IMC FACHHOCHSCHULE KREMS UNIVERSITY OF APPLIED SCIENCES

FACHHOCHSCHULE KREMS MEDICAL AND PHARMACEUTICAL BIOTECHNOLOGY

The role of adenosine and its receptors in fibrosis

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CONTENT

ABSTRACT

The aim of this project was to see how the application of an A_{2A} receptor antagonist affects the scar formation in an animal model. The theory behind this was based on a paper called 'Pharmacological blockade of A_{2A} receptors diminishes scarring' by Miguel Perez-Aso, Luis Chiriboga and Bruce N. Cronstein which was published in July 2012.

In this paper Perez-Aso et al showed that the blocking of the A_{2A} receptor via the antagonist ZM241385 leads to diminishing of scar formation.

The animal model which was used was 6 week old C57/BL6J wild type, male mice which had two wounds patterned on the dorsum. One wound was treated with the antagonist while the other one was treated with the vehicle. After 14 days the animals were treated every two days. If needed the hair was removed again.

The mice were sacrificed one month after the surgery and the scars were excised, dissected and fixed in 10% formalin to be later used for histology or were homogenized for mRNA and protein extraction or for the hydroxyproline assay.

The difference with this project is that here the animals are not scarred directly but are radiated. It is observed how the radiation affects the skin and also how the ZM241385 and the vehicle substance are affecting the skin. In this study not only wild type but also A_{2A} receptor knock-out mice were used. Also the animals were treated every day starting the day after radiation. Then mice were treated for 28 days before they were sacrificed and harvested. The results showed that in the WT animals the application of the ZM241385 lead to a higher skin thickness and breaking tension.

KURZFASSUNG

Das Ziel dieses Projekts war festzusellen ob die Appliaktion eines A_{2A} Rezeptor Antagonisten eine Auswirkung auf die Narbenbildung in einem Tiermodel hat. Diese Projekt wurde nach einem Paper mit dem Titel ‚*Pharmacological blockade oft he A_{2A} receptors diminishes scarring*' von Miguel Perez-Aso, Luis Chiriboga und Bruce N. Cronstein entworfen, welches Juli 2012 veröffentlicht wurde gefertigt.

In diesem Artikel zeigen Perez-Aso et al das die Blockade des A_{2A} Rezeptors Mithilfe des Antagonists ZM241385 zu einer Verminderung der Narbenbildung führt.

Die Tiere die in dieser Studie verwendet wurden waren sechs Wochen alte männliche C57/BL6J Wildtyp Mäuse denen zwei Wunden am Rücken zugefügt wurden. Nach 14 Tagen wurde eine der Wunden mit einem A_{2A} Rezeptor Antagonist behandelt während die andere mit DMSO behandelt wurde. Dies geschah alle 2 Tage und falls es notwendig war wurde der Rücken der Mäuse wieder enthaart.

Die Mäuse wurden einen Monat nach der Operation getötet und die Wunden wurden entnommen und ein Teil wurde in einer 10% Formalinlösung fixiert und später für Histology verwendet. Der andere Teil wurde homogenisiert und für mRNA und Protein Extraktion oder für ein Hydroxyproline Assay verwendet.

Im Gegensatz dazu wurden bei diesem Projekt die Tiere nicht direkt verletz sondern bestrahlt und es wurde dann beobachtet wie sich diese Bestrahlung auf die Haut der Tiere auswirkt und auch wie die ZM241385 und DMSO Substanzen sich darauf auswirken.

In diesem Projekt wurden außerdem nicht nur Wildtyp sondern auch A_{2A} Rezeptor Knock-Out Mäuse verwendet. Die Tiere wurden in dieser Studie am Tag nach der Bestrahlung beginnend, jeden Tag behandelt. Die Mäuse wurden 28 Tage behandelt bevor sie getötet und die Wunden analysiert wurden. Die Ergebnisse

zeigen, dass sowohl die Hautstärke und die Bruchspannung in den Wildtyp Tieren zugenommen hat.

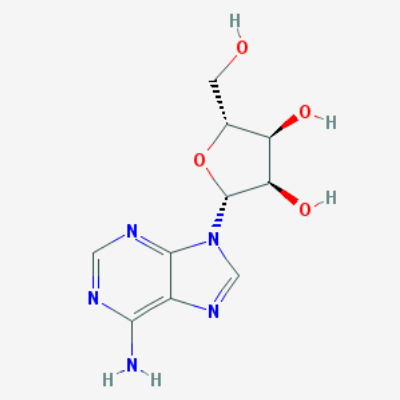
ABBREVIATIONS

1° AB	primary Antibody
2° AB	secondary Antibody
ADP	Adenosine Di-Phosphate
APC	Adenomatous Polyposis Coli
ATP	Adenosine Tri-Phosphate
BCA	Bicinchoninic Acid
BSA	Bovine Serum Albumine
β-Trcp	beta-Transducing Repeat-Containing Protein
cAMP	cyclic Adenosine Monophosphate
cDNA	complementary DNA
CK1	Casein Kinase 1
CTP	Cytidine Tri-Phosphate
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic Acid
Dvl	Dishevelled
FBS	Fetal Bovine Serum
Fz	Frizzled
GSK3	Glycogen Synthase Kinase 3
GTP	Guanosine Tri-Phosphate
KO mice	Knock-Out mice
LEP	Lymphoid Enhance Factor
LRP6	Low-density Lipoprotein Receptor related protein 6
$MgCl_2$	Magnesium Chloride
mRNA	messenger RNA
PDGF	Platelet Derived Growth Factor
PCR	Polymerase Chain Reaction
qPCR	quantitative PCR
RT	Reverse Transcriptase
SDS	Sodium Dodecyl Sulfate
SDS PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
S/P	Streptomycin / Penicillin

TBST	Tris Buffer Saline Tween20
TEMED	Tetramethylethylenediamine
TCF	T Cell Factor
TGF Beta	Transforming Growth Factor Beta
TTP	Thymidine Tri-Phosphate
Tris	Tris(hydroxymethyl)-aminomethan
WB	Western Blot
WT mice	Wild Type mice

INTRODUCTION

Adenosine



Pic 1: 2D Model of Adenosine consisting of an adenine molecule (in blue) and a d-ribose (in red)[2].

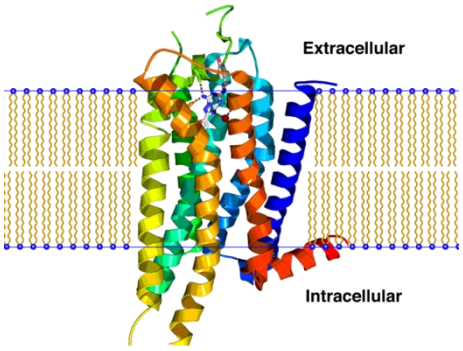
Adenosine is an endogenous nucleoside which is present in all cells in the body and is a crucial part in processes like energy transfer, as ATP and ADP. It also plays an important role in signal transduction as cAMP. It is an intermediate product of the adenine nucleotide metabolism and a potent physiological mediator which role it is to regulate a variety of physiological processes[1]. This nucleoside, which is composed out of adenine and d-ribose is a component of both DNA and RNA, plays an important role many biological processes and is also a neurotransmitter which is said to play a role in promoting sleep and also suppressing arousal[2].

When a tissue or organ is in stress, adenosine can act as a cytoprotective modulator, which is a chemical compound that provides protection to cells against harmful agents[3]. It is generated when ATP is catabolized, which happens when the energy demand increases or oxygen demand decreases at sites of tissue stress, injury and local hypoxia. This results in increased Adenosine concentrations in the interstitium where it exerts its actions via binding to four types of extracellular G-protein coupled adenosine receptors called A_1,A_{2A}, A_{2B} and A_3 [4,5].

These receptors are targets for caffeine, which is the most commonly consumed drug in the world, which acts as a prototypical antagonist against them and its stimulant action occurs mainly through that mechanism. Each of the four adenosine receptor has its own set of agonists and antagonists, which have been clinically evaluated but none of them have yet received regulatory approval[3].

There has been evidence that the receptors can also be used as therapeutic targets for a wide range of conditions like sleep disorder, cancer and immune and inflammatory disorders.

Each of the four Adenosine receptors is encoded by its own gene and has a different set of functions, even though some of them overlap[6].



Pic 2: Structure of the A_{2A} receptor showing the seven transmembrane alpha helices[28]

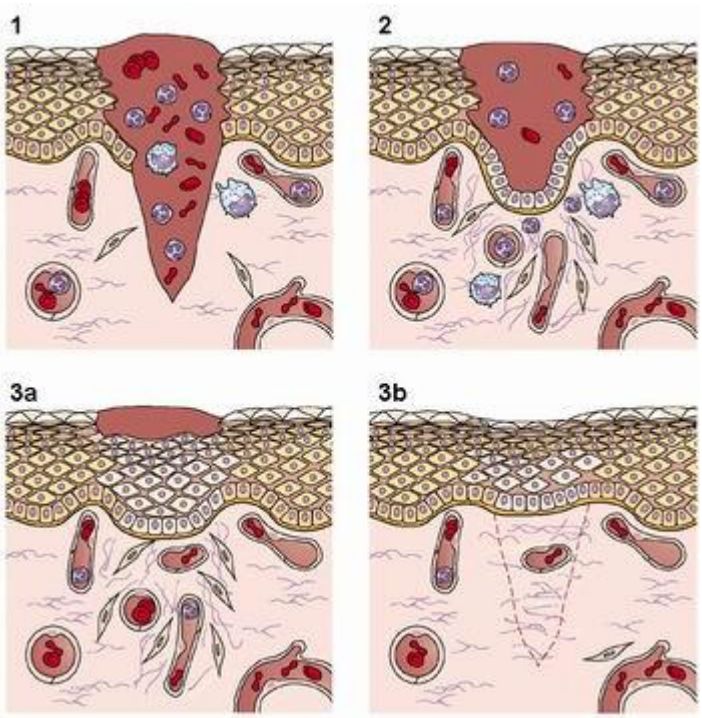
The A_{2A} receptor is a as stated above a member of the G-protein coupled receptors which consist of seven transmembrane alpha helices. This receptor is also shown to interact with the Dopamine receptor D2 and decreases its activity[7].

It has been shown that A_{2A} receptor stimulation promotes wound healing and that it is required for the development of fibrosis in the murine model of scleroderma and cirrhosis. It was shown that the application of the selective A_{2A} receptor ZM241385, and therefore the blockade of that receptor, decreased scar size and enhance tensile strength in a murine model. Also the collagen alignment and

composition of the scar and was improved by the application of ZM241385. It also reduced the number of myofibrobalsts and angiogenesis in the scar but not the number of macrophages.

Wound healing

Wounds normally heal in a very efficient manner which consists of four distinct but also overlapping phases. These are: hemostasis, inflammation, proliferation and remodeling.



Pic 3: Steps of wound healing[29]:

1: Inflammatory Phase; 2: Proliferative Phase;
3a:Maturation Phase; 3b: Remodeling Phase

Normally the healing process begins the moment the injury occurs by the blood spilling into the site where the injury took place like it can be seen in Pic.3. This in turn leads to the platelets coming in contact with exposed collagen and other parts of the extracellular matrix. When the platelets get into contact with these factors it causes them to release clotting factors, growth factors and cytokines, like for example TGF-beta and PDGF.

After this neutrophils start entering the wound and start the process of phagocytosis, in which they remove bacteria, foreign materials and injured tissue. Macrophages appear in this phase and continue phagocytosis and also produce additional PDGF and TGF-beta.

