis: loss of epithelial cell adhesion, *de novo*  $\alpha$ -SMA expression and actin re-organization, tubular basement membrane disruption and enhanced cell migration and invasion (Zeisberg and Kalluri, 2008).

#### 1.1.5.4.4 Endothelial Mesenchymal Transition

Zeisberg and Kalluri, 2008 argued that the endothelium is the origin of myofibroblasts. Their theory was based on their observation that 30-50% of fibroblasts expressed the endothelial marker CD31 as well as markers of fibroblasts and myofibroblasts ( $\alpha$ -SMA and FSP-1) in 3 different models of renal fibrosis. Another study supported this finding (Xavier *et al*, 2015).

#### 1.1.6 Treatment

At present there is no specific treatment for IgA nephropathy. Some general renal disease interventions are beneficial for patients with IgAN. Controlling blood pressure is vital to slow the progression of kidney damage from any cause. Renin-angiotensin system (RAS) blockers are recommended for proteinuria > 1 g/d (KDIGO). Tonsillectomy might be beneficial for IgAN patients with recurrent tonsillitis. Corticosteroids produce remission of proteinuria in patients with nephrotic-range proteinuria. However, there were serious adverse events in IgAN patients treated with corticosteroid (Lv *et al*, 2017). Cyclophosphamide may be beneficial for patients with crescentic IgAN. (Xiao *et al*, 2017; O'Shaughnessy and Lafayette, 2017). Mycophenolate mofetil and other immunosuppressants are still waiting to be further investigated. ACE inhibitors have been accepted as antifibrotic therapies (Wuhl and Schaefer, 2008; Stefoni *et al*, 2014), however the results remain unsatisfactory.

### **1.2 Calcium-Activated Potassium Channels**

Potassium channels are crucial membrane proteins found in most cell types. There are several types of potassium (K<sup>+</sup>) channels. Most of them are voltagedependent, others are activated by Na<sup>+</sup>, ATP, fatty acid, or Ca<sup>2+</sup>. Calcium activated potassium channels undergo opening by a high concentration of cytoplasmic Ca<sup>2+</sup>. They are widely expressed in cells throughout the body including the hematopoietic system, neurons, smooth muscle cells, bone, epithelia, and endocrine cells, and have a central role in cell activation, proliferation and differentiation. (Ishii *et al*, 1997).

The first description of Ca<sup>2+</sup> ions regulating the K<sup>+</sup> channels was provided by Gardos in work on red blood cells in 1958 (Ref). A rise in intracellular Ca<sup>2+</sup> in red blood cells opened the Gardos channel, causing potassium loss which led to cell dehydration. The Gardos channel was described as an intermediate conductance Ca<sup>2+</sup>-activated K<sup>+</sup> channels (also known as IK1, IKCa<sup>1</sup>, KCa<sup>4</sup>) (Schroder *et al*, 2000).

#### 1.2.1 Types and Function of Calcium Activated Potassium Channel

The human genome contains 8 genes coding for Ca<sup>2+</sup>-activated K<sup>+</sup> channels, which are divided into 3 classes based on differences in amino acid sequences and pharmacological profiles. Large conductance calcium-activated K<sup>+</sup> (BK) channels are activated by intracellular Ca<sup>2+</sup> and membrane potential and have a unit conductance between 100 and 220 pico-Siemens (pS). Small conductance (SK) Ca<sup>2+</sup>-activated K<sup>+</sup> channels have a unit conductance of 2-20 pS whereas intermediate conductance (IK) Ca<sup>2+</sup>-activated K<sup>+</sup> channels have 20-85 pS. Both SK and IK are more responsive to calcium than BK (Jorgensen *et al*, 1999).

#### 1.2.1.1 Large Conductance K<sub>Ca</sub> Channels

Large conductance (BK) Ca<sup>2+</sup>-activated K<sup>+</sup> channels, also known as K<sub>Ca</sub>1.1, consist of an S4 voltage-sensing element and C-terminal region. They are gated by voltage and Ca<sup>2+</sup> and are found in sarcolemma of skeletal muscle, neurons and smooth muscle cells. K<sub>Ca</sub>1.1 directly binds to Ca<sup>2+</sup>. These channels can be blocked by scorpion peptides, charybdotoxin, and iberitoxin. (Ishii *et al*, 1997)

#### 1.2.1.2 Small Conductance K<sub>Ca</sub> channels

Small conductance Ca<sup>2+</sup>-activated K<sup>+</sup> channels comprise the 3 structures, namely K<sub>Ca</sub>2.1 (SK1), K<sub>Ca</sub>2.2 (SK2), and K<sub>Ca</sub>2.3 (SK3) and have a calmodulinmediated gating mechanism. These channels have an important role in the activation and proliferation of excitable cells. Activation of these channels requires a concentration of intracellular Ca<sup>2+</sup> between 200-500nM. They are more sensitive to Ca<sup>2+</sup> than K<sub>Ca</sub>1.1. Apamin and bee venom peptide are the selective inhibitors of these channels (Wulff *et al*, 2007).

#### 1.2.1.3 Intermediate Conductance K<sub>Ca</sub> channels

The intermediate conductance Ca<sup>2+</sup> activated K<sup>+</sup> channel (also known as K<sub>Ca</sub>3.1, SK4, IK, IK1, IKCa1) is widely expressed throughout the body. K<sub>Ca</sub>3.1 is coded for by the KCNN4 gene located in chromosome 19q13.2. Similar to K<sub>Ca</sub>2, this

channel is associated with calmodulin and activation relies on changes in the concentration of internal Ca<sup>2+</sup> ions, causing membrane hyperpolarization and promoting Ca<sup>2+</sup> influx. Calmodulin senses internal Ca<sup>2+</sup> levels inducing channel opening with Ca<sup>2+</sup> concentration ranging from 95 to 350 nM. K<sub>Ca</sub>3.1 proteins are distributed in the cell membrane and mitochondria of many cell types except the excitable cells; neurons, cardiac myocytes, and skeletal muscle (Auguste *et al*, 1992; Thompson-Vest *et al*, 2006). Intracellular calcium increases result in negative membrane potential, which sustains calcium entry through CRAC or transient receptor potential (TRP) channels. KCa3.1 ultimately drives cellular proliferation (Figure 1-8)

### 1.2.2 Structure of K<sub>Ca</sub>3.1

 $K_{Ca}3.1$ , cloned in 1997, comprises 427 amino acids. It has 6 trans membrane domains and an intracellular calmodulin sensing domain. This channel does not have a voltage sensing domain, which means it does not activate at negative membrane potentials. The structure of  $K_{Ca}3.1$  is illustrated in Figure 1-9



**Figure 1-8. Illustration of K**<sub>ca</sub>**3.1 physiology.** The channel is activated by increases in intracellular calcium following calcium influx through CRAc or TRP channels and/or calcium release from endoplasmic reticulum (ER). CAM, calmodulin; EDHF, endothelium derived hyperpolarizing factor; ER, endoplasmic reticulum; IP3, inositol triphosphate; PLC, phospholipase C. Adapted from Wulff and Köhler (2013).



**Figure 1-9. Structure of the intermediate-conductance calcium-activated potassium channel**. S1-S6 are the transmembrane domains with the pore shown between S5 and S6. N-linked glycosylation is shown following S5. ERS: endoplasmic reticulum retention signal; CBD: putative calmodulin-binding domain. Sites for phosphorylation by protein kinases are denoted by asterisks; number 1 to 4 are PKA, PKG, PKC, and tyrosine kinase sites, respectively. The C-helix is the horizontal gray box, with leucines in triangles. Adapted from Tharp and Bowles (2009).

#### 1.2.3 Pharmacology of K<sub>Ca</sub>3.1

K<sub>Ca</sub>3.1 modulators are fairly well-developed pharmacological tool compounds which have greatly contributed to understanding the role of this channels in EDH-type dilator responses and other physiological functions. Hence, this channel is a promising therapeutic target.

#### 1.2.3.1 K<sub>Ca</sub>3.1 Activators

The only known physiological opener of  $K_{Ca}3.1$  is  $Ca^{2+}$ . Benzimidazolones and Benzothiazoles have been suggested as  $K_{Ca}3.1$  activators by increasing the sensitivity of calmodulin. There are four other  $K_{Ca}3.1$  activators, 1- ethyl-2-benzimidazoline (1-EBIO) activates the channels with an EC<sub>50</sub> value ranging from 30-100 µM in the presence of 100 nM free Ca<sup>2+</sup>. However, 1-EBIO is not a specific activator of  $K_{Ca}3.1$  as it also activates  $K_{Ca}2$  channels. 5,6-dichloro-1-ethyl-1,3-dihydro- 2H-benzimidazol-2-one (DC-EBIO), which has similar composition to 1-EBIO, is reported to be a more potent activator. SKA-20 and SKA 31 are also selective  $K_{Ca}3.1$  openers. The newest activator, namely NS309, has been reported to be even more potent than DC-EBIO (Sankaranarayanan *et al*, 2009). Their chemical structures are shown in figure 1-10.

#### 1.2.3.2 K<sub>Ca</sub>3.1 Blockers

The classical blocker for  $K_{Ca}3.1$  is charybdotoxin. This 37 amino acid peptide is a typical  $\alpha$ -KTX scorpion toxin. Charybdotoxin binds to the external surface of the channel. However, charybdotoxin is not a specific blocker for  $K_{Ca}3.1$  as it also inhibits  $K_{Ca}1.1$ .

An inhibitor which has been tried in a clinical setting, is an azole antimycotic (clotrimazole). This blocker inhibits  $K_{Ca}3.1$  channels and cytochrome P450 enzymes. It has been tested as a treatment for sickle cell anemia and rheumatoid arthritis. However, this drug was not effective because it was being poorly absorbed, had a short half-life and many side effects were reported. Therefore, this drug is not widely used (Ataga *et al*, 2008). There have been 2

other derivatives of clotrimazole which have less side effects and are more tolerable.

TRAM-34 (1-[2-Chlorophenyl) diphenylmethyl]-1H-pyrazole) is a highly specific blocker of  $K_{Ca}3.1$ . The main difference between clotrimazole and TRAM-34 is the switch of imidazole to pyrazole. This exchange improves stability of this agent. Unlike charybdotoxin which binds to  $K_{Ca}3.1$  extracellularly, this drug binds to the intracellular portion of the molecule (Wulff and Castle, 2010).

ICA-17043 (bis(4-fluorophenyl) phenyl acetamide) (also called Senicapoc®) is a more recent derivative of clotrimazole and is more potent than TRAM-34. This drug has been used in phase 2 and phase 3 clinical trials for sickle cell anaemia. ICA-17043 has been reported safe and tolerable. The structure of TRAM-34 and ICA-17043 is shown in figure 1-11 (Wulff and Castle, 2010).

#### 1.2.4 The Role of $K_{Ca}$ 3.1

#### 1.2.4.1 Basic functions of the $K_{Ca}$ 3.1 channel and its role in cellular processes

 $K_{Ca}3.1$  is widely expressed throughout the body, in hematopoietic cells (erythrocytes, platelets, monocytes/macrophages, mast cells, T cells, B cells), epithelial tissues (lung and gastrointestinal tracts), vascular endothelial cells and fibroblasts. Initially,  $K_{Ca}3.1$  was thought to be completely absent in excitable tissue but latterly has been described to be expressed in cerebellar Purkinje cells (Wulff and Köhler, 2013).

Although the basic function of  $K_{Ca}3.1$  is the control of cellular processes by regulating  $Ca^{2+}$  signalling and cell volume,  $K_{Ca}3.1$  has been linked to a plethora of cell processes including; cell activation, migration, apoptosis, inflammation, proliferation, differentiation and fibrosis in various cell types.  $K_{Ca}3.1$  mediates basic cell processes by opening the channel as a response to increased cytosolic calcium with rectifying unit conductance ranging from 50 pS at 2120 mV to 13 pS at 120 mV, causing cell hyperpolarization and ultimately promoting activation of  $Ca^{2+}$ -dependent-signalling pathways.

Identifying selective inhibitors of  $K_{Ca}3.1$  have enabled more information about its function in disease to be gathered. After Gardos (1958) observed that
internal calcium ions regulate potassium flux in red blood cells, further studies identified  $K_{Ca}3.1$  as the IK channel, involved in potassium loss and cell dehydration in people with hereditary sickle cell disease. Subsequent studies led to the finding that inhibiting  $K_{Ca}3.1$  with the selective blocker, Senicapoc<sup>®</sup> reduces haemolysis and numbers of dense erythrocytes, supporting the argument that  $K_{Ca}3.1$  contributes to the pathophysiology of sickle cell disease.

The gene coding for  $K_{Ca}3.1$  (IK1, SK4) was cloned in 1997 (Ishii *et al*, 1997). Two years later, Khanna *et al* (1999), reported that  $K_{Ca}3.1$  was expressed in lymphocytes. This led to work which revealed the ability of TRAM-34 to supress lymphocyte activation (Wulff *et al*, 2000).  $K_{Ca}3.1$  expression has been found to be up-regulated in memory T-cells and IgD<sup>+</sup>B-cells activated with antigens or mitogens. After activation the number of  $K_{Ca}3.1$  channels per cell rose to 200-500, while resting human T cells and B cells only expressed about 5-8  $K_{Ca}3.1$ channels per cell. As a result of this work  $K_{Ca}3.1$  has been proposed as a target for the treatment of autoimmune disease as well as transplant rejection (Chandy *et al*, 2004).

Previous studies identified a role for  $K_{Ca}3.1$  in tumor angiogenesis (Grgic *et al*, 2005), vascular restenosis after angioplasty (Kohler *et al*, 2003), vascular calcification (Freise and Querfeld, 2014), epidermal growth factor induced corneal angiogenesis, human fibrocyte migration (Cruse *et al*, 2011), and activation of human mast cells (Duffy M *et al*, 2004).

In animal models,  $K_{Ca}3.1$  selective inhibitors, TRAM-34 or Senicapoc<sup>®</sup> have been reported to alleviate the symptoms in mice with autoimmune encephalomyelitis (Reich, 2005) and to reduce the severity of colitis in a human inflammatory bowel disease mouse model (Hansen, 2014).

Studies demonstrated that  $K_{Ca}3.1$  plays various roles in mast cell function, including human lung mast cells and showed that inhibiting this channel with  $K_{Ca}3.1$  selective blockers affecting migration and degranulation responses, but didn't supress cytokine-induced proliferation (Cruse *et al*, 2011). Moreover, this channel appears to play a role in asthma and other inflammatory airway disorders.

The contribution of  $K_{Ca}3.1$  in cell activation and proliferation can be further emphasized with the evidence that  $K_{Ca}3.1$  plays a role in fibroblast proliferation. Basic fibroblast growth factor (bFGF) or transforming growth factor (TGF)- $\beta$ 1 leads to an increase in KCa3.1 expression through activation of the Ras/MEK/ERK pathway (Pena *et al*, 1999).

 $K_{Ca}3.1$  has been also reported to be involved in macrophage migration.  $K_{Ca}3.1$  blockers were proven to inhibit the migration of macrophages towards chemoattractant factors.

 $K_{Ca}3.1$  channels have further been shown to contribute to the development of various forms of cancer, from work in LNCaP and PC-3 prostate cancer cells, MCF-7 breast cancer cells, leukemic HL-60 and glioblastoma GL-15 cells. Benzaquen *et al* (1995) reported that  $K_{Ca}3.1$  blocker, clotrimazole, was able to reduce the number of lung metastases in mice with human melanoma cells.

#### 1.2.4.2 K<sub>Ca</sub>3.1 in Fibrosis

Recent evidence showed that  $K_{Ca}3.1$  plays a role in fibroblast proliferation. Grgic *et al* (2009) reported that TRAM-34 effectively reduced renal fibrosis in a unilateral ureteral obstruction (UUO) mouse model. Wang *et al* (2017) demonstrated that oxidative stress induced by Angiotensin II increased  $K_{Ca}3.1$ expression and promoted myocardial fibrosis in AGT-REN double transgenic hypertensive mice.



**Figure 1-10. The structure of K**<sub>Ca</sub>**3.1 activators.** 1-ethyl-2-benzimidazolinone (EBIO), 5,6dichloro-1- ethyl-1,3-dihydro-2H-benzimidazol-2-one (DC-EBIO), NS- 309 and Riluzole. Adapted from (Sankaranarayanan *et al*, 2009)



**Figure 1-11. The chemical structures of TRAM-34 and ICA-17043, K**<sub>Ca</sub>**3.1 blockers**. Adapted from (Ataga *et al*, 2008)

#### 1.2.4.3 K<sub>Ca</sub>3.1 in Kidney Disease

Many studies have demonstrated glomerulosclerosis and renal tubular interstitial fibrosis to be the hallmark of progressive kidney disease. In IgAN, progression has been found to be independent of IgA glomerular deposition but linked to interstitial fibrosis caused by proinflammatory and profibrotic mediators in mesangial cells (Barratt *et al*, 2004). Despite much work in the field, the biological mechanisms which lead to IgAN are still unclear. We do know that IgA deposition in the glomeruli leads to dysfunction of MC and podocytes (Po) and therefore damage to the glomerular filtration barrier. Furthermore, the disruption of the glomerulus leads to plasma protein leakage into the tubular lumen and subsequent pathological changes in proximal tubular epithelial cells (PTEC), which will lead to tubular interstitial fibrosis (Baines and Brunskill, 2008).

Several studies have highlighted the potential role of  $K_{Ca}3.1$  blockers in ameliorating renal fibrosis. Grgic et al (2009) reported  $K_{Ca}3.1$  to be involved in renal fibroblast proliferation and the ability of TRAM-34 to inhibit renal fibrosis after unilateral ureteral obstruction in wild-type mice. A recent study by Huang *et al*, (2013) found that TRAM 34 could attenuate extracellular matrix synthesis in diabetic nephropathy mouse models. Furthermore, they observed that the blockade of  $K_{Ca}3.1$  reduced proteinuria in STZ-induced diabetic mice (Huang *et al*, 2014). However, the role of  $K_{Ca}3.1$  in interstitial inflammation and fibrosis in IgAN has not been widely studied.

#### 1.2.4.4 Non-conducting effect of K<sub>Ca</sub>3.1

Emerging evidence suggests that ion channels can have non-conducting effects, in addition to their ability to pass ions through membrane channels. Potassium channel proteins interact with cell signalling pathways to directly regulate biochemical events, leading to cell activation and proliferation. Millership *et al* (2011) demonstrated that  $K_{Ca}3.1$  (hIK1) may directly regulate cell signalling pathways such as JNK and ERK1/2 to promote proliferation. Further evidence reported by Hegle *et al* (2006), observed that the *ether-à-go-go* (EAG)

K<sup>+</sup> channel regulates fibroblast proliferation via activation of the p38 mitogenactivated protein kinase (MAPK) pathway.

