UNIVERSITY OF SOUTHAMPTON

FACULTY OF MEDICINE, HEALTH AND LIFE SCIENCES

School of Medicine

ADAM33: A Cellular Model of Over-expression. From Full Length to Soluble.

by

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ABSTRACT

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Polymorphic variation in the *A Disintegrin And Metalloprotease 33* (*ADAM33)* gene has been associated with asthma and bronchial hyperresponsiveness. The ADAM family of proteins has a multi-domain structure and has diverse biological functions which can include growth factor shedding, cell migration and cell adhesion. Evidence for a soluble form of ADAM33 has been found in BAL fluid of asthmatic patients and there is evidence that ADAM33 has a function in angiogenesis.

To determine the possible function(s) of the full length ADAM33 protein, it was over expressed in HEK293 that do not normally express ADAM33 and the phenotypic consequences analyzed. Cells stably expressing full length ADAM33 under the control of a CMV promoter were obtained by selection with the antibiotic, G418. The expression of ADAM33 was confirmed by western blot analysis. Control cells were transfected with an empty vector control encoding G418 resistance only. Cells were examined for changes in cell phenotype and ability to produce soluble ADAM33.

Cells stably expressing ADAM33 grew more quickly than mock transfected cells but this effect was lost over time. However, processed (ie. active) ADAM33 protein could not be detected by western blot. In order to determine whether a subpopulation of cells was able to produce active ADAM33, the cells were cloned and re-cloned by limiting dilution. This allowed isolation of a number of ADAM33 clones that produced processed protein. Furthermore, these experiments revealed that ADAM33 expression increased the clonogenic potential of the cells. Supernatant from cloned cells was pulled down with ConA and samples analysed by western blot which showed a band at 50-55kDa in the ADAM33 clones using an antibody to the metalloproteinase domain. This soluble ADAM33 was active and promoted angiogenesis using a HUVEC capillary tube forming assay. Soluble ADAM33 was upregulated in the presence of TGF-β2 and its shedding is unliky to be autocatalytic.

Over expression of ADAM33 in HEK293 cells alters the clonogenic potential and survival of HEK293 cells in low density cultures. This property may contribute to the behaviour of mesenchymal cells in asthmatic airways. Soluble ADAM33 is released into culture media and has functional activity. Its release is up regulated in the presence of TGF-β2.

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Declaration of Authorship

I Anna Harvey

declare that the thesis entitled:

ADAM33: A Cellular Model of Over-expression: From Full Length to Soluble

and the work presented in the thesis are both my own, and have been generated by me as the result of my own original research. I confirm that:

- this work was done wholly or mainly while in candidature for research degree at this University;
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Signed.....

Dated.....

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Abbreviations

ADAM: a disintegrin and metalloprotease

APP: β-amyloid precursor protein

BAL: bronchoalveolar lavage fluid

BHR: bronchial hyper responsiveness

CD14: Cluster of differentiation 14

ConA: Concanavalin A

COPD: chronic obstructive pulmonary disease

DMEM: Dulbecco's Modified Eagle Medium

DPP10: Dipeptidyl peptidase 10

ECGS: Endothelial Cell Growth Supplement

EGF: epidermal growth factor

EGFR: epidermal growth factor receptor

EMTU: epithelial mesenchymal tropic unit

$FEV_{1:}$ forced expiratory volume in 1 second

FcεRI-β: beta chain of high affinity receptor for IgE

FRET: Fluorescence Resonance Energy Transfer

GPRA: G protein-coupled receptor for asthma

GM-CSF: granulocyte monocyte colony stimulating factor

HBEGF: Heparin-binding EGF-like growth factor

HBSS: Hanks balanced salt solution

HEK293: Human Embryonic Kidney 293

HRP: Horseradish peroxidase

HUVEC: Human umbilical vain endothelial cells

IFN-γ: interferon- γ

IgE: immunoglobulin E

IgG: immunoglobulin G

IgA: immunoglobulin A

IL-: interleukin

IL-4Rα: interleukin 4 receptor

KL-1: Kit-ligand-1

MMP: matrix metalloproteases

MP: Metalloprotease domain

PHF11: PHD finger protein 11

PKC: Protein Kinase C

PMA: phorbol 12-myristate 13-acetate

PTGDR: prostaglandin D receptor

RANTES: Regulated on Activation, Normal T Expressed and Secreted

SNPs: single nucleotide polymorphisms

SPINK5: serine peptidase inhibitor, Kazal type 5

sRaw: plethysmographic measurement of specific airway resistance

SV: snake venom disintegrins

TGF-β: transforming growth factor

Tim1: T cell Ig and mucin

TLR2: Toll like receptor 2

TNF-a: Tumor necrosis factor-alpha

3' UTR: three prime untranslated region

Chapter 1 - ADAM 33: Its Functions and Role in Asthma

Asthma

Asthma is a common respiratory disease with genetic and environmental causes and interactions. It is characterised by intermittent airflow obstruction, chronic inflammation of the lungs and bronchial hyper-responsiveness (BHR). In addition, respiratory symptoms such as wheezing, tightness of chest, cough, and breathlessness occur along with pathological changes of the airways Cohn et al 2003. Triggers of asthma include respiratory virus infections, cold air, smoke or exercise Rich et al 2000 and P Fireman 2003. The underlying causes of asthma are thought to be the inter-related pathological processes of inflammation and airway remodelling.

These are changes in structural cells of asthmatic patients and infiltration of the airways with immune and inflammatory cells. The structural changes that are characteristic of asthma are epithelial detachment, mucus gland hyperplasia in the epithelial layer and subepithelial fibrosis, airway smooth muscle hyperplasia, thickening of lamina reticularis, collagen deposition and vascular changes in the deeper layers of the lungs Q Hamid 2003. Asthmatic airways are also infiltrated with specific immune and inflammatory cell types: CD4 T-cells, eosinophils, mast cells, macrophages and plasma cells T Williams 2004 . In severe disease neutrophils are also found in increased numbers. Chronic inflammation of the airways by T_H2 type cells producing cytokines such as interleukin-4 (IL-4), IL-5, IL-9, and IL-13 have been found to be relatively increased in asthmatics compared to normal D Fernandes et al 2003. Along with many other factors such as interferon- γ (IFN-γ) N ten Hacken et al 1998,

transforming growth factor (TGF-β) A Redington et al 1997, TNF-a M Berry et al 2006, IL-10 L Borish et al 1996, IL16 N Krug et al 2000,and IL18 are important in the progression of disease C Wong et al 2001.

In normal individuals the bronchial epithelium has a stratified structure that protects the airway by secreting mucus and cytoprotective molecules. The interaction of the epithelium with the immune systems promotes tissue repair by cell signalling with cytokines, chemokines and cell adhesion molecules when damage occurs G Toews 2001.

Injury and damage to the bronchial epithelium including shedding of the epithelial cells is a characteristic feature of asthma but not other airway diseases. The injury of epithelial cells causes the release of proinflammatory mediators, fibroproliferative and profibrogenic growth factors. These include: TGF-β_2, fibroblast growth factor, platelet-derived growth factor, endothelin 1, fibronectin, RANTES, eotaxin, TNF-α, IL-1β, IL-5, IL-6, IL-8, IL-10, IL-11, IL-13, IL-16, IL-18 and GM-CSF C Wong et al 2001 and Tang et al 2006.

IL-4 and IL-13 are both major inflammatory cytokines in asthma and are increased compared to normal C Wong et al 2001. IL-4 perpetuates T_H2 cell differentiation and supports their survival. IL-13 induces sub epithelial fibrosis and smooth muscle proliferation which links this cytokine with airway hyper-responsiveness. Both IL-4 and IL-13 cause the production of IgE and IgG4 by activating B-cells. They are both involved in modulation of goblet cell metaplasia and mucus hypersecretion. Both are involved in the regulation of inducible nitric oxide synthase, eotaxin and IgA secretory component, which in turn can activate eosinophils and can also cause the production of many cytokines including RANTES, IL-6, IL-8 and GM-CSF. C Doucet et al 1998a, C

Doucet et al 1998b, C Perkins et al 2006, M Wills-Karp 2004, S Romagnani 2002 and P Fireman 2003.

TGF-β is an important growth factor in asthma because it causes the differentiation of fibroblasts to myofibroblasts which secrete interstitial collagens which lead to collagen deposition in the submucosa and thickening of the *lamina reticularis*. In addition the myofibroblasts produce growth factors such as vascular endothelial factor and endothelin 1 that are mitogens for smooth muscle and endothelial cells, leading to smooth muscle hyperplasia and vascular changes in asthma. The increase in the number and size of vessels can add to the thickening of the airway wall. This along with the increase in collagen deposition, the increase in fibroblasts and smooth muscle cell mass, leads to a thickening of the airway wall and therefore narrowing of the bronchial lumen. This leads to bronchial restriction when bronchial smooth muscle contraction occurs. The thickening of the airway walls also makes the airways stiffer, making it harder to breathe E Minshall et al 1997, A Vignola et al 1997 and C Duvernelle et at 2003.

In asthmatic airways EGFR is increased in areas of damage but also in areas of intact epithelium. In asthma however, there is no evidence of an increase in epithelial cell proliferation as would be expected with an increase in EGFR. This is probably due to the high expression of $p21^{waf}$ which is a negative regulator of G1 cyclins. This is induced by TGF-β_1 and β_2 and can extend the period of epithelial repair and may keep the pro inflammatory phenotype of the epithelium maintained. This could either mean that in asthmatic airways the EGFR can highlight epithelial damage and indicate that the damage in the epithelium is wide spread. Alternatively, EGFR may not be appropriately down

regulated after repair has been completed and the cells are held abnormally in the repair phenotype S Holgate et al 1999. EGFR could potentially add to the pro inflammatory response directly as it has been shown to cause the expression of IL-8 from epithelial cells M Monick et al 2005.

The interaction between the epithelium and mesenchymal cells in the submucosa through cytokines and growth factors has led to the hypothesis that the epithelial mesenchymal tropic unit (EMTU) is reactivated in susceptible individuals. The EMTU is required for lung development during embryogenesis and is the physiological process that regulates airway growth and branching. The balance of EGF and TGF-β and therefore communication between the epithelium and the mesenchymal cells causes the growth of the airways. Its reactivation in asthma is due to a similar balance of TGF-β and EGF (and the prolonged epithelial repair which maybe the cause of increase EGFR in the epithelium) being established in asthmatic airways causing the remodelling seen in asthma; this would mean the increased proliferation of fibroblasts, smooth muscle, microvessels and nerves along with increases in chemokines and cytokines D Davies and S Holgate 2002.

Treatments available so far affect the symptoms of asthma and help to reduce inflammation in the airways but have little or no impact on airway remodelling. The treatments used include: inhaled corticosteroids that relieve the symptoms of asthma and target the inflammation. Alternatively oral corticosteroids can be given in the short term to bring asthma under control as well as in more severe asthma when inhaled corticosteroids are ineffective. Long acting β agonists are inhaled and can keep the airways open for 12 hours or longer. They act by relaxing the muscles in the airways and therefore keeping

them open and reducing the resistance to exhaled airflow and making it easier to breathe. Short acting β agonists are used during an asthma attack to open the airways, they can act within minutes. Leukotriene inhibitors are used to reduce inflammation as they work by inhibiting leukotrienes which promote the inflammatory response seen during an acute asthma attack. These treatments and others are successful in most cases but for some individuals they are ineffective, therefore new treatments are needed for these individuals Drazen et al 2000 and Djukanovic 2002.

It has been shown that changes in the airways (remodelling) may be present in young children. Biopsy studies have found these remodelling changes to be present before the onset of asthma symptoms. These physiological changes could interact with factors conferring susceptibility to the environment such as diesel particles or house dust mites. Children with decreased lung function and early wheeze could be affected. These studies could mean that in susceptible individuals remodelling occurs before or at the same time as the predisposition to T_H2 inflammation and is central to chronic inflammation and chronic asthma J Bousquet et al 2000, I Fedorov et al 2005, A Barbato et al 2006 and S Saglani and A Bush 2007. Asthma is likely to be caused by the interaction of an individual's genes that predispose people to the disease with the environment.

Asthma susceptibility

Asthma has a strong genetic component, and has been associated with several genes thought to increase susceptibility to the disease. Of those genes, some are associated with the inflammation processes in asthma and others such *ADAM33* are likely to be involved in the airway remodelling aspect of asthma and BHR.

As ADAM33 is expressed in fibroblasts and smooth muscle cells not epithelial immune or inflammatory cell types P van Erdewegh et al 2002 and S Umland et al 2003. And SNPs in ADAM33 have been shown to be associated with BHR P van Erdewegh et al 2002 and decline in lung function including in 3 and 5 year old children A Simpson et al 2005 . Genes that have been found to confer susceptibility include the interleukin-13 (*IL13)* gene which is an asthma positional candidate gene, its involvement is supported by linkage studies, genetic association and function studies T Howard 2002. The Dipeptidyl peptidase 10 (*DPP10)* gene has been identified by positional cloning; it encodes a peptidase gene that might be involved in the immunological aspects of asthma such as cytokine processing in T cells M Allen 2003 and R Yamada and K Ymamoto 2005. On chromosome 13q14 the gene for PHD finger protein 11 (*PHF11)* was identified as a locus for immunoglobulin E levels in asthma R Yamada and K Ymamoto 2005 and G. Malerba and F Pignatti 2005. Also one of the Tim gene family, T cell Ig and mucin (*Tim1)* was identified, and seems to be involved in the differentiation of TH2 cells Q Liu et al 2007. The G protein-coupled receptor for asthma (*GPRA* or *GPR154)* gene on chromosome 7p14-p15 is a G-coupled receptor and polymorphisms of this gene may have an effect on bronchial epithelial and smooth muscle where it is expressed as a cell surface receptor T Laitinen et al 2004 and R Yamada and K Ymamoto 2005.
ADAM33 is one asthma susceptibility gene. A genome wide scan and multipoint linkage analyses found a region on chromosome 20p13 which contains the *ADAM33* gene. ADAM33 has many single nucleotide polymorphisms (fig 1.1). Several of these single nucleotide polymorphisms (SNPs) within the *ADAM33* gene have been associated with asthma and BHR. These include several in the

transmembrane and cytoplasmic domain S1, S2, T1 and T2 that cause coding changes in the ADAM33 protein Van Eerdewegh 2002. The SNPs associated with asthma are not the same in the different populations studied and indeed there are some studies that have found little or no association between these SNPs in the *ADAM33* gene and asthma, though some studies have looked at different sets of SNPs. For example in a study of Mexican and Puerto Rican patients no significant associations were found Lind et al 2003. A meta-analysis of existing data on ADAM33 polymorphisms found that overall in both transmission disequilibrium and case control studies that ADAM33 polymorphisms are associated with asthma even when the negative studies are included in the analysis. The F+1 and ST+7 SNPs were found to be most significantly associated with asthma. It was also noted in this meta analysis that the early studies on ADAM33 were small and therefore may lack power and may have overestimated the locus at a population level J Blakey et al 2005. Studies carried out after this meta-analysis also show contradictory information on the association of asthma and ADAM33 SNPs and haplotypes of the ADAM33.

However, it seems likely that some ADAM33 polymorphisms are associated with asthma susceptibility. Furthermore ADAM33 polymorphisms have been shown to be associated with decline in lung function over time in asthmatics Jongepier et al 2004 as well as in the general population van Diemen et al 2005 and in COPD Gosman et al 2006 as detailed below.

In early life some ADAM33 polymorphisms are associated with impaired lung function. Recent studies have associated some SNPs that predict impaired early life lung function. For example in a study by A Simpson et al children at age 3 and 5 had their lung function tested using sRaw (plethysmographic measurement

of specific airway resistance) and their SNPs analysed. At age 3 only the F+1 SNP showed association with decreased lung function (higher sRaw). At age 5 four SNPs F+1, S1, ST+5 and V4 showed association with decreased lung function. In the study the children that were homozygous for the A allele of F+1 the risk of early wheezing was more than doubled. Simpson *et al* predict that the functionally relevant SNP is likely to be at the 5' end of the gene between BC+1 and F1 SNPs A Simpson et al 2005. A study over 20 years by Jongepier et al showed that polymorphisms in ADAM 33 were associated with accelerated lung function decline in asthma. They found that the S2 polymorphism (a relatively rare genotype which affected approximately 7% of their patients) was most significantly associated with decline in FEV_1 (forced expiratory volume in 1 second). They suggest that S2 is the main locus or is in linkage disequilibrium with the main locus that determines excess decline in FEV_1. Van Diemen et al 2005 have found that polymorphisms in ADAM 33 in the general population and in chronic obstructive pulmonary disease (COPD) were associated with accelerated lung function decline. It therefore seems likely that some polymorphisms in ADAM33 have an effect on lung function. This indicates that remodelling changes in the airways may also be influenced by ADAM33.

If normal ADAM33 function protects the airway from increased repair processes, altered ADAM33 function conferred by SNPs could lead to airway remodelling, resulting in subepithelial fibrosis and may contribute to accelerated loss of lung function shown in the studies by Simpson et al 2005, Jongepier et al and Van Diemen et al 2005.

ADAM33 SNPs may interact together at different locations on the gene that may produce diverging functional changes. In addition, one SNP may interact with

polymorphisms on a different gene and this may result in accelerated decline in lung function or BHR. Furthermore, it could be that the SNP that confers susceptibility to asthma may only interact with other genes that are induced by exposure to environmental factors and that this interaction may lead to lung function loss.

Asthma susceptibility is unlikely to be caused by one gene alone but through many interactions with both genetic and environmental factors.

Table 1.1: Genes associated with asthma.

Gene Name	Function	
IL13	TH2 cytokine and key molecule asthma pathology	Polymorphisms increase risk of asthma and atopy.
IL-4 and *IL-4Rα*	IL4 perpetuates TH2 cell differentiation and supports their survival	Polymorphisms in both increase risk of asthma and atopy.
PHF11	PHD finger protein 11	On locus for increase IgE in asthma.
Tim1	T cell Ig and mucin domain protein. type I transmembrane proteins	involved in differentiation of TH2 cells
GPRA	Cell surface receptor	Elevated IgE levels, atopy and asthma.
ADAM33	Airway remodelling	Asthma and BHR
TLR2 Toll like receptor 2	Innate immunity receptor with microbial molecules as ligands	Reduced levels of asthma in farmers children with T allele
PTGDR Encodes DP receptor for PDG_2 (prostaglandin)		Asthma and Atopy
SPINK5	Encodes serine protease inhibitor LEKTI	Atopy, asthma, and eczema
CD14	Multifunctional receptor on macrophages, monocytes, neutrophils. And main LPS	IgE levels and inflammation

	receptor	
FcεRI-β the beta chain of high affinity receptor for IgE	Amplifying element in receptor and stabilises surface expression of the receptor	Polymorphisms related to atopy, asthma, BHR and severe atopic dermatitis.
IFNG. Interferon IFN-γ	Involved in differentiation and function of TH2 cells	Polymorphism associated with atopic asthma.

T Howard et al 2002, C Hersh et al 2007, M Bracken 2002, M Blumenthal 2005, Q Liu et al 2007, R Yamada and K Ymamoto 2005, F Nakao et al 2001, M Allen et al 2003, W Eder et al 2004, I de Faria et al 2008, G. Malerba and F Pignatti 2005, M Freidin et al 2003, H Zhang et al 2007, T Laitinen et al 2004 and S Weiss and B Raby 2004.

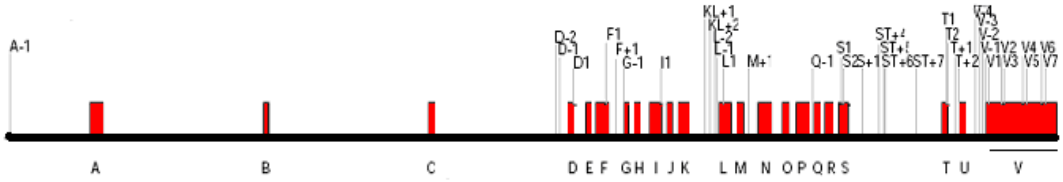
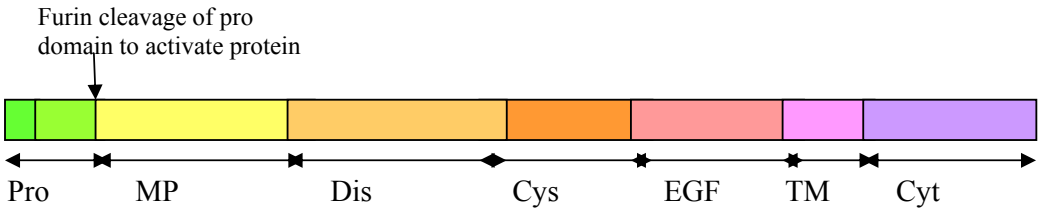


Figure 1.1: ADAM 33 gene map, with introns and exons (A-V) with the polymorphisms marked. Adapted from Van Eerdewegh et at 2002.

The ADAMs, Domains and Functions

ADAM33 is a member of the ADAM family of proteins (a disintegrin and metalloprotease). There is little functional information on ADAM33. Its possible functions are based on similarities of structure with other ADAM family members and functional studies of these ADAMs. ADAMs are multifunctional transmembrane proteins that are involved in diverse biological processes and comprise approximately 40 members in various species. All ADAMs comprise of a 7 domain structure as shown below.

Figure 1.2: domains of a disintegrin a metalloprotease (ADAM) protein



Pro: Pro domain
MP:Metalloprotease domain
Dis: Disintegrin domain
Cys: Cysteine rich domain
EGF: EGF like domain
TM: Transmembrane domain
Cyt: Cytoplasmic domain

Each of the domains will be discussed in turn starting with the N-terminus pro-domain.

Pro domain

The N terminus of the protein is an area known as the pro domain. It contains a signal sequence that directs these proteins into the secretory pathway and a regulatory area that primarily keeps the metalloprotease site inactive, through a cysteine switch mechanism. A conserved cysteine in the pro-domain coordinates with the zinc atom that is needed in the active site of the metalloprotease domain (MP). The processing of ADAMs from immature to mature form occurs in the trans-golgi network D Seals and S Courtneidge 2003 . The cleavage of the pro-domain is carried out by pro-protein convertase (PCs) such as furin or furin-like proteases. Cleavage by furin has been shown *in vitro* in a study of ADAM 15 L Lum et al, 1998. A conserved RX(RK)R motif is the site of cleavage. This release of the pro-domain switches the zinc atom coordination to the MP domain and the zinc ion becomes available for catalytic activity. If this motif is mutated, processing of ADAMs to their active forms is blocked as shown in studies of ADAMs 10, 12 and 19 D Seals and S Courtneidge 2003.

There is also evidence that some ADAMs are autocatalytic. In a study of ADAM 8 the process of pro-domain removal by proteolytic mechanisms is blocked in a mutant ADAM 8 where Glu^{330} is changed to Gln in zinc binding motif in the MP domain, yet the protein is still trafficked to the cell surface U Schlomann et al 2002. Another study has also suggested that ADAM 28 is at least partly autocatalytic as the mutation of Glu to Ala produces a similar response to ADAM 8 when protease cleavage is blocked L Howard et al 2000.

Secondary functions of the Pro-domain are firstly that it is needed for proper folding of ADAMs, particularly for the MP domain. For example, studies with ADAM 10 have shown that *in vivo* a construct lacking the pro domain is catalytically inactive, due to improper folding of the protein. Also a form of ADAM 12-S without the pro-domain is not secreted from the cell whereas the pro-domain containing form is secreted. The pro-domain is therefore necessary for folding of the catalytic active site, and the proper transit of ADAMs through the secretory pathway enabling mature ADAMs to be translocated to the extracellular and/or intracellular membrane surfaces D Seals and S Courtneidge 2003.

Metalloprotease domain (MP)

The MP domain's active site contains a zinc ion and water molecules that are needed for hydrolytic processing. The zinc ion is coordinated by 3 conserved histidine residues and downstream methionine and is in a Met turn motif which loops around to face the consensus sequence HEXXHXXGXXH. ADAMs with this consensus sequence are predicted to be proteolytically active R Black and J White 1998. These ADAMs are involved in the proteolytic release of cell surface membrane proteins like cytokines, growth factors and receptors. ADAM17 is TNF-α converting enzyme. It is needed for normal release of soluble TNF-α by directly cleaving the precursor D Seals and S Courtneidge 2003, R Black and J White 1998 and P Primakoff and D Myles 2000. ADAM 10 is involved in the neuronal development of Drosophila by the cleavage of NOTCH a cell surface receptor R Black and J White 1998. ADAM33 is one of the other ADAMs that is proteolytically active, though the substrates are not known. J Zou et al have

tested the catalytic properties of ADAM33 metalloproteinase domain against 40 synthetic peptides. Of these 4 were cleaved by the ADAM33 MP domain. They identified two of these peptides as the most relevant: KL-1 (Kit-ligand-1) and APP (β-amyloid precursor protein). Although these were identified as possible candidate physiological substrates, with full length APP no shedding was observed while KL-1 was only weakly shed by ADAM33. It is likely that there are other substrates to be identified that are more efficiently cleaved by ADAM33 J Zou et al 2004. J Zou *et al* have more recently expanded these studies to explore peptide specificity. They used the APP peptide YEVHH*QKLAF as a template sequence for amino acid replacement studies. They found that the most stringent residues and therefore most likely to be directly in contact with substrate binding pockets in ADAM33 are the P3 Val, the P2 Ala and P1' Gln. This led them to generate improved substrates for ADAM33 using FRET peptides. The best peptide substrate was FRET-P2 [K(Dubcy1)YRVAF*QKLAE(Edans)K], which was approximately 100 fold more efficiently cleaved than the wild type APP peptide and had a K_{cat}/Km value similar to other ADAMs with known substrates.

Another interesting point is that ADAM33 has alternative splicing events which include splice variants without the MP domain that is found within the cell R Powell et al 2004. ADAM12 and ADAM28 also have splice variants L Howard et al 2000. ADAM12 has two variants; a membrane bound form and a secreted form B Gilpin et al 1998. It is possible that the different variants of ADAM33 have different functions. It could therefore be possible that an ADAM33 variant with the MP domain is catalytically active and only a small amount is needed or that proteolytic cleavage of the yet unknown substrate is infrequent.

Disintegrin domain

In ADAMs the disintegrin domain is approximately 90 amino acids long. The ADAM family have homology to the snake venom (SV) disintegrins that bind platelet integrin receptors. SV disintegrins mimic the ligand site of matrix proteins like fibronectin. Most SVs have a RGD consensus sequence within a 13 amino acid loop-disintegrin, that interact with integrin receptors, the RGD is a sequence within fibronectin itself. ADAM15 is one of few ADAMs that also has the RGD sequence. Most ADAMs have a different sequence to their disintegrin loop and many share this RX6DEVF sequence. There is a subfamily of integrin receptors α4/α9 that do not recognise RGD, but aspartic acid containing sequences. In addition to this consensus sequence some charged residues in the disintegrin loop are crucial for integrin recognition. There are also other residues involved in integrin recognition that are out side of the disintegrin loop D Seals and S Courtneidge 2003 .