When the wound is clean fibroblasts are introduced into the wound. These cells then start the proliferation phase and also the production of extracellular matrix.

This the last phase of this process, the remodeling phase, the new collagen matrix gets cross linked and organized. There are multiple cell-signaling pathways playing together in order for this phase to function correctly.

If this process is not working correctly it can lead to pathological conditions such as non-healing pressure ulcers, which means that the ulcers stay in constant state chronic inflammation and that the healing can only continue once the inflammation is controlled[8].

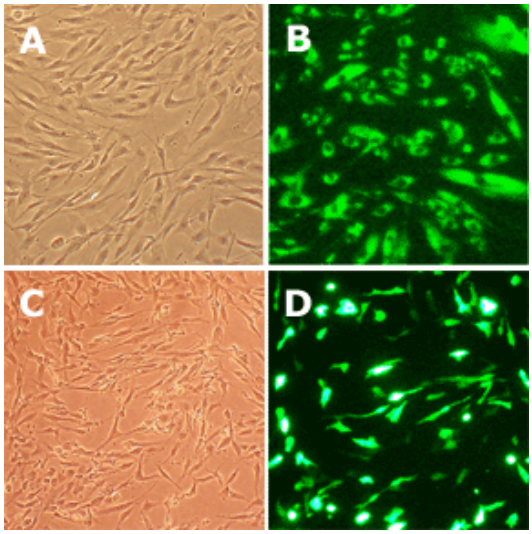
Fibrosis

Fibrosis is one form of wound healing in which there is a formation of excess fibrous connective tissue at a tissue or organ. This means that the wound fails to heal properly and therefore the remodeling of the tissue is more extensive and also involves heightened extracellular matrix production and the abnormal deposition in the tissue. This can eventually lead to the loss of tissue function[9].

It is thought that tissue fibrosis is the result of a dysregulation of the mentioned process which comes from a continual local injury or impaired control mechanism and inflammation can’t resolve and this leads to a chronic inflammation response[10].

There are different sites where fibrosis can occur like skin, lung, heart, kidney and liver. It is also estimated that about 45% of death occurring in the western world can be brought back to diseases where fibrosis plays a major role[11].

Normal Human Dermal Fibroblast Cell Line (NHDF)



Pic 4: A: Human Dermal Fibroblasts in culture. B: Stained by using Immunocytochemistry. C&D: Cells transfected with GFP plasmid DNA[13].

Dermal fibroblasts which are shown in Pic.4.are in general found in the dermis layer of the skin and generate connective tissue and therefore allow the skin to recover from injury[12].

In case of an injury fibroblast migrate towards the wound, where they dispose extracellular matrix, organize the substratum and also contract the wound. Growth factors, extracellular matrix and other molecules that are present at wounds have both chemokinetic and chemotactic effects on dermal fibroblasts[8].

These cells are either derived from the dermis of a normal human neonatal foreskin or the skin of an adult and are found in all connective tissue where under cell culture conditions, they synthesize and secrete extracellular matrix proteins. They are also often used for *in vivo* analysis of fibroblast growth, collagen metabolism and migration in wound healing[13].

Animal Model

The animal strain which was used in these experiments was the C57BL6 strain. It is a very common inbred strain which is most widely used in animal research. They are bred by a sibling system which means that the male and female are brother and sister. This strain has a black coat as pictured in Pic.5, is of average built and long-lived.

This type of mouse was the second mammal after the human to have its complete genome sequenced. It is used in a wide variety of research areas including developmental biology, genetics, immunology and neurobiology.

This mouse strain is also often used to produce transgenic mice and they are known to breed well, live long and have a low susceptibility to tumors. In later life they develop severe and progressive hearing loss and they are also more susceptible to noise-induced hearing loss during life due to the age related hearing loss 1 mutation. This phenomenon starts after the animal reaches 10 months of age[14].

Pic 5: Wildtype of a C57BL/6J mouse: This shows the characteristic black fur color of this mouse strain[14].

Knock-out mice

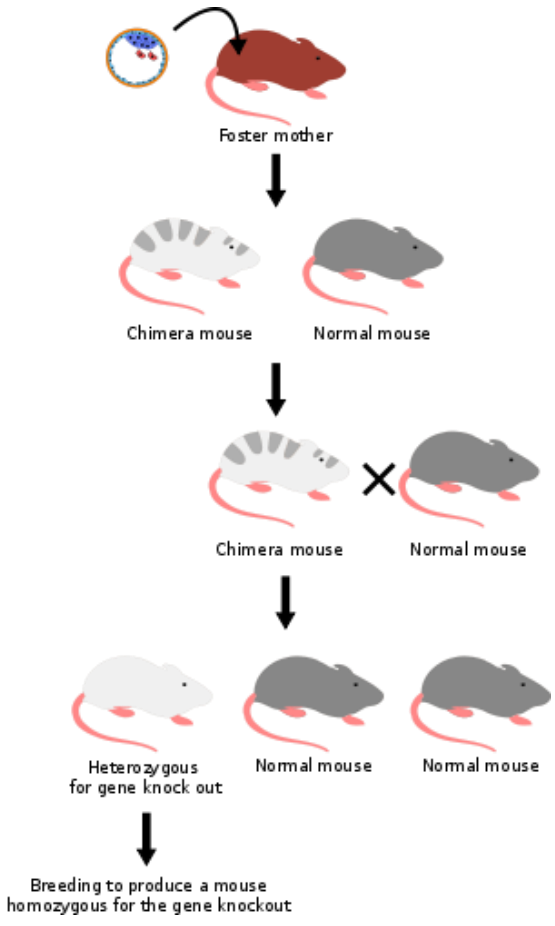
Knock-out mice are genetically altered animals that are used in research to better understand the function and importance of certain genes. This is done by breeding mice that have that certain gene inactivated or are lacking that gene by replacing or altering it by adding artificial DNA.

They are generated by taking embryonic stem cells from a pregnant donor mouse and using the cells from the inner cell mass of the embryos of that mouse. These cells are than placed into a media and grown like a tissue culture.

To create a modification in a gene, a piece of DNA from that gene is taken and a neomycin cassette into it which makes the gene unable to express normal proteins. To this vector a second modification is made by adding a toxic agent to the end of it.

This DNA vector is then added to the embryonic stem cells by just adding it to the tissue culture and giving them a small electric shock. This process is called electroporation and leads to three possible reactions of the cells.

The first is that the cells take up the complete DNA vector with the toxin still attached to it. If this is the case these cells are destroyed by the toxin. This type of reaction is called random integration.



Pic 6: Breeding Model of Knock-out mice[30]

In this picture the color of the fur is taken as indication if the animal is chimeric or normal.

The second possibility is that the cells don't take up the vector at all so the chromosomes are left untreated.

The third and most important reaction is that the vector finds the right gene inside the cell and integrates into that gene and replaces it. This is called homologous integration. If this happens it gets rid of the toxin and therefore the cells are able to survive.

It is important to separate the cells that have the new vector inside and those that do not. This is done by adding an antibiotic to the tissue culture to which the cells with the neomycin gene are resistant so it only kills the cells that carry the new gene.

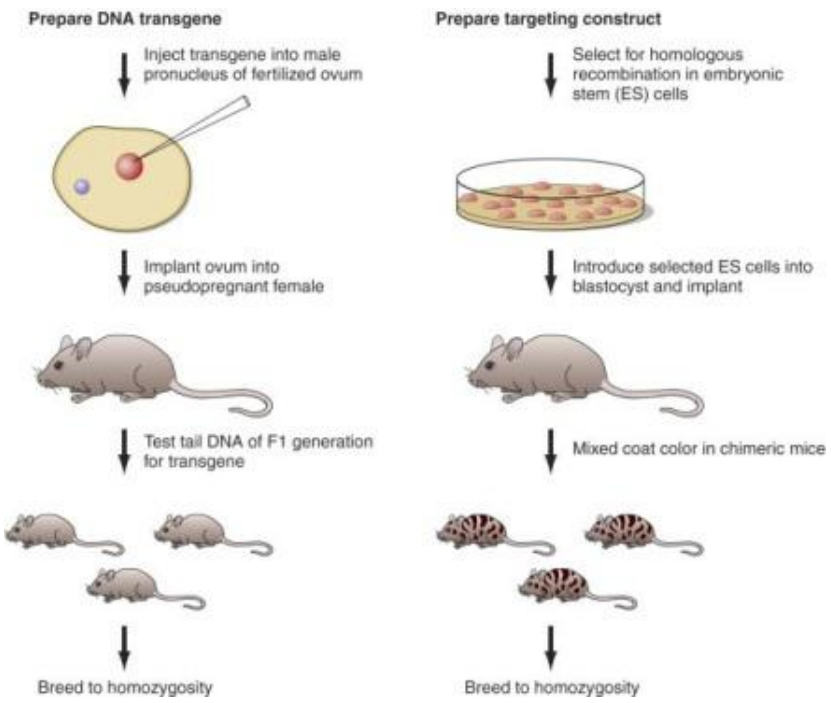
The cells that are now growing on the plate can be selected and injected into to

blastocyst of a donor embryo which is then in turn implanted into a pseudo-pregnant foster mother. From this point on the process is also shown in Pic 6. This mouse will now give birth to live pups which are either chimeric or normal mice. The chimeric mice are the ones that are used for further breeding since they carry the chromosomes from both the donor mouse and the embryonic stem cells.

These chimeric mice are now crossed with normal mice in order to get mice that carry the gene on one chromosome which means that they carry a heterozygous mutation in the gene of interest.

To get homozygous mutations two heterozygous mice are bred together and the offspring will have a 25% chance of being homozygous. 50% will be heterozygous and 25% will be normal mice[15].

Transgenic Mice



Pic 7: Simplification of the two methods to create transgenic mice[31]:

Right: Pronuclear Injection and implantation of the ovum into a pseudo-pregnant foster mother.

Left: Targeted mutagenesis. The altered embryonic stem cells are implanted into a blastocyst and chimeric pups are the outcome. Which can then be bred to reach homozygostity.

The transgenic mouse model is another animal model which can be used in research. These are made by adding additional foreign DNA to every cell of the animal. Foreign in this context means that the DNA comes from another organism. But this organism could also be another mouse. And with these animals it is possible to study the gene

function or regulation and also to help fashion a model for human diseases.

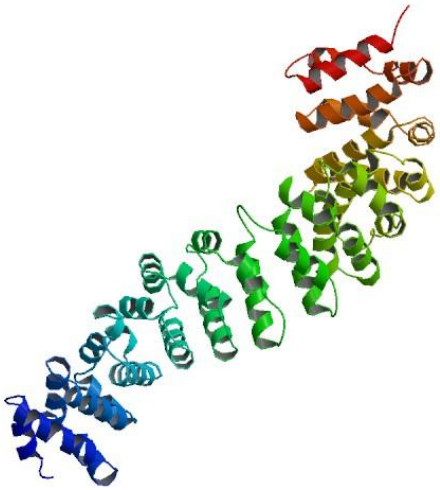
There are two methods which can be used to get a transgenic mouse. Both these methods are shown in Pic 7.

The first one is that the foreign DNA is introduced into the fertilized egg right at the beginning. This procedure is called pronuclear injection. The DNA is put into the large male pronucleus, which is derived from the sperm, by using a very fine needle. There is a possibility that the resulting mouse is only partly transgenic. If the transgenic cells are part of the germ line, then the sperm or the eggs of the mouse will be transgenic and therefore the next generation will be fully transgenic, which means that each cells has a copy of the foreign DNA.

