universität wien

DIPLOMARBEIT

Titel der Diplomarbeit

„Identifikation und Isolation von Cardiosphären aus dem Organismus Notophthalmus viridescens“

>1 von 1<

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Table of contents:

1. Introduction:

1.1 General:

The heart has always been given a special role in human history. It was said to be the center of emotions and in some cultures they offered it, as one of the most valuable possessions of men, to the gods.

Nowadays we know that the heart is a pump, which drives blood throughout our body and therefore is important to deliver oxygen and nutrients to all parts of the body and transport waste products out of the metabolism [1]. But even though we learn more and more about the heart and it's functions, irregularities of the functionary heart result in high mortality. Diseases of the heart have come to be the number one cause of death worldwide and the number of casualties is predicted to increase even further (WHO 2011).

Even though the heart can heal infarcted tissue, this tissue is non- functional because cardiomyocytes and smooth muscel cells are replaced with scar tissue. The inability of this scar tissue to contract consequently diminishes overall function of the heart, which can lead to heart failure and eventually death [2].

In order to compensate for the loss of tissue, mammalian hearts respond with a hypertrophic response, resulting in functional cells getting larger to cope with the overall cellular deficit [3]

The only available clinical means of replacing lost myocardium remains whole- organ transplantation. But the demand by patients with end- stage heart failure greatly exceeds the supply of suitable donor hearts. [4]

In the last few years cardiac stem cells were found which may provide a solution forr this problem. Even though there are already clinical trials on humans there are still some problems which remain like cell delivery, homing of the cells to damaged areas of the heart and electrical coupling to the host myocardium [5].

In order to overcome these problems we have utilized a model organism that has great intrinsic ability to regenerate. The organism in question is the red- spotted Newt (*Notophthalmus viridescens)*.

1.2 Introduction to the heart:

The heart is a myogenic muscular organ found in all animals, which possess a circulatory system. It is responsible for the blood flow throughout the body, which supplies all organs and tissues with oxygen as well as all the necessary nutrients.

It has been show that the heart is the first organ to get developed in early embryogenesis, where development already starts in the third week of embryogenesis [6].

In mammals the heart is formed out of the mesoderm germ- layer that, after gastrulation, then differentiates into mesothelium, endothelium and myocardium. The outer lineage of the heart is formed by the mesothelian pericardium [7].

The myocardium is the actual heart muscle that contracts and relaxes in order to pump blood through the body.

The cardiac tissue holds a group of specialised cells, called the electrical nodes [8].

The sinoatrial node is commonly referred to as the pacemaker and can be found in the wall of the right atrium of the heart. This node experiences rhythmic excitation with the impulse rapidly spreading throughout the atria leading to their contraction and the pumping of blood from the atria to the ventricles [9,10,11].

The antrioventricular node is a kind of relay station of the sinoatrial node. The antrioventricular node delays the signal of the sinoatrial node to preventing the atria and ventricle contracting at the same time [12,13].

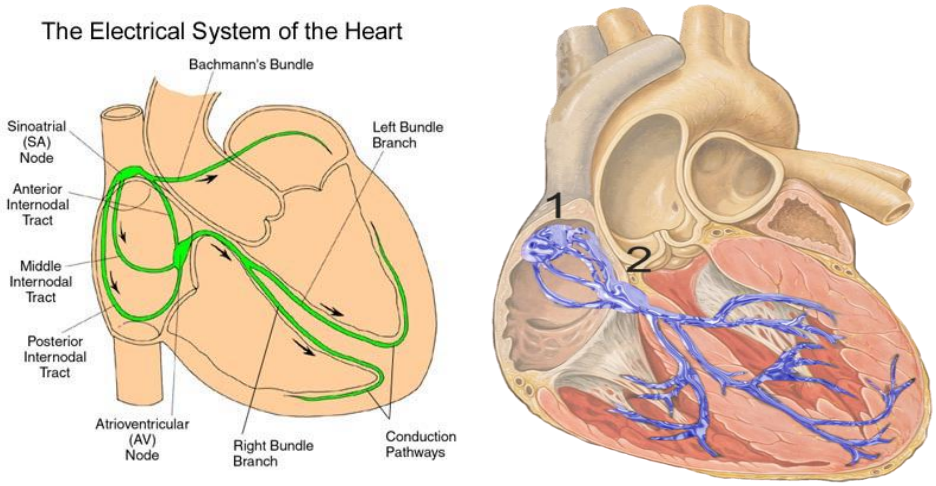

Figure 1,2: Display of electrical signal pathway in an human heart. In the right picture the number 1 indicates the sinoatrial node while number 2 shows the antrioventricular node.

The heart was long believed to be a terminally differentiated organ and therefore does not possess the ability to regenerate cardiomyocytes, which form the main power house of the heart [14]. This opinion has been challenged not so long ago. In order to do so the age of cardiomyocytes was verified through the use of C^{14} – isotopes that have integrated into the DNA. The source of this isotopes were nuclear bomb tests during the cold war after which all the produced C^{14} was taken in by many different organisms and got integrated into the DNA. [15]

1.3 The blood cycle

In mammals, deoxygenated blood enters the right atrium through the superior and the inferior vena carva from where it then is pumped into the right ventricle. The blood leaves the right ventricle through the pulmonary artery to the lung, where it is oxygenated and returned to the left atrium via the pulmonary vein.
The oxygen rich blood is pumped into the left ventrican and from there it is passed through the whole body through the aorta [16,17].
On its way the blood sustains the whole body with oxygen and nutrients while it also picks up metabolic waste products and CO_2. The metabolic waste will then be transported to specific tissues in the body where it will be processed before it is transported out of the body.
The CO_2 on the other hand will be transported back to the lungs where it will be exchanged with oxygen again at the start of a new cycle.
If the passage of the blood is blocked, for example by clots, so called thrombosis or plaques, there is a high possibility of an infarct [18].
If the blood flow is inhibited in the vessels of the heart this leads to a heart attack while the blocking of blood flow in the brain leads to a stroke. Blood flow constriction in the muscle can lead to so called muscle death (necrosis) [19].
Another very important task of the blood stream is immune response by relaying signals to the immune system that there is a threat and the following transport of lymphocytes, antibodies and other factors to the site of infection [20].

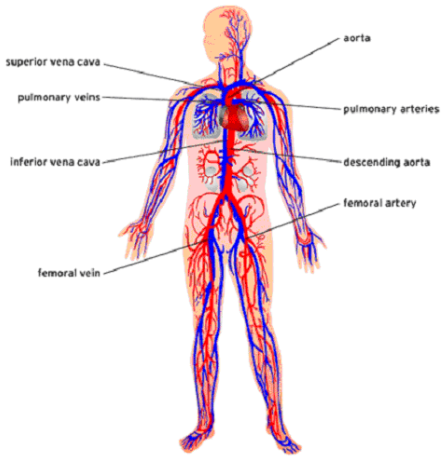

Figure 3: Rough outline of the circulatory system in humans. Red indicates the flow of oxygen rich blood while blue shows the path of the deoxygenated blood.

1.4 Different forms of vertebrate hearts:

Until now the focus was put on mammalian hearts but the morphology of the heart can vary between different vertebrate species.
Note, that figure 5 gives a rough schematic to show the differences between mammals, amphibians and fish hearts.

1.4.1 The amphibian heart:

The heart of amphibians is considered to be a three chambered system while mammals possess a 4 chambered heart. Amphibians have a heart with two atria, which enter one ventricle [21]. This fact should lead to a mixture of oxygenated and deoxygenated blood within the ventricle. However, it has been shown, that this kind of mixing process only happens in very trace amounts within the amphibic organism but this is still a matter of further scientific investigation.[52]
Another big difference between mammals and amphibians lies in the fact that the heart rate of amphibians is strongly dependant on the environmental temperature, while mammals have the ability to uphold their heart rate independent of the outer temperature [22].

1.4.2 The Fish heart:

Another form of vertebrate heart, which is worth mentioning in terms of heart regeneration, is the heart of the fish or to be more precise the heart of the Zebrafish that has become the favourite model organism of the "British heart foundation" due to its ability to regenerate up to 10% of their total heart after injury or resection as it can be seen in the top pictures of figure 4.

The zebrafish heart consists of two chambers, one atrium and one ventrical. Some scientists however suggest, that it is a four chambered system also including sinus venosus and bulbus arteriosus while others consider these two as vessels. However these four components, vessels or chambers, work together because they are connected in series. The heart pumps desaturated venous blood to the ventral aorta leading to the gill arches where oxygenation occurs, and from where it is distributed to the rest of the body.[23]. An big difference between the hearts of mammals and ambhibians can be found in the fact, that fish do not possess lungs but breath through gills. This leads to fish not having a cardio- pulmunary system but an altered version adepted to gills.

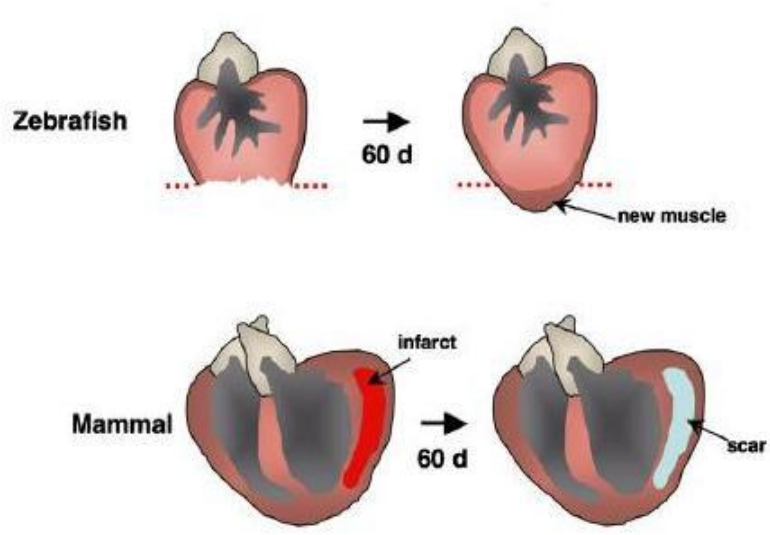

Figure 4: Difference of Zebrafish and mammals in therms of heart regeneration. While the zebrafish heart can regenerate A great part of its lost tissue the mammalian heart heals by the formation of scar tissue (indicated in gray)

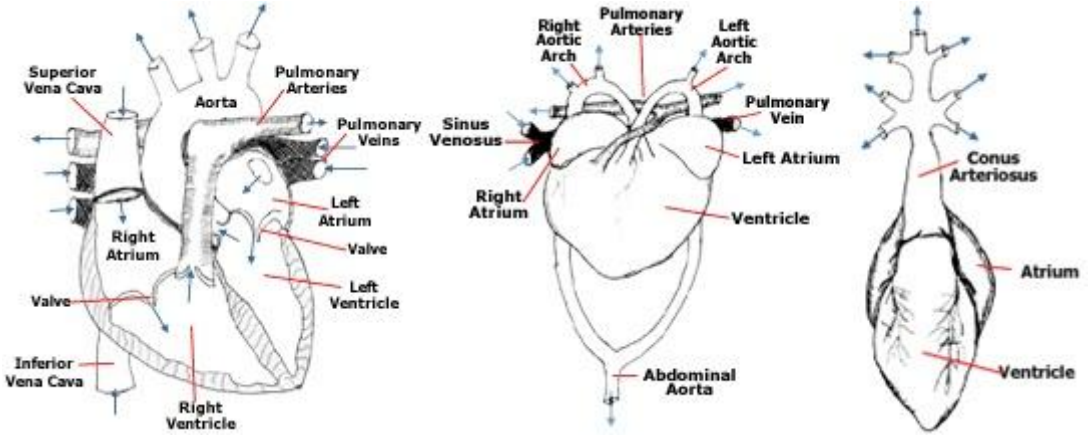

Figure 5: Comparrison between the human heart, an amphibian heart and a fish heart (from left to right)

