Mechanisms of aging induced decline in wound healing

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Introduction

The impaired wound healing is a significant concern affecting millions of people worldwide. The increased incidence is mainly caused by growing number of aged individuals, whose potential to repair tissues and healing of wounds has decreased. The aim of this study was to investigate gene functions affecting aging related decline in wound healing. In addition, the aim was to study the potential of these genes as drug targets and the effect of targeting drugs to improve wound healing.

Methods

This study was performed with fast-aging turquoise killifish (*Nothobranchius furzeri*) by comparing wound healing between young and old fish. A piece of tail fin of the fish was cut, and the fin regeneration was measured during several days. The gene expression and histological samples were also analysed. Finally, two drugs, which activate (roxadustat) and inhibit (semaxanib) blood vessel growth related genes, were administered to old fish. The differences between these drugs on wound healing process were observed.

Results

The fin regeneration was decreased with old fish compared to young fish, and no difference was found between males and females. After drug administration, growth length and rate were declined with semaxanib group, whereas growth rate was increased with roxadustat group compared to control group.

Conclusion

Tissue regeneration and wound healing is decreased with old fish and several genes participate to wound healing. However, searching of potential drug targets from these genes and drugs to improve the wound healing process needs more investigation.

Key words: wound healing, aging

1 INTRODUCTION

The impaired healing of wounds has become a significant concern, which affects millions of people worldwide. The increased incidence is mainly caused by growing number of aged individuals, whose potential to repair tissues and healing of wounds has decreased. Other reasons for impaired wound healing include certain diseases, such as diabetes. In addition, the mechanisms of tissue repair are not fully discovered, current treatments are limited, and efficient animal models are missing. The aim of this study was to investigate gene functions, which affect aging related decline in wound healing. In addition, the aim was to study if these genes could be used as potential drug targets, search targeting drugs and study their effect to improve the wound healing process.

1.1 Aging related impaired wound healing

Millions of traumatic injuries and surgical wounds are treated every year in Europe and in the United States. In addition, impaired healing of wound concerns wide range of people annually worldwide. Usually small injuries heal well in healthy people. However, large injuries, age and certain diseases such as diabetes and vascular disease may inhibit the healing process. Due to the aging of population, the incidence on impaired healing of chronic wound is growing, because the potential to repair tissues decrease with age. (Eming et al., 2014; Cañedo-Dorantes and Cañedo-Ayala, 2019)

The molecular mechanisms that affect repair and regeneration of tissues in healing process are not yet fully discovered, and currently used treatment methods are not effective enough. In addition, efficient animal models for studying tissue regeneration that are comparable with humans are limited. Therefore, treatment of chronic skin wounds and decreased wound healing has become a major concern in clinical health care and new treatments are needed to enhance the regeneration of tissue in impaired wound healing. (Eming et al., 2014)

The burden of skin diseases has increased significantly during the last ten years. Chronic wounds are found mostly with aged people and more often with women than men. In addition, chronic wounds are mainly leg ulcers. (Martinengo et al., 2019) The estimated prevalence of chronic wounds is 1-2 % in developed countries and for example, 6.5 million people have chronic wounds in the United States (Nussbaum et al., 2018).

1.2 Wound healing process

Skin forms a barrier around the body for protection from outer environment. When the skin is injured, a wound is formed on a skin or deeper in subcutaneous tissue, and muscles, nerves and bones may be also damaged. The healing process begins immediately after wounding and acute wounds heal normally during few days or within few weeks, complete recovery may last even a year. Wound healing is a continuous and complex process with different overlapping phases, which are haemostasis, inflammatory, proliferation and remodelling phases. Several cell types and molecular mediators, such as cytokines, chemokines and growth factors, have specific roles and interactions during the wound healing phases. For example, the major cell types in regenerating wound include neutrophils, macrophages, keratocytes, fibroblasts, endothelial cells and monocytes (Velnar et al., 2009; Cañedo-Dorantes and Cañedo-Ayala, 2019).

Phases of the wound healing process are presented in Figure 1. The haemostasis phase begins immediately after injury. During haemostasis the vascular system is protected, and bleeding stopped by forming a clot with platelets, fibrin and other components on the wound. The inflammation phase begins few hours after injury, and during the inflammation phase the wound is protected against pathogens by destroying micro-organisms and preventing infection with several immune cells, such as neutrophils, monocytes and macrophages. Tissue repair begins within few days after injury during the proliferation phase, which main functions include formation of granulation tissue (fibroplasia), keratinocyte reepithelialisation to cover the wound, formation of blood vessels (angiogenesis) and regeneration of peripheral nerves. Finally, few weeks after injury the formation of new epithelium and functional tissue begins by remodelling the tissue in the remodelling phase (Velnar et al., 2009; Cañedo-Dorantes and Cañedo-Ayala, 2019).

Wound healing phase	Haemostasis	Inflammation	Proliferation	Remodelling
Beginning	Few seconds	Few hours	Few days	Few weeks
Purpose	Protection of vascular system	Protection against pathogens	Beginning of tissue repair	Complete tissue remodelling
Main functions	Platelet aggregation to stop bleeding	Preventing infection with several immune cells	Angiogenesis, fibroplacia, keratinocyte reepithelialisation	Formation of new epithelium and functional tissue

Figure 1. Phases of the wound healing process with the beginning, purpose and main functions of each phase.

For example, keratinocyte reepithelialisation to cover the wound is one of the most important steps in the proliferation phase, and it begins usually within 16-24 hours after the injury (Cañedo-Dorantes and Cañedo-Ayala, 2019). In addition, dysfunction in keratinocyte migration is one of the reasons for chronic wounds (Eming et al., 2014). Therefore, isolated fish keratocyte cells are widely used in vitro model to study epithelial wound closure in the early stages of wound healing. Furthermore, isolated fish keratocytes can be used to study mechanisms of single cell motility, interactions between cells and collective migration of group of cells during wound healing. Keratocytes are isolated from fish scales by removing few scales from anesthetised fish and placing the scales on cell culture medium. During removal, some epithelial cells stay attached to the scale, and these cells, mostly keratocytes, start rapidly migrating from the scale on culture medium. Within the first hours keratocytes move collectively with stable speed and direction. Later the speed and collective direction decrease, when cells are spreading due to changes in gene expression. (Rapanan et al., 2015)

Epidermal keratocytes are simple shaped semi-circular cells. They move by gliding with lamellar extension, called lamellipodia, which is at the leading edge of the cell. Lamellipodia contains plenty of actin filaments, which have essential role in migration of the cells. (Lee et al., 1993; Svitkina et al., 1997)

The migration rate of keratocytes on culture medium resembles the migration rate of cells across a wound during the wound closure. In addition, the results are comparable with the migration of human keratinocytes, because both cells from fish and human are primary epithelial cells, which function similarly during the wound healing process. Furthermore, the organization and structure of keratocytes are highly similar between fish and mammalians. The study of migration of keratocytes is easy, rapid and inexpensive to perform. (Rapanan et al., 2015)

1.3 Formation of blood vessels (angiogenesis)

Formation of new blood vessels, called angiogenesis, is significant step during the proliferation phase in the wound healing process. Blood vessels in the circulatory system are responsible for constant oxygen and nutrient supply, and essential for the development and function of cells, tissues and organs. In addition, oxygen is needed for ATP production in mitochondrion and multiple reactions and oxygen homeostasis in eukaryotic cells. Therefore, angiogenesis has significant role in generating the constant transport of oxygen and nutrients to cells during regeneration of tissues in the wound healing process and during organism development. (Befani and Liakos, 2018; Zimna and Kurpizs, 2015).

In angiogenesis blood vessels are developed from already existing vessels, including remodelling and creation of networks of blood vessels with different sizes in adults and embryos. However, the development of primary circulatory system in embryonic development phase is called vasculogenesis. Angiogenesis is a complex process, which begins with a specific signal, such as blood vessel damage, low oxygen level or inflammation. Then, the signal activates endothelial cells and release of angiogenesis related growth factors, such as vascular endothelial growth factor (Vegf). (Befani and Liakos, 2018; Zimna and Kurpizs, 2015)

1.3.1 Genes regulating angiogenesis

Among several angiogenesis regulating factors, vascular endothelial growth factor A (Vegfa), a member of Vegf family, is the most significant. The main function of Vegf is vascular development by activating endothelial cell growth in arteries, veins and lymphatics with adults and during embryonic development. The expression of Vegf is mostly activated by low oxygen levels, called hypoxia, and release of wide range of growth factors and inflammatory cytokines. In addition, specific transcription factors, called hypoxia inducible factor (Hif), regulates the expression of Vegf. (Ferrara et al., 2003)

Hypoxia is one of the most important issues that activate the expression of Vegf. Hypoxia is mainly induced by low blood circulation or heavy consumption of oxygen. For example, decreased blood supply and increased cell metabolism during tissue repair in wound healing causes hypoxia.

Furthermore, hypoxia activates angiogenesis and finally the formation of Vegf. (Cañedo-Dorantes and Cañedo-Ayala, 2019) During hypoxia the expression of several genes are mainly regulated by the transcription factors, hypoxia-inducible factor-1 and -2 (Hif-1 and Hif-2). The genes regulated by Hif are involved in multiple cellular functions, such as cell survival and proliferation, angiogenesis and erythropoiesis. In addition, Hif is responsible for regulating functions during angiogenesis, such as blood vessel formation and cell migration. Additionally, Hif-1 and Hif-2 are potential drug targets. Activation of Hif-1 and Hif-2 could be used for the treatment of ischemia and cardiovascular diseases and inhibition of Hif-1 and Hif-2 to treat cancer. (Befani and Liakos, 2018; Zimna and Kurpizs, 2015)

Hif is a dimeric protein with two subunits α and β, in which α subunit is translated and degraded and β subunit is expressed continuously. Hif is regulated by certain hydroxylases, prolyl hydroxylase enzymes (Phd), with complex processes. Under normal oxygen conditions, Hif-α subunit is hydroxylated by Phd, which leads to degradation of Hif-α. During hypoxic conditions, the activity of Phd is blocked, which leads to reduced hydroxylation and increased stabilisation of Hif-α. Finally, Hif-α is translocated into nucleus, where it binds to Hif-β subunit and starts to regulate gene expression. (Befani and Liakos, 2018; Zimna and Kurpizs, 2015).

The effect of Vegf begins when Vegf binds to specific tyrosine kinase receptors and activates them. The most important tyrosine kinase receptors are fms-related tyrosine kinase 1 (Flt1) and kinase insert domain receptor (Kdr), which are mainly presented on the surface of vascular endothelial cells. Both receptors have different roles in regulating angiogenesis. (Ferrara et al., 2003) Kdr is a positive regulator of vascular development and Flt1 is a negative regulator by preventing the activation of Kdr (Park et al., 2018).

Several other genes are also important factors in angiogenesis. For example, vascular endothelial cadherin (Cdh5) has significant role in regulating cell adhesions between endothelial cells maintaining integrity of endothelial cell layer of blood vessels. The stable barrier of endothelial cells is important for several functions, such as wound healing, angiogenesis and inflammation. In addition, Cdh5 can operate as a target for cell signalling of these functions. (Abu Taha and Schnittler, 2014)

Interactions between Vegf and its receptors are important in regulating tissue repair during wound healing. Therefore, the number of Vegf, Flt1 and Kdr is increased during tissue repair. In the healing wound Vegf regulates several functions, such as vascular permeability, migration of inflammatory cells and rapid increase of endothelial cells. (Eming and Krieg, 2006)

The regulation of Vegf and its receptors have significant role also in the development of chronic wounds. The main function of Kdr is to promote angiogenesis, and Flt1 mainly mediates

inflammation. Immediately after wounding Vegf is secreted, although the secretion is declined or inhibited in chronic wounds. Inflammation and migration of macrophages after wounding is activated by Flt1. In addition, Flt1 decrease angiogenesis by inhibiting Kdr. For example, chronic wounds with normal circulation have increased levels of Flt1, which leads to inflammation. In addition, decreased levels of Kdr and declined angiogenesis have been found from patients who have chronic wounds with poor circulation, such as diabetic and venous leg ulcers. (Zhou et al., 2015)

1. 3.2 Drugs affecting angiogenesis

Dysfunction of angiogenesis regulating genes may cause several diseases either by increasing or inhibiting the gene regulation. The higher level of Vegf leads to increased angiogenesis, which is related to specific pathological conditions, mainly several types of cancer. Therefore, Vegf inhibitors has been mostly studied for the treatment of cancer, where they inhibit angiogenesis and decrease blood flow to the cancer cells. In addition, Vegf inhibitors could be used to treat other diseases, such as visual loss caused by vascular leakage, aging induced macular degeneration and several inflammatory diseases, such as rheumatic arthritis and psoriasis. (Ferrara et al., 2003; Park et al., 2018)

The dysfunction of Hif pathway, which regulates angiogenesis and other cellular functions, may lead to decreased level of Vegf and other factors. For example, anaemia with chronic kidney disease is caused by dysfunction of Hif pathway, which leads to decreased level of erythropoietin. The treatment with drugs that activate Hif pathway regulating genes finally leads to increased level of renal erythropoietin. (Locatelli et al. 2017) In addition, the potential to increase angiogenesis by activating these genes is being studied in order to increase the level of Vegf with tissue transplantation (Zhou et al., 2019).

Currently potential drugs targeting specific angiogenesis regulating genes have been widely investigated, and some of them are in clinical phase or already on the market (Park et al., 2018). For example, roxadustat is angiogenesis activating and semaxanib is angiogenesis inhibiting drug, and these drugs were investigated in this study.

