1.INTRODUCTION

1.1 The respiratory system

The respiratory system is an anatomic structure with the aim of supporting the gas exchange process. This process allows the exchange between oxygen and the carbon dioxide in the lungs with the help of hemoglobin and plasma. The respiratory system can be divided into two parts (1 Gray): the first part consists of the airway, the channel (main pipe) that the air passes through during the inspiration and expiration processes; the second part consists of the lung. The principal function of the lung is to allow the transport of oxygen from the atmosphere into the bloodstream and to release carbon dioxide from the bloodstream into the atmosphere. This exchange of gases is accomplished by specialized cells that form millions of tiny, exceptionally thin-walled air sacs called alveoli. From the anatomic point of view (Figure 1.1), the respiratory system is constituted by: nose, oral cavity, pharynx, larynx, trachea, main bronchi, intermediate bronchi and alveoli, lungs and pleura.

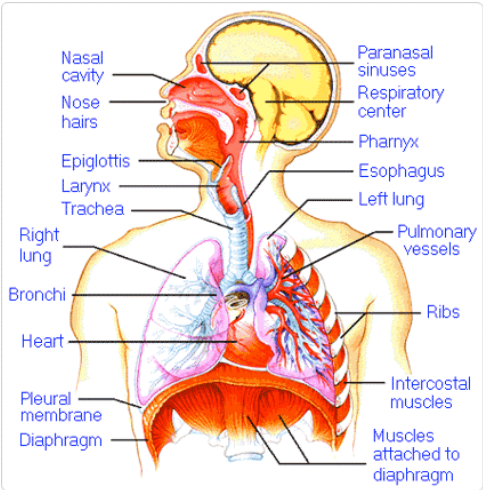


Figure 1.1: the anatomy of the respiratory system (natural medicine 2009)

The nose is divided into two regions: the external nose and the internal nasal cavity; the nasal cavity is divided by a midline nasal septum, which opens posteriorly into the nasal pharynx via internal nares; the ethomid and sphenoid bones form the roof, and the floor is formed by the hard and soft palates

(Figure 1.2).

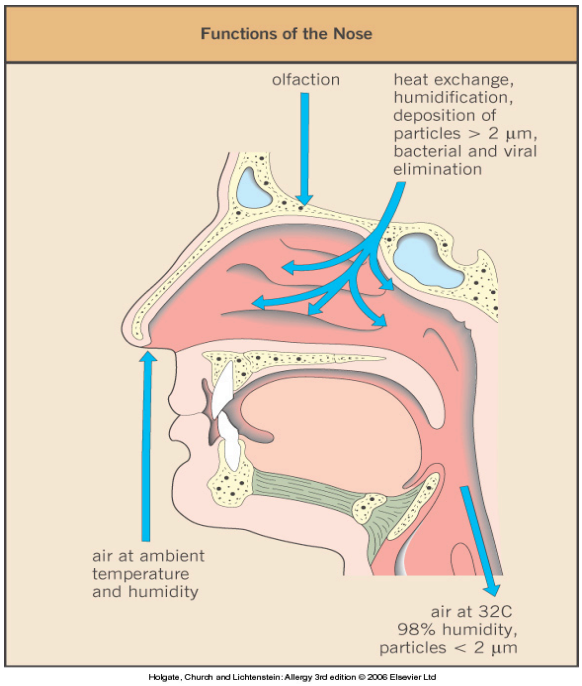


Figure 1.2: Holgate, Church and Lichenstein, Allergy 2006

The oral cavity connects the nose to the pharynx. The pharynx is part of the respiratory system as well as the digestive system because both air and food pass through it. The pharynx is divided in three parts: the nasopharynx, the oropharynx and the laryngopharynx. The larynx connects the inferior part of laryngopharynx to the trachea. The larynx consists of nine cartilages; three paired and three non-paired. The three paired are arytenoid, corniculate and cuneiform; the three non-paired are thyroid, cricoid and epiglottic. At the end of the larynx is the trachea, a tube formed by 20 C-shaped rings of cartilage, which

then bifurcates in the two main bronchi, the left bronchus and the right bronchus. The two main bronchi successively branch, within the lungs, into five lobar bronchi. The lobar bronchi then divide into 20 segmental bronchi, which then branch into primary bronchioles and finally into terminal bronchioles, each of which then has several respiratory bronchioles. The respiratory bronchioles branch into 2 to 11 alveolar ducts, each of which is associated to 5 or 6 alveolar sacs, the anatomical unit of gas exchange in the lung. There are two lungs in total which are placed in the thorax and separated from each other by the heart and other contents of the mediastinum. The mediastinum is a group of structures in the thorax cavity surrounded by connectivity tissue. Each lung is conical in shape, and presents for an apex, a base, three borders and two surfaces. The lungs are not identical, they are separated into lobes by fissures, with three lobes on the right and two on the left. The lobes are further divided into segments and then into lobules. The lungs structure consists of an external serous coat which folds back to make an interior lining for the chest cavity. The inner lung contains subserous areolar tissue with elastic fibers interspersed over the surface of the organ. The parenchyma, or functional part of the organ, is composed of secondary lobules (alveolar ducts) that differentiate into primary lobules (alveoli) consisting of blood vessels, lymphatics, nerves, and an alveolar duct that connects with air space.

1.2 Embryogenesis

Embryogenesis is the process by which the embryo is formed and developed into the foetus. The process begins when fertilization takes place, that is when a sperm meets and fuses with an ovum. The fertilized ovum is known as zygote, and it undergoes rapid mitotic division with no significant growth, and cellular differentiation that leads to the development of the embryo. The embryonic period comprises 56 days, that is eight weeks from the moment of fertilization. During the embryonic period most of the organ systems are established and this happens with an enormous rapidity. Cell division, movement and differentiation are the basic processes taking place during this phase. Following this phase there is the foetal period where the organs that formed during the embryonic period grow and differentiate.

Embryogenesis of the lung

The development of the lungs (Figure 1.3), in comparison with the development of other organs during the prenatal period, occupies a special position. The reason for this is because breathing organs are unnecessary for intrauterine existence. Nevertheless they must developed and be ready to function immediately following birth. This explains why the entire development of this organ extends from the embryonic period through the foetal period up to birth. However, during the intrauterine life, the lungs are an important source of amniotic fluid, which is "inhaled" and "exhaled" by the fetus. It is essential for this fluid to be breathed into the lungs in order for them to develop normally. This fluid is also very important for several other reasons such as mechanic protection of the foetus and as a source of proteins, carbohydrates, lipid and

phospholipids, urea and electrolytes which all aid and contribute to in the growth of foetus.

The lung development is divided in five phases: the embryonic phase (3rd week-8th week), the pseudoglandular phase (8th week -16th week), the canalicular phase (16th week – 24th week), the saccular phase (24th week – 36th week) and the alveolar phase (36th week- 1.5 years after birth).

The embryonic phase begins with the formation of a groove in the ventral lower pharynx, the sulcus laryngotrachealis. From the lower part of this sulcus, the true lung primordium will form, and in the further division it will form the main bronchi with the asymmetry they present in adults. This will start the subdivision of lobes and it will also form the pulmonary vessels.

During the pseudoglandular phase, the development of the entire bronchial tree up to the terminal bronchiole occurs, which means that at this point the respiratory ducts will have already formed. The ducts are coated by cuboidal epithelial cells which are the precursor cells of ciliated epithelium and of secretory cells. After the 10th week, cartilage and smooth muscle cells as well as bronchial glands can be found in the wall of bronchi. Also during this phase, the lung begins cyclical contraction at a rate of approximately 1 contraction per second.

The canalicular phase is characterized by the formation of the lung acinus, the invasion of capillaries into the mesenchyme, the differentiation of the epithelial cells that start the production of the amniotic fluid and by surfactanct. Between

the 20th week and the 22nd week the epithelial cells start the differentiation into type I and type II pneumocytes.

The saccular and the alveolar phases are characterized by the final development of the alveolus, which is now able to perform the gas exchange, and by the specialization of the II type of pneumocytes which are able to produce the mature surfactant. The surfactant (abbreviation for surface active agent) consists of glycerophospholipids, specific proteins, neutral fats and cholesterol. It covers the alveolar surface and reduces the surface tension, meaning that it prevents collapse of the alveoli during the expiration.

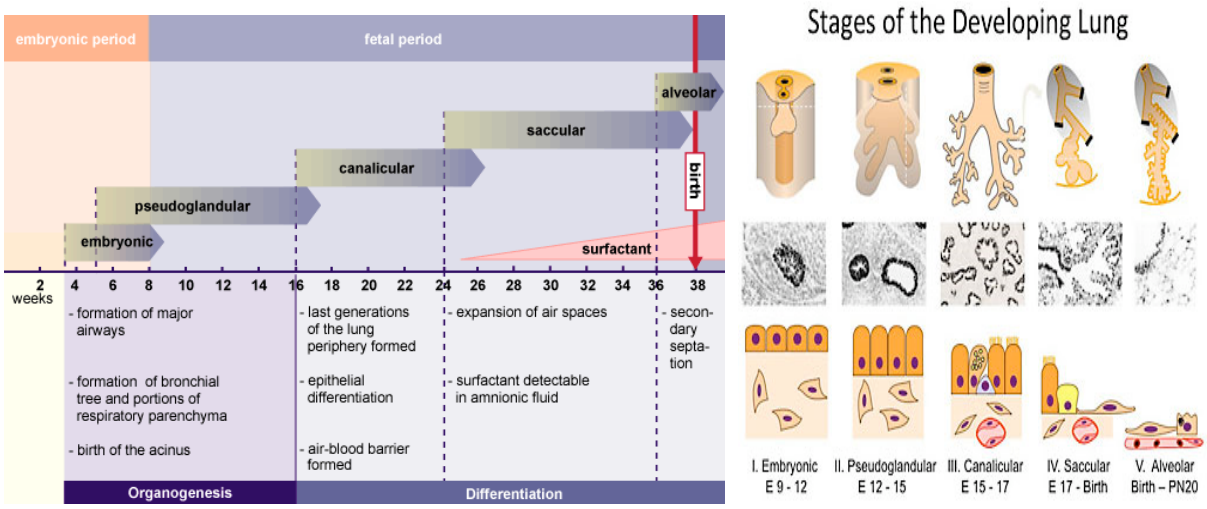


Figure 1.3: Human embryology and organogenesis 2009

1.3 Histology of the bronchi

The histological structure of the bronchus starting from inside to outside is constituted by three layers: mucosa, sub mucosa and adventitia (Figure 1.4).

Mucosa consists of a columnar epithelium pseudostratified ciliated and a lamina propria: the columnar psudostratified epithelium consists of several cell types: ciliated cell, globet cell and basal cell, the lamina propria is constitued of connective tissue where there are serous-mucous glands.

Sub-mucosa is situated below the mucosa, which contains collagen fibers, elastic fibers, fibroblasts and smooth muscle cells.

Finally, adventitia is constituted of cartilage and connective tissues which separates the bronchial tree from the others organs.

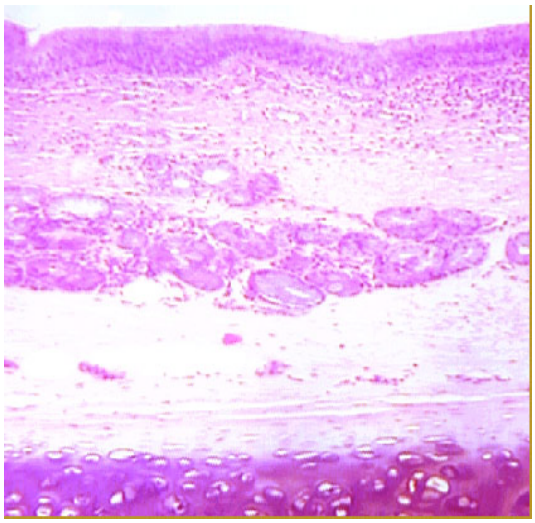


Figure 1.4:bronchial section stained with hematoxylin (nucleus) and eosin (cytoplasm). It is possible to distinguish the three layers: mucosa (top), sub mucosa and adventitia.

1.4 Asthma

One of the most common lung diseases is asthma. It is a chronic inflammatory disease of the airways characterized by variable airflow obstruction, bronchial hyper-responsiveness (BHR) and recurring symptoms such as wheezing, coughing, chest tightness, and shortness of breath (91,7,34). Asthma affects nearly 300 million people world wide, prevailingly in the US and-UK where the number is higher in respect to most of the other countries in the world (Figure 1.5 (Gina, 2009,)).

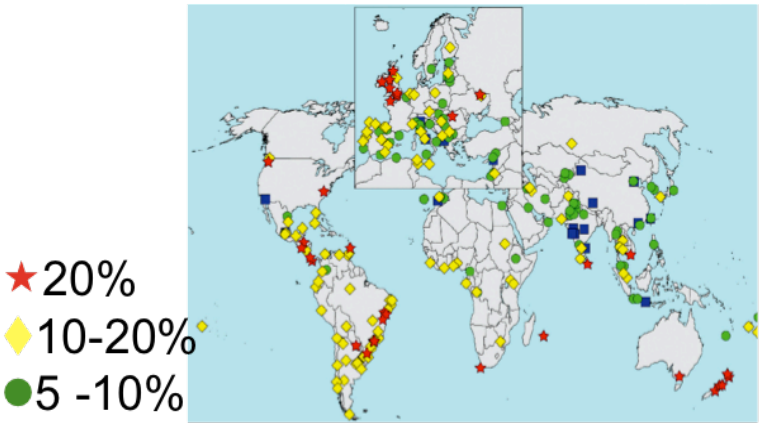


Figure 1.5: ISAAC thorax 2009

Asthma prevalence also differs between populations of the same ethnicity living in different places (1). For example, US-born Mexican populations have higher asthma rates than non-US born Mexican populations that are living in the US (2)

Other studies show that the incidence of asthma is highest among low-income populations both nationally and worldwide. In England, an estimated 261,000 people were newly diagnosed with asthma in 2005 (Gina 2009). In the last 20 years the prevalence of asthma has increased considerably in many countries, especially in children, with a consequent increase in cost for organizations such

as the UK National Health Service. Nowadays the guidelines for classifying asthma severity are based on two parameters: the FEV_1 (forced expiratory volume in 1 second) and the peak expiratory flow rate (3).

According to this clinical classification, asthma can be classified in terms of the frequency of symptoms which can be intermittent, mild persistent, moderate persistent and severe persistent. Asthma may also be classified as atopic or non atopic, according to the reaction to allergens (atopic) or not (non atopic). In this disease many cells play different roles, and like an orchestra they all play a crucial role in the evolution of the disease. Analyzing biological features of the disease, it is clear that it is characterized by a polarized Th-2 (T helper 2) cell response with an accumulation of CD4+ (T helper) lymphocytes, eosinophils and mast cells in the airway mucosa (4,8,9,34).