1.5 Stem cells for cardiac repair

Stem cells are defined as unspecialized cells with the intrinsic ability to renew themselves through cell division even if they have been inactive for a longer period of time.
The two most interesting forms of stem cells regarding science are embryonic stem cells which are pluripotent and non- embryonic stem cells referred to as somatic stem cells which are either multi- or mono- potent.[54]
Somatic stem cells have only a small ability to differentiate into different cell types. Embryonic stem cells have a far greater greatest ability of differentiation and have been shown to be able to differentiate into cardiomyocytes and other cell lineages necessary for the formation of new blood vessels [25].
The problem with embryonic stem cells, beside ethical issues, is that there is a high possibility of teratoma formation, making clinical trials using these cells a highly debatable issue.
Still some pluripotent and even somatic stem cells have been shown to posses the ability to differentiate into cardiomyocytes and/or lead to neovascularisation.
Bone marrow derived stem cells have already been applied in clinical studies, as well as cardiac tissue specific stem cells. Even though some of the results were in conflict with each other, the overall outcome seemed to be quite promising.[29, 30, 31]

1.6 Cardiac stem cells:

"Cardiac myocytes have been traditionally regarded as terminally differentiated cells that adapt to increased work and compensate for disease exclusively through hypertrophy. However in the past few years, compelling evidence has accumulated suggesting that the heart has regenerative potential." [24]

Cardiac stem cells, as primarily described by Beltrami et al. in 2003, have been shown to be multipotent and supportive to cardiac regeneration. Not only are they self- renewing, they are also capable of giving rise to myocytes as well as vascular cells. [25]

Cardiac stem cells can be characterised by the use of different surface markers such as the proto- oncogene c-kit, stem cell antigen 1 (Sca-1), MDR1, Islet1 (Isl-1) [25]. These markers are not specific for cardiac stem cells but are present in haematopoietic stem cells and other cell types as well.

Cardiac stem cells however differ greatly from other stem cells due to the fact that they are $CD34^-$ and $CD45^-$. CD45 has been considered a marker of haematopoietic specification for even the most primitive stem cells [26]

Because most $c\text{-}kit^+$ cells of bone marrow origin are also CD45+, it is possible that cardiac stem cells either represent a different sub- population or that they have resided in the myocardium long enough to have lost the epitopes of the blood cell lineage [25]

By now 5 apparently different cardiac stem and/or progenitor cells have been described by different groups [27]. This changed the view of the heart as a static tissue to an organ with the highest number of tissue- specific stem cell populations.

1.7 Cardiospheres:

If heart cells are cultured under the appropriate conditions, they start to proliferate and can give rise to cardiosphere progenitor cells. The isolation of cardiopsheres from heart explants was first described by Mesina et al. in 2004.[24]

These cardiosphere progenitors appear as bright rounded cells under the microscope and seem to be situated on top of the differentiated cells that are adhered to the plate. Cardiospheres are self-adherent clusters of progenitor cells, which can be isolated approximately three days after the progenitor cells are harvested from the explant culture.

They are clonogenic, express stem and endothelial progenitor cell antigens/markers and appear to have the properties of adult cardiac stem cells [25]
Cardiospheres, both *in vitro* and *in vivo*, have the ability to differentiate into the three major specialized cell types of the heart.
After injection into the injured heart, cardiospheres exhibit multilineage differentiation and confer functional benefits [28].
If these cardiospheres are expended in monolayer culture, Cardiosphere- derived cells (CDC's) can be obtained, which then can be put to use in clinical applications regarding post- myocardial infarction [28,53]

1.8 The newt as model organism

The red spotted newt (*Notophthalmus viridescens)* is known to have outstanding intrinsic regeneration abilities and therefore seems very well suited to research the process of heart regeneration.
In general this species has a lifespan of 12- 15 years in its natural environment, which is wet forests with small lakes or ponds in eastern North America. They can reach a length of 12.5 cm from head to tail and they are able to secret a poisonous substance from their skin if threatened or injured.
The red spotted newt, goes through 3 different stages of life after it is hatched. The first step is the aquatic larva stage followed by the red eft or terrestrial juvenile state. The adult animals undergo a last stage of metermorphosis after which they return into the water like ponds and lakes to reproduce.
The most important trait of this model organism is the fact that it can regenerate as an adult, a rare trait in vertebrates.
Some of the disadvantages of this organism lie in the fact that it is not possible to get transgenic offspring and that its genome has yet to be sequenced. In regard to this disadvantages one might argue that another organism with regenerative capabilities should be used instead, like for example the Zebrafish. Still the newt seems more suitable because in terms of evolution it seems to be more closely related to humans then the Zebrafish. In regards to investigating specific markers and/or proteins, newts often share homologies with mammals, which makes it easy to apply new findings in this organisms.

1.9 Regeneration

The term regeneration is understood in many different ways, always depending on the person one is talking to.

In "*Stedman's Medical Dictionary"* regeneration is defined as the "reproduction or reconstitution of a lost or injured part" or "a form of asexual reproduction".

This definition includes a broad spectrum of natural phenomena that work under dramatically different mechanisms.[32]

Regeneration is one of the oldest fields of biology, considering, that already Aristotle wrote about this special topic. The first scientific observation concerning regeneration however were made and reported by Rene- Antoine Ferchault de Reaumur in 1712.

Due to the discovery of stem cells, and their special properties, the interest in rebuilding lost or injured tissue through bioengineering or stem cell therapies has increased a great deal in recent years and formed a new field called "regenerative medicine".

There a many different types of regeneration known to us today but their classification is still a matter of discussion to scientists working in this field. "Such classifications got their value, when well understood processes are delineated and compared, but unfortunately, many regenerative processes are so poorly understood that it is difficult to assign them to any particular category."[32, page 2 bottom].

An attempt to give a rough overview over the different forms of regeneration is given in figure 6.

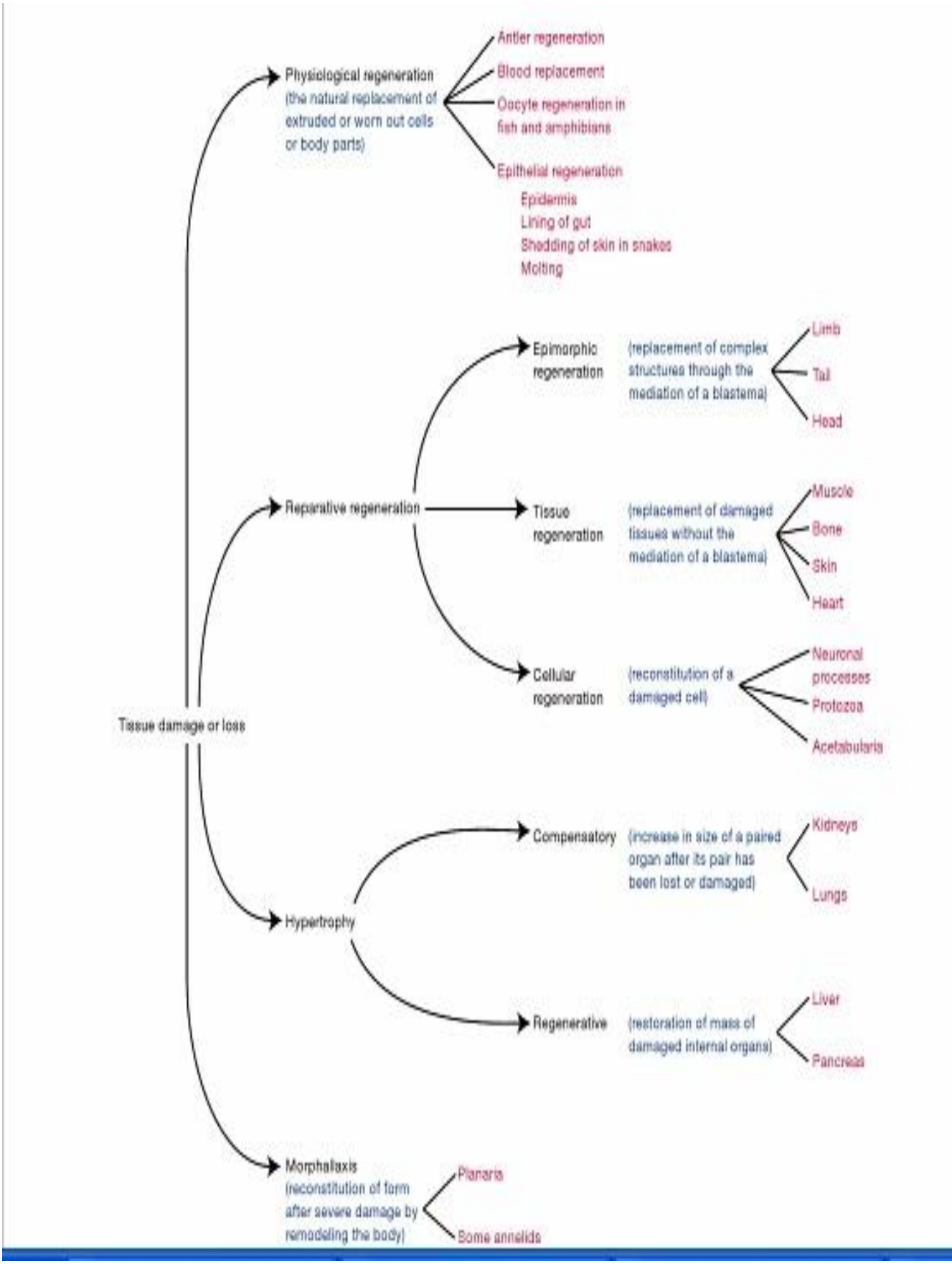

Figure 6: Diagram illustrating the major types of named regenerative phenomena [Bruce M. Carlson, Principles of regenerative Biology]

1.9.1Physiological regeneration

The term physiological regeneration describes the replacement of extruded or worn- out body parts. This process is known to occur in many different tissues all over the body and is generally discussed in terms of cell- turnover.
In mammalian physiology the best known examples of physiological regeneration are the shedding of the epidermis and the epithelial cell lining of the gut, the replacement of blood cells and the renewal of the endometrium after a menstrual period. [32]
Even though these examples involve quite different cellular processes and control mechanisms they all have one thing in common - they are all needed for the maintenance of the cellular or tissue equilibrium in the body.
A very important characteristic of physiological regeneration is the fact, that the intensity of this process can be adjusted to meet physiological needs [32]. As example one could state the increased output of red blood cells after ascent into high altitudes, which is often used by athletes before a championship.
As one can easily understand, the term physiological regeneration is not meant to define a specific type or set of mechanisms. The mechanism and the complexity of the mechanism can differ greatly between the different forms of physiological regeneration. The replacement cycle of the epidermis for example is a relatively simple mechanism, while the replacement of a deer antler, which is also a form of physiological regeneration, involves by far more complexity.
To sum up, physiological regeneration should not be considered in terms of mechanisms but in terms of the variety of processes designed to maintain the equilibrium in the body tissue.