Integrins are a cell-cell and cell-matrix receptor superfamily (5 groups). They are composed of non-covalently linked heterodimers and have broad and significant biological role in many processes such as haematopoiesis, embryonic development, wound repair and in the immune response . Many ADAMs have been shown to associate with integrin receptors. Multiple ADAM disintegrins are recognised by some integrin families e.g. $\alpha_6\beta_1$ integrin interacts with ADAMs 2, 3 and 9. The $\alpha_4\beta_1$ interacts with ADAMs 2, 3 and 28. And $\alpha_9\beta_1$ interacts with ADAMs 2, 3, 12, 15 and 33 and is also thought to interact with all proteolytic ADAMs. Multiple integrins can interact with the same ADAM for example ADAM9 is recognised by $\alpha_6\beta_1$ and $\alpha_v\beta_5$, and ADAM15 by $\alpha_9\beta_1$, $\alpha_v\beta_3$ and $\alpha_5\beta_1$. Multiple integrins expressed on some cells can also facilitate cell adhesion to the

single ADAM disintegrin domain D Seals and S Courtneidge 2003, L Bridges et al 2004 and J White 2003. ADAM12 and the integrin $\alpha_9\beta_1$have been shown to interact and mediate cell-cell attachment C Thodeti 2005. In addition there is evidence from P Lafuste *et al* that during the differentiation of human myogenic precursor cells, ADAM12 and $\alpha_9\beta_1$ are expressed together. This causes cell fusion and the growth of myotubes. A major reduction in cell fusion occurred by blocking ADAM12 and $\alpha_9\beta_1$ together or individually P Lafuste et al 2005.

Cysteine Rich Domain and EGF like Domain

ADAMs 1, 3, 12 and 14 have a motif that is similar to sequences found in viral fusion peptides. The cysteine rich domain is thought to complement binding capacity of the disintegrin domain and possibly bring specificity to the disintegrin domain mediated interactions D Seals and S Courtneidge 2003. For example in ADAM12 this domain produces adhesion of fibroblasts and myoblasts A Zolkiewska 1999. In ADAM13 this domain along with the disintegrin domain binds both fibronectin and β1 containing integrin receptors, but is inhibited with antibodies to the cysteine rich domain therefore indicating that the cysteine rich domain is needed D Seals and S Courtneidge 2003 and K Smith et al 2002.

The cysteine rich domain also acts as a ligand for the group of cell-adhesion molecules called the syndecans. The binding of cell surface syndecans with the cysteine-rich domain leads to an integrin- dependent spreading of cells. Iba et al suggest that initial binding of the ADAM12 cysteine rich domain by syndecan on mesenchymal cells produces a conformational change in the

cysteine rich domain that may allow integrins to bind to the disintegrin domain and then allows the cells to spread K Iba et al 2000.

ADAMs contain an EGF like repeat, which has not been widely studied, but has been identified as having a possible role in cell adhesion. A study by J Evans *et al* 1998 indicates that the EGF like domain of ADAM1 may have a role in cell adhesion. Recombinant proteins of ADAMs 1 and 2 (also known as fertilin α and β) comprising cysteine-rich domain and the EGF like domain with or without the disintegrin domain. It was found that recombinant proteins containing the disintegrin, cysteine-rich and EGF like domains inhibited sperm-egg binding. It was also found that the ADAM1 (but not ADAM2) that the cysteine-rich and EGF like domain recombinant also inhibited sperm-egg fusion though to a lesser degree than the disintegrin, cysteine-rich and EGF like recombinant. This suggests that either the cysteine-rich or EGF domain may participate in sperm-egg adhesion J Evans et al 1998.

Transmembrane and Cytoplasmic Domains

ADAMs are transmembrane proteins that span the plasma membrane or other internal membranes. This area of an ADAM protein spans the membrane once D Seals and S Courtneidge 2003.

The cytoplasmic regions are highly variable in length and sequence. These regions have specialized motifs that have been postulated to be involved in inside-out regulation of metalloprotease activity. Those regions are also involved in outside-in regulation of cell signalling, along with control of maturation and subcellular localization. One of the most common motifs found is the PxxP binding site for SH3 domain containing proteins, found in ADAMs 7, 8, 9, 10,

12, 15, 17, 19, 22, 29 and 33. Some ADAMs also have potential phosphorylation sites for serine-theonine and/or tyrosine kinases. This could regulate ADAM function directly or the resulting phosphotyrosine residues could provide ligands for SH2 domain containing proteins D Seals and S Courtneidge 2003 and E Shimizu et al 2003 the motifs contained within cytoplasmic region can give an idea of function.

The Function of ADAM 33 and its Role in Asthma.

From the study of other ADAM proteins it is known that ADAMs can have protease activity, can be involved in membrane fusion and can be involved in cell adhesion. ADAM33 could have one of these functions or it could be involved in all of them D Seals and S Courtneidge 2003. It is known that ADAM33 is proteolytically active although its substrates are not known and that ADAM33 could be involved in cell adhesion J Zou et al 2004. There are several possible functions of ADAM 33; it could be that ADAM33 is involved in cytokine stimulation. If the MP domain is functional as predicted ADAM33 could act as a sheddase G Weskamp et al 2006 and L Killar et al 1999. In addition it could be involved in cell adhesion through the disintegrin, cysteine rich and EGF-like domains Evans et al 1998 as ADAM33 has been shown to interact with the α9β1 integrin L Bridges et al 2005. The function of ADAM33 is still unknown and its role in the pathology of asthma also unknown.

To investigate the function of ADAM33 C Chen *et al* produced ADAM33 knock-out mice. However they found that the lack of ADAM33 did not affect the growth or reproductive ability, nor did it alter the phenotype of the mice in any way even after allergen challenge C Chen et al 2006.

It seems likely to be involved in the remodelling side of asthma rather than in inflammation as it is expressed in fibroblasts and smooth muscle cells P Van Eerdewegh et al 2002 and S Umland et al 2003. There is also some evidence for ADAM33 being expressed in epithelial cells of severe asthmatics S Foley et al 2007. However this finding by S Foley et al may not be conclusive as other groups have not found this S Umland et al 2003. And Y Yang et al 2008 have found the ADAM33 promoter to be methylated in epithelial cells and the gene is therefore silenced in this cell type. They also found that this methylation is maintained under disease conditions Y Yang et al 2008. ADAM33 SNPs could have many effects on protein function. SNPs in the promoter region could lead to an up or down regulation of gene expression. However in the *ADAM33* gene no polymorphisms are found in the promoter region and there is little evidence that there are changes in the amount of ADAM33 in normal individuals and those with asthma P Van Eerdewegh et al 2002 .

Splice variants of ADAM33 have been detected and it was found that the vast majority of these variants do not contain the MP domain. A variant of the EGF domain-to the C-terminus was the most abundant form detected. This may mean that the main function of ADAM33 may not be proteolytic or that its proteolytic function is an uncommon event and little of the protein is needed on the surface to carry out that function R Powell et al 2004. SNPs in introns of genes can have an effect on the splicing of genes and could affect the splice variants of ADAM33. SNPs in the intron regions are found in ADAM33 and some are associated with asthma such as F+1. Whether SNPs in the introns of ADAM33 cause changes in function or in splice variant requires further study P Van

Eerdewegh et al 2002 and there is no evidence that there is any difference in the splice variants expressed in asthmatics compared to non-asthmatics.

SNPs in the coding region of ADAM33 are also found, for example S1 and S2 could directly affect function. Also SNPs could be found in regulation sites and could affect the regulation of the gene and increase or decrease its function.

SNPs can also interact together at different locations on the gene that may produce diverging functional changes. It could be that one SNP may interact with polymorphisms on a different gene and this may result in accelerated decline in lung function. Or the SNP that confers susceptibility to asthma may only interact with other genes that are expressed due to environmental exposure and then lead to lung function loss P Van Eerdewegh et al 2002.

There is also a soluble form of ADAM33 found in bronchoalveolar lavage fluid (BAL) with a molecular weight of approximately 55kDa. It was found that soluble ADAM33 was increased in asthmatics compared to non asthmatics. Increasing levels of soluble ADAM33 were found as asthma severity increased. Lee and Parks et al 2006. This is the first evidence that the levels of ADAM33 are altered in asthma.

A purified MP domain of ADAM33 has been shown to induce the differentiation of endothelial cells *in vitro* and to cause formation of new vessels *ex vivo* and *in vivo*, implicating that soluble ADAM33 has a function in angiogenesis I Puxeddu et al 2008. Increased levels of soluble ADAM33 in asthmatics implicates ADAM33 as a remodelling gene that could contribute to airflow obstruction and lung function through its function in angiogenesis.

There are several possible functions of ADAM33 and ways that SNPs may affect the function.

Aims and Objectives

1. To determine the possible function or functions of ADAM33 the full length molecule will be examined to look at the possible catalytic function of ADAM33. By producing stably transfected cell lines containing full length ADAM33 along with mock transfected line as control. These stable cell lines will then be used to determine any changes in phenotype elicited from full length ADAM33 and gain information on the functions of this protein. Such changes could be a change in the size of cells or a change in the growth rate of the cells.
2. It is predicted that ADAM33 is an active protease, but as yet no substrate has been found but it is known that ADAM33 is involved in angiogenesis and in particular endothelial cell tube formation. Xenopus ADAM13, the closest homologue of ADAM33, is known to be shed from the cell surface. Since soluble ADAM33 is increased in asthma, I will also investigate shedding of the ADAM33 ectodomain and determine whether it is catalytically active.

My hypotheses are 1. that full length ADAM33 will alter the phenotype of cells that contain it, by changing the rate of proliferation or size of the cells. 2. That soluble ADAM33 is produced by ectodomain shedding.

Material and Methods

Tissue culture

Growth Conditions and Passaging of Cells

All media and supplements were purchased from Invitrogen (Paisley, UK) unless otherwise specified. HEK293 cells were routinely cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% v/v heat inactivated foetal bovine serum (FBS), 50IU/ml penicillin, 50μg/ml streptomycin, 1x non-essential amino acids,1mM sodium pyruvate and 2mM L-glutamine. Prior to use, all media and trypsin were pre-warmed to room temperature. Trypsin-EDTA concentrate (x10) was diluted to a 1x solution in balanced salt solution (HBSS) without Ca^{2+} and Mg^{2+}. Confluent cell monolayers were washed in HBSS to remove traces of serum, Ca^{2+} and Mg^{2+}. Trypsin was added to culture flasks (2ml for a T75 flask). Cells were removed by incubation with trypsin at $37^{o}C$ for 1-2 minute, followed by sharp tapping of the flask. To this 4ml of culture medium with 10% FBS was added to halt the action of the trypsin. The cells were then spun down at $1000min^{-1}$ for 4 minutes to remove trypsin and the cell pellet was resuspended in the appropriate volume of growth medium. On passage, non transfected HEK293 were typically split 1:10. The transfected (ADAM33 and Mock) HEK293 cells were typically split by cell number, $1x10^{6}$ cell/T25 flask or $3x10^{6}$cell/T75flask and cultures supplemented with 600mg/ml G418 (Calbiochem (Merck KGaA), Darmstadt, Germany).

Human umbilical vain endothelial cells (HUVEC) were harvested by Dr I Puxeddu from 5 samples by collagenase digestion. Umbilical cords were

collected following ethical approval from Southampton and southwest Hampshire local research ethics committee and informed consent from the donor. The cells were grown on 0.1% gelatin-coated culture flasks in M199 medium supplemented with Endothelial Cell Growth Supplement (ECGS) (30µg/ml), 10% heat-inactivated FBS, 10% newborn calf serum and heparin (0.1mg/ml) 50IU/ml penicillin, 50µg/ml streptomycin, 1x non-essential amino acids,1mM sodium pyruvate and 2mM L-glutamine. Cells incubated at 37^{o}C in a humidified atmosphere with 5% CO_2. All experiments were conducted with HUVECs from passage 2 to 7.

Cell counting

After trypsinization cell clumps were disrupted by aspiration through a 25 gauge needle, in order to obtain a single cell suspension. Cells were counted using the Trypan blue exclusion method. This method is based on the exclusion of dye from live cells whereas the dye can enter non-viable cells and colour them blue as their cell membranes are not intact. 20µl of cell suspension was added to 30µl HBSS and 50µl 0.4% (w/v) Trypan blue. The cells were counted using an improved Neubauer haemocytometer (depth 0.1mm, area 400mm^2). The mean cell number per square was calculated and used to calculate the number of cells per ml of cell suspension by multiplying the mean count by 5 (dilution factor) x 1x10^4.

Transfection

HEK293 cells were transfected with the pCDNA 3.1 vector backbone (figure 2.1) containing full length ADAM33 (without the 3'UTR) (.This construct was a gift from Prof G. Murphy, University of Cambridge.

Transfection using Effectene lipid reagent

HEK293 cells were cultured in 3 wells of a six well dish in D-MEM with 10% FBS until 80% confluent. When 80% confluent, one well was transfected with full length ADAM33pcDNA3.1 and one well with pcDNA3.1 (for mock transfection) and one left as a control. The pcDNA3.1 vector contains the neomycin resistance gene which allowed for selection of transfected cells with the antibiotic G418.

Transfections were carried out using Effectene reagent kit (Qiagen). Transfection media were prepared; 1µg of each DNA was incubated with 8µl of enhancer for 5min at room temperature, then 10µl of effectene was added and incubated for 10min at room temperature and then, 600µl of serum free DMEM was added. Each DNA/Effectene mixture was put on to well cells that had been washed in HBSS and 600µl of serum free D-MEM added. This was incubated at 37^0C and 5% CO_2 for 4 hours. After 4 hours, the media were changed to D-MEM with 10% FBS. After 24 hours, the media were replaced (on transfected and control wells) with DMEM (with 10% FBS) containing 600µg/ml G418 to select for cells transfected with the antibiotic resistance marker. Media were changed every two days and when the control cells had died, the transfected cells were trypsinized and seeded into a six

well dish and cultured in complete DMEM containing 600μg/ml G418 (for stable cells).

Transient transfections were carried out at as above except that cells were lysed into sample buffer 48 hours post transfection.

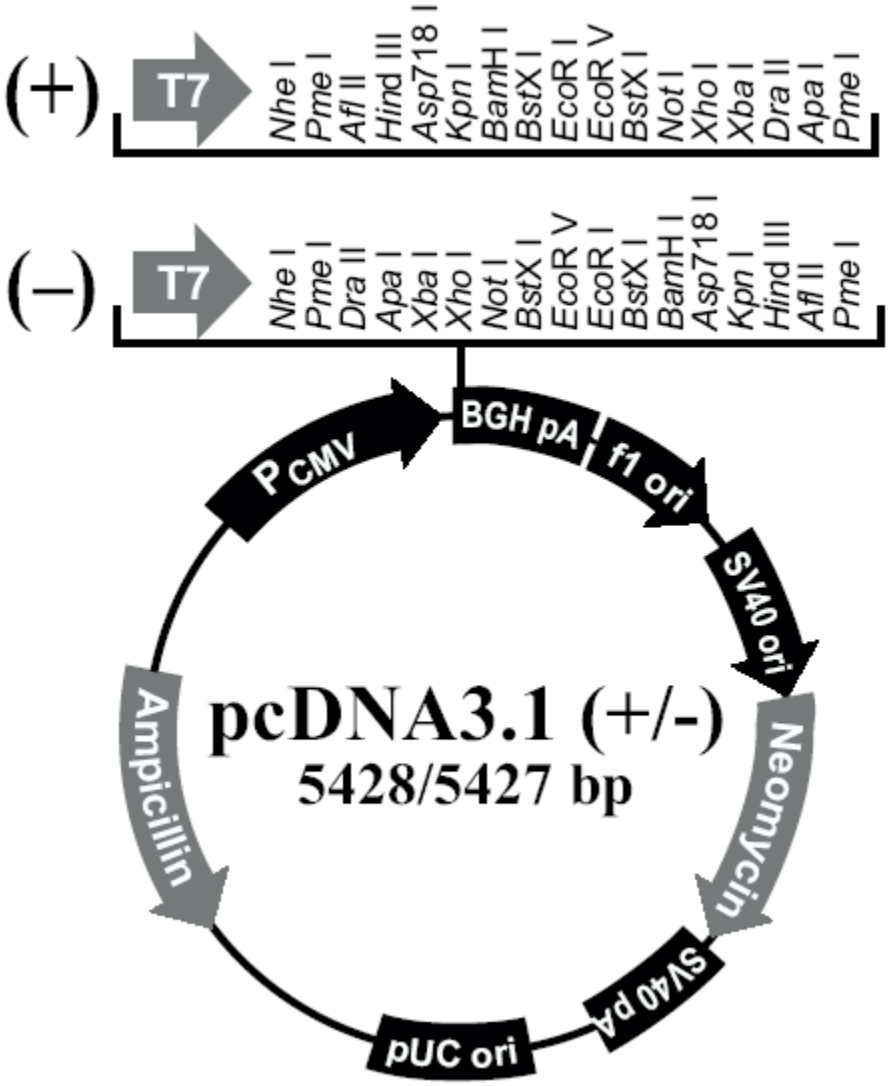


Figure 2.1: Diagram of pcDNA3.1 vector backbone.

Cloning of Transfected Cells

Mock and ADAM33 transfected HEK293 cells were cloned by two rounds of limiting dilution. Cells were seeded into the first row of a 96 well plate and serially diluted across the plate. Plates were observed and photographed. When single colonies could be identified in a well, it was marked and when a confluent monolayer had formed, the cells were removed and serially diluted for a second time. When confluent wells from a single colony were identified on this second round, they were plated into a 12 well plate and grown until confluent and then expanded into a T25 flask. At this stage assays were carried out on these cells.

SDS PAGE Electrophoresis and Western Blotting

Samples were prepared by lysing cells in 1x SDS (reducing) sample buffer (0.31525M Tris HCl pH 6.8, 10% SDS, 25% 2-mercaptoethanol, 0.01% bromophenol blue and 50% Glycerol). Sample buffer was boiled at 95^0C for 10min; and 200µl sample buffer was added per $2x10^6$ cells or 50µl per 40µl of ConA beads see ADAM33 Pull Down Assay section. Cell lysates were sonicated and kept at -20ºC until analysis.

Gels were prepared using the BioRad mini protean II system. 1mm thick SDS-PAGE gels were prepared using 10ml gel separation stock solution (for a 10% acrylamide gel, this was made from 30ml (w/v) 30% acrylamide/0.8%(w/v) bis acrylamide (Bio Rad), 22.5ml 1.5M TrisHCl pH 8.8, 37.1ml dH_2O and 0.45ml 20% (w/v) SDS), 33µl 10% (w/v) ammonium persulphate (AMPS) and 5µl N,N,N',N',-

tetramethylenediamine (TEMED) (Sigma, Poole, UK) were added per 10 ml gel stock (for 2 gels) to initiate polymerisation. Gel solution was immediately poured into the gel frame and overlaid with water saturated isopropanol to exclude oxygen and aid polymerisation. When set, the gels were washed with dH_2O and moisture removed using filter paper. 5ml stacking gel solution was prepared (stacking gel stock 12.5 ml (w/v) 30% acrylamide/0.8%(w/v) bis acrylamide, 25ml 0.5M Tris HCl pH 6.8, 62ml dH_2O and 0.5 ml 20% (w/v) SDS) with 16.7µl AMPS and 3.8µl TEMED. Stacking gel solution added on top of the polymerised separation gel and a 10 or 15 well comb inserted and gel left to set.

The samples to be analysed were defrosted and 20µl per sample applied to the gel along with 5µl of Kaleidoscope Precision Plus (Bio-Rad) protein marker. Separations on 10 or 12.5 % SDS-PAGE gels were run for approximately 1 hour at 160V (constant voltage), in running buffer (0.025M Tris, 0.192M glycine and 0.1% (w/v) SDS pH 8.3)

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After electrophoresis, transfer of the separated proteins onto Hybond P-PDVF membrane (Amersham biosciences) was carried out using electrophoretic transfer for 2 hours at 90V constant voltage as follows: the gel was removed from glass plates and placed into Tris/glycine transfer buffer (25mM Tris, 0.192M glycine, 20% (v/v) methanol, pH 8.0-8.3) for 15min to remove salts and preshrink the gel. A PVDF membrane was cut to size and hydrated sequentially in methanol for 3min, dH_2O for 3min and transfer buffer for 15min. The cassette was assembled with a sandwich of layers comprising, pre soaked scotch brite pad, 2 pieces of pre soaked

filter paper, SDS gel, PVDF membrane, 2 pieces of filter paper and another scotch brite pad. The cassette was placed into the transfer electrode assembly in transfer buffer with an ice pack. The tank was placed on a magnetic stirrer, surrounded with ice. The transfer was carried out using electrophoresis for 2 hours at 90V (constant volts) with the membrane nearest to the anode and the gel nearest the cathode. After transfer, the blots were air dried and stored at + 4^{o}C until analysis.

Blots were rehydrated in methanol and washed in dH_2O, and blocked in blocking buffer (PBS, 0.5% Tween (Sigma, Poole, UK), 5% milk power) for 1 hour on an orbital shaker. Blots were then washed 3 times for 5 min in wash buffer (PBS and 0.5% Tween). The appropriate primary antibodies (see table 1) were then incubated with the blots for 1hour at room temperature. Blots were washed as before and then incubated with the appropriate secondary antibody (see table 1) for 1 hour before washing again. The blots were then treated with ECL Plus western blotting Detection reagent (Amersham Biosciences) which is optimised for used with PVDF membranes. ECL Plus uses the substrate Lumigen PS-3 Acridan which is oxidised by Horseradish peroxidase (HRP) conjugated to the secondary antibody and peroxide, forming acridinium ester intermediates. These react with peroxide under slight alkaline conditions producing sustained chemiluminescence with maximum emission at a wavelength of 430nm. Detection with ECL plus was carried out according to the manufactures instructions. Briefly the reagents were warmed to room temperature. Solutions A and B were mixed at a ratio of 1:40, in this case 2ml solution A with 50µl of solution B for each blot. The blot was drained of excess wash buffer and placed on a sheet of cling film (protein side up) and the solution

immediately added onto the blot. The blot was then incubated for 5min at room temperature, the excess detection solution was drained off and then carefully covered in cling film and air bubbles gently smoothed out. The blot was then placed and secured by tape in a cassette for film exposure. Hyperfilm ECL (high performance chemiluminescence film) (Amersham biosciences) was exposed for the times indicated in each figure legend in a dark room. Hyperfilm ECL was developed using fotospeed PD5 print developer diluted 1:10 with H_2O for 1min and fixed in fotospeed FX20 rapid fixer diluted 1:4 with H_2O for 1min (Fotospeed, Corsham, UK), the film was then washed for 1min in H_2O, air dried and the protein standard markers were labelled by overlaying the dry film on to the blot in the cassette and marking the positions of the coloured protein standards with a permanent marker pen.

Table 2.1: Western Blot Antibodies

Primary Antibody	Species and isotype	Dilution	Supplier	Secondary Antibody	Dilution Secondary	Supplier
RP1 (prodomain of ADAM33)	Rabbit IgG Polyclonal	1:5000	Abcam Cat# ab39190	Goat anti Rabbit IgG HRP	1:10000	Jackson laboratory
RP2 (MP domain of ADAM33)	Rabbit IgG Polyclonal	1:5000	Abcam Cat# ab39191	Goat anti Rabbit IgG HRP	1:10000	Jackson laboratory
RP3 (cytoplasmic domain of ADAM33)	Rabbit IgG Polyclonal	1:5000	Abcam Cat# ab39193	Goat anti Rabbit IgG HRP	1:10000	Jackson laboratory
B-Actin	Mouse IgG Monoclonal	1:20000	Sigma-Aldrich	Rabbit anti mouse IgG HRP	1:10000	DAKO

Additional information for RP antibodies

RP1 (ab39190) was raised against a synthetic N terminal peptide from human ADAM33. (peptide sequence unavailable from Abcam)

RP2 (ab39191) was raised against a synthetic peptide from the N terminal region of active ADAM33. (peptide sequence unavailable from Abcam)

RP3 (ab39193) was raised against a synthetic peptide from human ADAM33 based on the cytoplasmic domain. (peptide sequence unavailable from Abcam)

Methylene Blue Proliferation Assay

Methylene Blue is a basic dye that is positively charged at pH 8.5. It binds electrostatically to negatively charged nucleic acids and some charged groups in proteins. By lowering the pH to less than 2 using HCl the dye is liberated, by causing the acidic groups to become protonated. This elution step must be carried out in a solution of ethanol and 0.1M HCl (1:1) to ensure there is only one absorption peak at 650nm. Ethanol prevents the formation of methylene blue dimmers at low pH (Oliver *et al* 1989). The absorbance at 650nm can then be used as a surrogate measure of cell number.

Cells were plated out at $6x10^4$ cells/ml in 96 well plates with the appropriate media. In some experiments the wells were pretreated with collagen (Vitrogen®100 diluted 1:100 with dH_2O), 100µl per well for 1hour at room temperature to coat the wells. One plate of cells had the media removed and each well fixed in 100µl formal saline (9g NaCl, 100ml 40% formaldehyde, 900ml H_2O) after 4 hours incubation at 37^oC and 5% CO_2 to provide data for the initial seeding density. The remaining plates were left in the incubator and one plate removed and fixed each day up to 5 or 7 days. When all the plates were fixed, the formyl saline was removed from each well and the plates air dried. When dry, 100µl of methylene blue (1% w/v methylene blue in 10mM borate buffer pH 8.5) was added to each well and incubated at room temperature for 30min. The dye was removed from the plates and blotted onto tissue paper to remove excess dye. The plates were then washed in water until the water ran clear. The plates were blotted on to tissue paper to remove excess moisture and the plates were left to air dry upside-down. When dry, the bound dye was eluted

with 1:1 Ethanol: 0.1M HCl, 100µl per well. Wells containing 100µl of elution buffer were similarly processed as blanks. The absorbance values on each plate were read at 630nm on a Multiskan Ascent plate reader. Calibration curves were carried out using triplicate wells of, cells seeded into 96 well plates at the following densities: $2x10^6$, $1x10^6$, $5x10^5$, $1x10^{5,}$ $5x10^4$, $1x10^4$, $5x10^3$ and $1x10^3$ per ml. Cells were incubated at 37^{o}C, 5% CO_2 for 4 hours, and fixed in formal saline overnight. Following Methylene blue treatment and elution the absorbance of each well was determined. A typical standard curve showing A_{630} vs cell number (assuming 100% plating efficiency) is shown in figure 2.2.