Also, neutrophils play an important role in the pathogenesis of the disease, especially in severe and mild conditions (5).

Based on this knowledge, the target to challenge has been cells such as the eosinophil and neutrophil (6,7,27,53,85).

However, targeting these components with selective therapies such as anti IL5 (7,43) has been disappointing. The current drug therapy used is based on anti-inflammatory drugs such as corticosteroids that switch off the inflammation process and bronchodilators that relax the bronchial smooth muscle. This general approach is helpful to treat the majority of asthmatic patients, but not all of them, and it does not resolve the underlying mechanism(s) of the disease. Also, there are other subgroups of the disease which do not follow a clear

method of classification, a problem derived from the difficulty in finding a therapy to control their symptoms (9,60,90).

Finding a way to identify subgroups that respond well to different types of treatments is a current critical goal of asthma research, and to achieve this, the underlying basis of asthma has to be identified.

In order to accomplish this, it is important to understand the complex genetic and environmental influences that are involved in the disease, and to develop appropriate treatment strategies.

Over one hundred genes have been associated with asthma or related phenotypes and just in 2006 and 2007, 53 novel candidate genes associations were reported. Also, many environmental factors have been associated with asthma development, such as environmental tobacco smoke, (especially maternal cigarette smoking), low air quality from traffic pollution or high ozone levels, viral respiratory infections and psychological stress (28,32,54,62).

Due the complexity of asthma, it is difficult to understand the single effect of each component and how it leads to the disease (Figure 1.6a, 1.6b). Some studies have confirmed without any doubt that asthma has an important genetic component, but that there is no clear pattern of inheritance with a variable heritability rate between 36-79% (10,55).

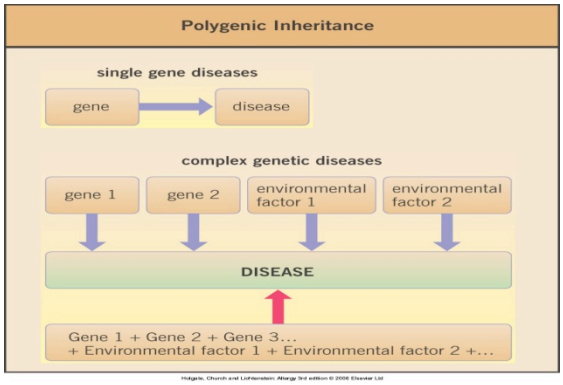


Figure 1.6a: Holgate, Church and Lichenstein, Allergy 2006

In order to be confident that a single gene or a group of genes are linked with a pathology, it is important to confirm the replication of genetic associations and also to demonstrate a functional mechanism for the associated variants. Efforts to identify asthma genes have been carried out in various laboratories around the world with two general approaches: positional cloning and a candidate gene approach. The first method is based on the isolation of partially overlapping DNA segments from genomic libraries to progress along the chromosome towards a specific gene. (This is a method of gene identification where only its approximate chromosomal location is known but not the function). This method is also known as the candidate region. Initially, the candidate region can be defined using techniques such as linkage analysis, and then with the positional cloning method it is possible to narrow the candidate region until the gene and its mutations are found. The second method is based on the approach that involves assessing the association between a particular allele of a gene that may be involved in the disease (a candidate gene) and the disease itself. This approach is limited by knowledge of the biology of the disease being investigated.

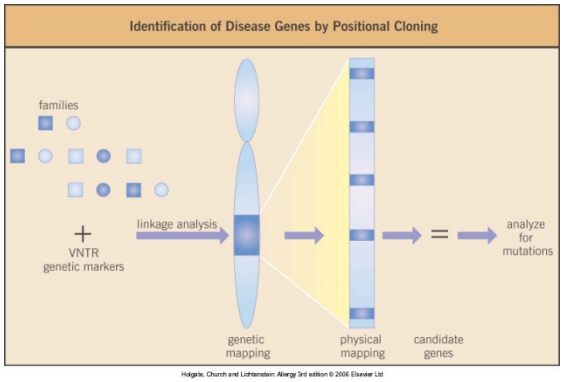
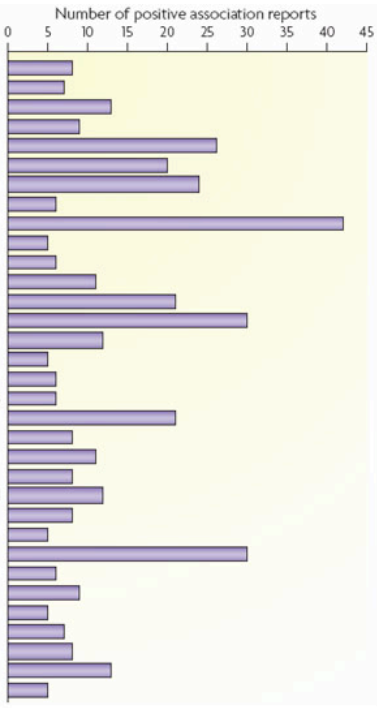


Figure 1.6b: Holgate, Church and Lichenstein, Allergy 2006.

Multiple genome-wide linkage studies for asthma and allergy have been performed to date, and show that many of the genes are related to the immune system or involved in the modulating inflammation or in the remodelling processes (Figure 1.7).

Gene	Chromosome	Function and pathway	Common variants
GSTM1	1p13.3	Environmental and oxidative stress — detoxification	+/null
FLG	1q21.3	Epithelial barrier integrity	Arg510X, 2282del4
IL10	1q31-q32	Immunoregulation	−1082A/G, −571C/A
CTLA4	2q33	T-cell-response inhibition and immunoregulation	−318C/T, 49A/G
IL13	5q31	T_H2 effector functions	−1112C/T, Arg130Gln
IL4	5q31.1	T_H2 differentiation and IgE induction	−589C/T, +33C/T
CD14	5q31.1	Innate immunity — microbial recognition	−1721G/A, −260C/T
SPINK5	5q32	Epithelial serine protease inhibitor	Glu420Lys
ADRB2	5q31-q32	Bronchial smooth-muscle relaxation	Arg16Gly, Gln27Glu
HAVCR1	5q33.2	T-cell-response regulation — HAV receptor	5383_5397del
LTC4S	5q35	Cysteinyl leukotriene biosynthesis — inflammation	−444A/C
LTA	6p21.3	Inflammation	*Nco*I (intron 1)
TNF	6p21.3	Inflammation	−308G/A, −857C/T
HLA-DRB1	6p21	Antigen presentation	Multi-SNP alleles
HLA-DQB1	6p21	Antigen presentation	Multi-SNP alleles
HLA-DPB1	6p21	Antigen presentation	Multi-SNP alleles
GPRA	7p14.3	Regulation of cell growth and neural mechanisms	Haplotypes
NAT2	8p22	Detoxification of drugs and carcinogens	Slow acetylation SNPs
FCERIB	11q13	High-affinity Fc receptor for IgE	Ile181Leu, Gly237Glu
CC16	11q12.3-q13.1	Epithelium-derived anti-inflammatory protein	38A/G
GSTP1	11q13	Environmental and oxidative stress — detoxification	Ile105Val
IL18	11q22.2-q22.3	Induction of IFNγ and TNF	−656T/G, −137G/C
STAT6	12q13	IL-4 and IL-13 signalling	2964G/A, (GT)n exon 1
NOS1	12q24.2-q24.31	Nitric oxide synthesis — cell–cell communication	3391C/T, 5266C/T
CMA1	14q11.2	Mast-cell chymotryptic serine protease	*Bst*X1, −1903G/A
IL4R	16p12.1-p12.2	α-chain of the IL-4 and IL-13 receptors	Ile50Val, Glu551Arg
CCL11	17q21.1-q21.2	Epithelium-derived eosinophil chemoattractant	Ala23Thr, −1328G/A
CCL5	17q11.2-q12	Monocyte, T-cell and eosinophil chemoattractant	−403A/G, −28C/G
ACE	17q23.3	Inactivation of inflammatory mediators	In/del
TBXA2R	19p13.3	Smooth-muscle contraction, inflammation	924T/C, 795T/C
TGFB1	19q13.1	Immunoregulation, cell proliferation	−509C/T
ADAM33	20p13	Cell–cell and cell–matrix interactions	Multiple SNPs
GSTT1	22q11.23	Environmental and oxidative stress — detoxification	A/null



Nature Reviews | Immunology

Figure 1.7: Nature Reviews Immunology 8, 169-182 (March 2008)

Susceptibility to asthma can be influenced both by genes and environmental factors which can have an impact at different stages in life. A genome-wide association study involving over 10,000 people with asthma and more than 16,000 controls identified variants at the ORMDL3/GSDMB locus were found to be associated only with childhood-onset disease (11,68,69).

Similarly, Simpson et al. (12) demonstrated an association between ADAM33 and asthma in childhood. Polymorphisms (SNPs) were associated with reduced lung function at both 3 and 5 years of age. All of the SNPs associated with altered lung function in this study were previously associated with asthma and bronchial hyperresponsiveness in adults in U.K., U.S., Dutch, and German populations. These important founding give effort to hypothesized that asthma has an important genetic component and that there are early life environmental influences which induce the disease development. From this point of view, it could be important to study the pattern of expression of specific asthma genes during lung development.

1.5 ADAM33

ADAM33 (A Disintegrin And Metalloproteinase 33) was the first asthma susceptibility gene to be identified by positional cloning using a cohort of 460 Caucasian family in US and UK (Figure 1.8) (13,86). It is an asthma and chronic obstructive pulmonary disease (COPD) susceptiblity gene (16,17,25) with single nucleotide polymorphism strongly associated with asthma, bronchial hyperresponsivness (BHR), and decline in lung function (14,15,23).

The gene is found on the short arm of chromosome 20 (20p13) and it consists of

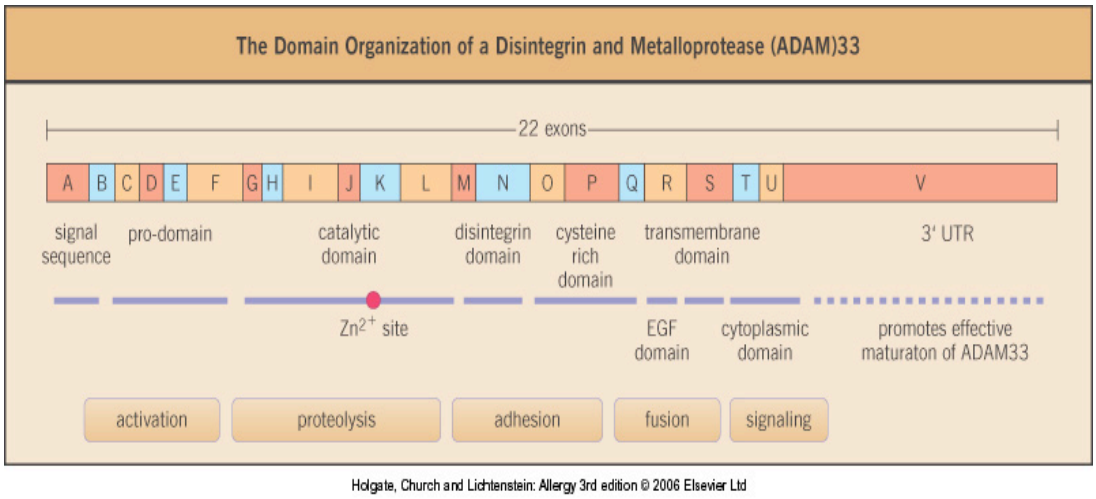


Figure 1.8: The domain organization of ADAM33, Holgate, Church and Lichenstein, Allergy 2006

22 exons (Association of ADAM33 gene with asthma and bronchial hyperesponsiveness, Nature 2002). The full-length protein consists of several domains: signal sequence, pro-domain, catalytic domain, disintegrin domain, cysteine-rich domain, EGF-domain, transmembrane-domain and cytoplasmic domain (16,17). ADAM33 belongs to the type 1 transmembrane metalloprotease family and it is a member of the ADAM protein family of zinc-dependent metalloproteases. The protein family comprises over 40 members and their main function is to act as enzymes

that cleave peptide bond proteins. ADAM33 is formed as pro-enzyme in which the catalytic activity of the enzyme is inactive due to the presence of the prodomain which binds to the catalytic site via a cysteine switch mechanism (18,19,20,21). The enzyme is activated by furin-type proteases that cleave the prodomain. Adjacent to the metalloprotease domain, there is the disintegrin-like domain, which is named to distinguish from the true disintegrins of the snake venom metalloprotease (SVMPs). Functionally, they have the same action, and many ADAMs can bind integrins via the conserved RGD motif (20). Close to the disintegrin-like domain, there are the cysteine-rich and EGF domains, which are involved protein-protein interactions (eg. in binding syndecans), and cell fusion respectively. Finally, is the transmembrane domain and cytoplasmic tail which has SH3 binding sites involved in intracellular signalling (22).

The ADAM name comes from two functions of the protein, disintegrin and metalloprotease, but based on the complex structure of the protein, it may have several other functions: such as cell proliferation, differentiation, migration and embryogenesis (24).

mRNA analysis has shown that ADAM33 occurs as multiple alternatively spliced isoforms (Figure 1.9). Through alternative use of the various domains of ADAM33, the splice variants may give rise to distinct forms of ADAM33 that have several functions. Surprisingly the metalloprotease domain is present only in 2% of the isoforms detected (24).

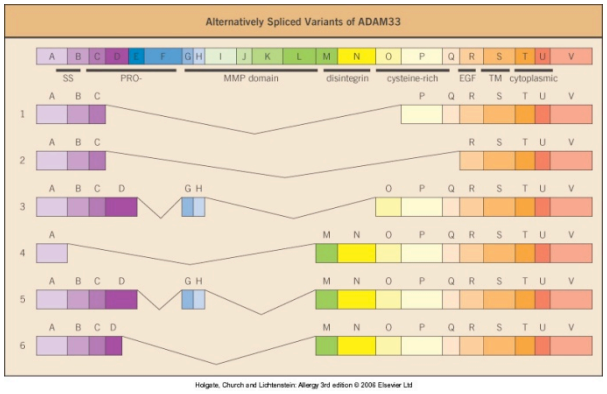


Figure 1.9: Holgate, Church and Lichenstein, Allergy 2006

It is known that ADAM33 mRNA is preferentially expressed in mesenchymal cells, such as smooth muscle, fibroblasts and myofibroblasts. It is also present in mesenchymal progenitor cells in the embryonic lung (26,30).

From the latter finding is possible to hypothesize a role for ADAM33 in lung development. Several ADAM33 protein isoforms have been found in adult bronchial smooth muscle and in human embryonic bronchi and surrounding mesenchyme, strongly suggesting its importance in smooth muscle development (26).

