Hypoxia Inducible Factor (HIF)-1 blockade attenuates early graft dysfunction in a model of rat orthotopic lung transplantation

INAUGURAL-DISSERTATION

for the acquisition of the doctoral degree

at the Faculty of Veterinary Medicine

of Justus-Liebig-University, Giessen

Germany

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Giessen 2012

With permission of the Faculty of Veterinary Medicine of Justus Liebig University, Giessen

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Dedication

The most exciting phrase to hear in science, the one that heralds the most discoveries, is not "Eureka!" (I found it!) but "That's funny..."

Isaac Asimov

This work is dedicated to the greatest heroes of scientific research:
the laboratory animals.

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1 Introduction

In the history of medicine, there was always the wish to delay the natural process of decay or malfunctions in the body caused by normal ageing processes, dysfunction by diseases or injury of important organs.

A steadily increasing average age of the society in 1. world countries shows the success of medical treatment, made possible by a constant growth of knowledge about mechanisms associated with pharmacological agents and ongoing development of improved surgical methods and medical equipment.

But, especially in a population of growing age, the number of so called end-stage diseases where all therapies fail is rising. For these patients, a solid organ transplantation is a necessity.

1.1 Transplantation – Data and Facts

The history of lung transplantation (LTX) began 1940 with the Russian scientist Vladimir Demikhov who made the first attempts to transplant a lung from one animal to another and showed that it was technically possible. But the lack of understanding in the process of rejection and the absence of immunosuppressive treatment left no chance in a real long-term survival.[1] In 1960, Sir Frank Burnet and Peter Medawar shared the Nobel Prize in medicine for their pioneering work in the understanding of the immunological process upon rejection.[2] Due to improvements like the invention of the heart-lung machine and immunosuppressive drugs, the first successful lung transplantation with an adequate long-term survival was performed in 1983 by Dr. Joel Cooper at the University of Toronto.[3] From this time on, LTX became a therapy for endstage pulmonary diseases.

In an overview from the Association of Heart and Lung Transplantation in 2010, 183,222 persons were recorded in available Organ Procurement and Transplantation Network (OPTN) data as living with a functional transplanted organ – this means an increase of 1.7% compared to 2006 and a 56.6% increase since 1999.[4]

In 2008 100,597 patients were registered in organ waiting lists in the United States. This is an increase of 3.7% people waiting for an organ since the end of 2007. The lung - waiting list increased even by 8.8%.[4]

Unfortunately, the donation of organs has not kept pace with this rising demand.[5]

Beside this, the LTX has always occupied a special place among all other solid organ transplantations, as it results in poorer graft and patient survival rates than most other transplantations. Nevertheless, the postransplant one - year patient and graft survival increased to 75% in 1995 and became over 80% in 2008.[4] Despite that improvement, this value is still low compared to survival rates of other solid organ transplants of approximately 90%. The situation of recipients of lung transplants leaves and even more dramatic impression when an average 5 year graft survival of only 54.4% is considered.[4] This phenomenon has a major cause: the lung is believed to be very prone towards being damaged. Often, it is already pre-damaged due to trauma, aspiration, resuscitation attempts or complication on the Intensive Care Unit (ICU).[6]

As a result, more than 60% of potential lungs have to be discarded by reason of poor organ function. Studies repeat that more than 60% of potential lungs have to be discarded.[5, 7] A study from Victoria, Australia has shown that only 17% of all lung donors were transplantable whereas 88% of these donors could still donate a kidney.[7] Therefore, many attempts have been made to enhance utilization of donor lungs and to maximize LTX opportunity. Even 'extended criteria' organs with history of asthma, airway secretion or smoking donors are thus transplanted with more or less acceptable short-term results.[8-10] In some centres, living related donor lungs or donation-after-cardiac-death (DCD) donor lungs have also been used.[11-13] The addressed studies imply that a main problem is finding the 'right' donor in the 'right' time to enable an optimal preparation of an organ.