Roxadustat activates angiogenesis with complex mechanism of action, which is presented in Figure 2. Roxadustat stabilizes Hif, which stimulates gene expression during hypoxia. With normal oxygen levels Phd enzyme hydroxylates Hif, which finally leads to degradation of Hif. With low oxygen levels or with agents mimicking hypoxia, such as roxadustat, Phd is blocked. Therefore, Hif activity

is promoted, which stimulates the expression of its target genes, such as *vegf*. Finally, increased level of Vegf leads to activation of angiogenesis. (Zhou et al., 2019)

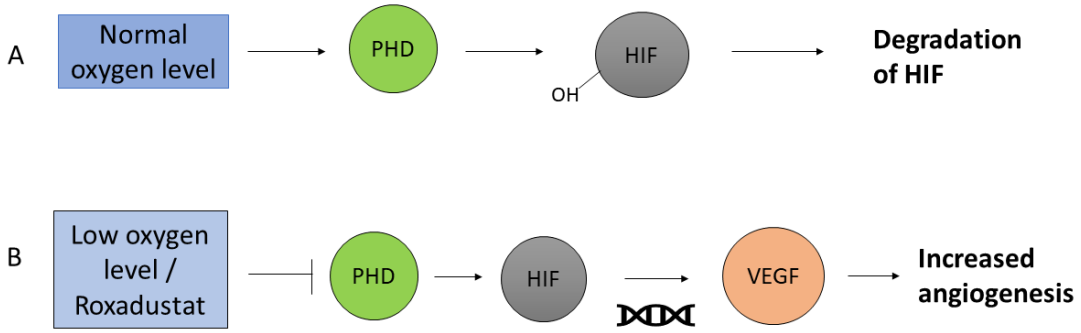

Figure 2. Mechanism of action of roxadustat is mediated by stabilization of hypoxia-inducible factor (HIF), which stimulates genes during hypoxia. A) With normal oxygen levels prolyl hydroxylase inhibitor (PHD) enzyme hydroxylates HIF, which finally leads to degradation of HIF. B) With low oxygen levels or with agents mimicking hypoxia, such as roxadustat, PHD is blocked. Therefore, HIF activity is promoted, which stimulates the expression of its target genes, such as *vegf*. Finally, increased level of VEGF leads to activation of angiogenesis. Figure was modified from Zhou et al. 2019.

Roxadustat is a small molecule orally administered drug, which is indicated for anaemia with chronic kidney disease. The inhibition of Hif-Phd stabilises Hif, which leads to production of erythropoietin and finally increased haemoglobin. Roxadustat is approved in China in December 2018. (Dhillon, 2019) One study of roxadustat has been performed with zebrafish embryos, using concentration of 2.5 µM. In this study, known Hif targets, glucose transporter 1 and lactate dehydrogenase A, were upregulated in zebrafish embryos after the treatment with roxadustat. (Jain et al., 2016).

Semaxanib inhibits angiogenesis by blocking receptor tyrosine kinases, which are receptors for several cytokines, hormones and growth factors, such as Vegf. Semaxanib inhibits Vegf receptors, Flt1 and Kdr, which leads to reduced angiogenesis. Increased activation of receptor tyrosine kinases is often involved in the development of cancer, and semaxanib is also designed for the treatment of cancer. Currently the investigation of semaxanib has been discontinued. (Haddad, 2012) For example, studies with zebrafish embryos has been performed with semaxanib, and severe defects in formation of blood vessels has been observed, when using concentrations of 1-10 µM (Farooq et al., 2018).

1.4 Chronic wounds

Acute wounds heal with normal time frame. However, wound becomes chronic, if the wound healing process is prolonged due to some complications during the healing process. The complications may be caused by several issues, such as impaired formation of blood vessels, decreased activity of growth factors, dysfunction of stem cells or prolonged inflammation. For example, impaired formation of blood vessels leads to lack of oxygen and nutrients in the healing wound, which delays the healing process. Additionally, concurrent infection with several microbes in the wound may form biofilms, which impairs the healing process significantly. Three major groups of chronic wounds are arterial and venous leg ulcers, pressure sores and diabetic foot ulcers. In addition, treatment of chronic wounds is challenging and time-consuming. Therefore, the finding of chronic nonhealing wounds as early as possible is important, and methods to distinguish patients with acute and chronic wounds has been developed. (Velnar et al., 2009; Lindley et al., 2016)

1.4.1 Indicators to predict poor wound healing (biomarkers)

Specific indicators, called biomarkers, that can be used to measure and predict biological conditions objectively, are important for detecting chronic wounds. Samples from wound fluids, tissue specimen and serum from patients with both well healing and chronic nonhealing wounds have been collected. The samples have been used to study pathology, gene functions and composition of proteins, lipids and microbes during wound healing. Furthermore, the samples have been used to search biomarkers for chronic nonhealing wounds. Multiple processes or molecules that inhibit or activate healing process, can be used as a biomarker for monitoring the progression of wound healing. For example, cytokines, proteases, macrophages, neutrophils, fibroblasts and growth factors are potential biomarkers. (Eming et al., 2014; Lindley et al. 2016: Patel et al., 2016)

Specific wound healing biomarkers could be used for targeting specific therapies for each patient, predict outcome of healing, follow progression of the disease and monitor response to therapy. In addition, novel biomarker should be accurate, easy to use and cost-effective. Although several diagnostic biomarkers are currently in clinical use, more investigation is needed to discover novel biomarkers that could predict healing outcome more accurately. However, the process of developing new biomarkers is time-consuming, including biomarker discovery, developing of clinical assay and biomarker validation in clinical trials. (Lindley et al. 2016; Patel et al., 2016)

1.4.2 Treatment of chronic wounds

Efficient treatments for chronic skin wounds are limited. The main principals in the treatment of chronic wounds are cleaning the wound from necrotic tissue, preventing infection and keeping the wound moisture in balance. The current treatments include for example wound dressings with moisture-retentive and antimicrobial agents, pressure therapy and hyperbaric oxygen. In addition, treatment with biologic dermal substitutes with human or animal skin cells, such as keratocytes or fibroblasts, and topical gel with recombinant human growth factor has been approved by FDA (Powers et al., 2016).

Potential novel treatments of chronic wounds include mainly drugs for specific molecular targets and modulation of stem cells. In addition, angiogenesis regulating molecules could be modified to increase angiogenesis during wound healing. Investigational drugs for impaired wound healing include wound dressings with recombinant growth factors and live cells. For example, systemic and local administration of Vegf has been widely studied to increase the growth of blood vessels during impaired healing of wounds. (Eming et al., 2014)

Wound healing is a complex process, and several molecules are participating the process. Due to the changes of these molecules in chronic wounds, treatments are often ineffective. Therefore, more efficient treatment could be a combination of several active ingredients. In addition, due to the significant variation of chronic wounds between individual patient, personalized treatments could be most efficient. (Eming et al., 2014) In addition, the tissue regeneration process of some specific species, such as salamanders and flatworms, has been studied to discover potential therapies for tissue injuries (Tal et al., 2010).

1.5 Tissue regeneration

During the tissue regeneration process specific body parts are replaced after injury. The regeneration potential varies among animal species and organs. For example, some nonmammalian vertebrates, such as fish and salamanders are able to regenerate whole organs. However, numerous mammalians have a low potential for regeneration. The mechanisms of regeneration are poorly understood. (Eming et al., 2014).

In most organisms the physiological mechanisms to regenerate tissues decrease during aging. For example, regeneration potential has been observed in humans only with embryo skin, and this ability is missing from human adults (Lorenz et al., 1992). The repairment of tissues in human adults is most significant in outer layer of a skin epidermis, epithelium of gut and hematopoietic system. In addition, due to the decreased potential of regeneration, chronic wounds are mostly observed among aged individuals. (Eming et al., 2014)

The regeneration potential has been studied with stem cell based and nonmammalian animal models to discover pathways, which activate the regeneration process. The final aim is to develop potential therapies, which could be used to treat injured tissues caused by for example aging or specific diseases. Several species have similar molecular signalling pathways to create limbs and appendages. In addition, similar pathways have been observed to be activated during both limb creation and regeneration. (Sehring et al., 2016; Tal et al., 2010)

1.6 Animal models for aging and wound healing

Novel animal models that resemble aging and wound healing processes with humans more accurately are needed. Species with potential to regenerate tissues provide relevant information of the wound healing process. Wound healing and tissue regeneration have been previously studied with pigs because of the similarities between pig and human skin. However, due to the complex procedures and high expenses related to pigs, rodents, especially mice, have become more popular models. Several transgenic and knockout mouse lines have been developed to study wound healing process more effectively. Although studies with mice consume plenty of time and money, they are still most widely used animal models in wound healing studies. (Eming et al., 2014)

Other animal models are also used to study aging, such as yeast, *Caenorhabditis elegans* worm, *Drosophila* fly and zebrafish (*Danio rerio*). The mechanisms of aging have been widely studied with invertebrates, such as worms and flies. However, vertebrates, such as mice and zebrafish have certain benefits. For example, vertebrates have more specialized systems affected by aging, such as complex nervous system and adaptive immune system. (Eming et al., 2014; Hu and Brunet, 2018)

Zebrafish has potential to regenerate several organs, such as fin, heart and liver. Therefore, zebrafish is a widely used model for studying regeneration after wounding. The most widely used experiment is to study regeneration after cutting a piece from caudal fin, because it is a simple and easy procedure and fin regeneration occurs fast. In addition to adult zebrafish, embryos can be also used to study

regeneration. Advantages of the use of embryos include rapid obtaining of large number of embryos, non-invasive wounding and better possibility for genetic manipulation than with adult fish. (Tal et al., 2010)

Caudal fin of zebrafish consists of several bony fin rays connected by inter-rays with soft tissue, and the fin is covered with epithelial cells. In addition, caudal fin includes several other cell types, such as blood vessels, nerves and fibroblasts. The caudal fin regenerates when ray components grow to the distal edge of the fin. The regeneration process includes several phases, such as formation of an apical epithelium over the wound and development of blastema with progenitor cells. Furthermore, progenitor cells differentiate into specific cell types, which are needed for tissue regeneration. In addition, regeneration includes other parallel processes, such as growing of blood vessels, nerves, epidermis and pigmentation. The fin of zebrafish grows back to its original length within two weeks. (Tal et al., 2010) Other fish species, such as turquoise killifish, (*Nothobranchius furzeri*), can be also used as animal models to study aging and wound healing (Platzer and Englert, 2016).

1.6.1 *Nothobranchius furzeri* in nature

N. furzeri is a suitable model for studying aging related wound healing due to the short lifespan and rapid aging of the *Nothobranchius* species. In addition, the size of these fish is small, with total length usually less than 7 cm. In the nature *N. furzeri* lives in African countries, especially in Zimbabwe and Mozambique. The fish live in small temporary ponds, which are filled with rainwater during the wet periods and desiccates in dry periods due to the monsoon seasons. Rainy seasons are usually short, lasting about 4 months, and the length of dry seasons are usually longer, 6-8 months. *N. furzeri* has been adapted to the specific natural environment by growing, reproducing and aging rapidly. Therefore, the lifespan of *N. furzeri* is naturally extremely short, which is usually from 4-6 months. For example, the lifespan of mouse and zebrafish is longer, more than three years. (Cellerino et al., 2016; Hu and Brunet, 2018; Platzer and Englert, 2016)

N. furzeri becomes sexually mature with early age, at the age of 3-4 weeks. The fish lays their embryos into the soft mud of the ponds, where the embryos survive during the dry season in developmental diapause state even more than a year. Hatching of the embryos is activated when the ponds are filled with rainfall during the next rainy season. (Cellerino et al., 2016; Hu and Brunet, 2018; Platzer and Englert, 2016)

1.6.2 *Nothobranchius furzeri* as animal model

Genus *Nothobranchius* contains over 60 species, in which *N. furzeri* is the mostly used species in laboratory. The *N. furzeri* GRZ strain with very short lifespan was presented in 2003 and it is the most widely used strain today. In addition, many other laboratory strains with differing lifespans are used. The lifespan of *N. furzeri* in the laboratory is usually 3-18 months, which is the shortest lifespan reported in vertebrates in laboratory conditions. (Cellerino et al., 2016; Hu and Brunet, 2018) The image of *N. furzeri* strain MZCS222 is presented in Figure 3.

Figure 3. Turquoise killifish, *Nothobranchius furzeri*, strain MZCS222. The larger and colourful fish are males and the smaller and pale blown fish are females.

Due to the extremely short lifespan of *N. furzeri*, the aging occurs also rapidly and the mechanisms of aging in molecular level resemble other vertebrates, also humans. In addition, sequence for *N. furzeri* genome have been established and transgenic lines have been developed. Based on these advantages, *N. furzeri* is suitable laboratory model to study ageing and age-dependent dysfunctions, genes and pathways, which resemble those with humans. (Cellerino et al., 2016; Hu and Brunet, 2018; Platzer and Englert, 2016) In addition, the maintenance of *N. furzeri* is performed easily and with low cost, and standardized housing protocols are available (Harel et al., 2016; Polačik et al., 2016).

Several aging related dysfunctions have been observed with *N. furzeri*. For example, reduced behavioural and learning performance, increasing of aging related biomarkers, mitochondrial dysfunction and shortening of telomeres, which affects the aging of muscle and skin have been observed. In addition, phenotypic changes and reduction in regenerative capacity of caudal fin caused by aging have been reported. (Platzer and Englert, 2016; Hu and Brunet, 2018)

1.7 Summary

The impaired aging related wound healing is a significant burden, which affect millions of people in the world. The main reason for the increased incidence is the aging of the population, because the potential of tissue repair and wound healing decrease within aging. In addition, the mechanisms of tissue repair are not fully discovered, current treatments are limited, and efficient animal models are missing. For example, *N. furzeri*, is a novel animal model for aging research due to its rapid aging and short lifespan.

Wound healing is a complex process with several overlapping phases. One of the most important phases is formation of blood vessels (angiogenesis), because the transport of oxygen and nutrients to the cells is essential for tissue repair during wound healing. Several factors have significant roles in angiogenesis, such as Vegf and its specific tyrosine kinase receptors, Flt1 and Kdr. Dysfunction of angiogenesis regulating genes may cause impaired wound healing and several diseases. Therefore, potential drugs targeting specific angiogenesis regulating genes has been widely investigated.

The aim of this study was to investigate these angiogenesis regulating genes, which affect aging related decline in wound healing, using fast-aging *N. furzeri* as a research model. In addition, the aim was to observe if these genes could be used as drug targets and to study potential drugs for these targets to improve the wound healing process.

2 RESULTS

2.1 Survival analysis

Survival data of the maintained *N. furzeri* strain MZCS222 (n = 110) was analysed with GraphPad Prism 8.4.0 to determine the median lifespan of the fish. The survival curve is presented in Figure 4. The median lifespan of *N. furzeri* strain MZCS222 was 34 weeks.

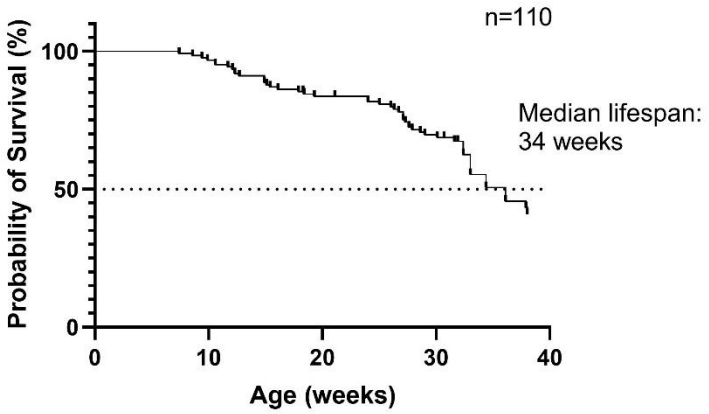

Figure 4. Survival curve of *N. furzeri* strain MZCS222. Median lifespan was 34 weeks.