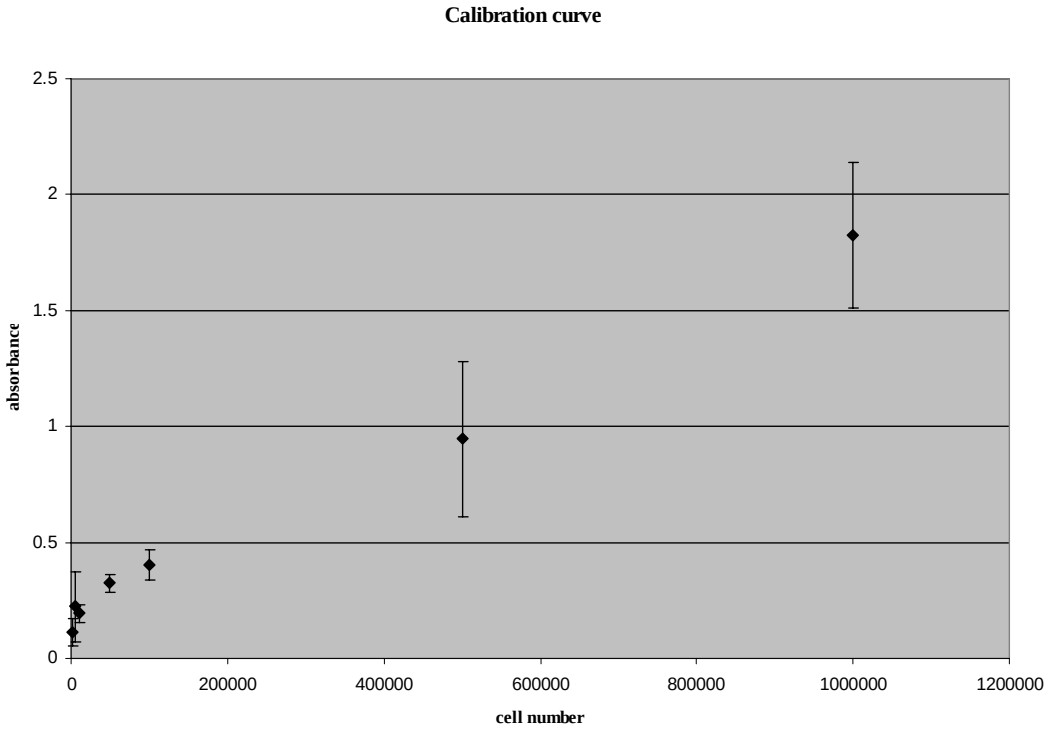


Figure 2.2: A typical standard curve was generated using ADAM33 transfected cells seeded at a series of known densities in a 96 well plate. Cells were allowed to adhere for 4hours and then fixed in formyl saline overnight. The plate was treated with Methlyene blue, eluted and absorbance readings were carried out at 630nm on a Multiskan Ascent plate reader.

CellTiter 96® AQueous One Solution Proliferation Assays

CellTiter 96 (Promega) is a solution that contains the tetrazolium compound MTS that can be reduced by cells into a soluble coloured formazan product. This change is caused by NADPH or NADH produced by dehydrogenase enzymes in cells that are metabolically active (viable cells). This assay was carried out directly in the culture media of adherent or suspended cells. Calibration curves were carried out

using cells seeded into 96 well plates using 100µl per well in triplicate along with media only controls at the following densities: $2x10^6$, $1x10^6$, $5x10^5$, $1x10^5$, $5x10^4$, $1x10^4$, $5x10^3$ and $1x10^3$ per ml. The cells were incubated for 4h to allow them to adhere to the plate. 20µl of CellTiter 96® solution was added to each well and incubated for 1 hour, the plate was then read at 492nm on a Multiskan Ascent plate reader. A typical standard curve is shown in figure 2.3 For proliferation assays ADAM33 transfected and mock transfected HEK293 cells were seeded at a density of $5x10^4$ cells/ml ($5x10^3$ cell per well). Each condition was tested in triplicate or quadruplicate in each 96 well plate. Several plates were made up for analysis of time points up to 144h. After 4 hours 1 plate was incubated with 20µl of CellTiter 96 for one hour and the absorbance read, this was repeated at each time point.

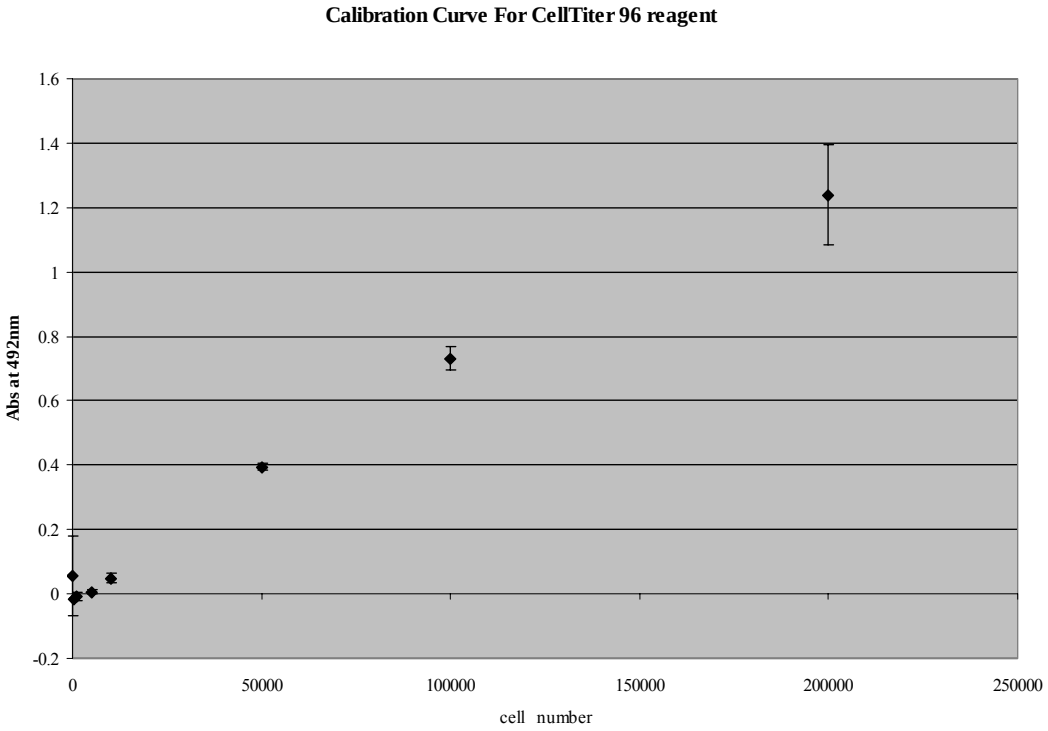


Figure 2.3: A typical standard curve using ADAM33 transfected cells seeded at series of known densities in a 96 well plate. Cells were allowed to adhere to plastic for 4hours and 20µl CellTiter 96 (Promega) added to culture medium for 1hour the plate was then read at 492nm on a Multiskan Ascent plate reader.

Soluble ADAM33 Pull Down With ConA beads

Concanavalin A-Sepharose® 4B beads (Sigma) bind glycosolated proteins with a binding capacity of 20-45µg/ml. These were used to pull down soluble ADAM33 from the supernatants of ADAM33 clones using supernatant from mock transfected clones as a negative control. The supernatant was adjusted to contain 0.5M NaCl and 20mM Tris pH 7.4; protease inhibitors (without EDTA) were added to achieve a 1x concentration (complete protease inhibitor cocktail tablets without EDTA (Roche)

were dissolved in dH_2O (1 tablet in 2ml) to produce an x25 stock solution, according to the manufacturer's instructions. ConA beads were washed and activated using 5 x volume wash buffer (0.5M NaCl, 5mM $MgCl_2$, 5mM $CaCl_2$ and $MnCl_2$ in dH_2O). Beads were spun down and re-suspended in equilibration buffer (20mM Tris HCl and 0.5M NaCl at a pH of 7.4) at a ratio of 1:1. 20µl of beads (i.e. 40µl of beads in equilibration buffer) were added per ml of supernatant unless otherwise stated. The supernatant/bead samples were rotated overnight (16h) on a blood mixer. The samples are spun down and the supernatant discarded. The beads were washed twice in cold equilibration buffer and were then washed in 20mM Tris HCl pH7.4 to remove excess salt. The beads were then lysed into SDS sample buffer (0.31525M Tris HCl pH 6.8, 10% SDS, 25% 2-mercaptoethanol, 0.01% bromophenol blue and 50% methyl-α-D-mannopyranoside (instead of Glycerol)) for analysis by western blot (see SDS PAGE Electrophoresis and Western Blotting section) or the protein was eluted from the beads using the sugar methyl α-D-mannopyranoside (0.5M methyl α-D-mannopyranoside in 20mM Tris HCl and 0.5M NaCl at a pH of 7.4) for assay of protease activity (see Activity assays of Soluble ADAM33 section). All the procedure involving ConA beads were carried out at 4^oC.

Activity assays of Soluble ADAM33

Fluorescence Resonance Energy Transfer (FRET) Peptide cleavage assay

A FRET peptide cleavage assay is an assay used to assess enzyme activity, in this case the soluble form of ADAM33 which was postulated to comprise the MP-Dis-EGF-Cys rich domains. A short peptide known to be cleaved by the proteinase of

interest is modified so one terminus is linked to a fluorescent reporter dye and the other is labelled with a quencher dye. When the peptide is intact the quencher absorbs the fluorescence emitted by the reporter dye, so little reporter fluorescence is detectable. When the peptide is incubated with the potential proteinase and is cleaved, the peptide is split into two and the reporter dye and quencher dyes are separated. This separation of the dyes prevents the quencher from absorbing energy from the reporter, allowing the reporter dye's fluorescence to be detected. A FRET peptide cleavage assay based on the study by Zou *et al.* was developed by Yun Pang and used to assess the activity of soluble ADAM33 released into the supernatant of transfected HEK293 clones eluted from ConA beads. All FRET peptide assay measurements were made in real time using the Bio-Rad iCycler, using the FAM filter (490nm) and required a well factor plate to be run before the reaction plate to ensure that the Bio-Rad iCycler ran correctly. Typically 20µl reactions containing 4.4µM FRET peptide (DABCYL-YRVAFQKLAE(FAM)K-NH_2) were incubated with soluble ADAM33 (pulled down using ConA from the supernatant of HEK293 clones) at 37°C in assay buffer containing 20mM HEPES pH7.0, 0.5M NaCl, 10mM $CaCl_2$, 10µM $ZnCl_2$, and 0.2mg/ml BSA. Recombinant ADAM33 MP domain (a gift from Yun Yun Pang) was used as a positive control. Reactions were carried out in typical polystyrene PCR plates used for qPCR (Abgene), sealed with transparent plate seals. The enzyme activity was determined by plotting the background subtracted relative fluorescence units (RFU) against time and calculating the rate at which RFU changes from a line of best fit. Rates were determined in the initial linear phase of the assay.

HUVEC differentiation in response to ADAM33 clone supernatant

96-well-plates were pre-coated with 50µl of Growth factor reduced Matrigel Matrix solution (BD Biosciences, MA, USA) and the gel was allowed to solidify at 37 °C for 1 hour. Endothelial cells were then plated at a density of 8×10^3 cells/well and further incubated with 100µl supernatant from ADAM33 HEK293 clones with mock transfected clones diluted 1:2 with M199 medium (1%FBS); M199 medium (1% FBS) alone was used as negative control and rhVEGF (10 ng/ml) (R&D Systems) in M199 was used as a positive control. After 18-hours, the 3-D organization was examined using an inverted, phase-contrast microscope and recorded digitally. The topological parameters of the capillary mesh (mesh areas, vessel length and mesh branching points) were measured by computed image analysis as previously described Guidolin *et al* 2004.

Densitometry

The ADAM33 TGF-B time and dose response blots were scanned on a flat-bed scanner. And densitometry was carried out in Photoshop. The grayscale option was selected and the image colours inverted. The lasso tool was used to draw a line all the way around the edges of each band and the histogram screen generated. The 'mean' and 'pixel' values were recorded. A measurement of the film background was also taken.

Data analysis: The mean value was multiplied by the pixel value for each band.

Giving the absolute intensity of each band. The relative intensity was determined by dividing the absolute intensity of each sample band by the absolute intensity of background (as no positive sample was available for comparison).

Statistics

T-tests were carried out using Microsoft Excel. The tests for proliferation assays and population means were compared ADAM33 cells vs Mock cells. Densitometry analysis tests were compared treated samples against untreated sample for each time point.

Chapter 3 Results

Effect of Transfected Full Length ADAM33 on the phenotype of HEK293 cells.

It is known that polymorphisms in the *ADAM33* gene confer susceptibility to asthma and bronchial hyper-responsiveness. The normal function of ADAM33 is as yet unknown. Work to characterize the function of ADAM33 has revealed that it is has proteolytic active and contains the ADAM consensus sequence HEXXHXXGXXH that predicts proteolytic activity. As yet a physiological substrate has not been found, although Zou *et al* have undertaken an investigation to find a substrate of ADAM33. Using a panel of peptides, Kit-1 and APP were identified as potential substrates, however when the complete proteins were tested little or no shedding was detected Zou et al 2004. ADAM33 could also have a role in cell adhesion through the disintegrin domain, which has been shown to interact with the integrin $\alpha_9\beta_1$ L Bridges et al 2005. In a mouse knockout model of ADAM33 there was no change in survival or development of the ADAM33 null mice, nor did it alter allergen induced responsiveness in the airways compared to wildtype C Chen *et al* 2006. The following experiments have been carried out to determine the phenotypic effect of the over expression of full length ADAM33 on HEK293 cells that do not express endogenous ADAM33 and to relate these findings to the possible functions of ADAM33.

In asthma there is an increase in smooth muscle mass and the number of fibroblasts in the airways. Since ADAM33 is expressed in these cell types it could be that ADAM33 affects the proliferation rate of these cells. My hypothesis is that the over-

expression of full length ADAM33 in HEK293 cells will alter the growth phenotype of these cells.

Generation of Transfected HEK293 cells

HEK293 cells can be transfected efficiently so have been utilized in these experiments. These cells are of epithelial origin and TaqMan analysis performed by Hans-Michael Haitchi found that ADAM33 is not endogenously expressed by these cells.

This is consistant with the studies of Van Eerdewegh *et al* 2002 and Umland *et al* 2004 found that ADAM33 was expressed in bronchial smooth muscle and fibroblasts but not in epithelial cells. Both transient and stable transfections were carried out on HEK293 cells.

Transient Transfections

Initial experiments were performed using transient transfections with an expression vector encoding full length ADAM33 under the control of a CMV promoter, which was used to drive constitutive expression of recombinant ADAM33 in order to determine if the full length ADAM 33 plasmid could be successfully transfected into HEK293 cells (virally transformed). Western blotting was carried out to examine ADAM33 protein expression. In an initial transient transfection of virally transformed HEK293 cells, two bands were observed at 100kDa and 120 kDa see fig 3.1. This indicates that ADAM 33 was being cleaved from the pro-protein (120kDa) to the processed form that may be on the cell surface (100kDa).

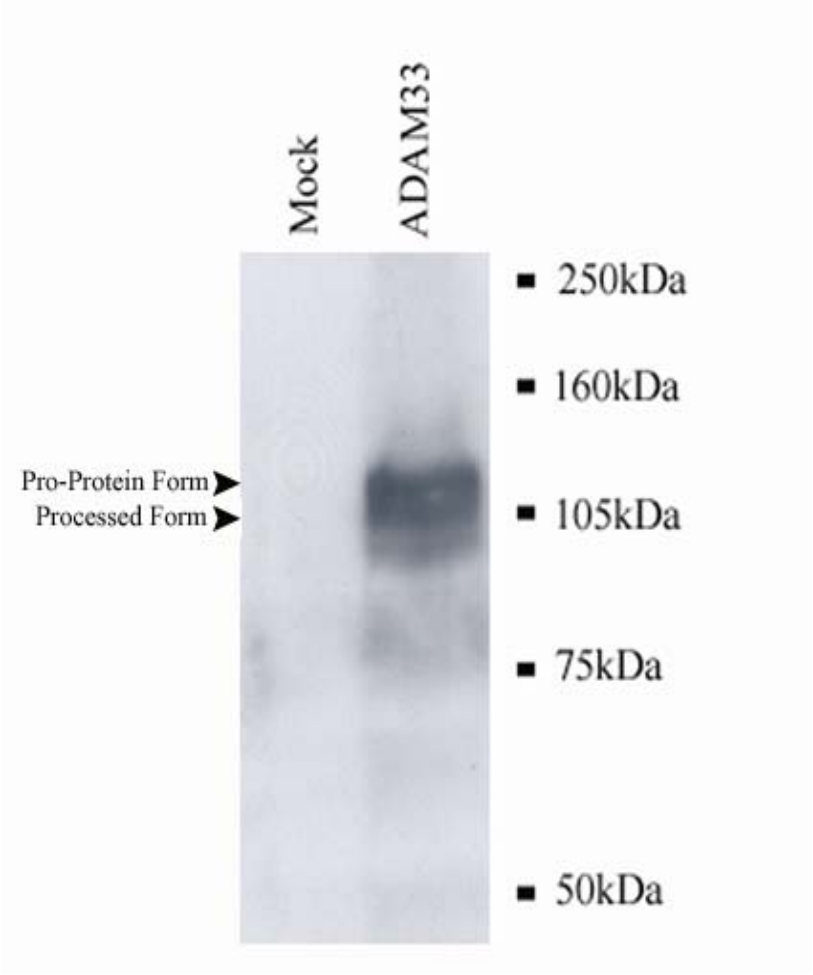


Figure 3.1: Transient Transfection of ADAM33 was Successful.

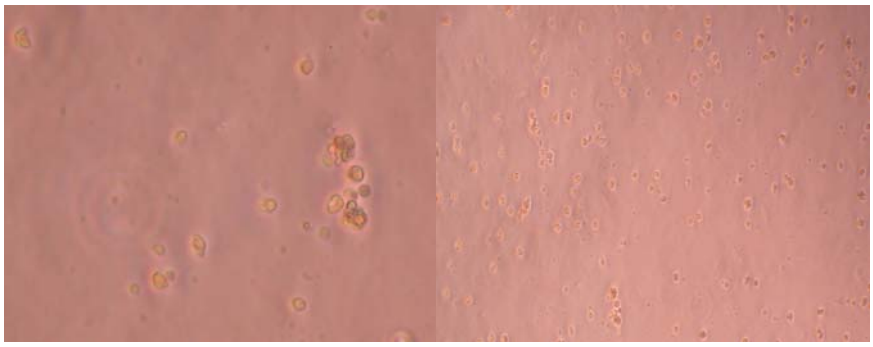
Western blot of cell lysates from transiently transfected HEK 293 cells (virally transformed) showing a double band at approximately 120kDa and 100kDa. The Blot was performed using the RP3 antibody to the ADAM 33 cytoplasmic domain.

Production of Cell Lines Stably Expressing ADAM33

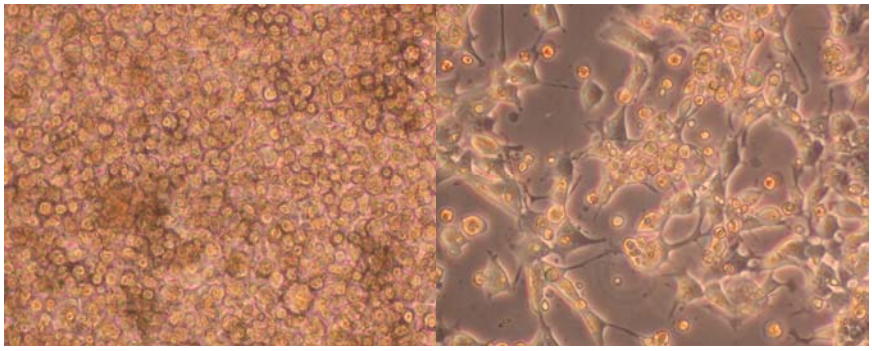
In order to have a reproducible and convenient supply of cells stable transfectants expressing full length ADAM33 cDNA and mock transfected (empty plasmid vector) HEK293 cells were produced after treatment of HEK293 cells with G418. In this case the HEK293 cells used were not the virally transformed line used in transient transfections as these were already G418 resistant. As can be seen in fig 3.2a, non-transfected HEK293 cells were killed by G418. In fig 3.2b, the cells transfected with the empty pcDNA3.1 plasmid vector (mock transfected cells) and in fig 3.2c, cells transfected with full length ADAM 33 were similar and were not all killed by treatment with G418, as successfully transfected cells are resistant to the antibiotic. There were areas of the well that had more dense cell growth and others that showed growing cells with projections/processes, as well as regions of dead cells (fig 3.2).

The stable transfected cells were cultured in the presence of G418 and a sample of cells taken for western blotting to confirm the presence or absence of ADAM33 protein. ADAM33 protein was detected in the transfected full length ADAM33 HEK293 cells but not the mock transfected HEK293 cells using the RP3 antibody which recognises the cytoplasmic tail of the ADAM33 protein and can therefore detect both the processed and unprocessed forms of ADAM33. An anti-β-Actin antibody was also used to show that the amount of protein loaded onto the gels was

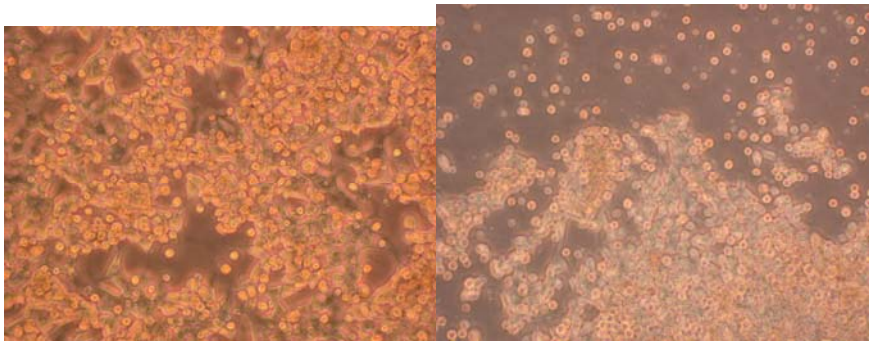
similar (fig 3.3). The transfected cells were cultured in the presence of G418 and samples taken for western blotting up to P13 (passage numbers start post



A - Control well



B - Mock transfected well



C - Full length ADAM33 transfected well

Figure 3.2: Selection of ADAM33 and Mock Transfected Cells using G418

Photo micrographs of HEK293 cells after treatment with G418 to select for successfully transfected cells, A - images of control (non-transfected well) at two different positions within one well showing

complete loss of cells caused by the G418 selection, B - images of mock transfected cells in different areas of the same well, C- images of ADAM 33 transfected cells in different areas of the same well.

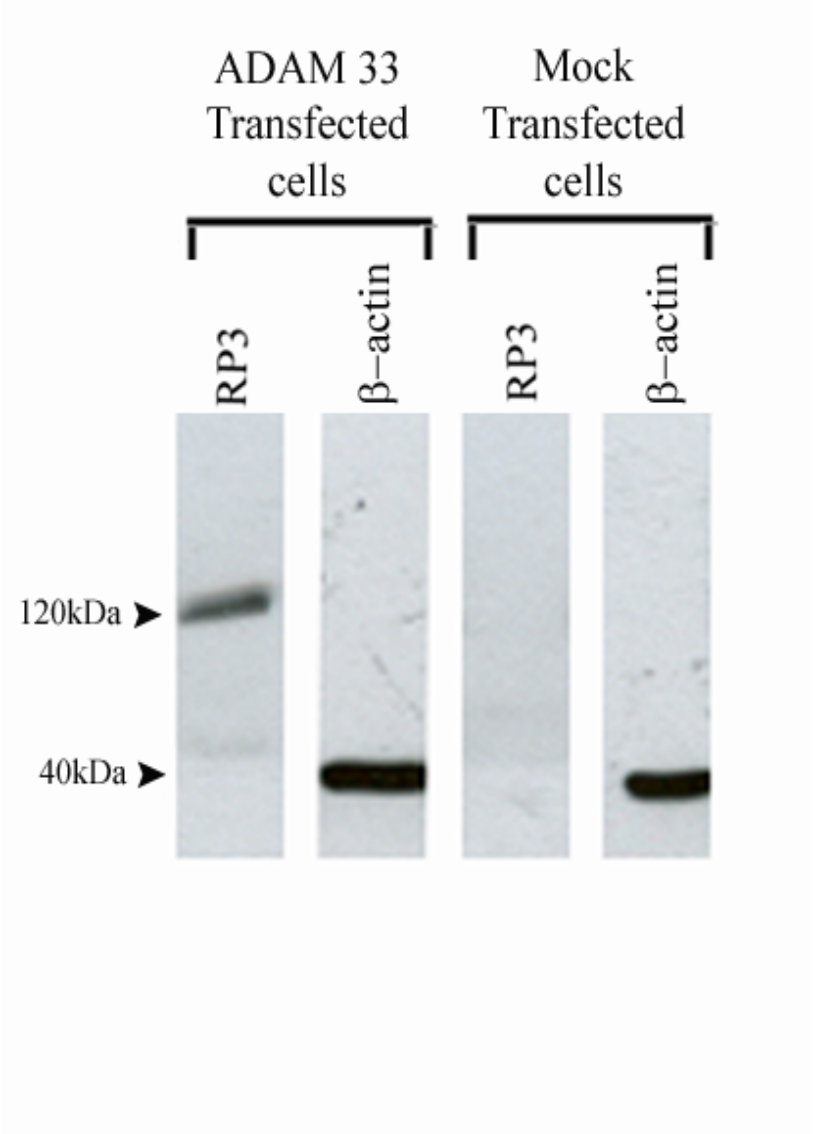


Figure 3.3: Expression of ADAM33 by Stably Transfected HEK293 Cells

Western blotting was used to determine the success of the transfection of HEK293 cells with full length ADAM33 cDNA. ADAM33 was seen in cell lysates from HEK cells transfected with full length ADAM33 cDNA but not in the Mock transfected cells by western blotting with RP3 antibody (cytoplasmic tail). Equivalent protein loading was verified using an anti-β-actin antibody.

transfection and selection). In the ADAM33 transfected cells, it was observed that up to P4 there was an increase in ADAM33 protein levels but not β-actin protein levels. From P5 to 11 there was a gradual loss of ADAM33 protein but not β-actin, with only a very faint band a P11. However at P12 and 13 the ADAM33 protein levels appear to have recovered to similar levels to samples taken at P6 and 7 (fig 3.4). However only a single ADAM 33 band was detected with the approximate molecular weight of 120kDa using the RP3 antibody.

Initial Observations of Transfected Cells

As the effects of ADAM33 overexpression are unknown, cells were observed for any change in phenotype between the HEK293 cells expressing full length ADAM33 and mock transfected HEK293 cells.

Morphologically no difference was observed between the two transfected cell cultures and indeed no difference was seen when compared with non transfected HEK293 cells as can be seen in figure 3.5. The only differences seen were possible differences in growth rate. It was observed by microscopy that in the first passage, the growth of ADAM33 transfected cells was faster when compared to mock transfected cells. In addition to this, direct cell counts were taken on passage of the cells which showed when ADAM33 and mock transfected cells were seeded at the same density the ADAM33 cells grew to a greater density at passage 2-5 but this effect was lost by passage 6 (figure 3.6).