### **Aims and Hypothesis**

The primary aim of this thesis is to investigate the hypothesis:  $K_{Ca}3.1$  plays a role in tubular and interstitial fibrosis in IgAN

This thesis covers the following area:

- 1. K<sub>Ca</sub>3.1 expression in human renal cells
- 2. IgA1 modulation of  $K_{Ca}$ 3.1 expression
- 3. The renal cell response to IgA1 is inhibited by selective  $K_{Ca}$ 3.1 blockers
- 4. K<sub>Ca</sub>3.1, a potential biomarker for IgAN.

### Chapter 2: Material and Methods

#### 2.1 Human subjects

Serum and urine samples were obtained from healthy individuals, biopsy proven IgAN patients and patient with other kidney diseases patients aged over 18 years. All subjects gave their informed consent. These studies were approved by Leicestershire, Northamptonshire and Rutland Research Ethics Committee and University of Leicester Ethics committee (Ref: UHL 09873)

#### 2.1.1 Serum samples

Venous blood was taken by phlebotomists and allowed to clot at room temperature for 30 minutes before being centrifuged at 1000 X G for 10 minutes. The serum was aspirated into a fresh container and used immediately or stored at -20°C.

#### 2.1.2 Urine samples

Fresh urine or 24-hour urine samples were used in this study. Fresh urine was stored on ice immediately. To separate cells from solute, whole urine was spun at 500 X G for 5 minutes. Samples were aliquoted and stored at -20°C for further use.

#### 2.2 In vitro studies

#### 2.2.1 Cell Cultures

#### 2.2.1.1 Human mesangial cells

Primary human mesangial cells (HMCs) were obtained from Lonza. These cells were grown in Mesangial cell basal medium (MsBM) containing 5% foetal calf serum in 5% CO<sub>2</sub> at 37°C. Cells at passage number 4-10 were used for the experiments. For the stimulation experiments, 24 well tissue culture plates were seeded with 20,000 cells per well in 500  $\mu$ L starvation media (MsBM with 0.5% foetal calf serum), incubated overnight to reach 80-90% confluency and incubated with fresh starvation media containing 50  $\mu$ g/mL human serum IgA1

with and without TRAM-34, TRAM-85, ICA-17043, or TGF- $\beta$  for 24 hours. Supernatant was collected, and cells lysed in TRIZOL or western blot reducing loading buffer for further analysis.

#### 2.2.1.2 hTERT/PTEC

hTERT/PTECs were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). The cells are proximal tubule epithelial cells extracted from normal human renal cortex and immortalized by plxsn-htert retroviral transfection. This cell line is positive for the epithelial marker pan-cytokeratin, as well as for epithelial cell adhesion molecule, E-cadherin. hTERT/PTECs also express both Aminopeptidase N and  $\gamma$ -Glutamyl Transferase (GGT) which are located in the brush border of the renal proximal tubular epithelium (Wieser *et al*, 2008).

hTERT/PTEC growth media was DMEM: F12 with 5 pM triiodo-L-thyronine, 10 ng/mL recombinant human EGF, 3.5 mg/mL ascorbic acid, 5.0  $\mu$ g/mL human transferrin, 5.0  $\mu$ g/mL insulin, 25 ng/mL prostaglandin E1, 25 ng/mL hydrocortisone, 8.65 ng/mL sodium selenite, 0.1  $\mu$ g/mL G418, 1.2 mg/mL sodium bicarbonate. Cells at passage number 6-10 were grown at 37°C in a humidified 5% CO<sub>2</sub> incubator. 100,000 cells were seeded in to wells of a 24 wells tissue culture plate and incubated overnight to achieve 80-90% confluency before 50 $\mu$ g/mL human serum IgA1 (total, monomer (mIgA1) or polymer plgA1) with and without DMSO, TRAM 34, TRAM 85 or ICA-17043, or TGF- $\beta$ 1 (2 ng/mL), siRNA or albumin (50 mg/mL or 5g/mL) were added for 24 hours. Supernatant was collected, and cells lysed in TRIzol or SDS PAGE reducing loading buffer for further analysis.

#### 2.2.1.3 HK-2 Cells

HK-2 (human kidney 2) is a proximal tubule epithelial cell line derived from normal adult kidney and immortalized by E6/E7 genes from human papilloma virus 16 (HPV16) (Ryan et. al, 1994). These cells have phenotypic characteristics of well-differentiated PTECs and express normal PTEC markers (alkaline phosphatase, gamma glutamyltranspeptidase, leucine aminopeptidase, cytokeratin, acid phosphatase, and fibronectin). They also retain functional characteristic of PTECs including Na<sup>+</sup>-dependent phlorizinsensitive sugar transport, adenylate cyclase responsiveness to parathyroid hormone, but not to antidiuretic hormone. These cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). They were grown in DMEM-F12 (Life Technologies, Paisley, UK), supplemented with 10% foetal calf serum, 2 mM L-Glutamine (Gibco, Fischer Scientific), penicillin ( $10^2$  IU/mL) and streptomycin ( $100 \mu g/mL$ ) at  $37^{\circ}$ C in a humidified 5% CO<sub>2</sub> atmosphere. For experiments, HK-2 cells were used between passages 10-20. 90% confluent 25 m<sup>2</sup> flask were trypsinized and used for patch clamping analysis.

#### 2.2.1.4 Podocytes

Immortalised human podocytes were a gift from Dr. Moin A. Saleem, University of Bristol. A conditionally immortalized human podocyte cell line has been developed by transfection with the temperature-sensitive SV40-T gene. These cells proliferate at the "permissive" temperature (33°C) (Saleem A.M, 2002). The cells were cultured in RPMI 1640 medium (gibco, Island, NY) containing insulin transferrin and selenium (ITS) 1:100, 10% foetal calf serum and penicillin-streptomycin (10,000 unit/ml penicillin). Undifferentiated podocytes were grown in 75 cm<sup>2</sup> flasks to confluency then 20,000 cells were transferred to each well of a 24-well tissue culture plate. The podocytes were incubated at 33°C to confluence before being incubated at 37°C for 10-14 days to allow the cells to differentiate into terminally-differentiated mature podocytes. The cells were incubated with; media only, recombinant human TGF-β1 (2ng/mL) or total serum IgA1 (50µg/ml) with or without 1µM TRAM-34 for 24 hours. The supernatant was removed, and the cells were washed three times with phosphate buffer solution (PBS) and lysed in Trizol for RNA extraction or SDS PAGE reducing loading buffer.

#### 2.2.2 IgA1 purification

Total serum IgA1 was purified from 20 ml serum of healthy individual with high Gd-IgA1 level by Jacalin-agarose affinity chromatography. High molecular

weight serum proteins were precipitated using 45% ammonium sulphate. The pellet was resuspended in 0.175 M Tris-HCl buffer, pH 7.5. Jacalin-agarose (Vector laboratories) was added to the protein solution and incubated to allow the binding of the IgA1 to the jacalin. The jacalin agarose was washed with 0.175 Tris HCl buffer, pH 7.5 to remove the unbound protein. IgA1 was eluted from the jacalin with 1M galactose in 0.175 M Tris-HCl. The IgA1 was dialysed overnight using Slide-A-Lyzer<sup>TM</sup> G2 dialysis cassettes (Thermo Fischer Scientific) in PBS to remove the galactose, then concentrated using an Amicon centrifugal filter unit (Millipore) and stored at -20°C

#### 2.2.3 Fast protein liquid chromatography (FPLC)

Total IgA1 was separated by size-exclusion chromatography into monomeric and polymeric fractions using a Hiload 16/600 superdex 200 preparative column (GE healthcare, Little Chalfont, UK) on an AKTA purifier (GE Healthcare). 1 mL of 2-3mg/ml IgA1 was applied to the column at a rate of 1 mL/min. IgA1 fractions were pooled into pIgA and mIgA and concentrated. KM55 was determined using Elisa method. The concentration of KM55 was 0.73 mg/mL (mIgA) and 2.66 mg/mL (pIgA). Fractions were assessed by the presence of IgA1 using SDS PAGE and western immunoblotting.

#### 2.2.4 Microculture Tetrazolium Assay

The selective K<sub>Ca</sub>3.1 blocker, TRAM-34 is water insoluble and so must be dissolved in DMSO before being added to culture media. *Microculture tetrazolium* (MTT) assay was performed to assess the effect of 0.1% DMSO, the final concentration of DMSO with 1 $\mu$ M TRAM-34, on cell viability. MTT is an enzyme-based method which relies on the reduction of a colouring reagent by dehydrogenase in viable cells to determine cell viability. The cells were incubated with media only, media plus 0.1% DMSO, or IgA1 (50 $\mu$ g/ml) or TGF- $\beta$ 1 (2ng/ml) with or without TRAM-34 for 24 hours, as described previously. The supernatant was removed, and the cells were washed with PBS. 50  $\mu$ L of MTT (2  $\mu$ g/mL in PBS) were added directly to the cells in each well and incubated for ½-1 hour at room temperature, checking every ½ hour for colour change. The cells were solubilized with 250  $\mu$ L DMSO per well. The absorbance at 540nm in

each well was measured. No change in cell viability was seen with the addition of 0.1% DMSO or any of the other incubation conditions.

#### 2.2.5 Wes – simple Western blot

Conceptually, simple Wes (ProteinSimple, USA) technique is the same as a traditional Western transfer and immunoblot. However, the Wes system is automated. Samples are mixed with Wes sample master mix containing dithiothreitol, and a mixture of three fluorescent standard dyes and heat denatured. The samples are then loaded on to a Wes assay plate with primary (Sigma) and secondary antibodies (Wes, ProteinSimple, USA), chemiluminescence substrate and wash buffers. Once the assay plate is prepared, it is placed into the Wes system machine with the capillary set.

#### 2.2.6 Small interfering RNA (siRNA)

hTERT-PTECs were seeded at 100,000 cells per well in 12 well plates and incubated at 37°C, 5% CO<sub>2</sub> until 60-80% confluent. For transfection, lipofectamine RNA/iMAX reagent plus 10  $\mu$ M TGF- $\beta$  siRNA (TGFB1 silencer select validated siRNA, Ambion) or 100 mM negative control (Silencer Cy 3 labelled, negative control siRNA, Ambion) was diluted in Opti-MEM medium and incubated for 5 minutes at room temperature to form siRNA-reagent complexes. The final concentration of TGF- $\beta$  siRNA was 30 pmol/mL for 12 wells plate. These complexes were added to hTERT/PTEC medium containing 0.2% foetal bovine serum and added to the cells for 24 hours before incubation with media containing total IgA1 at 50 µg/mL.



Figure 2-1 Small interfering RNA (siRNA) protocol.

#### 2.2.7 TGF-β Neutralizing antibody

hTERT/PTEC were seeded with 100,000 cells per well in 12 wells until 60- 80% confluent then incubated with 10  $\mu$ g/mL TGF- $\beta$  pan specific antibody (AB-100-NA, R&D system, UK) for 24 hours.

#### 2.2.8 Isolation RNA and protein from cell lysate

RNA and protein were isolated from the cell lysates using Trizol reagent from Life Technologies using the protocol from the manufacturer. RNA was resuspended in RNase-free water and stored at -80°C. Protein was stored in 1% SDS at 20°C. A protein quantitation assay from Bio-Rad was used to quantitate the protein.

#### 2.2.9 Selection of endogenous control (housekeeping gene)

An endogenous control plate (Taqman Array Gene Signature Sets 96-Well Plate) from Life Technologies was used to select the appropriate housekeeping

gene for qPCR. RNA isolated from mesangial or hTERT/PTEC cell lysates grown under the following conditions: media only, TGF- $\beta$ , and IgA (50 µg/mL) was used. cDNA was synthesised using 1 µg of RNA from each sample. RNase-free water and Taqman mastermix (Life Technologies) were added to each cDNA prep to a total volume of 720 µL. 20 µLof each sample was placed in each of the 32 wells of the endogenous control plate. The PCR program used was; hold 10 minutes 95°C, PCR (40 cycles), melt 15 minutes 95°C, anneal 1 minute 60°C. Housekeeping gene was selected when CT values differences in media only, TGF- $\beta$  and IgA were less than 1.

Gene Symbol	1	1 2	2 3	3 4		5	5	7 8		9 10	) 1	1 12
A	18S	GAPDH	HPRT1	GUSB	185	GAPDH	HPRT1	GUSB	18S	GAPDH	HPRT1	GUSB
В	АСТВ	B2M	HMBS	IPO8	АСТВ	B2M	HMBS	IPO8	АСТВ	B2M	HMBS	IPO8
С	PGK1	RPLPO	твр	TFRC	PGK1	RPLPO	твр	TFRC	PGK1	RPLPO	твр	TFRC
D	UBC	YWHAZ	ΡΡΙΑ	POLR2A	UBC	YWHAZ	ΡΡΙΑ	POLR2A	UBC	YWHAZ	PPIA	POLR2A
E	CASC3	CDKN1A	CDKN1B	GADD45A	CASC3	CDKN1A	CDKN1B	GADD45A	CASC3	CDKN1A	CDKN1B	GADD45A
F	PUM1	PSMC4	EIF2B1	PES1	PUM1	PSMC4	EIF2B1	PES1	PUM1	PSMC4	EIF2B1	PES1
G	ABL1	ELF1	MT-ATP6	MRPL19	ABL1	ELF1	MT-ATP6	MRPL19	ABL1	ELF1	MT-ATP6	MRPL19
н	POP4	RPL37A	RPL30	RPS17	POP4	RPL37A	RPL30	RPS17	POP4	RPL37A	RPL30	RPS17

Figure 2-2 Taqman<sup>®</sup> Array Gene Signature Sets 96-Well Plates to screen for the housekeeping gene

#### 2.2.10 qPCR

cDNA from podocytes, PTECs and HMCs was synthesised using ImProm-II Reverse Transcription System (Promega). Gene expression was analysed with TaqMan gene expression assays (Applied Biosystems by Thermo Fisher Scientific) Hs00158470\_m1 KCNN4. Sc04146268\_s1 ALT1, Hs00164004\_m1 COL1A1, Hs00943809\_m1 COL3A1 were used for KCa3.1, FSP1, type I collagen and type III collagen gene expression, respectively. The endogenous control gene for HMC was Rn03296710\_s1 MT-ATP6 and Hs00897727\_g1 PES1 FAM for hTERT/PTEC. All expression data was normalized to either MT-ATP6 or PES1. All gene expression assays were carried out in TaqMan Universal PCR Master Mix (Applied Biosystem by Thermo Fisher Scientific).

#### 2.2.11 Western blotting

Protein isolated from cell lysates was run through 10% acrylamide SDS PAGE, at 200V for 30 minutes, the proteins were transferred to nitrocellulose membrane (Amersham Hybond ECL, GE) in transfer buffer (500nM Glycine, 20% methanol) at 100 V for 1 hour. The membranes were washed with T-TBS 0.01% TWEEN and then blocked with 5% dried skimmed milk in T-TBS overnight, at 4°C. The membranes were incubated with anti-human K<sub>Ca</sub>3.1, anti-human fibronectin,  $\alpha$ -SMA in T-TBS for 2 h at room temperature before being washed three times with T-TBS, for 5 minutes each. The membranes were further incubated with Horseradish peroxidase (HRP) conjugated goat-anti rabbit (Dako) for anti-human K<sub>Ca</sub>3.1 and  $\alpha$ -SMA, Horseradish peroxidase (HRP) conjugated human anti-mouse (Dako) for anti-human fibronectin for 2 hours at room temperature, then washed three times with T-TBS for 10 minutes each before visualizing using ECL on a Chemidoc Touch Imaging System (Bio Rad). The membrane was reprobed, without stripping, using anti-human  $\beta$ -actin (Abcam) and appropriate secondary antibody (HRP, human anti-mouse, Dako).

#### 2.2.12 Elisa

IL-6 and TGF- $\beta$  concentrations were determined using Duo sets from R&D systems and protocols from the manufacturer.

#### 2.2.13 Multiplex cytokines assays

Luminex is an antibody-bead based method which is able to quantitate multiple cytokines and chemokines in a small volume of fluid (serum, plasma or cell culture supernatant). In this study, cell culture supernatants from three experiments were analysed. The method used was; 50  $\mu$ L microparticle cocktail (Luminex beads) were added to each well with 50  $\mu$ L standard or sample and incubated for 2 hours at room temperature on a horizontal orbital microplate shaker (0.12" orbit) set at 800±50 rpm. After washing the plate with the supplied wash buffer, 50  $\mu$ L biotin antibody cocktail was added to each well and incubated for 1 hour at room temperature on the shaker at 800±50 rpm. The microparticles were resuspended by adding 100  $\mu$ L of wash buffer and

incubated for 2 minutes on the shaker set at 800±50 rpm. The plates were analysed using a Bio-rad analyser. Concentrations were determined by comparison with a standard curve.

#### 2.2.14 Acetone protein precipitation

Acetone protein precipitation technique was used to precipitate protein from cell culture (hTERT/PTECs) supernatant. The method was as follows; 1.7 mL cold acetone (-20°C for 10 minutes) was added to 200  $\mu$ L supernatant and incubated overnight at -20°C before centrifugation at 14,000 rpm for 40 minutes. The pellets were resuspended in 20  $\mu$ L 0.1% SDS. The protein concentration was measured using a nanodrop spectrophotometer at A<sub>280</sub>.

#### 2.2.15 Microparticle isolation

Differential centrifugation was used to isolate microparticles (MP) from conditioned media (stored in -20°C before processing). Conditioned medium was centrifuged at 1000 X G for 15 minutes at 4°C in order to remove intact and broken cells, cell debris, and large cellular organelles. The supernatant was then centrifuged again at 18,000 X G for 30 minutes at 4°C and the supernatant was removed and kept for exosome isolation. 500  $\mu$ L of MP buffer (145 mM NaCl, 2.7 mM KCl, 10 mM Hepes, pH = 7.4) was added to the MP pellet and then centrifuged at 18,000 X G for 20 minutes. After centrifugation, 90% of this supernatant was removed and the microparticle containing pellet was resuspended with 100 $\mu$ L of MP buffer and frozen at -80°C for further use.



Figure 2-3 Diagram illustrating micropartile isolation.

#### 2.2.16 Exosome Isolation

Exosomes are small small vesicles (30-120 nm) which contain RNA and protein. Exosomes are secreted by different type of cells in culture and body fluids, such as urine, saliva, blood and breast milk (ref). Exosomes function as extracellular messengers. However, their biological pathways remain unclear.

Using 1 mL of supernatant of IgA1 stimulated PTECs kept from the prior microparticle isolation protocol, then 500  $\mu$ L exosome reagent (4478359, Invitrogen by Life technologies) was added, followed by mixing and incubating samples overnight at 2°C-8°C. After incubation, samples were centrifuged at 10,000 x g for 1 hour at 2°C-8°C. The pellet was then resuspended with 25 $\mu$ L exosome buffer. Isolated exosomes were kept at ≤20°C until further use.