2.2 Isolation of keratocytes

Isolation of keratocytes from fish scales is a widely used method to study aging related changes of reepithelialisation and cell migration during early stages of wound healing. Two young (age of 6 weeks) and two old (age of 24 weeks) fish were used in this study, and both age groups included one male and one female fish. Maximum of 10 scales were removed from each anesthetised fish and the scales were placed on 8-well plate with cell culture medium. The migration of isolated keratocytes was imaged with Nicon Eclipse Ti2-E time-lapse microscope for 8 hours with 15 minutes intervals. Furthermore, the migration rate was calculated from the measured lengths of the migrating keratocytes related to the migration time. The differences of the migration rate between young and old fish was analysed. Example of imaging the migration rate of isolated keratocytes is presented in Figure 5. As a result of this study, no significant difference was found between the migration rate of isolated keratocytes from young and old fish (Figure 6).

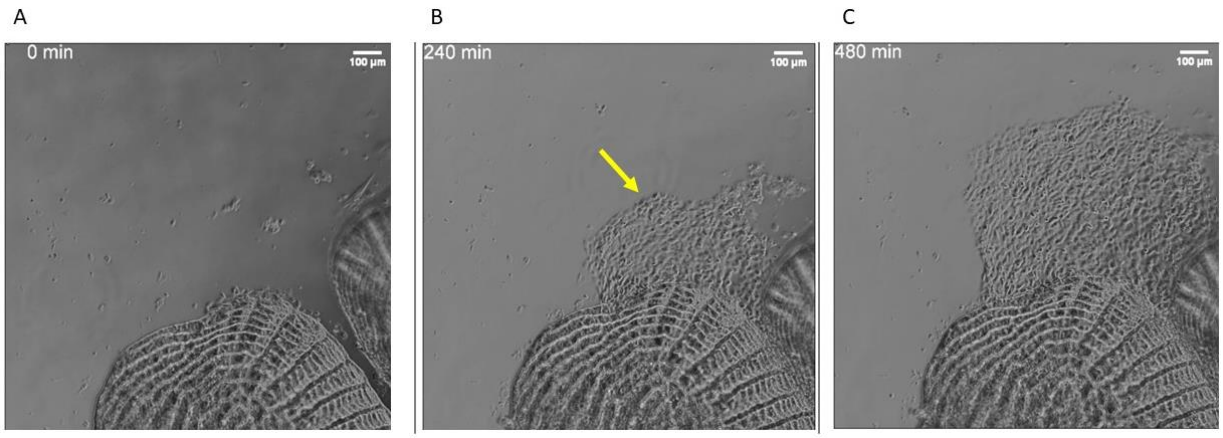

Figure 5. Example of imaging the migration rate of isolated keratocytes, where the keratocytes grow out form the scale collectively. The images were taken with Nicon Eclipse Ti2-E time-lapse microscope every 15 minutes for 8 hours using Hamamatsu phase camera with 10x magnification. A) The scale on cell culture at the beginning of the experiment. B) Cell migration out form the scale after 240 minutes (4 hours). Yellow arrow is pointing the edge of migrating keratocytes. C) Cell migration out form the scale after 480 minutes (8 hours).

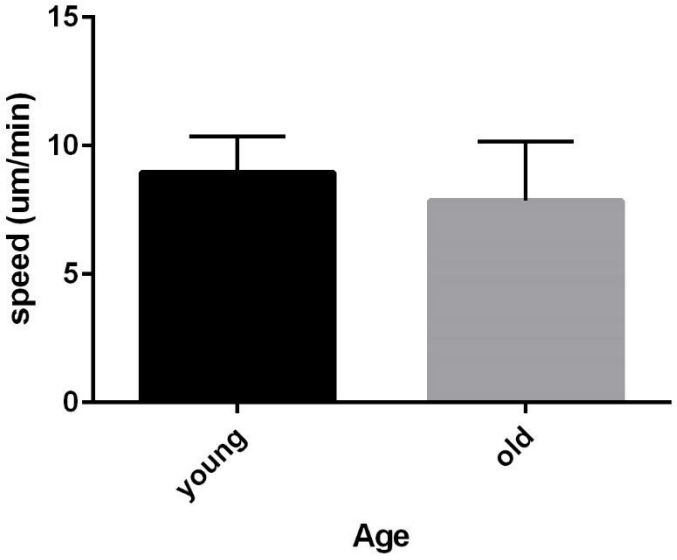

Figure 6. Migration rate of keratocytes that outgrow from the scales on cell culture. The scales were removed from two young (age 6 weeks) and two old (age 24 weeks) fish, and both age groups included one male and one female fish. The number of removed scales was maximum of 10 scales from each fish. The migration length of the keratocytes was measured at each timepoint from the images taken with Nicon Eclipse Ti2-E time-lapse microscope for 8 hours with 15 minutes intervals. The migration rate was calculated by relating the migration length to the migration time of young and old fish.

In addition, number and organization of actin filaments was observed with Leica TCS SP5 confocal microscope to study changes of the actin filaments between young and old fish. The cells were first

fixed with 4 % paraformaldehyde and then the cells were stained with Draq5 nuclear dye and phalloidin-555 to make the actin filaments visible. Example of fluorescence staining of keratocyte cells from young and old fish is presented in Figure 7.

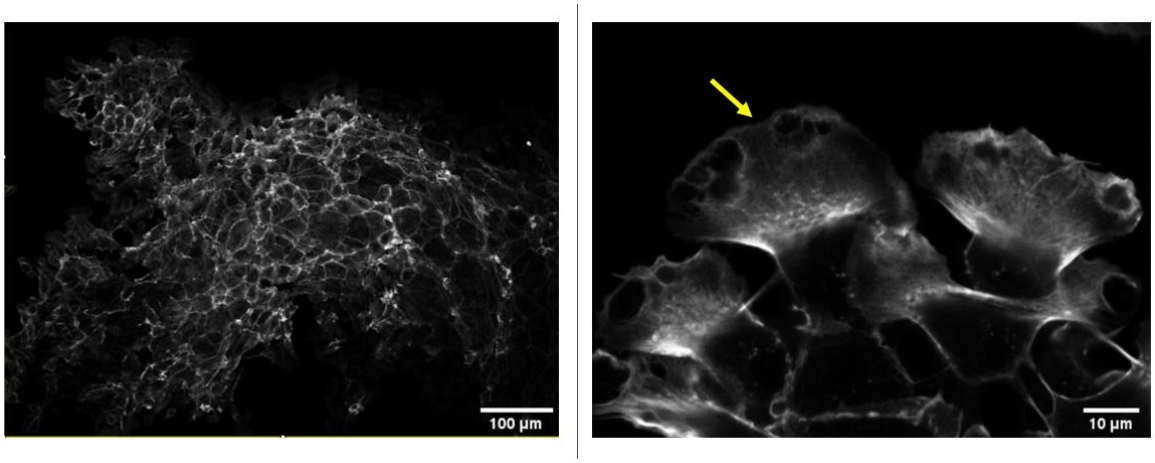

Figure 7. Example of fluorescence staining of keratocyte cells from old (age 24 weeks) fish. The cells were stained with Draq5 and phalloidin-555. A) Group of keratocyte cells grown out from scale on cell culture medium. The image is taken with 20x magnification. B) Individual keratocyte cells grown out from scale on cell culture medium. The image is taken with 63x magnification. Lamellipodia with actin fibres is marked with yellow arrow.

2.3 Wound healing study

In the wound healing study, the differences of fin growth length and rate between two age groups of *N. furzeri* strain MZCS222 was investigated. The age of young fish was 5 weeks and the age of old fish was 12 weeks. Both age groups included one group of eight males and one group of eight females. The study was performed by first cutting a piece of the caudal fin from the fish. Then, the regeneration of the fin was measured on days 1-4 and 7-8 and the growth length was compared between the age groups. The growth length of the fin was analysed with two measuring techniques, by measuring the length of the fin and thickness of the fin at each timepoint. First, the length of the whole fin was measured. Second, the thickness of the fin was determined by measuring only the regenerated part of the fin. Then, the measurement results were related to the cut biopsy on day 0. The results are presented in percentage, because the fish are different sizes depending on the age and gender. Examples of caudal fin regeneration of both age groups and genders on day 0 before and after biopsy and on day 8 are presented in Figure 8.

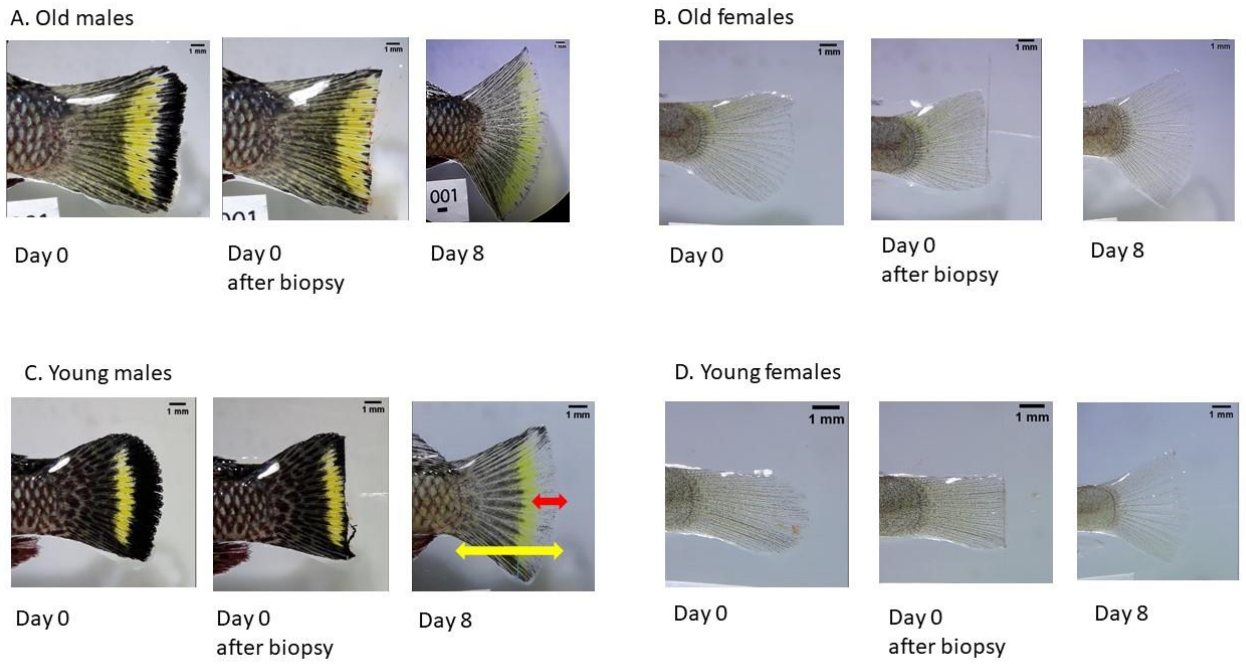

Figure 8. Examples of caudal fin regeneration of the *N. furzeri* strain MZCS222 on day 0 before and after biopsy and regenerated fin on day 8. A-B) Regeneration of caudal fin from old (age 12 weeks) male and female fish. C-D) Regeneration of caudal fin from young (age 5 weeks) male and female fish. In figure C is presented examples of measuring the length of fin regeneration with yellow arrow and measuring the thickness of fin with red arrow.

The results of this study are presented in Figures 9 and 10. The results show that the regeneration of the fin was declined with old fish and significant difference of the growth length between young and old fish was observed on days 7 and 8 ($p<0.001$). In addition, no difference was found in the growth length between males and females. The wound healing process began immediately on day 1, when the average of mean growth length in both groups was 4 %. Both measuring techniques showed similar results. By measuring the length of the whole fin, the mean growth length was 34 % with old and 64 % with young fish on day 7 and 43 % with old and 77 % with young fish on day 8. By measuring the thickness of the regenerated part of the fin, the mean growth length was 30 % with old and 56 % with young fish on day 7 and 37 % with old and 65 % with young fish on day 8. The most significant differences on the growth length (34 %) and on the fin thickness (28 %) between two age groups were observed on day 8.

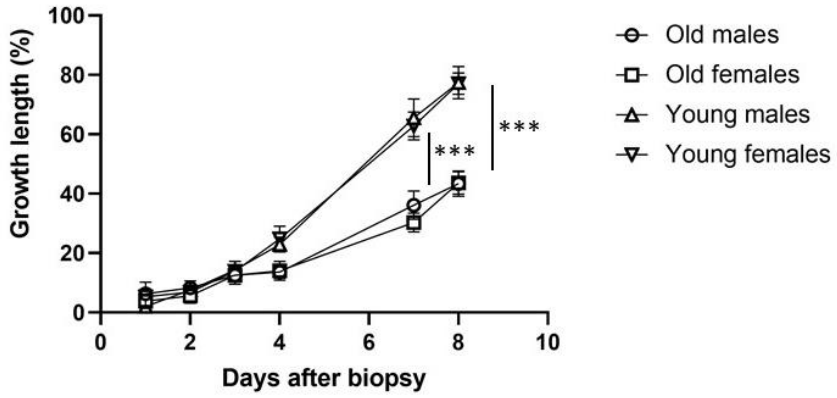

Figure 9. Growth length of fin regeneration after biopsy of young males and females (age 5 weeks) and old males and females (age 12 weeks) fish within 8 days. The sample size was eight fish in each group. The growth length was determined by measuring the whole fin at each timepoint, which was related to the cut biopsy on day 0. The regeneration of the fin was declined with old fish and significant difference between the two age groups was observed on days 7 and 8, *** $p<0.001$.

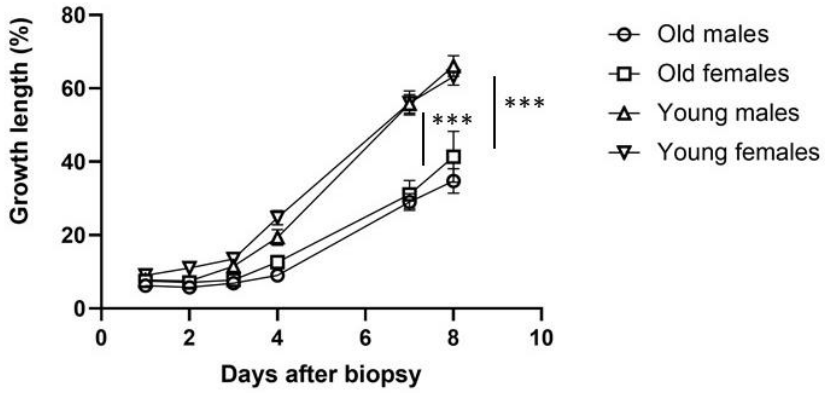

Figure 10. Growth thickness of the regenerated fin after biopsy of young males and females (age 5 weeks) and old males and females (age 12 weeks) fish within 8 days. The sample size was eight fish in each group. The growth thickness was determined by measuring the regenerated part of the fin at each timepoint, which was related to the cut biopsy on day 0. The regeneration of the fin was declined with old fish and significant difference between young and old fish was observed on day 7 and 8, *** $p<0.001$.