Another big issue occurs in the preparation itself, as it causes and intrinsic ischemia and thus a probable reperfusion injury. Amongst all other organs, the lung has the lowest ischemia tolerance. Thus, the most important criteria for successful lung transplantation is time! In a study from Victoria, Australia 16% of all donor lungs were unusable due to logistical limitations.[7] A critical point is the extended cold ischemia during the period of conservation that correlates with a high risk for development of primary graft dysfunction (PGD).[14]

The following chapters will give an overview about the leading causes of lung graft injury.

1.2 Ischemia and Reperfusion

Ischemia is defined as the insufficient or complete absence of blood flow in the tissue.[15] An ischemic state is usually followed by reperfusion when blood circulation is partially or completely restored.[16]

As a matter of fact, oxygen is the livelihood for every single cell. Thus, a lack of oxygen induces a powerful alarming signal that causes a critical and deleterious situation for the affected organ or even the whole organism.[17-20] Functional accommodation of oxygen is secured *via* the wide network of arterial and venous vessels that supply the tissue with oxygenated blood from the lung. If the normal blood flow is cut or strongly restricted in a part of the circulation system, the tissue will suffer from oxygen deprivation, resulting in hypoxia and complete ischemia if this state is kept.[17, 20] In transplantation medicine, ischemia as absolute or relative shortage of blood flow is a state, that can't be avoided during transplantation. The organ has to be taken out of the donor, and therefore cut from the donor's blood system prior to readaption to the recipient's blood circulation.

Ischemia in the lung can cause lipid peroxidation and oxidant injury despite the presence of oxygen.[21, 22]

Two kinds of ischemia are differentiated: cold and warm ischemia:

The ischemia is defined as 'warm', if the tissue or organ is kept at body temperature during this state. Warm ischemia occurs in organ harvest and transplantation to the recipient as the condition of the organ due the detachment from blood circulation and the time during surgery until the recipient's vessels are adapted.

The ischemia is defined as 'cold', if the tissue or organ is cooled down. This happens usually during storage and transport and can't be avoided. Since hypothermia decreases energy requirement and metabolic rate of the organ, temperature seems to play an important role during ischemia. It has been shown that the inflicted tissue damage distinguishes between cold and warm ischemia due to different types of cells being affected.[23, 24]

As both forms of ischemia lead to tissue damage, ischemia-time has to be kept as short as possible. Many different factors have to be considered like storage time, donor and recipient preparation or duration of surgery. In organ storage, a cold ischemic time, which is defined as the period of cooling the organ until reestablishment of the blood flow, of 4-8 hours are accepted. Warm ischemic time depends mostly on the surgeon's organ preparation and preservation experience.[17]

1.2.1 Ischemia and Reperfusion Injury

Many approaches have been developed to optimize the methodology of lung preservation over the past decade. The prevention and treatment of ischemia reperfusion injuries (IRI) lead to a reduction of PGD incidence of approximately 30-15%.[25, 26] Due to the decreasing num-

bers of donor organs and the extended donor selection criteria it is even more important to understand the mechanism of IRI and to develop better preservation and prevention possibilities. Especially in lung transplantation and the major use of brain dead donor lungs, the understanding of ischemia and reperfusion (I/R) is essential.

Ischemia leads to a lack of oxygen, hence hypoxia and was shown to increase the risk of inflammation in grafts that may lead to graft failure or rejection.[27] Hypoxia occurs in the lung, when alveolar oxygen pressure drops below 7 mmHg during ischemia, and it induces inflammatory response when perfusion is initialized after hypothermia. [28,29,30, 31,17,32, 33, 20, 33-41]

One important biochemical entity that responds to hypoxic conditions on a molecular level is the hypoxia inducible factor-1 (HIF-1). On previous studies it was reported to reach high levels in inflammatory tissue and is the central research topic of this work.[42]