After the finding of various alternatively spliced variants of ADAM33, a soluble form of ADAM33 protein (sADAM33) was found in BAL fluid and was shown to be associated with reduced lung function in asthma.

sADAM33 is a 55kDa protein containing the metalloprotease domain. A recent study has shown that the soluble form of ADAM33 promotes angiogenesis (29) and that sADAM33 is produced from full length ADAM33 by TGF-β mediated ectodomain shedding. These recent findings help to support the concept that ADAM33 as a modeling/remodeling gene in asthma or other respiratory diseases (31).

In addition to the recent evidence of ADAM33 expression in developing lung , the genetic findings of Simpson et al. (12) who demonstrated the association between ADAM33 and asthma in childhood, also suggest a role for ADAM33 in pre- or post-natal lung development. Polymorphisms (SNPs) were associated with reduced lung function at both 3 and 5 years of age. These important findings support the hypothesis that ADAM33 plays a role early in life to contribute to disease development. From this point of view, it could be important to study the pattern of expression of ADAM33 during lung development and in the perinatal period. It is also know that the maternal environment during pregnancy is one of many factors (immunological polarized Th2 because it helps to the foetus development), including cigarette smoke, alcohol, toxins or drugs and diet, that can have an influence on embryonic and foetal lung development and can result in early-life wheezing, which might continue as childhood or adult asthma.

There is approx 70% homology between the murine and human *Adam33/ADAM33* genes (64).

During the pseudoglandular phase of murine lung development, there is a hundred fold increase in Adam33 expression which then undergoes a second increase at birth when breathing begins. (Figure 1.10) (64).

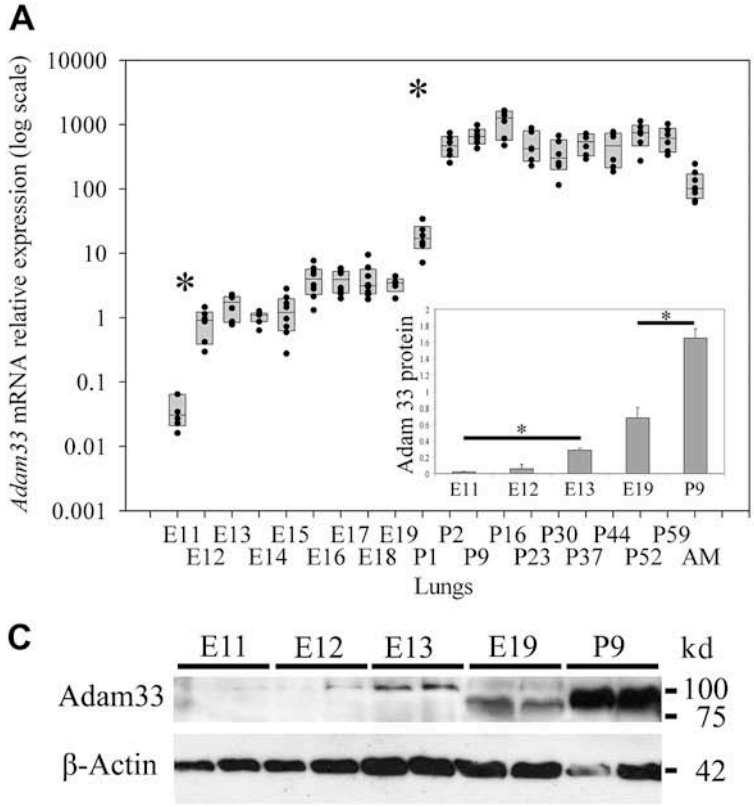


Figure 1.10: Induction of Adam33 during lung development Haitchi HM.2009

The early induction of Adam33 during the pseudoglandular stage of lung development corresponds with the start of peristaltic contractions of the airway, which are important for normal growth and development of the airways. Based on this finding in mouse and also on the presence of ADAM33 expression in human embryos, it has been suggested that Adam33/ADAM33 may be involved in airway wall "modeling" where it might have a role in the early life origins of asthma.

1.6 Aims and hypothesis

Stretch is part of the natural environment of the lung, with spontaneous peristaltic contractions occurring during lung development, beginning in the first trimester and continuing to birth (65).

Fluid within the foetal lung also creates transpulmonary pressure, aiding the stretching of the lungs (66) which is thought to be essential for proper growth and maturation. It is been shown that Adam33 mRNA expression increases in mice lungs during the pseudoglandular phase of development as spontaneous peristaltic contraction begins (64), suggesting that there is maybe a link between these two events.

Based on the finding that ADAM33 is expressed in mesenchymal cells and its expression increases during the pseudoglandular phase of lung development, we hypothesized that **cyclical mechanical strain of human foetal lung fibrobasts induces ADAM33 expression.**

Therefore the aims of this study were evaluate and characterize an in vitro mechanical strain model that mimic the spontaneous contraction during the pseudoglandular stage, and also to evaluate the effect of the cyclical mechanical strain on ADAM33 mRNA expression in foetal lung fibroblasts

2. MATERIALS AND METHODS

2.1 Cell cultures

Embryonic Fibroblast cell line

Human foetal fibroblasts (MRC 5) were obtained from the European Collection of Animal and Cell Cultures (ECACC). The cells were originally isolated from normal lung tissue of a 14-week-old male foetus by J.P.Jacobs in September 1966. They were used between passage 19 up to 25. MRC5 cultures were established in T25 flasks with 5 ml of Dulbecco's modified Minimum Essential Medium (DMEM) supplemented with between 1 and 10% of foetal bovine serum (FBS), penicillin 25000 Units G sodium, streptomycin 25 mg sulfate/ml, 1x non-essential amino acids, 1mM sodium pyruvate and 2mM glutamine (DMEM Complete). The MRC5 cells were incubated in a humidified Heraeus incubator at 37°C 5% CO_2.

Primary human embryonic fibroblasts

Human foetal fibroblasts were obtained from embryonic lung in the pseudoglandular stage, and used between passage 3 and 7. Primary fibroblast cultures were established by seeding embryonic lung tissue into culture dishes with 5 ml of DMEM Complete with 1-10% FBS. The tissue was chopped with a sterile scalpel and the tissue fragments scored onto the petri dish, to aid fibroblast attachment. The tissue was incubated in a Heraeus incubator at 37°C 5% CO_2, the growth medium (DMEM complete) were changed every two days until the cells became in confluent (cover almost 90% of the petri dish) in almost 2 weeks.

Trypsinisation of confluent cell monolayers

Fibroblasts were washed twice with Hank's balanced salt solution (HBSS) without Ca^{2+} and Mg^{2+} (calcium and magnesium interfere with the action of the trypsin), and then enough 1x trypsin-EDTA was added to cover the surface of the flask (2 mL for a T75 flask). This was removed immediately and then the cell monolayer incubated for 30 seconds before sharply tapping the flask to aid detachment. The action of the trypsin was stopped by the addition of DMEM Complete supplemented with 10% FBS. The cells were harvested by centrifugation for 5 minutes at 1100 xg and suspended in DMEM Complete.

Cryogenic Storage

The cells were stored according to Cryogen Brooke Lab protocol. After trypsinisation, cells were chilled and then resuspended in ice-cold DMEM complete containing 10% FBS and 10% DMSO (dimethyl sulfoxide), which acts as a cryo-protectant. Approximately $1x10^6$ cells in 1 mL were added to each cryovial and frozen at -80ºC overnight. The vials were then transferred into liquid nitrogen vapour at – 150ºC for long term storage.

Regeneration of Frozen Cell Stocks

The cryogenically-stored cells were regenerated by adding warm DMEM Complete supplemented with 10% FBS and placed into T75 flask. After around 6 hours, the medium was replaced to remove all traces of DMSO, as it is toxic for the cells.

2.2 Treatments

Cyclic Mechanical Strain

The Flexercell FX-4000 system (Flexer International Corp, FIC) was used to apply cyclic mechanical stretch to cell monolayers grown on collagen-1 coated wells with flexible membranes (Bioflex plate, FIC).

The Flexercell FX-4000 is a computer based system that uses a vacuum to strain cells adhered to flexible collagen-I coated membranes (Bioflex plates) arranged in six-well plate format (Figure 2.1).

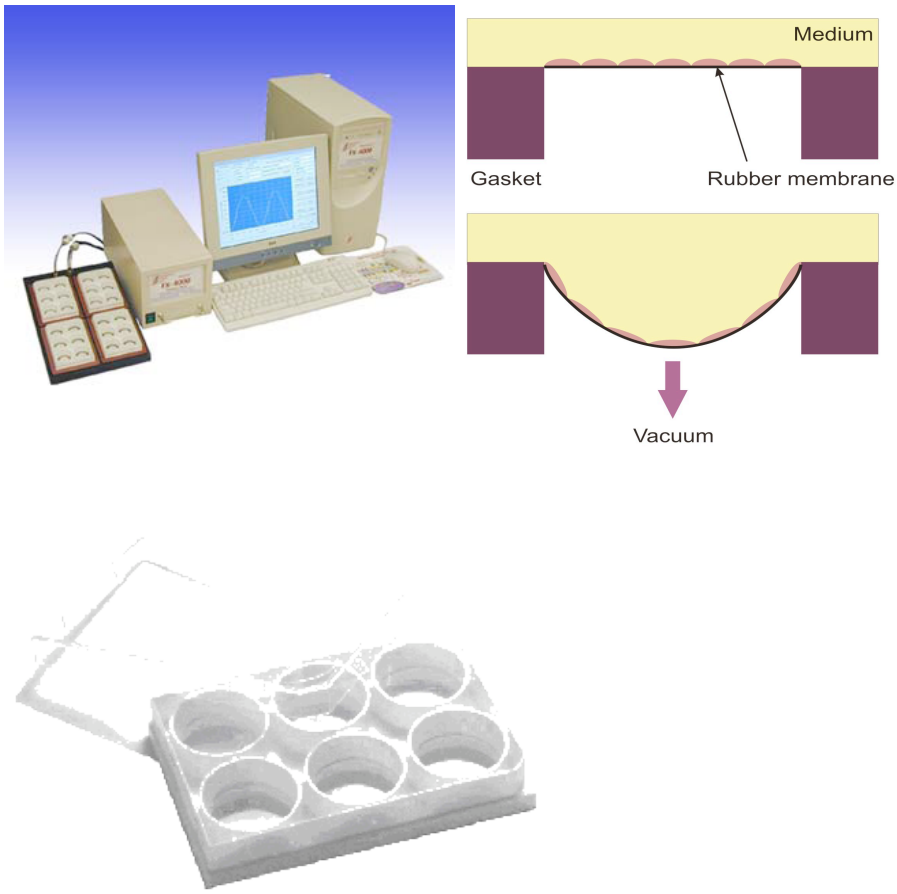


Figure 2.1: The FlexerCell FX4000 strain unit, the machine produces a negative pressure pulling down the flexible membrane where the cells are attached.

The duration, magnitude, frequency of the negative pressure, and desired strain profile were programmed via a dedicated computer using software provided by the manufacturer. Cells were strained using the vacuum method with the computer controlling application of negative pressure to the underside of silicone-elastic flexible based wells of the six wells plate.

Stretch Features

The Flexercell system was tested using a range of parameters. We programmed the stretch machine using the Vacuum method with the range of power between 8% and 30% (the maximum for the machine). The configuration of the vacuum method means that the elastic membrane of the Bioflex plate is deformed freely downward, resulting in a non-uniform strain profile on of the membrane. The duration of the stretch varied from 24 hours to up to 1 week. During the experiment time points were taken to evaluate the stretch feature.

We started using the MRC-5 fibroblast cell line and the stretch machine at the power of 8% for 48 hours, seeding at 500,000 cells for each well. We also investigated the effects of the TGF-β_2 on MRC-5 cells with and without stretch, as control experiments to demonstrate responsiveness of the fibroblasts. The power of the stretch was then increased to 16% for 48 hours using MRC-5 cells. Following these pilot studies, we used primary fibroblasts from embryonic lungs taken during the pseudoglandular stage. We set a range from 10% to 30% stretch, seeding cells from 100,000 to 250,000 cells/well; we also tested different concentration of FBS from 0%-10%. Cells were stretched for a maximum time of one week.

Calculation of cell number

Cells counting were performed using Barker counting chambers, in order to evaluate and compare the cell numbers in the sample controls and the samples treated with the stretch machine.

mRNA quantification using RT-qPCR

Total RNA was extracted using Trizol (Invitrogen, UK) reagent according to Brooke Lab RNA extraction protocol. Trizol reagent is a mono-phasic solution of phenol and guanidine isothiocyanate that allows the disruption and homogenisation of cells (1ml Trizol per sample). The samples were treated with 200ul of chloroform per ml of Trizol in order to separate the RNA from the DNA; after this step the samples were spun, and the top layer was treated with isopropanol to precipitate the RNA; this was left at -20°C overnight and the next day the samples were spun, washed with ethanol 75%, spun and air dried. The RNA was then treated with DNase which removes any trace contamination of genomic DNA. The quantity and the quality of RNA were analysed using a Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific, UK) by the ratio of absorbance at 260nm and 280nm a ratio of ~ 2.0 is generally accepted as"pure" for RNA. 1 ug of RNA was reverse transcribed to cDNA using Moloney murine leukemia virus reverse transcriptase enzyme (MMLV), random hexamers and oligo dNTP primer (PrimerDesign, UK). After reverse transcription, the cDNA was subjected to a quantitative polymerase chain reaction (qPCR) using an iCycler (Biorad, UK). The samples were loaded into 96 well plates, in conjunction

with the appropriate primers and probes, and either Precision MasterMix or SYBR green MasterMix (3-9ul of sample, 1 ul of primers and probe, 7.5 ul of Mastermix) depending on whether or not the assay was probe based (All reagent were from PrimerDesign UK and recommended PCR protocols were used). The data generated from the iCycler were analysed using the ΔΔCT method and normalised to the geometric mean of housekeeping genes UBC (ubiquitin C), A2 (phospholipase A2) and GAPDH (Glyceraldehyde 3-phosphate dehydrogenase). Also, T24h control values were used in order to understand the variation within samples.

Target	**Primers and probe**	**Dye**
ADAM33	F: 5'-GGCCTCTGCAAACAAACATAATT-3'	Fam
3'UTR	R: 5'-GGGCTCAGGAACCACCTAGG-3' P: 5'-CTTCCTGTTTCTTCCCACCCTGTCTTCTCT-3'	Fam
⟨SMA	F: 5'-GACAGCTACGTGGGTGACGAA-3' R: 5'-TTTTCCATGTCGTCCCAGTTG-3'	Sybr
IL8	F: 5'-CAGAGACAGCAGAGCACAC-3' R: 5'-AGCTTGGAAGTCATGTTTACAC-3'	Fam
A2/UBC Multiplex house Keeping gene kit	F: PrimerDesign R: PrimerDesign P: PerfectProbe™, PrimerDesign	Cy5 Fam

F: Forward primer

R: Reverse primer

P: Probe.