The growth rate of the fin was analysed by calculating the increase of the fin length from the measured length of the whole fin at each timepoint, related to the cut biopsy on day 0. Most of the measurements of the fin were performed daily. However, the measurements were not performed between day 4 and 7. Therefore, the daily growth rate on day 7 was calculated by dividing the growth rate on day 7 with the number of days when the measurements were not performed. Because no differences were observed in the growth length and thickness of the fin between males and females, the growth rate

was analysed by combining both genders and observing the difference of fin regeneration only between the two age groups, young and old. The results of fin growth rate are presented in Figure 11.

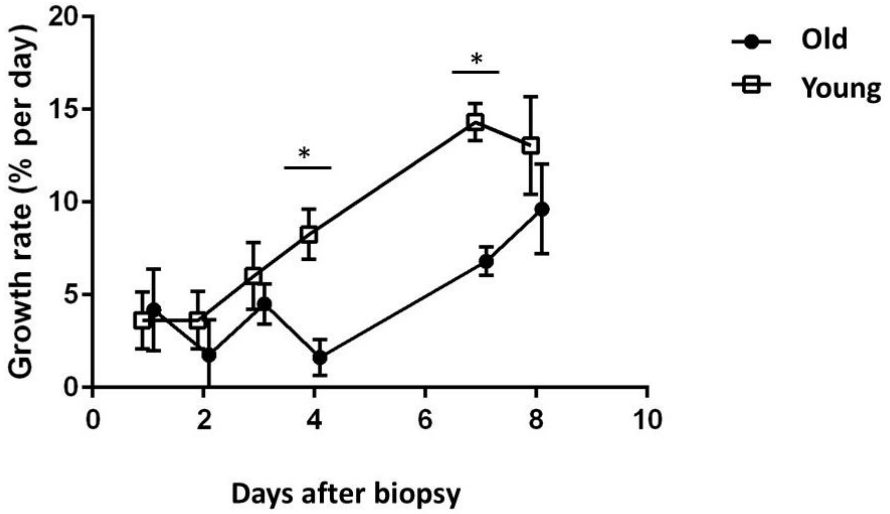

Figure 11. Growth rate of the fin regeneration after biopsy of young (age 5 weeks) and old (age 12 weeks) fish within 8 days. Both genders of each age groups have been combined and the difference of fin regeneration is analysed only between young and old fish. The sample size was 16 fish in both groups. The growth rate was analysed by calculating the increase of the fin length at each timepoint, related to the cut biopsy on day 0. The daily growth rate on day 7 was calculated by dividing the growth rate on day 7 with the number of days when the measurements were not performed. The regeneration rate of the fin was declined with old fish and significant difference between the two age groups was observed on day 4 and 7, *$p<0.05$.

Significant difference in the growth rate between young and old fish was observed on day 4 and 7. The growth rate was declined with old fish on day 4 ($p<0.05$) and on day 7 ($p<0.05$). The mean growth rate on day 4 was 2 % with old fish and 8 % with young fish and on day 7 the growth rate was 7 % with old fish and 14 % with young fish. Furthermore, the difference of the growth rate between the age groups was 6 % on day 4 and 7 % on day 7. Therefore, the most significant difference on the growth rate between young and old fish was observed on day 7.

2.3.1 Gene expression analysis

The aim of the gene expression analysis was to study the differences in gene expression between young and old fish at different timepoints. Ribonucleic acid (RNA) was isolated from fin biopsies that were cut on day 0, 4 and 8. The selected genes, *cdh5*, *flt1*, *kdr* and *vegfa*, are related to

angiogenesis, which is essential in the wound healing process. In addition, two genes with reported changes in the expression levels during aging in *N. furzeri* were analysed. Collagen type X alpha 1 (*col10a1*) is known to downregulated and cytochrome P450 family 1 subfamily B polypeptide 1 (*cyp1b1*) is known to be upregulated during aging. Internal control gene was *insr* and negative control included no sample.

The expression levels of angiogenesis related genes, *cdh5*, *flt1*, *kdr* and *vegfa*, between the two age groups on days 0, 4 and 8 from male fish are presented in Figure 12. The highest expression levels of *cdh5*, *flt1* and *kdr* were with old fish on day 0 and the expression level was significantly higher compared to the young fish ($p<0.001$). No other significant differences on the expression levels of these genes were found between the age groups. The highest expression levels were observed with *flt1*. The expression of all these genes decreased from day 0 to day 8 with both age groups. Significant difference between days 0 and 4 was found in the expression of *cdh5*, *flt1* and *kdr* with old fish ($p<0.001$). No other significant differences in the expression levels of these genes between the timepoints were observed. The highest expression level of *vegfa* was found with young fish on day 0. However, no significant differences on the expression levels of *vegfa* were found either between the age groups or between each timepoint.

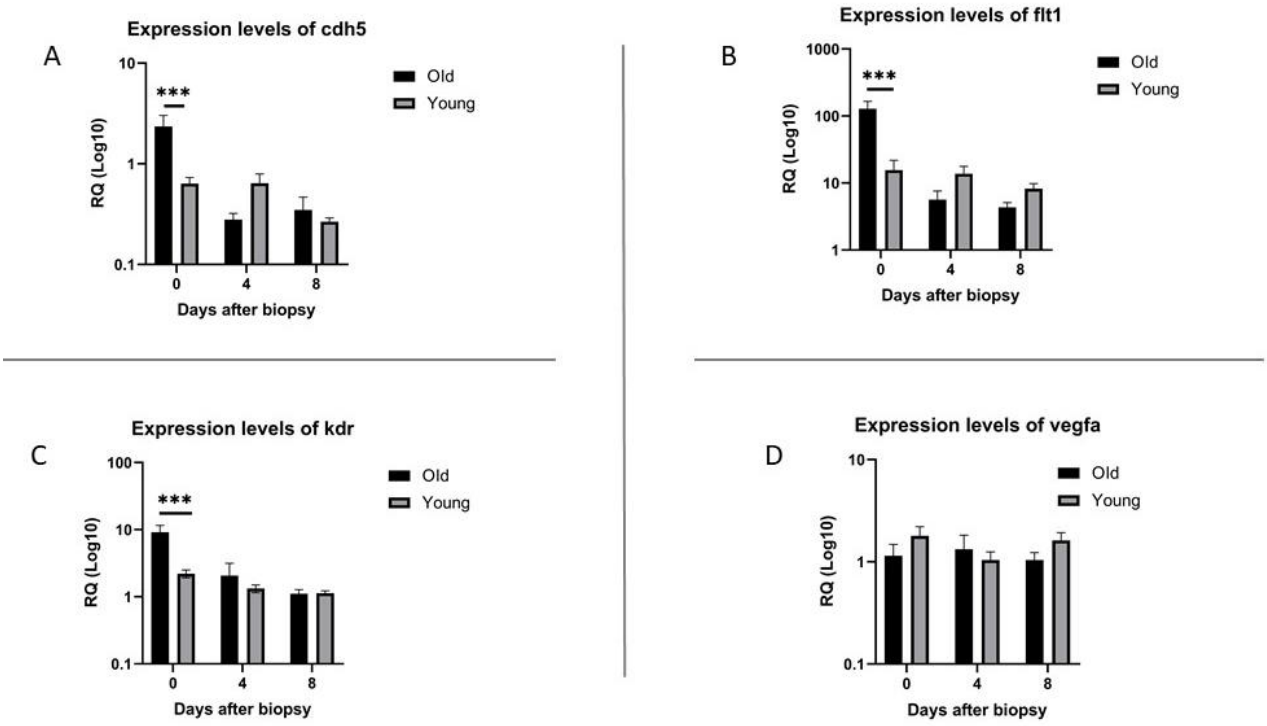

Figure 12. Expression levels of angiogenesis related genes *cdh5*, *flt1*, *kdr* and *vegfa* on day 0, 4 and 8 after fin biopsy of young (age 5 weeks) and old (age 12 weeks) male fish. The sample size was eight fish in each group. A-C) The expression levels of *cdh5*, *flt1*and *kdr* of old fish on day 0 were significantly higher than with young fish (***p<0.001). In addition, significant difference between days 0 and 4 with old fish was observed in the expression of *cdh5*, *flt1* and *kdr* (p<0.001). D) The highest expression level of *vegfa* was observed with young fish on day 0. No significant differences on the expression level of *vegfa* was observed either between the age groups or between each timepoint. RQ = relative quantification.

The expression levels of genes *col10a1* and *cyp1b1b* are presented in Figure 13. Significantly higher expression levels of *col10a1* on days 0 and 4 were observed with young fish compared to old fish (p<0.05). However, on day 8 the expression level was slightly higher with old fish, but no significant difference was observed. In addition, the expression level of *col10a1* was significantly increased from day 0 to day 8 with old fish (p>0.01) and decreased from day 4 to 8 with young fish (p>0.01). The expression level of *cyp1b1* was significantly higher with young fish on day 0 compared to old fish (p<0.05). The expression level of *cyp1b1* was significantly decreased from day 0 to 4 with young fish (p<0.05).

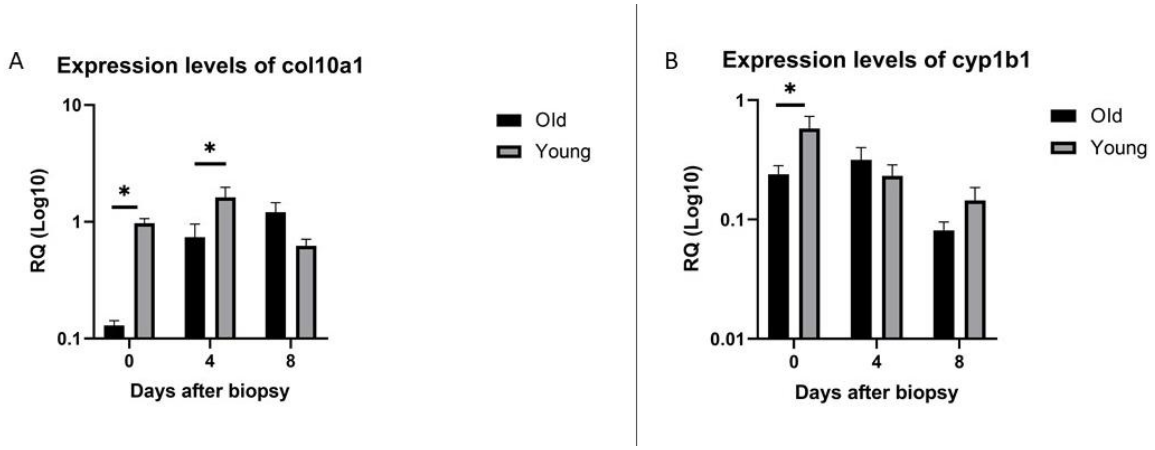

Figure 13. Expression levels of *col10a1* and *cyp1b1* genes on day 0, 4 and 8 after fin biopsy of young (age 5 weeks) and old (age 12 weeks) male fish. The sample size was eight fish in each group. Gene *col10a1* is known to be downregulated and *cyp1b1* upregulated in *N. furzeri* during aging. A) Significantly higher expression level of *col10a1* was observed with young than old fish (*p<0.05) on day 0 and 4. On day 8 the expression level was slightly higher with old fish, but no significant difference was observed. The expression level was significantly increased from day 0 to day 8 with old fish (p>0.01) and decreased from day 4 to 8 with young fish (p>0.01). B) The expression level of *cyp1b1* was significantly higher with young fish on day 0 compared to old fish (*p<0.05). The expression level was significantly decreased from day 0 to 4 with young fish (p<0.05). RQ = relative quantification.

2.3.2 Histological analysis

Histological analysis was performed to study differences on cell structures during fin regeneration between young and old fish. Tissue samples were taken from cut fin biopsies on day 0 and the regenerated part of the fin on day 8 from young and old fish. Then, the tissue samples from male fish on day 0 were processed into sections and stained with haematoxylin and eosin, because the samples from female fish on day 0 and all samples on day 8 were too small to be processed into sections. The differences in cell structures between young and old fish was observed using Pannoramic P1000 slide scanner microscope. Example of tissue samples from young and old male fish on day 0 is presented in Figure 14.

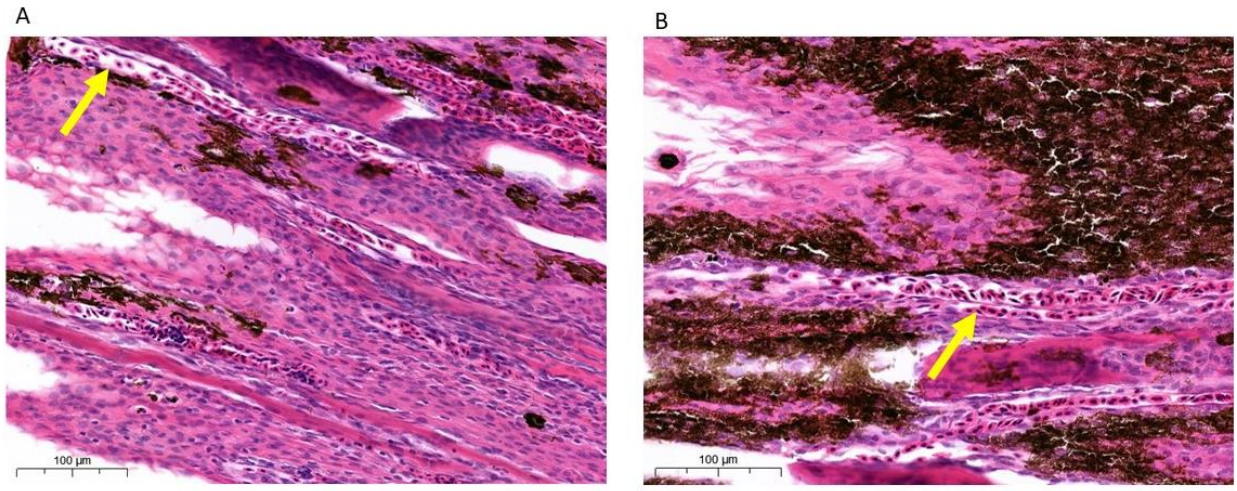

Figure 14. Example of tissue samples from cut biopsies of caudal fin from male fish on day 0. Blood vessels are marked with yellow arrows. Fish erythrocytes have nucleus, although human erythrocytes have no nucleus. A) Fin tissue from old (age 12 weeks) fish with 14.3x magnification. B) Fin tissue from young (age 5 weeks) fish with 15.1x magnification.