1.9.2. Reparative regeneration:

Reparative regeneration is the term that is commonly applied to most form of post traumatic regeneration. This form of regeneration can occur at levels from single cells to major parts of the body. [32]. One of the best known and most dramatic examples of

reparative regeneration is the regeneration of a whole limb following amputation in salamanders or newts.
The term “reparative regeneration” can be sub- divided into three specific forms of regeneration, which share the common element of replacement of damaged parts of the body.
This “sub- forms” of reparative regeneration will be explained below.

1.9.2.1 Epimorphic regeneration

“The term epimorphic regeneration was coined by Thomas Hunt Morgan (1901) to refer to “cases of regeneration in which a proliferation of material precedes the development of the new part.”
Nowadays the term epimorphic regeneration is commonly applied to phenomena that are characterised by the formation of a blastema.[32].
The blastema arises through epithelial mesenchymal interactions and contains and expresses intrinsic morphogenetic signals. The best known example of epimorphic regeneration and blastema formation is the regenerating amphibian limb. Approximately 21 days after the limb was amputated from the salamander or newt, the blastema appears as a small bud like structure at the tip of the limb stump.

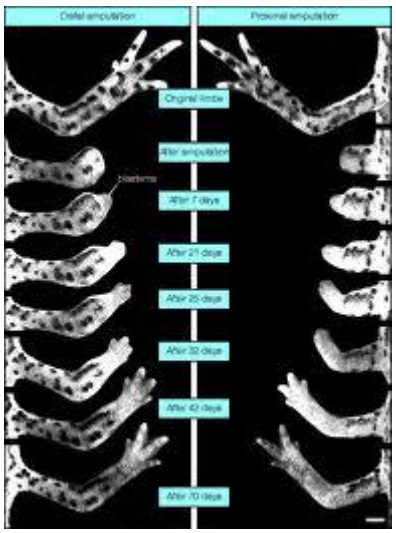

Figure 7: Regeneration of a newts limb

1.9.2.2 Tissue regeneration

The term tissue regeneration generally states a form of regeneration that does not involve the formation of a blastema. Tissue regeneration can be initiated through a variety of traumatic means, where a mechanic trauma is probably the most common example. But extremes of heat and cold and chemical insults, for example toxins, can also lead to a damage of tissue.
After the trauma an inflammatory response takes place and the nature of this inflammatory response bears a critical relation to the success of tissue regeneration. Especially the macrophage- based phase of inflammation that follows the initial acute phase has been found out to be important [33,34].
In general tissue regeneration can be considered as the intrinsic ability of an animal to heal wounds after different kinds of trauma.

1.9.2.3 Cellular Regeneration

"Cellular regeneration refers to the reconstruction of s single cell that has been traumatized" [32]. Classic examples of tissue regeneration are the reconstitution of protozo after resection and the regeneration of transected, or otherwise damaged, axons of peripheral nerves.
The topic of cellular regeneration will not be discussed any further here because it has no relevance to the project and was just stated to give a complete overview to the topic of regeneration.

1.9.3 Hypertrophy

Hypertrophy can generally be divided into two forms. The first form is compensatory hypertrophy, which indicates the increase of size of a paired internal organ, after its pair got lost or was damaged. Good examples for this kind of hypertrophy are the kidneys and the lungs.

The other form of hypertrophy is termed regenerative hypertrophy and describes the capacity of some internal organs to increase their mass after damage or partial removal of tissue to make up for a portion of the lost function.
Some of these organs also got the ability to increase their size in order to respond to increased functional demands.[32]
In contrast to other forms of regeneration, there is no replacement of the lost or damaged tissue but an increase in size of the undamaged tissue in order to compensate for the function of the lost tissue [35,36].
Hypertrophy can work in several ways. Either the level of individual cells or the internal functional unit, for example the alveoli in the lung and the nephrons in the kidney, can be increased in size (hypertrophy) or increased in number (hyperplasia) or they can do both [37]. Looking at a whole organ, the number of functional units can remain the same, but an increase in in these units can be caused by cellular proliferation within this functional unit. Additional to that an increase in fluid and/or an increase in the amount of extracellular matrix can lead to an overall increase in mass of the organ [32].

1.10 Used markers and their abriviations

1.10.1 Tyrosin kinase KIT (ckit)

This kinase, also termed CD117, is a protein found in the membrane of different cells and plays a key role in the differentiation of stem cells through the binding of *stem cell factor* (SCF). One of the best known locations of cKit are the haematopoetic stem cells, but it can also be found in tissue types. cKit plays a key role in many body functions, including angiogenesis, pigmentation of the skin and spermatogenesis.
As a proto oncogene a mutation in cKit can lead to a variety of cancers such as leukaemia, melanomas and germ cell tumours.
[38, 39]

1.10.2 Insulin gene enhancer protein (Isl1)

Isl1, better known as ISLET1, is an important factor for the neural tube motor neuron differentiation as well as a valuable marker for heart progenitor cells of the second heart

field, which forms the right ventricle as well as the outflow tract. In mouse it was shown, that a deficiency in Isl1, leads to a severely deformed heart.
[40,41,42]

1.10.3 Nkx

Nkx factors belong to the family of homeodomain transcription factors, that have been shown to be critical regulators of organ development and the tissue specific expression of genes responsible for cell differentiation.[43]
It has also been shown to be a key player in the determination of the temporal and spatial patterns of development.[55]

1.10.4 Wilms tumor protein1 (wt1)

Wt1 has an essential role in the development of the urogenital system and was found to bind a host of regulatory factors as for example p53, which is known to be a tumor suppressor [44].
On the C- terminus this protein got 4 zinc- finger motives and a proline / glutamine- rich DNA- binding domain at the N- term. The mutated form of this protein leads to the disease Wilms' tumor which is the proteins namesake.[56]

1.10.5 Cardiac troponin

Troponin is a complex of three regulatory proteins that are all involved in the contraction of skeletal and cardiac muscle. Troponin is often used as a diagnostic marker for various heart diseases [45, 46]

1.10.6 Myosine heavy chain (MHC)

MHC is a major component of the contractile machinery within the heart that provides structural integrity. Its isoform is a major determinant for contractile and functional properties of the myocardium [47, 48]

1.10.7 Smooth muscle actin (SMA)

Smooth muscle α- actin is the predominant isoform of actin found within smooth muscle cells where they participate as a part of the contractile machinery. Other isoforms like the β- actin have been found not to be involved directly with the contraction but that they are polymerising just below the plasma membrane in the presence of a contractile stimulant and thereby assist the mechanical tension [49,50]

1.11 Aims of the project

This project had the goal to find and establish a reproducible protocol for the isolation and cultivation of cardiospheres in the red spotted newt. As basis for this project we relied on two published works who were able to isolate cardiospheres from mammalian model organisms [25,51].

The first step in this process was to find a way to isolate heart stem or heart progenitor cells directly from the newt heart in a cell culture dish. This step includes the adaption of medium used by the groups mentioned above to our needs. In addition to this, other parameters needed to be determined such as, observing the right time indices for treating the heart explants, the feeding and refeeding of the explants and harvesting of cells.

The primary goal of the second part of the project was to identify the desired cardiospheres from all the different cell structures obtained in part one, as well as finding the right culture conditions and the best time points for passaging these cells or taking them for other experiments.

The last part of the project was meant to verify, that the cells we obtained really were cardiospheres. To prove that the obtained structures really are cardiospheres we decided to use two different experiments. First hand we used an immunocytochemical approach and stained the obtained structures with different markers specific for either stem cells or cardiac specific cells. In the second experiment we wanted to show that these structures we see as cardiospheres possess the ability to form the three major cell lines of the heart, which are cardiomyocytes, smooth muscle cells and epithelial cells.

Figure 8 shows the findings of other groups concerning cardiospheres and were therefore our indicator on which we measured the progress of our project and where we took hints on how well we proceeded.

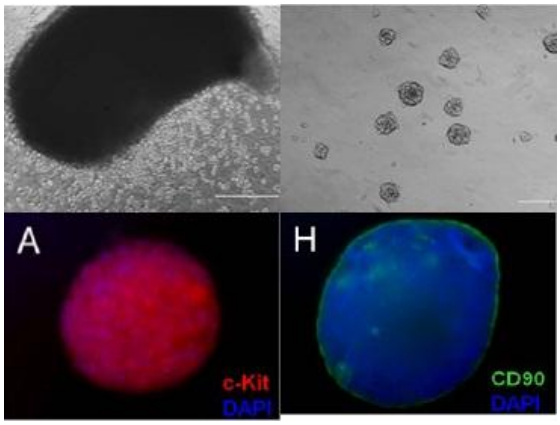

Figure 8: The letters of the different pictures merged together here were given by the groups that published this pictures and are stated here
to give an overall idea what we were looking for during our project.
The top pictures were taken from [38] and show a fragment of a mouse heart surrounded by the desired cardiac progenitor cell on the left while the picture on the right shows the desired cardiospheres.
Pictures A and H were taken from [28] and show two different stainings of cardiospheres with stem cell specific markers.

2. Materials and Methods

2.1 Animal care and sample

All experiments were performed according to European Community and local ethics committee guidelines. Adult red- spotted newts, *N. viridescens,* were supplied by Charles D. Sullivan Co., Inc. and maintained in a humidified room at 15- 20° C.
Newts were anesthetized by placing them in an aqueous solution of 0,1% ethyl 3- amino benzoate methansulfonate salt (Sigma- Aldrich) for 15- 20 min). Newts were positioned supine and an incision was made into the ventral body wall exposing the pericardium. After the thoracic cavity was exposed fully a blunted 5.0 forceps were placed under exposed aorta and lifted in order to expose the heart exteriorly [Nevin Witman et al. 2010]. The aorta of the exposed heart then was severed with an iridectomy scissor in order to remove the heart from the newt. The atria were removed with the help of the scissors and the entire ventricle was cut into four pieces within amphibian ringers solution.

2.2 Explant culture

The obtained newt heart pieces were further processed in a cell culture hood to guarantee a sterile environment, in order to prevent bacterial and fungal contamination. The pieces of the heart were transferred to 0,05% EDTA- Trypsin and cut up into even smaller pieces by a sharp scalpel. These pieces then were transferred into CEM (Cardiac Explant Medium) [see Media and Solutions] to stop the trypsinisation reaction. The obtained heart fragments were then transferred to a fibronectin coated 35mm dish with the smallest amount of medium possible, so there was a gap of approximately 1 cm between explants. To achieve that, the fragments were put on the plate in a rectangular shape as it is indicated in figure 9.