2.3 Immunofluorescent staining for F-actin.

Control cells and those subjected to mechanical stretch were fixed in 4% paraformaldehyde in PBS (Phosphate Buffered Saline) for 15 minutes at room temperature. They were then permeabilized with 0.1% Triton in PBS for 5 minutes and blocked with 1% BSA and, 0.1% Triton-X in PBS. The immunofluorescent staining for F-actin was performed using Phalliodin conjugated with (1:150) FITC (Fluorescein isothiocyanate). Using this antibody it is possible to probe the cytoskeleton arrangement.

2.4 Soluble Collagens assay

Cell media were analysed in order to evaluate collagen production from the control cells or those subjected to stretch. The Sircol Collagen Assay is a dye-binding method for the analysis of acid and pepsin-soluble collagens. The sircol dye reagent contains sirius red which is an anionic dye with sulfonic acid side chain groups. These groups react with the side chain of the basic amino acids presents in newly synthesised collagen. The range of the kit, using the standard curve, was from 0 to 50 ug/ml collagen. The lower limit of detection was 1 ug/ml; a typical standard curve is shown in Figure 3b typical standard curve of soluble collagen assay.

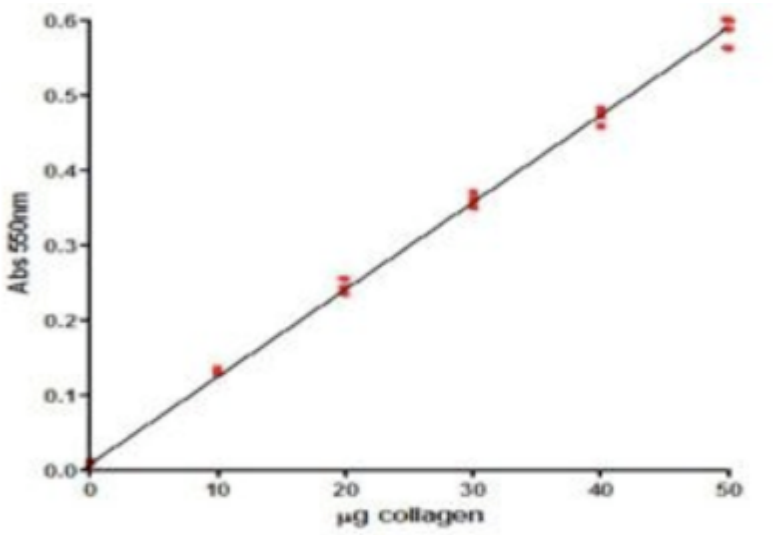


Fig. 3b: Collagen Reference Standards, 0 – 50.0 µg
using 1000 µl of Alkali Reagent to recover the collagen bound dye.

2.5 Western Blot

The proteins produced by the cells subjected to mechanical stretch and the control cells were extracted using SDS lysis buffer (Brooke Lab protocol, Tris-HCl, EDTA, SDS and 1%Triton X100), sonicated and heated at 95ºC for 5 minutes before electrophoresis (160V, 1 hour) on 12.5% SDS polyacrylamide gels (SDS-PAGE) (stacking gel mix for 3-4 gels: 12.5 ml of 30% acrylamide, 25 ml of 0.5 M Tris-HCL pH 6.8, 62ml dH_20, 0.5ml 20% SDS, 25ul of APS (0.1 gr/ml), 5 ul of Temed; separation gel mix: 37.5ml 30% acrylamide, 22.5ml 1.5 M Tris-HCL pH 8.8, 62.5ul of APS (0.1 gr/ml), 6.25 ul of TEMED; 1x running buffer pH 8.3 for 0.025M, 0.192M glycine 0.1% (w/v) SDS, PH 8.3 with HCL. The total proteins were separated according to their size and their charge. SDS (sodium dodecyl sulphate) is a detergent that dissolves cell membranes allowing the cell proteins to be solubilised by the detergent. As SDS has a negative charge, all the proteins with SDS bound will then have a net negative charge, which means they will all migrate towards the positive pole when placed on an electric field. After the SDS-PAGE, the proteins were transferred, by electrophoresis, to a nitrocellulose membrane (transfer buffer pH 8.3 for 5 litre: 25uM Tris, 192uM Glycine, 1000ml of 20% methanol; wash buffer pH7.4 for 2 litres: 1XPBS, 0.05% tween 20) and then the membranes were blocked at room temperature in PBS containing 3% BSA and 0,5% Tween before being incubated overnight at 4ºC with primary antibody specific for ADAM33 (ABCAM UK, Rabbit polyclonal: RP1 recognizing the ADAM33 prodomain, RP2 against the metalloprotease domain or RP3 against the cytoplasmic domain (see Figure 2.3), each diluted 1:5000,). After being washed with PBS-T (PBS, 0,05% Tween), the membranes were incubated

with a peroxidise-conjugated secondary antibody (Jackson Immunoresearch, Goat anti-rabbit, 1:10000) for 1 hour at room temperature. After washing, protein bands were visualised by enhanced chemiluminescence (ECL) for autoradiography following the manufacturer's instructions. To confirm equivalent protein loading, membranes were stripped and re-probed for GAPDH. Protein bands were quantified using a densitometer.

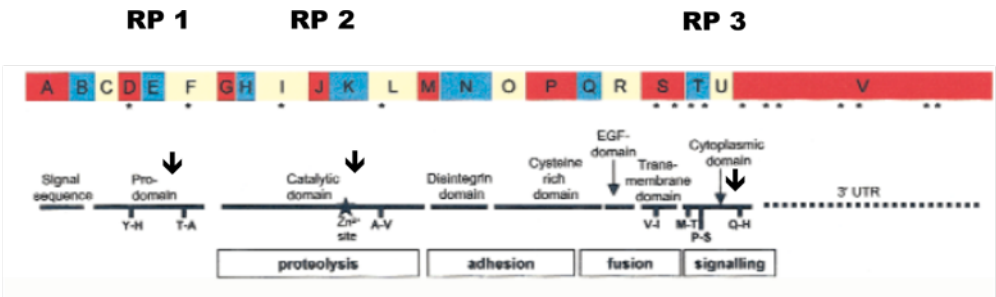


Figure 2.3: ADAM33 domains structure: prodomain RP1, catalytic domain RP2, cytoplasmic domain RP3

2.6 Protein A/G pulldown and Concanavalin A assay

The aim of this protocol was to pull down ADAM33 which is a glycosylated protein using Concanavalin A (Con A, Sigma-Aldrich, Poole UK). However, the samples were first precleared using Protein A/G plus-Agarose (Santa Cruz Biotechnology) to remove imnumoglobulins in the culture medium. 20 ul of resuspended Protein A/G beads were used per 1ml of cell growth medium. In order to prepare the beads they were spun for 5 minutes and the supernatant discarded. After this step the beads were washed with cold phosphate buffered saline (PBS), then they were spun and the supernatant discarded. The beads were subsequently suspended with an equal volume to the initial beads volume

used of PBS. 20 ul of this mixture were added to the samples and then incubated overnight on a rotating mixer at 4°C to allow binding to the immunoglobulins. The following day the mixture was spun and the supernatant used for pulldown with Con A. Con A is a lectin protein which binds to the glycosylated sugars, such as ADAM33, this can increase the concentration of ADAM33 found in the cells growth medium. We were using 20ul of CON A per 1ml of sample. After pulling down the glycosylated proteins and washing the CON A beads 3x to remove non-specifically bound proteins, the beads were processed in order to perform a Western Blot assay.

2.7 IL-8 ELISA

The BD Human IL-8 ELISA Kit is for the quantitative determination of human IL-8 in serum, plasma, and cell culture supernatant. The BD OptEIA test is a solid phase sandwich ELISA (Enzyme-Linked Immunosorbent Assay). It utilizes a monoclonal antibody specific for IL-8 coated on a 96-well plate. Standards and samples are added to the wells, and any IL-8 present binds to the immobilized antibody. The wells are washed and streptavidin- horseradish peroxidase conjugate mixed with biotinylated anti-human IL-8 antibody is added, producing an antibody-antigen-antibody "sandwich". The wells are again washed and H_2O_2 substrate solution is added with TMB which acts as a chromogen to produce a blue color in direct proportion to the amount of IL-8 present in the initial sample. The Stop Solution changes the color from blue to yellow, and the absorbances of each well are read at 450 nm using 540 nm reference filter. A typical standard curve is shown in Figure 2.4; the lower limit of detection was 0.8 pg/ml.

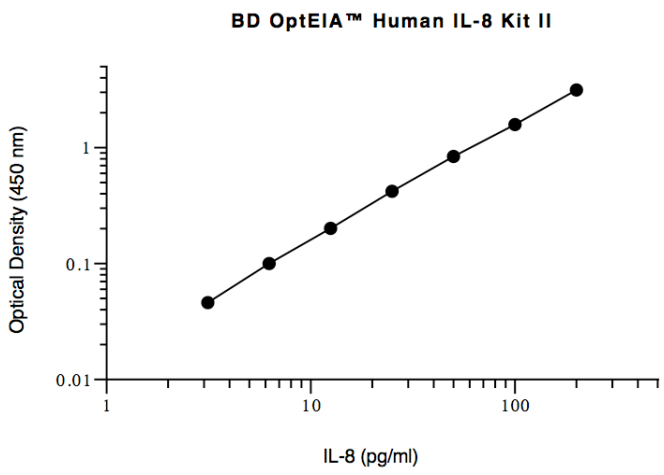


Figure 2.4: IL8 standard curve BD kit

2.8 Phase Contrast Microscopy

Cells were routinely photographed before and after treatment to record any morphological change. An inverted light microscope equipped with phase contrast rings (LEICA DM-IRB) was used to visualise changes in cell size, shape and orientations and this was recorded by digital photography.

2.9 Statistic analysis

The data were analyzing using Student t-test in order to analyze the difference in mRNA expression and protein level measured by ELISA between stretched and non stretched cells. Results were considered statistically significant at p levels <0.05.

3. RESULTS

3.1 Human foetal fibroblasts treated with TGF B_2

The first part of this chapter aimed to confirm previous experiment made using human foetal lung fibroblasts.

Before testing the effect of the cyclical mechanical strain, a control experiment was carried out to test the effect of myofibroblast differentiation induced by TGF-β_2 on the expression of ADAM33 and alpha smooth muscle actin (α-SMA) with the purpose of confirming results found previously. (40,41) . Both ADAM33 and aSMA are expressed in mesenchymal cells and previous work has shown that TGF-β_2 induces a phenotype switch of the cells from fibroblasts to myofibroblasts, characterised by ADAM33 suppression and aSMA induction (32 Wicks J. 2005). In order to do these studies, a human foetal lung fibroblast cell line called MRC-5 was used (Figure 3.1a, 3.2b). The MRC-5 is a cell line obtained from the normal lung tissue of a 14 weeks old Caucasian male foetus which corresponds to the pseudoglandular stage of lung development.

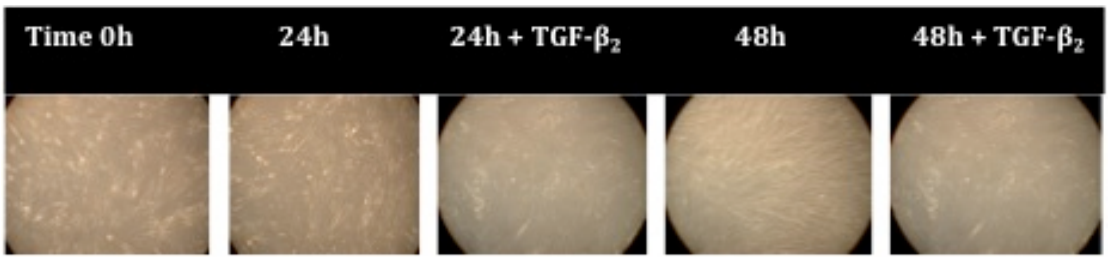


Figure 3.1a: Morphological appearance of MRC5 cultured in the absence or presence of TGF-β_2 up to 48 hours as viewed by phase contrast microscopy 40X magnification.

Foetal lung fibroblasts (MRC5) were grown on a 6 well plate until ~90% confluent using DMEM 10%FBS (foetal bovine serum) as growth medium. The

cells were serum starved for 24 hours and then either treated in the absence or presence of 10ng/ml TGF-B2 in UltraCulture serum free media for 24 and 48 hours. RNA was extracted using the Trizol method and then Real Time PCR was performed. The PCR results of a-SMA and ADAM33 were compared to the geometric mean of two housekeeping genes: UBC and A2.

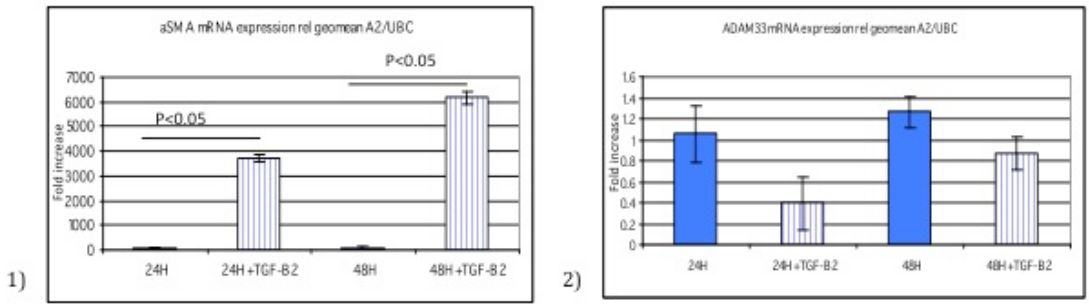


Figure 3.2b: the effect on MRC-5 cell line of TGF-B2 10ng/ml on aSMA (1) and ADAM33 (2) mRNA expression. aSMA was significantly induced in cells treated with 10ng/ml of TGF-B2 compared with not treated cells.

As previously reported, the expression of aSMA increased in response to treatment with TGF-B2, while, in contrast, ADAM33 expression tended to be suppressed due the treatment with TGF-B2. These results confirm previous results and gave me the confidence to proceed with the evaluation of the effect of the cyclical mechanical stretch on ADAM33 expression.

3.2 Cyclic strain machine optimization

In order to find the best setting of the bio-engineering stretch machine (Figure 3.3), several experiments were performed with the aim of optimizing the conditions, including percentage of membrane elongation, frequency, time, seeding density of the cells and the choice of the growth medium. Previous studies (70,73,74) used between 1-30% of elongation, a frequency of 0.1-1 Hz , 24-144 hours of strain time and between 10^4 and $5x10^5$ cells per well, with 1-10% FBS supplemented in the growth medium.