#### 2.2.17 Patch Clamping

The whole-cell variant of the patch-clamp technique was used (ref). Pipettes made manually from borosilicate fiber-containing glass (Clark were Electromedical Instruments, Edenbridge, UK), and their tips were heat polished. The standard pipette solution contained KCI, 140 mM; MgCl<sub>2</sub>, 2 mM; Na<sup>+</sup>-ATP, 2 mM; GTP, 0.1 mM; HEPES, 10 mM (pH 7.3). The external solution contained NaCl, 140 mM; KCl, 5 mM, MgCl<sub>2</sub>, 1 mM; CaCl<sub>2</sub>, 2mM; HEPES, 10 mM (pH 7.3). For recording, HK-2 cells were detached using trypsin-EDTA solution, centrifuged, and resuspended in fresh media and aliquoted. The cell suspension was placed in 35 mm dishes containing external solution. EBIO was used as channel opener. Whole-cell currents were recorded using an Axoclamp 200A amplifier (Axon Instruments, Sunnyvale, CA, USA), and currents were evoked by applying voltage commands to a range of potentials (-120 to 100 mV) in 10 mV steps from a holding potential of -20 mV and analyzed using pClamp software (version 10.3; Molecular Devices, Sunnyvale, CA, USA). This work was carried out by M. Duffy.

#### 2.3 Ex vivo studies

#### 2.3.1 Urine trichloroacetic acid (TCA) protein precipitation

Trichloroacetic acid was used to precipitate proteins from urine. 250  $\mu$ L of TCA was added to 1 mL total urine or urine supernatant and incubated at 4°C for 10 minutes then centrifuged at 14,000 rpm for 5 minutes. The supernatant was discarded, and the pellet was washed 3 times with cold acetone. The pellet was dried at 95°C in a heat block for 5 minutes to remove any remaining acetone. The pellet was suspended in 100  $\mu$ L 0.1% SDS

#### 2.3.2 Bioassay urine creatinine and protein

QuantiChrom creatinine assay kit (DICT-500) was used to measure the creatinine concentration in urine samples. Urine and standard 5  $\mu$ L each in duplicate were transferred to wells of a clear bottom 96-well plate. Working reagent A and B were mixed and 200  $\mu$ L were added to the wells. The plate was tapped briefly to mix and the optical density was read immediately (OD<sub>0</sub>)

and then at 5 minutes (OD<sub>5</sub>) at 490-530 nm (peak absorbance at 510 nm). The creatinine concentration of the samples was calculated by

$$= \frac{OD_{SAMPLE 5} - OD_{SAMPLE0}}{OD_{STD 5} - OD_{STD 0}} \times [STD] (mg/dL)$$

Total protein concentration was measured using QuantiChrom protein assay kit (QCPR-500) according to the manufacturer's instructions. Using 10  $\mu$ L diluted samples with the detection range 0.06-1.0  $\mu$ g/mL protein in 96-well plate. OD was measured at 570-630 nm (peak absorbance is at 595 nm).

#### 2.4 Statistical analysis

Results were analysed using GraphPad prism version 7.0c for Mac (GraphPad Software, La Jolla, CA, USA) and expressed as mean ± standard error of the mean (SEM). Comparisons between means were made by the one-way analysis of variance (ANOVA) test with the Greenhouse-Geisser correction, combined with Tukey's multiple comparison test with individual variances computed for each comparison. Two-way ANOVA was used for group analysis. A P value <0.05 was considered statistically significant.

# Chapter 3: The Role of K<sub>Ca</sub>3.1 in Human Mesangial Cells by IgA1 stimulation

#### 3.1 Introduction

The defining characteristic of IgAN is deposition of undergalactosylated IgA1 containing immune complexes in the renal mesangium. The renal mesangium is composed of fibroblast-like mesangial cells and extracellular matrix proteins. Deposition of IgA1 triggers mesangial cell proliferation and extracellular matrix synthesis which leads to breakdown of the glomerular filtration barrier and, in 30% of cases end stage renal disease. It is known that progression of IgAN is not linked to the amount of IgA deposited in the mesangium (Magistroni *et al*, 2015). However, the Oxford classification of IgAN identified mesangial hypercellularity, as an indicator of IgAN progression (Xie *et al*, 2012), suggesting that the mechanisms regulating mesangial changes in the presence of IgA1 are pivotal in the pathogenesis of IgAN and therefore could be a target for new treatments for the disease (Magistroni *et al*, 2015)

Previous studies reported that  $K_{Ca}3.1$  is found in mesangial cells and that  $K_{Ca}3.1$  is associated with inflammation, proliferation and activation of lymphocytes, migration of macrophages, and fibrosis of endothelial cells, fibroblasts and fibrocytes through regulation of calcium entry into cells by controlling membrane potential (Wulff *et al*, 2004; Kohler *et al*, 2003; Grgic *et al*, 2009; Wulff and Castle, 2010; Cruse *et al*, 2011). TRAM-34 is a highly selective blocker of  $K_{Ca}3.1$ , and it has been shown to block the effects of stimulation of cells through this channel.

This chapter will investigate whether  $K_{Ca}3.1$  is functioning in human mesangial cells, if expression of the protein is altered by exposure to IgA1 and if blocking  $K_{Ca}3.1$  alters the mesangial cell response to IgA1.

#### 3.2 Results

### 3.2.1 Primary human mesangial cells contain functioning K<sub>Ca</sub>3.1 which can be opened using 1-EBIO and blocked by TRAM-34

Patch clamping was used to measure changes in membrane current in the presence of the  $K_{Ca}3.1$  channel opener (1-EBIO) with and without TRAM-34. Primary human mesangial cells (HMCs) were suspended in growth arrest media (0.2% FCS) with and without 1-EBIO (100 $\mu$ M) or TRAM-34 (200nM) and subjected to 100ms voltage commands ranging from -120 to +100mV from a holding potential of -20mV and membrane currents were recorded. This work was carried out by Mark Duffy.

The results in Figure 3.1 show increased membrane current in HMCs from baseline with the addition of 1-EBIO, indicating opening of  $K_{Ca}3.1$  in the cells. The opening of  $K_{Ca}3.1$  is blocked by the subsequent addition of TRAM-34. These results clearly demonstrate constitutive expression of active  $K_{Ca}3.1$  in primary human mesangial cells.



Figure 3-1 Representative current-voltage relationships from single patch-clamped human mesangial cells. HMCs were subjected to 100ms voltage commands ranging from - 120 to +100mV from a holding potential of -20mV. Membrane currents during these commands were recorded and the current amplitude plotted against command potential. (1) baseline recording, (2) recording made following addition of  $100\mu$ M 1-EBIO (KCa3.1 channel opener), and (3) recording made following the subsequent addition of 200nM TRAM-34 (KCa3.1 channel blocker). N=1 Results presented with kind permission of M. Duffy

#### 3.2.2 IgA1 increases K<sub>Ca</sub>3.1 protein by HMCs in a dose dependent manner

If K<sub>Ca</sub>3.1 plays a role in the mesangial cell changes initiated by IgA1 deposition *in vivo* then it can be assumed that similar changes will be seen when HMCs are incubated with IgA1 *in vitro*. To investigate this, confluent primary HMCs were serum starved (0.2% FCS) for 24 hours and incubated with TGF- $\beta$ 1 (2 ng/mL, positive control) or pooled human serum IgA1 (25, 50 or 100 µg/mL) for 24 hours. The supernatant was removed, and the cells were lysed in SDS-PAGE reducing sample buffer before proteins were separated on a 10% SDS PAGE gel, Western transferred and immunoblotted with an anti-human K<sub>Ca</sub>3.1 antibody.

Figure 3.1A shows a  $K_{Ca}3.1$  band running at the correct size for  $K_{Ca}3.1$  (48 kDa). An increase in  $K_{Ca}3.1$  was seen in the presence of 2 ng/mL TGF- $\beta1$  or human serum IgA1 (25, 50 or 100 µg/mL) (Figure 3.1B). Analysis by densitometry showed the increase in the presence of IgA1 was dose dependent, being greatest and reaching significance at 100µg/ml.

Cell proliferation was analysed by MTT assay and no difference was seen between experimental conditions.



**Human Mesangial Cells** 



Figure 3-2 HMCs increase K<sub>ca</sub>3.1 protein in the presence of human serum IgA1. Confluent primary HMCs were incubated in serum free media with 2 ng/mL TGF- $\beta$ 1 or IgA1 at 25, 50, or 100 µg/mL. The cells were lysed in SDS PAGE reducing sample buffer before being passed through a 10% polyacrylamide gel. Western blotting was performed and the membrane was incubated with rabbit anti human K<sub>ca</sub>3.1 antibody and then HRP conjugated goat-anti rabbit. (A) A K<sub>ca</sub>3.1 band was seen at approx. 48 kDa. The density values for the K<sub>ca</sub>3.1 bands were normalized against β-actin. (B) HMCs synthesis of K<sub>ca</sub>3.1 is upregulated in the presence IgA1 in a dose dependent manner. N=3 Data is shown as mean +/- SEM and was analysed by one way ANOVA, \*p<0.05

### 3.2.3 IgA increases $K_{Ca}$ 3.1 expression by HMCs and this is blocked by TRAM-34

To investigate if the IgA dependent changes in K<sub>Ca</sub>3.1 protein by HMCs can be modulated by the K<sub>Ca</sub>3.1 blocker, TRAM-34, confluent, growth arrested primary HMCs were incubated in starvation media (0.2% FCS) with TGF- $\beta$ 1 (2 ng/mL) or IgA1 (50 µg/mL) for 24 hours with or without TRAM-34 (1 µM). The increase of K<sub>Ca</sub>3.1 protein in the presence of IgA1 was inhibited by the presence of the selective K<sub>Ca</sub>3.1 inhibitor, TRAM-34 (Figure 3.3).



Figure 3-3 HMC increased K<sub>ca</sub>3.1 protein in the presence of human serum IgA1 is inhibited by TRAM-34. Confluent primary HMCs were incubated in serum free media with TGF- $\beta$  (2 ng/mL) or IgA1 (50 µg/mL) for 24 hours with or without TRAM-34 (1 µM). The cells were lysed in SDS PAGE reducing sample buffer before being passed through a 10% polyacrylamide gel. Western blotting was performed and the membrane was incubated with rabbit anti human K<sub>Ca</sub>3.1 antibody and then HRP conjugated goat-anti rabbit. The band densities were normalized with  $\beta$ -actin and the results are expressed as fold change compared to media only. K<sub>Ca</sub>3.1 protein by the HMCs increased in the presence IgA1 and the increase was modulated by TRAM-34 (1 µM), N=3. Data is shown as mean +/- SEM and was analysed using one way ANOVA. \*\*p<0.005 \*\*\*\*p<0.0001

## 3.2.4 IgA specifically increases KCNN4 expression by HMCs and this is blocked by TRAM-34

To determine if the IgA dependent increase in K<sub>Ca</sub>3.1 protein synthesis seen in HMCs was controlled at the gene level, and was specific to K<sub>Ca</sub>3.1, mRNA for the gene coding for K<sub>Ca</sub>3.1 (KCNN4) was quantified and compared with levels of mRNA from the gene coding for K<sub>Ca</sub>2.3 (KCNN3). RNA was isolated from HMCs incubated with and without 50 $\mu$ g/ml IgA1 or 2ng/ml TGF- $\beta$ 1. cDNA was synthesized and expression of KCNN4 and KCNN3 were determined. KCNN4 expression increased in the presence of IgA1 and TGF- $\beta$ 1. These increases were not seen with KCNN3 (Figure 3-4A). The significant decrease in KCNN4 expression seen in the presence of TRAM-34 was not seen with KCNN3 (Figure 3-4B). These findings suggest that the presence of IgA1 in the media specifically induces the gene coding for K<sub>Ca</sub>3.1 in HMCs and that this change is modulated byTRAM-34.



Figure 3-4 HMCs specifically overexpress KCNN4 in the presence of human serum IgA1. Confluent primary HMCs were incubated in growth arrest media (0.2% FCS) with or without TGF- $\beta$ 1 (2 ng/mL) or IgA1 (50  $\mu$ g/mL) without or with TRAM-34 for 24 hours. RNA was extracted from the cells and cDNA synthesised using the Improm-II Reverse Transcription system. qPCR was performed using the Taqman gene expression kits for KCNN4 (K<sub>Ca</sub>3.1) or KCNN3 (K<sub>Ca</sub>2.3). Ct values were normalised to the housekeeping gene MT-ATP6 and the results are shown as fold increase compared to media only. (A) HMCs increased expression of the KCNN4, but not the KCNN3, gene in the presence of human serum IgA1. (B) TRAM-34 partly blocked KCNN4 overexpression by HMCs in the presence of human serum IgA1 but had little effect on the expression of KCNN3. N=3. Data is shown as mean +/- SEM and was analysed using two-way ANOVA. \*p<0.05 \*\*p<0.005 \*\*\*\*p<0.0001

Α

#### 3.2.5 IgA1 induces IL-6 release by HMCs which is dependent on K<sub>Ca</sub>3.1

IL-6 is recognised as a marker of inflammatory processes in HMCs. To establish the role of K<sub>Ca</sub>3.1 in the production of IL-6 by HMCs, primary HMCs were incubated with IgA1 (50µg/ml) or TGF- $\beta$ 1 (2ng/ml) with or without TRAM-34 (1µM or 4µM). Levels of IL-6 in the supernatant were determined using ELISA. An increase in IL-6 in the supernatant was seen with TGF- $\beta$ 1 (Figure 3.5A) and IgA1 (Figure 3.5B). This increase was reduced with the addition of 1µM and abrogated with the addition of 4µm TRAM 34 (Figure 3.5A and 3.5B). As TRAM-34 selectively blocks K<sub>Ca</sub>3.1, this result demonstrates that K<sub>Ca</sub>3.1 is involved in the mechanism linking incubation of IgA1 and synthesis of IL-6 by HMCs.

Levels of other cytokines in the supernatant produced when HMCs are incubated with IgA1 were measured using Luminex technology. A literature search was carried out to choose the cytokines which may play a role in the pathogenesis of IgAN and fibrosis. Levels of TNF-alpha, IL-18BPa, C5a, IP-10/CXCL10, IL-10, MCP-1, IL-1 beta, IFN-gamma, RANTES, IL-1 alpha, Fas Ligand, IL-17A, GM-CSF, TIM-1/KIM-1, ICAM-1, Endothelin-1, GRO alpha/CXCL1, MCP-2/CCL8, and MMP-7 were found to be below the sensitivity of the Luminex method. The levels of Serpin E1/PAI-1 were found to be above the standard curve. Levels of MMP-9MCP-1/CCL2, IL-2, Collagen IV, BMP-2 did not change with the presence of IgA in the culture media.

Incubation with IgA1 resulted in a non-significant increase in levels of MCP-3/CCL-7, MIF, IL-1 ra/IL-1F3, IL-8/CXCL and PDGF-AA. This increase was reduced by 1 $\mu$ M TRAM-34 and to a lesser extent by 4 $\mu$ M TRAM-34 (results in appendix, Chapter 3-Figure 1)



Figure 3-5 HMCs increase synthesis of IL-6 protein in the presence of total serum IgA1 or TGF- $\beta$ 1. Confluent primary HMCs were incubated in growth arrest media (0.2% FCS) with (A) TGF- $\beta$ 1 (2 ng/mL) or (B) IgA1 (50 µg/mL) for 24 hours with or without TRAM-34 (1 µM or 4 µM). Supernatant was collected and ELISA was used to determine levels of IL-6. IL-6 synthesis by the HMCs increased in the presence of (A) TGF- $\beta$ 1 and (B) IgA1, the increase was modulated by TRAM-34 (1 µM or 4 µM). N=3. Data is shown as mean +/- SEM and was analysed using one way ANOVA. \*p<0.05 \*\*p<0.005

Α

## 3.2.6 Total serum IgA1 induces COL1A1 mRNA expression by HMCs which is dependent on $K_{Ca}3.1$

Expression of the gene coding for type 1 collagen (1 $\alpha$ 1, COL1A1) was measured to establish the role of KCa3.1 in extracellular matrix synthesis by primary HMCs. The cells were incubated with IgA1 (50 µg/mL) or TGF- $\beta$ 1 (2 ng/mL) with or without TRAM-34 (1 µM and 4 µM) for 24 hours. RNA was extracted and cDNA synthesised. qPCR was performed using a Taqman gene expression kit for COL1A1. Ct values were normalised to the housekeeping gene MT-ATP6 and results presented as fold increase compared with media only.

Figure 3.6A shows that HMC COL1A1 gene expression was significantly increased in the presence of human serum IgA1, this increase was modulated by TRAM-34, similar results were seen with TGF- $\beta$ 1 (Figure 3.6A). These results were specific to type 1 collagen, no change in type 3 collagen gene (COL3A1) expression was seen when HMCs were incubation with IgA1 (results in appendix, Chapter 3 Figure 2). A small but non-significant increase in alpha smooth muscle synthesis by HMCs incubated with IgA1 was seen. This increase was abrogated with the presence of TRAM34 (results in appendix, Chapter 3).



Figure 3-6 Increased COL1A1 expression by HMCs in the presence of human serum IgA1 is modulated by TRAM-34. Confluent primary HMCs were incubated in growth arrest media (0.2% FCS) with A. IgA1 (50  $\mu$ g/mL) or B. TGF- $\beta$ 1 (2 ng/mL) with or without TRAM-34 (1  $\mu$ M and 4  $\mu$ M) for 24 hour. The cells were lysed in TRIZOL. RNA was extracted and quantified before cDNA synthesised using Improm-II Reverse Transcription system. qPCR was performed using the Taqman gene expression kits for COL1A1. Ct values were normalised to the housekeeping gene MT-ATP6 and results are presented as fold increase compared with media only. HMC COL1A1 gene expression was significantly increased in the presence of human serum IgA1, this increase was modulated by TRAM-34 (1  $\mu$ M and 4  $\mu$ M), similar results were not seen with TGF- $\beta$ 1. Data is shown as mean +/- SEM and was analysed using one-way ANOVA. N=3 \*p<0.05

Α

В

## 3.1.1 IgA1 does not affect KCNN4, FSP-1, COL1A1 gene expression and $\alpha$ -SMA, fibronection protein synthesis in Human Podocytes

To investigate whether K<sub>Ca</sub>3.1 plays a role in podocyte remodeling by IgA1 in vitro, undifferentiated podocytes were grown in 75 cm<sup>2</sup> flasks to confluency then 20,000 cells were transferred to each well of a 24-well tissue culture plate. The podocytes were incubated at 33°C to confluence before being incubated at 37°C for 10-14 days to allow the cells to differentiate into terminally-differentiated mature podocytes, then incubated with TGF- $\beta$ 1 (2 ng/mL, positive control) or human serum IgA1 (50 µg/mL) for 24 hours. The supernatant was removed, and the cells were lysed in Trizol<sup>R</sup> and SDS-PAGE reducing sample buffer and used accordingly.

cDNA was synthesized and expression of KCNN4, the gene coding for K<sub>Ca</sub>3.1, FSP-1 and COL1A1. There was no change in the expression of KCNN4, FSP-1 or COL1A1 in the presence of TGF- $\beta$ 1 or IgA1 50  $\mu$ g/mL (graphs in appendix).