Number of blood vessels per millimeter from young and old male fish were calculated from the tissue samples on day 0 and the results are presented in Figure 15. The number of blood vessels with old fish was signicantly higher ($p<0.001$) compared to young fish. The mean number of blood vessels with old fish was 9 blood vessels per millimeter and with young fish 4 blood vessels per millimeter.

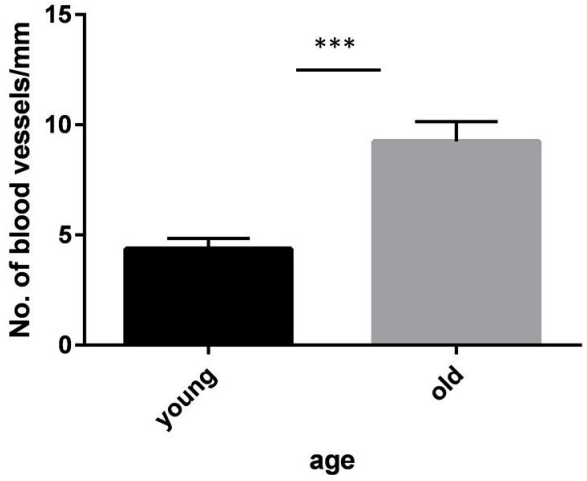

Figure 15. Number of blood vessels on tissue samples from cut biopsies of caudal fin of young (age 5 weeks) and old (age 12 weeks) male fish on day 0. The sample size of both groups was 8 fish and 2-8 measurements were performed from each sample. The mean number of blood vessels was signicantly higher with old fish compered to young fish, *** $p<0.001$

2.4 Drug study

The drug study was performed to investigate the effect on wound healing of two drugs, angiogenesis inhibiting semaxanib and angiogenesis activating roxadustat with old fish (age of 32 weeks). Semaxanib inhibits angiogenesis by blocking Vegf receptors, such as Flt1 and Kdr. Roxadustat activates angiogenesis by mimicking hypoxia in order to stabilize Hif, which stimulates its target genes, such as *vegf,* during hypoxia. The drugs were diluted into dimethyl sulfoxide (DMSO), which was also used as negative control. The drugs and DMSO were administered to the fish by mixing them with tank water of each fish. All three study groups included eight fish, both males and females. The drugs were administered to the fish after cutting a piece of the caudal fin from the fish. The growth length of the fin was measured every second day. The results of the drug study are presented in Figure 16. The results show that the growth length of the fin was significantly declined with the semaxanib group on days 6 and 8 compared to the control group ($p<0.001$). On day 6 the mean growth length of the fin was 2 % with the semaxanib group and 16 % with the control group. On day 8 the mean growth length was 11 % with the semaxanib group and 24 % with the control group. In addition, no significant difference was found in the growth length of the fin between roxadustat and control groups.

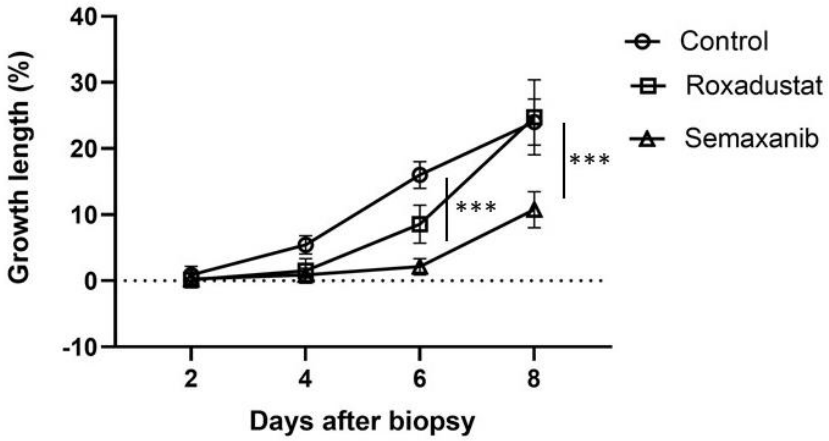

Figure 16. Growth length of the fin regeneration of old (age 32 weeks) male and female fish after drug administration within 8 days. The sample size was eight fish in each group. Roxadustat activates and semaxanib inhibits angiogenesis, and DMSO was used as negative control. Growth length of the fin was significantly declined with the semaxanib group on days 6 and 8 compared to the control group, *** $p<0.001$. In addition, no significant difference was observed in the growth length of the fin between roxadustat and control groups.

The growth rate of the fin after drug administration was analysed by calculating the increased length from the measured length of the whole fin at each timepoint related to the cut biopsy on day 0. The results of the growth rates at each timepoint and with all study groups are presented in Figure 17. The growth rate was significantly declined with the semaxanib group on day 6 compared to the control group ($p<0.001$). The mean growth rate on day 6 was 1 % with the semaxanib group and 11 % with the control group. However, the growth rate was significantly increased with the roxadustat group on day 8 compared to the control group ($p<0.01$). The mean growth rate on day 8 was 16 % with the roxadustat group and 7 % with the control group.

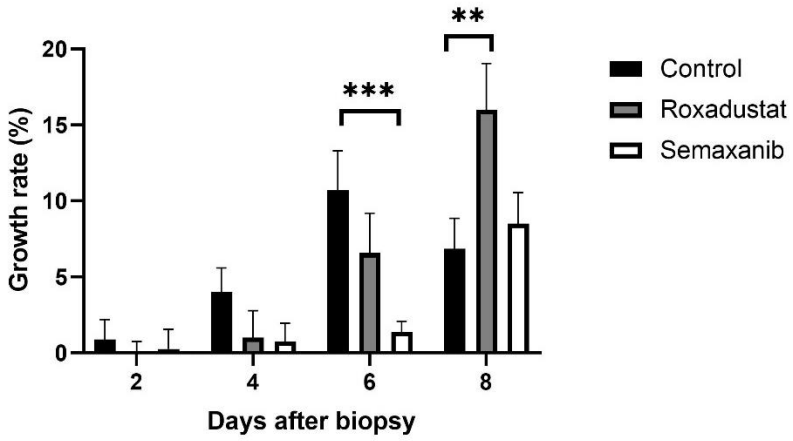

Figure 17. Growth rate of the fin regeneration of old (age 32 weeks) male and female fish after administration of semaxanib and roxadustat within 8 days. The sample size was eight fish in each group. Roxadustat activates and semaxanib inhibits angiogenesis, and DMSO was used as negative control. The growth rate was significantly declined with the semaxanib group on day 6 compared to the control group, ***$p<0.001$. The growth rate was significantly increased with the roxadustat group on day 8 compared to the control group, **$p<0.01$.

2.4.1 Weight measurements

The possible toxicity of the investigated drugs was observed by calculating weight loss of each fish from the weight measured at the beginning and at the end of the study. Furthermore, the changes in the weight loss between the study groups and individual fish during the study were analysed. The weight loss is presented in percentage, because sizes of the fish are variable. The results of the weight loss between the study groups are presented in Figure 18. The weight loss was significantly higher in the semaxanib group ($p<0.05$) compared to the control group and no significant difference was observed between the roxadustat and control groups. The mean weight loss in the control group was 11 %, in roxadustat group 15 % and in semaxanib group 23 %. In addition, one fish from each group died during the experiment.

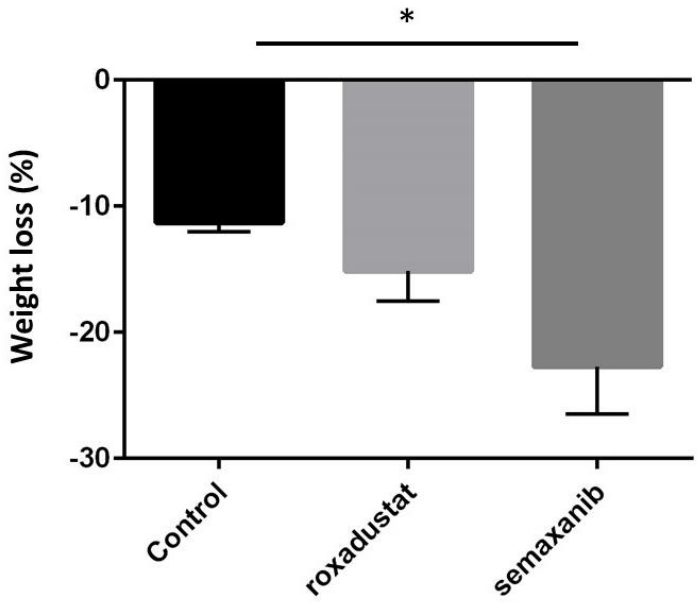

Figure 18. Weight loss of the old (age 32 weeks) male and female fish between each study group during the drug study. The sample size was eight fish in each group. Roxadustat activates and semaxanib inhibits angiogenesis, and DMSO was used as negative control. The weight loss was significantly higher in the semaxanib group (*$p<0.05$) compared to the control group and no significant difference was observed between the roxadustat and control groups.

The correlation of the growth length of the fin during the experiment and weight loss of each fish was analysed to further observe possible toxicity of the investigated drugs. The results are presented in Figure 19. No significant correlation between weight loss and poor fin regeneration was observed in any of the study groups.

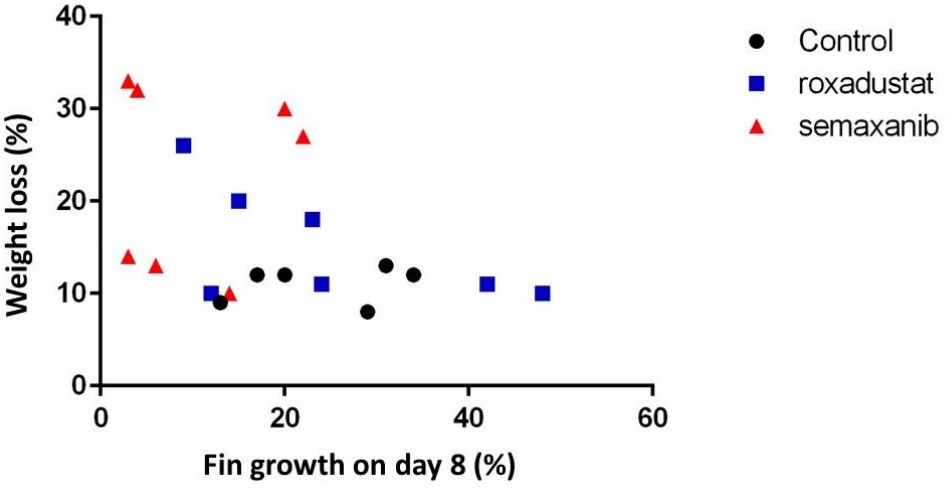

Figure 19. Correlation between fin regeneration and weight loss of the old male and female fish (age of 32 weeks) during the drug study. The sample size was eight fish in each group. Roxadustat activates and semaxanib inhibits angiogenesis, and DMSO was used as negative control. No significant correlation between weight loss and poor fin regeneration was observed in any of the study groups.

2.5 Summry

In this study the aging related impaired fin regeneration during wound healing was investigated. The study was performed by investigating the migration of keratocyte cells isolated from fish scales, and differences on fin regeneration and gene functions during the regeneration process. In addition, the effect of two wound healing affecting drugs was investigated. When studying the migration of isolated keratocytes, no significant difference was found between the migration rate of the keratocytes from young and old fish.

Based on the results from the wound healing study, the tissue regeneration and wound healing was significantly decreased from day 7 after the biopsy with old fish compared to young fish. In addition, no difference was observed between male and female fish. Furthermore, the growth rate was declined from day 4 with old fish compared to young fish. Several angiogenesis regulating genes have a role in the wound healing process, although searching of potential drug targets from these genes and drugs to improve the wound healing process needs more investigation.

The results from the drug study show that fin regeneration was declined from day 6 after biopsy with fish receiving angiogenesis inhibiting drug, semaxanib. In addition, fin regeneration rate was increased on day 8 after biopsy with fish receiving angiogenesis activating drug, roxadustat. Weight loss was highest in the semaxanib group, but no correlation with weight loss and poor fin regeneration was observed in any of the study groups.

3 DISCUSSION

The aging induced decline on wound healing was investigated in this study with *N. furzeri* fish. First, the early stages of wound healing were studied on cell culture using isolated keratocyte cells from scales that were removed from young and old fish. Second, differences on fin regeneration and gene functions during the regeneration process with gene expression analysis was studied between young and old fish. Finally, specific wound healing affecting drugs were administered to the fish and the effect of the drugs was studied. In addition, the median lifespan of the research model, *N. furzeri* fish, was determined.

The median lifespan of *N. furzeri* strain MZCS222 at the facility was determined from the analysed survival data, and the median lifespan was found to be 34 weeks. However, the determined median lifespan was higher than the earlier reported median lifespan, which is 18 weeks (Blažek et al., 2017). The lifespan of the fish in the facility may have been increased due to lower temperature of tank water of the fish (+ 26 °C), because usually the temperature of tank water of *N. furzeri* is + 28 °C, and aging of *N. furzeri* is delayed with lower temperature and restricted food administration (Polačik et al., 2016).

3.1 Isolation of keratocytes

Isolation of keratocytes from fish scales is a widely used method to study aging related changes of reepithelialisation and cell migration during early stages of wound healing. In this study the method optimization was extremely challenging and time consuming, because protocols for isolation of keratocytes from *N. furzeri* has not been reported previously. However, isolation of keratocytes has been previously published with several other fish species, such as zebrafish (Rapanan et al., 2015), goldfish (*Carrasius auratus*) (Lee and Jacobson, 1997) and black tetra (*Gymnocorymbus ternetzi*) (Svitkina et al., 1997). Therefore, this isolation of keratocytes study was performed by following and combining these protocols for different fish species.