Initial experiments started with 8% membrane elongation, a frequency of 0.1 Hz (1 elongation every 10 seconds) applied for 24 and 48 hours. Cells were seeded at $5x10^5$ cells on a collagen coated elastic membrane (MRC-5 passage 21) in DMEM with 10% FBS during cell growth, but starved in serum free medium 24 hours before beginning the stretch experiment. We also included treatment with TGF-B2 (10ng/ml) under stretched and non-stretched conditions in order to evaluate the effect of the TGF-B2 on the ADAM33 expression during the cyclical mechanical strain experiment. These parameters were used to see if there were any changes in the cells morphology and gene expression, without matching the exact physiological condition of airway contraction in the pseudoglandular stage of lung development.

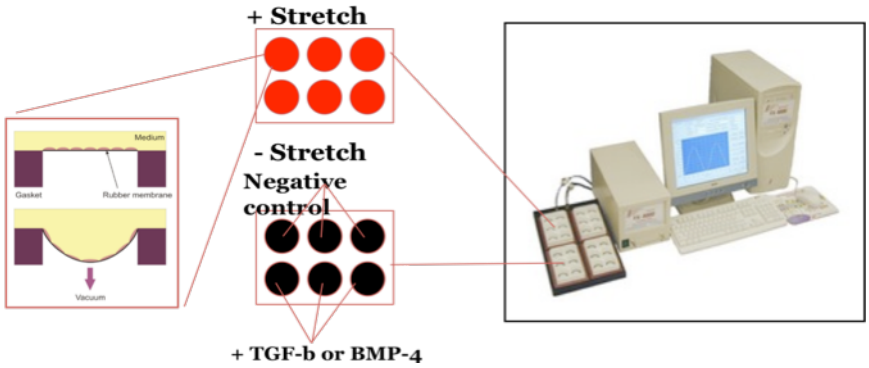


Figure 3.3: experiment configuration, red plate represents the cells stretched, black plate the control

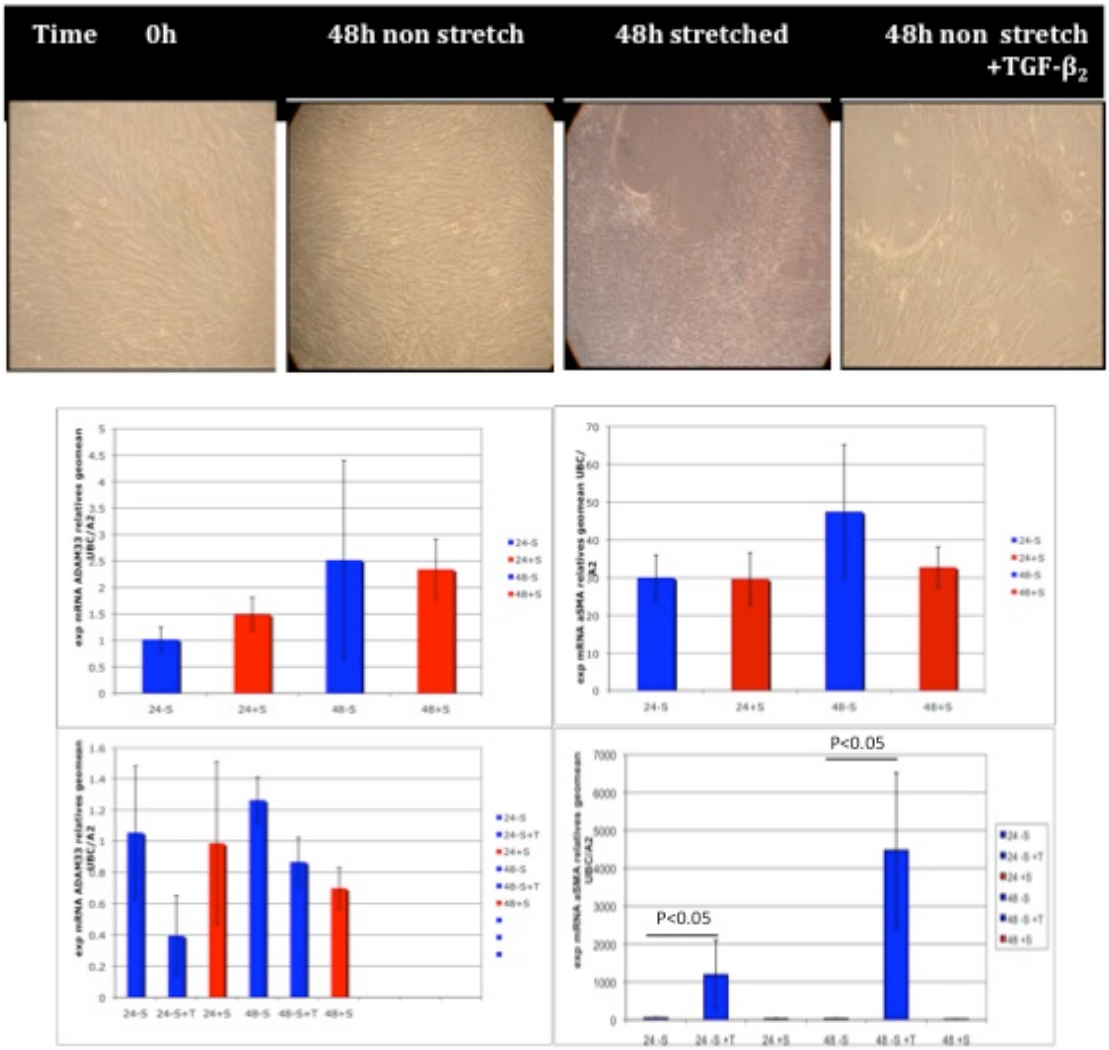


Figure 3.4: Morphological appearance of MRC5 cultured in the absence or presence of TGF-β_2 up to 48 hours as viewed by phase contrast microscopy 40X magnification. aSMA and ADAM33 mRNA expression due the cyclical mechanical strain of MRC-5 (human foetal lung fibroblast) and due 10ng/ml of TGF-β_2 treatment.

After the first cyclical mechanical strain experiment, morphological changes were evident in non-stretched TGF-B_2 treated cells consistent with a switch to a myofibroblast phenotype. Cells exposed to 8% stretch also formed a more contracted cell structure, possibly due to failure of the cells to remain attached to the collagen coated elastic membrane. The cells stretched in the presence of TGF-B_2 had totally detached, and appeared not to be able to tolerate both stretch and TGF-B_2.

Analysis of aSMA and ADAM33 mRNA expression revealed no significant difference between stretched cells and non stretched at either 24 or 48hr. Nevertheless, ADAM33 expression did fall in non-stretched cells in response to TGF-B_2, as observed previously.

Cyclic strain machine power optimization

The application of 8% stretch to the MRC-5 cells in serum free medium did not appear to be enough to affect the expression of our target gene (ADAM33). For this reason, we tried to increase the percentage of elongation up to 16%, keeping the other parameters, as in the previous experiment, except that the concentration of TGF-B2 was reduced to 5ng/ml instead of 10ng/ml. In addition, 30ng/ml of BMP-4 (bone morphogenetic factor 4), another polypeptide belonging to the TGF-B superfamily of proteins, was also tested (Figure 3.5a, 3.5b).

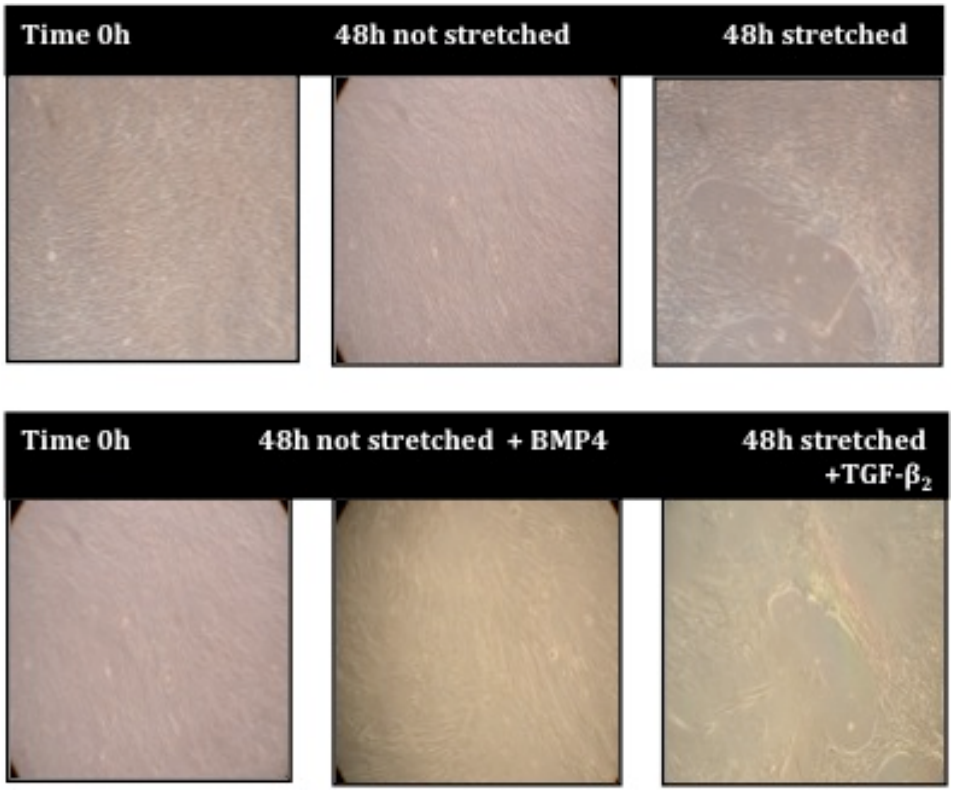


Figure 3.5a: Morphological appearance of MRC5 cultured in the absence or presence of BMP4, TGF-β_2 up to 48 hours as viewed by phase contrast microscopy 40X magnification.

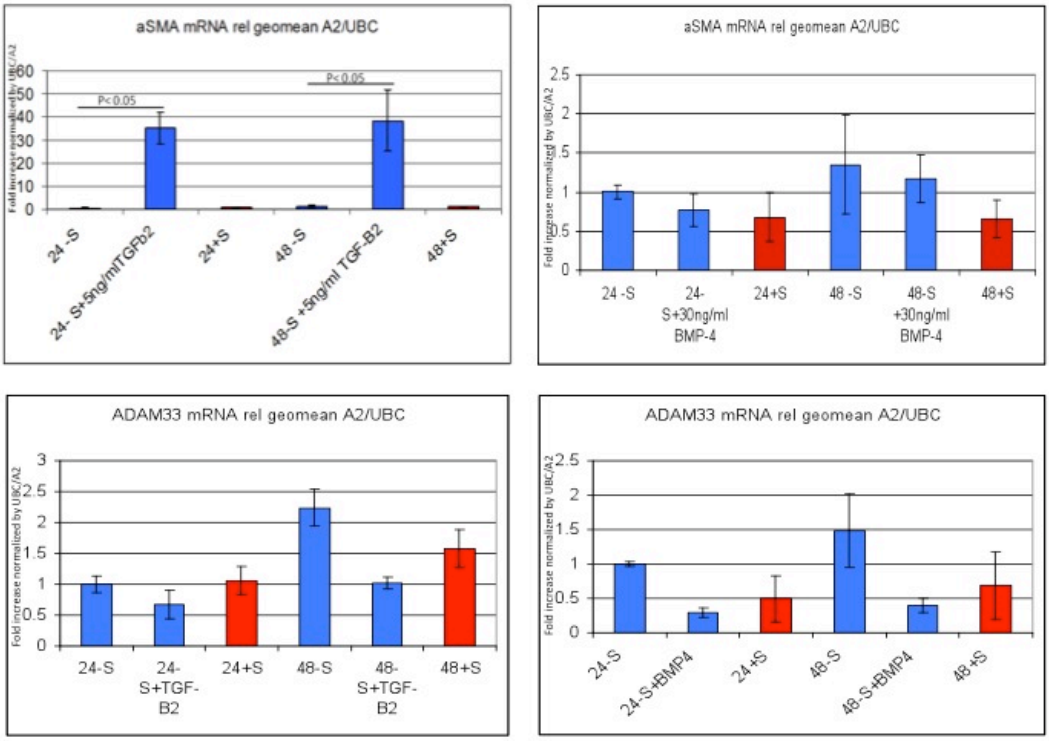


Figure 3.5b: aSMA and ADAM33 mRNA expression due cyclical mechanical strain of MRC-5 or treatment with 5ng/ml TGF-B2 or 30ng/ml of BMP4

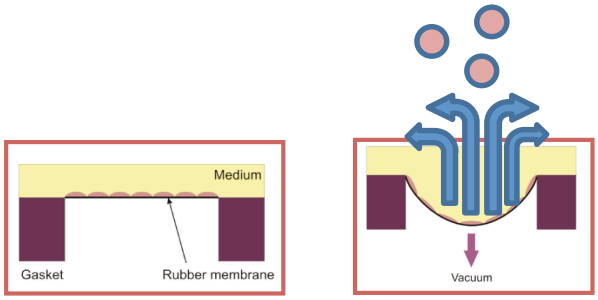
As in the first experiment, there were morphological changes due the effect of mechanical stretch, with the cells forming tube-like structures and with TGF-B2 causing morphological changes as the fibroblasts switched phenotype to myofibroblasts. BMP-4 30ng/ml did not produce any morphological change.

RTqPCR confirmed that 5 ng/ml TGF-B2 increased a-SMA mRNA expression and reduced ADAM33 expression. Despite these results, there was no significant difference between stretched and non stretched cells in ADAM33 mRNA expression. Previous studies have reported that mechanical stretch induces muscle differentiation (73), therefore analysis of genes such as myosin, serum response factor, versican and collagen I was also undertaken to explore the phenotype of the stretched cells. However, there was no evidence of smooth muscle differentiation due the cyclical mechanical strain (data not shown). BMP4 did not induce any myofibroblast or smooth muscle differentiation.

Cyclic strain machine cells optimization

From these initial experiments, we were concerned whether the MRC-5 cell line was the best choice of cell and we also noted that we were losing too many cells from our plates (Figure 3.6). However one of the reasons could be the high number of cells seeded ($5x10^5$).

Figure 3.6:



To address these two issues, we switched to the use of primary embryonic lung fibroblasts from two 14th week donors, and evaluated the effect of seeding at $1x10^5$, $2x10^5$, $5x10^5$ cells per well (also in order to understand the right number of cells per well) using DMEM 10%FBS (foetal bovine serum) as growth medium. We decided to start with 8%-16% membrane elongation, 0.1 Hz of frequency (1 elongation every 10 seconds) for 24 and 48 hours time, cells were serum starved 24 hours before beginning the experiment.

The aim of this study was to explore the morphological changes of the fibroblasts due the cyclical mechanical strain. In order to do it immediately after the stretch experiment, the cells were fixed in paraformaldehyde 4% and stained with Phallotoxin and PET nuclear stain Hoechst 2018, phallotoxin labels F-actin one of the major component of cytoskeleton (Figure 3.7).