Cell lysate with SDS-PAGE was passed through a 10% SDS PAGE gel, Western transferred and immunoblotted with  $\alpha$ -SMA and fibronectin antibody. Neither  $\alpha$ -SMA nor fibronectin altered in the presence of TGF- $\beta$ 1 or IgA150  $\mu$ g/mL (graphs in appendix Chapter 3, Figure 4-8).

#### 3.3 Conclusion

Fu *et al* (2014) showed the presence of  $K_{Ca}3.1$  in rat mesangial cells and that overexpression of the gene coding for  $K_{Ca}3.1$ , in the presence of TGF- $\beta$ 1, leads to synthesis of markers of cell injury and fibrosis. Furthermore, Fu demonstrated that  $K_{Ca}3.1$  is distributed in the rat mesangial plasma membrane and that changes in expression of  $K_{Ca}3.1$  leads to cell phenotype trans-differentiation and proliferation of mesangial cells.

The findings in this report show, for the first time,  $K_{Ca}3.1$  in primary human mesangial cells and that  $K_{Ca}3.1$  is active in this cell type. The results also show that expression of the gene coding for  $K_{Ca}3.1$  and subsequent expression of the protein in HMCs are increased in the presence of IgA1 isolated from human serum. Results from our laboratory show that HMCs grown with IgA1 release the proinflammatory marker, IL-6. The results presented in this project show that the  $K_{Ca}3.1$  selective blocker, TRAM-34, is able to modulate this IL-6 release suggesting that  $K_{Ca}3.1$  is important in the mechanism linking IgA1 deposition and glomerular inflammation *in vivo*.

These results also show increased expression of the gene coding for the biomarker of fibrosis,  $1\alpha 1$  collagen (COL1A1) by HMCs in the presence of total serum IgA1. This effect is negated by blocking K<sub>Ca</sub>3.1 with TRAM-34, suggesting that this drug might have potential therapeutic efficacy in preventing glomerular fibrosis.

TRAM-34 is able to reduce IL-6 synthesis and COL1A1 expression at 1 $\mu$ M and 4 $\mu$ M, but no effect was seen when TRAM-34 was added at 20nm and 200nm (results not shown). It should be noted that (Wulff *et al*, 2000) showed the selectivity of TRAM-34 is compromised at higher concentrations: at 4 $\mu$ M TRAM-34 impacts on Kv1 family channels, and Roy et al (2009) revealed increased cell proliferation of breast cancer cells with 3 $\mu$ M TRAM-34. At 1 $\mu$ M both groups showed the inhibitor to be selective. However, for TRAM-34 to be useful in inhibiting inflammation and fibrosis in the mesangium in IgAN a more potent KCa3.1 blocker may be needed.

Unlike the HMCs, there were no changes in  $K_{Ca}3.1$  synthesis by podocytes following IgA1 or TGF- $\beta$  stimulation (results were displayed in the appendix). The role of podocyte damage in the pathogenesis of IgAN has been reported by Lai *et al* (2003) with scar tissue formation and the detachment of cells from the glomerular basement membrane. However, Lai *et al* (2009) found that IgA has no detectable effect on podocytes. They were also unable to detect the presence of any known IgA1 receptor in podocytes. Podocytes possibly require a mediator to undergo fibrosis. There is no direct effect of TGF- $\beta$  and IgA on podocytes in vitro. Therefore, these findings are worth repeating with incubating podocytes in IgA1 stimulated HMC media.

# Chapter 4: The Role of KCa3.1 in Proximal Tubular Epithelial Cells

#### 4.1 Introduction

The development and validation of the Oxford classification of IgAN revealed tubular interstitial fibrosis to be the most important marker to affect the development and prognosis of IgAN (Working Group of the International IgA Nephropathy Network and the Renal Pathology Society *et al*, 2009; Trimarchi *et al*, 2017) The mechanisms leading to this fibrosis are not clear but it has been reported that renal tubular epithelial to mesenchymal transition (EMT) is an essential mechanism in tubulointerstitial fibrosis. (Zeisberg and Kalluri, 2008; Rodemann and Muller, 1991; Couser and Johnson, 1994). Renal tubular EMT is thought to be initiated by the presence of unfiltered proteins in the tubules. In IgAN, these proteins contain the undergalactosylated polymeric IgA1 present at raised levels in serum from IgAN patients.

Our group have observed synthesis of the pro-fibrotic mediator, TGF- $\beta$ 1 by proximal tubule epithelial cells incubated with IgA1 isolated from human serum and that this synthesis is greater with undergalactosylated polymeric IgA1 (unpublished data). Huang et al (2014) showed that immortalized human proximal tubule cells (HK2) responded to incubation with TGF- $\beta$ 1 by initiating a large potassium current, which could be abrogated by the selective K<sub>Ca</sub>3.1 blocker, TRAM-34, suggesting the involvement of K<sub>Ca</sub>3.1.

This chapter will investigate the role of  $K_{Ca}3.1$  in the response of an immortalized human proximal tubule cell line, hTERT/PTECs, to incubation with IgA1 and whether any changes can be moderated by  $K_{Ca}3.1$  blockers.

#### 4.2 Results

### 4.2.1 Proximal tubular epithelial cells contain functioning K<sub>Ca</sub>3.1 which can be opened by 1-EBIO and blocked by TRAM-34

Proximal tubular epithelial (HK-2) cells were suspended in growth arrest media (0.2%) with and without the K<sub>Ca</sub>3.1 channel opener (1-EBIO, 100 $\mu$ M) and TRAM-34 (200nM) and subjected to 100ms voltage commands ranging from - 120 to +100mV from a holding potential of -20mV and membrane current were recorded. This work was carried out by Mark Duffy.

The results in Figure 3.1 show increased baseline membrane current of the HMCs from baseline with the addition of 1-EBIO, indicating opening of  $K_{Ca}3.1$  in the cells. The opening of  $K_{Ca}3.1$  is blocked by the subsequent addition of TRAM-34. These results clearly show the presence of functioning  $K_{Ca}3.1$  in human proximal tubule cells, confirming results by Huang et al (2014).



Figure 4-1 Representative current-voltage relationships from single patch-clamped HK-2 cells. Immortalised human proximal tubule cells (HK2) were subjected to 100ms voltage commands ranging from -120 to +100mV from a holding potential of -20mV. Membrane currents during these commands were recorded and the current amplitude plotted against command potential. (1) baseline recording, (2) recording made following addition of  $100\mu$ M 1-EBIO (KCa3.1 channel opener), and (3) recording made following the subsequent addition of 200nM TRAM-34 (K<sub>Ca</sub>3.1 channel blocker). N=1 Results presented with kind permission of M. Duffy

## 4.2.2 IgA1 increases $K_{Ca}$ 3.1 protein synthesis by hTERT/PTEC and this is blocked by TRAM-34 and ICA-17043

To investigate the effect of human serum IgA1 on levels of KCa3.1 in human proximal tubule cells, hTERT/PTECs were incubated in serum free media with TGF- $\beta$ 1 (2 ng/mL) or IgA1 (50 µg/mL) for 24 hours with or without the K<sub>Ca</sub>3.1 blockers TRAM-34 (20nm, 200nm or 1 µM) or ICA-17043 (10nm or 100nm) or the inactive, TRAM-85 (20nm, 200nm or 1 µM). The cells were lysed and K<sub>Ca</sub>3.1 levels were measured by western transfer and immunoblotting.

Figure 4.2A shows an increase in  $K_{Ca}3.1$  synthesis by hTERT/PTECs incubated with IgA1 or TGF- $\beta$ 1. This increase was blocked by TRAM-34 in a dose dependent manner. No such reductions were seen with the inactive TRAM-85 (Figure 4.2B). ICA-17043 significantly reduced the increased  $K_{Ca}3.1$  synthesis seen in the presence of IgA1 at a lower concentration than TRAM-34 (10nm and 100nm compared with 20nm and 200nm, Figure 4.2C).

Interestingly, in hTERT/PTECs the IgA1 dependent increase in  $K_{Ca}3.1$  synthesis is blocked by a lower concentration of TRAM-34 compared with that seen with primary HMCs, 200nm compared with 1µM, respectively.

Cell proliferation was analased by MTT assay and No difference was seen between experimental condition.



В

Α



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Figure 4-2 Increased hTERT/PTEC synthesis of K<sub>ca</sub>3.1 with IgA1 is blocked by TRAM-34 and ICA-17043. Confluent hTERT/PTECs were incubated with (A) total serum IgA1 (50 µg/ml) or TGF- $\beta$ 1 (2ng/ml), (B) total serum IgA1 (50 µg/ml) or TGF- $\beta$ 1 (2ng/ml) with or without TRAM-34 or TRAM-85 or (C) total serum IgA1 (50 µg/ml) with or without ICA-17043 for 24 hours. Cells were lysed in Western blot loading buffer and passed through a 10% SDS PAGE gel. The proteins were Western transferred and immunoblotted with an anti-human KCa3.1 antibody. ECL was used to visualize the bands. K<sub>Ca</sub>3.1 band intensity was normalized using  $\beta$ -actin. N=3 Data is expressed as mean +/- SEM and was analyzed using one-way ANOVA. \*p<0.05 \*\*p<0.005

# 4.2.3 IgA1 specifically increases expression of the gene coding for $K_{Ca}$ 3.1 (KCNN4) by hTERT/PTEC and this is blocked by TRAM-34 and ICA-17043

To determine if the changes in  $K_{Ca}3.1$  protein synthesis by hTERT/PTECS incubated with IgA1 or TGF- $\beta$ 1 are controlled at gene level, RNA was isolated from hTERT/PTEC incubated with 50 $\mu$ g/ml IgA1 or 2ng/ml TGF- $\beta$ 1 plus or minus TRAM-34 or ICA-17043 or the inactive TRAM-85. cDNA was synthesized and expression of KCNN4, the gene coding for  $K_{Ca}3.1$ , and KCNN3, the gene coding for  $K_{Ca}2.3$ , were determined by qPCR. PES1 was the housekeeping gene used to normalize the data.

As seen with the HMCs, KCNN4 expression increased in the presence of IgA1 and TGF- $\beta$ 1. These increases were not seen with KCNN3 (Figure 4.3A). The increase in KCNN4 expression seen with IgA1 was blocked in the presence of TRAM-34 in a dose dependent manner and no change was seen with KCNN3 (Figure 4.3B). TRAM-85 did not significantly decrease the raised expression of KCNN4 seen with IgA1 (Figure 4.2C). The increased sensitivity of hTERT/PTECS to TRAM-34 compared to HMCS is also seen at gene level (Figure 4.3B and C). As with the changes in K<sub>Ca</sub>3.1 protein synthesis, ICA-17043 significantly blocked the IgA1 dependent increase in KCNN4 expression at 10nM and 100nM (Figure 4.3D).

These findings show that control of the IgA1 dependent increase in  $K_{Ca}3.1$  synthesis is at the gene level and that TRAM-34 and ICA-17043, directly or indirectly, block the expression of KCNN4.



В

Α











С

## 4.2.4 IgA1 induces $\alpha$ -smooth muscle actin protein synthesis by hTERT/PTEC which is dependent on K<sub>Ca</sub>3.1

Alpha–smooth muscle actin ( $\alpha$ -SMA) is a marker of the EMT seen in renal fibrosis (Gong *et al*, 2018). To determine if the changes in K<sub>Ca</sub>3.1 synthesis have an effect on EMT in hTERT/PTECs,  $\alpha$ -SMA synthesis was measured in cells incubated with IgA1 (50µg/ml) of TGF- $\beta$ 1 (2ng/ml) with and without the K<sub>Ca</sub>3.1 inhibitors TRAM-34 and ICA-17043 or the inactive TRAM-85 for 24 hours. The cells were lysed and  $\alpha$ -SMA was measured by Western transfer and immunoblotting.

Figures 4-4A shows a significant increase in synthesis of  $\alpha$ -SMA by hTERT/PTECs in the presence of IgA1 or TGF- $\beta$ 1. The IgA1 dependent upregulation of  $\alpha$ -SMA is modulated by TRAM-34 in a dose dependent manner, but not by the inactive TRAM-85 (Figure 4-4B). As with the K<sub>Ca</sub>3.1 synthesis and KCNN4 expression, ICA-17043 blocks the IgA1 dependent increase in K<sub>Ca</sub>3.1 synthesis at 10nM and 100nM (Figure 4-4C).



В

Α





Figure 4-4 Synthesis of  $\alpha$ -SMA by hTERT/PTEC after treatment with IgA or TGF- $\beta$ 1 +/-TRAM-34, TRAM-85 or ICA-17043. Confluent hTERT/PTECs were incubated with (A) total serum IgA1 (50 µg/ml) or TGF- $\beta$ 1 (B) total serum IgA1 (50 µg/ml) with or without TRAM-34 or TRAM-85 or (C) total serum IgA1 (50 µg/ml) with or without ICA-17043 for 24 hours. Cells were lysed in Western blot loading buffer and passed through a 10% SDS PAGE gel. The proteins were Western transferred and immunoblotted for  $\alpha$ -SMA. ECL was used to visualize the bands.  $\alpha$ -SMA band intensity was normalized using  $\beta$ -actin. N=3 Data is expressed as mean +/- SEM and was analyzed using one-way ANOVA. \*p<0.05 \*\*p<0.005

## 4.2.5 IgA1 induces fibronectin protein synthesis by hTERT/PTEC which is dependent on K<sub>Ca</sub>3.1

Fibronectin is an important extracellular protein which is upregulated in renal fibrosis (Cho *et al*, 2016). Analysis of fibronectin levels in hTERT/PTEC incubated with IgA1 (50  $\mu$ g/mL) with or without the K<sub>Ca</sub>3.1 inhibitors TRAM-34 or ICA-17034 or the inactive TRAM-85 was made using Western transfer and immunoblotting.

Figure 4.4A shows a significant increase in fibronectin synthesis in the presence of IgA1 or TGF- $\beta$ 1. The IgA1 dependent increase is blocked in the presence of TRAM-34 (Figure 4.5B) or ICA-17043 (Figure 4.5C) in a dose dependent manner. TRAM-85 did not significantly block the IgA1 dependent increase in K<sub>Ca</sub>3.1 synthesis (Figure 4.5B).

No changes in the expression of the genes coding for the extracellular matrix proteins  $1\alpha 1$  collagen and  $3\alpha 1$  collagen, COL1A1 or COL3A1 respectively, were seen in hTERT/PTECs incubated with IgA1 or TGF- $\beta 1$  (see appendix chapter 4)









Figure 4-5. Synthesis of fibronectin protein by hTERT/PTEC after treatment IgA1 or TGF- $\beta$ 1 +/-TRAM-34, TRAM-85 or ICA-17043. Confluent hTERT/PTECs were incubated with (A) total serum IgA1 (50 µg/ml) or TGF- $\beta$ 1 (B) total serum IgA1 (50 µg/ml) with or without TRAM-34 or TRAM-85 or (C) total serum IgA1 (50 µg/ml) with or without ICA-17043 for 24 hours. Cells were lysed in Western blot loading buffer and passed through a 10% SDS PAGE gel. The proteins were Western transferred and immunoblotted for fibronectin. ECL was used to visualize the bands. K<sub>Ca</sub>3.1 band intensity was normalized using  $\beta$ -actin. N=3 Data is expressed as mean +/- SEM and was analyzed using one-way ANOVA. \*p<0.05 \*\*p<0.005

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## 4.2.6 IgA1 induces FSP-1 expression by hTERT/PTEC which is dependent on K<sub>Ca</sub>3.1

Fibroblast-specific protein 1 (FSP-1) is a recognized marker of fibroblasts in tissues undergoing tissue remodeling. It is used to identify fibroblasts derived from EMT. (Österreicher et al 2011, Kalluri and Weinburg 2009) To add to the evidence that IgA1, through synthesis of K<sub>Ca</sub>3.1, promotes EMT in PTECs, hTERT PTECs were incubated with IgA1 ( $50\mu$ g/ml) or TGF- $\beta$ 1 (2ng/ml) with and without the K<sub>Ca</sub>3.1 inhibitors TRAM-34 and ICA-17043 or the inactive TRAM-85 for 24 hours. The cells were lysed and RNA extracted. qPCR was used to measure expression of the gene coding for FSP-1 (ALT-1) using PES1 as the housekeeping gene.

An increase in FSP-1 gene expression was seen when hTER/PTECs were incubated with TGF- $\beta$ 1 or IgA1 (Figure 4-6A). This increase in expression was blocked by the presence of TRAM-34 in a dose dependent manner but not by TRAM-85 (Figure 4-6B). ICA-17043 was also able to block FSP-1 gene expression in a dose dependent manner but the concentration required for inhibition was lower than that needed for TRAM-34 (Figures 4-6B and C).



В



Α



Figure 4-6 FSP-1 gene (ALT-1) expression by hTERT/PTECs after incubation with IgA1 or TGF- $\beta$ 1 +/- TRAM-34 or ICA-17043. qPCR analysis of ALT-1 mRNA from hTERT/PTECs incubated with (A) IgA1 or TGF $\beta$ 1 (B) IgA1 with and without TRAM-34 or TRAM-85 (C) IgA1 with or without ICA-17043. N= 3 Results are shown as mean ±SEM of Ct values normalised to PES1 and media only. Data was analysed by one-way ANOVA. \*p<0.05 \*\*p<0.005.

## 4.2.7 The role of TGF- $\beta$ in modulation of K<sub>Ca</sub>3.1 synthesis by hTERT/PTECs in the presence of IgA

Previous studies from our laboratory have shown synthesis of TGF- $\beta$ 1 by PTECs when they are incubated with IgA1. The results in this chapter show an increase in K<sub>Ca</sub>3.1 when PTECs are incubated with IgA1 and Huang et al (2014) have shown an increase in K<sub>Ca</sub>3.1 synthesis by PTECs with incubation with TGF- $\beta$ 1. Taken together, these results could mean that the IgA1 dependent increase in K<sub>Ca</sub>3.1 by hTERT/PTECs is caused by IgA1 stimulating the cells to synthesize TGF- $\beta$ 1 which then upregulates the K<sub>Ca</sub>3.1 synthesis. TGF- $\beta$ 1 small interfering RNA (siRNA) and TGF- $\beta$  neutralizing antibody were used to investigate the role of TGF- $\beta$ 1 in IgA1 stimulation of K<sub>Ca</sub>3.1 synthesis by hTERT/PTECs. The cells were incubated with IgA1 (50µg/ml) alone or with TGF- $\beta$ 1 siRNA, scrambled siRNA (-ve control) or TGF- $\beta$ 1 neutralising antibody. ELISA was used to measure TGF- $\beta$ 1 levels in the culture supernatant and Western transfer and immunoblotting was used to measure K<sub>Ca</sub>3.1 protein levels in the cell lysates.

Figure 4-7A shows a significant increase in the level of TGF- $\beta$ 1 in the supernatant of hTERT/PTECs incubated with IgA1. This increase is completely blocked when expression of the gene coding for TGF- $\beta$ 1 is inhibited by the TGF- $\beta$ 1 siRNA. This effect is not seen when the negative control (scrambled) siRNA is added to the media with IgA1. Significantly less TGF- $\beta$ 1 is seen in the supernatant when the cells are incubated with IgA1 and TGF- $\beta$ 1 neutralising antibody.