The second method of producing transgenic mice is similar to the production of knockout mice. The DNA is introduced into embryonic stem cells of an animal which have been taken from the animal in a very early stage of the embryo. That means that the cells can still differentiate into every cell of the body.

The chances are that in this procedure the DNA gets integrated at the right point of the DNA and is may therefore undergo homologous recombination. The DNA is then put into a host embryo where is develops. The resulting mouse will be chimeric and since embryonic stem cells are often part of the germ line this will result in some sperm carrying the additional DNA. When these transgenic mice are bred with a normal mouse, the transgenic mouse being the father, a transgenic mouse will be produced that carries the foreign DNA in every cell[16].

Beta Catenin



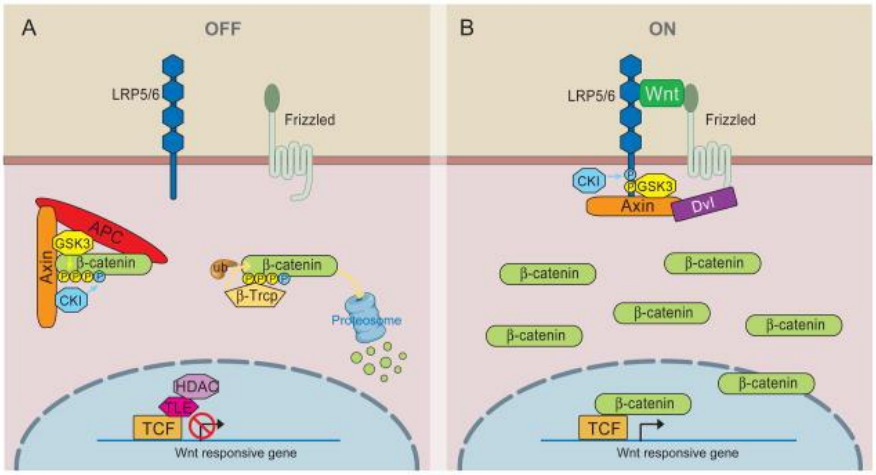
Pic 8: 3D structure of Beta-Catenin[32]

Beta Catenin (Pic 8) is a protein that is encoded by the CTNNB1 gene complex in humans and the homologous protein in drosophila is called armadillo.
Since it has been discovered to be a protein which was associated with the cytoplasmic region of E-cadherin, beta-catenin has also been shown to have two seemingly unrelated functions. It is known that it has a very crucial role in cell-cell adhesion and also that it plays a signaling role in the Wnt/wg pathway[17].

This kind of signaling controls embryonic development and also adult homeostasis. So a mutation in the Wnt pathway, which is shown in Pic 9, can be linked to human birth defects, cancer and other diseases[18].

When Wnt is not present, the cytoplasmic β-catenin protein is degraded by the work of the Axin complex. This complex is made up of the scaffolding protein Axin, a tumor suppressor gene product called APC, CK1 and GSK3.
CK1 and GSK3 both phosphorylate the amino terminal region of β-catenin, which results in the recognition of β-catenin by β-Trcp, which is an E3 ubiquitin ligase subunit. This subsequently leads to β-catenin ubiquitination proteosomal degradation[19]. Because β-catenin is eliminated on a continual basis, β-catenin is

prevented from reaching the nucleus and therefore the Wnt target genes are repressed by the DNA-bound TCF/LEF family of proteins.



Pic 9: The Wnt/β-catenin pathway[33]

When a Wnt ligand binds to the Fz receptor, which is a seven pass-transmembrane receptor, and its co receptor LRP6, the Wnt/ β-catenin pathway gets activated. A Wnt/Fz/LRP6 complex is and the scaffolding protein Dvl is recruited which leads to a phosphorylation and activation of LRP6 and a recruitment of the Axin complex to the receptors. This leads to a stabilization of β-catenin, by inhibiting the Axin mediated β-catenin phosphorylation. β-catenin then accumulates and travels to the nucleus to form complexes with TCF/LEF and therefore activate Wnt target gene expression[33].

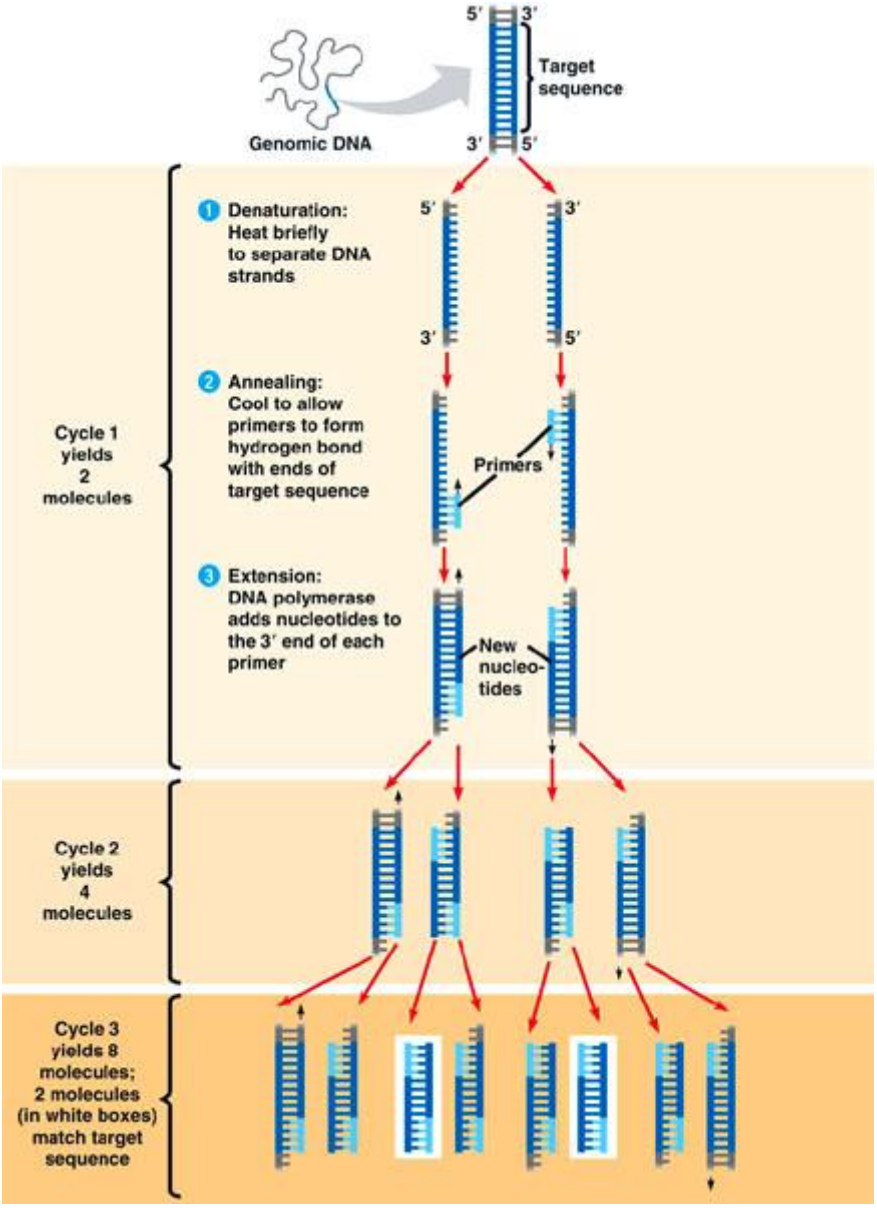
Polymerase Chain Reaction

Polymerase Chain Reaction or PCR is a method which is used in research to amplify piece of DNA over several orders of magnitude which in turn generates thousands of copies of that particular sequence of DNA.

In 1983 Kary Mullis developed this method and therefore was awarded the Nobel Prize for chemistry in 1993. His concept was further improved over the years until by several researchers[20].

PCR is a widely used technique in research labs and can be used for many different procedures. These include function analysis of genes, DNA cloning for sequencing, genotyping and the identification of genetic fingerprints.

The theory behind this method is that DNA polymerase is able to synthesize a new complementary strand of DNA to the given template strand. Important with this technique is that there has to be a primer present since DNA polymerase can only add nucleotides onto a pre-existing 3'OH group. So the primer is used to add the first nucleotide to it. This makes it possible to design a specific region of template that then can be amplified. When the PCR reaction is finished this DNA sequence will be present in billions of copies which are called amplicons[21].



Pic 10: Basic PCR cycle[34]

The basic procedure of a PCR cycle is shown in Pic. 6. The process starts with denaturation in order to get the double stranded DNA to convert into two single stranded DNA strains. This is done by heating the DNA briefly up to about 95°C. After that the primers are added to the 3’ ends of the strands and the temperature is lowered to approximately 55°C. This makes it possible for the primers to form hydrogen bonds with the DNA sequence. When this is done the temperature rises again and the primers start synthesizing the complementary strand to the specific DNA template. After this first cycle there will be two strands of double stranded

DNA. This process is then normally repeated multiple times until there are millions of copies of the original DNA strand.

There are many different types of PCR procedures but one of the most commonly used is the Reverse transcription Polymerase Chain Reaction or RT-PCR.

This type of PCR is used to detect mRNA expression levels. Before the PCR cycle can start in this method the mRNA has to be reversely transcribed into complementary DNA by adding a primer to the mRNA and transcribing a complementary DNA strand. This cDNA is then used as a template for the PCR reaction[22].

Another type of PCR that is often used in research labs is the real time Polymerase Chain Reaction (qPCR). This method is used to detect and quantify specific DNA sequences. It quantitatively measures the starting amounts of either DNA, RNA or cDNA in the sample. In this method the product of the reaction is detected at the end of the PCR cycle with the help of either non-specific fluorescent dyes that interact with any double stranded DNA, or with sequence specific DNA probes. These consist of with a fluorescent reporter labeled oligonucleotides. This form of detection method only functions after the probe is hybridized with its complementary DNA target[22].

Western Blot

Western Blot is used in research as an analytic method to analyze the proteins in a given sample. This method which is also called Immuno blotting was first reported in 1979. It got its name as a reference to the southern blot which was developed by Edwin Southern and is a technique to detect DNA. There is also a procedure called Northern Blotting, which is the detection of RNA[23].

In this procedure the proteins are separate with the help of polyacrylamide gel electrophoresis, where charged protein travel through the gel when an electric field is applied[24].

If an ionic agent like Sodium Dodecyl Sulfate is used, the distance the proteins travel through the gel is inversely proportional to the molecular weight of the protein[24]. It is also important that the proteins are unfolded via denaturation before they are loaded so that is possible for the antibody to bind to the epitope. This is a small part of the protein which often lies within the proteins 3D structure. In order to achieve this, the samples are mixed with loading buffer and then boiled before loading them on the gel. It is important to add a marker to each gel because it will show bands at known molecular weights and therefore prove that the protein is in the sample. Also the antibodies often stain more than one protein and therefore not all the bands in the gel and membrane are of interest.

The proteins are normally transferred from the gel to a membrane. This is normally either a nitrocellulose or a PVDF (positive charged nylon) membrane. There are two different ways of transferring the protein. Either a wet or semi dry method can be used: the semi dry method is faster but less secure because there is the risk of letting the membrane dry out[25].

Immunocytochemistry

It describes a highly specific interaction between an antigen and its antibody. Therefore the use of labeled antibodies has proved very useful in localizing and identifying specific proteins.