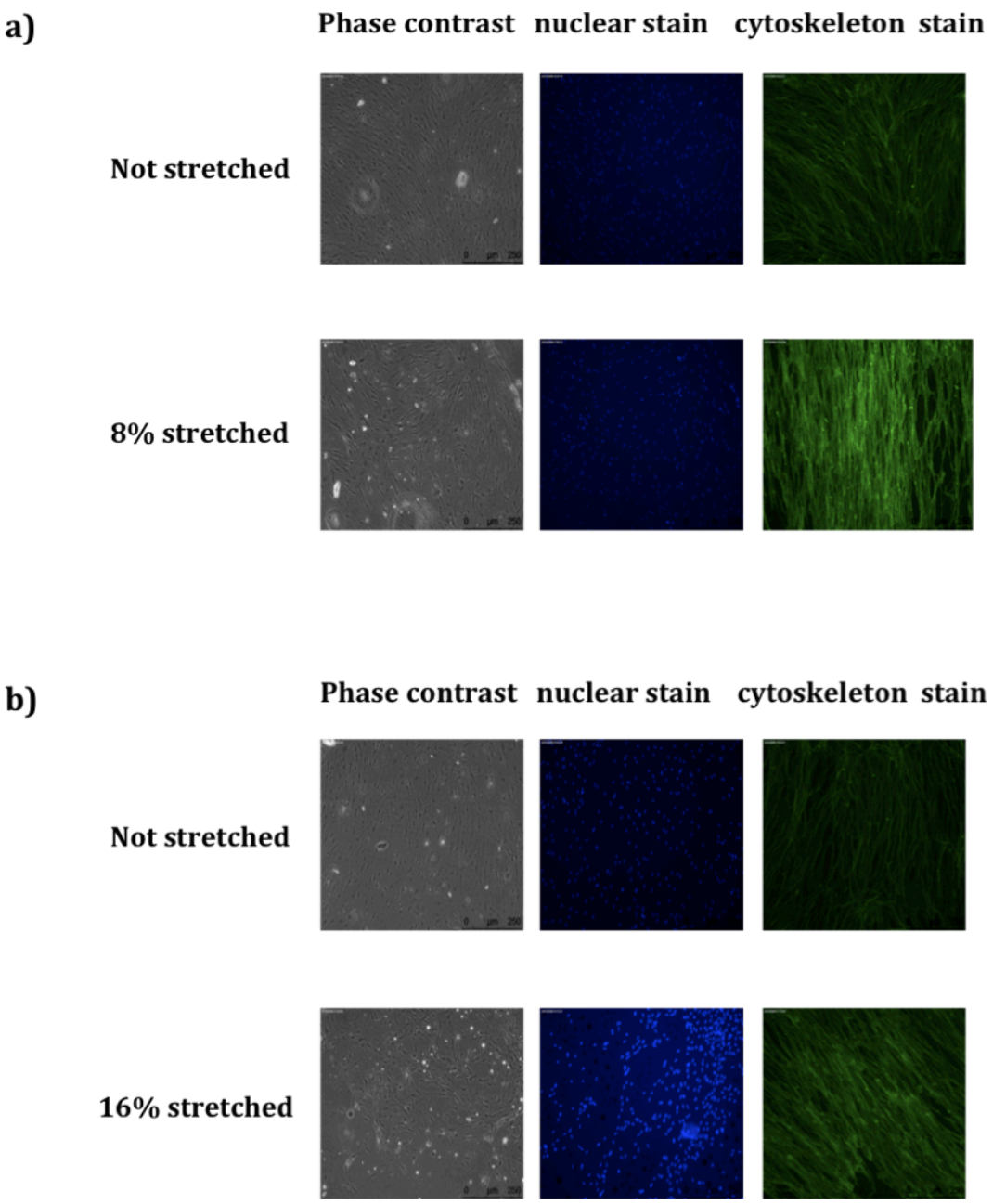


Figure 3.7: phallotoxin and pet stain in foetal lung fibroblasts after 8%(a) -16%(b) cyclical mechanical strain of human foetal lung fibroblast.

Using the Phallotoxin and PET stain we could see the cytoskeleton rearrangement according to the force of the stretch, the cells were also morphologically smaller and elongated in shape. However, after this study we still found (in the 16% experiment) a large number of cells lost from each well.

Next, we decided to investigate the role of the fetal bovine serum (FBS) because it is well know that cells need it for attachment and growth (75,78,79,80) although it is also known that FBS contains TGF-B which down regulates our target gene (ADAM33).

As in the previous experiment, we used $2x10^5$ primary foetal lung fibroblasts from a 14th week donor, the cells were left to grow for one day using DMEM 10%FBS as growth medium. The cells were divided into two groups: the first group was tested with DMEM supplemented with 1% FBS while the second used DMEM supplemented with 10% FBS. As usual, non-stretched cells were grown under the same conditions as control. The stretch experiment lasted for 24 and 48 hours using 16% stretch. At the end of the experiment, the morphology of the cells was examined microscopically and RNA was extracted using the Trizol method and then Real Time PCR was performed. The PCR results of a-SMA, ADAM33 were compared to the geometric mean of two housekeeping gene UBC and A2 (Figure 3.8a, 3.8b).

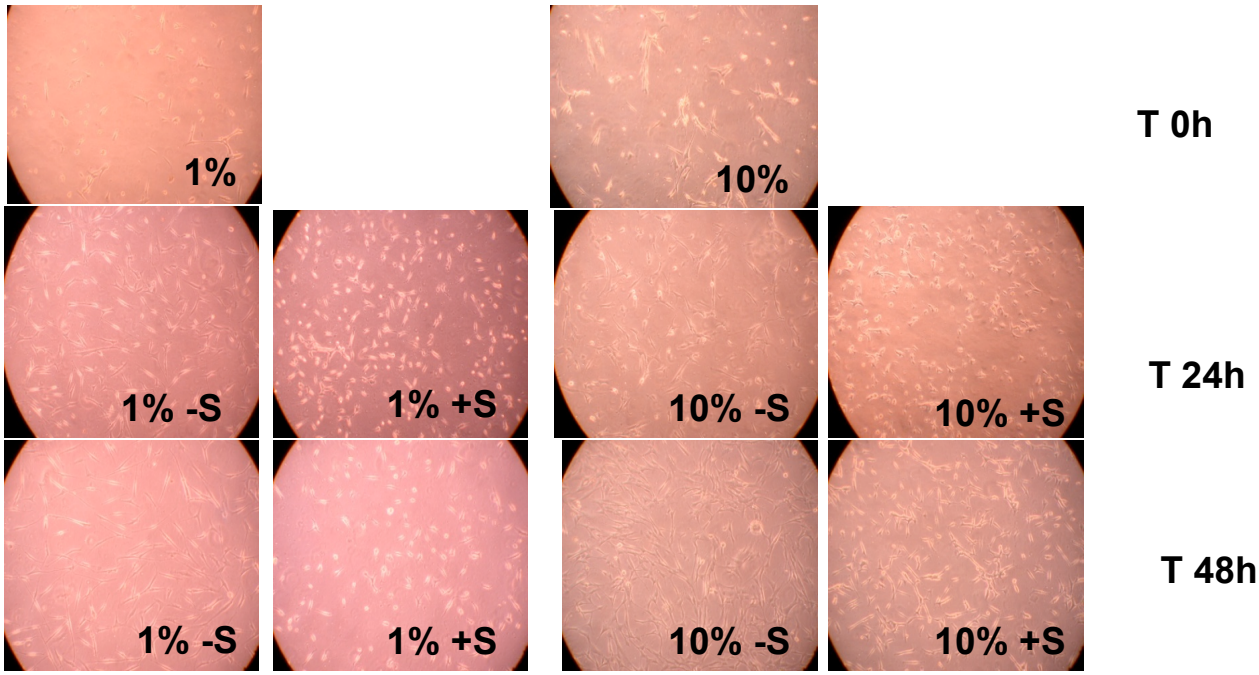
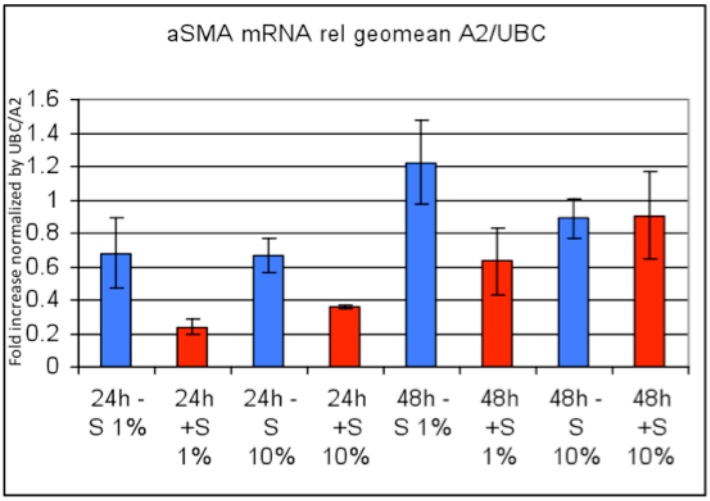


Figure 3.8a: Morphological appearance of foetal human primary lung fibroblast cultured in DMEM with different concentration of FBS 1% and 10% as viewed by phase contrast microscopy 40X magnification.



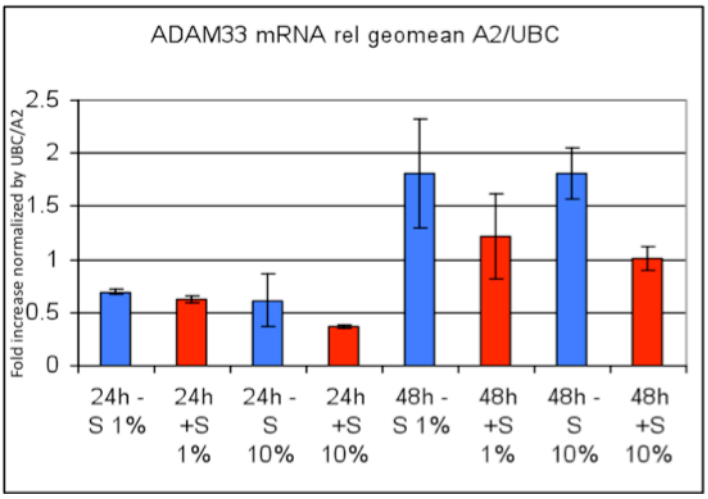
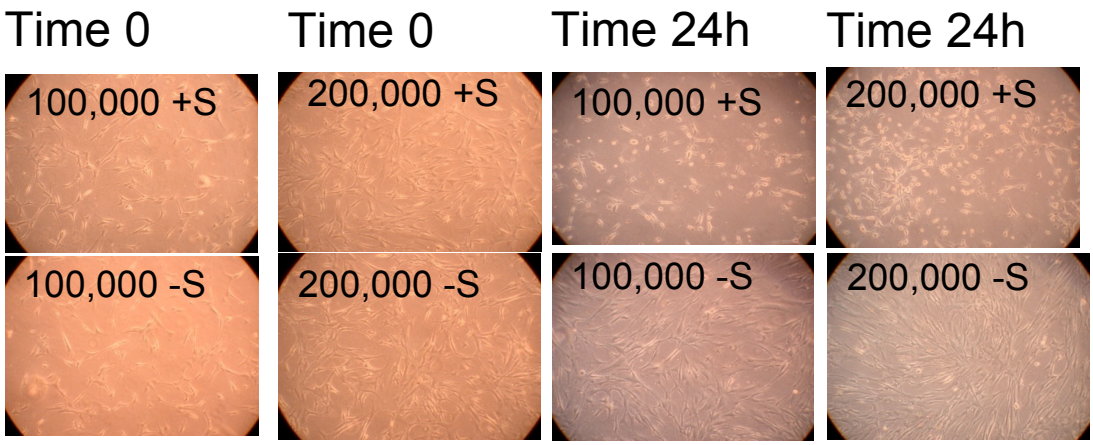


Figure 3.8b: two groups on the left 1%FBS, on the right 10%FBS growth for 48 hours. aSMA and ADAM33 mRNA expression were analyzed using RT-PCR after a 16% cyclical mechanical strain experiment.

Using the higher FBS concentration (10%), more cells remained attached to the collagen coated elastic membrane than using the lower concentration (1%). However, despite the improvement in cell adhesion, there were no significant differences between cells stretched and non stretched for ADAM33 mRNA expression, while it appeared that αSMA expression tended to decrease due to the cyclical mechanical strain.

These findings gave us the confidence to increase the power of the stretch from 20% of elongation up to the maximum of 30% that the machine can achieve. The frequency was tested at 6, 13 and 18 cycles per minute, with the aim of finding the tolerance limit of the cells before the detachment. In order to perform these experiments, we used $1x10^5$ and $2x10^5$ human primary foetal lung fibroblasts. The cells were left to grow for one day using DMEM 10%FBS (foetal bovine serum) as growth medium. During the stretch experiment DMEM 10% FBS was used, and, as usual, non-stretched cells were used as control.

The stretch experiment lasted 24 hours and a cell count was performed at the end of the experiment in order to determine the number of cells lost and to correlate this to the magnitude and frequency of the stretch applied (Figure 21).



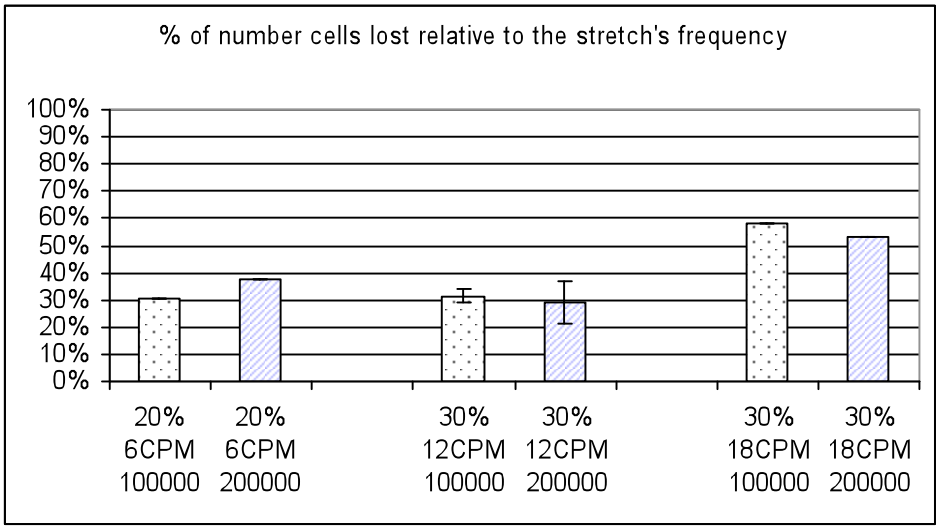


Figure 3.9: Pictures of two groups of cells used in order to study the correlation between the number of cells lost with the power and frequency of the stretch machine, as viewed by phase contrast microscopy 40X magnification.