At the beginning of this study the fish scales were collected to isolate keratocyte cells from the scales. Collection of the scales was first performed at the same time when setting up mating with male and female fish in mesh bottomed tank, where the fish usually loose some scales. After few hours of mating the detached scales were collected by pouring the tank water through a plastic tea sieve. However, only few scales were received and low number of keratocyte cells were grown out from

these scales. The main reason for the low number of keratocytes was probably too long delay between scale detachment from the fish and transferring of the scale to culture medium, which reduced cell survival. The purpose of the scale collection simultaneously with mating was to avoid harming the fish with anaesthesia, which is a widely used method of removing scales, as described in the protocols used in this study. When the scales are removed from anesthetized fish, transferring of the scales to culture medium is faster.

Cell culturing was also challenging in this study. First, the scales were placed on a dish into a droplet of culture medium, and a coverslip was added on top of the scales to let the cells attach to the bottom of the dish more efficiently. This method is described in previously published protocols (Lee and Jacobson, 1997; Barnhart et al., 2011). After overnight incubation, coverslips and scales were removed, and incubation was continued for few hours to let the cells attach again to the bottom of the dish. To observe individual cells, the cells were detached from the bottom of the dish and each other with Trypsin-EDTA (Sigma) according to previously published protocols (Svitkina et al., 1997; Barnhart et al., 2011). Then, the cells were collected by centrifuging, resuspending into culture medium and incubating overnight. However, low number of keratocytes were grown out from the scales, which may have been caused by several reasons. For example, some of the cells have probably been attached to the scales and removed within the removal of the scales. In addition, the cells may have been died during centrifuging or treatment with Trypsin-EDTA. Therefore, Cell Dissociation buffer (Gibco), which is enzyme-free, phosphate buffered saline (PBS) -based and more sensitive to the cells, was tested instead of Trypsin-EDTA. However, the treatment with Cell Dissociation buffer did not increase the number of isolated keratocytes. Therefore, culturing of the cells was further optimized by simplifying the method.

One of the most important issues in the cell culturing is that the scales are well attached to the bottom of the dish, which leads to successful migration of keratocytes. Therefore, in this study the most successful method of cell culturing was by placing the scales to an empty dish and let them attach to the bottom of the dish for few minutes until the scales were almost dry before adding the culture medium. This method is described in the previously published protocols (Svitkina et al., 1997; Rapanan et al., 2015). Adding the culture medium at the right time is critical step in the procedure. If the culture medium is added too soon, the scales may not attach to the bottom of the dish and start floating. If the culture medium is added too late, the scales and the attached cells may become too dry and no keratocytes grow out from the scales. In addition, sometimes only few epithelial cells are attached to the scale, which prevents the migration of keratocytes from the scales (Rapanan et al., 2015). In this study keratocytes were grown out from approximately 64 % of the scales that were

placed on cell culture. The number is lower than previously reported mean outgrowth of keratocytes from 75 % of the scales with zebrafish (Rapanan et al., 2015). However, these are different species, which may have differences between the mechanisms of the outgrowth of keratocytes, and therefore the numbers are not completely comparable.

Migration of keratocytes and number and organization of actin filaments in the cells was studied with microscopic techniques, and some difficulties were observed with staining of the cells. For the keratocyte migration study, the cell nucleus was stained with live cell fluorogenic DNA labelling probe, SiR-DNA SC007 (Spirochrome), to detect live cells more easily. However, the signal of the nucleus staining was too weak, although it became stronger during the experiment. Probably the incubation period of 30 minutes for staining was too short. In addition, the use of this dye has not been reported previously with *N. furzeri*. According to the SiR-DNA (SC007) Live Cell Fluorogenic DNA Labelling Probe (SpiroChrome Probes for Bioimaging) product information (February 2017), the penetration of the dye into the cells may vary between different species and may be poor due to high number of efflux pumps in cells. To visualize actin filaments, the keratocyte cells were stained with fluorescent phalloidin and immunostaining of the cells was performed with primary antibody pTyr PY-20 (mouse monoclonal) and secondary antibody a-mouse 488 for staining of focal adhesions. However, the use of these antibodies has not been previously reported with *N. furzeri*, and the signal of mouse antibodies was found to be too strong with nonspecific staining.

No difference on the migration srate was observed between the two age groups in this study. The migration of the cells from young fish was expected to be faster, because keratocytes participate to the wound healing process and wound healing is declined during aging (Eming et al. 2014). However, the unexpected results are probably caused by the several challenges during the method optimization and technical problems with the microscope. In addition, the migration rate of keratocytes in *N. furzeri* may be similar with young and old fish.

More investigation is needed for studying isolated keratocytes from *N. furzeri*. Therefore, the differences of keratocyte migration between isolated cells from young and old fish should be studied further. In addition, the differences of number and organization of actin filaments from the isolated keratocyte cells between young and old fish should be further studied.

3.2 Wound healing study

Differences on fin regeneration and gene expression during the regeneration process between young and old fish was investigated in the wound healing study. Based on the results of this study the fin regeneration was declined with old fish compared to young fish. The results are consistent with the previously published results, where fin regeneration after biopsy was declined with old *N. furzeri* fish compared to young fish (Wendler et al., 2015). Only this one study of aging related decline in fin regeneration with *N. furzeri* by Wendler et al. has been published previously.

Decreased aging related tissue regeneration has been studied also with other species. For example, impaired aging related fin regeneration has been previously reported with zebrafish (Anchelin et al., 2011; Shao et al., 2011; Tsai et al., 2007). However, in one study no difference in the fin regeneration between young and old zebrafish was observed (Itou et al., 2012). The variable results may have been caused by differences in the duration of each of these studies and differences between the age groups of young and old fish in each study. The mechanisms of aging related impaired tissue regeneration have been also widely studied, especially with mice. For example, decreased activity of specific muscle stem cells, which leads to declined regeneration of muscles with aged mice after muscle injury has been reported (Conboy et al., 2005¸ Sousa-Victor et al., 2014¸ Chakkalakal et al., 2012).

In the study by Wendler et al. significant difference of the growth length between young and old fish was observed already on day 3, and the difference increased during the study. However, in this study the significant difference of growth length was observed on day 7 and 8. The main reason for the variable results received from this study is probably caused by the smaller difference between the age groups in this study (age of young fish 5 weeks and age of old fish 12 weeks), compared to the difference in the study by Wendler et al. (age of young fish 8 weeks and age of old fish 50-60 weeks). In addition, in this study the measurements of fin growth length were not performed between days 4 and 7, and therefore the difference in growth length and rate could have been present earlier than on day 7.

Significant difference in the growth rate between young and old fish in this study was observed on day 4 and 7, but not on day 8. However, in the study by Wendler et al., the difference of the growth rate increased during the study starting from day 3. In this study the difference in growth rate between young and old fish was not observed on day 8 probably because the mean growth length of young fish was already 77 % on day 8. Therefore, the fin growth rate of young fish may have been already decreased on day 8 in this study.

Some differences between the results of the growth length and rate in this study was observed. Significant difference of growth length between young and old fish was observed on day 7 and 8,

although significant difference of growth rate was observed between young and old fish on day 4 and 7. The differences may have been affected by the changes in the study groups when analysing the results of the growth rate. The fin growth length was analysed by using four study groups, including young males and females and old males and females. However, the fin growth rate was analysed by combining both genders into two study groups of young and old fish, and the increased number of fish in each study groups decrease the variance of the groups.

The expression levels of specific angiogenesis related genes, *vegf*, *flt1*, *kdr* and *cdh5*, were analysed in this study. Generally, the number of Vegf and its receptors, Flt1 and Kdr, is increased during tissue repair process (Eming and Krieg, 2006). In addition, Cdh5 has a significant role during the wound healing process (Abu Taha and Schnittler, 2014). However, no significant difference on the expression levels of these genes between young and old fish or between different timepoints was observed during this study. Except expression levels of *cdh5*, *flt1* and *kdr* were decreased between days 0 and 4 with old fish. In addition, expression levels of *cdh5*, *flt1* and *kdr* were significantly higher on day 0 with old wish compared to young fish. The expression levels of these genes during wound healing has not been previously reported with *N. furzeri*. Therefore, it is possible that these genes do not have significant role in the wound healing process of *N. furzeri*. In addition, the results of this study may have been influenced by the challenges of cutting the regenerated piece from the caudal fin of the fish, due to the small size of the fish. Furthermore, female fish are smaller than males and they have less pigment in the caudal fin, which complicates the cutting of the regenerated piece of the fin. When tissue samples were cut from the small regenerated part of the fin, some tissue from the fin before regeneration may have been also cut at the same time. Therefore, samples from day 4 and 8 may include also genes from the fin tissue before regeneration, which may have affected to the results.

The expression levels of specific aging related biomarker genes, *col10a1* and *cyp1b*, were also analysed. *col10a1* is known to be downregulated and *cyp1b1* upregulated during aging in the samples of skin and brain from *N. furzeri* (Petzold et al., 2013). In this study, significantly lower expression levels of *col10a1* were observed with old fish compared to young fish on day 0 and 4, as previously reported by Petzold et al. However, the expression level of *cyp1b1* was also significantly lower with old fish compared to young fish, and no other significant difference between the age groups was observed. Therefore, the analysed expression level of *cyp1b1* in this study was not consistent with the results from Petzold et al. Probably the reason for the unexpected expression levels of *cyp1b1* is that the old fish in this study were not old enough (age of 12 weeks) and the difference in gene expression of this gene may be seen only with older fish. In addition, the wound healing process may

affect to the expression levels of these biomarker genes, and therefore the differences between the age groups at different timepoints were not observed. The number of study subjects, which was 8 fish in each group, may have also been too low for detecting the difference between young and old fish.

Tissue samples were taken from the fin of the fish before cutting and after regeneration for histological analysis to compare cell structures from young and old fish. However, the samples from old females on day 0 and from all the fish on day 8 were too small to process into sections. In addition, tissue samples from young female fish were used only for gene expression analysis and no samples for histological analysis was taken. Therefore, histological analysis by comparing tissue samples from the fins before cutting and after regeneration could not be performed. Only tissue samples from young and old males on day 0 was analysed. However, measuring and analysing of these tissue samples was extremely difficult, and therefore the results are probably not completely reliable. The tissue samples from young and old male fish on day 0 were difficult to compare, because the samples were not similar. For example, all the samples were different sizes, and the samples from young fish were smaller than the samples from old fish, due to the smaller size of young fish. Furthermore, different parts of the fins were presented in some of the tissue samples, and some of the samples included only small pieces of the fin tissue instead of whole part of the fin. Therefore, variable number of blood vessels were probably presented in the samples.

3.3 Drug study

In the drug study the effect on wound healing of two drugs, angiogenesis inhibiting semaxanib and angiogenesis activating roxadustat, was investigated. Semaxanib inhibits angiogenesis by blocking Vegf receptors. Roxadustat activates angiogenesis by mimicking hypoxia to stabilize Hif, which stimulates its target genes, such as *vegf*, during hypoxia. This study was performed with old fish with age of 32 weeks.

The results of this study show that the fin growth length was significantly declined with the semaxanib group on day 6 and 8 compared to the control group. This is probably caused by the effect of the drug, because semaxanib decreases angiogenesis by inhibiting the Vegf receptors, Flt1 and Kdr (Haddad, 2012). In addition, studies with zebrafish embryos has been performed with semaxanib, which caused severe defects in the formation of blood vessels (Farooq et al., 2018). Treatment with semaxanib has been shown to inhibit fin regeneration in zebrafish embryos and adults by inhibiting fibroblast growth factor receptor, which is important for fin regeneration (Kawakami et al., 2004; Mathew et al., 2006;

Poss et al., 2000). However, studies with semaxanib in *N. furzeri* has not been reported previously. Semaxanib may inhibit angiogenesis also with *N. furzeri*, and therefore the fin regeneration was declined on day 6 and 8 in this study.

The growth rate was declined significantly with semaxanib group compared to the control group on day 6, but not on day 8 in this study. The reason may be that the effect of semaxanib is most significant at the beginning of the treatment. For example, declined fin regeneration has been reported already four days after wounding with adult zebrafish that have received semaxanib, compared to the fish that have not received semaxanib (Poss et al., 2000).

No significant difference in the growth length with the roxadustat group compared to the control group was observed in this study. However, the growth rate with roxadustat group was increased significantly on day 8 compared to the control group. The increased fin growth rate is probably caused by the effect of roxadustat, which activates angiogenesis, leading to increased tissue repair (Zhou et al., 2015). In addition, the effect of roxdustat has been reported with zebrafish embryos in one study, where known Hif targets, glucose trasporter 1 and lactate dehydrogenase A, were upregulated in zebrafish embryos after treatment with roxadustat (Jain et al., 2016). Although the effect of roxadustat has not been reported previously with *N. furzeri*, roxadustat may increase angiogenesis and tissue repair also with *N. furzeri* leading to increased fin regeneration rate observed in this study. The increased growth rate was observed only on day 8, which may have been caused by the low effect of roxadustat on fin regeneration with *N. furzeri* or the effect of the drug may begin slowly.

In this study the mean fin growth length in the control group was 24 % on day 8, which is lower than the mean fin growth length with the old fish observed in the wound healing study (43 % on day 8). The reason for the lower growth length observed in the drug study is probably because the old fish in this study (age of 32 weeks) were significantly older than the old fish in the wound healing study (age of 12 weeks), and because fin regeneration declines within aging. However, the fish in this study were not too old, because differences in fin regeneration between the study groups were observed. In addition, in this study the fish of the control group were treated with DMSO, which may also affect wound healing process, because the effect of DMSO with *N. furzeri* has not been reported previously.

The weight of each fish was measured at the beginning and at the end of the study to investigate possible toxicity of the treatments. In this study the weight loss was higher in the semaxanib group compared to the control and roxadustat groups. The higher weight lost may have been caused by the effect of the drug, because semaxanib inhibits angiogenesis (Haddad, 2012), although studies of semaxanib with *N. furzeri* has not been reported previously. However, no unusual behaviour was

reported in one study with adult zebrafish when treated with semaxanib for four days at concentrations of 1.7 – 17 µM (Poss et al., 2000). In addition, high variance in the weigh changes was observed in this study between individual fish in each of the study groups, and the highest variance was observed in the semaxanib group. The high variance in the wight changes may have been caused by the small number of fish in each study groups. Additionally, the high weight loss of individual fish may be related to poor eating of the fish during this study.

The weight of the fish in each of the study groups was decreased, which is probably due to the once daily food administration during this study. Normally, the fish are fed twice a day in the facility. In addition, at the beginning of the study the fish were fed before the weight measurements, and at the end of the study the fish were not given food before the last weight measurements, which have probably caused lower weight measurements at the end of the study.