Beside its beneficial adaptive effects on long-term hypoxia, it also has the ability to regulate several functions of myeloid cells to enhance their stimulation, aggregation, motility and invasiveness.[43, 44] It also prolongs the lifespan of neutrophils and has an influence on the adaptive immunity[45, 46]

In studies on mountaineers suffering from mountain sickness, it was demonstrated that HIF-1 influences inflammatory processes and pulmonary edema which was demonstrated [47-49]

Under hypoxic conditions HIF-1 has a great effect on the cell *via* regulation of Vascular Endothelial Growth Factor-A (VEGF). VEGF leads to increased permeability of the endothelium and a formation of *fenestrae* in the capillary sheet, which are relevant steps towards the development of edema.[50-52] This condition follows a swelling of alveolar interstitium and a flooding of the alveoli. Accumulated extravascular water and the swelling leads to a critical disturbance of the mechanical function and a disablement of the gas exchange which may end in PGD afterwards.[53-57]

Additionally, several mechanisms affect the graft during the hypothermic storage of organ transplants and may lead to activation of inflammatory mediators that lead to inflammation and injury at the time of reperfusion.[17] Hypothermia, for example, has an effect on the functionality of many enzymes, as most of them show a 1.5-2.0 fold decrease in their activity for every 10°C decrease in temperature.[58] Understandably this effect is favourable as it reduces the metabolic processes. But many downregulated or even stopped enzymic function results in dysfunction of critical mechanisms to keep the balanced state in the cell.[59]

One of these critical points is the inactivation of sodium pumps, like the $Na^{+/}K^{+}$ ATPase. This ATP dependent enzyme is accountable to perpetuate the intracellular electrolyte concentration by shuttling potassium ions (K^{+}) from the outside of the cell into the intracellular fluid and

sodium ions in the opposite direction. Thus, a high K^+ and a low Na^+ concentration inside the cell is maintained which has to be high in intracellular fluid and sodium ions ($Na^{+)}$. Consequently the $Na^{+/}K^+$ ATPase enables the adequate clearance of the alveolar cell fluids.[17]

Under hypothermic conditions, the function of the sodium pumps is diminished but can be re-established upon reaching a temperature of 37°C under the premise that there is no damage to the endothelial cells.[60] This temporary deficit leads to an accumulation of sodium in the intracellular fluid and a passive influx of chloride. This also causes cell swelling and accumulates with the swelling cause by HIF-1 upregulation in the time of repefusion.[17, 53-57]

One important consequence of ischemia and reperfusion injury is the upregulation of certain molecules on the cell surface membrane. These adhesion molecules play an important role in modulating the inflammatory response. They can be differentiated in three families: selectins, immunoglobulin superfamily and integrins. They are upregulated on pulmonary endothelial cells during ischemia and are important for leukocyte emigration. The emigration of leukocytes depends on different events mediated by adhesion molecules: rolling, adherence, activation and extravasation. The activation and proper adherence appears when leukocyte ß1 or ß2-integrin bind to endothelial cells which then express intercellular adhesion molecule-1 (ICAM-1) or vascular endothelial adhesion molecule-1(VCAM-1). The extravasation into the tissue is dependend on integrin-immunglobulin interactions which involves ICAM-1 and platelet endothelial cell adhesion molecule-1.[17]

Under hypoxic conditions, endothelial cells and macrophages develop inflammatory properties which lead to impaired blood rheology during reperfusion and cause critical damage in IRI.[61,62-64]

Another factor is Endothelin, a vasoconstrictor, 10 times more powerful than Angiotensin II. It is released *via* endothelial cells and its expression is predominant in the lung.[65] It stimulates the production of cytokines by monocytes/macrophages and promotes the retention of neutrophils in the lung.[66] It is known to accumulate in lung tissue before and during the first hours after reperfusion[67, 68] – high levels even lead to increased expression of VEGF and cause a higher vascular permeability.[69]