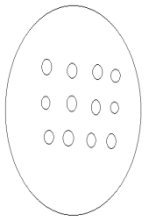

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Figure 9: Schematic of how the heart fragments should be positioned within the 35 mm dish indicated by the 12 small circles in the middle of the large circle (indicating the 35 mm dish)

After 3 hours at 25°C in an incubator with 2.0% CO_2 1 mL of fresh medium was added to the dish. The dish had to be under constant observation during this 3 hour period to prevent the expalnt fragments from drying out.
After 24 hours another 0.3 mL fresh medium were added and the dish was then let to rest in the incubator for one week so the explant fragments could settle down on the plate.
After the explants settled down the medium was changed completely three times a week.

2.3. Harvesting and culture of cardiospheres

After a three to five day incubation of the heart tissue the explant fragments were gently trypsinised and the cells were harvested.
For the trypsinisation the medium was removed from the well and collected into a 15mL falcon tube. The explant then was washed two times with A1 PBS. The A1 PBS was transferred into the same tube as the old media and 1mL of versene was added to the explant. After 2 min the versene was removed and again transferred to the tube already containing the Medium and A1 PBS. The explant fragments now were trypsinised with 0,05% Trypsin for 2 min 30 sec at room temperature before the addition of the same volume of CEM to neutralize the trypsin.
The mixture of Typsin and CEM was too transferred to the falcon tube before spinning down the cells at 300 x g for 10 min. The supernatant was carefully removed and 1 mL of CGM (Cardiosphere Growing Medium) was used to resuspend the cells before transfere to a poly-D- lysine coated 35mm dish. The cells were then incubated at 25°C and 2% CO_2 for several days.
After 7 days, the cardiospheres were passaged to new dishes. The passaging of cells also had the benefit that a good part of the old medium could be exchanged to fresh medium

without risking to lose too much of the unattached cardiosphere clusters. Due to the poly-D- lysine unwanted cell debries and already differentiated cells should have stuck down at this point and making collection of the cardiospheres in suspension much easier.
The dish was washed with the CGM it already contained, which was then transferred to a falcon tube. After the addition of fresh CGM (4mL total were obtained) the suspension was mixed by gently pipetting up and down. 2 mL were transferred to a new poly-D lysine coated 35mm dish giving two dishes total.

2.4 Differentiation Experiments

As cardiac progenitor cells, cardiospheres should be able to give rise to all 3 major cell types of the heart. Cardiospheres were transferred to fibronectin and poly-D lysine coated wells of a 24- well dish. The first two rows were fibronecin coated, the lower two wells were poly-D- Lysin coated. The first week the cardiospheres were held in CGM before changing the medium to CEM, so they got the growth factors from the FCS to start differentiation.
After the cardiospheres have stuck down and startet to differentiate the wells were stained with specific markers for the 3 different major cell types of the heart.

2.5. Cardiosphere staining

The majority of the cardiospheres do not stick down immediately, so all steps of the staining protocol had to take place under the microscope to prevent the loss of the desired cardiospheres.
The medium from an cardiosphere growing dish was transferred to a falcon tube and another 400 µL of CGM was added to get a total of 2.4mL medium. The suspension was evenly mixed by gentle pipetting and 300µL of the suspension was transferred to each well of a fibronectin coated 8-well chamber slide.
The chamber slide was then placed into the incubator to give the cardiospheres time to eventually settle down.
A list of all used solution can be found below under “Media and Solutions”

2.5.1 Fixation of cardiospheres

The next day all the medium was removed from the wells and the cardiospheres were washed once with A1 PBS before incubating them with 2% Paraformaldehyde (PFA) on ice for 20 minutes.
After aspiration of PFA the cardiospheres were washed with washing solution.

2.5.2 Permeabilization

The caridiospheres were incubated with freshly prepared permeabilization solution for 10 min at room temperature. This step was omitted when stainig for cKit, as cKit is a membrane marker.
After the removal of permeabilization solution the cardiospheres were washed three times with washing solution before an incubation with blocking solution for one hour at room temperature in an humidified chamber.

2.5.3. Incubation with primary antibody

The chosen antibodies were diluted in the blocking solution according to table shown below and kept on ice until use.
The blocking solution was aspired from cardiospheres and the antibodies were added to the designated wells.
By this time the majority of the cardiospheres had stuck down but because of the risk that they may detach again every step was still preformed under the microscope.
The cardiospheres were placed in a humidified chamber and incubated overnight at 4°C.

2.5.4 Incubation with secondary antibody

After the removal of the primary antibody solution, the cardiospheres were washed three times with A1 PBS before the adequate secondary antibody was added to the well according to the species the primary antibody was gained from. The dilutions of the different secondary antibodies can be seen in the table below.

The cardiospheres were incubated with the secondary antibody for one hour at room temperature in an humidified chamber.
After the incubation time the secondary antibody solution was aspired and the samples washed gently with A1 PBS.
The loci where the cardiospheres were sitting on the slide were marked prior to the removal of the chambers by circling them with a waterproof wax pen.
The chambers were removed according to manufacturer's protocol. The rubber which connected the chambers to the slide had to be removed with upmost care because any leftovers could have lead to an inconsistent mounting of the coverslip. The samples were mounted with Dako Flourescent mounting medium that contained To- Pro3 or DAPI.
After mounting, the slide was let to rest in the dark at 4°C overnight to guarantee that the coverslip sat firmly on the slide before analysis.

2.5.5 List of Antibodies

Primary Antibody	**Dilution**
Rabbit-α- Nkx	1 in 50
Mouse-α-Gata4	1 in 50
Rabbit-α-wt1	1 in 50
Mouse-α- Isl1	none (*)
Goat- α- cKit	1 in 50
Rabbit- α- H_3P	1 in 200
Mouse- α- MHC	1 in 200
Goat- α- Tropanin	1 in 200

(*) Antibody is in supernatant form and therefore did not have to be diluted

Secondary antibody	**Flourophor**	**Wavelength**	**Dilution**
Donky- α- Goat	Alexa Flour	488	1 in 400
Donky- α- Mouse	Alexa Flour	546	1 in 400
Donky- α- Rabbit	Alexa Flour	647	1 in 400
Goat- α- Rabbit	FITC	488	1 in 400

2.6 Microscopy

Pictures of the explant cultures and cardiosphere cultures were produced with a brightfield microscope (Zeiss) at 20X and 40X magnification with the use of Slidebook program. Pictures of stained cardiospheres were taken with a confocal microscope (Zeiss) and the program LSM 5.1.

2.7 Alternative strategies

The development of a new method, as we have done, always takes a path of trail and error before one can find the right parameters. Therefore it seems necessary to give a short overview of steps that had to be taken to reach our goal.

2.7.1 Explant culture

The first steps of the explant cultures were taken in 24- well dishes with one heart fragment per well. The heart was not cut with a scalpel but with a self-made construct using diatome blades. Due to the fact that the diatome blades could not be sterilised without getting a considerable amount of rust on them, which interfered greatly with the sterility and growth of the cultures and the problem of the construct not being as stable as we hoped it to be, we changed to using a scalpel after the first few tries.
In one of the first attempts to get cardiospheres the explant fragments were treated with collagens or remained untreated which showed to not make any great difference. The amount of cardiospheres gained by this procedure was very limited and only after the use of trypsin, instead of collagenase, we were able to harvest a satisfying amount of cardiosphere progenitor cells. Another point, which lead to a great improvement of the number of cardiospheres gained from the explant, was the change from the 24-well to a 35mm dish. The presence of other explants seems to have boosted the production of cardiosphere progenitor cells a great deal.
While in the first attempts of the project the explant fragments were transferred into a dish with the full amount of medium in it, it turned out, that the fragments seemed to adhere to the dish much faster if they were transferred in the lowest possible amount of medium.

For the medium different concentrations of FCS were tried out where we could observe, that 10% seemed to be the optimal concentration. In "Current protocols in cell biology" (Chapter 2C.3) a concentration of 20% FCS is proposed which does not seem to work for cells gained from the newt.

2.7.2 Cardiospher culture

The first cardiosphere cultures were done in a well of a 24- well dish due to the small amount of spheres obtained by the explants. After the yield of cardiospheres increased we changed to 35mm dishes and started to passage them once a week as described above. Another problem was that we took the wrong kind of cell clusters under observation at the beginning because we did not know what we had to look out for exactly.
After several weeks of observation we were able to distinguish between real cardiospheres and simple cell clusters before we proceeded with our work.

2.8 Media and Solutions

Many of the Media and solutions described in this section were diluted with 24% ddH_2O. The reason for this is that 24% water makes the solutions suitable for the use on newt cells. We generally refer to these solutions as A1 solutions.

Cardiac Explant Medium (CEM):

X mL	**I**scove's **M**odified **D**ulbecco's **M**edium (IMDM) [Sigma- Aldrich]*
10%	Fetal calf serum
24%	ddH_2O
100 U/mL	Penicillin
100 U/mL	Streptomycin
2 mmol/L	Glutamax
0,1 mmol/L	2- mercaptoethanol
1:2000	Fungozome

*The amount of IMDM should be chosen according to experimental needs. Under normal circumstances the freshly prepared CEM can be stored for around two weeks at 4°C.
The fungozome should always be added fresh to a recent amount of CEM according to need.

Cardiosphere Growing Medium (CGM):

35%	**I**scove's **M**odified **D**ulbecco's **M**edium (IMDM) [Sigma- Aldrich]
65%	DMEM- Ham's F-12 [Life Technologies]
24%	ddH_2O
2 %	B- 27 supplement [Life Technologies]
0,1 mmol/L	2- mercaptoethanol
10 ng/mL	**E**pidermal **G**rowth **F**actor (EGF) [Life Technologies]
20 ng/mL	**B**asic **F**ibroblastic **G**rowth **F**actor (bFGF) [BD Bioscience]
25 ng/mL	Cardiotrophin [Sigma- Aldrich]
5 Units	Thrombin [Sigma Aldrich]
1:2000	Fungozome

Thrombin stock solution (4µM)

33 µg of the Thrombine- powder (50 Units) were diluted in 230 µL A1 PBS and stored as 25 µL Aliquots at -20°C.
This stock then could be used in a 1:100 dilution.

Cardiotrophin stock solution (4µM):

10 µL of the cardiotrophin were diluted in 100 µL of A1 PBS to a 40 µM working solution and stored as 5 µL Aliquots at -20°C.

EGF stock solution:

10 µg of the EGF were dissolved in 1 mL of A1 PBS and stored in 20 µL Aliquots at -20°C

bFGF stock solution:

10 µg of bFGF were dissolved in 1 mL of ddH_2O and stored as 20 µL aliquots at -20°

A1 PBS:

The 10x PBS solution without magnesium and without calcium [Sigma Aldrich] had to be diluted two times in order to get the desired A1 PBS. At first the 10x solution was diluted to a 1x dilution of PBS. To make this solution suitable for the use on newt cells this 1x dilution had to be diluted again with 24% ddH_2O.

Fibronectin stock solution:

1 mg of the fibronectin was diluted in 1 mL A1 PBS and was stored in 10 µL Aliquots at -20°C

Poly- D- Lysin stock solution:

Poly- D- Lysin [Sigma Aldrich] was diluted to 1 mg/mL and stored as 1 mL aliquots at -20°C

A1 Trypsin (0,05%):

4 mL of 0,5% Trypsin were diluted in 26,4 mL of 1x PBS and 9,6 mL ddH_2O.

A1 Versene:

760 µL of versene were diluted with 240 µL ddH_2O in order to obtain A1 Versene.