The average cell loss using 20% and 30% stretch was approximately 30% when the stretch frequency was either 6 or 12cpm, however there was an increase in cell loss to about 50% when the frequency increased to 18 cpm. Based on these results we decided to use 30% as magnitude, 12 cpm as frequency and two hundred thousand cells per well.

Investigation of the morphological features of the fibroblasts due the cyclical mechanical strain (30% magnitude of elongation, 12 cpm as frequency 48 hours) showed differences in morphology which were related to their position in the well. Thus, the cells were more stretched on the border of the well than in the middle (Figure 3.10). This can be explained by the differences in stretch experienced by the cells as a result of the deformation of the silastic membrane (Figure 3.11)

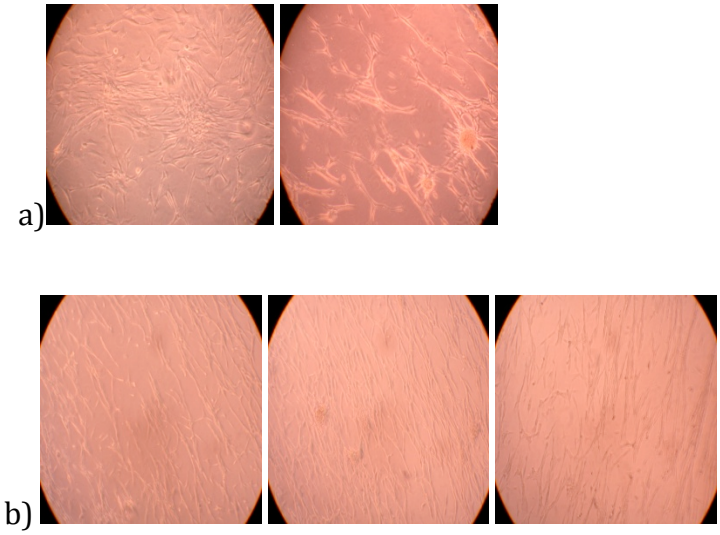


Figure 3.10: Morphological appearance of stretched cells a) in the middle of the well, b) on the border of the well. Cells were subjected to 30% stretch at 12cpm for 48hours, as viewed by phase contrast microscopy 40X magnification.

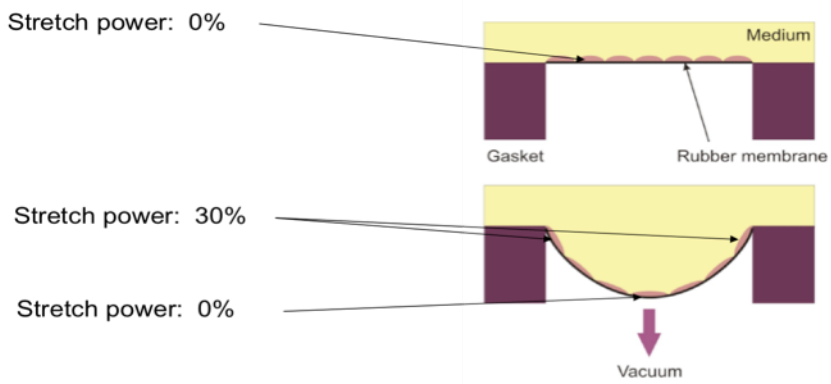


Figure 3.11:

The relatively low % of cells lost even when using the maximum power gave us the confidence to perform several experiment using this configuaration (30% magnitude of elongation, 12 cpm as frequency), and to extend the time course up to 96 hours.

3.3 Timecourse for the effect of cyclical mechanical strain at 30% stretch and 12 cycles/minute

It is known that the expression of a gene can be induced directly or secondary to a change in the expression of another gene. For this reason we investigated the expression of ADAM33 and aSMA at different time points up to 24, 48, 96 hours. As in the previous experiment, we used $2x10^5$ primary foetal lung fibroblasts and the cells were left to grow for one day using DMEM 10%FBS as growth medium. The cells subjected to cyclical mechanical strain were also cultured in DMEM supplemented with 10% FBS. As usual non-stretched cells were used as control and the stretch experiment was performing for 24, 48, 96 hours (Figure 3.12).

RNA was extracted using the Trizol method and then Real Time PCR was performed. The PCR results of a-SMA, ADAM33 were compared to the geometric mean of two housekeeping gene UBC and GAPDH. Cellular proteins were solubilized into SDS for western blot analysis and the growth medium was retained in order to explore the presence of the 55kda soluble form of sADAM33 (see material and method section).

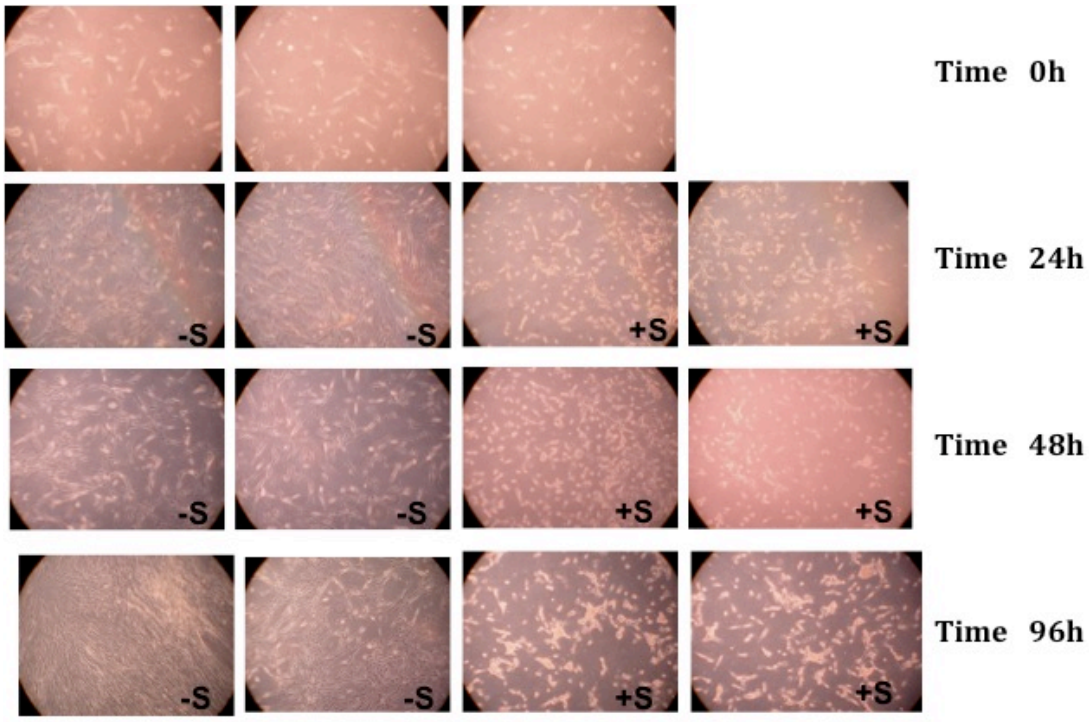


Figure 3.12: morphological appearance of foetal human primary lung fibroblast cultured in DMEM at each time point; cells were viewed in the middle of the well. Stretched cells were exposed to 30% stretch at 12cpm cyclical mechanical strain, as viewed by phase contrast microscopy 40X magnification.

Analysis of ADAM33 and αSMA expression by RTqPCR showed that after 48 hours (24 and 96 hours as well) cyclical mechanical strain failed to affect ADAM33 expression (Figure 3.13), however as observed previously αSMA expression decreased significantly in response to the cells being stretched. As changes in gene expression can be transient and do not always reflect changes in protein levels, we also examined ADAM33 protein expression by western blotting. As it has been shown previously that ADAM33 exists as multiple splice forms (all of which can be detected by the primers used for the RTqPCR), we used 3 antibodies directed at different domains of ADAM33 (Figure 3.14). As for the RTqPCR, no differences in ADAM33 expression was found in cell lysates comparing stretched and non-stretched cells. For αSMA, there appeared to be a small reduction in its expression following exposure to cyclical mechanical.

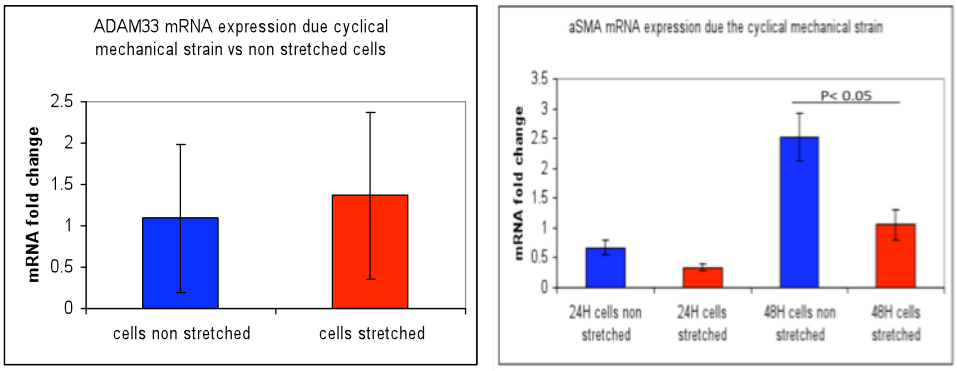


Figure 3.13: aSMA and ADAM33 mRNA expression were analyzed using RT-PCR after 3 experiments of 30% 12cpm 48 (24,96 data not showed) hours cyclical mechanical strain of human foetal lung fibroblast.

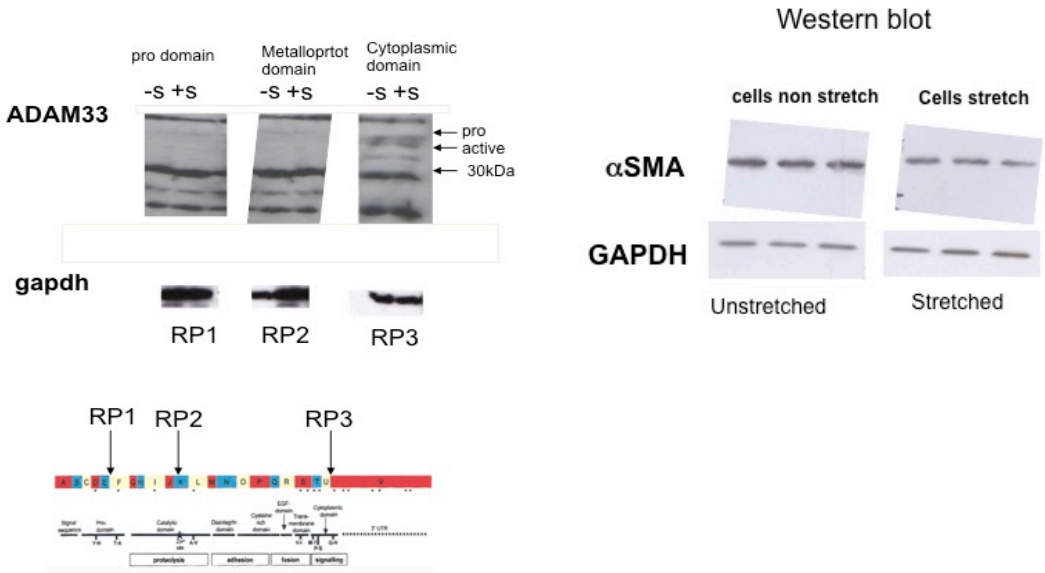


Figure 3.14: on the top left, western blot (for ADAM33 rp1, rp2, rp3) using protein extracted from the cells (as showed on the bottom left), there were not significant difference of ADAM33 protein expression between cells stretched and cells not stretched using 30% 12cpm 48 hours cyclical mechanical stretch of human foetal lung fibroblast. On the right, western blot for aSMA using protein extracted from the cells, there were not significant difference of aSMA protein expression between cells stretched and not stretched using 30% 12cpm 48 hours cyclical mechanical stretch of human foetal lung fibroblasts For αSMA, there appeared to be a small reduction in its expression following exposure to cyclical mechanical.

As ADAM33 can also be post translationally modified due to TGFβ_2-induced ectodomain shedding, we also investigated whether cyclical mechanical strain affected the release of sADAM33 into the cell culture media. As ADAM33 is glycosylated, this involved pulling down glycosylated proteins from the culture media using ConA; the harvested proteins were then solubilised and western blotted for ADAM33 (Figure 3.15). This revealed several immunoreactive fragments of ADAM33 detected using antibodies to the Pro and MP domains, however most were smaller than the 55kDa sADAM33 reported previously, suggesting degradation. Of interest, using the RP1 antibody, we also detected a strong band at 100kDa corresponding to the size of full length ADAM33. This might be explained by release of exosomes into the medium; in this case the ADAM33 would still be membrane anchored and full length. However, in none of the experiments did we note any effect of cyclical mechanical strain on ADAM33 isoforms.

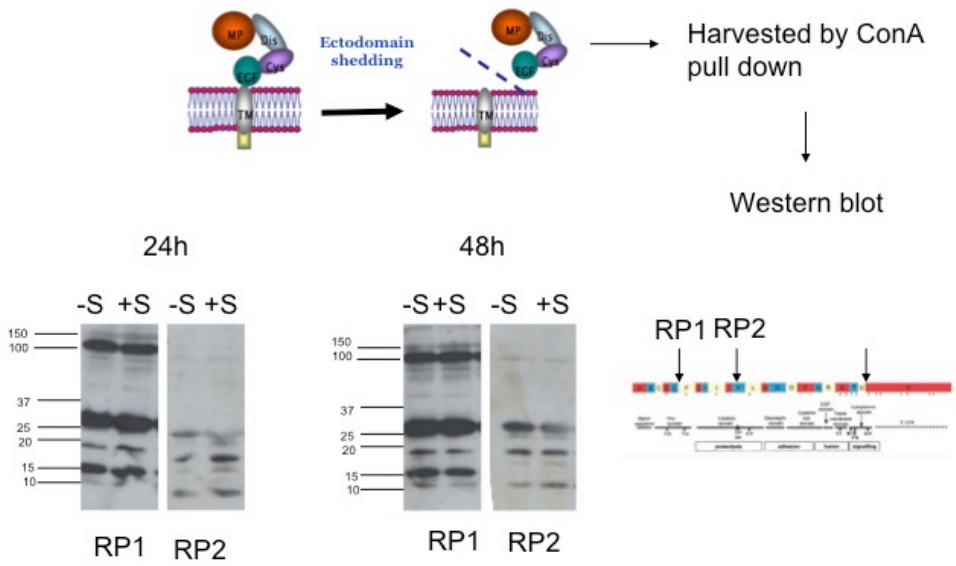


Figure 3.15: Western Blot (for ADAM33 rp1, rp2, rp3 and aSMA) using protein extracted from the cells and from the growth medium of the cells after several cyclical mechanical strain experiment using 30% as magnitude 12cpm as frequency.

Based on these results we can say that ADAM33 mRNA and protein expression were not significantly affected by mechanical strain. However, several ADAM33 isoforms are expressed in embryonic lung fibroblasts where they may be involved in airway wall modeling.