Proinflammatory factors are released in many organs after I/R and this has also been shown also for the lung.[70] Measurable amounts of pro- and anti-inflammatory cytokines like TNF-α, IFN-ɣ, IL-8, Il-10, IL-12 and IL-18 were found in human lung transplants during cold ischemia and after reperfusion.[71] Most of them decrease after reperfusion except IL-8 which shows a significant increase. While most donor parameter like oxygen tension, consequences of brain death or smoking history did not appear to influence the cytokine level, it was found

that IL-10 level, an anti-inflammatory cytokine, correlated inversely with donor age.[72] IL -10 and IL-4 play an important role as anti-inflammatory cytokines in lung transplantation patients. Especially IL-4 seems to take part in diverse immunomodulatory mechanisms – some are part of the inflammatory axis of chronic rejection, but interestingly also in anti-inflammatory ways.[73-75]

Another relationship was found between IL-8 and graft function in human lung transplants.[71] This potent chemokine promotes neutrophil migration which rapidly increases after reperfusion. The IL-8 concentration 2h after reperfusion is negatively correlated with signs of lung injury like the ratio of partial oxygen tension of alveolary to the fraction of O_2 in inhaled air (PaO_2/FiO_2) and mean airway pressure. With Acute Physiology and Chronic Health Evaluation Score, on the other hand, it has a positive correlation during the first 24 h on ICU.[71]

Besides that, the C-X-C chemokine receptor type 4 (CXCR4) has been demonstrated to be very active in the inflammatory process of acute lung injury and the development of *bronchiolitis obliterans*. The number of CXCR4 positive cells is increased in lungs of patients that suffer from this form of chronic rejection. It is also involved in several fibrogenic processes after lung injury. [76-78]

1.3 Primary Graft Dysfunction (PGD)

The primary graft dysfunction (PGD) is a multifactorial injury that occurs in the graft and developed within the first 72 h after transplantation. It presents itself with acute lung edema, severe hypoxemia and diffuse alveolar damage.[14, 79-81]

The transplantation process consists of many steps, that all represent bottlenecks in adequate organ function. Beside the harvesting process, that is critical, the selected organ has to undergo the preservation process, transport and implantation. All these manipulation steps may lead to PGD which affects an estimated fraction of 10-25% of lung transplants and is the leading cause of early post-transplantation morbidity and mortality.[79-87]

Unfortunately, these necessary procedures can damage the lung even when all of them are performed under optimal conditions just by the mere mechanical manipulation of the organ.[11-13]

Risk factors to develop a PGD are prolonged mechanical ventilation, aspiration pneumonitis/pneumonia, trauma, or hemodynamic instability after brain death.[88]

It is also known already, that brain death itself has a critical effect on organ function by interfering with the whole homeostatic regulatory system and producing a severe disorganization

in the endocrine function. The so called 'cytokine storm' leads to significantly higher levels of inflammatory cytokines.[89-94] This may end up in decreased tolerance to ischemic periods and therefore augment the susceptibility for ischemia reperfusion damage and, later on, an alloimmune response.[17]

Pathological mechanisms like the constitution after brain death, pulmonary ischemia, cold organ preservation in due to organ transport and reperfusion are also critical factors which lead in the end to PGD.

Especially important for this work and reported as an important driving force is the injury caused by cold or warm ischemia and following reperfusion (IRI) which is described in chapter 1.2.1.[17, 88, 61, 70, 95, 96].

It has been shown that there is a connection between PGD and chronic allograft rejection as persons suffering from PGD have elevated proinflammatory mediators during early post-transplant period and increased development of *de novo* anti-Human Leukocyte Antigens (HLA)/Multi Histocompatibility Complex (MHC) type II antibodies.[97, 98]

The worst form of PGD is described to occur after 48 hours. It is not only shown, that there is a relation between PGD and the *bronchiolitis obliterans* syndrome (BOS), which is the clinical form of chronic rejection.[99-101] Some studies also revealed that an increase in PGD severity is correlated with decreased short- and longterm survival.[102] 30-Days mortality rates are eightfold higher in patients with severe PGD compared to those without.