Fixing solution for staining (2% PFA):

270 µL of 37% PFA were diluted in 4730µL of ddH_2O and could be kept on room temperature for at least a week

Washing solution for staining:

X mL	A1 PBS
0,1%(v/v)	Tween – 20

Permeabilization solution for staining:

X mL	A1 PBS
0,1% (v/v)	Tween- 20
0,1%(v/v)	Triton-X

Blocking solution for staining:

X mL	A1 PBS
0,1%(v/v)	Tween- 20
10%(v/v)	Swine serum

3.Results

3.1 Explant cultures

During the project we were able to find a reproduceable protocol of bringing fragments of the newt heart into culture and induce them to produce bright rounded cells. Under the light microscope the bright rounded cells did not seem different from the ones found by other groups from mammalian organisms.

One of the obstacles we had to clear in the process was to get the heart fragments to stick down to the fibronectin coated dishes. We found out, that the ability of these fragments to settle down was greatly influenced by their size. A size of 1- 1.5 mm^3 proved to give the best results. That means, fragments of this size stuck down in the shortest amount of time and also seemed more likely to remain attached during trypsinisation process.

It also appeared to be of importance to seed the heart explants in close to proximity to each other. When we seeded the heart fragments in 24- well dishes, one fragment per dish, it took the explant fragments longer to stick down and the amount of bright rounded cells obtained was very low. Furthermore, we discovered that the treating of explants with trypsin gave a better outcome of bright rounded cells then the treatment with collagenase or leaving them completely untreated.

After approximately one week we could observe, that nearly all explants did settle down and started to produce bright rounded cells even before an outgrowth could be observed. After 2- 3 weeks there was an increasing amount of outgrowth to be seen and bright rounded cells.

Some of the heart fragments kept their ability to contract up to 8 weeks after explantation.

In general the explant cultures were kept only for 5- 6 weeks in order to do 3 or 4 cardiosphere harvests. After that there were no more cells to be obtained from the explants and the risk of fungal infection also rose a great deal.
Within the last days of the project we were also able to observe that some explants seemed to form bridges between each other through their outgrowth. This finding will be a matter of further investigation by another member of the group.

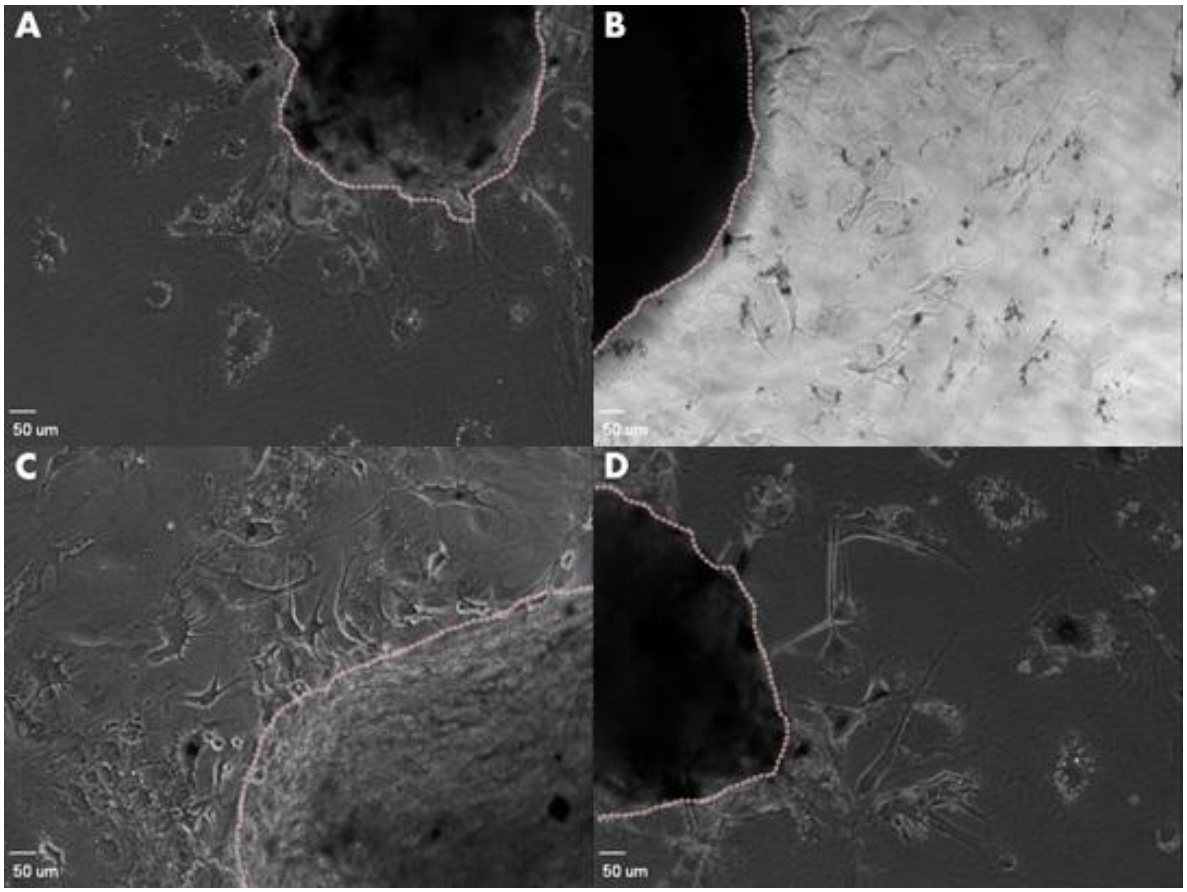


Figure 10: These pictures show heart explant fragments which were kept in a 24- well dish with one fragment per well at different timepoints.

- *A) Untreated fragment 3 weeks after it was put into culture. .Only a small amount of outgrowth can be seen and there do not seem to be any bright rounded cells*
- *B) Collagenase treated fragment 3 weeks after explantation. There is more outgrowth to be observed then in (A) but still no signs of bright rounded cells*
- *C) Collagenase treated explant 4 weeks after explantation. Outgrowth becomes more structured and very few bright rounded cells are to be observed.*

D) Untreated cell explant after 4 weeks. There is outgrowth to be seen even if it is still considerably smaller then in the treated explants. No signs of bright rounded cells

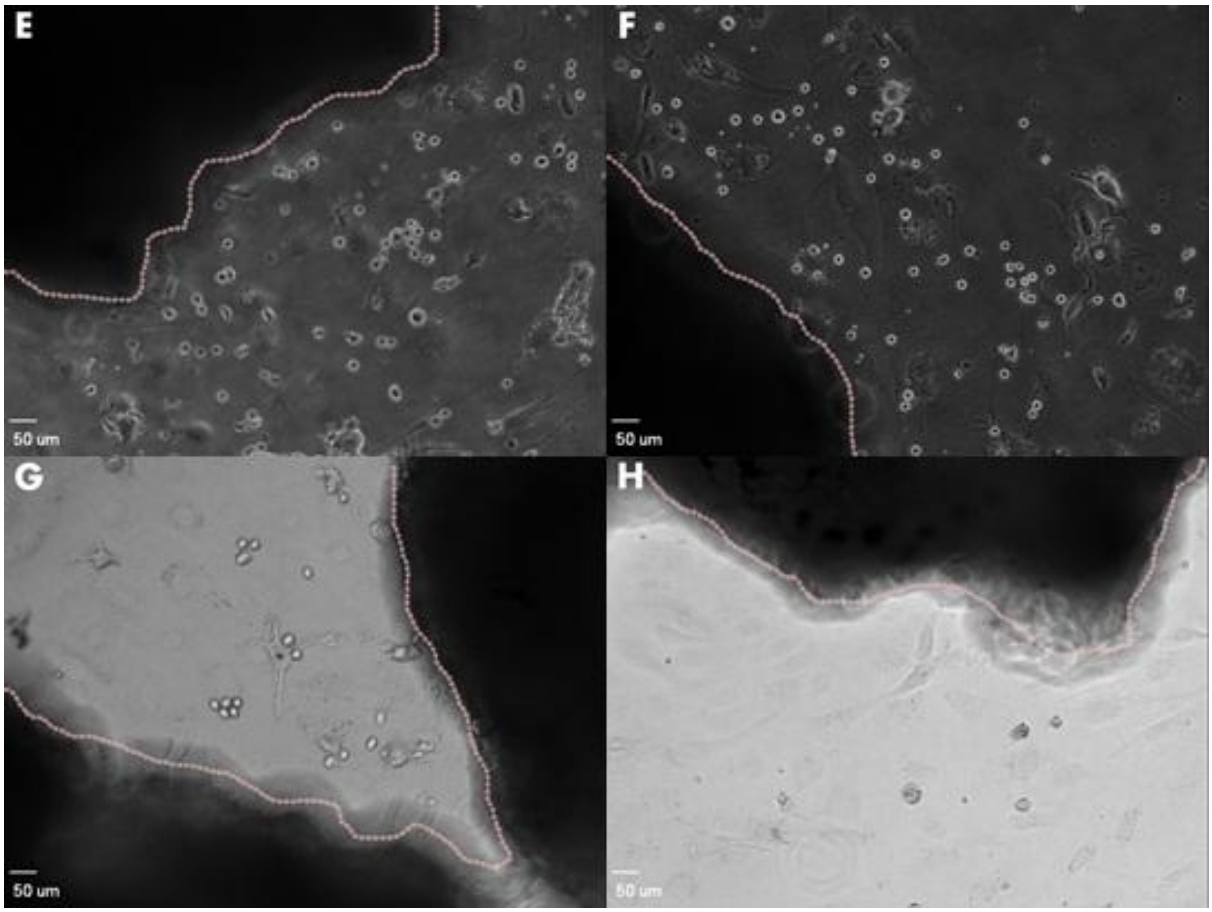


Figure 11: Different heart explant fragments of two different 35mm dishes cultured one month apart from each other. All explats were treated with trypsin prior to seeding.

E) Only little outgrowth can be observed but a recent amount of bright rounded cells can be see around the explant.

F) Another explant of the same dish shows a similar picture then (E).

G) Explant was taken one month after (E and F). While showing considerably less outgrowth there are still quite some bright rounded cells to be observed.

H) This pictures show an explant of the same 35mm dish as (G). At the bottom of the explant one can already see a round, ball like structure that could be a cardiosphere in development.