The correlation of weight loss and poor fin regeneration was analysed to further investigate possible toxicity of the treatments. No correlation was observed in any of the study groups, which indicates that the treatments have no toxic effects to the fish. However, the correlation may not have been observed due to the high variance of the results because of the small study groups and short duration of this study. In addition, one fish from each group died during this study. However, it was probably not related to any of the treatments, because same number of fish died in each group (13 %). Furthermore, the probability of survival for 32 weeks old fish was 65 % determined from the survival analysis.

A gene expression analysis should be performed to study the expression levels of specific angiogenesis related genes and to further investigate the effect of semaxanib and roxadustat on fin regeneration with *N. furzeri*. In addition, histological analysis to investigate differences on cell structures caused by these drugs should be performed.

3.4 Summary

The mechanisms of declined wound healing caused by aging were investigated in this study, because impaired aging related wound healing is a significant burden affecting millions of people in the world. The study was performed with *N. furzeri* fish, which is a novel animal model for aging studies due to its rapid aging and short lifespan. This study began by investigating the early stages of wound healing on cell culture using keratocyte cells, which were isolated from fish scales. Then, fin regeneration and gene functions during the regeneration process was studied. Finally, the effects of specific wound

healing affecting drugs was investigated. Several challenges with the methods were observed during this study, because only few similar studies with *N. furzeri* has been reported previously.

The isolated keratocytes were used to study differences on migration rate of the cells between young and old fish. However, no difference on the migration rate was observed between the two age groups. The results may have been affected by several challenges with the method optimization, because protocols for isolation of keratocytes from *N. furzeri* has not been reported previously. More investigation is needed with isolated keratocytes from *N. furzeri*. Therefore, the differences of keratocyte migration between isolated cells from young and old fish should be studied further. In addition, the differences of number and organization of actin filaments from the isolated keratocyte cells between young and old fish should be further studied.

The results from the wound healing study show that the tissue regeneration and wound healing was significantly decreased with old fish compared to young fish. In addition, no difference was found between males and females. The expression levels of specific angiogenesis regulating genes during the fin regeneration with young and old fish was also studied. However, no significant difference in the expression levels of these genes was observed between the two age groups and between each timepoint. Angiogenesis and the regulating genes are important in the wound healing process. Therefore, searching of the most significant angiogenesis regulating genes during the wound healing process for potential drug targets needs more investigation.

In the drug study the effect on wound healing of two drugs, angiogenesis inhibiting semaxanib and angiogenesis activating roxadustat was investigated. As a result of this study, the fin regeneration was declined with the fish receiving semaxanib. This indicates that semaxanib probably reduces fin regeneration and wound healing by inhibiting angiogenesis with *N. furzeri*. In addition, fin regeneration rate was increased with the fish receiving roxadustat, which indicates that roxadustat probably activates angiogenesis and tissue regeneration with *N. furzeri*. In future studies a gene expression analysis should be performed to further investigate the effect of semaxanib and roxadustat on fin regeneration with *N. furzeri*. In addition, histological analysis should be performed to investigate differences of these drugs on cell structures.

4 MATERIALS AND METHODS

This study was conducted with *N. furzeri* strain MZCS222 (NF222) with reported median lifespan of 18 weeks (Blažek et al., 2017). The strain was received from Institute of Vertebrate Biology, the Czech Academy of Science. The experimental design of this project includes three parts: *in vitro* cell migration and structure study with isolated keratocytes, wound healing study and drug study.

The authorization for this project has been obtained from Animal Experimental Board (ESAVI/16458/2019). The procedures performed during this study, including removal of a few scales, cutting a small piece from the distal part of the caudal fin and anaesthesia of the fish, are all considered as mild procedures. According to Xing et al., 2014, the similar cutting of caudal fin is a routine procedure performed during genotyping of zebrafish. In addition, the piece that is cut from the fin is fully regenerated, especially in young fish. The procedure of fin cutting is also comparable with routine ear marking of laboratory mice. The fish in the facility were maintained according to protocol from Polačik et al., 2016 by the trained personnel.

At the beginning of this study the needed reagents were prepared. The recipes of the reagents, Methylene blue stock solution (10 g/l), Tricaine stock solution (4 g/l) and Keratocyte culture medium, are presented in Tables 1-3. Recovery tanks and anaesthesia chambers were prepared before each study. Individual recovery tanks with 0.5 mg/l methylene blue (Sigma-Aldrich) were prepared by mixing 2.8 l system water with 140 µl methylene blue stock solution (10 g/l) in 4 l tanks. Methylene blue is added to the tank water to prevent parasite growth (Dodzian et al., 2018). The anaesthesia chambers with 400 mg/l MS222/Tricaine (Sigma) were prepared by diluting 40 ml Tricaine stock solution (4 g/l) with 400 ml system water in 500 ml beaker.

Table 1. Recipe of Methylene blue stock solution (10 g/l).

Component	**Concentration**
Methylene blue	1 %
MilliQwater	

Table 2. Recipe of Tricaine stock solution (4 g/l).

Component	**Concentration**
Tricaine	0.4 %
1 M ph=9 Tris solution	2.1 %
MilliQwater	

Table 3. Recipe of Keratocyte culture medium.

Component	**Concentration**
Leibovitz F-15	
Fetal bovine serum/Fetal calf serum	10 %
Glutamine	1 %
Penicilline-streptomycin	1 %
Gentamicin	0.1 %

4.1 Survival analysis

Survival data of the maintained *N. furzeri* strain MZCS222 (n = 110) was analysed by creating a survival curve using GraphPad Prism 8.4.0. In addition, median lifespan of the strain was determined from the survival curve.

4.2 Isolation of keratocytes

Isolation of keratocytes is widely used model to study aging related changes of reepithelialisation and cell migration during early stages of wound healing. The aim of this study was to investigate the differences in migration of keratocyte cells and number and organization of actin filaments of the cells between young and old fish. Both age groups included male and female fish. Keratocytes were isolated from scales that were collected from the fish of both age groups. The study was performed according to the protocol from Rapanan et al., 2015 with minor modifications. First, anaesthesia chamber with 400 mg/l tricaine and individual recovery tanks with 0.5 mg/l methylene blue were prepared as described previously.

The collection of scales was performed by removing few scales form anesthetized fish. Two young (age of 6 weeks) and two old (age of 24 weeks) fish were individually anesthetized by placing them into the anaesthesia chamber and let to anesthetize until they lose balance and gill movement slows, which takes usually 1-2 minutes. After anesthetized, the fish were places to a petri dish lid and maximum of 10 scales from each fish were removed by holding the caudal fin of the fish with one tweezers and plucking the scales with other tweezers under the microscope (Zeiss). Then, the scales were transferred into wells of 6-well plate with PBS (Biowest). After the procedure the fish were transferred to recovery tanks, where they recovered from anaesthesia in 1-2 minutes, and the tanks were transferred to + 26 °C room overnight. On the following day the fish were returned to their home tanks.

Cell culturing was performed in the laminar hood following mainly the protocol from Rapanan et al., 2015, with minor modifications. The scales were transferred from the wells with PBS into empty wells of µ-Slide 8 well IbiTreat (Ibidi) -plate with tweezers. The scales from each fish were placed in two separate wells, 5 scales were placed per well. After few minutes until the scales were almost dry and attached to the bottom of the wells, 300 µl Keratocyte medium was added into each well and the plates were incubated for two hours at room temperature. After the incubation the plates were ready for studying to motility of the cells of fixation and staining of the cells for imaging the actin filaments of the cells.

4.2.1 Imaging of keratocyte motility

The motility of live keratocyte cells was measured by using time-lapse microscope (Nikon Eclipse Ti2) to image migration of the cells at several timepoints. The differences of migration rate were compared between young and old fish. The scales were searched from each well of the 8-well plate using the time-lapse microscope and the positions of each scale was marked to observe the outgrowth of keratocytes from the scales. Multi-position experiment was performed with 10x objective and exposure time of 50 ms. Hamamatsu camera was used for photographing each position for 8 hours with 15 minutes imaging intervals. Each scale was photographed with 3x3 large images to determine the possible outgrowth of keratocytes cells around the whole scale in one image.

The images were analysed using ImageJ (FIJI) by measuring the migration length of the keratocyte cells from all positions at each timepoint. The migration rate was calculated by relating the migration length to the migration time. Finally, the results were compared between the samples from young and

old fish. The results were analysed using GraphPad Prism 8.4.0 and statistical analysis was performed with unpaired t test.

After the imaging of keratocyte migration, some of the cells were used for RNA isolation to further study the differences in the gene expression analysis between young and old fish. RNA of the keratocytes was isolated according to the NucleoSpin RNA Plus XS Kit (Machery-Nagel) user manual (February 2018), which is used for isolating RNA from samples containing small amounts of RNA. The homogenization of the cells was performed in the wells using the lysis buffer. After isolation, RNA content of each sample was measured with NanoDrop Lite (Thermo Scientific) spectrophotometer. Isolated RNA was converted to complementary DNA (cDNA) according to the Applied Biosystems High-Capacity cDNA Reverse Transcription Kit (Thermo Fischer Scientific) product information sheet (21. March 2016). Due to the variable RNA contents of each sample and based on the samples with smallest RNA content, 200 ng RNA were converted to cDNA from each sample. Program of the thermal cycling conditions was performed with T100 Thermal Cycler (Bio-Rad). However, the gene expression analysis with quantitative reverse transcription polymerase chain reaction (qRT-PCR) could not be performed.

4.2.2 Imaging of actin filaments

Some on the cells were fixed to stabilize the cells for later staining and visualization with microscope. First, keratocyte medium was removed from the 8 well IbiTreat -plates and the cells were briefly washed with PBS. Then, 300 µl 4 % paraformaldehyde (Thermo Scientific) in PBS was added to the wells and the plates were incubated for 20 minutes at room temperature. Finally, 4 % paraformaldehyde was removed, 300 µl PBS was added into each well, and the plates were placed into + 4 °C.

The keratocyte cells were stained with Phalloidin (Invitrogen) to make the actin filaments of the cells visible and with Draq5 nuclear dye (Thermo Scientific). The dyes were diluted according to the recommendations of the manufacturers with bovine serum albumin (BSA) (Sigma) and PBS. The cells were first permeabilized by incubating the plates with 300 µl 0.1 % Triton-X-100 (Sigma) in 2 % BSA/PBS for 15 minutes in room temperature. After permeabilization, the cells were blocked with 300 µl 2 % BSA/PBS for 30 minutes in room temperature. Then, 100 µl mixture of Phalloidin-555 dye 1:50 and Draq5 nuclear dye 1:1000 diluted into 2 % BSA/PBS was added and the plates were incubated for one hour in room temperature. Finally, the plates were washed with 3x300 µl PBS and

placed into + 4 °C in humidor, covered from light. The immunofluorescence staining was used to visualize the actin filaments of the cells. The number and organization of actin filaments between the samples of young and old fish was compared with Leica TCS SP5 confocal microscope.

4.3 Wound healing study

In the wound healing study, the differences in a wound healing process between young and old fish at different timepoints within eight days were investigated. The aim was to find the timepoint where the difference in wound healing between young and old fish begins and where the difference is most significant. Additionally, the possible variance in wound healing process between males and females and individual fish was studied. The results from this study were used to determine the gender and relevant number of fish per group, and the timepoint to investigate the healing process in the following experiments.

The study was performed according to the protocol from Wendler et al., 2015, with minor modification. Four groups of eight fish were used, including young (age of 5 weeks) and old (age of 12 weeks) fish of both genders. First, anaesthesia chamber with 400 mg/l tricaine and individual recovery tanks with 0.5 mg/l methylene blue were prepared as described previously.

On day 0 the fish were individually anesthetized by placing them into the anaesthesia chamber. After anesthetized, the fish were transferred to a petri dish lid and weighted on a scale (Sartorius) and the caudal fin of the fish were photographed using the Zeiss microscope. Then, a small piece of the caudal fin was cut using a scalpel and the fin biopsy was cut in half. One half was placed into an empty 1.5 ml Eppendorf tube for gene expression analysis and the other half was placed into 1.5 ml Eppendorf tube with 1 ml 10 % Neutral buffered formalin (FF-Chemicals) for histological analysis. After the fin biopsy, the caudal fins of the fish were photographed again under the Zeiss microscope. Finally, the fish were placed to recovery tanks, and then the tanks were transferred to + 26 °C room overnight.

Fin biopsies in empty 1.5 ml Eppendorf tubes were stored at -80 °C for gene expression analysis. Fin biopsies with 10 % Neutral buffered formalin were stored in room temperature for histological analysis and formalin was replaced with 1 ml of 70 % ethanol (VWR).

The caudal fins of the fish were photographed under the Zeiss microscope on days 1-4 and on day 7 after the fish were anaesthetized. In addition, the fish were fed on days 2-4 and 7 with frozen bloodworms and the tanks were cleaned by chancing nearly half of the water to filtrated + 26 °C water and removing waste with Pasteur pipet.

On day 8 after the fish were anesthetized, they were weighted on the scale and the caudal fin of the fish were photographed under the Zeiss microscope. The regenerated part of the fin was cut, and the fin biopsy of all males and old females were cut in half for gene expression and histological analysis as described on day 0. Due to the small size of young female fish their regenerated fin biopsies were not cut in half, but the biopsies were collected only for gene expression analysis. After cutting the fin biopsy, the caudal fins of the fish were photographed under the Zeiss microscope. Finally, the fish were transferred to new recovery tanks and the tanks were placed to + 26 °C room. On the following day the fish were returned to their home tanks.

The fin regeneration was analysed by measuring the growth length at each timepoint from the images using ImageJ (FIJI). The growth length was analysed with two measuring techniques, by measuring the length and thickness of the fin at each timepoint. First, the growth length was determined by measuring the length of the whole fin, related to the length of the cut biopsy of each fish. Second, the thickness of the fin was determined by measuring only the regenerated part of the fin, related to the cut biopsy of each fish. The growth rate was determined by calculating the increase of the fin at each timepoint from the measured length of the whole fin, related to the cut biopsy of each fish. In addition, the mean and standard deviations of the measurements from both age and gender groups were calculated. The differences of the length and rate of regenerated fin at each timepoint between each group were analysed using GraphPad Prism 8.4.0. Statistical analysis for growth length was performed with 2way ANOVA Tukey's multiple comparisons test and for growth rate with Sidak's multiple comparison test.

The wound healing study was repeated to further investigate the early timepoints of the wound healing process between young and old fish. The study was performed as described in the previous wound healing study with modifications based on the analysed results. The modifications included differences in the size of study groups, the duration of the experiment and analysing of the results. The number of the fish in the two study groups was eight young (age of 7 weeks) and eight old (age of 14 weeks) fish, including four males and four females in both groups. Additionally, the fin regeneration was measured daily within four days and the regenerated part of the caudal fin was cut on day 4. Furthermore, the fish were not weighted on the scale during the study and fin biopsies were collected only for gene expression analysis.