During organ injuries like PGD, selfantigens are exposed and lead to a potential activation of autoimmune response. A special protein, Collagen type V - a major collagen in the lung, seems to play a crucial role in this as it seems to be an antigen in the lung if released as a fragment.[97, 103]

The damage to epithelium and microvasculature leads to excessive fibroproliferation and tissue repair. The local ischemia caused by microvasculature damage stimulates HIF-1α and, therefore, angiogenesis which is required to support chronic inflammation and fibrooblitération.[104-106]

1.4 Rejection – different Faces, different Causes

Although this work concentrates on the early phase of ischemia and reperfusion injury and primary graft dysfunction in the first 48 h, aspects of longterm rejection triggered *via* the immune system will be shortly addressed with this section.

Following the cascade of events, not only complement cascade, cytokines and chemokines are lerted, but also other parts of the innate immune system like neutrophils and macrophages. The inflammatory reaction due to IRI is believed to be predominantly governed by an innate immune response.[19]

The immune reaction via leukocyte activation can be parted into two stages – an early and a delayed phase of reperfusion. The early phase is known to depend primarily on donor characteristic determinants, whereas the delayed phase is promoted by recipient dependent.[107] Neutrophils mediate the early phase by producing local microvascular and parenchymal damage. Later on monocytes and macrophages are infiltrating the tissue and may augment the injury phase.[41]

Despite the observation that the trafficking of T-cells into an organ was followed by IRI, it was believed that they only have the role of 'passive observers'. Nowadays many studies support the assumption that T-cells are direct mediators of IRI in many organ systems.[108-115]

But besides the destructive function of T-cells, new data show the potential role of lymphocytes in the healing and recovering process. It was shown that $CD8^+$ and $CD4^+$ deficiency was associated with lower healing response.[116-119]

Additionally evidence for the existence of regulator or suppressor T-cells which are capable of many different functions including the modulation of the immune system has been gathered.[119-122]

The most feared form of complication besides an infection due to the application of immunosuppressive agents is the rejection of the graft itself. Acute rejection affects almost 55% of all transplant patients within their first year after transplantation.[123]

The local innate immune activation and constant activation of immune response due to *noxae* from the environment contribute to the high rates of rejection of lung transplants.[123]

Rejection is a complex mechanism that is divided in different stages and severity, which are: hyperacute, acute, chronic and subclinical rejection. The classification into one of these categories depens on the time of onset after transplantation.[124]

Hyperacute rejection occurs minutes to hours after transplantation and is mainly caused by pre-sensitization of the tissue. It is the result of alloantibodies and complement fixation.[124] Some patients may already have developed anti-MHC/HLA antibodies due to pregnancy, transfusion or a previous transplantation. This antibodies react with donor antigens, leading to accelerated humoral rejection and *bronchiolitis obliterans* syndrome (BOS) or to hyperacute rejection with immediate graft loss.[124]

Another form distinguished from hyperacute rejection but showing similar clinical signs is acute antibody mediated rejection (AAMR) that is known to occur any time after transplantation.[124] It manifests in a rapid increase of serum creatinine concentration and shows a resistance to therapy with steroids or T- cell specific agents.[125, 126] AAMR has a poorer prognosis than a pure T- cell mediated acute rejection.