Figure 4-7B shows levels of  $K_{Ca}3.1$  in cell lysates from the experiment described above. A significant increase in  $K_{Ca}3.1$  was seen when the cells were incubated with IgA1 alone. This increase was abrogated with the addition of TGF- $\beta$ 1 siRNA but not the scrambled siRNA, mirroring the result seen with TGF- $\beta$ 1 in the supernatant. These results strongly suggest that the increase in KCa3.1 synthesis by hTERT/PTECs in the presence of IgA1 is dependent on the TGF- $\beta$ 1 synthesis initiated by IgA1. However, this is not supported by the results with the TGF- $\beta$ 1 neutralising antibodies which shows a lack of increase

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of TGF- $\beta$ 1 in the supernatant but a raised level of K<sub>Ca</sub>3.1 in the cells. Repeating this experiment with a gradient of concentrations of the neutralizing antibody may help to explain these results.



**Figure 4-7.** Changes in TGF-β1 and K<sub>Ca</sub>3.1 synthesis by hTERT/PTECs with IgA1 +/- TGF β1 siRNA and TGF-β1 neutralizing antibody. Confluent hTERT/PTECs were incubated with total serum IgA1 (50 µg/ml) alone or with siRNA TGF-β1, or TGF β1 neutralising antibody or scrambled siRNA for 24 hours. ELISA was used to measure TGF-β1 in the supernatant and Western transfer and immunoblotting was used to measure K<sub>Ca</sub>3.1 in the cell lysate. ECL was used to visualize the bands. KCa3.1 band intensity was normalized using β-actin (A) supernatant TGF-β1 increased with the presence of IgA1, this increase was blocked by siRNA TGF-β1 or TGF-β1 neutralising antibody but not by scrambled siRNA. (B) KCa3.1 synthesis increased with IgA1 alone, this increase was blocked in the presence of siRNA TGF-β1, while the TGF-β1 neutralising antibody and the scrambled siRNA had no effect on the IgA1 dependent increase in KCa3.1. N=3 Data is expressed as mean +/- SEM and was analysed using one-way ANOVA. \*\*\*p<0.0005 \*\*\*\*p<0.0001.

#### 4.3 Conclusions

Previous studies from our laboratory by Cheung *et al* (personal communication) demonstrated that IgA1 from IgAN patients triggers synthesis of proinflammatory and profibrotic phenotypic markers by PTECs, suggesting interaction between filtered IgA1 and PTEC might be a key factor in driving tubulointerstitial damage and progression of renal fibrosis in IgAN.

Results in this chapter confirm the presence of active  $K_{Ca}3.1$  in human PTECs. They also show that the presence of IgA1 causes increased synthesis of  $K_{Ca}3.1$  by hTERT/PTECs, the control of which includes changes of KCNN4 gene expression. The ability of  $K_{Ca}3.1$  blockers to prevent IgA1 dependent changes in expression and synthesis of markers of fibrosis and EMT in PTECS suggests involvement of this potassium channel in tubulointerstitial damage in IgAN. These findings are consistent with published data from Huang et al, 2013 who reported that pharmacological inhibition and gene knockdown of  $K_{Ca}3.1$  inhibited TGF- $\beta$ 1-induced MCP-1 expression in PTECs. They also observed that KCa3.1 blockade by TRAM-34, leads to downregulation of KCNN4 gene expression.

The ability of TGF- $\beta$ 1 siRNA to block increased synthesis of K<sub>Ca</sub>3.1 when PTECs were incubated with IgA1 indicates a pivotal role for TGF- $\beta$ 1 in IgA1 dependent upregulation of K<sub>Ca</sub>3.1 synthesis and thus markers of fibrosis and EMT, although more work needs to be carried out to confirm the findings.

The importance of interstitial fibrosis in renal disease, including IgAN, is well established. To date there is no way of reversing renal fibrosis, although therapies to control blood pressure and proteinuria (ACE inhibitors and ARBs) have proved moderately successful at slowing down the process. These data indicate that blocking  $K_{Ca}3.1$  activity in PTECs could prove useful in preventing interstitial fibrosis.

This data shows TRAM-34 blocks  $K_{Ca}3.1$  expression and synthesis in hTERT/PTECS at lower concentrations than in primary HMCs. This could be explained by PTECs being more sensitive to TRAM-34 or because HMCs are

incubated with IgA1 with or without TRAM-34 in media containing 0.2% FCS whereas hTERT/PTECS are grown in serum free media. TRAM-34 is known to bind serum proteins and this reduces its effectiveness. This explanation of the difference between sensitivity of TRAM-34 with the two cell lines could be confirmed by repeating the experiment and including 0.2% FCS in the hTERT/PTEC media. Primary HMCs will not survive in serum free media. The interaction of TRAM-34 with serum proteins and the effect on these proteins on the effectiveness of the ability of TRAM-34 to block  $K_{Ca}$ 3.1 means that ICA-17043 (Senicapoc) is more useful *in vivo*. Other studies have shown ICA-17043 and TRAM-34 to effective blockers of KCa3.1 in pulmonary myofibroblasts (Roach *et al*, 2013), monocytes (She *et al*, 2019) and corneal fibrosis (Anumanthan *et al*, 2018).

### Chapter 5: The Role of K<sub>Ca</sub>3.1 in polymeric and monomeric IgA induced Proximal Tubular Epithelial Cells

#### 5.1 Introduction

Yao et al (2014) reported that renal tubular EMT correlates with disease progression in IgAN. A number of studies have shown that plgA1 is more likely to induce a pro-inflammatory and pro-fibrotic response by renal cells compared to mlgA1 (Oortwijn et al, 2006; Moura et al, 2005; van der Boog et al, 2004). Most of this work has been carried out using HMCs. However, in our lab this effect is also found in proximal tubule epithelial cells, the largest response being from undergalactosylated plgA1. Allen et al (2001) characterized the lgA1 deposited in the mesangium and found it to be undergalactosylated and and others polymeric. Our group have seen raised levels of undergalactosylated plgA1 in serum from IgAN patients, with patients with the more progressive form of the disease having the highest levels. This accumulated evidence leads to the suggestion that upon the breakdown of the glomerular filtration barrier, in IgAN, unfiltered proteins, enriched with undergalactosylated plgA1, will come into contact with the epithelial cells lining the proximal tubules and start mechanisms which drive tubulointerstitial fibrosis. The process leading to this fibrosis is not fully understood.

Having shown in the previous chapter that human PTECs contain functioning  $K_{Ca}3.1$  and that  $K_{Ca}3.1$  is important in total IgA1-dependent synthesis of profibrotic and pro-inflammatory mediators, the work described in this chapter will investigate the role of this transport protein in stimulation of hTERT/PTEC with plgA.

#### 5.2 Results

# 5.2.1 Polymeric but not monomeric IgA1 increases $K_{Ca}$ 3.1 protein synthesis by hTERT/PTEC and this is blocked by TRAM-34 and ICA-17043

Confluent primary hTERT/PTECTs were incubated with serum plgA1 or mlgA1, isolated from a healthy individual with high Gd-IgA or albumin (both 50  $\mu$ g/mL) for 24 hours with or without K<sub>Ca</sub>3.1 blocker TRAM-34 or ICA-17043 and negative control blocker TRAM-85. The supernatant was removed, and the cells were lysed in SDS-PAGE reducing sample buffer before being passed through a 10% SDS PAGE gel, Western transferred and immunoblotted with an anti-human K<sub>Ca</sub>3.1 antibody.

Figure 5-1A shows upregulation of  $K_{Ca}3.1$  protein synthesis by hTERT/PTECs after incubated with plgA as well as with albumin 50 µg/mL, but not in the presence of mlgA. The upregulation of  $K_{Ca}3.1$  by plgA was modulated by TRAM-34 but not by the inactive, TRAM-85 (Figure 5-1B). The more potent  $K_{Ca}3.1$  blocker, ICA-17043, showed a greater inhibitory effect in a dose dependent manner. Neither inhibitor had an effect on  $K_{Ca}3.1$  synthesis in the presence of mlgA (Figure 5-1C).





Figure 5-1 Polymeric but not monomeric IgA1 increases K<sub>Ca</sub>3.1 protein synthesis by hTERT/PTEC and this is blocked by TRAM-34 and ICA-17043. Confluent hTERT/PTECs were incubated in serum free media with pIgA1 (50  $\mu$ g/mL), mIgA1 (50  $\mu$ g/mL) or albumin (50  $\mu$ g/mL for 24 hours with or without TRAM-34 (200 nM) TRAM-85 (200 nM), ICA-17043 (10nM and 100 nM). The cells were lysed in SDS PAGE reducing sample buffer before being passed through a 10% polyacrylamide gel. Western transfer and immunoblotting were performed. The band densities were normalized with  $\beta$ -actin. (A) K<sub>Ca</sub>3.1 synthesis by the hTERT/PTEC increased in the presence pIgA1 and albumin but not mIgA1. (B) The increased K<sub>Ca</sub>3.1 seen with pIgA1 was modulated by TRAM-34 or ICA-17043 in dose dependent manner, but not TRAM-85. (C) The presence of TRAM-34 or ICA-17043 with mIgA1 had no effect on K<sub>Ca</sub>3.1 synthesis. N=6. Results are shown as mean ±SEM and were analysed by one-way ANOVA. \*p<0.05 \*\*p<0.005

В

С

#### 5.2.2 Polymeric but not monomeric IgA1 specifically increases KCNN4 expression by hTERT/PTEC and this is blocked by TRAM-34 and ICA-17043

To determine if the IgA dependent increase in  $K_{Ca}3.1$  protein synthesis seen in hTERT/PTECs was controlled at the gene level, was specific to the gene coding for  $K_{Ca}3.1$  (KCNN4) and if blocking this channel can modulate the expression of KCNN4, mRNA for KCNN4 was quantified. mRNA was isolated from hTERT/PTECs incubated with  $50\mu$ g/ml pIgA1 or mIgA1 with or without TRAM-34, ICA-17043 or the negative control, TRAM-85. cDNA was synthesized and expression of KCNN4 were determined using qPCR. A significant increase in KCNN4 gene expression by hTERT/PTECs was seen with the presence of pIgA and albumin but not mIgA (Figure 5-2A). The pIgA dependent increase was abrogated by TRAM-34, and by ICA-17043 in a dose dependent manner but not by TRAM-85 (Figure 5-2B). Neither ICA-17043 not TRAM-34 had an effect on KCNN4 expression by hTERT/PTECS in the presence of mIgA (Figure 5-2C).





С



В

Figure 5-2 Polymeric but not monomeric IgA1 increases KCNN4 expression by hTERT/PTEC and this is blocked by TRAM-34 and ICA-17043. Confluent hTERT/PTEC were incubated in serum free media with pIgA1 (50  $\mu$ g/mL), mIgA (50  $\mu$ g/mL), albumin (50  $\mu$ g/mL) for 24 hours with or without TRAM-34 (200 nM), TRAM-85 (200 nM) and ICA-17043 (10nM and 100 nM). The cells were lysed in TRIZOL, RNA was extracted and cDNA synthesised using the Improm-II Reverse Transcription system. qPCR was performed using the Taqman gene expression kits for KCNN4 (K<sub>Ca</sub>3.1) Ct values were normalised to the housekeeping gene MT-ATP6; A. KCNN4 expression by the hTERT/PTEC increased in the presence of pIgA1 and albumin (50  $\mu$ g/mL); B. The increase in KCNN4 expression seen in the presence of pIgA1 was modulated by TRAM-34 and ICA-17043 in dose dependent manner, but not TRAM-85; C. hTERT/PTEC expression of KCNN4 was not changed by TRAM-34 or ICA-17043 when the cells where incubated with mIgA. N=6. Results are shown as mean ±SEM and were analysed using one-way ANOVA. \*p<0.05 \*\*p<0.005

## 5.2.3 Pro-inflammatory cytokine release by hTERT/PTECs incubated with human plgA1.

To determine whether the release of pro-inflammatory cytokines by hTERT/PTECs incubated with plgA1 can be modulated by blocking K<sub>Ca</sub>3.1, an IL-6 ELISA was performed. Confluent hTERT/PTEC were incubated in serum free media with plgA1 (50  $\mu$ g/mL), mlgA1 (50  $\mu$ g/mL) for 24 hours with or without TRAM-34 (200nM) TRAM-85 (200nM) and ICA-17043 (100nM). Supernatant was used to perform the ELISA.

IL-6 synthesis by the hTERT/PTEC increased in the presence of pIgA. This increase was abrogated by TRAM-34, and ICA-17043 in dose dependent manner, but not TRAM-85 (Figure 5-3A). No changes in IL-6 levels were seen in supernatants from cells incubated with mIgA1 whether TRAM-34 or ICA-17043 were present or not (Figure 5-3B).





Α

Figure 5-3 Polymeric but not monomeric IgA1 increases IL-6 synthesis by hTERT/PTEC and this is blocked by TRAM-34 and ICA-17043. Confluent hTERT/PTEC were incubated in serum free media with pIgA1 (50  $\mu$ g/mL) or mIgA1 (50  $\mu$ g/mL) for 24 hours with or without TRAM-34 (200nM) TRAM-85 (200nM), or ICA-17043 (10nM and 100nM). Supernatant was used to perform the Luminex method. IL-6 synthesis by the hTERT/PTECs increased in the presence of pIgA1 (A) but not monomeric IgA1. (B) The increase in IL-6 synthesis seen with pIgA was blocked by TRAM-34 and ICA-17043 in dose dependent manner, but not TRAM-85. (A) IL-6 synthesis by hTERT/PTECs did not change with the presence of mIgA with or without TRAM-34 or ICA-17043. (B) N=6. Results are shown as mean ±SEM and were analysed using one-way ANOVA. \*p<0.05 \*\*\*p<0.005 \*\*\*\*p<0.0005

### 5.2.4 Polymeric but not monomeric IgA1 specifically induces Endothelial Mesenchymal Transition by hTERT/PTEC and this is dependent on $K_{Ca}3.1$

5.2.4.1 α-SMA

 $\alpha$ -SMA is a well-recognized marker of endothelial mesenchymal transition (EMT) in renal and other cell types (Masola *et al*, 2109).

Having shown that  $K_{Ca}3.1$  mediates synthesis of  $\alpha$ -SMA by hTERT/PTECs incubated with total serum IgA1, the same cell line was incubated with mIgA1 or pIgA1 with and without  $K_{Ca}3.1$  inhibitors and  $\alpha$ -SMA synthesis was measured using Western transfer and immunoblotting.

Figure 5-4A shows an increase in  $\alpha$ -SMA synthesis with the presence of plgA1 or albumin but not mlgA1 (all at 50µg/ml). The increase in  $\alpha$ -SMA synthesis by hTERT/PTEC in the presence of plgA is blocked by the K<sub>Ca</sub>3.1 inhibitors TRAM-34 and ICA-17043, the latter in a dose dependent manner, but not by TRAM-85 (Figure 5-4B). No significant change in  $\alpha$ -SMA synthesis was seen in the presence of mlgA with or without TRAM-34, TRAM-85 or ICA-17043 (Figure 5-4C).



A



Figure 5-4 Polymeric but not monomeric IgA1 increases  $\alpha$ -SMA protein synthesis by hTERT/PTEC and this is blocked by TRAM-34 and ICA-17043. Confluent hTERT/PTEC were incubated in serum free media with 50 µg/mL pIgA1, mIgA1 or albumin for 24 hours with or without TRAM-34 (200 nM), TRAM-85 (200 nM), ICA-17043 (10nM and 100nM). The cells were lysed in SDS PAGE reducing sample buffer before being passed through a 10% polyacrylamide gel. Western transfer and immunoblotting was performed. The band densities were normalized with  $\beta$ -actin. (A)  $\alpha$ -SMA synthesis by the hTERT/PTEC increased in the presence pIgA and albumin but not mIgA1. (B) The increase seen with pIgA was blocked by TRAM-34, ICA-17043 in dose dependent manner, but not TRAM-85 (C)  $\alpha$ -SMA synthesis was not altered with the presence of mIgA with or without TRAM-34 or ICA-17043, N=6. Results are shown as mean ±SEM and were analysed using one way ANOVA. \*p<0.05 \*\*p<0.005

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## 5.2.5 Release of the pro-fibrotic markers by hTERT/PTEC incubated with IgA1

To determine whether release of pro-fibrotic cytokines and chemokines by pIgA induced hTERT/PTECs can be modulated by  $K_{Ca}3.1$  blockers, confluent hTERT/PTEC were incubated in serum free media with pIgA1 or mIgA (50  $\mu$ g/mL) for 24 hours with or without TRAM-34 (200 nM), TRAM-85 (200 nM) and ICA-17043 (100 nM). Supernatant was collected and analysed using ELISA (TGF- $\beta$ 1) or Luminex technology (GDF-15, PDGF-AA, GM-CSF, MMP-9, MCP-1/CCL2, MMP-2).

#### 5.2.5.1 TGF- $\beta$ 1 Concentration by ELISA

Figures 5-5A and 5-5B show increased TGF- $\beta$  synthesis by hTERT/PTECs in the presence of plgA but not mlgA. The increase seen with plgA was blocked by TRAM-34 and ICA-17043 in dose dependent manner, but not by TRAM-85 (Figure 5-5A). The presence of TRAM-34 or ICA-17043 did not alter TGF- $\beta$  synthesis in the presence of mlgA (Figure 5-5B).



Figure 5-5 Polymeric but not monomeric IgA1 increases TGF- $\beta$  synthesis by hTERT/PTEC and this is blocked by TRAM-34 and ICA-17043. Confluent hTERT/PTEC were incubated in serum free media with pIgA1 or mIgA1 (50 µg/mL) for 24 hours with or without TRAM-34 (200 nM), TRAM-85 (200 nM), ICA-17043 (10nM and 100 nM). The supernatant was collected and ELISA was used to measure TGF- $\beta$ 1 concentrations. (A) TGF- $\beta$ 1 synthesis by the hTERT/PTEC increased in the presence of pIgA1. The increase seen with pIgA was modulated by TRAM-34 and ICA-17043 in dose dependent manner, but not TRAM-85. (B) No significant change in TGF- $\beta$ 1 concentration was seen when hTERT/PTECS were incubated with mIgA1 with or without TRAM-34 or ICA-17043. N=6. Results are shown as mean ±SEM and analysed using one-way ANOVA. \*p<0.05 \*\*p<0.005

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#### 5.2.5.2 GDF-15 Concentration by LUMINEX

GDF-15 synthesis by the hTERT/PTEC increased in the presence pIgA (Figure 5-6A). This increase was reduced with TRAM-34 and ICA-17043 in a dose dependent manner, and unexpectedly with TRAM-85. No significant changes in GDF-15 levels in the supernatant were seen when hTERT/PTECs were incubated with mIgA with or without TRAM-34, TRAM-85 or ICA-17043 (Figure 5-6B).