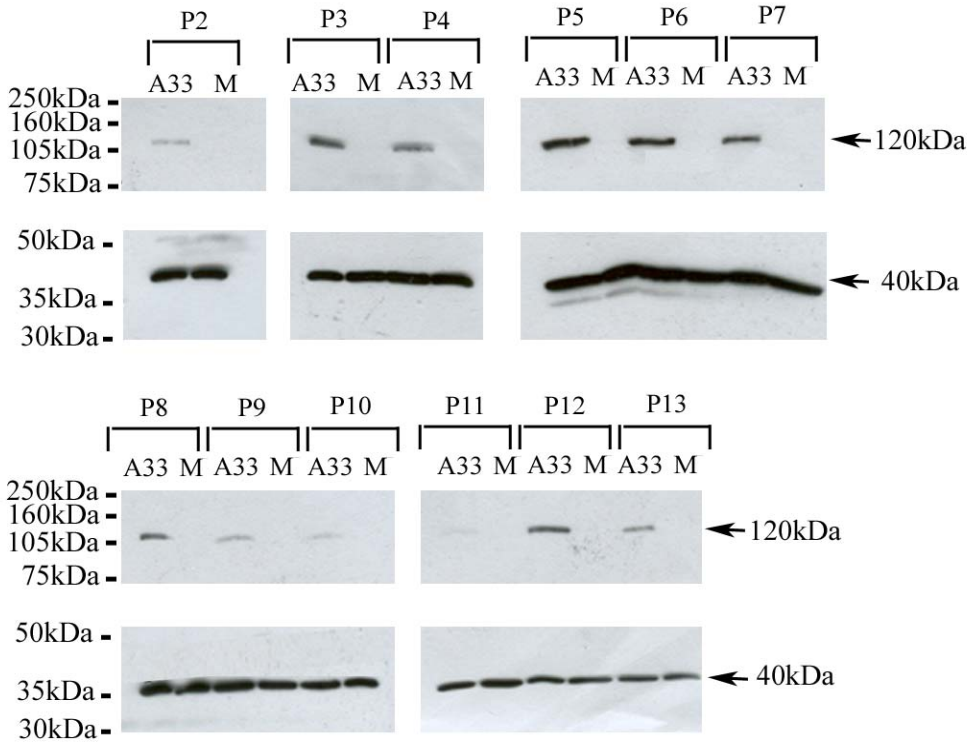
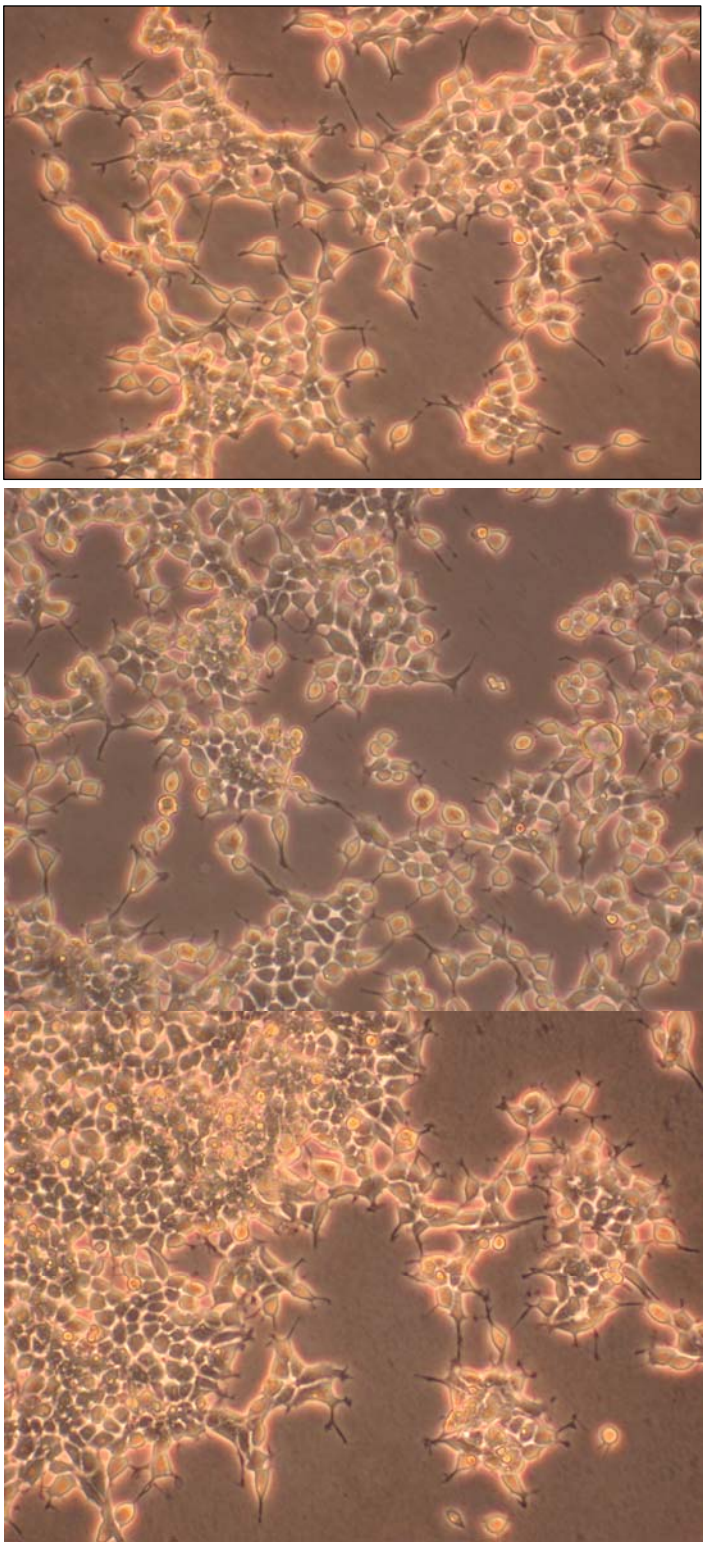


Figure 3.4: Expression of ADAM33 in HEK293 Cells stably transfected with full length ADAM33.

After HEK293 cells were transfected with full length ADAM33 or mock transfected with pcDNA3.1 empty vector and selection with G418 (passage numbers used start from this point) expression of ADAM33 was observed over time and compared to the mock transfected cells. Western Blot analysis using RP3 (cytoplasmic tail antibody) was used to determine relative protein levels over time from P1 to P13 (120kDa band), β-actin was used as a loading control (40kDa band). Samples contained $5x10^5$ cells per 100µl of SDS sample buffer.



A: Untransfected HEK293 cells

B: Mock transfected HEK293 cells

C: ADAM33 tansfected HEK293 cells

Figure 3.5: Full Length ADAM 33 does not Alter the Morphology of HEK293 Cells.

Photographs of the non-transfected, mock transfected and ADAM33 expressing HEK293 cells at P3 to examine any morphological effects resulting from the presence of ADAM33. The morphology of transfected cells does not alter in the presence of ADAM33.

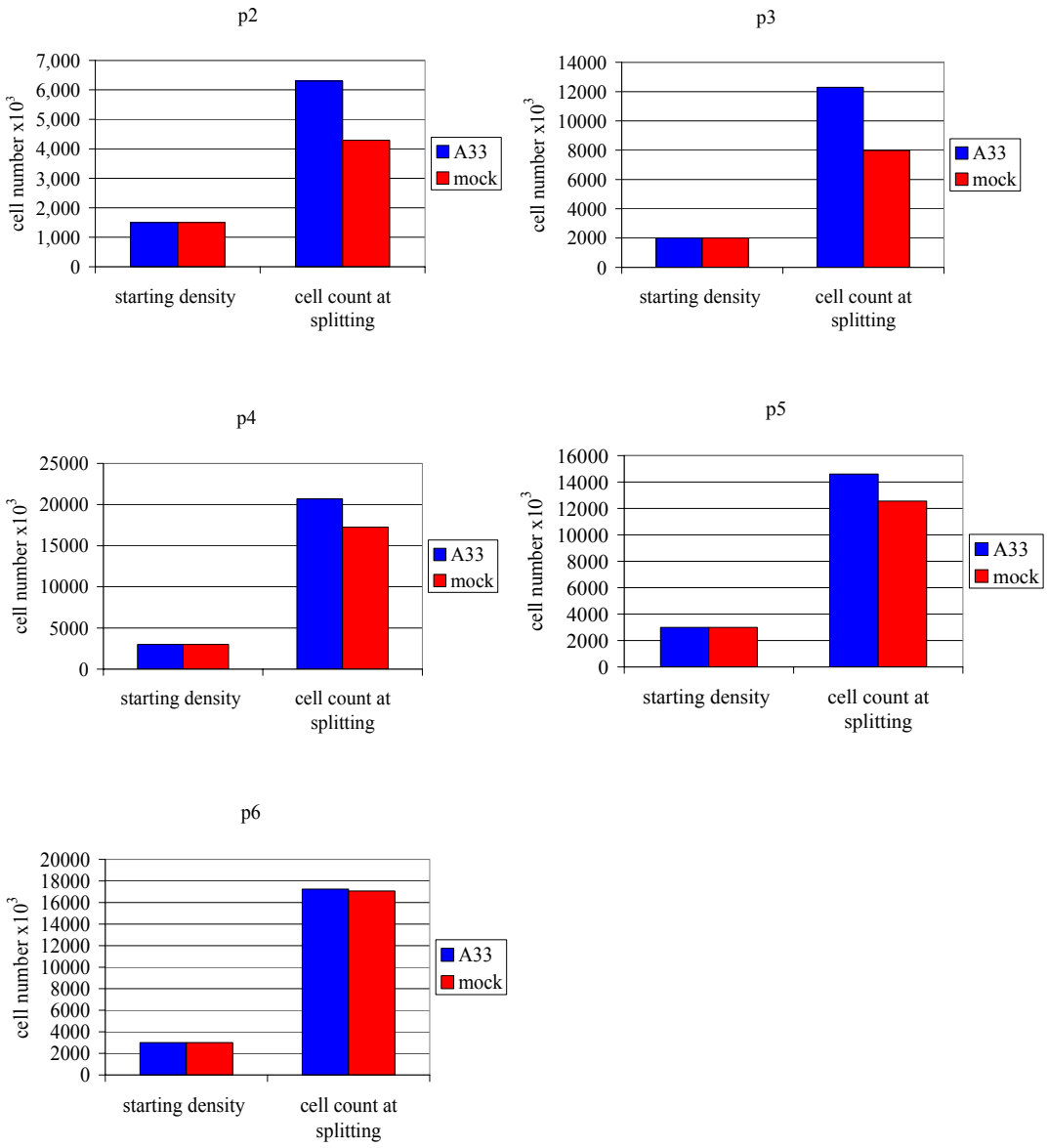


Figure 3.6: Direct Cell Counts of Stably Transfected HEK293 Cells.

Cell counts included here were from ADAM33 and Mock transfected HEK293 cells seeded at the same time using identical starting densities. Passage numbers are post selection with G418. Cell counts at P2 and P3 cells were taken at 5 days and at P4, 5 and 6 were grown at 7 days. Cells at P2 were grown in T25 flasks and P3 to P6 were grown in T75 flasks. N=1

Proliferation Assays

Methelyene Blue Assay.

Since it was observed that ADAM33 transfected cells may grow faster than the mock transfected cells, methylene blue assays were carried out to determine the proliferation rates of the different cells. Initial methylene blue assays were carried out at P6 and P7 (figure 3.7). Experiments were also performed to determine if pre-coating of plates with collagen was necessary and the effect of serum free conditions were examined. Serum free conditions were compared to serum containing conditions to assess any autocrine contribution of the HEK293 cells with or without ADAM33. It was found that there was no difference in rate of proliferation between the ADAM33 transfected cells and the mock transfected cells ($p=0.3$), which confirms the observations in figure 3.6. Under serum free conditions on plastic the proliferation rate dropped off after day 3 until day 5 whereas when the cells were plated on collagen this was not observed. It seems that when grown with serum there is no need for the cells to be on collagen coated plates.

The assays were repeated with a new set of stably transfected HEK293 cells and were carried out from P1. At P1 an increase in proliferation was observed in ADAM33 transfected cells compared to mock transfected cells in DMEM and Ultraculture figure 3.8a. However at P2 this was no longer observed figure 3.8b ($p=\geq 0.05$).

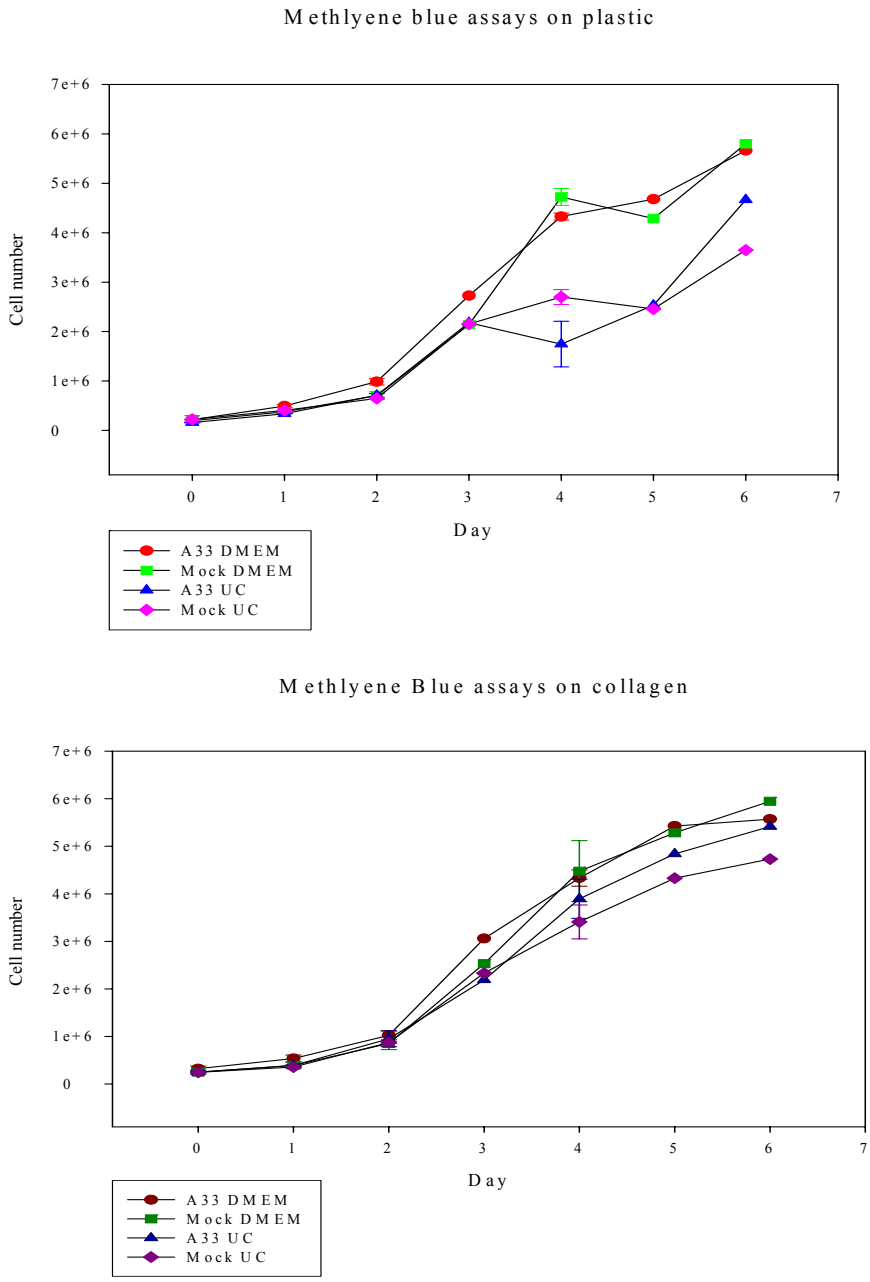
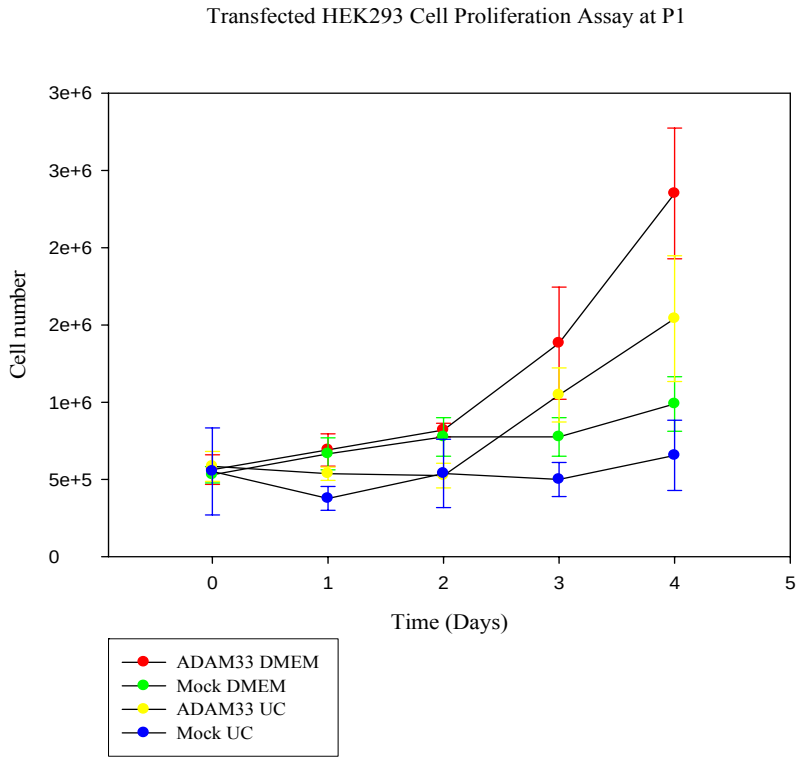


Figure 3.7: Proliferation Assays on ADAM33 Transfected Cells Grown on Plastic or Collagen

Proliferation assays using HEK293 cells transfected with or without ADAM33. Data are means of all numbers obtained at P6 and 7 with standard error. ADAM33 and mock transfected HEK293 cells were grown in either DMEM or Ultraculture medium. And either on plastic (top panel) or on collagen coated plastic (bottom panel).



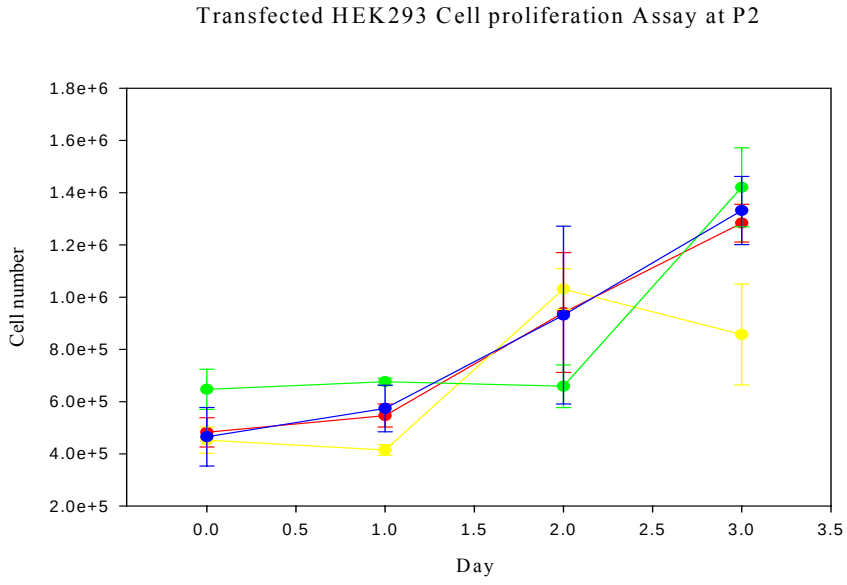


Figure 3.8: Proliferation Assays with Early Passage ADAM33 expressing cells

Methelyene blue proliferation assays at P1 (A) and P2 (B). At P1 the cells transfected with ADAM33 appeared to proliferate faster than the mock transfected cells, but at P2 there was little difference between the two.

CellTiter 96 one solution proliferation Assay

As the HEK293 cells were not very adherent there was concern over the reliability of the methlyene blue assay which involves washing and fixing the monolayer this led to evaluation of theCellTiter 96 one solution assay (promega), as it can be carried out directly in the culture medium and therefore eliminates the problem of cells being lost in washing and fixing stages. CellTiter 96 is a solution containing a tetrazolium compound MTS that is reduced by cells into a soluble coloured formazan product. It is thought that this change is caused by NADPH or NADH produced by dehydrogenase enzymes in cells that are metabolically active (Promega Technical Bulletin TB245).

Two series of proliferation assays were carried out on two batches of ADAM33 and mock Transfected HEK293 cells. Both sets came from frozen stocks stored in liquid nitrogen. In the first set of assays (figure 3.9) it was found that with P5 cells grown in serum containing DMEM, the ADAM33 HEK293 cells grew approximately 3 times faster than the mock HEK293 cells over 96 hours ($p=\leq 0.04$). The same pattern was seen when the cells were grown in Ultraculture with the ADAM33 cells growing approximately twice as fast as the Mock cells ($p=\leq 0.05$), though both grow more quickly than the mock cells in DMEM (fig 3.9a). At P6 additional time points were added to determine when a plateau was reached. Up to 48 hours the cells were in a lag phase and cells did not enter exponential growth until 72 hours which lasted until 120 hours, though the rate of proliferation of the ADAM33 cells dropped at 96h. At 144h, the cell number under all conditions declined, most likely because some of the cells died as there were no media changes during the experiment. The

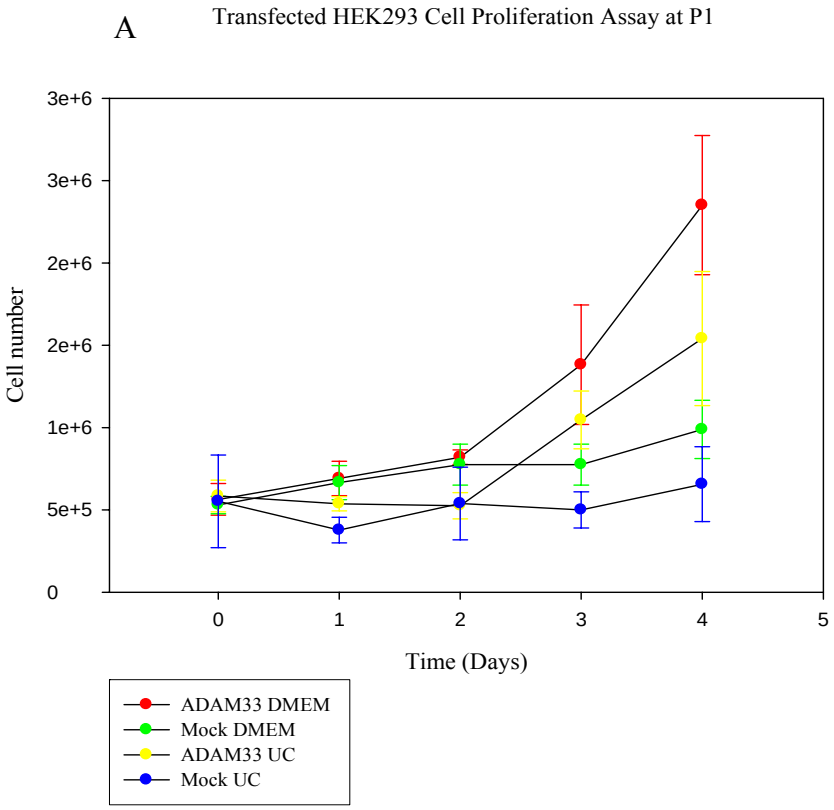
ADAM33 HEK293 cells again proliferated more quickly than the mock cells in DMEM (Fig 3.9b) after 72hours ($p=\leq 0.002$). At P7 there was little difference between the ADAM33 HEK293 cells and the mock HEK293 cells($p=\geq 0.32$). When these data were compared to the P6 data, the overall cell numbers in the ADAM33 cells have fallen, indicating that the proliferation rate of the ADAM33 cells was slower for the cells at P7 (Fig 3.9c). At P10 the ADAM33 cells in DMEM and Ultraculture were not proliferating more quickly than the mock cells as at P7 (Fig 3.9d). But there was a diffrence beween the ADAM33 cells and mock cells in both DMEM and Ultraculture ($p=\leq 0.03$) after 48hours.

A second batch of transfected cells were taken from stock supply so earlier passage number cells could be tested. This set of cells was tested from passage 3 to passage 6. At P3 the mock HEK293 cells appeared to proliferated more quickly than the ADAM33 cells and follow the same pattern under both conditions (Fig 3.10a) but this was no significant ($p=\geq 0.1$). At P4 the mock cells in DMEM were proliferating more quickly than any of the cells under the others conditions (Fig 3.10b) ($p=\leq 0.01$)and at P5 the same pattern was followed (Fig 3.10c) ($p=\leq 0.03$) in DMEM. At P6 the rates of proliferation were very similar up to 72h, though the mock cells were slightly faster compared to the ADAM33 cells under the same condition. At 96 hours the Mock cells in Ultraculture were above the others. But theses findings were not significant ($p=\geq 0.12$).

Overall the first set of cells show that at P5 and P6 the ADAM33 cells grow more quickly and that this rate is lost at P7 and P10 and was reduced to similar cell

numbers as the Mock cells. The second set of cells does not give the same picture; the overall cell numbers are comparable to the mock cells in the first set at the same time points.