Both, hyperacute rejection and AAMR present themselves clinical with parenchymal injury, fibrinoid arterial necrosis including capillaries that are congested with neutrophils, macrophages and thrombi consisting of platelets and fibrin.[127-130]

Acute lung allograft rejection, also known as lymphocytic bronchiolitis is defined as perivascular or peribronchiolar mononuclear inflammation.[123] It may occur after a few days but may also take years until onset.[123, 124] It is the result of a response of antigen specific T- cells and/or antibodies that show alloreactivity in the graft.[124] Its frequency and severity seem to be the most critical risk factor for the development of BOS, the clinical form of chronic rejection. This fact limits the survival to 50% at 5 years after transplantation.[131, 132]

One of the most problematic systems in transplantation is the recognition-system which enables the organism to differentiate cells into self and non-self. In vertebrates it has evolved into a very complicated system that almost every cell of a body is linked to. This Major Histocompatibility Complex (MHC), or in humans Human Leukocyte Antigene (HLA) is a protein complex encoded by a set of very closely linked genes. This complex presents antigenic peptides that regulate immunological response by presenting these antigens to T- cells.

MHC genes are traditionally divided into two groups – MHC I and II. MHC class I genes are expressed on the surface of most nucleated cells including epithelial cells. The molecules of these genes mainly present endogenous peptides to CD 8^+ T- cells.[123, 124]

MHC class II genes are constitutively expressed on B- cells, monocytes, dendritic cells and other antigen presenting cells (APCs). They are upregulated on many other cells under inflammatory conditions.[123]

After donor APCs die out or are destroyed, the dendritic cells of the recipient process present the alloantigens to the recipient T-cells.[133]

Another critical point relies within the blood group antigen that represents a distinctive mark for the immune system.[124]

Even if there are still many mechanisms not fully understood, commonly, acute rejection involves the recruitment and activation of recipient lymphocytes which are mainly effector T-cells. This cause lung allograft injury and later on, its loss.[133] They also provide help for B-cell memory, antibody class switching and affinity maturation. This is controlled by many

different T- cell derived cytokines and co-stimulatory factors, recognizing receptors at the surface of B- cells. B- cells produce long living plasma cells in the tissue and migrate to the bone marrow where they continue antibody production indefinitely without requiring T- cell help anymore.[134]

Chronic rejection, also known as *bronchiolitis obliterans* (BO) is a leading cause of morbidity and late mortality after one year in lung - transplantation patients.[100]

Normally the first sings are recognized clinically by sustained declined expiratory flow rate at least over three weeks when other reasons have been excluded.[135] In advanced BO there is a wide spectrum from partial to completely acellular fibrotic obliteration where only a scar remains of the airway lumen.[100, 135, 136] This small airway destruction may lead to accumulation of foamy macrophages and mucostasis in the distal airspace.[136] Alloimmune independent factors like primary graft dysfunction or alloimune dependent as acute rejection are thought to be associated with BO.[137-139]

Pro- and anti-inflammatory cytokines play a critical role in wound repair and the progression of BO. Different types of immune response interact during chronic rejection.

Type 1 immune response goes mainly together with cell-mediated immunity and results in production of IL-2, IL-12, IFN-ɣ, lymphotoxin that drives cytotoxic T-lymphocytes and delayed hypersensitivity response.[140]

Type 2 immune response is identified via production of IL-4, IL-5, IL-13 and goes along with humoral immunity.[140]

Type 17 immune response leads to IL-17 and IL-23 production and results in autoimmunity.[140]

The type of the three immune responses is considered to change *via* the nature of alloantigens and the pattern of cytokines released into the environment.[141] Chemokines also have a role in this complicated process as growth factors and humoral factors like the complement fragments.[140]

1.5 Hypoxia Inducible Factor - 1 (HIF-1)

Oxygen serves as an important reactant in every cell – it is crucial for metabolic processes in mammals and serves as an electron acceptor in the reaction during ATP formation. Therefore, the status of hypoxia, which is defined as a local or even general pathological status of deprived oxygen supply, is a severe condition that leads to cell damage if the status is not normalized quickly. In highly developed organisms like mammals, a very specific system of

oxygen sensing and regulation factors has evolved, to resolve or reduce the negative effects of hypoxia to the single cell but also to the complete organ.