Figure 5-6 Polymeric but not monomeric IgA1 increases GDF-15 synthesis by hTERT/PTEC and this is blocked by TRAM-34 and ICA-17043. Confluent hTERT/PTEC were incubated in serum free media with pIgA1 or mIgA1 (50 µg/mL) for 24 hours with or without TRAM-34 (200 nM), TRAM-85 (200 nM) and ICA-17043 (10nM and 100 nM). GDF-15 levels were measured using Luminex. (A) GDF-15 synthesis by the hTERT/PTEC increased in the presence of plgA1. This increase was abrogated by TRAM-34 and ICA-17043 in dose dependent manner and to a lesser extent by TRAM-85. (B) The presence of mlgA with or without TRAM-34 or ICA-17043 did not alter supernatant levels of GDF-15. N=6. Results are shown as mean±SEM and were analysed with one way ANOVA. \*\*\*p<0.0005 \*\*\*\*p<0.0001

В
# 5.2.5.3 PDGF-AA Concentration by LUMINEX

PDGF-AA synthesis by the hTERT/PTEC increased in the presence of plgA (Figure 5-7A). This increase was modulated by TRAM-34 and ICA-17043, but not TRAM-85 (Figure 5-7A). No change in PDGF-AA supernatant levels was seen when hTERT/PTECs were incubated with mlgA with or without TRAM-34 or ICA-17043 (Figure 5-7B).

No significant changes in levels of GM-CSF, MMP-9, MCP-1/CCL-2 or MMP-2 were seen in supernatants from cells incubated with pIgA or mIgA plus or minus TRAM-34, TRAM85 or ICA-17043 (results in Appendix Chapter 5, Figure 1-4).

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Α



Figure 5-7 Polymeric but not monomeric IgA1 increases PDGF-AA synthesis by hTERT/PTEC and this is blocked by TRAM-34 and ICA-17043. Confluent hTERT/PTEC were incubated in serum free media with pIgA1 or mIgA (50  $\mu$ g/mL) for 24 hours with or without TRAM-34 (200 nM), TRAM-85 (200 nM) and ICA-17043 (10nM and 100 nM). Levels of PDGF-AA in the supernatant were measured using Luminex technology. A. PDGF-AA synthesis by the hTERT/PTEC increased in the presence pIgA1, the increase was blocked by TRAM-34 and ICA-17043, but not TRAM-85 B. No changes were seen in levels of PDGF-AA in the supernatant from hTERT/PTECs incubated with mIg1A with or without TRAM-34 or ICA-17043. N=6. Results are shown as mean±SEM and were analysed using one-way ANOVA. \*\*p<0.005

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# 5.2.6 Polymeric but not monomeric IgA1 specifically induces K<sub>Ca</sub>3.1 dependent ECM protein synthesis by PTECs

Extracellular matrix synthesis by PTECs is important in the pathogenesis of kidney fibrosis in IgAN. FSP-1 and fibronectin are synthesised during tubular interstitium fibrosis.

# 5.2.6.1 FSP-1 mRNA expression

Confluent hTERT/PTEC were incubated in serum free media with plgA1 or mlgA (50  $\mu$ g/mL) or albumin (50  $\mu$ g/mL) for 24 hours with or without TRAM-34 (200 nM), TRAM-85 (200 nM) or ICA-17043 (10nM and 100nM). The cells were lysed in TRIZOL. RNA was extracted and quantified before cDNA was synthesised using the Improm-II Reverse Transcription system. qPCR was performed using the Taqman gene expression kits for ALT-1 (FSP-1). Ct values were normalised to the housekeeping gene MT-ATP6 and the results are shown as fold increase compared to media only.

Figure 5-8A shows a significant increase in FSP-1 expression by the hTERT/PTECs in the presence pIgA and albumin 50  $\mu$ g/mL but not mIgA. The increase with pIgA was blocked by TRAM-34 and ICA-17043 in dose dependent manner, but not by TRAM-85 (Figure 5-8B). The presence of mIgA did not change FSP-1 expression with or without TRAM-34 or ICA-17043 (Figure 5-8C).



Α



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Figure 5-8 Polymeric but not monomeric IgA1 increases FSP-1 expression by hTERT/PTEC and this is blocked by TRAM-34 and ICA-17043. Confluent hTERT/PTEC were incubated in serum free media with pIgA1 or mIgA or albumin (50  $\mu$ g/mL) for 24 hours with or without TRAM-34 (200 nM), TRAM-85 (200 nM) and ICA-17043 (10nM and 100 nM). The cells were lysed in TRIZOL. RNA was extracted and cDNA synthesised. qPCR was performed using the Taqman gene expression kits for ALT-1. Ct values were normalised to the housekeeping gene MT-ATP6 and the results are shown as fold increase compared to media only. (A) FSP-1 expression by the hTERT/PTEC increased in the presence of pIgA and albumin 50  $\mu$ g/mL, (B) The increase of pIgA was modulated by TRAM-34 or ICA-17043 in dose dependent manner, but not with TRAM-85 (C) mIgA was not modulated by TRAM-34 or ICA-17043, N=6. Results are shown as mean±SEM and analysed using one way ANOVA. \*p<0.05 \*\*p<0.005

### 5.2.6.2 Fibronectin protein synthesis

To investigate fibronectin protein synthesis, cells treated as described above were lysed in SDS PAGE reducing sample buffer before being passed through a 10% polyacrylamide gel. Western transfer and immunoblotting was performed. The band densities were normalized with  $\beta$ -actin and the results are expressed as fold change compared to media.

Figure 5-9A shows that fibronectin synthesis by the hTERT/PTEC significantly increased in the presence of pIgA1 and albumin. The increase in fibronectin synthesis seen with pIgA1 was modulated by TRAM-34 or ICA-17043 in dose dependent manner, but not by TRAM-85 (Figure 5-9B). No change was seen when hTERT/PTECs were incubated with mIgA1 with or without TRAM-34 or ICA-17043 (Figure 5-9C).





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Figure 5-9 Polymeric but not monomeric IgA1 increases fibronectin protein synthesis by hTERT/PTECs and this is blocked by TRAM-34 and ICA-17043. Confluent hTERT/PTEC were incubated in serum free media with pIgA1, mIgA1 or albumin (50  $\mu$ g/mL) for 24 hours with or without TRAM-34 (200 nM) TRAM-85 (200 nM) or ICA-17043 (10nM and 100 nM). The cells were lysed in SDS PAGE reducing sample buffer before being passed through a 10% polyacrylamide gel. Western transfer and immunoblotting was performed, The band densities were normalized with  $\beta$ -actin and the results are expressed as fold change compared to media. (A) Fibronectin synthesis by the hTERT/PTEC increased in the presence of pIgA and albumin. (B) The pIgA dependent increase was blocked by TRAM-34, ICA-17043 in dose dependent manner, but not by TRAM-85. (C) Fibronectin synthesis was not modulated by mIgA with or without TRAM-34 or ICA-17043. N=6. Results are shown as mean ±SEM \*p<0.05

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# 5.3 Conclusion

Tubular interstitial fibrosis is an important driver of renal damage in IgAN. Understanding the pathways which result in this fibrosis could prove valuable in the search for treatments to control the disease. In health, large proteins such as IgA1 do not pass through the glomerular filtration barrier and therefore do not come into contact with the cells lining the tubules of the nephron (proximal distal and collecting duct). As a result of glomerular damage, unfiltered proteins pass through the nephron and are lost in the urine. Included in these unfiltered proteins is IgA1 from serum. Serum IgA1 consists of 90% mIgA1 and 10% polymeric. Despite the low concentration in serum the work shown in this chapter clearly shows that it is the pIgA1 fraction of serum IgA1 which is most potent when it comes to driving inflammatory and fibrotic pathways in proximal tubule epithelial cells. It is important therefore, when considering blocking  $K_{Ca}3.1$  as a treatment to reduce interstitial fibrosis in IgAN that the treatment should be effective at blocking the effects of pIgA1.

The results in this chapter show human serum plgA1 induces human PTECs to increase synthesis of the intermediate conductance Ca<sup>2+</sup> dependent K+ channel, K<sub>Ca</sub>3.1. They also show that this increase in synthesis is controlled at the DNA level: expression of the KCNN4 gene is seen with incubation with plgA1. The lack of change in expression of KCNN4 and synthesis of K<sub>Ca</sub>3.1 with incubation with mIgA1 and the absence of any change in expression of the related KCNN3 gene with plgA1 suggests specific control of K<sub>Ca</sub>3.1 by plgA1. The increase in K<sub>Ca</sub>3.1 synthesis with plgA1 was seen in conjunction with an increase in synthesis in the marker of inflammation, IL-6 and markers of fibrosis and EMT, TGF- $\beta$ , GDF-15, PDGF-AA, FSP-1 and fibronectin, indicating the relationship between plgA1 and KCa3.1 is pivotal to the mechanisms driving inflammation and fibrosis in the tubular interstitium and that controlling this relationship my prove critical in the search for a therapy to slow down renal damage in IgAN. The ability of TRAM-34, and the more potent and more stable ICA-17043, to block plgA1 dependent synthesis of K<sub>Ca</sub>3.1 and markers of inflammation, fibrosis and EMT provides strong evidence for the possibility of using ICA-17043 as a treatment for IgAN.

# Chapter 6: Potential Use of K<sub>Ca</sub>3.1 as a Novel Biomarker of IgA Nephropathy

# 6.1 Introduction

The incidence of IgAN varies across the world, and the attitude of general practitioners or nephrologists towards urine abnormality may contribute to this difference. For example, children in Japan are screened for changes in urine markers yearly and have a renal biopsy if any abnormalities are detected; while in the UK, patients with IgAN most frequently present with hematuria and/or proteinuria (mostly mild) and a renal biopsy is performed only when further investigations indicate reduced renal function. Consequently, in the UK individuals with mild symptoms are frequently underdiagnosed. Although renal biopsy is not considered a high-risk procedure, it is not comfortable, and some patients are quite reluctant to undergo renal biopsy unless it is considered necessary. Therefore, there is a need for reliable, reproducible, non-invasive biomarkers to diagnose IgAN and to help monitor progression of the disease and efficacy of treatment regimens.

This chapter covers the discovery of  $K_{Ca}3.1$  in the urine which may have potential as a biomarker for IgAN diagnosis and progression.

# 6.2 Result

# 6.2.1 K<sub>Ca</sub>3.1 synthesis in supernatants of IgA1 stimulated hTERT/PTECs

Confluent hTERT/PTECs were incubated with TGF- $\beta$ 1 (2 ng/mL) or total serum IgA1 (50 µg/mL) for 24 hours with or without TRAM-34 (1 µM). To determine if K<sub>Ca</sub>3.1 is found in the supernatant, the supernatant was collected and proteins precipitated using cold acetone, centrifuged and the pellet was resuspended in 20 µL 0.1% SDS. 1 µg/mL samples were mixed with Wes sample master mix containing dithiothreitol, and a mixture of three fluorescent standard dyes and heat denatured. The samples were then loaded on to a Wes assay plate with rabbit anti human K<sub>Ca</sub>3.1 antibody and then HRP conjugated goat-anti rabbit antibody. The intensity of the K<sub>Ca</sub>3.1 band was determined using WES software. Cell lysates from the experiment described above were used to determine K<sub>Ca</sub>3.1 protein level in the cells.

Figure 6-1A shows, as seen in chapter 4, a significant increase in  $K_{Ca}3.1$  protein in cell lysates of hTERT/PTECs incubated with both TGF- $\beta$ 1 and IgA1. Figure 6-1B shows  $K_{Ca}3.1$  in supernatant from cells incubated with IgA1 but not TGF- $\beta$ 1 and a reduction in this  $K_{Ca}3.1$  when TRAM-34 is present.



 $K_{Ca}3.1$  protein from cell lysates inTGF- $\beta$  and IgA1 stimulation hTERT/PTEC

В

# KCa 3.1 protein synthesis from supernatants inTGF- $\beta$ and IgA1 stimulation hTERT/PTEC



Figure 6-1 K<sub>Ca</sub>3.1 protein in cell lysates and supernatants from hTERT/PTECs incubated with total serum IgA1 and TGF- $\beta$  +/- TRAM-34 1  $\mu$ M. Confluent hTERT/PTECs were incubated with TGF- $\beta$ 1 (2 ng/mL) or total serum IgA1 (50  $\mu$ g/mL) for 24 hours with or without TRAM-34 (1  $\mu$ M). Levels of K<sub>Ca</sub>3.1 in the cell lysates and supernatants were analysed using Wes. (A) Significantly more KCa3.1 protein was seen in hTERT/PTECs cell lysates incubated with IgA1 or TGF- $\beta$ 1 compared to media only. (B) K<sub>Ca</sub>3.1 was seen in the supernatant from hTERT/PTECs incubated with IgA1 but not from those incubated with TGF- $\beta$ 1. The presence of TRAM-34 blocks the increase in K<sub>Ca</sub>3.1 synthesis seen with IgA1 alone. N=4 Results are shown as mean +/- SEM and were analysed using one-way ANOVA. \*p<0.05 \*p<0.005

### 6.2.2 K<sub>Ca</sub>3.1 protein in microvesicles

#### 6.2.2.1 K<sub>Ca</sub>3.1 is not found in microparticles

K<sub>Ca</sub>3.1 is a membrane protein which crosses the plasma membrane multiple times and so it was surprising that the full-length protein was detected in the cell media. To determine where the K<sub>Ca</sub>3.1 in cell media from the hTERT/PTECs (incubated with total serum IgA1) originates, confluent hTERT/PTECs were incubated with total serum IgA1 (50 µg/mL) for 24 hours. The media were centrifuged at 1000 X G for 15 minutes to remove intact and broken cells, cell debris, and large cellular organelles. The supernatant was centrifuged at 18,000g for 30 minutes. The supernatant was retained and the microparticlecontaining pellet was suspended in 500 µL MP buffer (145 mM NaCl, 2.7 mM KCl, 10 mM Hepes, pH = 7.4). The retained supernatant was precipitated using cold acetone, centrifuged and the exosome-containing pellet was resuspended in 20  $\mu$ L 0.1% SDS. 1  $\mu$ g/mL samples from pellets and supernatant were mixed with Wes sample master mix containing dithiothreitol, and a mixture of three fluorescent standard dyes and heat denatured. The samples were then loaded on to a Wes assay plate with rabbit anti human K<sub>Ca</sub>3.1 antibody (1:1000) and then HRP conjugated goat-anti rabbit (1:5000).

Figure 6-2A shows  $K_{Ca}3.1$  in the cell media supernatant after centrifugation to remove microparticles but not in the microparticle pellet using WES, Figure 6-2B shows the same results using Western transfer and immunoblotting.



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Figure 6-2 K<sub>Ca</sub>3.1 protein is not detected in microparticles isolated from media from hTERT/PTECs incubated with total serum IgA1. Confluent hTERT/PTECs were incubated with IgA1 (50  $\mu$ g/mL) for 24 hours. Differential centrifugation was used to isolate microparticles (MP) from the media supernatant. K<sub>Ca</sub>3.1 was not seen in the microparticle pellet but was seen in the supernatant using A. WES and B. Traditional Western transfer and immunoblotting.

Α

#### 6.2.2.2. K<sub>Ca</sub>3.1 is present in Exosomes

#### 6.2.2.1.1 Exosome marker

Western transfer and immunoblotting with an antibody which binds the exosome specific protein CD63 was used to confirm the presence of exosomes in the acetone precipitated protein pellet. An aliquot of the exosome containing pellet and the supernatant were passed through an 8% SDS PAGE gel. The proteins were Western transferred, and the membrane immunoblotted for human CD63. Figure 6-3 shows CD63 protein in the exosome pellet but not in the supernatant.



Figure 6-3 CD63 protein expression in exosomes isolated from media from hTERT/PTECs stimulated by total human IgA1. Confluent hTERT/PTECs were incubated with total human IgA1 (50  $\mu$ g/mL) for 24 hours. Differential centrifugation and acetone precipitation was used to isolate exosomes from the media. The exosome-containing pellet and the supernatant was passed through an 8% SDS PAGE gel (1  $\mu$ g/mL total protein), Western transferred and immunoblotted with mouse anti human CD63 antibody (2  $\mu$ g/mL) and then HRP conjugated rabbit-anti mouse (1:1000). CD63 protein was seen in the exosome containing pellet but not in supernatant after the exosomes were removed.

#### 6.2.2.1.2 K<sub>Ca</sub>3.1 in exosomes

After confirming that the pellet contained exosomes, and the supernatant did not, by immunoblotting with antiCD63 the membrane from Figure 6-3 was stripped and then incubated with rabbit anti human  $K_{Ca}3.1$  antibody and then HRP conjugated goat-anti rabbit antibody. Figure 6-4 shows  $K_{Ca}3.1$  protein in the exosome containing pellet and not in the exosome free supernatant from hTERT/PTECs incubated with total serum IgA1.



Figure 6-4 K<sub>Ca</sub>3.1 protein expression in exosomes isolated from supernatants of hTERT/PTECs stimulated by total IgA1. Confluent hTERT/PTECs were incubated with IgA1 (50  $\mu$ g/mL) for 24 hours. Supernatant from the microparticle isolation step was used to perform exosome isolation. Using the membrane from figure 6-3, after stripping the membrane, the membrane was incubated with rabbit anti human KCa3.1 antibody (1:1000) and then HRP conjugated goat-anti rabbit (1:5000). KCa 3.1 protein was detected in exosomes, not in supernatant of IgA1 stimulated hTERT/PTECs.

# 6.2.3 Urinary $K_{Ca}$ 3.1 synthesis in IgA Nephropathy patients compared to Other Kidney Diseases

Urine was collected from patients with IgAN (n=20), other biopsy proved kidney diseases (n=20, Table 1) and healthy individuals (n=19). The levels of creatinine in the urine were determined. 1 mL urine was precipitated with 250  $\mu$ L TCA and centrifuged at 14,000 rpm for 5 minutes. The supernatant was discarded, and the protein pellet was washed and resuspended in a volume of SDS 1% which was normalised for creatinine. 1 $\mu$ g/mL samples (x $\mu$ I) were mixed with Wes sample master mix containing dithiothreitol, and a mixture of three fluorescent standard dyes and heat denatured. The samples were then loaded on to a Wes assay plate with rabbit anti human K<sub>Ca</sub>3.1 antibody (1:1000) and then HRP conjugated goat-anti rabbit (1:5000). The density of the K<sub>Ca</sub>3.1 bands was analysed using WES software. Figure 6.5 shows higher levels of K<sub>Ca</sub>3.1 from IgAN patients compared to other kidney diseases, and no K<sub>Ca</sub>3.1 in urine from healthy subjects. The presence of K<sub>Ca</sub>3.1 in urine did not correlate with level of proteinuria or haematuria.

No.		Ν
1	Adult type Polycystic kidney disease	3
2	Diabetic Nephropathy	3
3	Membranous Nephropathy	3
4	Focal Segmental Glomerulosclerosis	2
5	Chronic Glomerulonephritis	1
6	Granulomatosis with Polyangitis	1
7	Obstructive Nephropathy	2
8	Tubular interstitial nephritis	1
9	Systemic Sclerosis	1
10	Microscopic Polyangitis	1
11	Chronic Kidney Disease	2
	Total	20

Table 6-1 Disease characteristics from the other biopsy proved kidney disease patients.