A


Transfected HEK293 Cell Proliferation Assay at P1
Cell number
3e+6
3e+6
2e+6
2e+6
1e+6
5e+5
0
0
1
2
3
4
5
Time (Days)
ADAM33 DMEM
Mock DMEM
ADAM33 UC
Mock UC

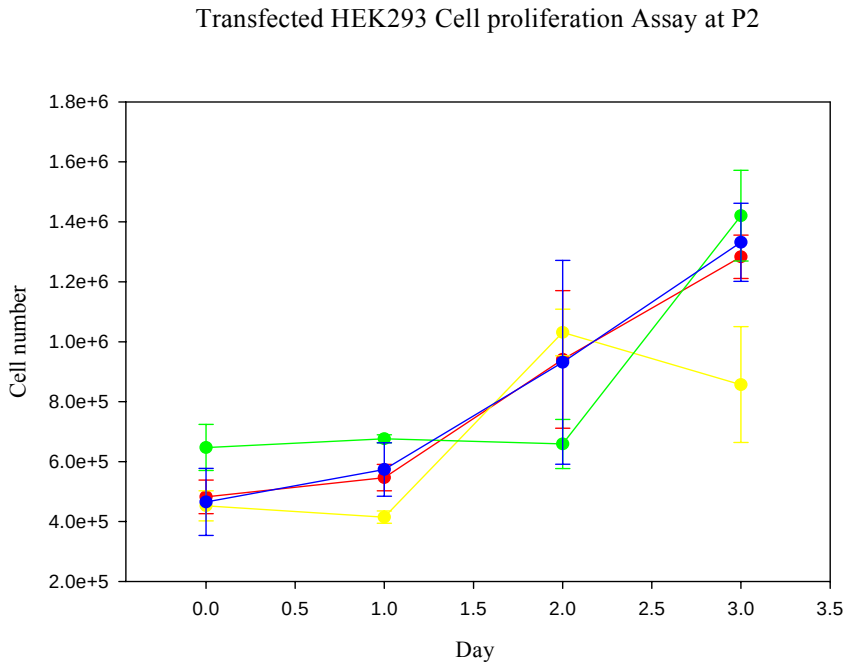
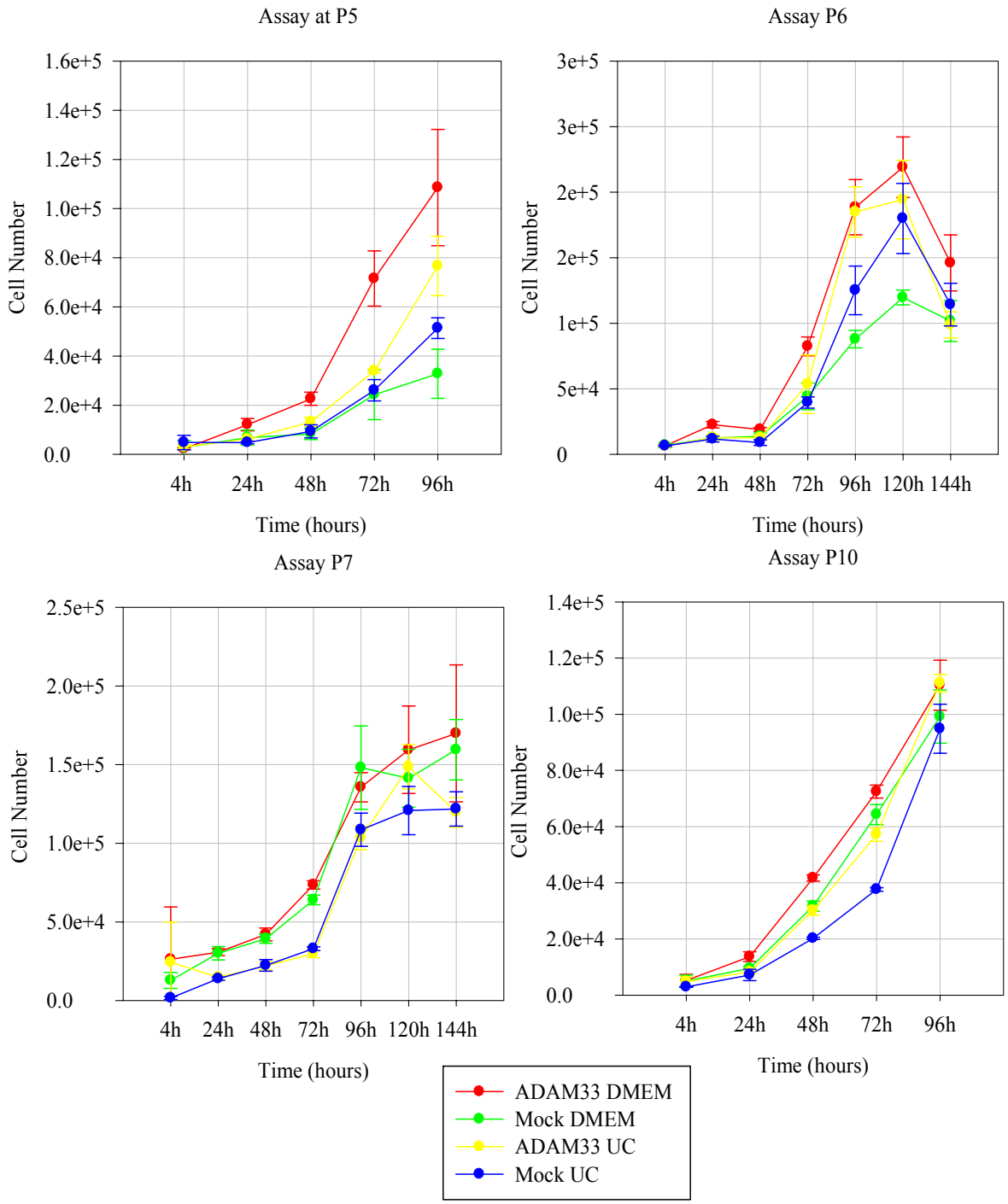


Figure 3.8: Proliferation Assays with Early Passage ADAM33 expressing cells

Methelyene blue proliferation assays at P1 (A) and P2 (B). At P1 the cells transfected with ADAM33 appeared to proliferate faster than the mock transfected cells, but at P2 there was little difference between the two.



3.9: Proliferation Assay on First Batch of Transfected Cells

Proliferation assays carried out on ADAM33 and Mock transfected cells at P5-7 and P10 using CellTiter 96 one solution. A P5 cells, B P6 cells, C P7 cells, D P10 cells.

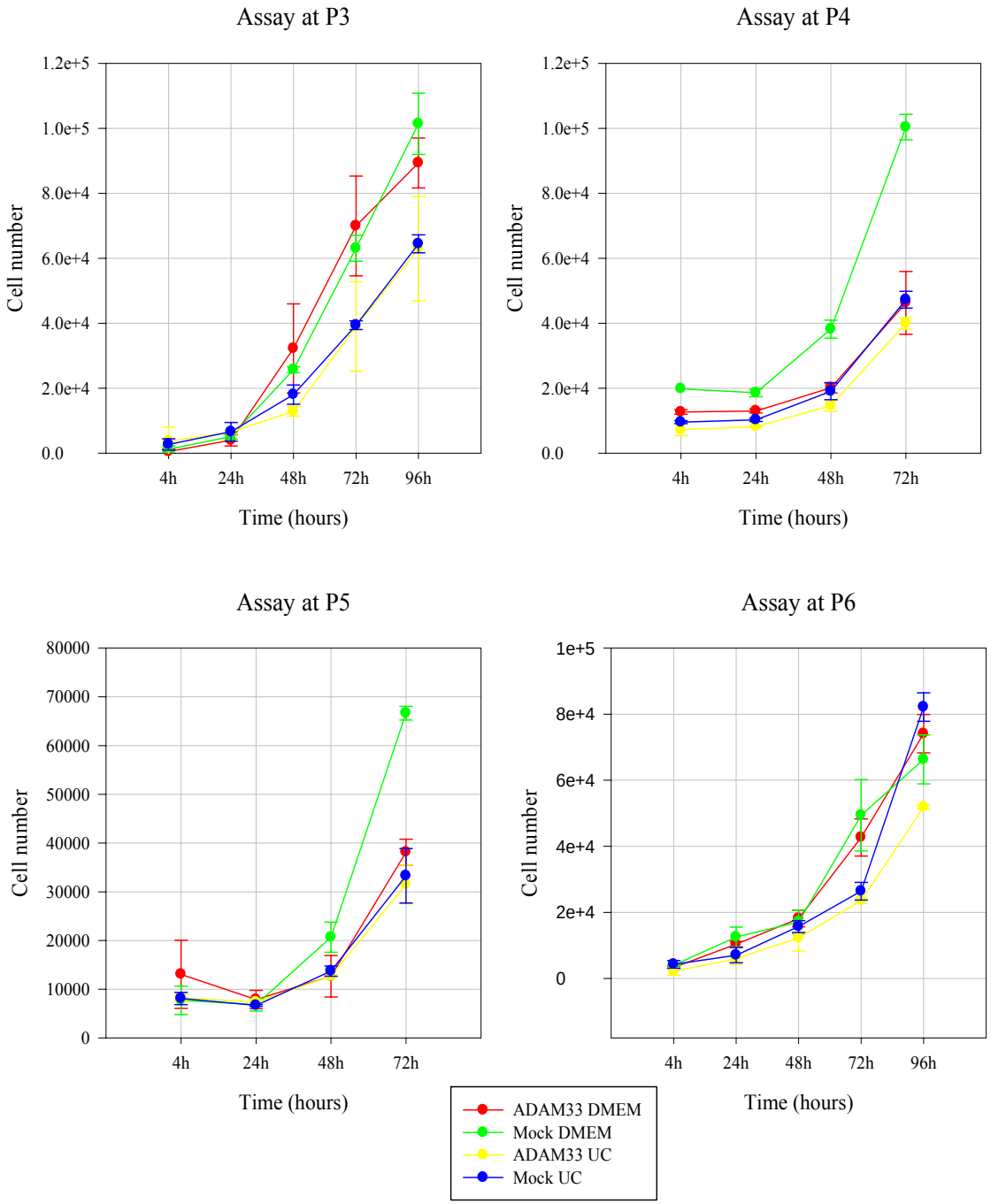


Figure 3.10: Proliferation Assay on Second Batch of Tansfected Cells

Proliferation assays carried out on ADAM33 and Mock transfected cells at P3-P6 using CellTiter 96 one solution. A P3 cells, B P4 cells, C P5 cells, D P6 cells.

Processing of ADAM33

As with other ADAM proteins, ADAM33 has a prodomain that keeps the protein inactive until it is cleaved by furin or furin-like proteins. It was expected that the transfected full length ADAM33 would be processed to the active 100kDa protein and a double band would be observed using the RP3 antibody. However this was not the case in the stable ADAM33 HEK293 cells. Figure 3.11 shows there was no double band and that the single band detected was at 120kDa indicating that it is the unprocessed protein. This was confirmed by western blotting with an antibody for the prodomain (RP1). A single band would be expected and is seen at 120kDa if the prodomain was still attached. This indicates that the protein is only produced in its inactive form with the pro-domain attached.

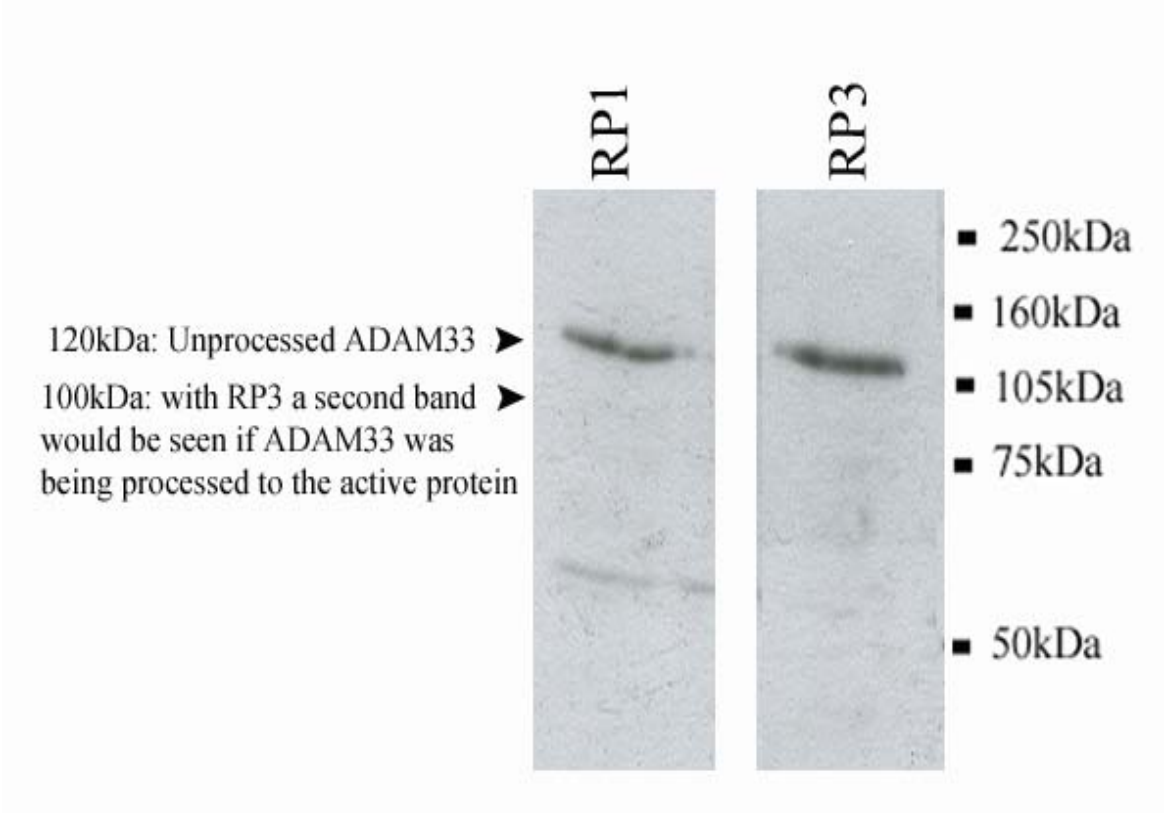


Figure 3.11: ADAM33 Transfected Cells do not Process the Protein to the Mature form

ADAM33 like other ADAM proteins are transcribed as pro-proteins that are inactive. Activation occurs with cleavage of the pro domain. It is therefore expected that when full length ADAM33 is analysed by western blot with the antibody to the cytoplasmic domain (RP3) two bands at 120KDa and 100KDa that correspond to the unprocessed protein with the pro-domain attached and a processed form without would be detected. However only a single band at 120KDa would be detected when using the pro-domain antibody (RP1).The above blot of ADAM33 HEK293 cells using RP1 (prodomain antibody) and RP3 (cytoplasmic tail antibody) shows that there no processing of ADAM33 to the mature and active form detected in comparison to figure 1.1 where a transient transfection did produce a double band.

Discussion

To determine any effect on the phenotype of cells containing ADAM33 compared to those that do not contain ADAM33, the first step was to produce two stable transfected HEK293 cell lines, one containing full length ADAM33 and the other a mock transfected cell line. The transfections where successfully achieved and the ADAM33 transfected cells did express ADAM33. However unlike transiently transfected cells only a single band was detected on the western blots at approximately 120kD indicating that only the unprocessed protein is present. This was shown in blots using the antibody to the cytoplasmic domain and confirmed using the antibody to the prodomain. As it has previously been shown that ADAM33 can be processed into an active protein of approximately 100kD, it was therefore expected that the ADAM33 in the transfected cells would be processed to an active form by cleavage of the pro-domain that keeps the metalloprotease site inactive, through a cysteine switch mechanism, as with other ADAMs such as ADAM17 that have been found in the pro and active forms D Seals and S Courtneidge 2003. In contrast with these cells which stably express ADAM33, initial transient transfections from this study showed that processing of ADAM 33 can occur and two bands were detected.

There are several possible explanations for this. It has been suggested by Umland et al 2003 that ADAM 33 requires its 3'UTR in order to be processed into mature protein effectively. Cells containing the 3'UTR were shown to have a 2-3 fold increase in ADAM33 processed to the mature protein. The construct used for the current study did not contain the 3'UTR so this may explain why no mature ADAM 33 was detected. However this was used for both the transient transfections (where

processing was observed) as well as the stable transfections (where no processing was observed).

The most likely difference is that the initial transient transfections were carried out in virally transformed HEK293 cells and that these had G418 resistant so could not be used for the stable transfections, and a G418 sensitive HEK293 cell line was used instead.

It is also possible that processed ADAM33 could have a different effect on cells to the unprocessed form of the protein and that, in turn, this leads to the cells that are processing ADAM33 to be overgrown by non-processing cells. Consequently during the selection process cells expressing ADAM33 but not processing it are favoured. It has been shown that HBEGF is found in two different forms in cells and that these have different functions: the membrane-anchored precursor form is pro-apoptotic and the mature secreted form is anti-apoptotic J Lin et al 2001. It is also possible that only a very small amount of ADAM33 is present in the stably transfected cells and that it cannot be detected by western blotting. Though this does not explain why ADAM33 is processed in transient transfections.

Western blots were carried out on samples of stably transfected cells from P1 to P13 to examine expression of ADAM 33 over time. It was found that the expression of ADAM33 did vary over time to some extent, though it was never completely lost due to the cells requiring the G418 resistance gene for survival. At P11 there was only a very faint band which increased again at P12 and 13 yet β-actin protein level were consistent between the samples, so similar levels of protein had been loaded, so for some reason the A33 transfected cells produced less ADAM33 protein at P11.

Observations on the initial stable transfected HEK293 cells at early passage numbers indicated that ADAM33 transfected cells are not morphologically different from the mock transfected cells. It was also observed that the ADAM33 cells appeared to grow more quickly than their mock transfected counter parts. Early cell count data indicated that the increase in proliferation seen in the ADAM33 transfected cells was lost over subsequent passage numbers (P2-6). Methylene blue proliferation assays were carried out to examine this observation further. A variety of conditions were tested including growth on collagen or on plastic, in ultraculture (serum free) or DMEM (10% FCS). Collagen coating of some of the wells was used as the transfected HEK293 were easily detached from plastic. There was however no difference between cells grown on plastic or collagen in DMEM (10%FCS). Both the mock and ADAM 33 cells did not grow as quickly in Ultraculture as those in DMEM. No difference was found between the ADAM33 cells and the mock cells at P6. Although this was not necessarily unexpected as by this time the initial cell counts data also indicated that the ADAM33 cells were no longer proliferating more quickly than the mock transfected cells. It seems possible that the effect of ADAM33 was lost over time.

The transfections were repeated to establish proliferation assays from P1 (post selection with G418). Proliferation assays were carried out at earlier passage numbers to determine if the increased proliferation of the ADAM33 cells was observed and if there was a difference and if this was lost over time. It was found that at P1 the cells transfected with ADAM 33 proliferated faster than the mock transfected cells but after this there was no difference between the ADAM 33 and mock transfected cells. There is a possibility that the P1 cells could have been

processing A33 that was not detectable by western blotting, that was lost by P2, although this seems unlikely. The loss of effect could be due to the cells that are producing active ADAM33 may cause of growth factor to be released into the media that causes the other cells (not processing ADAM33) to grow more quickly. These cells could then out-grow the cells processing ADAM33 so the effect is lost as not enough growth factor is being produced to increase the growth in the majority of cells present. Another problem is that methlyene blue assays have their limitations; they only give an estimate of cell number as it does not distinguish between cells of different sizes, so it could be that the ADAM33 transfected cells are bigger than the mock transfected cells only a direct cell count would give the actual cell number and confirm that there is a difference. In addition in this set of assays the transfected cells were very loose, so it is likely that some cells were lost in the fixing and washing stages causing the data to be inaccurate.

Due to the problems with the methlyene blue proliferation assay method, proliferation assays were also carried out using CellTiter 96 cell proliferation one solution. This eliminated the problem of cell loss because the assay is carried out directly in the cell culture media. Two different batches of cells were used to determine if ADAM33 transfected HEK293 cells proliferated more quickly than the mock transfected HEK293 cells. In the first set of experiments it seems that the ADAM33 cells were proliferating more quickly than mock cells even when the proliferation assays were carried out from P5, yet the rate of proliferation of the ADAM33 cells did drop at P7. However in the second set of cells that were removed

from stocks stored in liquid nitrogen, the rates of proliferation were very similar between the ADAM33 cells and mock cells. It seems most likely that due to the polyclonal nature of these cells that some batches of transfected cells may not express ADAM33 protein at the same levels and do not produce the change in proliferation shown in the data from the first batch of cells. It would therefore indicate that it is necessary to clone these cells.

Conclusion

HEK293 cells have been successfully transfected with full length ADAM33, but are not processing to mature protein in detectable levels or at all. ADAM33 in HEK293 cells may cause the cells to proliferate more quickly. However if the ADAM 33 protein is not being processed by HEK293 cells how can the presence of ADAM33 confer a growth advantage? The next stage will be to clone both the ADAM33 and Mock transfected cells.

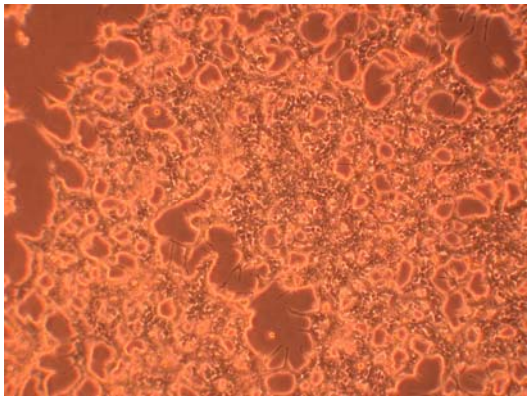
Chapter 4 Results

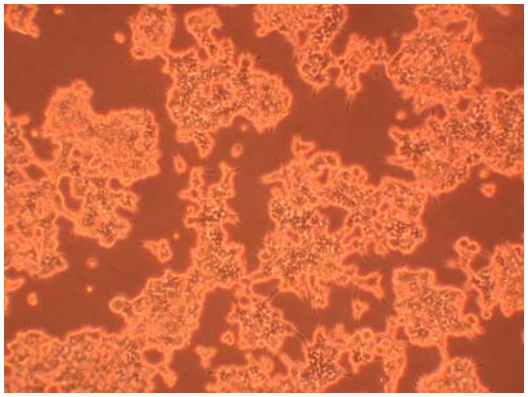
In the previous chapter HEK293 cells were successfully transfected with full length ADAM33, however no mature (processed) protein was detectable by western blotting and attempts to induce processing failed. Observations that ADAM33 transfection increased the proliferation of HEK293 cells compared to mock transfected HEK293 cells could not be confirmed by proliferation assay. One possible explanation for this could be that the polyclonal nature of the transfected cells does not produce sufficient cells that are capable of processing ADAM33 and there was therefore insufficient protein to be detected by western blotting. As HEK293 cells contain furin and other pro-protein convertases that are capable of cleaving ADAM17 and other ADAM proteins K Endres et al 2003 it would seem likely that this cell line would also process ADAM33.

Cloning of ADAM33 and mock transfected HEK293 cells.

To further assess the phenotypic effect of the over-expression of ADAM33 in HEK293 cells, both ADAM33 and mock transfected HEK293 cells were cloned by two rounds of limiting dilution. There was no change in morphology of the different clones or between A33 and mock as can be seen in Figure 4.1

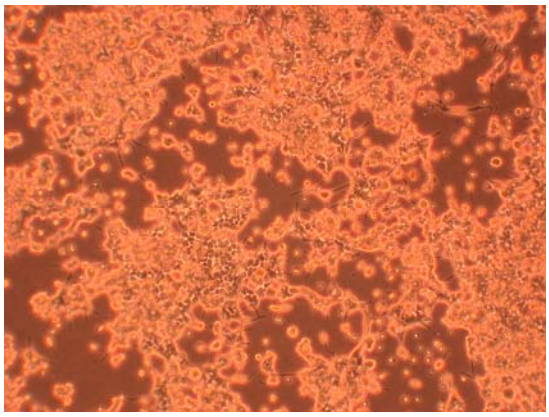
A33 G10 C3





A33 B9B10

Mock B7B3



A33 F8B4

Figure 4.1: The presence of ADAM33 does not alter the Morphology of HEK293 cells.

This figure shows a selection of ADAM33 clones grown in 6 well plates, in complete DMEM compared to mock transfected clones.

Differences in Number of Mock and ADAM33 clones generated

During the cloning process, the wells identified as containing single colonies were trypinsed when confluent and a second serial dilution carried out. Again wells containing a single colony were trypsinised when confluent and grown up into T25 flasks for further analysis. During this process it was noted that the HEK293 cells transfected with ADAM33 produced more single colonies that survived to be grown out. In the first round of dilution, it was found that from the two 96 well plates of mock and ADAM33 transfected cells, only 4 single colonies were observed from mock cells and continued into the second round of cloning compared to 15 from the ADAM33 cells. In addition to the difference in numbers during the first round of serial dilution it was noted that ADAM33 cells survived better at higher dilutions. ADAM33 transfected cells formed single colonies following 10 serial dilutions while in the mock transfected cells only went out to 7 dilutions when the cells were seeded at the same cell density (figure 4.2). In the second round of dilutions as seen in figure 4.3, 16 single colonies were identified in the ADAM33 cells and 6 single colonies in the mock cells. In this round of cloning the ADAM33 HEK293 cells again survived better at higher dilutions, such that 11 of the 16 ADAM33 clones were found past dilution 6, compared to just 1 out of 6 in the Mock clones. However the cells were not seeded at the same density in this round of dilutions.

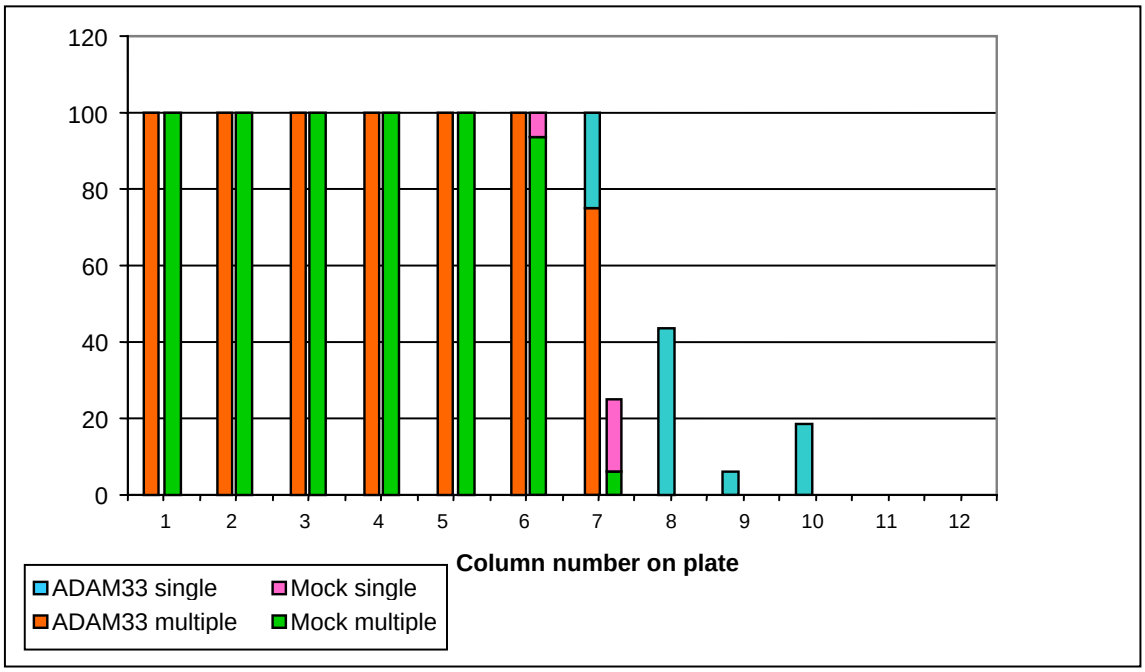


Figure 4.2: Distribution of wells single and multiple colonies form the first round of cloning.

During the cloning process it was noted that the ADAM33 transfected cells grew in columns further out in the 96 well plates. The cells were seed at $5x10^4$ cells per well in the first column of each 96 well plate, two plate for ADAM33 and Mock HEK293 cells.

Blue bars represent the percentage of wells that contained a single colony from ADAM33 cells. Pink bars represent the percentage of wells that contained a single colony from Mock cells. Orange bars represent the percentage of wells that contained multiple colonies from ADAM33 cells. Green bars represent the percentage of wells that contained multiple colonies from Mock cells.