This state of emergency is moderated by the transcription factor HIF-1, which was first described by Semenza in 1992.[142, 143] The first discovered HIF-1 effect was the induction of erythropoeitin (EPO) upon hypoxia thus increasing the O_2 carrying capacity of the blood.[144, 145]

HIF-1 consists of two subunits which form together the activated HIF-1 protein as a heterodimer. Both subunits were shown to be basic helix-loop-helix (bHLH) PAS-proteins who are known to be regulated in their expression by oxygen tension.[142] PAS-proteins (named after the first isolated proteins from this family of *Drosophila melanogaster* – PER-ARNT-SIM[146, 147]) always contain two internal homology units and are responsible for protein-protein interactions. The subunits form contacts to the DNA in the major groove[148-150] and were named as HIF-1α and the already known HIF-1ß.[151] HIF-1α and -ß units were detected in all tissues of rodents and humans.[143]

HIF-1α is a 120 kDa bHLH-PAS protein[142, 152] and is only detectable during hypoxic conditions in the cell of adult mammals.[143, 153] High levels of HIF-1α under non hypoxic conditions are only found in embryonic stem cells and are due to the special situation in the tissue before and during neovascularisation.[143] The half life of this protein is very short and below five minutes.[153] A detection of HIF-1α is possible after 30 minutes of exposure to hypoxia (1-2% O2) and it has a peak between 4-8h under hypoxic conditions. Endogenous HIF-1α is localized in the nucleus of hypoxic cells.[153]

HIF-1ß was also formerly known as ARNT (Aryl Hydrocarbon Receptor Nuclear Translator).Its mass differs between 92-94 kDa and different isoforms exist.[142] Unlike to HIF-1α, it is constitutively expressed and present in the nucleus of cell.[152, 154]

The regulation of HIF-1α and therefore, the functionable HIF-1 protein is performed *via* different steps of ubiquination and proteasomal degradation in which the von-Hippel-Lindau product (pVHL) plays a crucial role.[153, 155] Under normoxic conditions, HIF-1α is polyubiquinated *via* pVHL by binding to HIF-1α only when a conserved proline structure is hydroxilated and, thus, degraded by the proteasome. [155-159] Responsible for this reaction is the HIF-1α oxygen-dependent-degradation-domain (ODD).[155, 160-162] The hydroxylation of the proline is oxygen dependent and ceases to proceed under hypoxic conditions.[163] pVHL is known to be intact in HIF-1 overexpressing tumor cells and as the interaction between HIF-1α and pVHL is iron-dependent, it can be blocked by iron chelators.[153, 155, 163]

This leads to accumulation of HIF-1α, diffusion into the nucleus and dimerisation with the always expressed HIF-1ß subunit.[164, 165] The now functional HIF-1 transcription factor starts gene transcription by binding to the hypoxia respond element on HIF-1 regulated genes. HIF-1 binds to the hypoxia response element (HRE) on the target which contains functionally HIF-1 binding sites.[143, 152]