There are cells in the body, which are able to distinguish between self and foreign cells and producing proteins, called antibodies in response to be exposed to foreign molecules, called antigens. Antibodies are proteins of the immunoglobulin family and react specifically to a foreign substance.

In Immunocytochemistry cells are incubated in a solution containing the antibody of the protein is believed to be in the cell and which I want to show. The antibody binds specifically to that certain protein and therefore the location and quantity of that protein can be shown using either a light or electron microscope.

Antibodies against a specific protein in a certain animal are produced by injecting the protein into a different species. If the protein is different enough to the host organism it will be recognized as foreign and the animal will produce antibodies against it. These antibodies are then collected form the animals plasma and are purified and used in Immunocytochemistry

There are two different types of antibodies: polyclonal and monoclonal antibodies[26].

Polyclonal antibodies:

They are derived form a several clones of B-lymphocytes of the animal that has been injected with the foreign protein, as they are able to recognize different parts of the protein injected and therefore produce different antigens against each part. The antibodies produced contain a mixture of polyclonal antibodies. Polyclonal antibodies are able to recognize different epitopes on one antigen which means they can be used to help amplifying signals from target proteins that have a low expression level. This is caused by the target molecule being able to bind more than one antibody on multiple epitopes.

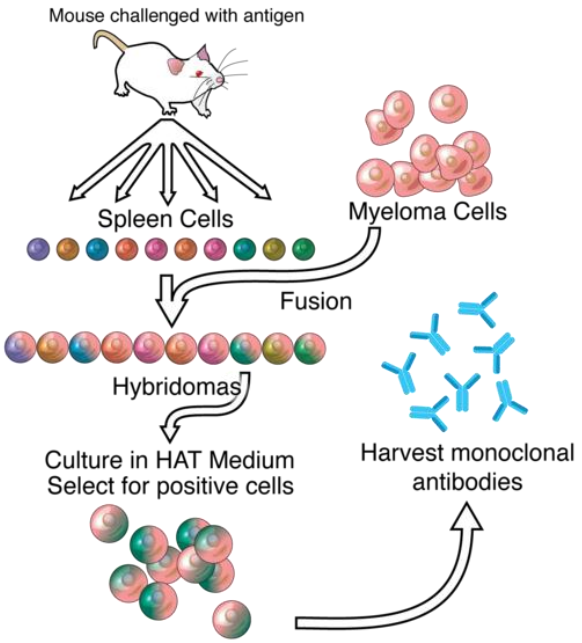
These kinds of antibodies are also more tolerant to small changes in the antigen for example, a slight denaturation or polymorphism than monoclonal antibodies.

Other advantages are that they are faster and cheaper to produce and due to the fact that they can bind to multiple epitopes the offer a more robust detection.

On the other hand one major disadvantage is that they are not that specific and this can sometimes lead to a large amount of background signal[27].

Monoclonal antibodies:

It is possible that different types of lymphocytes produce different antibodies against several parts of the injected protein. Each of these clones can be isolated and cultured on its own so that the different antibodies can be collected. Each of these specialized antibodies is a monoclonal antibody.



Pic 11: Production of monoclonal Antibodies using a mouse model[35]:

The animal was immunized with the specific antigen and cells form the spleen where taken. Which were than fused with Myeloma Cells. This generates Hybridomas which are then out in a special media which is called the HAT media. In there the hybridoma cells are able to grow indefinitely. The antibodies that are secreted by the cells are then separated by the different antibodies they secrete and are then tested by procedures like ELISA and the most productive and stable clone is selected for future use.

One major advantage of monoclonal antibodies over polyclonal is that they are highly specific and can be selected in order to bind strongly to only one epitope on the antigen. This has the advantage that there will be less unspecific binding which makes it easier to detect a certain protein and the risk of cross-reaction with other proteins is small.

Since they only consist of one antibody subtype it is required that a secondary antibody is used for the detection. This secondary antibody is usually an antibody against the correct subclass.

Monoclonal antibodies have a high homogeneity which makes it easier to reproduce results that were obtained using them.

One disadvantage is that they are more vulnerable to a possible loss of the epitome due to chemical treatment of the antigen. This can be prevented by using two or more monoclonal antibodies on the same antigen[27].

METHODS

Animal Work

A3

The aim of this experiment was to discover if the blocking of the A_{2A} receptor had a significant impact on the radiated skin and if the application of the A_{2A} receptor antagonist ZM241385 had a similar effect on the wild type and Knock-out animals.

Animals

Overall 30 animals were used for this experiment. They were C57B/6 male mice which were 6 weeks of age at the point of radiation. They were spilt into wild type and A_{2A} receptor Knock-out mice.

ZM241385 was used for the treatments in a concentration of 2.5g/ml and DMSO was used as a negative control in the same concentration.

Procedure

The animals were split in 6 equal groups of 5 mice each and were either radiated or not radiated and afterwards either treated with the A_{2A} receptor Antagonist ZM241385 or with DMSO which was acting as a vehicle substance.

The groups were:

01 KO Non- Radiated Vehicle

02 KO Radiated Vehicle

03 KO Non Radiated ZM

04 KO Radiated ZM

05 WT Radiated Vehicle

06 WT Radiated ZM

As the experiment was already done with WT mice there is no Non-Radiated control group for the WT mice.

Each animal was anesthetized and a section of the back was shaved and depilated in order to make sure that there was no hair left which could interfere with the radiation or the application of the treatment and therefore influence the experiment.

On the next day the animals were radiated at 40Gy for about 10 minutes. Start by clamping the hairless skin of the dorsum with chop sticks and cover the rest of the animal with a shield to protect it from getting radiated. This is important so that it is known exactly which area is radiated in order to be able to compare it later on with the non-radiated skin.

After radiation the animals were treated daily with either the A_{2A} receptor antagonist or the vehicle solution for 28 days.

During this procedure the mice were anesthetized and patches were attached to the skin so it was possible to the same area on each animal. The respective solution is then applied to the skin of the animal and a band aid is used to make sure the fluid stay on the skin.

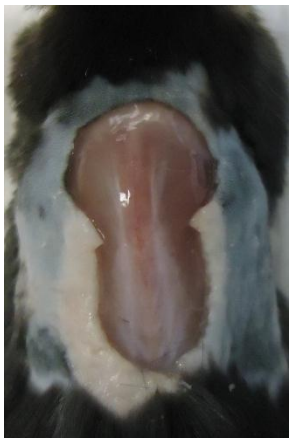
This is repeated every day for 28 days. When a difference in the animals could be observed pictures were taken of the mice.

After 28 days the animals were sacrificed and the treated skin was taken and measured for skin thickness, breaking tension and dermal thickness were measured. It was also determined which proteins and what amount of proteins was present in the skin.

A4

Overall 25 animals were used in this experiment. They were all male WT C57B/6 mice, which were between 6 and 8 weeks old.

20 animals were radiated at 40Gy (gray radiation) and were not stimulated. The animals were harvested after one, two, three or four weeks and the skin was tested for Adenosine content and CY content.



Pic 12: Mouse after the two skin samples were taken

If necessary the animals were shaved and depilated before the radiated skin from their back was taken. Using a 12mm punch the sink was marked and cut along this line. This can be seen in Pic12. Two samples were taken from the back of the mouse One for the testing of Adenosine and the other one to test for cytokine content. It is important to make sure that the skin samples all weigh the same. So a weight of 60mg was taken as standard.

The samples are then washed in Low-Glucose DMEM Medium, which contains S/P but no FBS. There is no FBS in the medium because it would interfere with the proteins in the skin but the S/P is needed to avoid contamination of the sample.

The next step has to be performed in the Tissue Culture Hood. The skin samples are cut into very small pieces and each sample is put into a separate chamber of a multiple chamber plate.

The Samples that are treated for Adenosine are incubated in the media for four hours at 37°C. Then the supernatant is taken off and the sample is centrifuged for 5 minutes at 2000rpm. Then 400µl of the supernatant are taken and 400µl of 10% TCA are added. Each sample is vortexed for about ten seconds and are then kept on ice for30 minutes. After that 800µl of Freon-octylamine solution is added and the samples are vortexed again to make sure that the two resulting layers mix well. Centrifuge the samples for four minutes at 13200rpm at 4°C. Take off the aqueous

layer and transfer it to a new eppendorf tube. The samples are then frozen at -80°C and can be kept there until they are needed.

The samples which are used for the cytokines are kept in the media overnight at 37°C and on the next day the supernatant is taken off and centrifuged for 5 minutes at 2000rpm. The supernatant is taken and frozen at -80°C.

mRNA Extraction

Preparation

The RNeasy Mini Kit from Qiagen was used for the extraction of mRNA.

Before starting the procedure, all the equipment has to be cleaned with RNA tissues to make sure that there is not residual RNA which could interfere with the RNA from the cells and therefore falsify the results.

10µl β-Mercaptoethanol for each milliliter of RTL buffer was added right before use. Also the RPS buffer is concentrated and must therefore be diluted by adding 4 volume of 100% ethanol to the RPS buffer stock solution. One volume is 350µl.

All steps were done at room temperature and the centrifugation steps were done between 20°C and 25°C in a standard micro centrifuge.

Procedure

Start the procedure by adding 350µl RLT Buffer to the cell pellet and vortex. Use the QIAshredder columns for homogenization by putting them into a 2ml eppendorf tube and centrifuging for 2 minutes at high speed.

Add 1 volume (=350 µl) of 70% ethanol and mix it thoroughly with a pipette. Transfer the whole sample to an RNeasy column and add a 2ml collector tube to it. Centrifuge for 15 seconds at 10.000rpm and discard the supernatant but keep the collector tube because it can be reused.

Add 1 volume of RW1 buffer to the RNeasy column and centrifuge again for 15 seconds at 10.000rpm. Discard the supernatant and reuse the collector tube.

Add 10µl DNase I and 70µl RDD buffer to the cells and mix everything by inversion. Incubate the sample for 15 minutes at room temperature.

Add again 1 volume of RW1 buffer to the RNeasy column and centrifuge for 15 seconds at 10.000 rpm. Discard the supernatant and transfer the RNeasy column to a new 2ml collector tube. Add 500µl RPS buffer to the sample and centrifuge for 15 seconds at 10.000 rpm. Discard the supernatant.

Add another 500µl RPE buffer and centrifuge for 2 minutes at 10.000rpm. Discard the supernatant.

Transfer column to a new 1.5ml tube and add 30µl RNase free water. Centrifuge 1 minute at 10.000rpm.

To concentrate the sample, transfer the sample to the same column and centrifuge 1 minute at 10.000rpm.

RNA quantification is done by using a nanodrop apparatus. This works by putting 1µl of the sample on the electrode and measuring the mRNA. Water is used as a blank.

Protein Quantification

One method to find out the amount of protein in a sample is using a bicinchoninic acid (BCA) protein quantification protocol. This procedure is used to establish the protein concentration of an unknown sample in order to be able to use it afterwards for further experiments like Western Blots or Gel Electrophoresis.

There are different kits on the market that make it easier to determine the right quantity of protein which all use different proteins as a standard.

One of the most common proteins used is bovine serum albumine. These kits come with different set concentrations of the protein which are used to get a standard curve with which it is then possible to find out the protein quantity in the sample.

When working with an unknown sample it is important to include a standard curve every time the assay is performed.

Procedure

Take the samples form the – 80°C freezer and wait for them to thaw and meanwhile turn on the water bath to 37°C. The BCA reagents A+B and the pre-diluted Protein Standards for the standard curve are needed. Dilute reagents A:B in a 50:1 ratio.