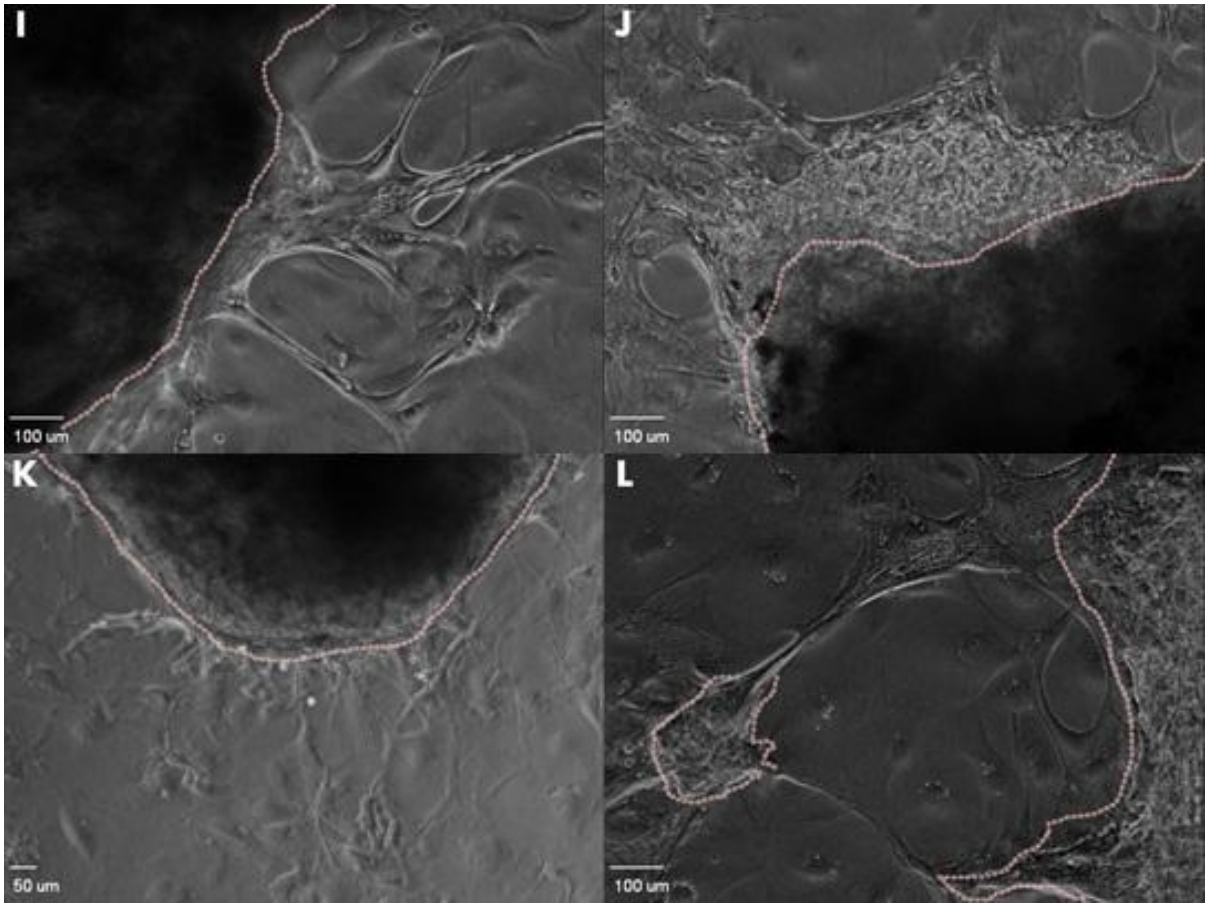


Figure 12: These explants were cultured for at least 3 weeks and already show considerable amounts of outgrowth.

While (I) and (J) seem to show nothing but the outgrowth one can observe some bright rounded cells at the bottom of explant (K) indicating, that there is still a production of these cells after 3 weeks and the first trypsinisation.

(L) shows two heart explant fragments which seem to form some kind of bridge between them. Under the microscope we were able to observe a contraction of both fragments even though this contractions were not in synch with each other.

3.2. Cardiosphere cultures

One problem we faced when harvesting the bright rounded cells and transferring them to the new culture dishes always was that the amount of cells was always too small to get a decent pellet after spinning them down. So great care was taken when removing the medium and resuspending the cells again.

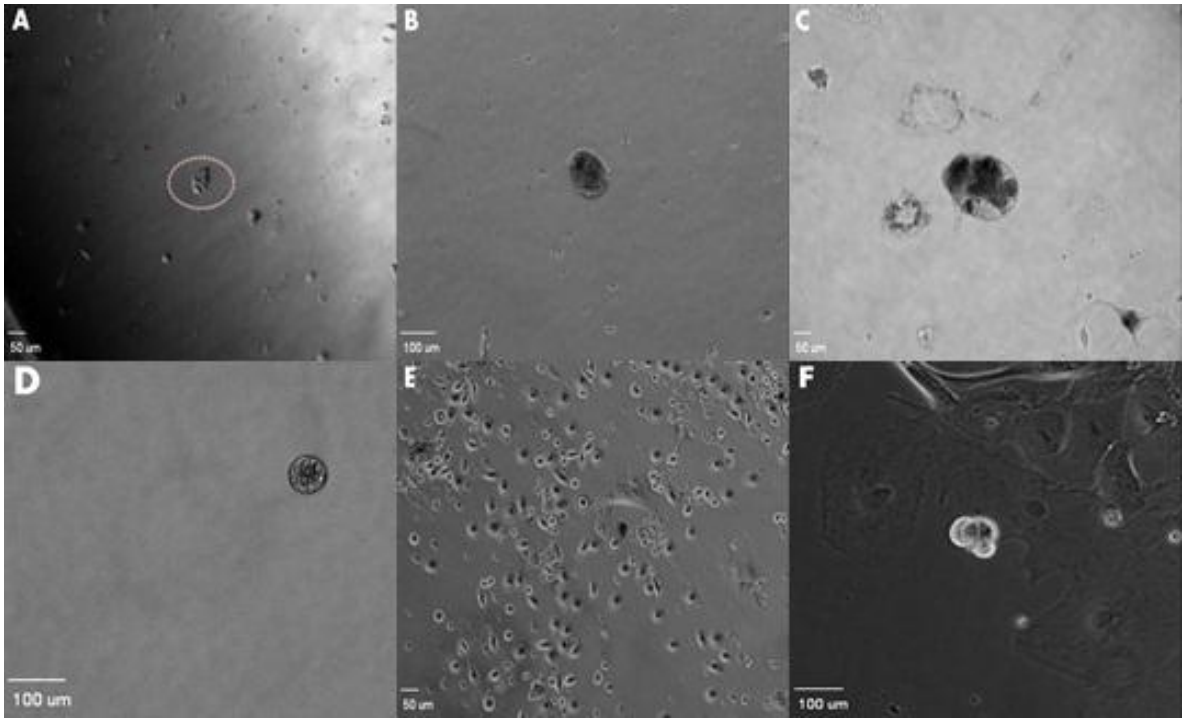
We discovered that a 35 mm dish was too large to serve our needs. The cells were at such a small concentration that they did not seem to aggregate and form clusters. After switching from a 35mm dish to one well of a 24- well plate we could observe a much quicker growth of the cells we consider to be cardiospheres and we also were able to see that these cells in average were larger than the ones obtained from 35mm dishes.

We also found, that cardiospheres derived from the newt seem to be able to stick down on poly- D- Lysine after around 3 weeks where we also could observe the onset of differentiation. In general, we can say that there is not much difference if the cardiospheres are plated out on fibronectin or poly- D- lysine. They seem to stick down on both coatings after some time and there did not seem to be a considerable time difference between the two surfaces used.

The passaging of these cardiospheres did not show the expected results. Due to the fact that we always passaged them into 35mm dishes it seems that we only diluted the cell suspension further and further without giving the cells the chance to form clusters and grow in considerable amounts.

The cell clusters observed varied greatly in size from 20 µm to around 120 µm. According to other groups, cardiospheres should have an approximate size of 50- over 200µm. It is possible that we weren't able to obtain a great number of clusters of the expected size due to the sparseness of the cardiosphere populations in the 35mm dish. In order to overcome this problem we thought about trying to cultivate cardiospheres in even smaller wells like a well of a 96 well dish for example.

We also realised, that the CGM and all its components were not strictly necessary for the growth ad proliferation of the cardiospheres, but that the simple CEM is enough to get the same results. To improve the yield of cardiospheres, the concentration of FCS might have to be adjusted by decreasing it. We already tried a concentration of 20% FCS and observed no real difference to 10% FCS medium. If anything, the outcome of this experiment with 20% FCS was slightly worse than usual.



Figures 13: These pictures represent different types of cardiospheres at different stages of their development. All this possible cardiospheres were plated out on poly- D- Lysin.

- *A) This cluster of single cells could well be the starting point for a cardiosphere. In theory the bright rounded cells from the heart explant should accongregate in such way before they form spherical structures.*
- *B) and C) represent cells or cell clusters, which appear to be cardiosphers because of their slightly round shape and their size which in both cases is around or above 100 µm*
- *D) This cluster of cells shows a nice inner structure like it was already observed by other groups and relays the impression, that this rounded structure realy is composed a lot of different cells.*
- *E) An overall look at the cardiosphere cultures, reveals, that there seem to be a whole lot of the desired rounded structures that we consider as cardiospheres. Between this rounded structers one can already see first signs of differentiating cells.*
- *F) This rounded structure with the little bubbles coming out represents a possible cardiospher which have stuck down to the dish prior to differentiation. According to other groups this is a common phenomena of cardiospheres which are starting to differentiate.*

3.3 Differentiation experiment

Due to problems with the CO_2 inlet at the incubator, the results of the differentiation experiments got adversely compromised. Another problem with the differentiation experiment was again the low concentration of cardiospheres in the cultures and the finding of the right time index of transferring the cells. It is possible that the cells or cell clusters transferred were left for to long in the previous culture and therefore lost much of their ability to differentiate.

The only time there was a clear sign of differentiating cardiospheres was seen in a 35mm cardiosphere culture dish. The differentiation of this cardiosphere was not expected for this culture conditions, but proved to us that there had to be cells with the ability to form heart structures.