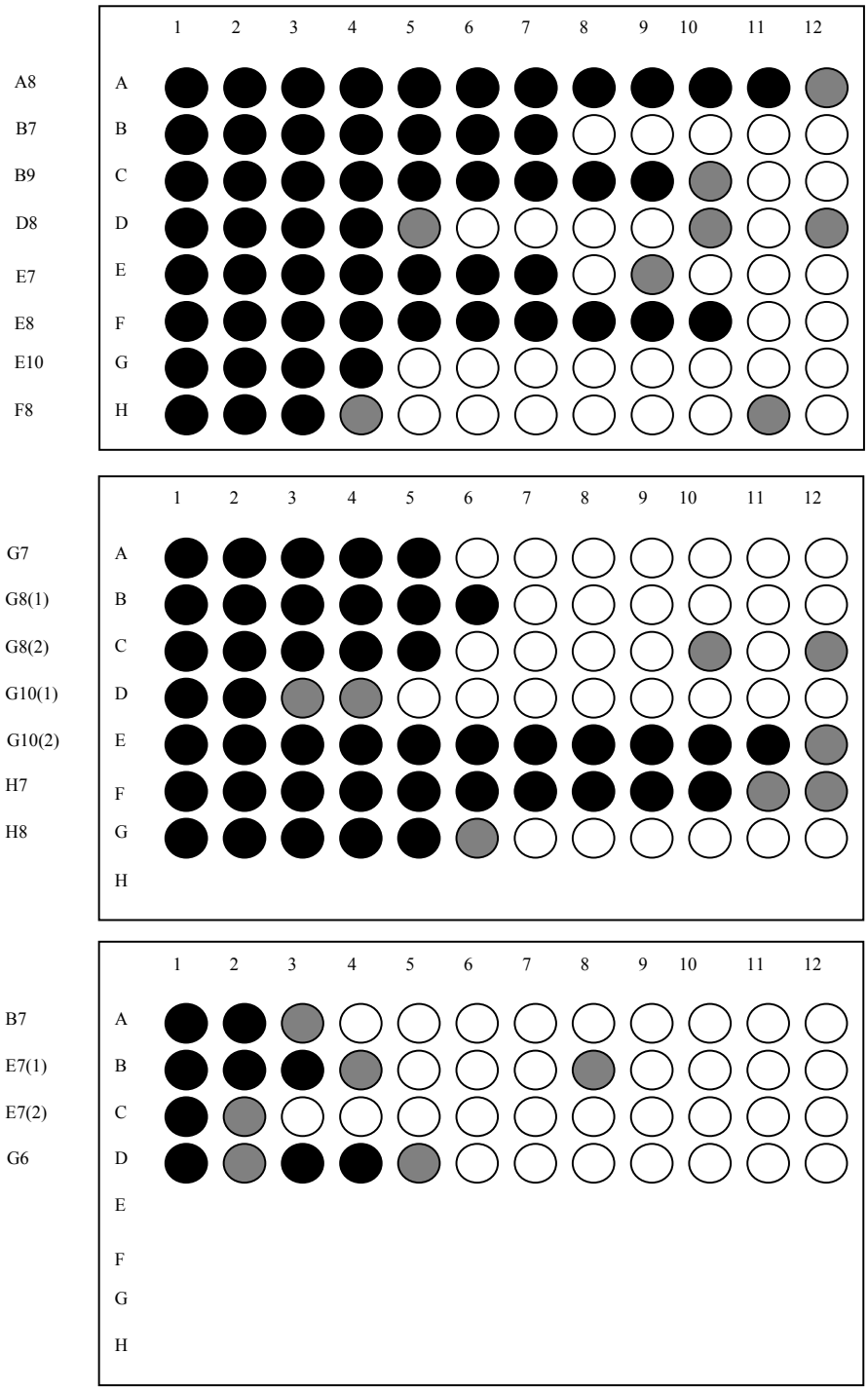


Figure 4.3: Second limiting dilution of ADAM33 and Mock HEK293 cells.
In the first round of dilution wells containing single colonies were identified and when confluent these cells were then trypsinised, spun down and resuspended in 200µl complete DMEM. This was then seeded into the first well of a 96 well plate and serial dilution carried out for a second time. In this case the cells seeded into the first column were not at the same cell density, which may account for the variation seen in the above figure. This is a pictorial representation of cloning plates, ○ denotes no cells growing, ◉ denotes a single colony and ● denotes multiple colonies. A8 etc. denote the position of the well the cells came from in the first round of dilutions. A and B are ADAM33 clones and C are Mock clones.

Western Blot Analysis of Clones

Western blot analysis was carried out to examine if the cloning process had produced any clones capable of processing ADAM33 to the mature 100kDa protein. It was also possible to identify clones that had different levels of ADAM33 expression when a fixed number of cells were put into SDS sample buffer.

It was found that some of the ADAM33 clones did produce mature ADAM33 which was detected as the lowerband in a doublet at 100-120kDa as seen in figure 4.4. This figure shows a selection of ADAM33 clones that were blotted with both the cytoplasmic domain (RP3) and pro-domain (RP1) antibodies. As expected only a single band at 120kDa was seen with RP1, however with RP3 at an exposure time of 15min, most of the ADAM33 clones shown had a double band at 100-120kDa. This was not the case in the majority of clones tested. Figure 4.5 shows a lower percentage of ADAM33 clones with a double band compared to ADAM33 clones without double bands.

In figure 4.4a there were other non-specific bands, most notably at 60kDa and 70kDa, which appeared in all the samples including the mock transfected clone. But also another band to 40kDa that is specific to the ADAM33 clones and appears to be more abundant in the clones with a clear double band. No such band is detected in the blot to the pro-domain (fig 4.4b).

There was one ADAM33 clone that has an unexpected result when blotted. In the first instance the RP3 antibody was used to blot for ADAM33, and the blots re-run for the RP1 antibody. When the clone ADAM33 A8F4 was blotted with RP3 no band was observed (figure 4.6a) and was assumed that it had lost the recombinant

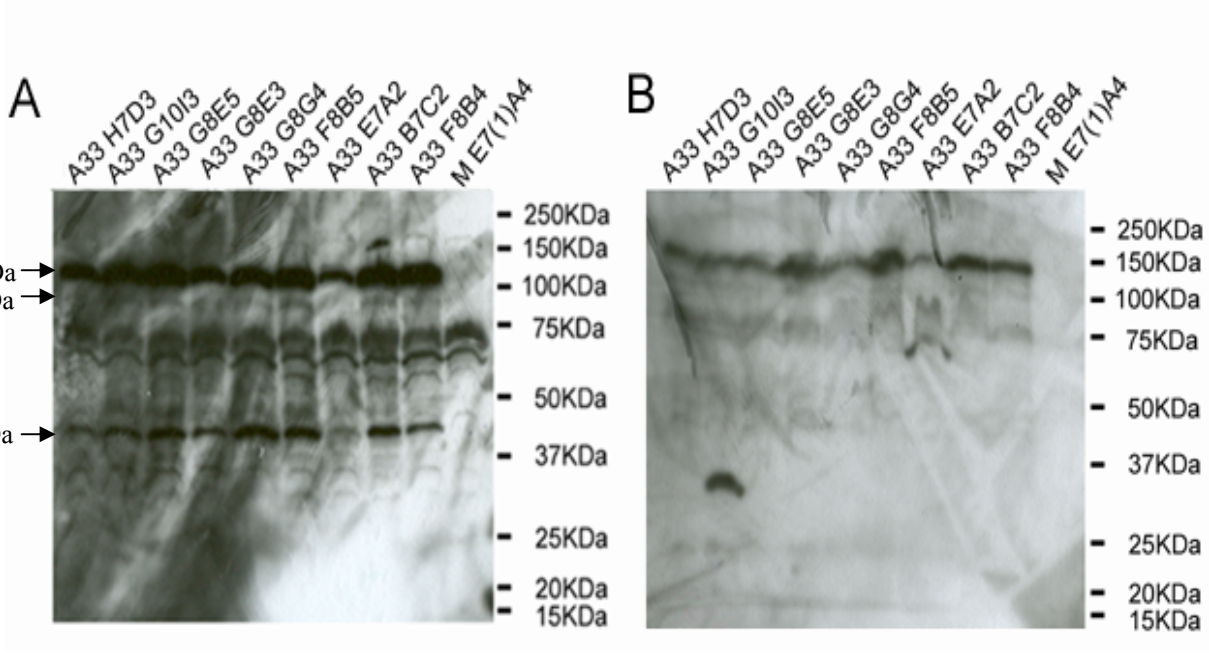


Figure 4.4: Some Clones Can Process the Recombinant ADAM33 Protein.

Western Blot analysis was carried out on samples of $1x10^5$ cells per 20µl of reducing SDS sample buffer. Blotting was carried out using the BioRad mini protean II system. Using antibodies to the pro-domain and Cytoplasmic domain and at 15 minute exposure for film.

A is a western blot film probed with RP3 antibody, it shows that some clones had a double band and therefore were producing mature ADAM33.

B is a western blot film probed with RP1 antibody; it shows that only single bands were found as would be expected with this antibody.

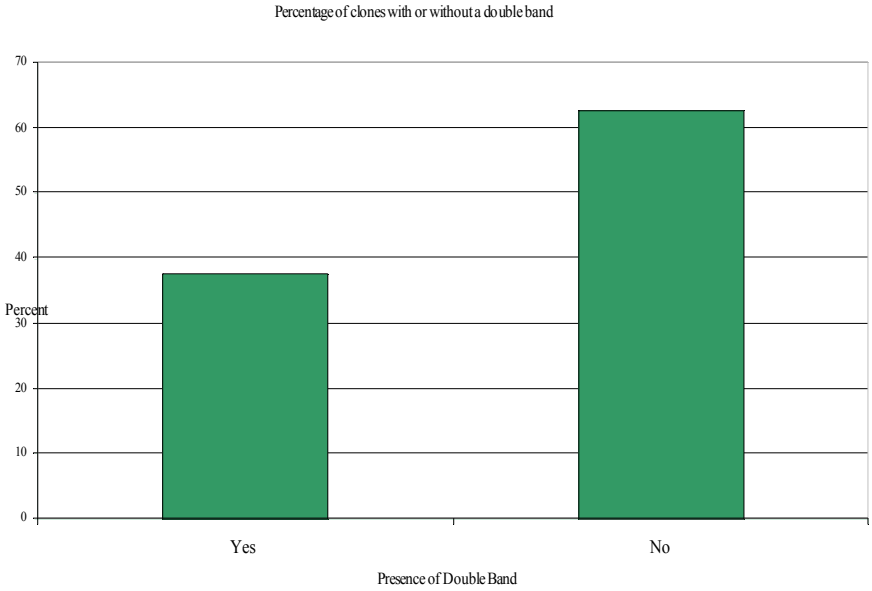


Figure 4.5: percentage of ADAM33 clones that had a double band compared to those that did not.

The presence of a double band was tested by western blot from a 15 minute film exposure time. This includes samples taken from wells with multiple colonies during the second round of dilution.

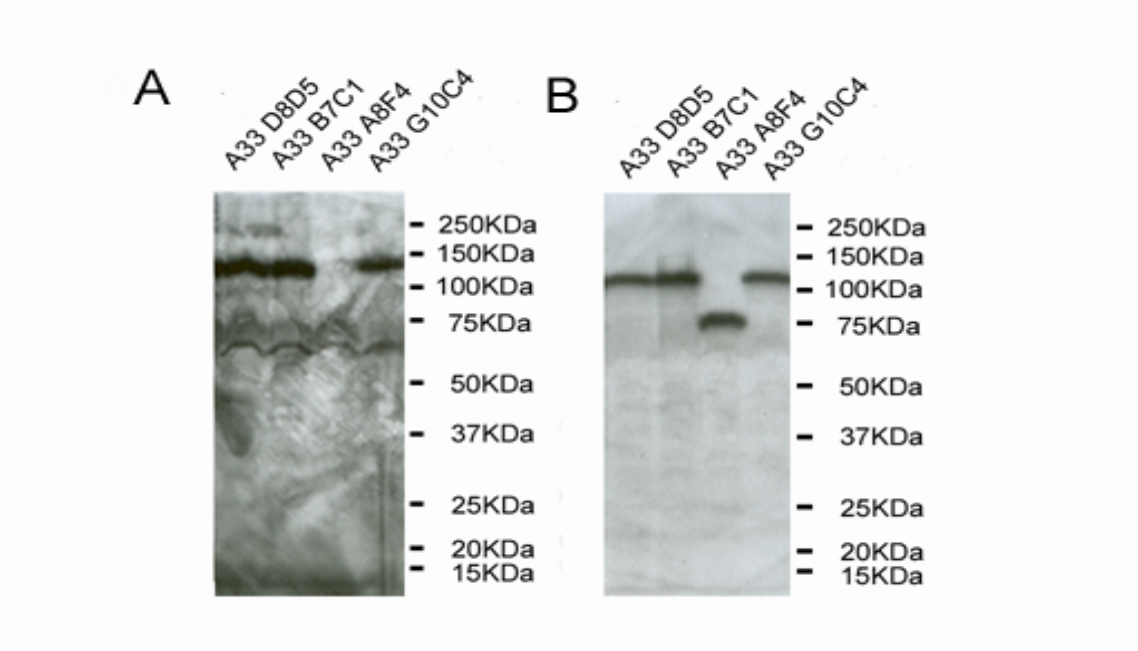


Figure 4.6: The ADAM33 Clone A8F4 has Lost Its Cytoplasmic Tail.

During the process of cloning the transfected HEK293 cells, a clone was spontaneously generated which had lost its cytoplasmic tail. **A** shows a western blot of a selection of ADAM33 clones using the RP3 antibody (cytoplasmic domain antibody), including the A8F4 clone which does not have a specific ADAM33 band with this antibody. However when blotted with the RP1 antibody (pro-domain antibody) as seen in **B** a band is detected at approximately 80-90KDa. Film exposure time for both blots was 15min.

ADAM33 or had a very low level expression. However when this sample was tested with the RP1 antibody a band was seen at 80-90kDa compared to bands at 120kDa with the other ADAM33 clones. It would seem that this clone had spontaneously lost its cytoplasmic tail as this antibody (RP3) does not produce a band.

CellTiter 96 Proliferation assays

Having generated clonal lines capable of processing ADAM33, cell proliferation assays were carried out using the CellTiter96 one solution proliferation assay as in the previous chapter. Analysis of proliferation assays on ADAM33 and mock clones was used to determine if there was a difference in growth rate between the mock and ADAM33 cell populations and between individual clones. In addition to this any differences in proliferation rate between ADAM33 clones in relation to ADAM33 protein expression levels (determined by western blotting) was examined.

When population means were taken across all clones tested it was found that up to 96 hours the overall trend was that ADAM33 clones proliferated more quickly but there was no significant difference between the ADAM33 clones or the mock transfected clones($p=\geq 0.12$) except at 24h ($p=0.01$). But with variation between individual clones, giving large error bars after the lag phase of the growth curve at 72 and 96 hours not unexpected (figure 4.7). After this at the 120 and 144 hour time points the cells seem to reach a plateau, with the mean mock cell number overall

achieving higher cells number at 120 hours but again no significant difference (p=0.13). At 144h the cell numbers between the mock and the ADAM33 clones

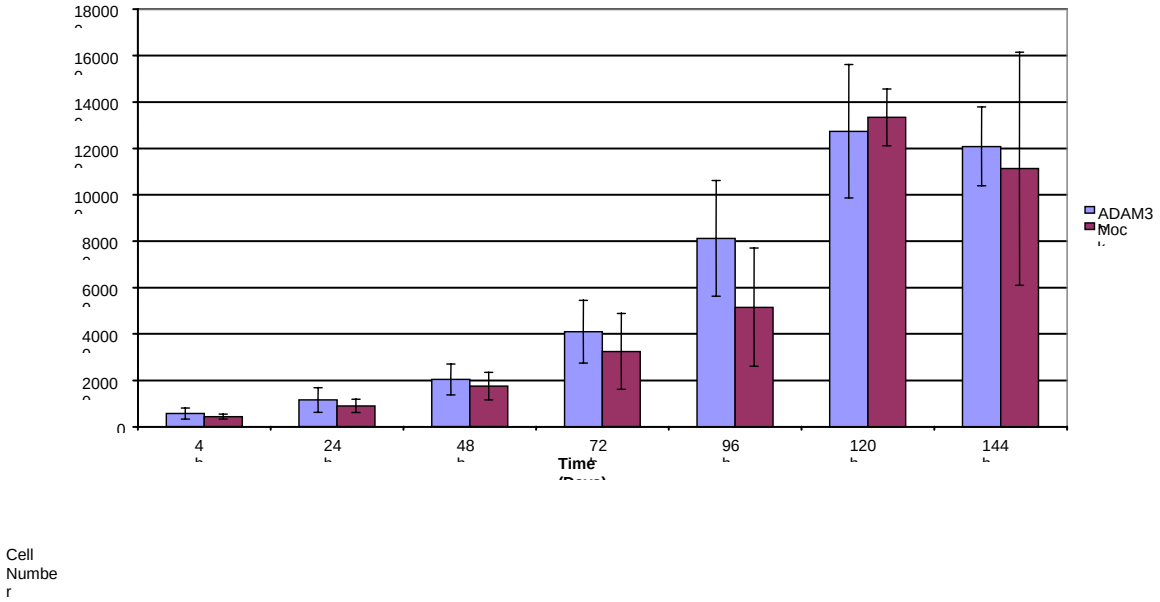


Figure 4.7: Population means from CellTiter 96 Proliferation Assays of Mock and ADAM33 HEK293 clones.

To look at the overall difference between the mock cell population and the ADAM33 cell population were analysed. Means and standard deviation of cell number were taken from CellTiter proliferation assay data carried out on 8 of 15 the ADAM33 clones and 6 of 6 the mock clones.

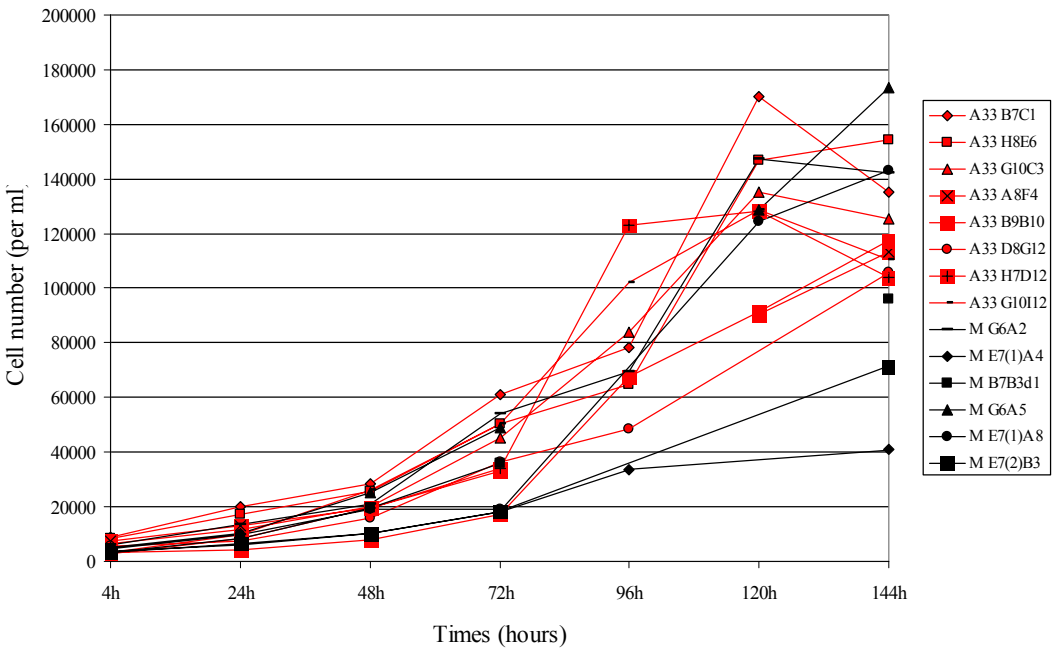


Figure 4.8: Proliferation Assays on Mock and ADAM33 Clones.

The graph above shows the growth curves of individual clones 8 ADAM33 clones and 6 mock clones (the same clones used for the population mean in figure 2.7). The curves in red indicate an ADAM33 clone and the curves in black a Mock clone. Cell were seeded at $5x10^4$ cells per ml in 96 well plates (100µl per well) and run in triplicate. N=3 or more.

were very similar (p=0.87). When individual plots were examined (figure 4.8), the 6 mock clones examined were split into two groups. The first group consisted of clones MG6A5, MG6A2 and E7(1)A8 this group have proliferation rates that are in the same range as the ADAM33 clones with the highest proliferation rates. The other group which was made up of clones MB7B3, E7(1)A4 and E7(2)B3. This group had proliferation rates that were lower than all other clones.

Overall the data put forward here suggests that the presence ADAM33 does not increase the proliferation rate of HEK293.

The second part of the analysis was to look at the ADAM33 clones and to determine if the differences between the ADAM33 clones could be attributed to their protein expression determined when the clones were analysed by western blot and ranked by eye into groups according to the presence of the double band and intensity of the

band. Figure 4.9 shows 5 clones which have different ADAM33 protein expression level from one with a double band to A8F4 which lacks the cytoplasmic domain. There is no real difference between the clones and the slight variations in the proliferation assay is not altered by protein expression levels. Figure 4.10 shows two clones that have a double band and two clones with a strong ADAM33 protein level. This figure also confirms that a double band and therefore mature ADAM33 does not increase the proliferation rate of HEK293 cells.

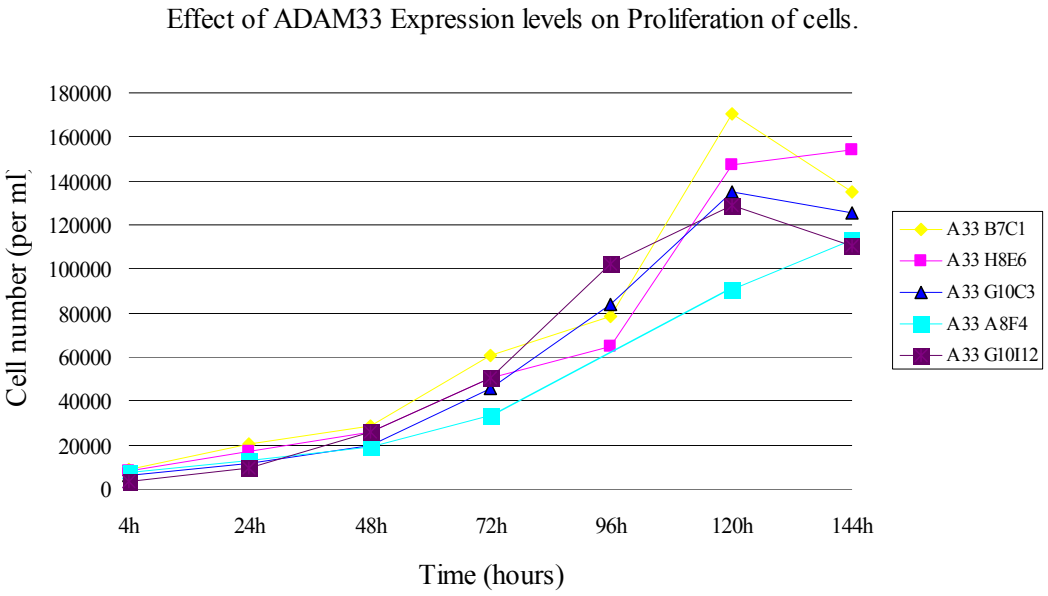


Figure 4.9: Effect of ADAM33 Expression levels on Proliferation of cells.

In addition to comparison of ADAM33 clones and mock clones, the ADAM33 clones were analysed to look at any evidence for a change in proliferation rate at different levels of protein expression.

Each plot is an example of a growth curve from cells that had a different ADAM33 expression level when analysed by western blot using RP3 antibody (cytoplasmic tail). The pink plot was from cells that had processed ADAM33 protein, the dark blue, yellow and purple lines were from cells expressing high, medium and low levels of ADAM33 respectively, while the cyan blue plot was from cells that lack the cytoplasmic tail of ADAM33 (clone A8F4).

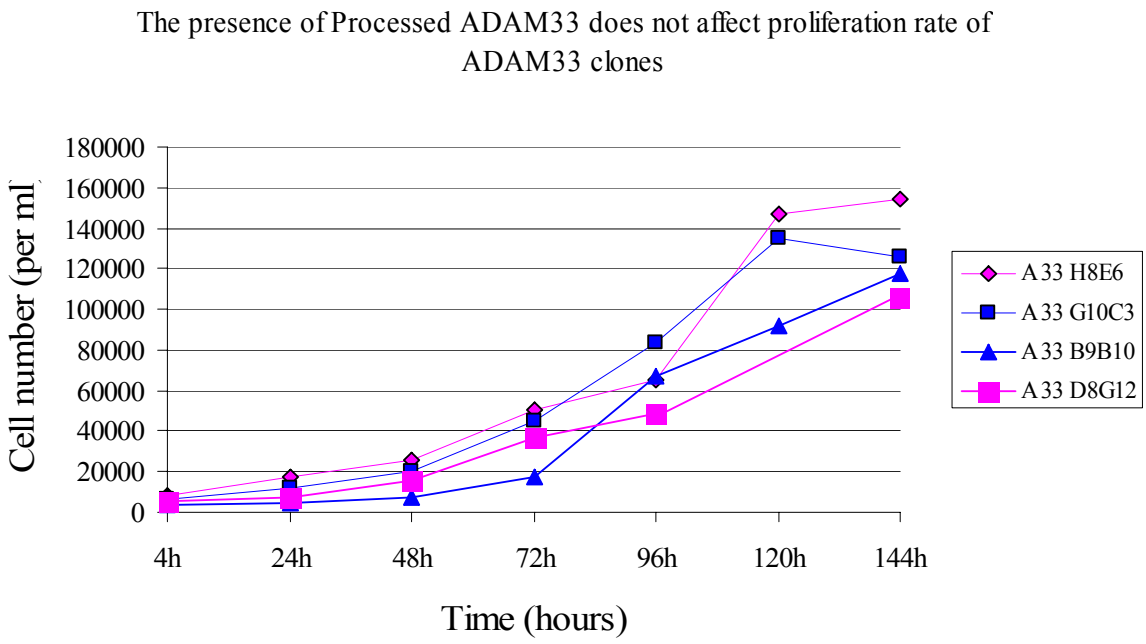


Figure 4.10: The presence of Processed ADAM33 does not affect proliferation rate of ADAM33 clones.

When the ADAM33 clones were analysed by western blot it was found that some clones had a double band at 100kDa and 120kDa indicating that ADAM33 has been processed to the mature form in these clones. Here 2 clones with detectable ADAM33 processing (pink) and two without evidence of processing (blue) were compared. The evidence suggests that there is no difference in proliferation rate when mature ADAM33 is detected in its mature form.

Discussion

To further assess any effect on the phenotype of cells containing recombinant ADAM33, the stable transfected HEK293 ADAM33 and Mock cells were cloned by limiting dilution. The process of cloning produced 6 mock cell lines and 15 ADAM33 cell lines that came from wells (in both the first and second round of dilution) that contained a single colony.

As in results chapter 1, there was no overall change in the morphology of the Mock and ADAM33 cells, although there were individual clones in both the mock and ADAM33 clones that were different from each other. The conclusion from this is that these changes are not related to the presence of ADAM33.

During the process of cloning these cells it was noted that ADAM33 cells were easier to clone and wells containing a single colony were more numerous, 16 compared to 4 in the first round. From these 15, ADAM33 and 6 Mock cell lines were generated. Therefore, it is possible that ADAM33 allows the cells to grow better at very low cell numbers.

When samples of the clones were assessed by western blot analysis, it was found that some of the ADAM33 clones were capable of processing ADAM33 to the

mature form of the protein. It would seem that previous analysis of the polyclonal ADAM33 transfected cells were not capable of producing enough mature protein to be detected by western blotting rather than that the HEK293 cells used for producing the stable transfected cells could not process ADAM33. However not all the ADAM33 clones show evidence of processing, the most likely explanation is that there are clones with lower expressions of the full length unprocessed ADAM33 protein. This could mean that the levels of processed ADAM33 are to low to be detected by western blot.

In addition an anomalous clone lacking the cytoplasmic tail with an approximate molecular weight of 90kDa was spontaneously generated and will merit further study.

In conclusion the HEK293 cells used to generate stably transfected cell lines are capable of processing full length ADAM33 to mature protein.