Pipette the samples first into a 96 well plate. Normally 10mcl in 1 ml and add the reagent solution. Do every sample in duplicate and start with the standard curve. If colour changes can be observed right away this means that the protein concentration in the sample is too high and will have to be diluted.

Put in the water bath for 30 minutes and afterwards use the Softmax Pro machine at wavelength 562 to get the values for the standard curve and the samples.

Check if the machine gives samples that are between the 0 control and the highest value of the standard curve. If the values are higher they need to be diluted and the dilution factor has to be taken into consideration when calculating the protein concentration.

Reverse Transcriptase

Since the starting product is mRNA there needs to be a step between measuring the amount of RNA and doing the PCR.

This step is the reverse transcriptase or RT. In this process the mRNA gets reverse transcribed into cDNA so that it can be then used for the PCR.

Materials

Tab. 1: Composition of the sample for the reverse transcriptase: The ingredients of the Master Mix in italic.

Component	Volume	Final concentration
25mM $MgCl_2$ Solution	4µl	5mM
10X PCR Buffer II	2µl	1X
DEPC Treated DI Water	2µl*	
dGTP	2µl	1mM
dATP	2µl	1mM
dTTP	2µl	1mM
dCTP	2µl	1mM
RNase Inhibitor	1µl	1U/µl
MuLV Reverse Transcriptase	1µl	2.5U/µl
Random Hexamers	1µl	2.5µM
Sample	1µl*	0.5ng
Total	**20µl**	

* The amount of water or sample in the mix can vary as long as the end concentration is 20µl.

Procedure

After the amount of mRNA in the sample is measured it can be calculated how much of my sample I have to add in order to get an end concentration of 0.5µg of mRNA in the final mix.

It is important to mix the four nucleotides before adding them in order to make sure that there is the same amount of each nucleotide everywhere in the solution. When everything is pipetted together start with the water, then the Mastermix and

at the end the sample is added. The samples are then spun down and are put into the machine for the RT:

The RT will run one cycle where the steps are: 15 minutes at 42°C, 5 minutes at 99°C and then 5 minutes at 5°C.

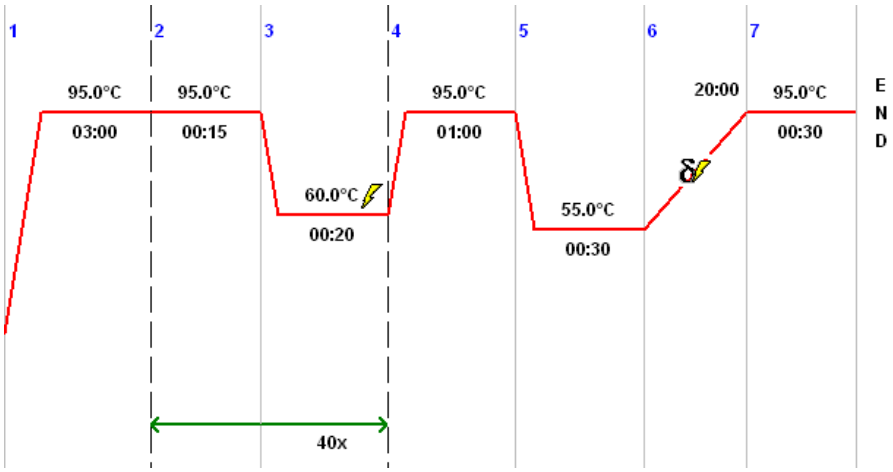
After that the mRNA in the samples have been reverse transcribed into DNA and the samples can be stored at -20°C until they are used.

PCR

After the RT is done the PCR can be started since the product to work with is cDNA. According to how many samples are used and how many genes we want to detect the Mastermix has to be prepared. It is advised to prepare for one more sample than the number of samples used in case of pipetting errors.

For each gene that is going to be detected, one Mastermix is prepared. It is important to also add a Housekeeping Gene which acts as a control. The Brilliant III Ultra-Fast SYBR® Green QPCR Master Mix was used and it comes finished. The forward primer, backward primer and the water have to be added to the mix and then 20µl of the mix and 5µl of the sample are pipetted into the 96 well plate.

The eppendorf realplex^2 Mastercycler epgradientS is then used for the PCR. The general principle behind the reaction is described above.



Pic 13: Program for the PCR. Steps 2and 3 are repeated 40 times

The result will be values of SyBr Green concentration. By comparing the values with the control it is possible to say if the gene was expressed in the sample.

Western Blot

Preparation

Tab. 2: Lower Gel: 10% Acrylamide Gel

Ingredients	**Amount**
1.5M Tris pH 8.8	2.5 ml
dH_2O	4 ml
30% Acrylamide	3.3ml
10% SDS	100µl
10% Ammonium Persulphate	100µl
TEMED	8µl
Total Amount	**10ml**

Tab. 3: Upper Gel

Ingredients	**Amount**
0.5M Tris pH 6.8	1.25 ml
dH_2O	3.2 ml
30% Acrylamide	0.5ml
10% SDS	50µl
10% Ammonium Persulphate	50µl
TEMED	5µl
Total Amount	**5ml**

Tab. 4: 10X Running Buffer

Ingredients	**Amount**
Tris Base	30.3g (per mole)
Glycine	144.2g (per mole)
dH_2O	Up to 1L
Total Amount	**1L**

Tab. 5: 1X Electrophoresis Buffer

Ingredients	**Amount**
10X running Buffer	100ml
10% SDS	10ml
dH_2O	Up to 1L
Total Amount	**1L**

Tab. 6: Transfer Buffer: 1X Nitrocellulose Buffer

Ingredients	**Amount**
10X running Buffer	100ml
Methanol	200ml
dH_2O	Up to 1L
Total Amount	**1L**

Tab. 7: TBST

Ingredients	**Amount**
5M NaCl	30 ml
1M Tris pH 7.4	20 ml
Tween	1 ml
dH_2O	Up to 1L
Total Amount	**1L**

Procedure

The first step is to make a gel with the correct percentage of acrylamide. The amount of acrylamide that has to be used depends in the protein for which the Western Blot is done.

Prepare samples with the concentrations that need to be tested and add the loading dye in the ratio 1:4

Boils the samples for 5 minutes to break open the bonds and also to defold the proteins so that now they can be sorted according to size and not depend on the folding.

Load the gel and always use 5µl of marker on each gel. It is important that there is the same amount of sample in each pocket in which there is the same amount needed. It is not that important that there is the given amount just make sure that there is the same amount of sample in the pockets.

Make sure that there is enough buffer in the camber, by looking at the markings on the electrophoresis chamber, so that the gel will not dry out during electrophoresis.

Start the electrophoresis (150V) for about 1.5 hours. The procedure has to be observed to make sure that the visible blue line reaches the bottom of the gel. When the voltage is applied small bubbles will surface. This means that the procedure is starting.

When the gels are the sponges, the paper and the membrane are soaked in the buffer for the transfer step and are then put into the holders for the Western Blot in following order:

Sponge – 2 papers – membrane – Gel – 2 papers – Sponge

It is important to start form the white side to the black side because the proteins will travel from the white to black side along the current.

When the holders are in the chamber there has to be enough buffer added to make sure that the whole procedure can run without the membrane drying out.

Since the process is generating heat, the chamber can be put in the fridge to avoid overheating. The chamber is then connected to a power source and it will run for 1 hour at 90V.

After that the membrane is put in blocking solution for one hour. It is important to mark the membrane so that it is known on which side the proteins are.

Prepare the primary antibody and apply it to the membrane and put it on the rolling machine (membranes in 50 ml falcon tubes) and leave it at 4 degrees over night

Next day pour off the primary antibody (can be reused) and wash the membrane with TBST three times.

Apply the secondary antibody in the right concentration and keep on rolling machine for one hour in dark when using fluorescent antibodies.

Wash again with TBST and take pictures of the membrane using a membrane scanner.

Cell Culture

Cell Splitting

Cell splitting is a procedure which is used that the cells are not getting too dense. If the cells would get too dense it would change the properties of the cells and how they behave to certain treatments.

Start by checking that the cells are 75% confluent, take off the media and add Trypsin. Trypsin is used to detach cells from the bottom of the flask by cleaving proteins that make the cells attach to the surface of the cell culture flask.

The flask is incubated until the majority of the cells are detached. This can be observed under the microscope. The detached cells look like spheres and are floating around when the flask is moved.

Add medium to counteract the Trypsin. If the Trypsin is not neutralized it will kill the cells so it is important that the cells are not in contact with them too long. Add enough media to split the cells in a certain ratio.

Mix well to make sure that the cells are evenly distributed in the media and then split distribute the cells into new flasks or plates and label them correctly consisting of the patch number and the date.

Put them into the incubator at 37°C and 5% CO_2.

The cells were used until they reached patch 5 because at that age they are losing the A_{2A} receptor.

Cell Starving

This is done by leaving the cells in a media without serum. Cell starving is done to get all of them to the same stage. This can also lead to the sensitizing of the cells if the cells are starved before treatment is started.

Take of the medium and add the same amount of starving medium to the cells. Let it incubate for 24 hours in the incubator. When adding the treatment afterwards do not change back to normal medium because that would be the biggest influence on the cells and the treatment would not take much effect in the cells.

Cell Thawing

Cells are kept in liquid nitrogen in order to keep them from proliferating and growing. To be able to work with them they have to be thawed and put into medium. As long as they are in liquid nitrogen the cells are kept in DMSO so it is important to thaw the cells quickly and re-suspend them in pre-warmed medium.

Incubate the cells over night at 37°C and 5% CO_2. The medium has to be changed on the next day in order to get rid of the DMSO.

Immunocytochemistry

Methods

Since the method that was normally applied in the lab did not work properly, different kinds of deviations of this method were used.

Method 1

When the cells reach 75% confluence they are split and plated into 12 well plates. Before that cover slides are washed in 70% EtOH and put into the wells of the 12 well plate.

The cells are incubated until they reach 75% confluence again which takes approximately 48 hours.

When the desired confluence is reached the media is changed and part of the cells are starved (as described before).

They are incubated for another 24 hours and after that CGS is added for 0-1-2-24 hours.

When the cells are ready, start by washing the wells once with PBS. After that use 4% Paraformaldehyde (PFA) to fix the cells. Leave it on for 15 minutes at room temperature. This step is done in order to fix the cells onto the slide and to make sure that the antibody has free access to its antigen on the slide by immobilizing the antigens and while keeping the authentic cellular and sub cellular architecture and therefore allowing the antibody to access all cells and sub cellular compartments.

Wash with PBS and continue by blocking and making the cells permeable by using 0.01% Trition X-100 plus BSA and PBS. Leave this solution on the slides for 90 minutes at room temperature.

Take off the blocking solution and add the desired concentration of the primary Antibody.

Leave it on overnight at 4°C in a humid chamber to make sure that the cells do not dry out.

Next day wash again with PBS and incubate with according secondary antibody for one hour at 37°C. It is important to work in dark environment if a fluorescent secondary antibody is use in order to avoid bleaching.

Put one drop of Flouroshield onto the cover slip and put the cover slide upside down onto it. The Flouroshield already has DAPI in it which is a reagent that stains the nuclei of the cells. Use one drop of Crazyglue on each side to fix it. The slides can now be stored at 4°C for later microscopy.

Method 2

Cells are coated in 0.1% Gelatin in dH_2O. As this is done on the bench the gelatin solution has to be filtered in the hood in case to avoid contamination of the cells. Apply 400µl to the wells and let it incubate for 10 minutes at room temperature. Take off the coating solution and let it air dry for 15 minutes. After that the slide can be stored at room temperature until they are used.