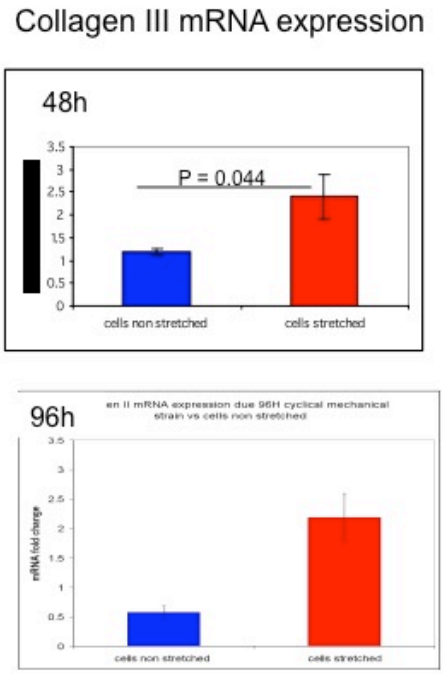
3.4 The effect of cyclical mechanical strain on IL-8 and Collagen mRNA and protein expression

It is known that fibroblasts are able to produce a large amount of different molecules such as collagen, an extra cellular matrix (35, 51) (ECM) protein, and IL-8 best known as a proinflammatory mediator. Collagen and ECM, for example, can be produced in response to an inflammatory process but we postulate that it is also produced during embryonic lung development as a requirement for stiffening of the airways as the tubular structures develop. IL-8 is one of the major mediators of the inflammatory response, also known as Neutrophil Chemotactic Factor. However, we postulate that IL8 (52) is not proinflammatory in the context of airway development, and may be a paracrine growth factor produced by mesenchymal cells for developing epithelial or endothelial cells in response to stretch during airway growth.

In order to test these hypotheses, we set up several cyclical mechanical strain experiments using $2x10^5$ primary foetal lung fibroblasts from a 14th week donor. The cells were left to grow for one day using DMEM 10%FBS as growth medium. We used 30% stretch and 12 cpm for 48 hours; non stretched cells were used as control.

RNA was extracted using the Trizol method and then Real Time PCR was performed. The PCR results of IL-8 and IL8 receptor (CXCR2) and Collagen III were compared to the geometric mean of two housekeeping gene UBC and GAPDH. Protein levels were analyzed using IL-8 ELISA kit and a soluble collagen assay.

After 30%12cpm 48hours cyclical mechanical strain experiment, analysis of Collagen III mRNA expression and soluble collagen revealed a significant difference between stretched cells and non stretched at either 48hr (Figure 3.16). Moreover, after 30% stretch at 12cpm for 48hours, analysis of IL8 mRNA expression and IL8 protein expression revealed a significant difference between stretched cells and non stretched at either 48hr (Figure 3.17). Also in order to confirm our hypothesis we studied the IL8 and IL8 receprtor (CXCR2) mRNA expression in human embryonic lung tissue which have both the epithelial and fibroblast components, and the results support our hypothesis, because we found an mRNA expression in foetal embryos that could explain the necessity of this cytokine as paracrine grow factor.



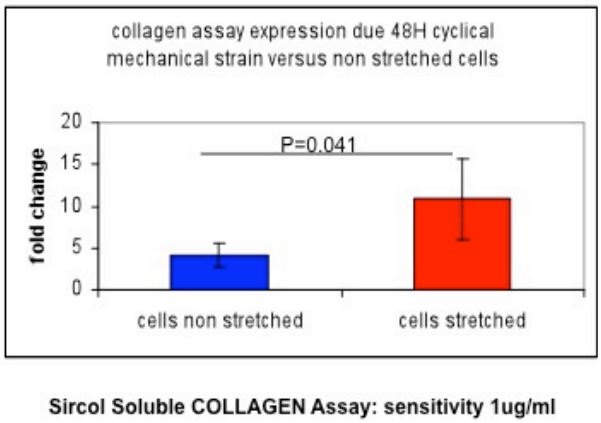


Figure 3.16: on the left Collagen III mRNA level due the 30%12cpm 48 hours cyclical mechanical strain. On the right soluble Collagen expression (using protein extracted from the growth medium of the cells) due the 30%12cpm 48 hours cyclical mechanical strain.

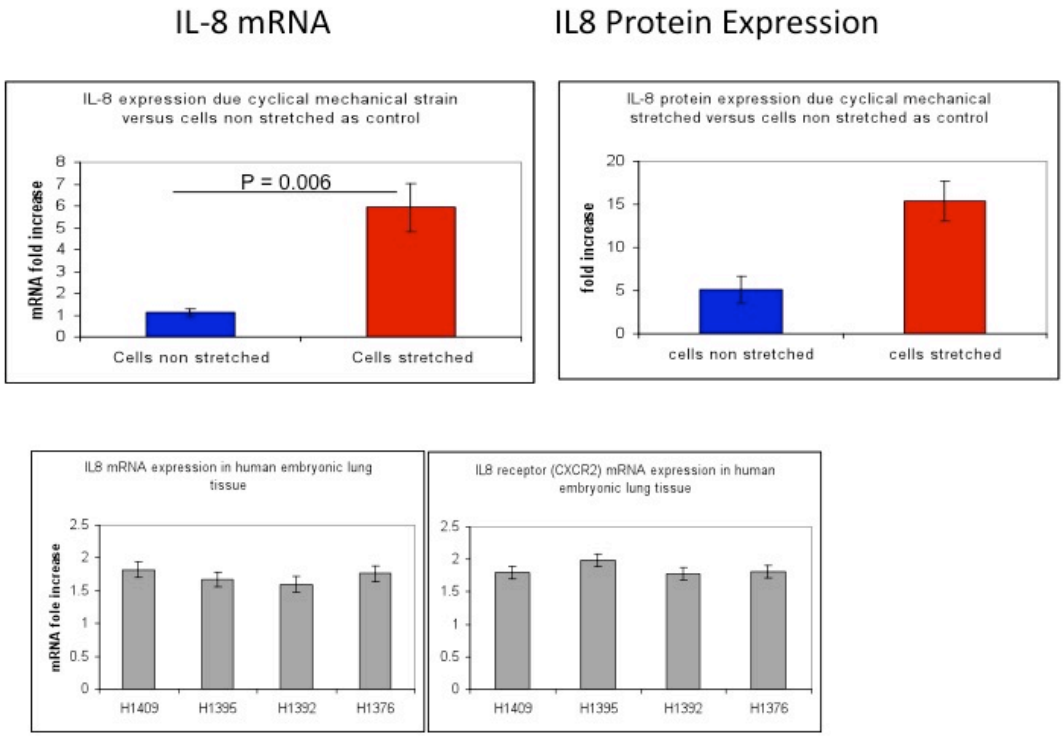


Figure 3.17: on the top left IL8 mRNA expression, on the top right IL8 protein expression due the 30%12cpm 48 hours cyclical mechanical strain. On the bottom left IL8 mRNA expression in human embryoinic lung tissue, on the bottom right IL8 receptor (CXCR2) mRNA expression in human embryonic lung tissue.

4. DISCUSSION

ADAM33 is a metalloprotease that is expressed in asthma (12) and has been implicated to play a role in airway remodelling (81,82,83,84).

Genetic polymorphism in ADAM33 has been associated with accelerated decline in lung function over time in asthma, suggesting a role for ADAM33 in chronic airway injury and repair.

Moreover ADAM33 mRNA expression is significantly higher in both moderate and severe asthma compared with mild disease and controls . It has recently been found that Adam 33 mRNA expression increases in murine fetal lung development (64), which may be due the mechanical strain of the airway during the peudoglandular stage of the embryonic development.

It is well known that embryonic lung development is a very complex process, it must be tightly controlled. Some of the controlling factors, which have been identified, include transcription factors, growth factors, extracellular matrix molecules, integrins and intercellular adhesion molecules. These interact along the proximal-distal axis of the respiratory tract influencing local gene networks which ultimately direct endodermal patterning and lung branching morphogenesis, vascularization and response to mechanical stress. Genetic analyses have demonstrated that cell-extracellular matrix and cell-cell interactions and growth/transcription factors can influence pulmonary development (42). A previous pilot study in our laboratory (using the same machine, same cell line and a magnitude of 4-8% stretch) had shown an increase between 5-15 fold in ADAM33 mRNA expression and between 2-8 fold in aSMA mRNA expression due the cyclical mechanical strain. These findings led us to

consider the relationship between the airway development and the influence of ADAM33 in on these modeling/remodeling processes. We hypothesized that the cyclical mechanical strain of human fetal fibroblats induced the expression of ADAM33.

In order to test our hypothesis, the first step was the optimization of the bio-engineer machine in terms of magnitude of elongation, frequency of strain and the time course experiment. We immediately found some technical limitations, such as the uneven stretch across the well, the impossibility to take pictures during the experiment (unless we stopped the experiment for few minutes) and that we lost a large number of cells during the experiment due the inability of the cells to remain attached on the collagen coated silicone membrane. However Nishimura et al. using the same machine failed to show any evidence of increased apoptosis in response to the stretch and suggested that the cell loss was due the decreased DNA synthesis (Nishimura K. et al. wound repair and regeneration 2007)

We started using a low % of elongation in order to reduce cell detachment (already seen in the preliminary study) (49,50) and also to mimic the physiological level in embryos. However the lower percent of stretch did not show any ADAM33 mRNA induction. In contrast, a previous study using adult fibroblasts was performed using a higher magnitude and frequency than used in the present study (47,71,72,87).

We also changed several parameters such as the density of cells seeded onto the collagen coated silicone membrane, the cell type (starting from a fetal lung fibroblast cell line and changing to primary fetal fibroblasts) and the composition of the growth medium in term of percentage of fetal bovine serum (FBS) added. FBS was found to be an important component of the culture

medium that helped us to keep the cells attached on the collagen coated silicone membrane. FBS is the most widely used serum-component for in vitro cell culture, as it has very low level of antibodies, high levels of grow factors and a rich variety of proteins (eg. Fibronectin) that allow cultured cells to survive, grow, divide and keep attached to plastic/silicone support. However, although it helped us to perform our experiments, its use was complicated because it contains TGF-B2 that is known to suppress ADAM33 mRNA expression.

Based on our initial negative findings, we decided to increase the frequency and magnitude of the elongation even though we knew that we would exceed the magnitude of strain experienced by cells during the peristaltic movements of the lung during embryonic development. However, it could give us more confidence about the bio-engineer strain machine in term of knowledge of the machine. Another limitation of our experiments was that we were using a monolayer (2-dimension) of fetal lung fibroblasts during the experiments, whereas physiologically the cells are surrounded by ECM in three dimensional space; they also are in contact with the epithelial layer in vivo where they are considered as a singular unit (58,88) (EMTU epithelial-mesenchimal trophic unit). This allows a continuous bidirectional exchange of signals between the layers, for example bronchial epithelial cells can produce many molecules, such as cytokines and growth factors in response to an inflammatory injury, and fibroblasts can respond to these molecules, perhaps by producing ECM or other mediators that propagate or amplify the response (43,56,57,59). From this point of view the optimal experiment could be the co-culture of both cell types (43,44,46).

Once we had optimized the conditions for mechanical strain of the fibroblasts, the target was to demonstrate the capacity of the fetal lung fibroblasts to induce

ADAM33 mRNA expression due the cyclical mechanical strain. After several experiments, we found that ADAM33 mRNA expression was not induced, however the reasons for this result could be the non-physiological environment, or the possibility that TGF-B2 in the FBS was suppressing expression of our target gene. However, inclusion of a Smad3 inhibitor, SIS3 (Calbiochem) at 1-3uM in several cyclical strain experiments had no effect on ADAM33 mRNA expression (data not shown). This suggested that TGF-B2 was not suppressing ADAM33 mRNA expression in our experiments. Therefore, it is possible that ADAM33 expression is not directly influenced by mechanical strain or that the effect may be complex and involve other cell types, eg. the epithelial layer, in order to activate a specific pathway.

In our studies, we also found that mechanical strain did not increase aSMA mRNA and protein expression, rather its levels were decreased. aSMA is a marker of myofibroblast differentiation, and is expressed in granulation tissue during wound repair and in airway remodeling and fibrotic processes (33,36,37,38,39,41,42). The failure to induce aSMA mRNA expression is consistent with an absence of a TGF-B_2–mediated response during mechanical strain, as inferred for ADAM33. Thus, based on our experiments we conclude that, under the conditions used, mechanical strain does not promote fibroblast to myo-fibroblast differentiation.

Despite a lack of effect on myofibroblast differentiation, we did find that cyclic mechanical strain of human fetal lung fibroblasts induced significant collagen III mRNA expression and collagen protein production, causing a 3-4 fold induction of mRNA expression and a 5 fold increase in protein production.

Collagens are important components of connective tissues, providing the

framework for various organs and they also give support and strength to the tissue. Collagen III is an interstitial collagen that is rapidly produced by fibroblasts before the tougher type I collagen is produced. Its production by embryonic fibroblasts in response mechanical strain may suggest that during embryonic development collagen is produced as a requirement for stiffening of the airways as the tubular structures develop.

In addition to increasing expression of collagen III, we found that cyclical mechanical strain of human fetal fibroblasts also induced a 5 fold increase in IL8 (52) mRNA expression and a 10 fold increase in IL-8 protein release into the medium.

Although IL-8 is best known as a neutrophil chemoattractant, it is unlikely that it plays such a role during lung development. In order to test whether IL-8 expression might be an experimental artifact due to the *in vitro* conditions, we also investigated whether it is expressed *in vivo* during lung development by analyzing mRNA extracted from human embryonic lung. Consistent with our *in vitro* findings, we found expression of IL8 mRNA, and also its receptor, CXCR2 in human embryonic lung tissue. Therefore, we postulate that the increased IL8 expression may act as a paracrine growth factor during epithelial or endothelial cell development. In support of this, previous studies using RT-PCR and immunohistochemical staining on lung from fetuses of 8 and 16+/-2 weeks post-conception have identified CXCR2 in bronchiolar epithelial cells. Furthermore, CXCR2 (59) has been shown to mediate the recruitment of endothelial progenitor cells and the angiogenic response in an animal model of allergic airway inflammation (89).

5. CONCLUSION

In conclusion, we have established a model in which have demonstrated that cyclical mechanical strain of human primary fetal fibroblasts induces collagen and IL8 mRNA and protein expression. In contrast cyclical mechanical strain does not increase ADAM33 and aSMA mRNA and protein expression compared with the control, unstretched cells. IL8 and Collagen III may be produced by fibroblasts in response to the peristaltic contractions of the developing airways to support their development. In contrast, the lack of effect of mechanical strain on ADAM33 expression, suggests that alternative mechanisms are responsible for its up-regulation during lung development; further studies would be required to identify these unknown mechanisms.