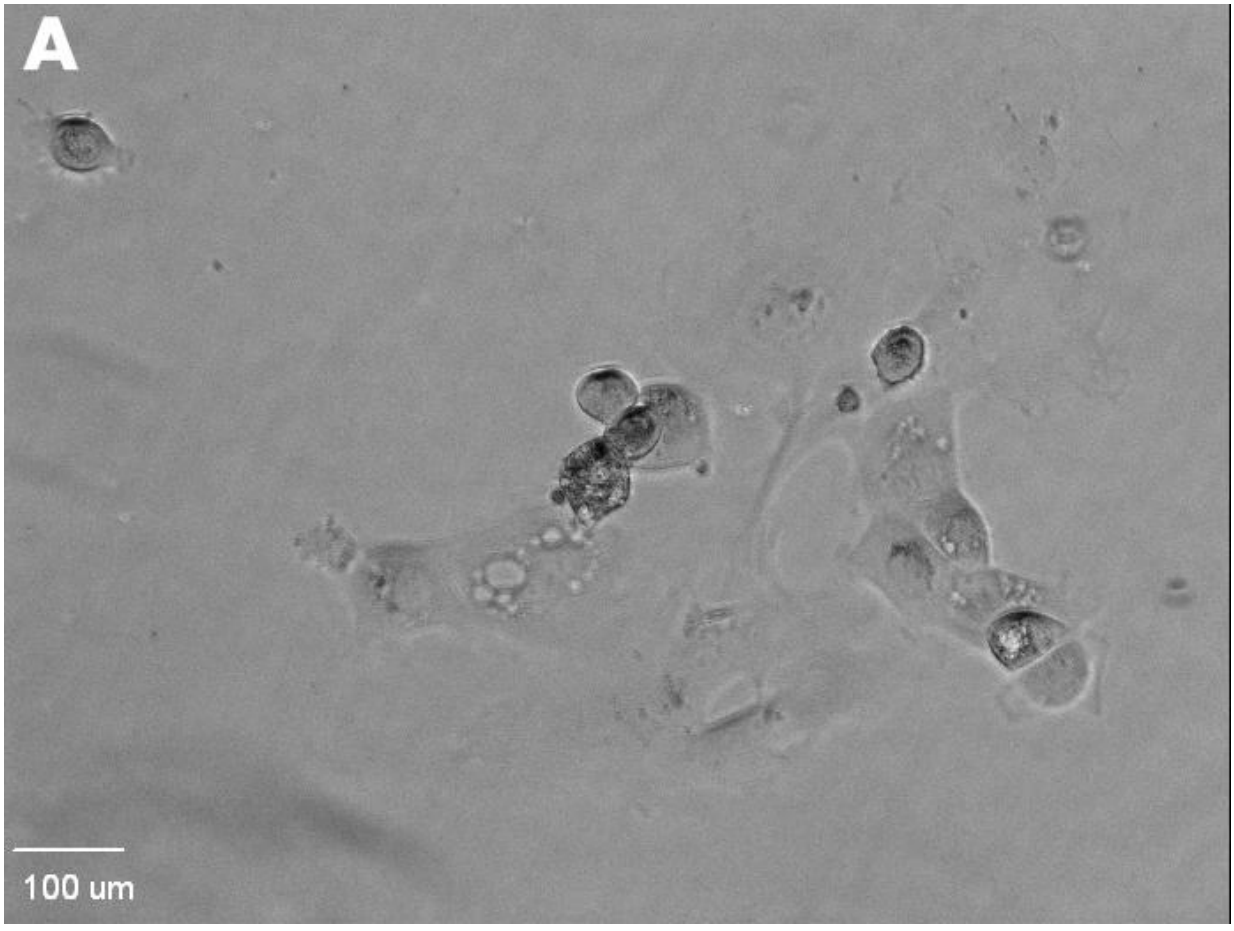


Figure 14.1: This microscope picture shows rounded structures that could have been cardiospheres next to cell structures that remind of epithelial cells.

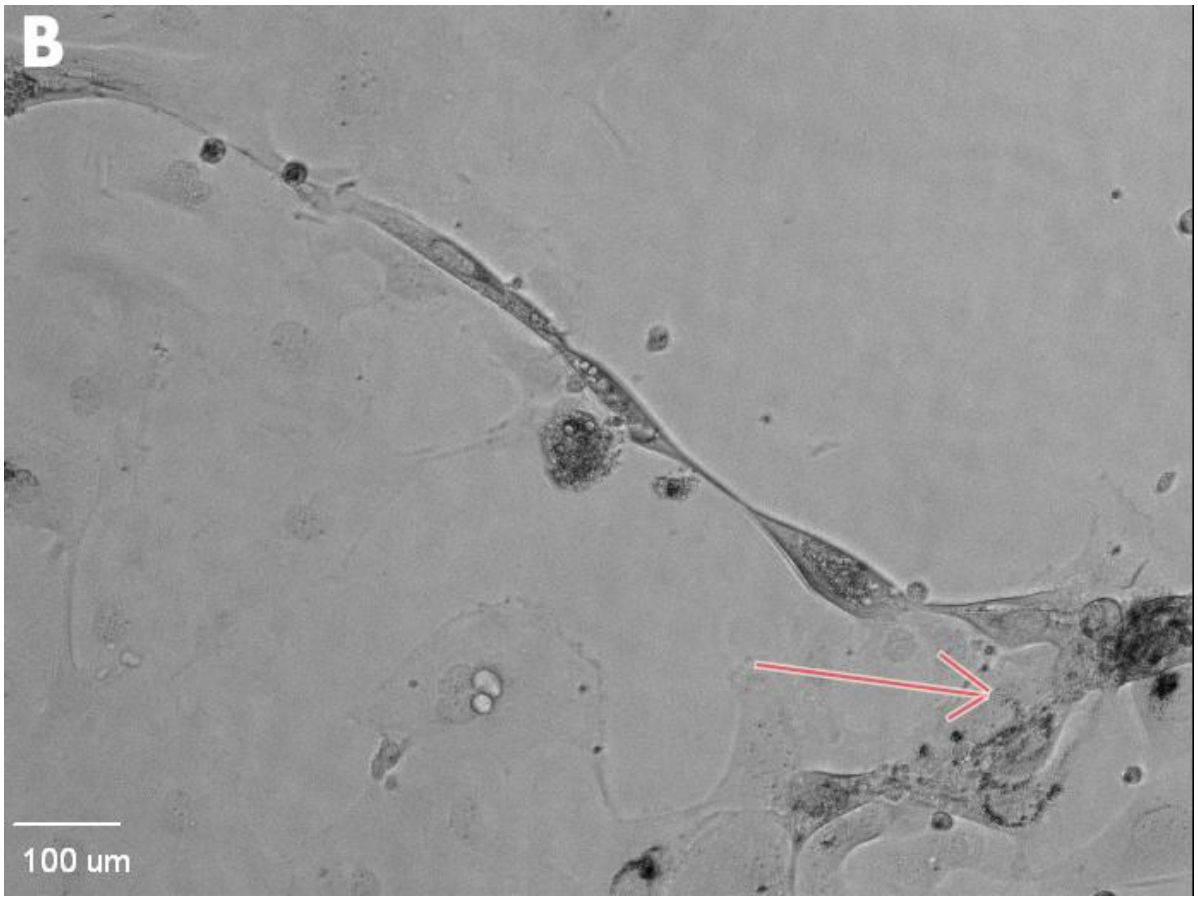


Figure 14.2: This fiber like structure got several attachment points to rounded, cardiosphere like structures. The arrow indicates a spot of this structure which was observed to contract under the microscope, indicating that this structure could be a cardiomyocyte. A video of this beating cardiomyocyte is available.

3.4. Immunocytochemistry:

The results from the immunocytochemical staining experiments were not totally conclusive. While some nice positive stains of the different markers could be obtained, the staining of the nucleus with DAPI or To- Pro gave inconsistent and not very convincing results.
Overall we observed cells or cell clusters we had stained seemed to have started to differentiate due to the fact that they were more likely to give a strong signal with markers that were specific for heart cell lines then with markers that were stem cell specific.
Each staining will be explained together with the pictures taken below.

3.4.1 Isl1 + NkX

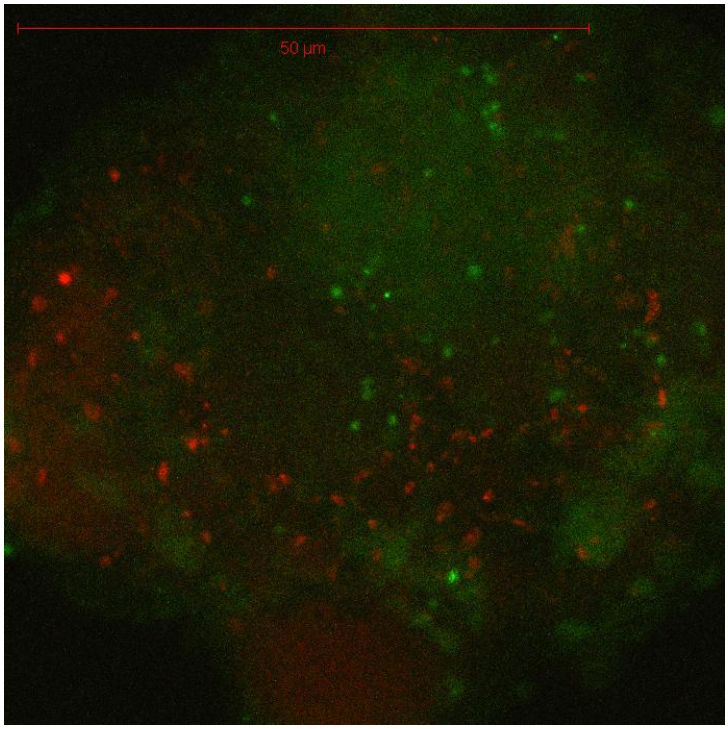


Figure 15.1: This figure shows a potential cardiosphere stained with Isl1 in red and Nkx in green. Note, that there does not seem to be any colocalisation between the Nkx and Isl1 signals. Due to the large size of the cluster observed we are quite positive to look at a real cardiosphere here. Because of problems regarding the use of To- Pro with cardiospheres we are unable to deliver nuclear stainings to this figure.

The different regions of the cardiosphere structure seem to home cells expressing either one marker or the other. There doesn't seem to be any colocalisation of the two markers in this particular cluster.
The many different signals indicate that this structure consists of a large number of cells, which is expected for cardiospheres. In contrast to many other cardiosphere like structures observed this one also shows a considerable large size (larger then 50 µm).
Even though the structure is not perfectly round, as one expects for cardiospheres, this might only be a consequence of the staining procedure. Note, that the clusters get squeezed during mounting maybe leading to a loss of form and structure.

3.4.2 GATA4 + wt1

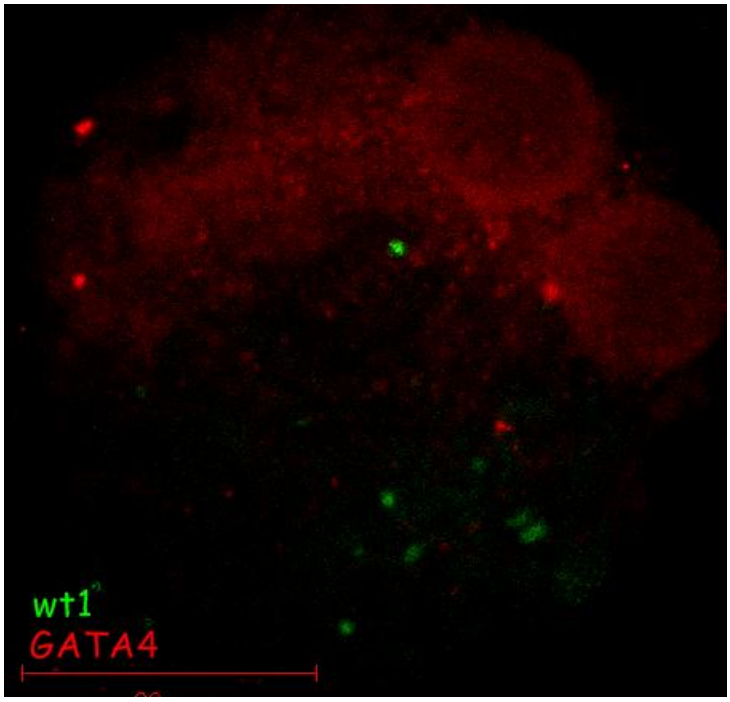


Figure 15.2: Picture of GATA4 in red and Wilms tumor protein in green. The scalebar at the very bottom left of the picture indicates a size of 20µm.

Here it can be seen, that the two different markers seem to be situated in two very distinct regions of the cardiosphere. Note the two rounded structures at the top right, which seem to be vacuoles, were observed in a number of different structures under the microscope.
It remains unclear, which factors lead to the expression of different markers in different parts of the cardiosphere structure.