Dissolve 2g of PFA in 100ml PBA and add 100µl of 10mM NaOH and when the powder is dissolved add 100µl of 11.6 HCl to adjust the pH to about 7.4 at the end.

The PFA solution can be stored at 4 degrees in the fridge.

Start by washing the cells once with PBS (by Life technologies) for 5 minutes. After that replace the PBS with PFA and leave it on for 10 minutes. Continue by washing twice with PBS for 5 minutes each. The next step is to permeabilize the cells with 0.1% Triton in PBS for 1 hour and follow this by washing the cells twice with PBS for 5 minutes each.

Incubate in 1% BSAPBS for one hour at room temperature. 1% BSAPBS is done by diluting 0.1g of BSA in 10ml of PBS.

Take off the blocking reagent and apply the primary antibody in the right concentration. The 1° Antibody is diluted in 1% BSAPBS and it is kept on overnight at 4 degrees in a humidified chamber so that the cells do not dry out.

On the next day wash three times in PBS for 5 minutes each and in the meantime thaw the secondary antibody.

Dilute the 2° AB in the right concentration in PBS and apply 300µl to each well and let it incubate for a minimum of 30 minutes at room temperature. Starting here the rest of the procedure has to be done in the dark in order to avoid bleaching of the secondary antibody.

Wash the cells 3 times in PBS for 5 minutes each. Follow this by applying Flouroshield containing DAPI and apply a cover slip and seal it off by using crazyclue. DAPI is used to stain the nuclei of the cells.

Method 3

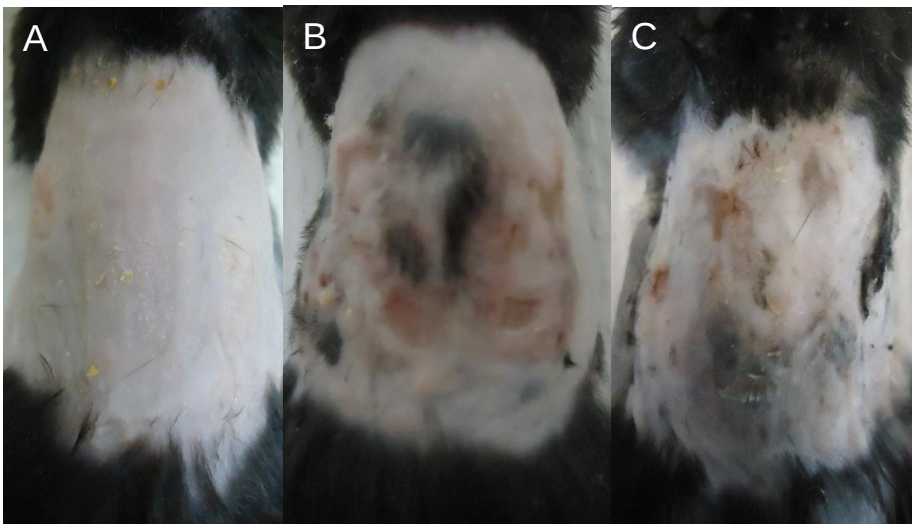
The main part of the method was the same as method 1 except that there was no Triton used for the blocking step.

RESULTS AND DISCUSSION

Animal Experiments

This experiment was based on another one which has already been done before I joined the lab. In that experiment they showed that the application of ZM, which is an antagonist of the A_{2A} receptor, leads to a decrease in scar size and enhances the tensile strength of the scar.

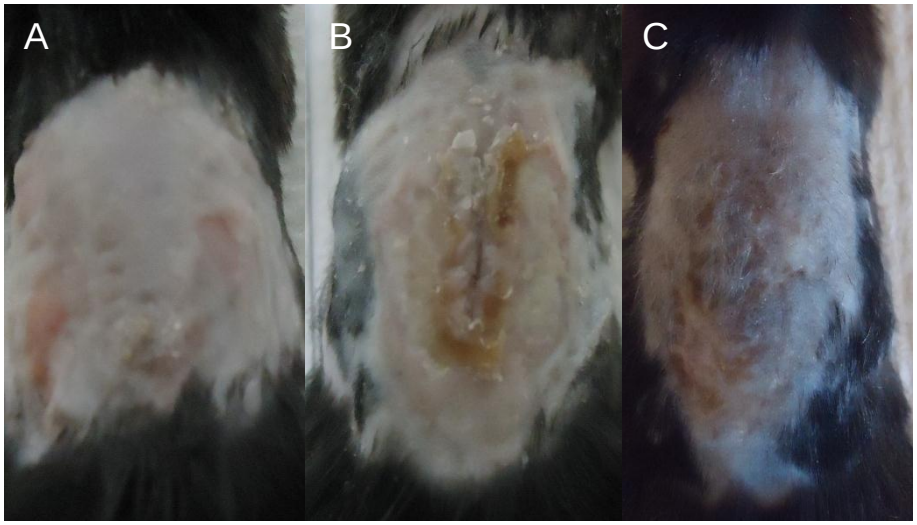
A3



Pic 14: KO, non-radiated, vehicle:

A: 9 days after radiation; B: 17 days after radiation; C: 28 days after radiation

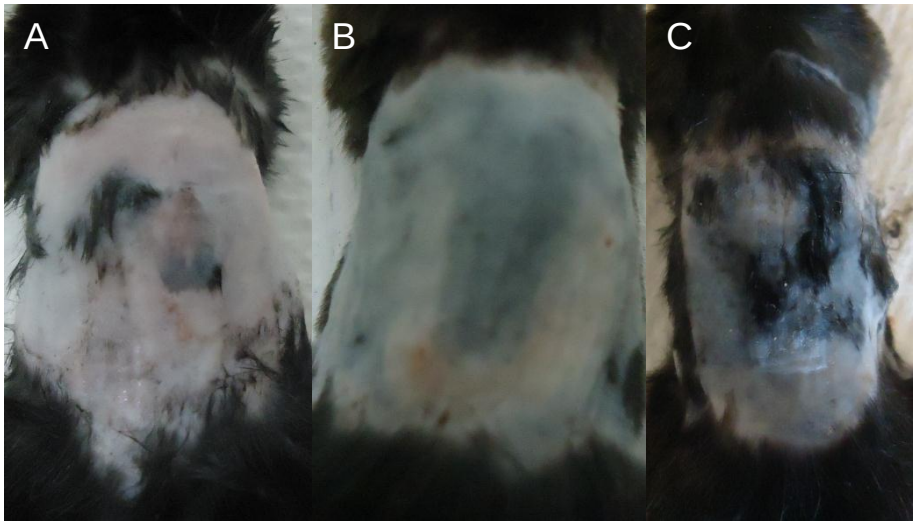
Even though these animals have not been radiated they still seem to have to have some kind of scar formation.



Pic 15: KO, radiated, vehicle:

A: 9 days after radiation; B: 17 days after radiation; C: 28 days after radiation

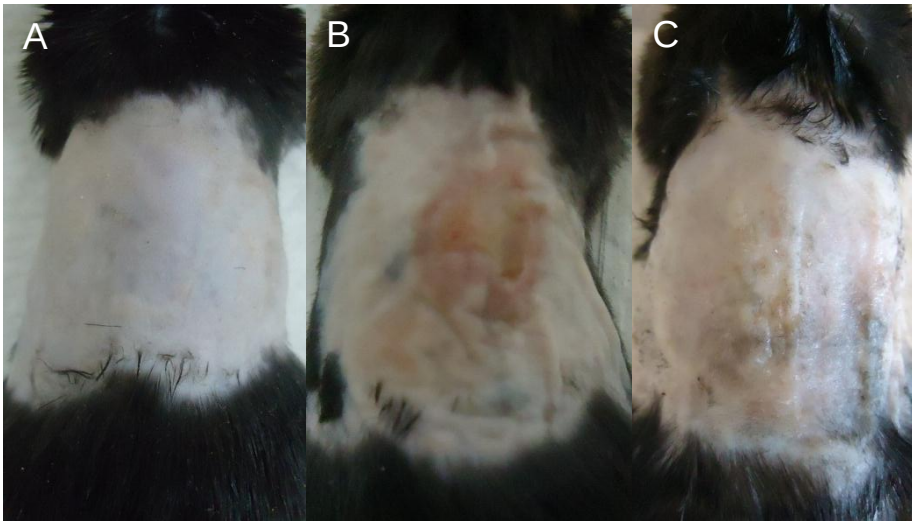
It can be seen that the wound has healed between day 17 and day 28. It can also be observed that the area that has been radiated is still free of fur after 28 days.



Pic 16: KO, non-radiated, ZM:

A: 9 days after radiation; B: 17 days after radiation; C: 28 days after radiation

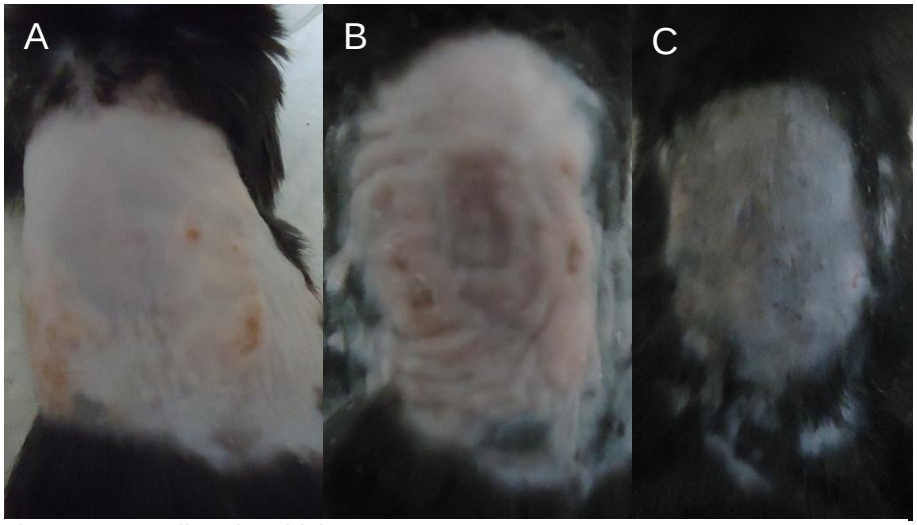
Even though the animals have not been radiated the skin still looks different than non radiated skin. Also the hair only grows in patches in the shaved area even though it should be growing normally.



Pic 17: KO, radiated, ZM:

A: 9 days after radiation; B: 17 days after radiation; C: 28 days after radiation

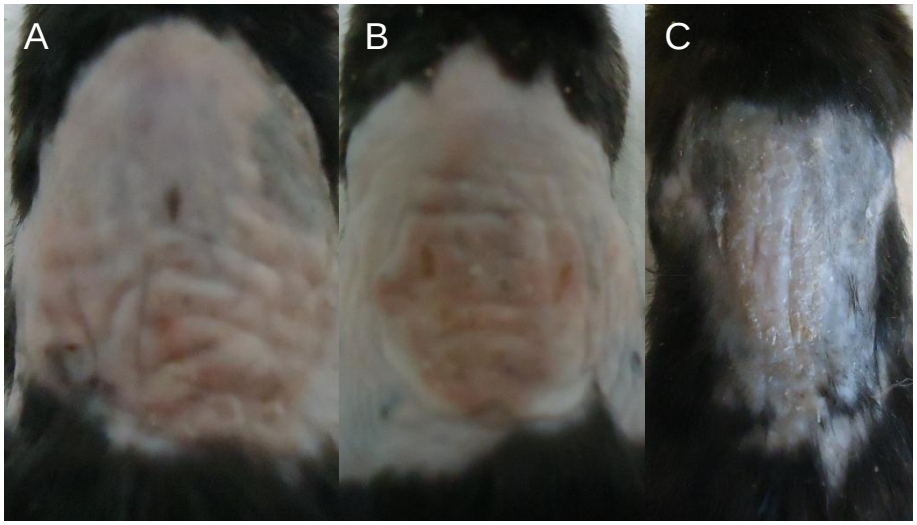
In these pictures it looks like the wound gets better between day 17 and day 28. In Pic 17C the wound looks like it is nearly healed.