A 40kDa band on blots that was specific to ADAM33 transfected cells in western blots of ADAM33 transfected and mock transfected clone cell lysate. The intensity of the band seems to increase with both, increased expression of ADAM33 protein and the presence of the processed ADAM33 band (100kDa). This band cannot be a splice variant of ADAM33 as the cells were transfected with full length cDNA construct which cannot undergo splicing. The most likely explanations are that either this protein is a degradation product of the full length protein or that arises due to the loss of the extracellular domain portion of ADAM33 left behind if a soluble form is cleaved from the cell surface. This may also explain why very little processed ADAM33 (100kDa) is detected.

The proliferation assay studies were carried out determin if there was any diffrence in proliferation rate. Firstly between the ADAM33 clones and Mock transfected clones and secondly between ADAM33 clones with different expression and processing of the ADAM33 protein. When the proliferation assays were carried out on a selection of ADAM33 and Mock transfected clones it was found that as a population there was very little difference between the ADAM33 group and mock transfected groups up to 96 hours except at 24hours, though with large variability between the clones. At 120hours a plateau was reached and overall the mock cells had a higher cells number but it could the ADAM33 cells had already reached the higher cell number and started to die and the cells to fall off the plates.

When the individual clones were analysed the ADAM33 clones had a wide range of growth curves, the mock cells however formed two populations, one group had low proliferation rates that are below all the other clones and do not reach an exponential phase like most of the ADAM33 clones and the other three mock clones. If the experiments were continued past 144h and the media changed it would be expected that the cells would become confluent in the wells and reach a cell number similar to the other clones. The other mock group have high proliferation rates that are comparable to the ADAM33 clones that had the highest proliferation rate. Possible explanations for this are that there were too few mock transfected clones to assess the range of proliferation rates in the same way as the ADAM33 clones. Ideally the same number ADAM33 and mock transfected clones should have been compared. The lack of difference between the mock transfected and ADAM33 clones could be

due to the selection pressure of the cloning process has selected only the most robust mock tansfected cells and may skew the proliferation data.

It is also possible that as ADAM33 is a large transmembrane protein that is highly expressed in the transfected cells due to continual transcription driven by the CMV promoter, this could reduce the proliferation rate of these cells and could be masking the true effects of ADAM33 on cell proliferation. As the control cells used an empty vector, further experiments could be undertaken by transfecting an irrelevant transmembrane protein of a similar size to ADAM33 that does not increase proliferation and to assess the proliferation rate of these cells compared to ADAM33 transfected cells. Alternatively full length ADAM33 could be inserted into a vector with an inducible promoter to control the level of expression.

When the ADAM33 clones were assessed for differences in proliferation rate by their ADAM33 protein expression levels (determined by western blotting) no trend was found and the level of expression did not correlate with differences in proliferation rate. When the high expresser's that did not process ADAM33 were compared with those that did there was no significant difference in there proliferation rate. Analysis was only carried out on a limited number of ADAM33 clones to determine if any trend was seen in the data. As no trend was found a full analysis of all the ADAM33 clones was not carried out.

In conclusion from the assays carried out it seems that ADAM33 does not confer a proliferation advantage. But that the presence of ADAM33 improves the survival of HEK293 cells compared to mock transfected HEK293 cells under the cloning conditions.

While no differences in proliferation were found, it does seem that ADAM33 may improve the cells ability to grow under the stressful process of cloning. The data presented in results chapters 1 and 2 show that ADAM33 does not alter cell morphology and although initial observations indicated that ADAM33 may increase the proliferation rate of HEK293 cells but when this was examined using methylene blue and CellTiter 96 one solution proliferation assays the differences could not be confirmed. This could perhaps be explored further by transfecting in a protein of similar size to ADAM33 and assess what effect this has on cell proliferation.

In addition, the cloning of the ADAM33 transfected HEK293 cells showed that ADAM33 can be processed to the mature form which had not been seen before in the stably transfected cells. Also the band at 40kDa detected using the antibody to the cytoplasmic domain antibody but not the pro domain antibody is unlikely to be a splice variant. A soluble form has been found in BAL fluid of normal and asthmatic patients, with increased levels of soluble ADAM33 found in BAL of asthmatics with a molecular weight of 55kDa which was thought to the putative soluble form as described above.

The next stage of this study will be to determine if my ADAM33 HEK293 clones release the soluble form of ADAM33 into their culture medium. The first step will be to determine if this form is present using the antibody to the MP domain of ADAM33 which should detect this form. If present, the objective is to assess what factors may increase the levels of this form of ADAM33 and to consider how it is cleaved from the cell surface or secreted from the cells.

Chapter 5 Results

Soluble ADAM33

In the previous chapter it was found that some of the cloned full length ADAM33 transfected HEK293 cells were capable of processing ADAM33 to its active 100kDa form. This however did not lead to confirmation that ADAM33 increases the proliferation of HEK293 cells compared to the mock transfected clones, although its expression appeared to improve the survival under the harsh conditions of limiting dilution. This chapter will explore the possibility of a soluble form of ADAM33 protein. It was observed previously that a 40kDa band is detected in blots of cell lysates from ADAM33 clones using the antibody to the cytoplasmic tail. This band could be a breakdown or cleavage product as it must contain the cytoplasmic domain. It can not be a non specific band as it is not contained in the mock transfected clones and is not seen in blots using the antibody to the pro-domain. As ADAM33 is not constitutively expressed in HEK293 cells this band cannot be a splice variant, though such a variant was described by Powell *et al* 2004. The observation of this 40kDa band leads to the the questions: What has happened to the remaining 50-60kDa of the protein? Could it be a soluble form of ADAM33 that is cleaved from the cell surface or secreted from the cells?

A soluble form of ADAM33 was described by Lee and Park *et al* 2006 in BAL fluid of normal and asthmatic people. They found a 55kDa band by western blotting with an affinity- purified rabbit anti-human ADAM33 Metalloprotease domain antibody (ASP2) in BAL fluid and in the supernatant of MRC-5 fibroblast cell line. They also found a significant increase of soluble ADAM33 between normal control subjects and asthmatics with a significant correlation between increased levels of soluble ADAM33 and reduced FEV_1 indicating a link with asthma severity.

Other ADAM proteins have also been found to have soluble variants, these include human ADAM12 B Gilpin et al 1998 and xenopus ADAM13 A Gaultier et al 2002 which is also the closest relative of ADAM33. These soluble forms of ADAMs have been found to contain the metalloprotease domain with or without the inhibitory prodomain, and the disintegrin and cysteine-rich domains. It is as yet unclear how these soluble forms function or if their specificity is the same as their membrane bound counterparts B Gilpin et al 1998 and A Gaultier et al 2002.

It is known that in wild type ADAM13 the MP, disintegrin and cysteine rich domains are shed from the cell surface where as a mutant form can not. This suggests that ADAM13 shedding may involve its own metalloprotease activity. It is also known that ADAM13 needs to be inserted into the cell membrane before it is cleaved for the resultant protein to be active. It is also thought that the released protease may interact with both integrins and extracellular matrix proteins A Gaultier et al 2002 . These findings are in contrast to ADAM12 where the soluble form is generated by mRNA splicing. When ADAM12 is generated as an artificial construct lacking the cytoplasmic and transmembrane domains the released soluble form is still active B Gilpin et al 1998 .

These previous studies lead to several points that need to be examined in relation to a possible soluble form of ADAM33. How is the soluble form released from the cell surface and is it active? Is ADAM33 involved in its own cleavage like ADAM13? Can the release of soluble ADAM33 be regulated?

The aims of this chapter are to determine if the ADAM33 transfected clones produce a soluble from of ADAM33 at approximately 55kDa. Can any release of soluble ADAM33 be regulated using non specific stimuli such as PMA and more specific agents that are associated with asthma such as TGF-B. Finally, to determine if this form

is active using HUVEC tube formation assay used by Dr Ilaria Puxeddu and a FRET assay used by Dr Yun Yun Pang.

Preliminary studies to determine the presence of Soluble ADAM33 in the Supernatant of ADAM33 HEK293 clones and optimum processing conditions.

To determine if a soluble form of ADAM33 is released by ADAM33 transfected HEK293 clones, the fact that the ADAM33 MP domain is glycosylated was utilised by using ConA that binds glycosylated proteins, to in effect, non-specifically concentrate any soluble ADAM33 for analysis by western blot. The clones were grown in DMEM containing 0.01% FBS. The supernatant was harvested after 96h and rotated overnight with different amount of ConA beads and the beads eluted in different volumes of reducing sample buffer.

In a pilot study, it was found that ADAM33 HEK293 clones did produce a soluble 55kDa form of ADAM33 protein that could be pulled down using the ConA beads and detected using the RP2 antibody to the MP domain of ADAM33. The next step was to determine how much ConA to use to pull down the soluble ADAM33 and if using more western blot sample buffer was possible. In figure 5.1 three different amounts of ConA beads were used and it was found that 20µl of beads per ml of supernatant pulled down as much soluble ADAM33 as 60µl of beads. Using the RP1 antibodies (to the pro-domain) a band at approximately 27kDa was obtained while the RP2 antibody (to the MP domain) yielded a band at approximately 55kDa and 27kDa. The smaller 27kDa band must contain the pro-domain though the predicted size of the pro-domain is 20kDa using the RP1 pro-domain antibody. But this band is also specific to the ADAM33 clones when blotted with RP2-MP antibody. Meaning that either this band contains the pro-domain and part of the MP domain or it is a different band of similar size that contains only the MP or catalitic domain (predictied size 23.5kDa) as described in Zou et al 2004,the larger than expected size is likely to be due to protein glycosylation (J

Zou et al 2004). Using 20µl of beads per ml of supernatant, the beads were lysed into either 50µl or 150µl of reducing sample buffer for western blotting and it was found that the larger volume neither increased nor reduces the amount of ADAM33 detected on western blot (figure 5.2).

To try and confirm the protein detected was the same as in the work done by Lee and Parks *et al.* Their antibody to the MP domain (Asp2) was used as a comparison to RP2. As can be seen in figure 5.3 with RP2 bands are only detected in an ADAM33 clone at 55kDa and not in the mock transfected clone, however with Asp2 a band is detected in all the samples including the mock transfected clones. It appears that this band is also slightly bigger. Further studies were not undertaken with this antibody due to limited availability.

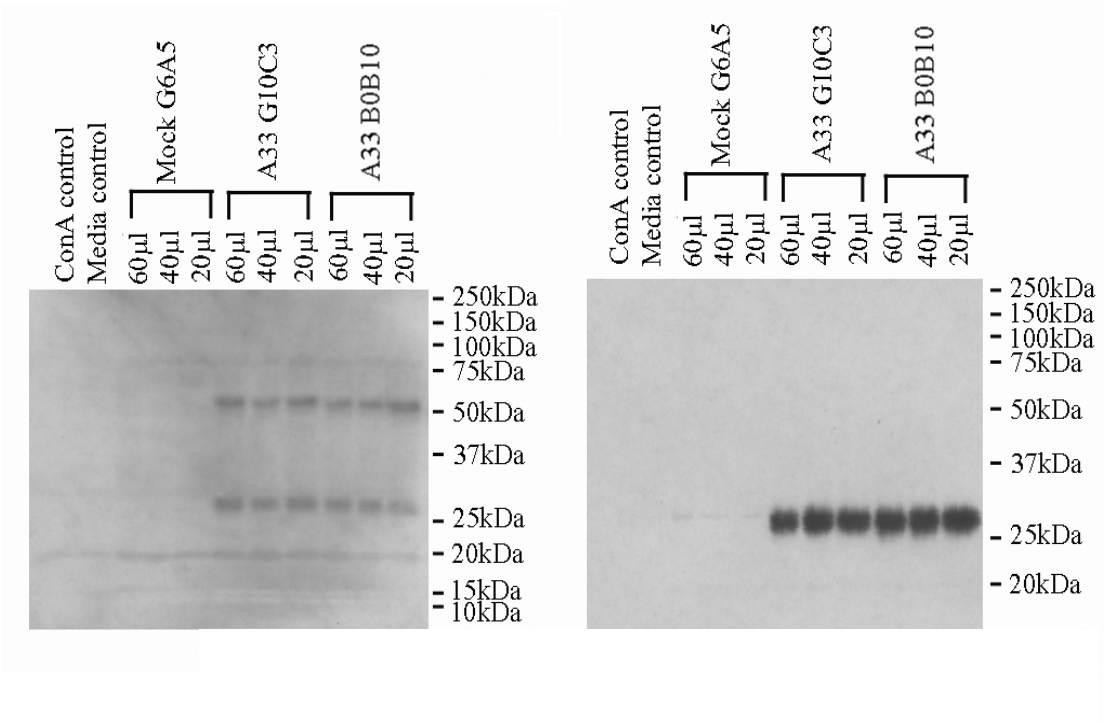


Figure 5.1 Western Blot Analysis to Determine the Presence of Soluble ADAM33 pulled down on to ConA beads using Antibodies to the MP and Pro domains.

To determine the presence of soluble ADAM33 and the best volume of ConA beads to use in the pull down procedure of soluble ADAM33 from supernatant of ADAM33 transfected HEK293 clones, 2ml of supernatant from the clones A33B9B10, A33G10C3 and the control clone MG6A5, were pulled down overnight using either 20, 40 or 60µl of ConA beads. The beads were then lysed into 50µl of reducing SDS sample buffer and 20µl blotted, using 12.5% SDS PAGE acrylamide gels. The PVDF membranes were probed with either RP2-MP domain antibody (a) and RP1-Pro domain antibody (b).

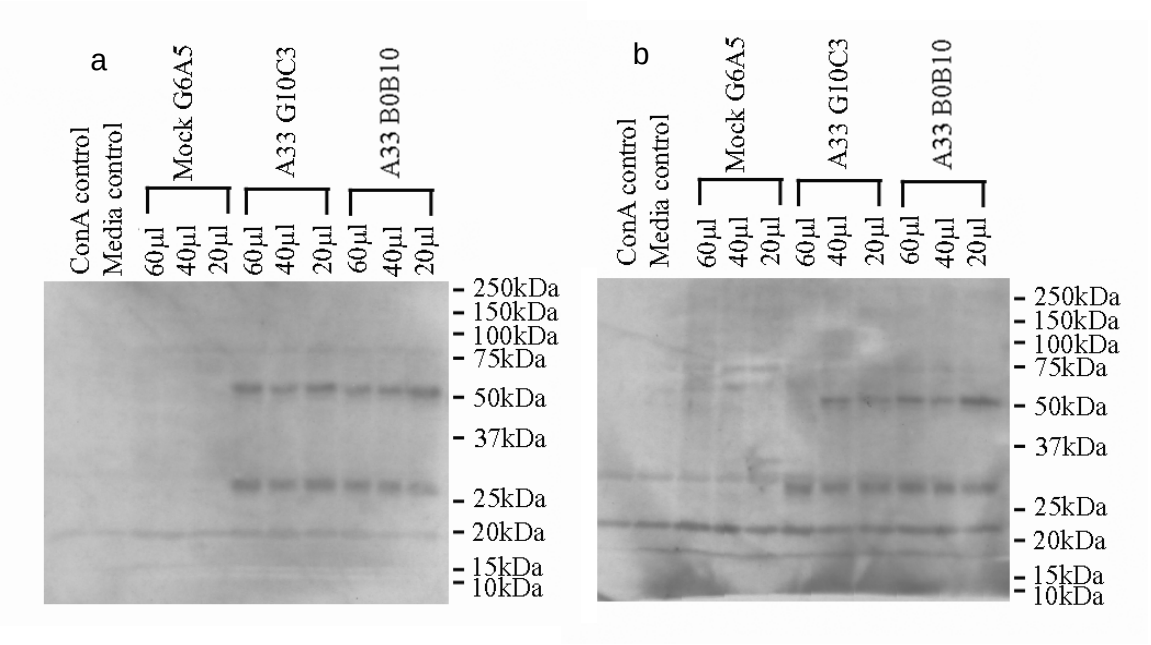


Figure 5.2 Western Blot Analysis of ConA pull down and the lysis of beads into two different volumes of SDS Sample Buffer.

Supernatant from ADAM33 transfected HEK293 clones (2ml) A33B9B10, A33G10C3 and the control clone MG6A5, were pulled down overnight using either 20, 40 or 60µl of ConA beads. The beads were then lysed into either 50µl or 150µl of reducing SDS sample buffer and 20µl blotted, using 12.5% SDS PAGE acrylamide gels. The PVDF membranes were probed with RP2-MP domain antibody. a- Blot of ConA beads in 50µl of SDS sample buffer and b- Blot of ConA beads in 150µl SDS sample buffer.

TGF-B up regulates the release of soluble ADAM33

To try and determine possible ways soluble ADAM33 is regulated. This was done using a variety of agents both non specific stimuli such as PMA and H_2O_2 to induce oxidative stress and more specific agents that are associated with asthma such as TGF-B. Of the agents used, only TGF-B induced a change in the amount of soluble ADAM33 released from the ADAM33 HEK293 cells. In figure 5.4 it can be seen that there appeared to be an increase in the amount of soluble ADAM33 released in a dose and time dependent manner at 24 and 48h at all doses except 20ng/ml of TGF-B. At 72h the effect was less clear though it appear that the untreated supernatant contained less sADAM33. And when three independent experiments were subjected to densitometry analysis figure 5.5 the following was found: at 24h all the TGF-B doses had a mean relative absorbance higher than the untreated except at 1ng/ml but none of the differences were significant. At 48h all the TGF-B doses had a mean relative absorbance higher than the untreated sample, the only significant difference was at 10ng/ml compared to the untreated ($p=0.04$). And at 72h only the 1ng/ml and 20ng/ml treated samples had a mean relative absorbance higher than the untreated sample but again none were significantly different. The 27kDa band seen in figures 5.1 and 5.2 did not seem to increase in response to TGF-B.

Inhibition of soluble ADAM33

As part of investigations into the release of soluble ADAM33 from transfected HEK293 cells, ADAM33 and Mock clones were treated with MMP8 inhibitor (MMP8 inhibitor I (3R)-(+)-[2-(4-methoxybenzenesulfonyl)-1,2,3,4-tetrahydroisoquinoline-3-hydroxymate)and MMP2/9 inhibitor (MMP2/MMP9 inhibitor 1 (2R)-2-[4(-Biphenylylsulfonyl)amino]-3-phenyl propionic acid) (Merck Chemicals Ltd, Nottingham, UK). The MMP8 inhibitor has been shown to inhibit ADAM33 Puxeddu et al 2008 and therefore has been used to determine if ADAM33 is involved in its own

cleavage from the cells and autocatalytic like ADAM13. The MMP2/9 inhibitor was used as a control to this. Figure 5.6 shows a western blot of cells treated with the MMP8 inhibitor which does not prevent the release of soluble ADAM33 from the ADAM33 HEK293 cells even at high concentrations. The MMP2/9 inhibitor did however appear to increase the amount of soluble ADAM33 released and increases with increasing concentration of the MMP2/9 inhibitor.

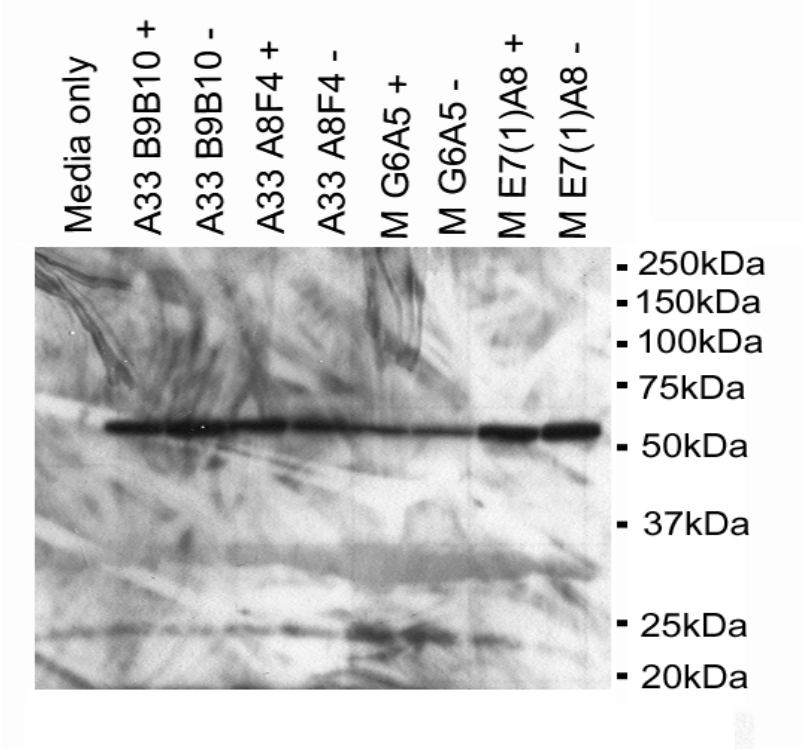


Figure 5.3: Western Blot Analysis of ConA pull down ADAM33 and Mock transfected clones, Probed with Asp2 an antibody to the MP domain.

Two ADAM33 clones and two Mock clones were treated with or without 10ng/ml TGF-B for 48h. Supernatant was collected and pulled down onto ConA beads. The beads were then lysed in to reducing SDS sample buffer and 20µl blotted using 12.5% SDS PAGE acrylamide gels. The PVDF membrane was probed with Asp2 an affinity purified antibody reported to detect the MP domain of ADAM33.

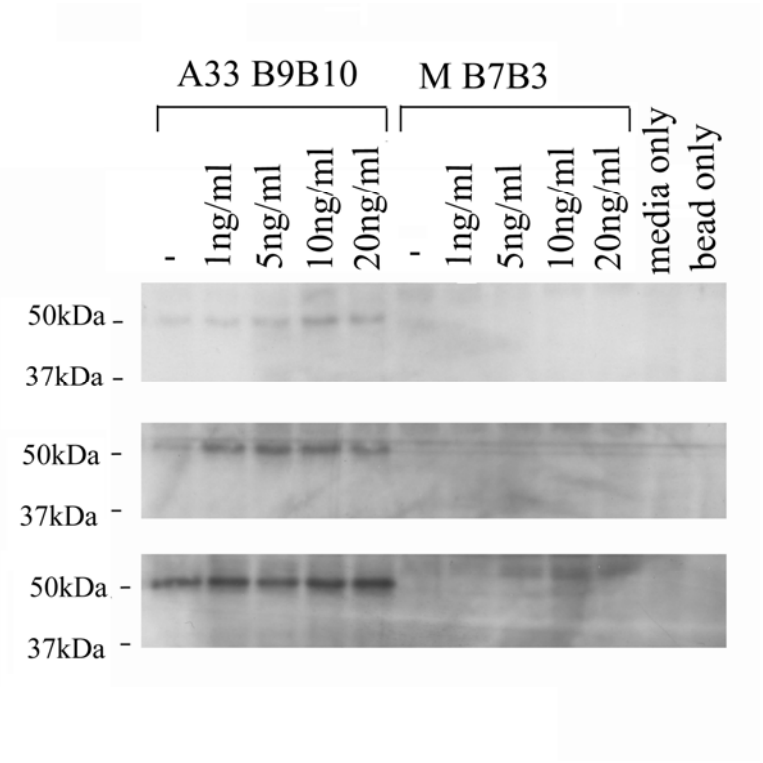


Figure 5.4 Time and dose response of the release of soluble ADAM33 in response to TGF-β.

A: The ADAM33 clone B9B10 and the Mock transfected clone B7B3 were treated with increasing amounts of TGF-β and supernatant collected at at 24h (top panel), 48h (middle panel) and 72h (bottom panel) and glycosylated proteins pulled down onto ConA beads. The beads were then lysed into 150µl of reducing SDS sample buffer and 20µl blotted, using 12.5% SDS PAGE acrylamide gels. The PVDF membranes were probed with RP2-MP domain antibody.

B: Cell lysates of ADAM33 transfected cells treated with 20ng/ml TGF-B compared to un-treated ADAM33 transfected cells.

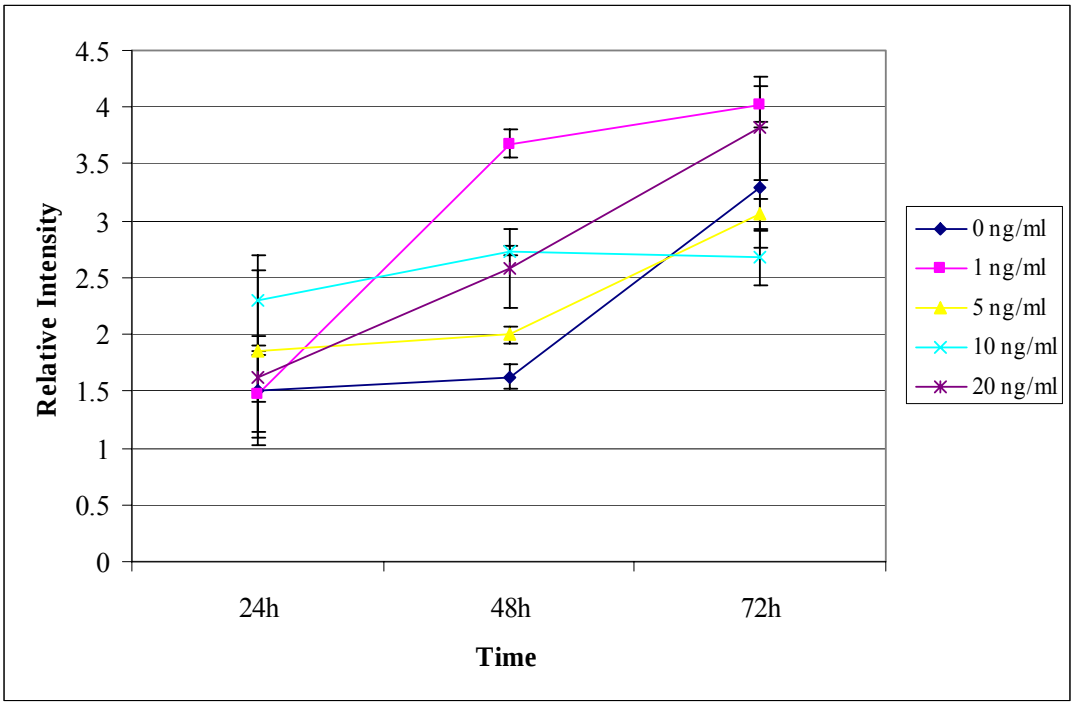


Figure 5.5: Densitometry Analysis of Time and dose response of the release of soluble ADAM33 in response to TGF-.β

The ADAM33 clone B9B10 and the Mock transfected clone B7B3 were treated with increasing amounts of TGF-β and supernatant collected at 24h, 48h and 72h and glycosylated proteins pulled down onto ConA beads. The beads were then lysed into 150µl of reducing SDS sample buffer and 20µl blotted, using 12.5% SDS PAGE acrlimide gels. The PVDF membranes were probed with RP2-MP domain antibody. This was analysed using densitometry analysis. Error bars indicate standard deviation from 3 independent experiments.

Activity Assays

To determine if the soluble ADAM33 released from the ADAM33 HEK293 clones was an active protease, two activity assays were carried out. The first was a FRET assay, using soluble ADAM33 pulled down and eluted from ConA beads. It was found that ADAM33 is capable of cleaving the FRET peptide with a significant difference when compared to ConA pull down supernatant from mock transfected clones $p= 0.02$ as shown in figure 5.7.