4.3.2 Gene expression analysis

Differences in the expression of wound healing related genes between young and old fish were investigated in the gene expression analysis, using isolated RNA from male fish. First, different types of cells and genes participating in the wound healing process were investigated to select relevant marker genes for this study. Genes from different cell types in regenerating wound were searched from the CellMarker database (Zhang et al., 2019, http://biocc.hrbmu.edu.cn/CellMarker/), which includes information of genes from different tissues of humans and mice used as biomarkers. Then, these genes were searched from the RNA-seq data from aging skin of *N. furzeri* (Baumgart et al., 2016) to determine which of these genes are expressed with *N. furzeri* and have different expression levels during aging.

Angiogenesis is one of the most important factors in tissue repair and wound healing. Therefore, four well characterized angiogenesis related genes, *cdh5, vegfa, flt1* and *kdr*, were selected as marker genes for this study. Three control genes, *β-actin*, insulin receptor (*insr*) and hypoxanthine guanine phosphoribosyl transferase (*hprt),* were selected as positive control genes for this study. The selected genes have been previously used in studies with *N. furzeri.* In addition, two genes with reported changes in the expression levels during aging in *N. furzeri*, Col10a1 and Cyp1b1, were also selected. According to Petzold et al., 2013, Col10a1 is known to be downregulated and Cyp1b1 upregulated during aging in the samples from skin and brain from *N. furzeri*. and these genes have been used for validation of RNA-seq results using qRT-PCR.

RNA of the fin biopsies was isolated according to the NucleoSpin RNA Plus XS kit (Machery-Nagel) user manual (February 2018). The tissue samples were homogenized with microtube Pellet Pestle Motor (Kontes) with 1,5 ml pestle (Kimble) and centrifuged with Eppendorf Centrifuge 5424. After isolation, RNA content of each sample was measured with NanoDrop Lite (Thermo Scientific) spectrophotometer.

Isolated RNA was converted to cDNA according to the Applied Biosystems High-Capacity cDNA Reverse Transcription Kit (Thermo Fischer Scientific) product information sheet (21. March 2016). Due to the variable RNA contents of each sample and based on the samples with smallest RNA content, 200 ng RNA were converted to cDNA from each sample. Program of the thermal cycling conditions was performed with T100 Thermal Cycler (Bio-Rad).

Sequences of the primers, which were previously used in studies with *N. furzeri*, were found from the published articles. The other primers for the selected genes were designed with NCBI primer design tool (Ye et al., 2012, https://www.ncbi.nlm.nih.gov/tools/primer-blast/). Sequences of selected primers are presented in Table 4. The primers were ordered from Integrated DNA Technologies,

Belgium. Before the PCR reaction, all primers were diluted to 100 µM concentration with sterile water.

Table 4. List of primer sequences used in this experiment to study the differences in gene expression of young (age of 5 weeks) and old (age of 12 weeks) male fish.

Primer	Forward sequence	Reverse sequence	Reference
β-actin	CTTTTCACGGTTGGCCTTAGGGTT	AGATTACTGCCCTGGCTCCTA	D'Angelo et al., 2014
cdh5	CTCTCTAGGCTGCGACTGCT	TTAGTCTCCGGTGGCACAAAA	
col10a1	CCACTGGAAAGGGGTATGTG	GGCAGACCAATTCCATTCTC	Petzold et al., 2013
cyp1b1	CGGACATATTTGGAGCCAGT	AGCTGTTGCTGGTCTTCGAT	Petzold et al., 2013
flt1	AGTCCACCATCCATTGGCCG	GCGGGAAATCACGCAACAG	
hprt	TGAGATCAGAGTGATCGGT	GCTACTTTAACCATTTTGGGAT	Hartmann et al., 2009
insr	TGCCTCTTCAAACCCTGAGT	AGGATGGCGATCTTATCACG	Wendler et al., 2015; Petzold et al., 2013; Hartmann et al., 2009
kdr	GCTCAGTTGGGTCACACGA	ATCCACCGGCAGGTTGTAGT	
vegfa	AAGCCGAGAAGATGAGAGCG	TGTCAGACCAAGGGGAATGC	

The selected genes were replicated from the cDNA samples with qRT-PCR according to the Applied Biosystems PowerUp SYBR Green Master Mix (ThermoFisher Scientific) user guide (4. February 2016). First, cDNA samples were diluted 1:10 with sterile water to receive 2 ng single-stranded cDNA for each reaction. Reaction volume was 10 µl and concentration of primers was 500 nM in each reaction. Wells with no RNA samples were used as negative controls.

Run files were prepared with standard cycling conditions and settings for Applied Biosystems instruments using QuantStudio 12kFlex -software. The plates were run by Turku Bioscience/FFGC Plate Running Service. After running the reactions, the data was analysed using Online software Thermo Fisher Cloud. First, the primers for each gene were tested by analysing the specificity of amplification signal from the amplification and melting plots of the genes. Primers are suitable, if the amplification curves are similar in amplification plot and only one peak of melting curves is seen in the melting plot. Poor primers have different amplification curves and several peaks in the melting

plot. Based on the testing of primers, *insr* and *hprt* were selected as control genes and *β-actin* was rejected. Examples of analysing the PCR reactions are presented in Figure 20.

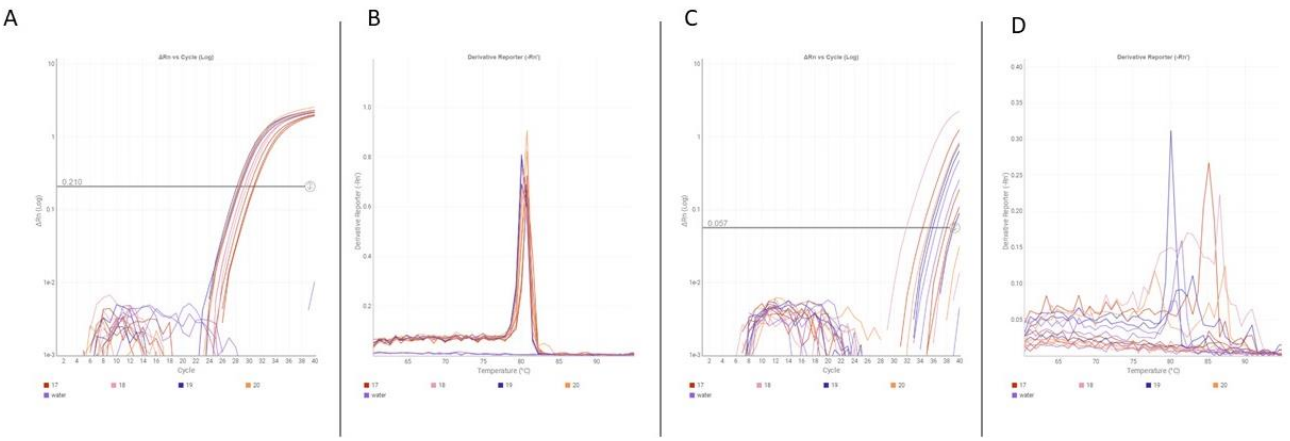

Figure 20. Examples of analysing the PCR reactions by testing primers for genes *insr* and *β-actin*. A) Good primers for *insr*, because the amplification curves are similar in amplification plot. B) Good primers for *insr*, because only one peak of melting curves is seen in the melting plot. C) Poor primers for *β-actin*, because amplification curves are not similar. D) Poor primers for *β-actin*, because several peaks are seen in the melting plot.

The differences of gene expression at each timepoint between the age groups were analysed using GraphPad Prism 8.4.0 and statistical analysis was performed with 2way ANOVA Sidak's multiple comparisons test.

4.3.3 Histological analysis

The aim of the histological analysis was to observe the differences in cell structures from fin tissue before biopsy and regenerated fin tissues between young and old fish. The tissue samples of the fins were processed into sections with haematoxylin and eosin staining by Histology core facility (Histocore), University of Turku. Then, the samples were scanned with Pannoramic P1000 slide scanner microscope to visualize the cell structures of the samples. However, the tissue samples from female fish on day 0 and all samples from day 8 were too small to process into sections. Therefore, only samples from young and old male fish on day 0 were analysed. The number of blood vessels were calculated from the scanned images with CaseViewer (3DHISTECH) program. The difference in the number of blood vessels per mm between the two age groups was compared. The results were analysed using GraphPad Prism 8.4.0 and statistical analysis was performed with Mann Whitney test.

4.4 Drug study

The drug study was performed to investigate two drugs targeting angiogenesis regulating genes, *vegfa, flt1, kdr* and *cdh5*, which were studied in the gene expression analysis. In addition, the aim was to investigate the effect of the drugs to improve the wound healing in old fish. The targeting drugs were searched from DrugBank database (Wishart et al., 2018 https://www.drugbank.ca/), which includes information of both approved and experimental drugs with drug target information. From the search results, two drugs with opposite effects for angiogenesis, semaxanib, SU5416 (Santa Cruz Biotechnology Inc.) and roxadustat, FG-4592 (Cayman Chemical Company), were selected. Semaxanib inhibits Vegfa receptors, Flt1 and Kdr, and roxadustat activates Vegfa by stabilizing Hif during hypoxic conditions.

The drugs were diluted with DMSO (Sigma), which is recommended by both drug manufacturers. Final drug concentrations were selected according to the previous publications, which are presented in Table 5. First, stock concentrations of each drug were performed based on the final concentrations, in order to receive same volumes of DMSO for the doses of both drugs. Furthermore, the same volume of DMSO was used as negative control.

Table 5. Concentrations of stock and final solutions for semaxanib and roxadustat.

Drug	Stock concentration	Final concentration	Reference
semaxanib	10 mM	1 µM	Farooq et al., 2018
roxadustat	25 mM	2.5 µM	Isha et al., 2016

Aged fish (age of 32 weeks) were selected for this study, using eight fish in each of the three study groups, including four fish of both gender in each group. The number of fish needed in each group was calculated using Power and sample Size Calculator (HyLown) by comparing two means with 2-sample and 2-sided equality (http://powerandsamplesize.com/Calculators/Compare-2-Means/2-Sample-Equality). Mean and standard deviation from the wound healing study were used with significance level of 0.05 and 80 % power. The drugs were administered to the fish by mixing them with the tank water of each fish. Two study groups received the investigated drugs, semaxanib and roxadustat, and the same procedure with DMSO was performed to the control group. Individual tanks for each fish were prepared by mixing 200 µl each drug or DMSO and 100 µl methylene blue with

2.0 l system water in 4 l tanks. In addition, anaesthesia chamber was prepared by mixing 45 ml Tricaine stock solution (4 g/l) into 400 ml system water in 500 ml beaker.

Day 0 was performed as described in the wound healing study. After the fish were anaesthetized, they were weighted and photographed, and a piece of their caudal fin were cut. After the biopsy the caudal fins of the fish were photographed again. The fin biopsies were placed into empty 1.5 ml Eppendorf tubes into -80 °C for gene expression analysis. However, eight of the fin biopsies from male fish were cut in half, from which one half was used for the gene expression analysis. The other half was used for histological analysis and placed into 1.5 ml Eppendorf tube with 1 ml 10 % Neutral buffered formalin and stored by replacing formalin with 1 ml of 70 % ethanol. The drugs were administered by transferring the fish to individual tanks, where the drug was mixed with system water with the final concentration presented in Table 5. After the procedure the tanks were transferred to + 26 °C room overnight.

The regeneration of the fin was observed by photographing the caudal fin of the anaesthetized fish every second day under the Zeiss microscope. The fish tanks were cleaned daily by removing 400 ml (20 %) of the tank water and adding 400 ml same concentration of each drug or DMSO mixed with system water. Therefore, the concentrations of each drug and control were stable in the tanks of each fish during the study. In addition, waste was removed from the tanks with Pasteur pipet and the fish were fed with frozen bloodworms daily.

On day 8 the fish were sacrificed with tricaine. Two anaesthesia chambers were performed by mixing 200 ml Tricaine stock solution (4 g/l) with 300 ml system water (first chamber) and 65 ml Tricaine stock solution (4 g/l) with 300 ml system water (final chamber). The fish were placed one at a time into the first anaesthesia chamber until the they lose balance and gill movement stops completely. Then, the fish were weighted on the scale, and photographed under the Zeiss microscope, and the regenerated piece of the caudal fin was cut. The fin biopsies were cut in half for gene expression analysis and histological analysis. After the procedure the fish were placed into the final anaesthesia chamber for 30 minutes to confirm the sacrifice. Finally, the whole fish were fixed by placing each group of fish into one 500 ml bottle with formalin for further analysis.

The length of the regenerated fins was measured from the images with ImageJ (FIJI) to determine both the growth length and rate. The growth length was calculated by comparing the measured length of the fin to the length of the cut biopsy of each fish. The growth rate was calculated by comparing the growth length between two time points to the cut biopsy of each fish. The differences of fin regeneration between both drug groups and controls group were analysed using GraphPad Prism 8.4.0

and statistical analysis was performed with 2way ANOVA Dunnett's multiple comparisons test. In addition, the mean and standard deviations for the measurements for each group were calculated.

RNA of the regenerated fin samples was isolated and converted to cDNA as described in the wound healing study. However, the gene expression analysis with qRT-PCR and histological analysis from tissue samples could not be performed.

4.4.1 Weight measurements

The possible toxicity of the investigated drugs, roxadustat and semaxanib, and control, DMSO, was observed by analysing the weight loss of the fish during the study. The weight loss was calculated from the measured weight of each fish at the beginning and at the end of the experiment. The differences of weight loss between the study groups were analysed using GraphPad Prism 8.4.0 and statistical analysis was performed with one-way ANOVA Dunnett's multiple comparisons test. In addition, the mean and standard deviation for the measurements for each group were calculated.

The correlation between weight loss and fin regeneration during the study was analysed to further observe the possible toxicity of the investigated drugs. The correlation was analysed using GraphPad Prism 8.4.0 and statistical analysis was performed with Spearman correlation test.

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6 ABBREVIATION LIST

Cdh5	vascular endothelial cadherin
Col10a1	collagen, type X, alpha 1
Cyp1b1	cytochrome P450, family 1, subfamily B, polypeptide 1
Flt1	fms-related tyrosine kinase 1
Hif	hypoxia inducible factor
Hprt	hypoxanthine guanine phosphoribosyl transferase
Insr	insulin receptor
Kdr	kinase insert domain receptor
Phd	prolyl hydroxylase inhibitor
Vegfa	vascular endothelial growth factor A

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8 APPENDICES

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