Pic 18: WT, radiated, vehicle:

A: 9 days after radiation; B: 17 days after radiation; C: 28 days after radiation

It can be seen that in the WT animals it looks like the radiation starts to show earlier and also the hair slowly starts to grow again.



Pic 19: WT, radiated, ZM:

A: 9 days after radiation; B: 17 days after radiation; C: 28 days after radiation

The healing process can be seen clearly in these animals. The difference between Pic 19B and Pic19C can be observed and that the wound looks much more healed in Pic19C.

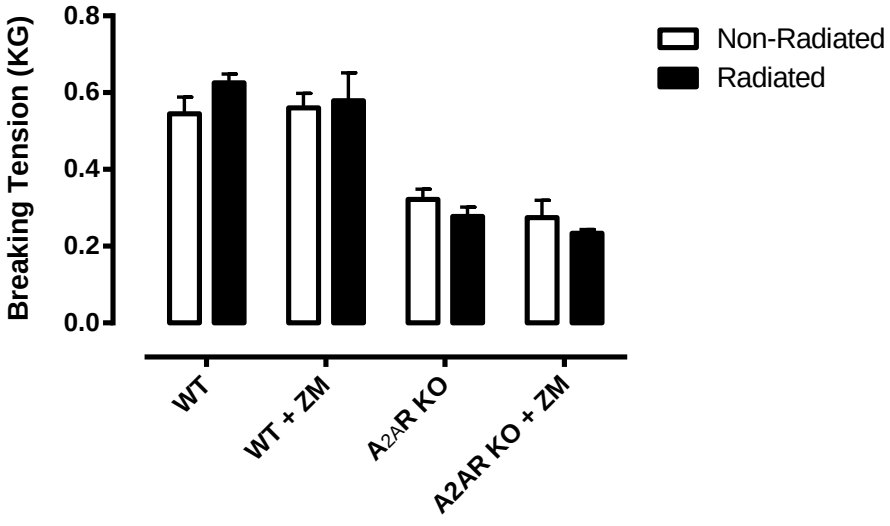


Figure 1: Breaking Tension of the WT and KO mice

It can be seen that the breaking tension is higher in the WT animals to begin with. Also that in the WT animals the breaking tension increases when radiated and in the KO animals it is the other way around.

What can be observed in Figure 1 is that the breaking tension of the scar in the WT mice generally was much higher than in KO mice. Also the results may indicate that the addition of ZM to the WT radiated mice did not significantly increase the breaking tension of the skin. On the other hand the radiated WT mice had a slightly higher breaking tension than the non-radiated ones.

With the KOs I looks as though the addition of ZM to the radiated mice decreased the breaking tension slightly. But the non-radiated KO mice that were not treated with ZM had a slightly higher breaking tension.

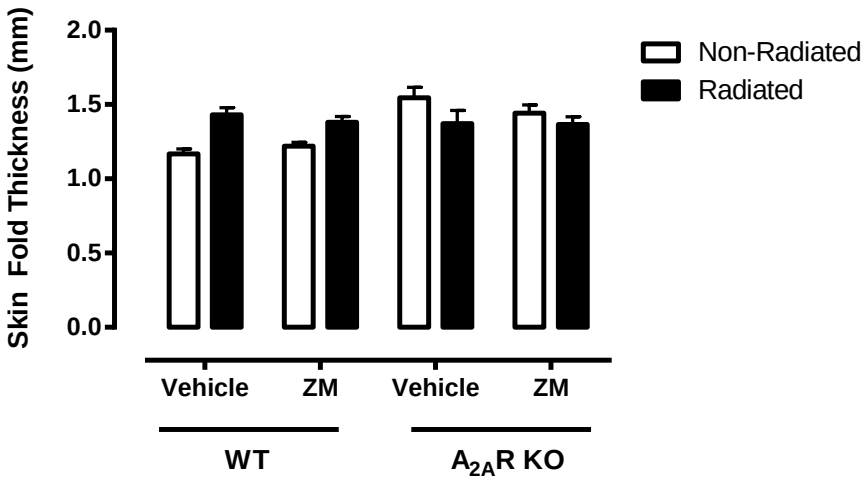


Figure 2: Skin Thickness WT and KO detail

It can be seen that the skin thickness in the radiated animals is higher in the WT animals and the other way around in the KO mice. This correlates with the results seen Figure 1.

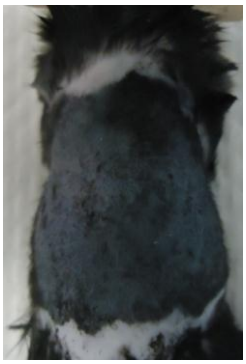
The skin thickness in the WT animals was slightly higher in the mice that had been radiated than in the ones that had not been radiated as shown in Figure 2. This explains why the breaking tension of the scars in these animals was higher.

In the KOs on the other hand the skin thickness of the animals that had been radiated is lower and also the breaking tension is lower.

When looking at the pictures is can be seen that the first group, in Pic. 14, behaved strange. These KO have not been radiated and also just treated with the vehicle substance and they still look like they have been radiated. On the other hand when looking at Pic. 15 and Pic.17 it can be seen that the scar looks much better in the animals which were treated with ZM which we expected.

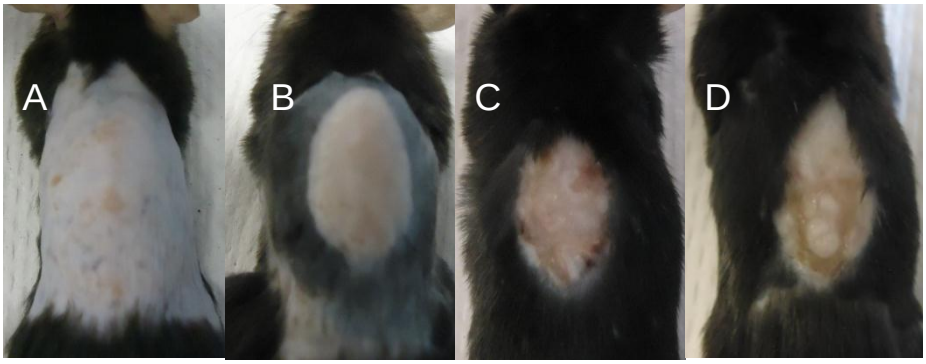
Since the KO animals in general acted not like expected there will be another study in the future where the skin of a non-radiated non treated animal will be biopsied to find out if there is something wrong with the skin of the KOs in general that has nothing to do with the radiation.

A4



Pic 20: Non-radiated; sacrificed after 2 weeks

It can be seen that the fur is growing back.



Pic 211: Radiated WT mice

A: 1 week after radiation; B: 2 weeks after radiation; C: 3 weeks after radiation; D: 4 weeks after radiation

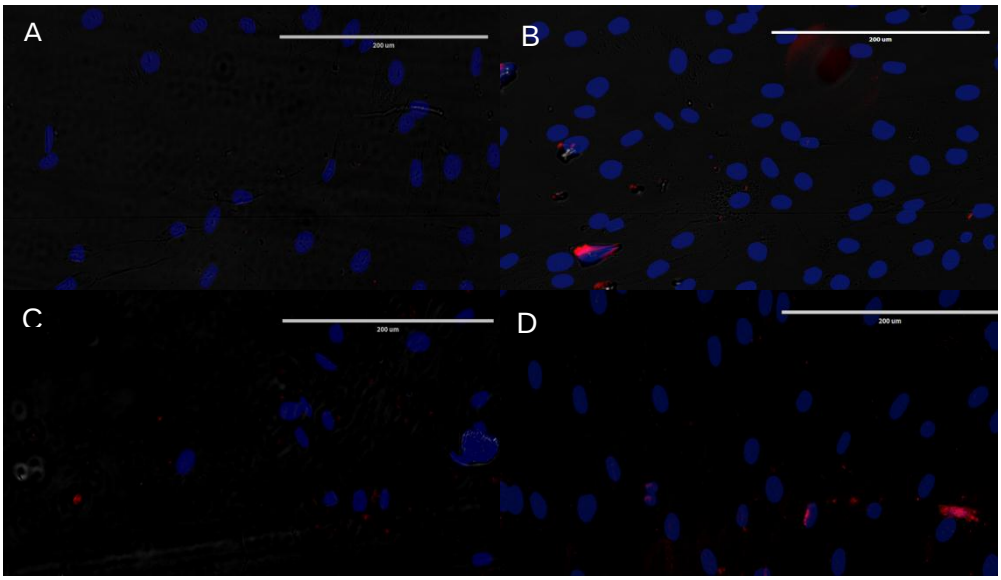
The radiated area can easily be seen since there is no hair growth and also the wound formation can easily be observed.

The aim of this study was to find out how the radiation is affecting the skin if it is not treated. As it can be seen in Pic.21 the area which has been radiated is easy to see and also the formation of the scar can be observed in this time course. It can be observed that there is no more hair growth on the affected area and also that the skin is forming scars. The sections that have been taken from the wounded skin should have been used for an HPLC but unfortunately we were not able to finish this experiment due to hurricane Sandy.

Cells

Immunocytochemistry

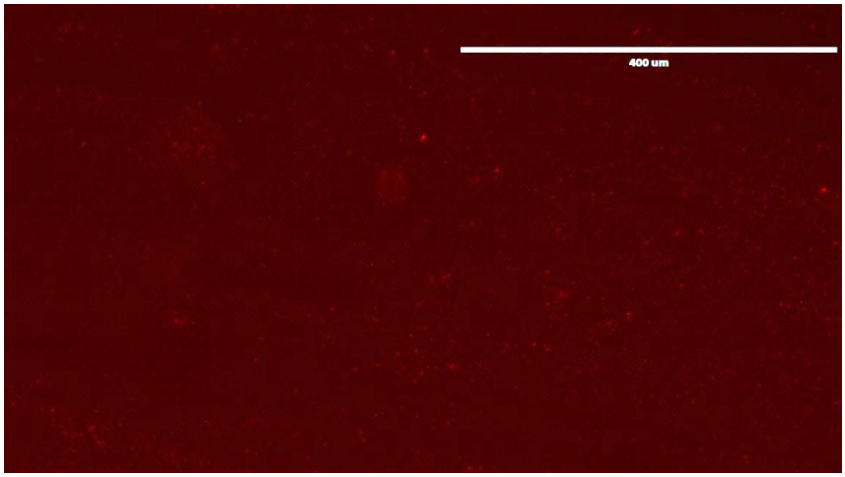
Method 1



Pic 22: The cells were stained with β catenin.

A: Control without 1°AB; B: No CGS added; C: CGS added for 2 hours; D: TGF-beta added for 2 hours

There seems to be no staining due to the primary antibody. The only things stained were the nuclei which have been stained by the DAPI flouro shield.



Pic 23: β-catenin staining.