The second was a HUVEC tube formation assay using low serum supernatant from Mock and ADAM33 clones were carried out by Ilaria Puxeddu. When HUVEC cells are grown in the presence of VEGF they are triggered to form tube structures when grown on Matrigel, as in Figure 5.8a. In contrast, without VEGF stimulation complete tubes are not formed Figure 5.8b. It has have previously shown that the purified MP domain of ADAM33 is also capable of causing tube formation Puxeddu et al 2008. Figure 5.8c shows HUVECs grown in supernatant from the A33B9B10 clone also form tubes. This clone has been shown to release soluble ADAM33 and can cleave the FRET peptide. Compared to supernatant taken from MB7B3 which like the media only control does not form complete tubes as shown by figure 5.8d.

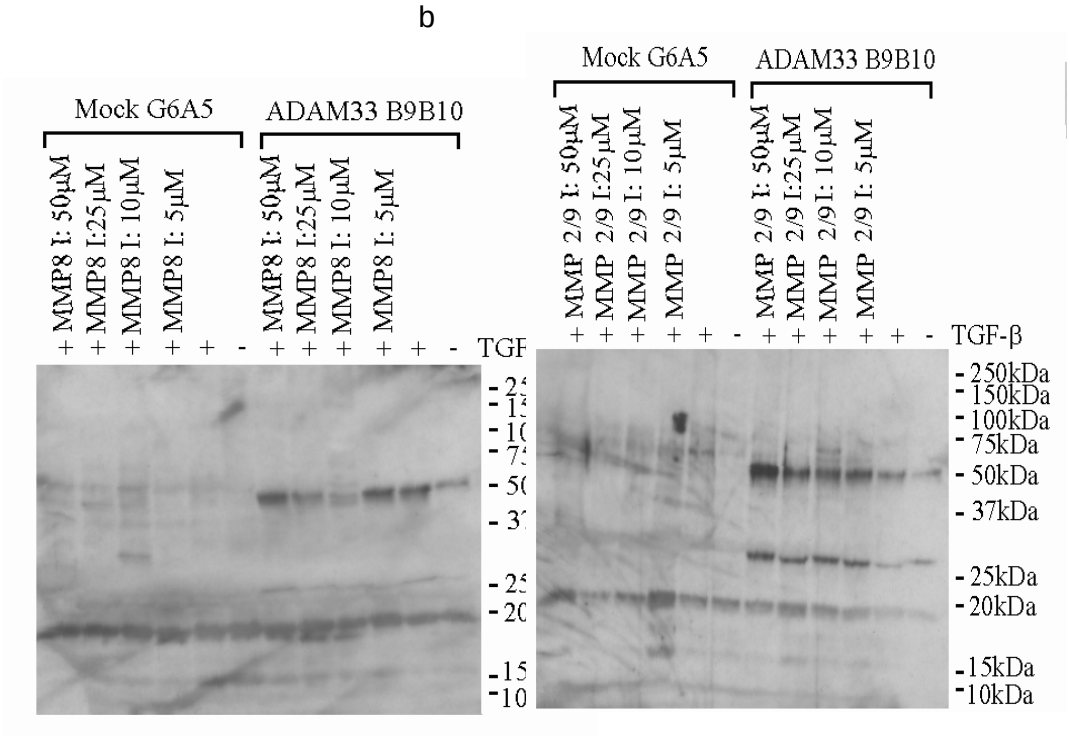


Figure 5.6 Western Blot analysis of the effect of MMP8 and MMP2/9 inhibitors on the release of Soluble ADAM33 from A33B9B10 clone.

A33B9B10 and MG6A5 clones were treated with 10ng of TGF-β and with increasing concentrations of either the MMP8 or MMP2/9 inhibitors for 72h in DMEM (0.01% FBS). a- shows the treatment with the MMP8 inhibitor at 5, 10, 25 and 50µM and TGF-β only and non treated as controls. b-shows the treatment with the MMP2/9 inhibitor at 5, 10, 25 and 50µM and TGF-β only and non treated as controls.

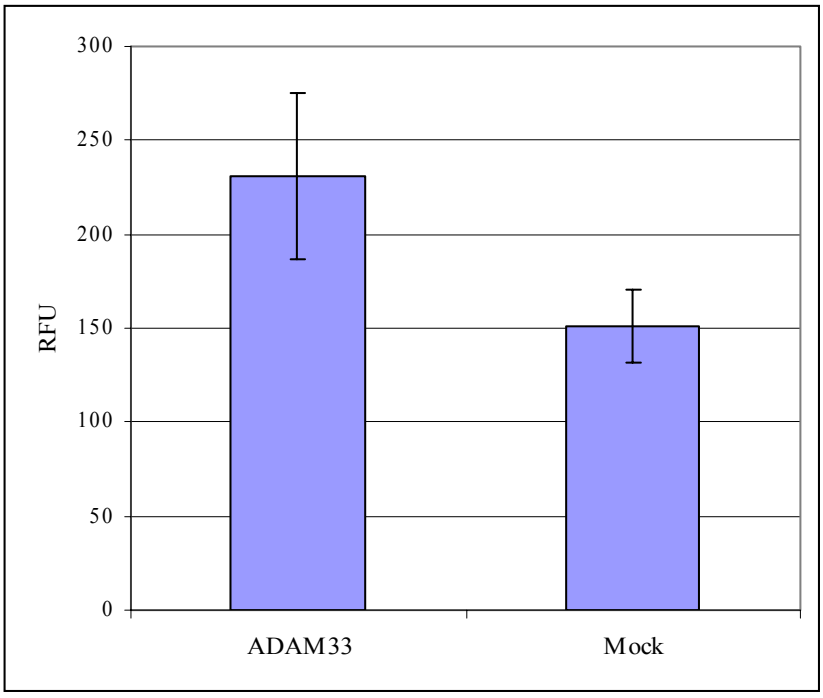


Figure 5.7: Cleavage Assay of ADAM33 and Mock transfected cell Supernatant Pulled Down onto ConA beads.

Supernatant from ADAM33 clones known to produce sADAM33 was pulled down with ConA beads and eluted as was supernatant from mock transfected clones. The FRET peptide used had the following sequence DABCYL-YRVAFQKLAE(FAM)K-NH2 which is known to be cleaved by ADAM33 MP domain. Relative florescence units have been used to compare the cleavage activity of supernatant from ADAM33 clones with supernatant from Mock transfected clones. P=0.02 (TTest).

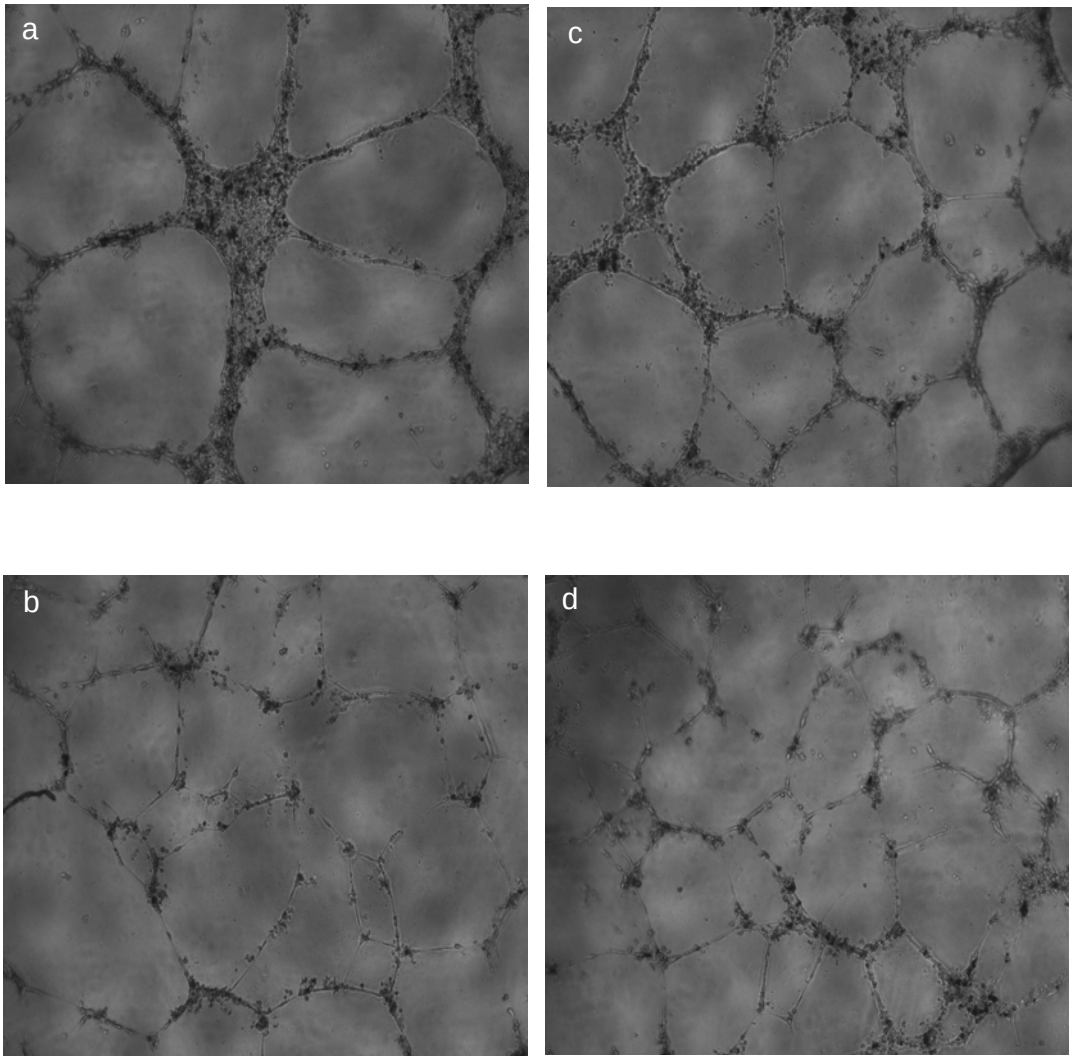


Figure 5.8 Digital Photographs of Endothelial Cell Tube Formation Assay using Supernatant from HEK293 transfected clones.

Human umbilical vein endothelial cells seeded at $8x10^3$ cells per well of a 96 well plate pre coated with growth factor reduced Matrigel matrix solution. Cells were the incubated with a- rhVEGF (10ng/ml) in M199 medium (1% FBS) as a positive control. b- M199 medium (1%FBS) as a negative control. c- Supernatant from the ADAM33 transfected HEK293 clone A33B9B10 (DMEM 0.1% serum) diluted 1:2 in M199 meduim (1% FBS). d- Supernatant from the Mock transfected HEK293 clone MB7B3 (DMEM 0.1% serum) diluted 1:2 in M199 medium (1% FBS).

Discussion

The studies in this chapter have shown that the HEK293 clones containing full length ADAM33 produce a soluble form of ADAM33 with a molecular weight of 55kDa. This indicates that ADAM33 may exert a function indirectly on other cell types that do not express ADAM33 or on components of the extracellular matrix. It is possible that in asthma, SNPs may effect the release of soluble ADAM33.

These data support the observations of Lee and Parks et al 2006 in that there is a soluble form of ADAM33. However, they also cast doubt on whether their antibody (Asp2) is detecting ADAM33. In my study, the data showed that both cell lysates and ConA pulled-down supernatant from mock transfected clones were positive using Asp2. In contrast to the RP antibodies where the mock transfacted clones were consistently negative for ADAM33. Future studies should include work to determine if Lee and Parks observation of increased sADAM33 in BAL fluid of asthmatics can be confirmed independently.

The next stage of the study was to find out how the release of sADAM33 is regulated. In attempts to up-regulate shedding of sADAM33 it was found that of all the agents tested only TGF-B2 had any effect. This up-regulation of sADAM occurred in a dose and time dependent manner. This is interesting because TGF-ß levels are known to be increased in asthmatics BAL fluid compared to normal subjects. Furthermore TGF-ß is involved in the differentiation of fibroblasts to myofibroblasts and changes in the airways of asthmatics A Redington et al 1997. Further studies could include analysis of ADAM33 cell lysates treated with the different doses of TGF-ß and a different time points using the RP1 and RP3 antibodies as well as ConA pulled down supernatant for RP1.

Inhibitor studies were then carried out to determine if ADAM33 like ADAM13 is involved in its own cleavage from the cell surface as the soluble ADAM33 cannot be

formed by splice variants A Gaultier et al 2002. The data contained here were inconclusive but, indicate that ADAM33 does not cleave itself from the cell surface as the MMP8 inhibitor should have blocked release of sADAM33 if this was the case.

Another important part of the study was to determine that the soluble ADAM33 was active. Both the FRET assay and the HUVEC tube formation assays were positive when tested using media derived from ADAM33 clones but not mock transfected clones. These studies show that in this transfected cell model, soluble ADAM33 is active and adds to evidence that ADAM33 is an active protease in the soluble form and is involved in angiogenesis I Puxeddu and YY Pang et al 2008.

Most ADAM33 SNPs associated with asthma are found in the cytoplasmic tail of the ADAM33 gene. It is therefore possible that one or more of these SNPs could cause an increase of soluble ADAM33 release from the cell surface. This could then lead to a gain of function which would contribute to obstruction of the the airways and loss of lung function in asthmatics. Alternatively polymorphisms in the 5' region of ADAM33 in the catalytic domains may have a direct effect on function. Since sADAM33 promotes angiogenesis ADAM33 is therefore implicated in remodeling aspects of asthma pathogenesis rather than inflammation aspects.

Since TGF-β2 has been shown to up regulate sADAM33 release from the surface, my data further suggests that, there must be some interaction with environmental factors. For example epithelium damage may lead to increased production of TGF-β. Alternatively eosinophils that are recruited into the airways following allergen exposure may also be a source of TGF-β.

In conclusion sADAM33 has a likely function in angiogenesis and can be upregulated in a time and dose dependent manner in the presence of TGF-B. These data are consistent with the idea that ADAM33 has a role in remodeling. Dis-regulation of the release of sADAM33 from the cell surface in asthmatics could arise from SNPs in the full length ADAM33 and the interaction of sADAM33 and TGF-B which is also up-regulated in asthma. Showing the interaction of underlying genetic changes in asthma and environmental factors involved in asthma.

Final Discussion

ADAM33: A Cellular model of over-expression. From Full Length to Soluble.

The objectives of this project were to produce a stable transfected cell line over-expressing full length ADAM33 as well as a mock transfected control cell line. These cell lines were then assessed for any phenotypic changes that may provide a clue concerning potential functions for ADAM33. This involved assessing 1) changes in proliferation rate between ADAM33 transfected cells and the mock transfected cells, 2) the processing of ADAM33 to the mature form and 3) the possibility of shedding of a soluble form of ADAM33.

HEK293 cells were successfully transfected with a full length ADAM33 construct and a stable cell line was produced along with a mock transfected control cell line. However the ADAM33 cell line did not seem to process ADAM33 from the 120kDa pro-protein into the mature 100kDa form. There were several explanations for this finding. These included lack of the 3' UTR, absence of furin which is required for processing of ADAM33, or simply that not enough was produced to be detected by western blot. Umland et al 2003 found that ADAM33 is 2-3 fold more effectively processed when the 3'UTR present but this is an unlikely explanation in the current study as in transient transfections processing was seen by western blotting. Another possibility was that HEK293 cells did not contain furin/furin like pro-protein convertases needed to cleave the prodomain from the MP domain of ADAM33. Again this is unlikely because of the transient transfections and is confirmed by K Endres et al 2003 who demonstrate the presence of furin in HEK293 cells. Alternatively it could be that the levels of processing were very low. This could have been the case because the selection process used to produce the stable cell line containing ADAM33 may have favoured cells that do not

process the pro form of ADAM to the mature and active protein. This means that while some of the transfected cells can process ADAM33, this population of cells was smaller than the cells that did not process ADAM33 and therefore the 100kDa mature form was not detected by western blot. In addition to this initial observations suggested that the ADAM33 transfected cells were growing faster than the mock transfected control cells. This phenotypic change could only have been caused by the mature form of ADAM33. The observation of increased proliferation in the ADAM33 cells was lost by passage 6 also indicating that ADAM33 cells capable of processing to the mature form were disfavoured.

This observation was the only phenotypic change to be seen between the ADAM33 cells and the mock transfected cells. To explore this further proliferation assays were carried out using firstly Methylene blue method and then using CellTiter 96 one solution. Using both these methods no definitive answer could be found. In part this was due to technical problems with the Methylene blue assay. However these problems were reduced by using CellTiter 96 one solution so it was concluded the technical problems were not the only issue. The most likely reason was the polyclonal nature of the cells and the likely disfavouring of ADAM33 cell capable of processing over time.

At this time it was decided that both cell lines should be cloned by limiting dilution as the problems of the ADAM33 protein not being processed and the inconclusive proliferation assays could be due to the polyclonal nature of these cell lines. After cloning some of the ADAM33 transfected clones were found to be processing the protein on western blot. However when proliferation assays were repeated with a selection of clones and for the ADAM33 clones that could or would not process ADAM33 no difference in proliferation was found between the cells processing and those not. Nor was there a significant difference when the ADAM33 population was compared to the mock transfected clones. However there was evidence that ADAM33

may improve cell survival when the cells were under stress during cloning.

Another suggestion was that the lack of consistent proliferation data may be because the true extent of the difference was masked due to ADAM33 being a large transmembrane protein driven by a CMV promoter for constant expression which could be reducing the true proliferation rate produced by the presence of ADAM33 because the cells have to commit much of their biosynthetic machinery to produce this protein. A good control would have been to transfect HEK293 cells with another large transmembane protein and to observe the proliferation of these cells and non-transfected HEK293 cells.

As well as showing that HEK293 cells are capable of processing ADAM33, western blots of the ADAM33 clones showed a band at 40kDa that was specific to the ADAM33 clones using the RP3 antibody that detects the cytoplasmic tail of ADAM33. This band also increased with expression of ADAM33 protein and with the ability to process the ADAM33 but was not found in the mock transfected clones. This band could have been a degradation product of the full length protein or could have been a part of the protein left behind if the extracellular domain of ADAM33 was cleaved from the cell surface producing a soluble form. If this was the case it could also explain why so little processing was detected. Indeed Lee and Parks et al 2006 have shown a soluble form of ADAM33 with a molecular weight of 55kDa in BAL fluid of asthmatics.

To determine whether ADAM33 ectodomain was shed from the transfected cells, the supernatants of ADAM33 HEK293 clones and a mock transfected HEK293 clone as a control and was extracted using ConA beads that bind glycosylated proteins, including ADAM33. Western blot analysis using the RP2 antibody to the MP domain found that there was a soluble form of ADAM33 with a molecular weight of 55kDa which is consistent with the soluble form found by Lee and Parks et al 2006. However when using their affinity purified Asp2 antibody a band at 55kDa was found in both ADAM33 clones and a mock transfected clone suggesting that this band was due to

non-specific antibody binding. While their antibody may not detect ADAM33, a soluble form of ADAM33 has been found in the current study. Further work on soluble ADAM33 would have to include a repeat of Lee and Parks work on the supernatant of MRC5 fibroblast cell line and BAL fluid from normal and asthmatic subjects using the RP2 antibody to confirm that soluble ADAM33 is present in these samples and that there is indeed an increase in sADAM33 with increasing severity of disease. It is unlikely that there will be as much soluble ADAM33 in these samples as in the ADAM33 transfected HEK293 as ADAM33 expression is controlled by a CMV promoter and therefore overexpressed. Therefore it will be necessary to optimise conditions for enrichment of soluble ADAM33 using the ConA pull down procedure.

One point worth considering is how sADAM33 could be released into BAL fluid as epithelial cells do not appear to express ADAM33. Although there has been some evidence that ADAM33 is found in epithelial cells, recent work by Y Yang et al has shown that the ADAM33 promoter is methylated in epithelial cells and this silences its expression, even in asthma Y Yang et al 2008. As ADAM33 is expressed in mesenchymal cells S Umland et al 2003, soluble ADAM33 could be released from fibroblasts, myofibroblasts and smooth muscle cells and then into BAL fluid through damaged epithelium. This would also explain the increase of sADAM33 seen as disease severity increases as damage to the epithelium is more extensive in severe asthma. Or by soluble ADAM33 binding to the integrins of inflammatory cells via its disintegrin domain. ADAM proteins have been shown to bind integrins found on lymphocytes L Bridges et al 2005.

The confirmation that ADAM33 was found in a soluble form led to further experiments to explore how the release of sADAM33 can be regulated and how cleavage from the cell surface was achieved. One possibility was that ADAM33 is capable of cleaving itself from the cell surface as is the case with ADAM13 A Gaultier et al 2002. This

appeared not to be the case as inhibitor studies to stop the activity of ADAM33 did not stop the release of ADAM33 from the cell surface. This suggests it must be cleaved by another protease as yet unidentified. The soluble form of ADAM33 cannot be a splice variant as the HEK293 were transfected with a construct of full length ADAM33 cDNA and expression of endogenous ADAM33 was not induced upon treatment. Regulation may involve MMP 2 or 9 and their inhibitors as in the inhibitor study here unexpectedly showed an increase in soluble ADAM33 in the presence an MMP2/9 inhibitor so less of these MMPs resulted in increased soluble ADAM33.

Activity assays were carried out to assess if soluble ADAM33 was an active protease. In a FRET assay soluble ADAM33 eluted from ConA beads was able to cleave the FRET peptide leading to an increase in fluorescence. In addition a HUVEC tube formation was carried out on cell supernatant and produced the same tube formation as purified ADAM33 MP protein which was used as a positive control I Puxeddu and YY Pang et al 2008. In contrast, supernatants from a mock transfected clone did not. Therefore there is a likely function of ADAM33 in angiogenesis. While a knock-out mouse model of ADAM33 showed no phenotypic abnormalities C Chen et al 2006 it is unlikely that ADAM33 knock-out alone would inhibit angiogenesis as it is likely that other metalloproteases would be capable of replacing the ADAM33 function in this vital process. It is also more likely that SNPs in the *ADAM33* gene lead to overexpression in asthmatics rather than a loss of function as there is no evidence of the loss of ADAM33 in asthmatics and so it is possible that ADAM33 could be associated with vascular changes seen in asthma. These changes include increases in the number and size of vessels due to the increased expression of angiogenic mediators and their receptors. This may support cell growth and add to the thickening of the airway wall. Angiogenesis has been shown to correlate with disease severity and an increased rate of lung function decline X Li et al 1997 and M Hashimoto et al 2005. It is therefore

possible that SNPs in the cytoplasmic domain that could cause gain of function changes where over-expression and release of soluble ADAM33 results in excess cleavage of substrates that cause angiogenesis.

Studies were also carried out to assess if the release soluble ADAM33 could be upregulated. PMA and calcium ionophore were used as general cell stimuli. PMA is used to experimentally activate Protein Kinase C (PKC) and has been shown to stimulate the expression of other ADAM proteins, for example Worley et al showed upregulation of ADAM8, 9, 17 and 28 in THP-1 cells (human monocyte-like cells) treated with PMA J Worley et al 2003. Calcium ionophore A23187 can mimic the stimulation that cells receive from cytokines and can cause increases in cytoplasmic Ca^{2+} leading to cell activation, cell differentiation and proliferation. In a study of ADAM3, A23187 removed a soluble form of ADAM3 from sperm heads during the acrosome reaction (which allows sperm to fuse with the egg) E Kim et al 2004. However neither of these agents had any effect on the levels of soluble ADAM33 under the conditions used.

When molecules that have links to asthma were tested only TGF-B was shown to upregulate the release of ADAM33. TGF-ß levels are known to be increased in bronchoalveolar lavage fluid of asthmatics compared to normal subjects Duvernelle et al 2003. TGF-β causes the differentiation of fibroblasts to myofibroblasts which secrete interstitial collagens and therefore with increased levels in asthmatic airways there is collagen deposition in the submucosa and thickening of the laminar reticularis.

TGF-β(1 and 2) are implicated in processes that are thought to extend the period of epithelial repair and that may keep the proinflammatory phenotype of the epithelium maintained E Minshall et al 1997, A Vignola et al 1997 and Holgate et al 1999. The balance of TGF-β and EGF in asthmatics has led to the hypothesis that the epithelial mesenchymal tropic unit (EMTU) is reactivated in susceptible individuals and that

interaction between the epithelium and mesenchymal cells in the submucosa leads to the airway remodelling seen in asthma D Davies and S Holgate 2002.

Soluble ADAM33 is increased in the presence of TGF-β suggesting that under conditions with increased TGF- β more soluble ADAM33 can be produced. This may lead to a potential gain of function in individuals susceptible to asthma. Abnormal regulation of ADAM33 due to polymorphisms in the *ADAM33* gene could interact with TGF-β to produce more sADAM33 and lead to a gain of function. Based on current evidence that increased sADAM33 could contribute to vascular changes seen in asthma by increasing vessel formation this may contribute to thickening of the airway walls and therefore the decline in lung function and worsening severity of asthma.

ADAM33 is implicated in the remodelling aspect of asthma. Several studies have shown that SNPs in the *ADAM33* gene are associated with asthma and in particular with BHR. Other studies have associated SNPs with decline in lung function both in early life (3 and 5 year old children) A Simpson et al 2005 and accelerated lung function decline in asthmatics over a 20 year period Jongepier et al 2004. Polymorphisms in the gene have also been associated with accelerated lung function decline in people with COPD Gosman et al 2006 and in the general population van Diemen et al 2005. Changes in the *ADAM33* gene in asthmatic individuals could interact with changes in the environment that cause damage to the airways which increases levels of growth factors. Further work is required to discover the effect of ADAM33 SNPs in this process. This could be done by site directed mutagenesis of ADAM33 plasmids with SNPs associated with asthma. Firstly this would be SNPs that cause coding changes in the transmembrane and cytoplasmic domains S1, S2, T1 and T2. To determine if SNPs in this area lead to a loss of regulation and an increase in sADAM33. S1 is also associated with impaired lung function in early life (aged 5) and S2 with accelerated lung function decline in asthmatics. It would also be useful to examine the effect of the

F+1 and ST+7 SNPs that were most significantly associated with asthma and BHR in a meta analysis J Blakey et al 2005 and are located in introns suggesting they may affect splicing It would also be useful to determine what domains of ADAM33 are included in the soluble form and in particular if the disintegrin domain is included as this could have implications in ADAM33 function. Another aspect of soluble ADAM33 is how it is cleaved from cell surface and the mechanism involved in the release of ADAM33 and the mechanism of its proteolytic activity. Inhibition of soluble ADAM33 may be an effective target for asthma treatment and could potentially be a successful disease modifier rather than relieving symptoms like current treatments.

Conclusions

ADAM33 and in particular the soluble form are implicated strongly in remodelling of asthmatic airways by its function in promoting angiogenesis and the vascular changes in asthma.

This study has identified that the ectodomain of ADAM33 is shed from the cell surface and that this is enhanced by TGF-B. The shed ectodomain was functionally active in angiogenesis assays suggesting a potential role for ADAM33 in asthma.

Further studies are required to determine if ADAM33 is shed by fibroblasts or smooth muscle cells and if more soluble ADAM33 is released from cells of asthmatics and if so is there a correlation with disease severity. It will also be important to investigate what cleaves ADAM33 from the cell surface and which domains are included in the soluble protein. Another important area to study would be the role of SNPs in the release and function of soluble ADAM33. Finally there is a need to determine when soluble ADAM33 acts, whether it is important in early life changes in asthmatics before any asthma symptoms are evident or whether it acts in response to epithelial damage, allergens or to cytokines or growth factors.

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