Urine K<sub>Ca</sub>3.1 protein



Figure 6-5 K<sub>ca</sub>3.1 protein levels in urine from IgAN patients, renal disease controls and healthy subjects. Urine was donated from 20 patients with IgAN, 20 patients with other kidney diseases and 19 healthy subjects. 1 mL urine was precipitated with 250  $\mu$ L TCA and centrifuged at 14,000 rpm for 5 minutes. Supernatant was discarded and pellet was washed, heated and resuspended in a volume of 1% SDS which normalised the samples according to creatinine levels. 1  $\mu$ g/mL samples are mixed with Wes sample master mix containing dithiothreitol, and a mixture of three fluorescent standard dyes and heat denatured. The samples were then loaded on to a Wes assay plate with rabbit anti human K<sub>Ca</sub>3.1 antibody (1:1000) and then HRP conjugated goat-anti rabbit (1:5000). The density of the K<sub>Ca</sub>3.1 protein bands was determined using WES software. Urine from IgAN patients contained higher levels of KCa3.1 compared with urine from patients with other kidney diseases and healthy subjects. Results are shown as mean +/- SEM and analysed using one-way ANOVA. \*p<0.05

#### 6.2.4 The location of K<sub>Ca</sub>3.1 in urine

To determine whether the K<sub>Ca</sub>3.1 found in urine is located in the cellular or soluble fraction of urine, urine from 20 IgAN patients and 20 healthy subjects was collected and immediately centrifuged at 3,000 rpm for 5 mins and the creatinine levels of the urine were determined. The cell debris pellets and supernatant were separated. The pellets were dissolved in 100 µL 1%SDS. The proteins in 1 mL urine supernatant were then precipitated with 250 µL TCA and centrifuged at 14,000 rpm for 5 minutes. Supernatant was discarded and pellet was washed, heated and resuspended in a volume of SDS 1% which normalised the samples to creatinine. 1 µg/mL samples from supernatant and pellets were mixed with Wes sample master mix containing dithiothreitol, and a mixture of three fluorescent standard dyes and heat denatured. The samples were then loaded on to a Wes assay plate with rabbit anti human K<sub>Ca</sub>3.1 antibody (1:1000) and then HRP conjugated goat-anti rabbit (1:5000). The density of the K<sub>Ca</sub>3.1 bands was measured using WES software. No significant differences were seen between K<sub>Ca</sub>3.1 levels from the cellular and soluble fractions from urine from IgAN patients and healthy subjects (Figure 6-6).

1 ml of urine from an IgAN patient which had been shown to contain  $K_{Ca}3.1$  was centrifuged to remove cell debris. The cell debris pellet was suspended in 500µl Western blot reducing loading buffer. The supernatant was further centrifuged to pellet microparticles. The microparticle pellet was suspended in 500µl Western blot reducing loading buffer. The supernatant was again centrifuged to pellet exosomes. The exosome pellet was suspended in 500µl Western blot reducing loading buffer. The supernatant was again centrifuged to pellet exosomes. The exosome pellet was suspended in 500µl Western blot reducing loading buffer. 10µl of each of whole urine, each of the pellet suspensions and of the supernatants were run through an SDS PAGE and  $K_{Ca}3.1$  content was visualised using Western transfer, imunoblotting with antibody which binds human  $K_{Ca}3.1$ . Figure 6-7 shows  $K_{Ca}3.1$  in urine, in the cell debris free supernatant but very little in the cell debris, in the microparticle free supernatant but not in the microparticles, and in the exosome containing pellet but very little in the exosome free supernatant. This data reveals that, as with the *in vitro* results exposing hTERT/PTECS to IgA1,  $K_{Ca}3.1$  in urine is not

free in solution but is embedded into exosomes. The cellular origin of the exosomes found in urine is unclear.

Urine K<sub>Ca</sub>3.1 protein



**Figure 6-6. Urine K<sub>Ca</sub>3.1 protein expression in IgAN patients**. Total 40 individuals were involved, consisting of 20 patients with IgAN and 20 healthy subjects. Urine was collected and directly centrifuged at 3,000 rpm for 5 mins. Pellets and supernatant was separated. Pellets were resuspended with 100  $\mu$ L SDS 1%. 1 mL urine supernatant was then precipitated with 250  $\mu$ L TCA and centrifuged at 14,000 rpm for 5 minutes. Supernatant was discarded and pellet was washed, heated and resuspended in 100  $\mu$ L SDS 1%. 1 ug/mL samples from supernatant and pellets were mixed with Wes sample master mix containing dithiothreitol, and a mixture of three fluorescent standard dyes and heat denatured. The samples were then loaded on to a Wes assay plate with rabbit anti human K<sub>Ca</sub>3.1 antibody (1:1000) and then HRP conjugated goat-anti rabbit antibody (1:5000). There was no significant different between pellets and supernatants. However, K<sub>Ca</sub>3.1 protein was mostly found in supernatants of IgAN patients. \*p<0.05



- 3= cell debris pellet 4= cell debris free supernatant 5= microparticle containing pellet
- 6= microparticle free supernatant
- 7= exosome containing pellet

8= exosome free supernatant

**Figure 6-7** K<sub>Ca</sub>**3.1** in subfractions of urine. 1 ml of urine from an IgAN patient which had been shown to contain K<sub>Ca</sub>**3.1** was centrifuged to remove cell debris. The cell debris pellet was suspended in 500µl Western blot reducing loading buffer. The supernatant was further centrifuged to pellet microparticles. The microparticle pellet was suspended in 500µl Western blot reducing loading buffer. The supernatant was again centrifuged to pellet exosomes. The exosome pellet was suspended in 500µl Western blot reducing loading buffer. 10µl of each of whole urine, each of the pellet suspensions and of the supernatants were run through an SDS PAGE and K<sub>Ca</sub>**3.1** content was visualised using Western transfer, imunoblotting with a K<sub>Ca</sub>**3.1** antibody.

# 6.3 Conclusions

As  $K_{Ca}3.1$  is a water insoluble membrane protein with the majority of the protein embedded in the phospholipid bilayer. It was an unexpected finding to detect this protein in cell media. The results in this chapter show  $K_{Ca}3.1$  in the exosome fraction of the media from hTERT/PTECs incubated with IgA1 and not in the microvesicle free supernatant. How and why these  $K_{Ca}3.1$  containing exosomes are formed in the presence of IgA1, and interestingly not with TGF- $\beta$ 1, requires further work. This work used total IgA1 which had a high level of undergalactosylated forms isolated from serum taken from a healthy subject. It should be noted that in health large serum proteins such as IgA1 do not cross the glomerular filtration barrier and so do not come into contact with PTECs. As tubulointerstitial fibrosis is an important driver of progression in IgAN and patients with the progressive form of IgAN have higher levels of undergalactoyslated IgA1 in their serum, finding out the impact of IgA1 containing different levels of undergalactosylated forms may reveal more information about the pathogenesis of IgAN.

Having shown that PTECs incubated in the presence of IgA1 increase their synthesis of  $K_{Ca}3.1$  and then shed exosomes with  $K_{Ca}3.1$  embedded in the membrane led to the suggestion that in IgAN unfiltered proteins, including under galactosylated IgA1, arrive in the tubules of the nephron, and may initiate up-regulation of  $K_{Ca}3.1$  and the release of exosomes containing the protein. If this is the case,  $K_{Ca}3.1$  will be found in the urine and may prove to be a biomarker for IgAN. At present the only method for predicting disease progression in IgAN is the Oxford classification of IgAN which requires a renal biopsy. A procedure which is often uncomfortable and comes with a certain amount of risk. Although a renal biopsy is necessary for diagnosis of IgAN, patients are not keen to agree to more biopsies. An easily accessible, robust and reliable biomarker is needed to enable monitoring of disease progression in IgAN. This would be especially useful in assessing the efficacy of clinical trials. At present level of proteinuria is used as a surrogate marker for renal damage, however proteinuria does not always correlate with renal damage.

This work shows  $K_{Ca}3.1$  in urine from 14 out of 20 IgAN patients, and 3 out of 20 patients with other renal diseases, but it is not found in urine from healthy subjects. Detailed clinical data about the patients who donated these urines was not available.  $K_{Ca}3.1$  is found in the cell membrane of erythrocytes but the lack of correlation between urinary  $K_{Ca}3.1$  and haematuria suggests that the protein does not originate from red blood cells. The lack of  $K_{Ca}3.1$  in urine from healthy subjects links the presence of the protein with renal damage. More work needs to be carried out using urine samples with detailed clinical information to explain why  $K_{Ca}3.1$  is present in urine from some and not all, IgAN patients.

 $K_{Ca}3.1$  was detected in a smaller proportion of urines from patients with non-IgAN renal diseases. The presence of the protein in urine was not disease dependent in the samples from the non-IgAN renal disease patients. It will be interesting to investigate if presence of  $K_{Ca}3.1$  is linked to serum levels of undergalactosylated IgA1.

# Chapter 7: Discussion

# 7.1 Summary of results

The aim of this study was to investigate the role of  $K_{Ca}3.1$  in the response of renal cells (mesangial cells, podocytes, proximal tubular epithelial cells) to incubation with serum IgA1, and to determine how this response is altered when the channel is selectively blocked. The following hypothesis was tested " $K_{Ca}3.1$  has a direct effect on cellular and fibrogenic activation of renal cells following IgA1 deposition in IgAN"

For the first time, it has been demonstrated that primary human mesangial cells express  $K_{Ca}3.1$ , and that blocking this channel inhibits proinflammatory cytokine release and extracellular matrix formation. These results suggest that  $K_{Ca}3.1$  is involved in the process of renal damage in IgAN, starting in the mesangium following IgA1 deposition.

The second part of this project focused on the contribution of  $K_{Ca}3.1$  in IgA1 induced tubulointerstitial inflammation and fibrosis. IgA1 stimulated PTECs to increase gene expression and protein synthesis of KCa3.1, both of which were blocked by selective  $K_{Ca}3.1$  blockers. Furthermore, KCa3.1 blockers prevented IgA1 dependent changes in proinflammatory and profibrotic cytokine release, as well as expression and synthesis of markers of EMT and fibrosis by PTECs. These results suggest the active involvement of this specific channel in tubulointerstitial damage.

In light of evidence which has shown that patients with the more aggressive form of IgAN have higher levels of undergalactosylated pIgA1 in their serum, and that PTECs incubated with undergalactosylated pIgA produce higher levels of the profibrotic mediator, TGF- $\beta$ 1, the effect of undergalactosylated pIgA1 on PTECs was investigated. Incubation of PTECS with pIgA1 led to a greater increase in expression and synthesis of K<sub>Ca</sub>3.1 compared with mIgA. Selectively blocking K<sub>Ca</sub>3.1 attenuated pIgA1-induced increases in EMT and fibrosis markers. This suggests that KCa3.1 has a role in the response of PTECs under

more pathologic conditions. The reduced pIgA1-dependent synthesis of markers of fibrosis in the presence of the ICA-17043 suggests that repurposing this drug as a treatment from IgAN is worth further investigation.

The existence of  $K_{Ca}3.1$  in supernatant from IgA1-induced PTECs was an unexpected incidental finding, which led to investigations to explain how this hydrophobic membrane protein exists in supernatant, and if it is found in urine. It was interesting that this channel is more often found in urine from patients with IgAN compared to other kidney diseases and is not found in urine from healthy subjects. The observation that the presence of  $K_{Ca}3.1$  in urine did not correlate with proteinuria or haematuria suggests that the route by which it arrives in urine is not generic to all proteins. However, the number of patients involved in this study was relatively small and the clinical information was limited. Further investigations will reveal if  $K_{Ca}3.1$  has the potential to be a useful biomarker for IgAN.

# 7.2 Limitations of the study

#### 7.2.1 Limitations to the in vitro studies

This project set out to investigate if  $K_{Ca}3.1$  is involved in the pathogenesis of IgAN. As IgAN is characterized by the deposition of IgA1 in the renal mesangium, I first looked at how  $K_{Ca}3.1$  gene expression and synthesis changed in HMCs with incubation with IgA1. The first limitation of this part of the project relates to the mesangial cells. These were primary human mesangial cells and so would be expected to have a phenotype very similar to HMCs *in vivo* but they were from a single donor. Ebefors et al (2016) found that mesangial cells isolated from patients with IgAN have increased susceptibility to poorly galactosylated IgA1. The donor from whom the HMCs in this study were derived had no history of IgAN, and so this study could be improved by using HMCs from more than one donor, including cells from patients with IgAN. This might give an insight as to whether differences in  $K_{Ca}3.1$  in HMCs can explain why some people have been found to have mesangial IgA deposits but no renal damage (Suzuki et al, 2003).

The IgA1 used in this study was from a single healthy person with a high level of poorly galactosylated IgA1. Including IgA1 from healthy subjects with different levels of poorly galactosylated IgA1 and patients with IgAN with different prognoses might reveal if  $K_{Ca}3.1$  is involved in the increased stimulation of HMCs by undergalactosylated IgA1 and the pathways which lead to the variable prognosis seen in IgAN patients (Ebefors et al, 2016). Using both HMCS and IgA1 from multiple donors might define the role of  $K_{Ca}3.1$  in IgA1 stimulation of HMCs.

The HMCs in this project were incubated with IgA1 suggesting that the changes in  $K_{Ca}3.1$  gene expression and synthesis were IgA1 specific: adding IgG, the polymeric IgM or albumin as controls will reveal if these changes are actually IgA1 specific or are brought about by the presence of an antibody or even just a protein. Much of this work investigates the amount of  $K_{Ca}3.1$  in HMCs, work by M. Duffy shows  $K_{Ca}3.1$  to be active in HMCs however, the activity of  $K_{Ca}3.1$  in the presence of IgA1 was not investigated. Repeating the patch clamping experiments using HMCs and IgA1 with or without  $K_{Ca}3.1$  blockers would reveal if IgA1 stimulated  $K_{Ca}3.1$  activity as well as synthesis.

In IgAN, glomerular injury occurs when pathogenic IgA1 and immune complexes deposit in the mesangium and the response of the mesangial cells causes a breakdown of the glomerular filtration barrier. The loss of the selective barrier in the glomerulus leads to the presence of unfiltered proteins (including IgA1) in the proximal tubules. This work shows increased K<sub>Ca</sub>3.1 expression and synthesis by hTERT PTECs after incubation with IgA1. hTERT PTECs are an immortalized cell line. This work could have been improved by using primary PTECs from patients with IgAN or healthy subjects. Using PTECs from different donors and IgA1 with different levels of poorly galactosylated IgA1 from different donors could reveal if KCa3.1 participates in the mechanism which causes the interstitial fibrosis seen in IgAN biopsies. The differences in the response of PTECs to incubation with pIgA1 or mIgA1 indicate that the changes seen in PTECs are dependent on the presence of plgA1 and not just a protein or an immunoglobulin. However, using polymeric IgM would rule out the suggestion that the changes are the result of incubation with a polymeric immunoglobulin. Carrying out patch clamp experiments on PTECS in the presence of plgA1 or mlgA1 would have shown if the increased synthesis of  $K_{Ca}3.1$ , seen in the presence of plgA1, had an effect on the influx of Ca<sup>2+</sup>.

Although mesangial cells, podocytes and proximal tubular epithelial cells can be tested and manipulated *in vitro* to identify the basic principles of behaviour of each cell type, such experiments cannot represent the complex model of the disease. This is the major limitation of this and all *in vitro* studies. To bridge the gap between cell assays and the human system, animal models are often used. However, IgAN does not occur in animals, other than humans and hominoid primates. IgAN is difficult to create in animal models because only humans and upper primates have the IgA1 subclass. Several studies have developed a mouse model for IgAN, however no mouse model can represent all aspects of the disease, ranging from galactose deficient IgA1 deposition to pro-inflammatory cytokines release by mesangial cells, podocyte injury, glomerular damage, tubulointerstitial fibrosis, and manifestation of hematuria and proteinuria (Eitner *et al*,2010)

#### 7.2.2 Limitations to the ex vivo studies

In this study  $K_{Ca}3.1$  was detected for the first time in the urine. The lack of the protein in the cell debris pellet suggests the protein does not derive from bladder cells. The presence of  $K_{Ca}3.1$  in urine with or without proteinuria or hematuria means that the protein does not come from leucocytes or erythrocytes. Urine from a limited number of patients were included in this experiment. No basic clinical data (blood pressure, eGFR, histological diagnosis (Oxford classification MESTC score) or treatment record was available. In future work comorbidities such as urinary tract infection and diabetes will be taken into account and eventually excluded.  $K_{Ca}3.1$  was detected in 14 out of 20 patients with IgAN. This could be due to lack of sensitivity of the WES or because the patients with  $K_{Ca}3.1$  were experiencing active disease while those without the protein have a more benign pathology. More sensitive methods of measuring  $K_{Ca}3.1$  may be required, using urine collected at biopsy, so that the disease state is known. The potential of  $K_{Ca}3.1$  as a biomarker of IgAN needs to be further investigated.

# 7.3 Hypothesis

#### 7.3.1 The role of K<sub>Ca</sub>3.1 in renal progression in IgA nephropathy

The study performed in this thesis has examined the role of  $K_{Ca}3.1$  in the process of renal damage which contributes to the progression of renal fibrosis in IgAN. Evidence in this work shows that  $K_{Ca}3.1$  is involved in cell activation by triggering; the release of pro-inflammatory and pro-fibrotic cytokines, cell remodelling, and increased synthesis of extracellular matrix.

 $K_{Ca}3.1$  is known to be involved in the mechanisms which lead to cellular activation and proliferation (Panyi, 2005). How this channel is linked to cell proliferation is still unclear. Several studies reported that  $K_{Ca}3.1$  inhibition inhibits cell proliferation by membrane potential depolarization (Wonderlin WF, 1996; Ouadid-Ahidouch and Ahidouch, 2008).

In the human mesangial cell experiments included in this study, an upregulation of  $K_{Ca}3.1$  gene expression and protein synthesis was seen after the cells are incubated with total IgA1. These observations are in accordance with those from a study by Huang *et al* (2013). They demonstrated up-regulation of  $K_{Ca}3.1$  in kidneys from a mouse model of diabetic nephropathy, and diabetic nephropathy patients. The trigger that initiates this dysregulation is not known. There may be an interaction between a receptor which binds IgA1 on the surface of HMCs, this needs further investigation.

The increase in IL-6 seen when HMCs are incubated with IgA1 suggests an active inflammatory response. IL-6 is a pleotropic cytokine which induces leucocyte infiltration, enhances proliferation and increases MCP-1 synthesis and matrix accumulation in mesangial cells (Coletta et al, 2000). The IL-6 increase was modulated by a  $K_{Ca}3.1$  blocker (TRAM-34), suggesting the involvement of  $K_{Ca}3.1$  in this inflammatory response. The mechanism which links IL-6 and  $K_{Ca}3.1$  was regulated by Ca<sup>+</sup> (Chan *et al*, 2004). Ultimately, this process leads to increase in extracellular matrix accumulation (COL1A1) and this was dependent on  $K_{Ca}3.1$ .