CGS added for 2 hours

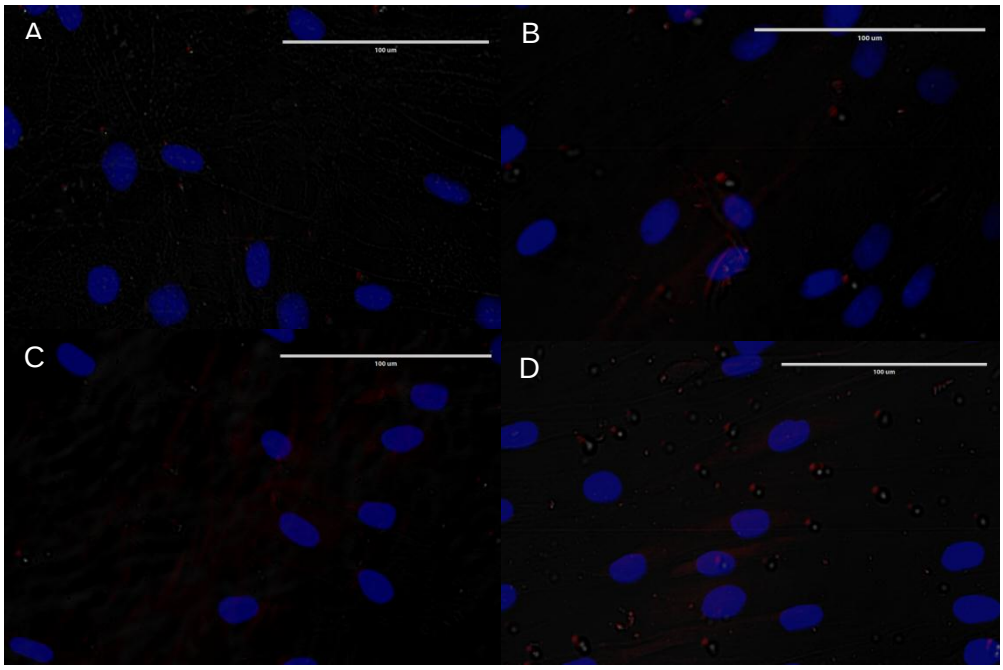
This picture shows that the secondary antibody stained everything and not specific in for the cell membrane. That means that it did not really bind to the primary antibody.

In this first method it can be observed that the staining with the primary antibody did not really work as expected. The β-catenin should stain the cell membrane and since the secondary antibody is the Alexa 594 which should give a red signal when it binds to the primary Antibody it can be seen in the picture that there was no cell specific binding.

On the other hand it can be seen in Pic.23 that there is a huge background which means that the secondary antibody stained everything and not the cells specifically like it was supposed to do.

We tried this method with different concentrations of primary and secondary antibody but the result was always the same. One of the reasons this happened could be that in the blocking step the Triton X-100 penetrates the cell wall and therefore breaks it a little bit. That is the reason why later on we tried method 3.

Method 2



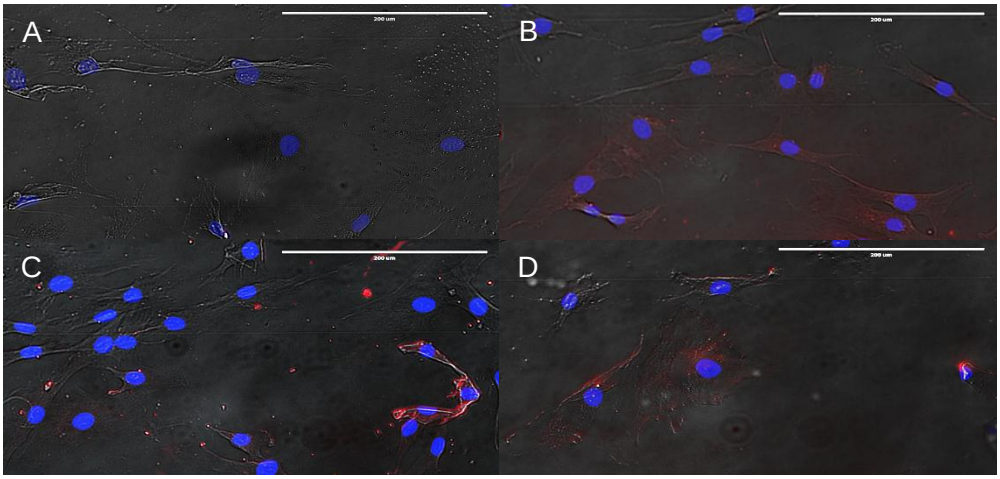
Pic 24: A: Control without 1° AB; B: no CGS added; C: CGS added for 2 hours; D: TGF-beta added for 2 hours

There is more specific binding with this method than with method 1. In Pic24B-D it can be seen that there is some red cell staining than in Pic.25. There was still a lot of background with this method.

In the second method the slides were covered with a 0.1% gelatin solution before the cells were grown on them. The idea was that some cells like to grow in the coating better than on the glass of the slide.

It did not work exactly as expected but it can be seen that in Pic.24D it looks like the primary antibody did stain the cells a little better than in the first method. But also for that method the background was really strong and it took a lot of adjusting the microscope to get pictures where the background did not shine through that much. But it can also be seen that there is really no difference between the cells that were treated with CGS and the cells without CGS. So this method was not used again since it did not show the expected results.

Method 3



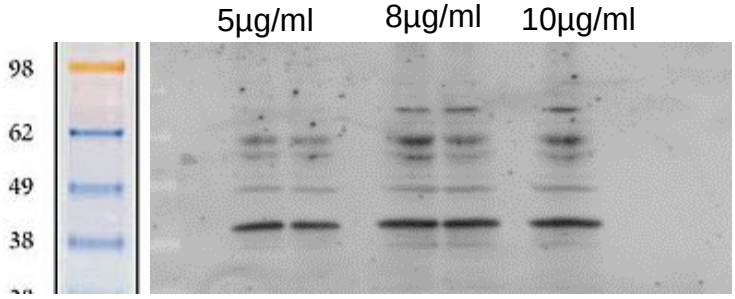
Pic 25: A: control without 1°AB; B: Without CGS; C: CGS added for 2 hours; D: TGF-beta added for 2 hours

With this method it can be observed that there is more cell specific cell staining. And also that there can be no red seen in the control, which is what was expected.

In this method it can be clearly seen that the control has no red staining and the other slides due. Even though it is very faint and there was still a lot of background this method worked the best and would have been used for further immunocytochemistry staining. It can be clearly seen in Pic.25B,C,D that the cells did get stained with the primary antibody. The difference between this method and method 1 was that in the blocking step there was no Triton used. So this could be

the reason that this method worked the best. Since the cell membrane was not penetrated by the Triton it was therefore possible to stain the cell more clearly. It looks like the staining worked better on the cells that have not been treated with CGS and the ones treated with TGF-beta. I would have liked to confirm this further but due to unforeseen circumstances in form of Hurricane Sandy I had to pause my work for two months and when I could start working again the Antibody was not useable.

Western Blot



Pic 26: WB for Actin in an unknown sample

It can be seen that the band is at the expected height of the gel according to the molecular weight of the protein that was stained for. Also the thickness of the bands changes due the amount of protein loaded.

The molecular weight of Actin is approximately 42kDa and as it can be observed in Pic.26 the lanes are in the right place so it can be said that the Western Blot worked as expected. All the samples were done in duplicates with one lane free between the different concentrations. This Western Blot was only done to train the procedure this is why an antibody like Actin was taken. Since Actin in present in all eukaryotic cells and therefore it could be expected that there would be bands on the gel.

Since the concentrations of the different samples are not very different there is not a lot change in the bands so it cannot really be said if there really is twice the amount of protein in the lanes with the concentration of 10µl/ml.

The reason that there are only these three concentrations on there is that due to the high amount of protein in the given sample it was not possible to dilute it to concentrations higher than 10µl/ml. Also, since this Western Blot was only done as a training exercise it was not that important.

This method should have been also used in other cell samples which have been treated with different concentrations of CGS and also with different antibodies. The reason that this did not happen is because of the Hurricane Sandy. The samples which would have been used for further Western Blots were ready but when the Hurricane struck they got lost when they were transferred from the lab.

Polymerase Chain Reaction

Tab. 8: Results from the nanodrop; samples 1-9 are the non-starved cells and samples S1-S9 are the cells that have been starved for 24 hours.

#	Sample	RNA concentration [ng/µl]	260/280 ratio
1	No AB / No CGS	28.5	2.13
2	No AB / CGS 10^{-6}	31.9	2.16
3	No AB / CGS 10^{-5}	45.6	2.10
4	CTGF / No CGS	43.4	2.16
5	CTGF / CGS 10^{-6}	53.0	2.11
6	CTGF / CGS 10^{-5}	39.8	2.14
7	Control / No CGS	24.6	2.14
8	Control / CGS 10^{-6}	37.0	2.05
9	Control / CGS 10^{-5}	30.5	2.15
S1	No AB / No CGS	18.6	2.25
S2	No AB / CGS 10^{-6}	23.3	2.10
S3	No AB / CGS 10^{-5}	20.1	2.17

S4	CTGF / No CGS	25.7	2.14
S5	CTGF / CGS 10^{-6}	22.6	2.18
S6	CTGF / CGS 10^{-5}	17.0	2.24
S7	Control / No CGS	18.1	2.16
S8	Control / CGS 10^{-6}	17.5	2.18
S9	Control / CGS 10^{-5}	21.5	2.15

These results show that the amount of mRNA in the samples is very small which could be normal for these cells or another reason could be that the mRNA extraction did not work properly. Since there is so little amount of mRNA in the sample we decided to use the same volume of samples for the RT and not the volume we calculated we would need in order to have the same amount or RNA in each sample. We were able to do the RT but not the PCR due to Sandy. What can be seen in Tab.8is that in general there seems to be more mRNA present in the cells that have been treated with CGS. It also looks like there is more RNS in the samples that have not been starved which was also expected due to the fact that when the cells are starved, they start to die.

CONCLUSION

It can be seen in Pic.18 and 19 and as well in Fig.1and 2 that the WT animals did behave as expected in the A3 experiment. For some reason the KO mice behaved strange. Even the ones that have not been radiated and just treated with the vehicle substance had scars in their backs and the skin looked as if it had been radiated. To make sure if that is a trait of that strain or if it was just something that happened in this experiment there is going to be a study with non-treated KO mice and their skin is going to be dissected and stained to see if they show the same characteristics.

In the A4 study the animals behaved as expected and it can easily be seen where they have been radiated. The skin is scarred and also there is no hair growth in this region. It can also be observed that the scaring of the skin gets worse over the weeks and that the animals from week 4 have significantly more scaring than the animals from week 2. Unfortunately this experiment could not be finished due to hurricane Sandy and therefore the HPLC could not be done, which would have been the last part of this study.

The three methods used for Immunocytochemistry show that even small changes in the procedure show significant differences in the results. As is can be seen between method 1 and method 3 only one reagent was changed and the results look very different. Even though method 3 does not give the results that were expected, because there was still a huge background, it can still be seen that the antibody stained the cell wall.

Due to the hurricane I was forced to take a break from work for nearly two months. And after these two months we were also not able to just continue working like before because during the storm our samples were taken to Long Island for safekeeping and also some got lost during the trip. So I had to start all over again which is also the reason why I was no able to get any real results during the last weeks.

So it can be said that these are the result from approximately three months of lab work. And for most of the experiments we were not able to finish them the way it was planned.

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