3.4.3 cKit

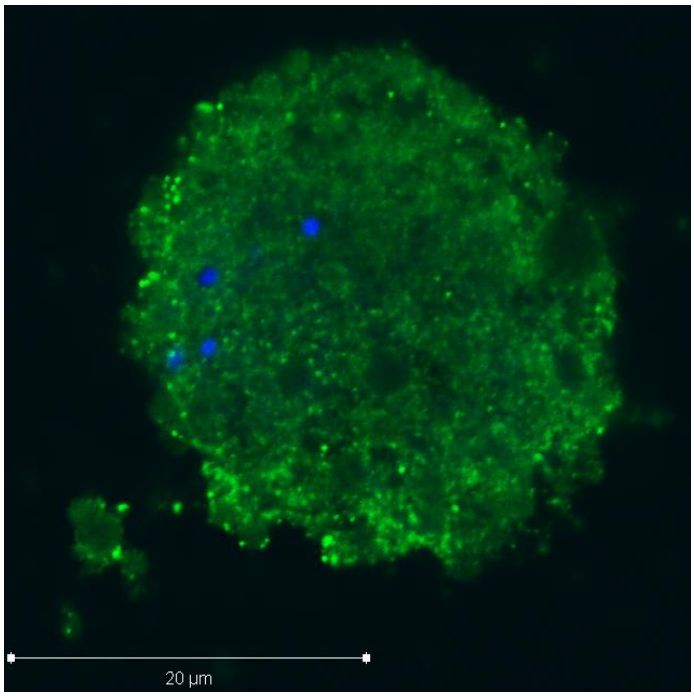


Figure 15.3: Flourescent picture of a potential cardiosphere stained with ckit in green and To- Pro in blue (indicating the nuclei).

This picture shows that the structures assumed to be cardiospheres show more than one nucleus. In addition, c-kit staining seems to be predominantly situated on the very edge of the multi cellular structures.
On the very right of the picture one can see some kind of vacuole, which can be observed in a large number of pictures taken.

3.4.4 MHC

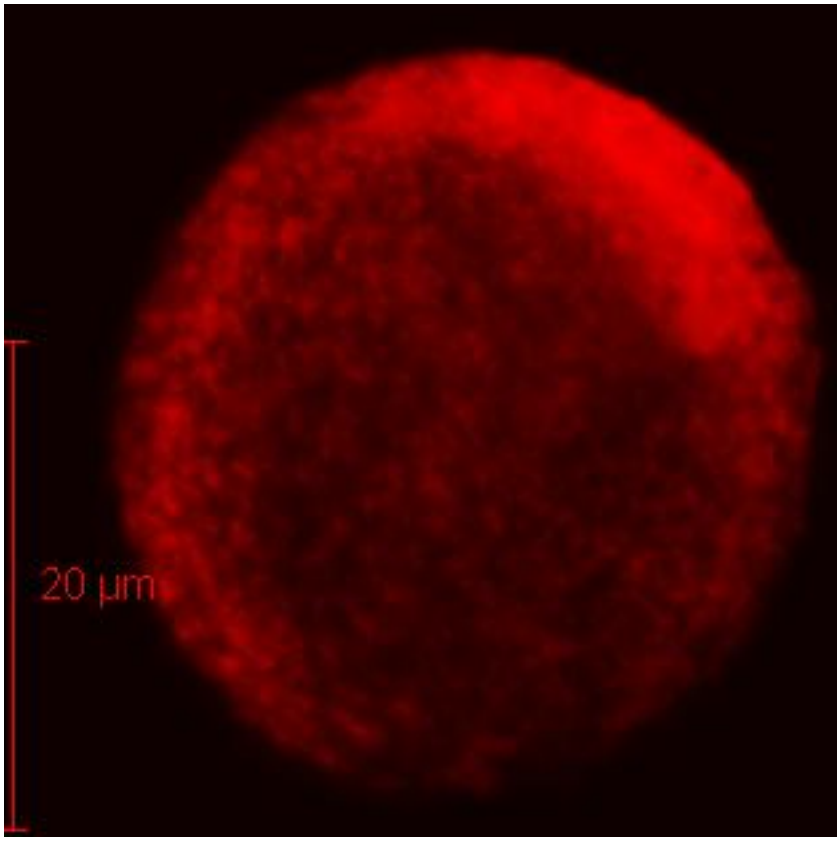


Figure 15.4: Cardiosphere like structure stained with MHC in red. Note the perfectly round shape of the structure. Due to a problem with the nucleus marker To- Pro there is no nuclear staining to be observed

The MHC staining of the potential cardiosphere shows a very strong signal at the edge of the structure, especially the upper right section. This goes along perfectly with the finding of other groups that the differentiation of cardiospheres to cardiomyocytes always starts at the periphery of the clusters [25].

The cardiosphere had been cultured for several weeks at time the staining, so it seems realistic that there was already a lot of differentiation going on in this cluster of cells.

3.4.5 H_3P

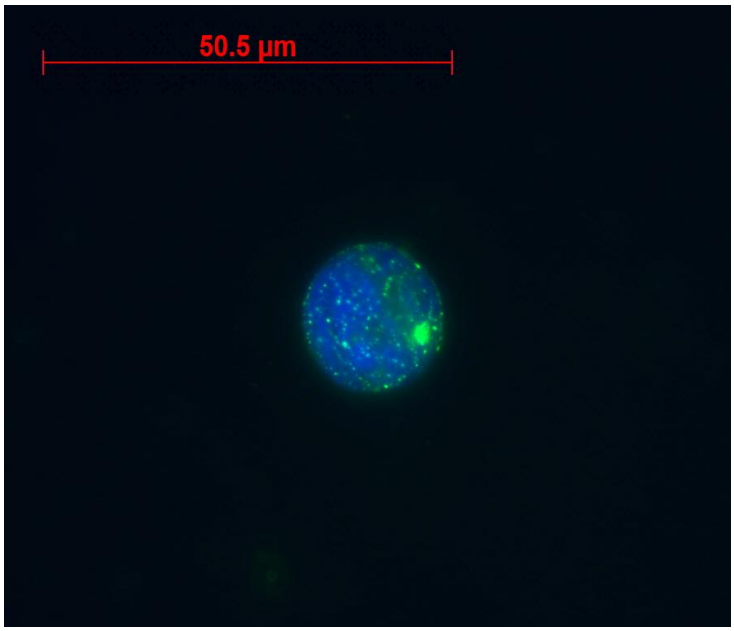


Figure 15.5: Picture of a small cardiospher like structure stained with DAPI in blue and H_3P in green. Very nice to see here is the perfectly round structure which is typically for cardiospheres. Also note the many different spots with H_3P signal indicating, that there are many different nuclei deviding within the same structure.

The phosphorylated Histone marker shows dividing cells or to be more precise, dividing nuclei. The picture presented here shows that there are variable spots where nuclei are dividing, again indicating that the structure consists of numerous nuclei. This again strengthens the claim that the observed structure represents a cardiosphere. Sadly, the staining with To- Pro again isn't all that conclusive, which might be due to the fact that the To- Pro did not seem to work so well on cardiosphere structures.

4. Discussion:

Cardiospheres might be the future for the therapy of ishemic heart disease and are therefore studied in many different mammalian species such as mice, pig and rats. Even though this cell structures are already used for clinical trials, with promising results, there are still obstacles that need to be resolved in order to establish an effective therapy.
In order to overcome these obstacles, such as the homing of the cells and the effectiveness of the delivered cell clusters, it might be worthwhile reproducing the experiments already done on mammalian organisms in an organism that possesses the intrinsic ability to regenerate.
The project aimed to reproduce findings from other groups concerning the use of cardiospheres to heal damaged/ infarcted hearts.
In contrast to other groups we have chosen a model organism that is known to be capable of regenerating parts of its heart after injury.
Findings in this organism might lead to hints how to apply cardiospheres in mammals more efficiently and therefor leading to a valid therapy for ishemic heart desease.
As for every project that tries to establish a new method there were a lot of setbacks to be faced and the time the project lasted was not enough to get everything done.

4.1 Explant cultures:

We managed to get the fragments of the heart explant quite quickly and to get a considerable amount of bright rounded cells emerging from them. Still it seems that we were not getting enough of these rounded cells for our needs. Changing medium might be a good starting point in order to optimize the amount of cells that can be harvested from these cells. As it seems, newt cells seem to prefer more simple media. So a change from IMDM to MEM might give slightly better results.
In general, newt cells prefer quite different culturing conditions compared to mammalian cells. The concentration of FCS in the medium might also be another thing to be adjusted in order to get the explants to produce more of the desired bright rounded cells.
All in all, we were able to find a reproducible protocol of bringing fragments newt explanted hearts in culture. Even though there are a plethora of things to try to optimize the

protocol, we were able to get enough bright rounded cells to advance the project to the next level.

4.2 Cardiosphere culture

We were able to show that the newt forms cardiospheres just like mammalian organisms do and that we are able to culture and stain them. The number and size of cardiospheres can vary greatly making it hard in the beginning to determine which clusters really were cardiospheres.
During the immunocytochemistry procedures, we were able to observe that these cardiospheres really had a ball like structure, because they showed a rolling movement in the direction of the suction of the pipette.
Even though we made quite some progress in culturing cardiospheres, it appears that the perfect conditions still haven't been met and there is still work to be done before the cardiospheres can be used to decipher what cell types contribute to the regeneration seen in the newt heart.

4.3 Further Perspectives

The work on the newt heart cardiospheres has just begun and beside the need to optimise procedures, media and culture conditions there are many things to be done.
One important step would be to re- inject cardiospheres into a regenerating newt heart in order to observe the homing and integration of these cell clusters. Therefor a method has to be found which enables us to permanently stain these cardiospheres.
Another great project would be the comparison of RNA and protein levels of this newt cardiospheres to different other cell types.
These cell types could be mammalian cardiospheres, mammalian heart cells and/or newt heart cells.
This knowledge then again could be used to regulate the activity of cardiospheres in mammals and therefore optimise the outcome of cardiosphere therapies in humans.

5. Aknowlegements:

There are many people to be thanked for giving me the chance to work on this project.

First of all I want to thank Jamie Morrison for taking me into his group and for patiently helping me with the obstacles I faced in course of my project.

Also to be thanked here is Friedrich Propst who took the responsibility as my supervisor from Vienna University for this project and helped me a great deal to get everything that had to be done, done.

I also want to thank Nevin Witman for his steady help and support in the lab as well as off hours when I just needed someone to talk to.

Furthermore I give great thanks to my parents and relatives whose support and care made it possible for me to work on such a project away from home and who always believed in me and the way I chose for my live.

Also great thanks are to be given to my fiancé and my son who showed great patience and understanding for me going abroad for such a long time and whose love kept me going even when I was not all too well.

Finally I want to thank all my other colleagues from Stockholm University who helped me out and made my trip to Sweden a nice and pleasurable experience.

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7. List of figures

Figure 1: http://heart-consult.com/articles/electrical-conduction-system-heart

Figure 2: http://mothe.us/what-makes-the-heart-to-continue-to-beat/

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Figure 6: Bruce M. Carlson, Principles of regenerative Biology, Chapter 1, Page 3, Figure 1-1

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Figure 8: Merged image from reference papers 25 [page 6, figures 5A & 5H] and 51 [page 2C.3.4, figures 2C.3.1 B&C]

Figure 9: Self made image drawn with Microsoft Paint