In these experiments a relatively high concentration of TRAM-34 was required to suppress the IgA1-dependent changes in KCa3.1 gene expression and synthesis in HMCs. Several studies have demonstrated an antiproliferative effect of TRAM-34 at concentrations of  $4\mu$ M and above (Wulff et al, 2000; Ghanshani et al, 2000; Jager et al, 2004; Grgic et al, 2009; Wang et al, 2007; Toyama et al, 2008; Lallet-Daher et al, 2009; Roy et al, 2010). Roy et al, 2010 reported a biphasic effect of TRAM-34 on MCF-7 cells: they found that at moderate concentrations, TRAM-34 increase cell proliferation, but in high concentration it inhibits proliferation. In light of this evidence, the attenuation of synthesis of  $K_{Ca}3.1$ , IL-6 and COL1A1 with 4µM TRAM-34 should be viewed with some caution. Of note, TRAM-34 is a high plasma protein bound agent. In these experiments mesangial cells were grown in a medium containing 5% FCS and then starved with 0.2% FCS before incubating with IgA1 and TRAM-34. The need for higher concentrations of TRAM-34 to observe abrogation of IgA1dependent changes in mesangial cells may be due to the serum in the HMC media.

Active  $K_{Ca}3.1$  was seen in human mesangial cell and proximal tubular epithelial cells (hTERT/PTEC) using patch clamping. The latter confirmed results by Huang, et al, 2014 who reported that TGF- $\beta$ 1 induces a large  $K_{Ca}3.1$  dependent potassium current by human proximal tubular cells, which is inhibited by TRAM-34.

It is known that rate of decline of renal function and long-term clinical outcome in IgAN correlates with severity of tubule-interstitial damage but not the amount of IgA deposition, so patients with severe tubular atrophy and interstitial fibrosis, are more likely to progress to end stage renal disease (Schena, 1990). Questions have been raised about the mechanism of tubulointerstitial damage in IgAN. Chan et al, 2005 reported that tubulointerstitial damage in IgA was a consequence of glomerular-tubular cross-talk due to increase mediators (cytokines, chemokines, growth factors, extracellular matrix components) passing through the glomerular barrier. Contrasting with the findings from our laboratory (unpublished data), they observed a lack of IgA receptors and PTEC activation. They showed that mediators released by HMC after mesangial IgA deposition led to activation of PTECs and subsequent production of local cytokines, chemokines and extracellular matrix, which ultimately leads to tubular atrophy and interstitial fibrosis.

Our results showed that IgA1 induced hTERT/PTECs to produce proinflammatory and profibrotic cytokines, and this was dependent on  $K_{Ca}3.1$ . However, the pathways linking  $K_{Ca}3.1$  with IL-6 and TGF- $\beta$  in PTECs requires further investigation.

Several studies have reported the phenomenon of EMT in renal fibrosis (Zhou and Liu, 2015). EMT has been described in a wide range of organs. In the kidney, EMT is a process in which renal tubular epithelial cells differentiate by losing their epithelial markers (E-cadherin, cytokeratin, laminin-1) and expressing mesenchymal cell markers such as fibronectin, vimentin,  $\alpha$ -SMA, N-cadherin) (Liu, 2004). We observed changes in protein synthesis of  $\alpha$ -SMA, FSP-1 and fibronectin which mirrored that of K<sub>Ca</sub>3.1 in the presence of IgA1 with and without K<sub>Ca</sub>3.1 blockers, which indicates the process of EMT in PTECs is dependent on K<sub>Ca</sub>3.1. However, further studies investigating other markers of EMT are needed to confirm these findings, as these markers are not exclusively expressed in fibroblasts.

Having shown that total serum IgA1 induces PTECs to synthesise  $K_{Ca}3.1$  and markers of fibrosis, the effect of pIgA1 and mIgA1 on PTECs was investigated. The IgA1 deposited in the renal mesangium in IgAN is predominantly poorly galactosylated pIgA1 (Trascasa *et al*, 1980; Monteiro *et al*, 1985). It has been demonstrated by many studies that pIgA1 induces more markers of inflammation and fibrosis in renal cells compared to mIgA1 and therefore this form of IgA1 is said to be pathogenic (Reterink *et al*, 1996; Leung *et al*, 2002; Leung *et al*, 2003; Nakamura *et al*, 2004). Results in this thesis showed that pIgA1, but not mIgA1, induced PTECs to increase  $K_{Ca}3.1$  gene expression and protein synthesis. Alongside this pIgA1-dependent increase in  $K_{Ca}3.1$  was an increase in synthesis of markers of inflammation and pro-fibrosis and EMT (IL-6, TGF- $\beta$ , GDF-15, PDGF-AA, FSP-1 and fibronectin), all of which were shown to be modulated by blockers of  $K_{Ca}3.1$ , thus showing the pivotal role of  $K_{Ca}3.1$  in tubular interstitial fibrosis.

The ability of blockers of  $K_{Ca}3.1$  to abrogate IgA1, and most notably pIgA1dependent, synthesis of pro-inflammatory and profibrotic factors indicates that these blockers may be effective as a novel treatment in IgAN.

# 7.3.2 Potential use of K<sub>Ca</sub>3.1 as a biomarker in IgA nephropathy

Several studies have demonstrated proteins and peptides as a potential biomarker for IgAN. However, none has been validated in a large cohort study. Interleukin-6 (IL-6) and IL-8 were reported to be found in the urine of IgAN patients (Huang *et al*, 2001; Harada *et al*, 2002) Other studies include chemokines (monocyte chemoattractant protein-1), growth factors (epidermal growth factors [EGF]), ratios of urinary IL-6, EGF, and EGF monocyte chemoattractant-1 (Grandaliano *et al*, 1996; Ranieri *et al*, 1996; Torres *et al*, 2008). However, these biomarkers are also found in other forms of glomerulonephritis.

Recently, several studies have tried to identify specific biomarkers for IgAN such as urinary concentration of immune complexes containing Gd-IgA1 and IgG, CD89 and TG2 (Matousovic *et al*, 2006; Moresco *et al*, 2016; Delanghe *et al*, 2013). The discovery of a biomarker which could predict disease severity in IgAN would be invaluable in helping clinicians decide treatment strategies in this very variable renal disease.

The incidental finding of  $K_{Ca}3.1$  in urine from IgAN patients seen in this work have been very interesting.  $K_{Ca}3.1$  was found in the exosome fraction from media from IgA1 induced PTECs and urine from 14 out of 20 IgAN patients, 3 out of 20 other kidney diseases, and no healthy individuals. The mechanism driving the shedding of  $K_{Ca}3.1$  from PTECs incubated with IgA1 but not with TGF- $\beta$ 1 *in vitro* and which lead to the presence of the protein in urine from IgAN patients needs to be further investigated and some questions should be investigated. Is it possible to distinguish which cells release  $K_{Ca}3.1$  exosomes? Do these exosomes play a role in IgAN disease pathogenesis, particularly in cell crosstalk? Considerations should be made about the method of urine handling performed in this experiment. The finding of this channel in urine from 15% of other kidney diseases raises some questions. In this study urine from IgAN patients and healthy individuals was collected and immediately centrifuged to remove cell debris. However, the urine from patients with other renal diseases did not have cell debris removed before freezing.

Another interesting finding was the lack of correlation between urine protein creatinine ratio (PCR), haematuria and  $K_{Ca}3.1$  in urine. This suggests that  $K_{Ca}3.1$  was not a generic tubulointerstitial marker. Further work is needed to confirm this finding, including using large numbers of patients.


Figure 7-1. A schematic diagram demonstrating the proposed role of KCa3.1 in the pathogenesis of IgA nephropathy. Glomerular IgA1 immune complexes bind to mesangial cell receptors leading to opening of cell surface  $K_{Ca}3.1$  channels (1). The resultant potassium flux causes an increase in intracellular calcium concentration through opening of voltage-gated calcium channels (2), which, in turn, mediates upregulation of KCa3.1 expression (3), IL-6 release and ECM synthesis (5). With continued mesangial cell activation there is progressive damage to the glomerular basement membrane (6) and increasing passage of cytokines, IgA1 immune complexes and albumin into Bowman's space. Direct interaction between these cytokines and IgA1 immune complexes cause both podocyte (7) and proximal tubule epithelial cell (PTEC) injury (8). KCa3.1 is also important in mediating PTEC activation. When PTEC are exposed to IgA1 immune complexes in the early urine there is induction of TGF- $\beta$ 1 synthesis (9) which acts in an auto/paracrine manner to increase KCa3.1 expression by PTEC (10), increasing intracellular calcium ion levels, and triggering release of cytokines and pro-fibrotic mediators (11), ultimately causing tubulointerstitial fibrosis.

# Appendix

# Chapter 3

Cytokine and Chemokine release in IgA1 stimulated human mesangial cells (LUMINEX)

Α



IL-1 ra/IL-1F3

В





IL-8/CXCL8

D



С

# **PGGF-AA**



**Figure 1.** Cytokines and chemokines Expression in the presence of IgA plus +/- TRAM-34. Levels of **A**. MIF; **B**. IL-1 ra/IL-1F3; **C**. MCP-3/CCL-7; **D**. IL-8/CXCL8; **E**. PDGF-AA showed an upward trend in the presence of IgA1. This change was moderated in the presence of TRAM34. Results are shown as mean +/- SEM, N=3. NS=not significant.

COL3A1 by HMCs does not change with the presence of human serum IgA1





В



133

Figure 2. COL3A1 by HMCs does not change with the presence of human serum IgA1. Confluent primary HMCs were incubated in serum free media with A. IgA1 (50  $\mu$ g/mL) or B. TGF- $\beta$  (2 ng/mL) with or without TRAM-34 (1  $\mu$ M and 4  $\mu$ M) for 24 hour. The cells were lysed in TRIZOL. RNA was extracted, cDNA and qPCR was performed using the Taqman gene expression kits for COL3A1. Ct values were normalised to the housekeeping gene MT-ATP6 and results are presented as fold change compared to media only. HMCs do not increase COL3A1 gene expression in the presence of human serum IgA1, N=3, results are shown as mean +/- SEM. Results were analysed using one-way ANOVA, N=3. NS=not significant. Alpha-SMA by HMCs does not change with the presence of human serum IgA1

Α



В



**Figure 3.** α-**SMA synthesis by HMCs in the presence of human serum IgA1 or TGF-**β1. Confluent primary HMCs were incubated in serum free media with A. IgA1 (50 µg/mL) or B. TGF-β1 (2 ng/mL) for 24 hour with or without TRAM-34 (1 µM or 4µM) in duplicate. The cells were lysed in SDS PAGE reducing sample buffer before being passed through a 10% polyacrylamide gel. Western blotting was performed, and the membrane was incubated with rabbit anti human alpha smooth muscle actin (1:1000) and then HRP conjugated goat-anti rabbit (1:5000). The band densities were normalized with β-actin and the results were presented as fold change compared with media only. HMCs do not increase α-SMA protein synthesis in the presence of human serum IgA1, N=3, results are shown as mean +/- SEM. Results were analysed using one-way ANOVA, N=3. NS=not significant.



Figure 4. Expression of KCNN4 by podocytes. Q-PCR analysis of mRNA coding for KCNN4 podocytes incubated with TGF-  $\beta$  or IgA1 with and without TRAM-34 or DMSO. N=6, NS = not significant

IgA1 does not affect FSP-1 gene expression in Human Podocyte



Figure 5. Expression of FSP-1 by podocytes. Q-PCR analysis of mRNA coding for FSP-1 (ALT-1) in podocytes incubated with TGF-  $\beta$  or IgA1 with and without TRAM-34 or DMSO. N=4, NS = not significant

IgA1 does not affect COL1A1 gene expression in Human Podocyte



Figure 6. Expression of COL1A1 by podocytes. Q-PCR analysis of mRNA coding for COL1A1 in podocytes incubated with TGF-  $\beta$  or IgA1 with and without TRAM-34 or DMSO. N=4, NS = not significant.



Figure 7.  $\alpha$ -SMA protein synthesis by IgA1 stimulated podocytes. Western blot of  $\alpha$ -SMA protein synthesis by podocytes. There was no difference in the expression of  $\alpha$ -SMA by podocytes in the presence or absence of IgA1 nor with TRAM-34. n=3. NS = not significant

# IgA1 does not affect fibronectin protein synthesis in Human Podocyte



**Figure 8. Fibronectin protein synthesis by IgA1 stimulated podocytes.** Western blot of fibronectin protein synthesis by podocytes. There was no difference in the expression of fibronectin by podocytes in the presence or absence of IgA1 nor with TRAM-34. n=3. NS = not significant

# Chapter 4

Expression of COL1A1 was not attenuated by  $K_{Ca}$ 3.1 blockade in hTERT/PTECs







Figure 1. COL1A1 gene expression by hTERT/PTECs after incubation with IgA1 or TGF- $\beta$ 1 +/- TRAM-34 or ICA-17043.. qPCR analysis of COL1A1 mRNA from hTERT/PTECs incubated with (A) IgA1 or TGF $\beta$ 1 (B) IgA1 with and without TRAM-34 (C) IgA1 with or without ICA-17043. N= 3 Results are shown as mean ±SEM of Ct values normalised to PES1 and media only. Data was analysed by one-way ANOVA. NS = not significant

Expression of COL3A1 was not attenuated by  $K_{Ca}$ 3.1 blockade in hTERT/PTECs







Figure 1. COL3A1 gene expression by hTERT/PTECs after incubation with IgA1 or TGF- $\beta$ 1 +/- TRAM-34 or ICA-17043.. qPCR analysis of COL3A1 mRNA from hTERT/PTECs incubated with (A) IgA1 or TGF $\beta$ 1 (B) IgA1 with and without TRAM-34 (C) IgA1 with or without ICA-17043. N= 3 Results are shown as mean ±SEM of Ct values normalised to PES1 and media only. Data was analysed by one-way ANOVA. NS = not significant

# Chapter 5

Cytokine and Chemokine release in mlgA1 and mlgA stimulated hTERT/PTEC (LUMINEX)

# 1. GM-CSF

Α







Figure 1. Polymeric and monomeric IgA1 do not increase GM-CSF synthesis by hTERT/PTEC. Confluent hTERT/PTEC were incubated in serum free media with pIgA1 or mIgA (50  $\mu$ g/mL) for 24 hours with or without TRAM-34 (200 nM) TRAM-85 (200 nM), ICA-17043 (10nM and 100 nM). Levels of GM-CSF in the supernatant were measured using Luminex technology. No changes were seen in levels of GM-CSF in the supernatant from hTERT/PTECs incubated with A. pIgA and B. mIg1A with or without TRAM-34 or ICA-17043. N=6. Results are shown as mean ±SEM and were analysed using one-way ANOVA. NS=not significant.

- 2. MCP-1/CCL-2
  - Α







Figure 2. Polymeric and monomeric IgA1 do not increase MCP-1/CCL2 synthesis by hTERT/PTEC. Confluent hTERT/PTEC were incubated in serum free media with pIgA1 or mIgA (50  $\mu$ g/mL) for 24 hours with or without TRAM-34 (200 nM) TRAM-85 (200 nM), ICA-17043 (10nM and 100 nM). Levels of MCP-1/CCL2 in the supernatant were measured using Luminex technology. No changes were seen in levels of MCP-1/CCL2 in the supernatant from hTERT/PTECs incubated with A. pIgA and B. mIg1A with or without TRAM-34 or ICA-17043. N=6. Results are shown as mean ±SEM and were analysed using one-way ANOVA. NS=not significant.

3. MMP-2





150

Figure 3. Polymeric and monomeric IgA1 do not increase MMP-2 synthesis by hTERT/PTEC. Confluent hTERT/PTEC were incubated in serum free media with pIgA1 or mIgA (50  $\mu$ g/mL) for 24 hours with or without TRAM-34 (200 nM) TRAM-85 (200 nM), ICA-17043 (10nM and 100 nM). Levels of MMP-2 in the supernatant were measured using Luminex technology. No changes were seen in levels of MMP-2 in the supernatant from hTERT/PTECs incubated with A. pIgA and B. mIg1A with or without TRAM-34 or ICA-17043. N=6. Results are shown as mean ±SEM and were analysed using one-way ANOVA. NS=not significant.

4. MMP-9





NS

Figure 4. Polymeric and monomeric IgA1 do not increase MMP-9 synthesis by hTERT/PTEC. Confluent hTERT/PTEC were incubated in serum free media with pIgA1 or mIgA (50  $\mu$ g/mL) for 24 hours with or without TRAM-34 (200 nM) TRAM-85 (200 nM), ICA-17043 (10nM and 100 nM). Levels of MMP-9 in the supernatant were measured using Luminex technology. No changes were seen in levels of MMP-9 in the supernatant from hTERT/PTECs incubated with A. pIgA and B. mIg1A with or without TRAM-34 or ICA-17043. N=6. Results are shown as mean ±SEM and were analysed using one-way ANOVA. NS=not significant.

# **General buffers**

## Phosphate buffered saline (PBS)

8 g NaCl, 1.15 g anhydrous Na<sub>2</sub>HPO<sub>4</sub>, 0.2 g KCl, 0.2 g KH<sub>2</sub>PO<sub>4</sub> Dissolved in 1 L distilled water, adjusted to pH 7.4

# Tris buffered saline (TBS)

6.06 g Tris base, 8.77 g NaCl Dissolved in 1 L distilled water, adjusted to pH 7.6

## Saturated ammonium sulphate (4.1M)

21.67 g ammonium sulphate in 40 mL PBS

#### **ELISA**

## Coating buffer (0.05M carbonate/bicarbonate, pH 9.6)

0.189 g Sodium hydrogen carbonate, 0.027 g Sodium carbonate Dissolved in 50 mL distilled water

#### Wash buffer (PBS/0.3M NaCl/0.1% Tween 20)

20.75 g NaCl, 1 mL Tween-20 Dissolved in 1 L PBS

## **OPD** substrate

2 OPD tablet (o-phenylenediamine dihydrochloride) (Dako) in 6 mL distilled water. 2.5  $\mu$ L hydrogen peroxide solution 30% (w/w) added immediately before use.

# Sample buffer, 8 mL

4 mL water, 1 mL 0.5M Tris-HCl pH 6.8, 0.8 mL Glycerol, 1.6 mL 10% (w/v) SDS, 0.4mL  $\beta$ -mercaptoethanol, 0.05% (w/v) Bromophenol blue.

# Running buffer (10x), 1 L

30.3 g Tris Base, 144 g Glycine, 10 g SDS, dissolved in 1 L distilled water.

# Transfer buffer (10x), 1 L

30.3 g Tris Base, 144 g Glycine, dissolved in 1 L distilled water.

## **De-staining buffer**

40% methanol, 10% acetic acid, 50% water

## PCR TAE buffer:

2 M Tris-HCI, 50 mM EDTA-Na2, 1 mM glacial acetic acid

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