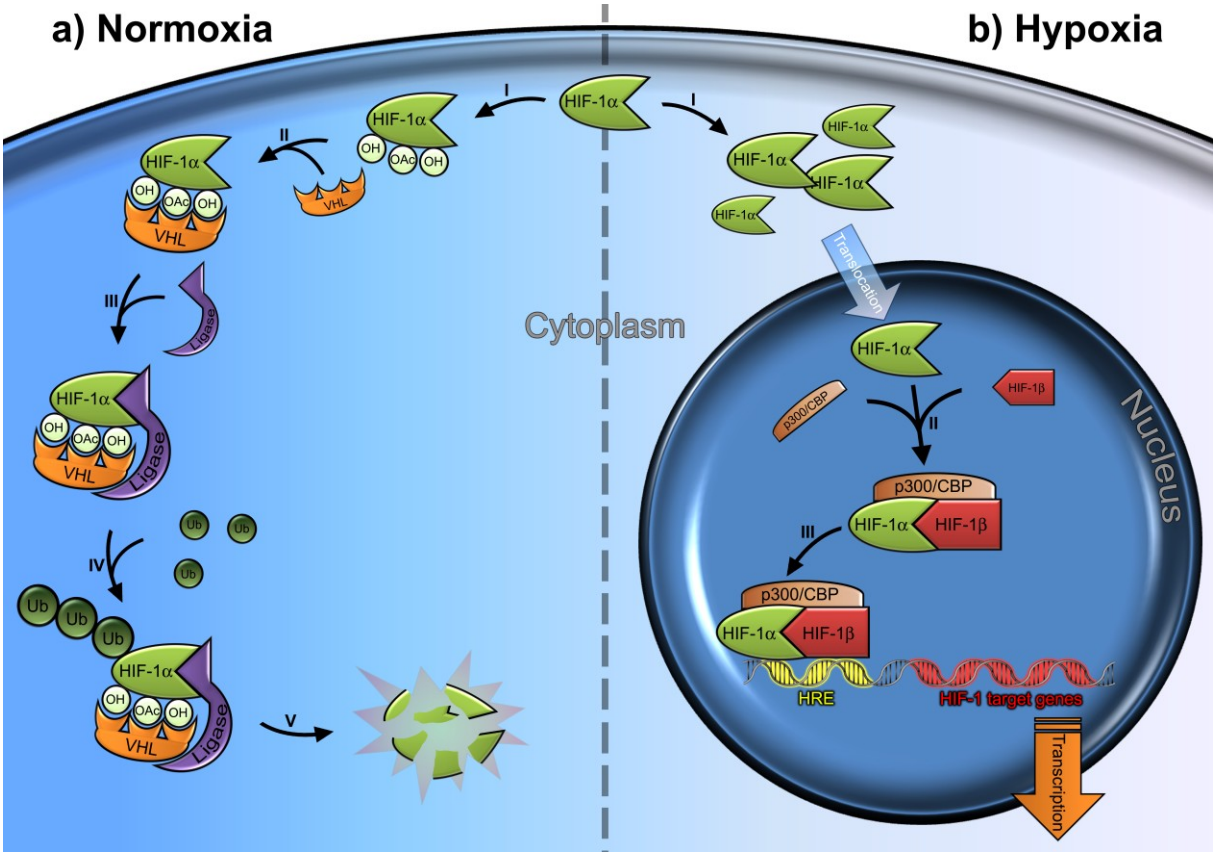

Figure 1: HIF-1 pathway (Created according to literature 166, 167). a) Normoxia: HIF-1α is hydroxylated via prolyl hydroxilases (PHD 1,2,3) in presence of oxygen, iron and other factors (I). The hydroxylated HIF-1α is now recognized by pVHL which binds to it (II). A multisubunit ubiquitin ligase complex binds and tags HIF-1α with polyubiquitin (IV). This enables recognition by the proteasome and HIF-1α is degradated (V) b) Hypoxia: The proline hydroxylation is inhibited and pVHL is not able to bind HIF-1α. Proteasomal degradation is blocked and HIF-1α accumulates in the cytosol (I) HIF-1α now translocates into the nucleus where it dimerises with HIF-1ß and recruits transcriptional co-activators like p300/CBP for its full transcriptional activity (II). This complex binds to the hypoxia response element (HRE) in the promoter of target genes and activates the transcription of HIF-1 regulated genes that fulfil many different functions as cell survival and proliferation, proteolysis, angiogenesis, erythropoiesis, pH regulation, apoptosis and glucose metabolism. VHL = protein product of von Hippel Lindau transcription factor; HIF = hypoxia inducible factor; HRE = hypoxia response element.

The list of identified target genes regulated by HIF-1 is steadily growing and they are encoding important products for hypoxia response, oxygen homeostasis, angiogenesis, glycolysis and tumor growth, but according to new studies, also inflammatory processes.[143, 155, 168] HIF-1

regulated genes can be divided in two groups – fast acting and long acting genes. The fast acting genes are regulated within minutes.[169]

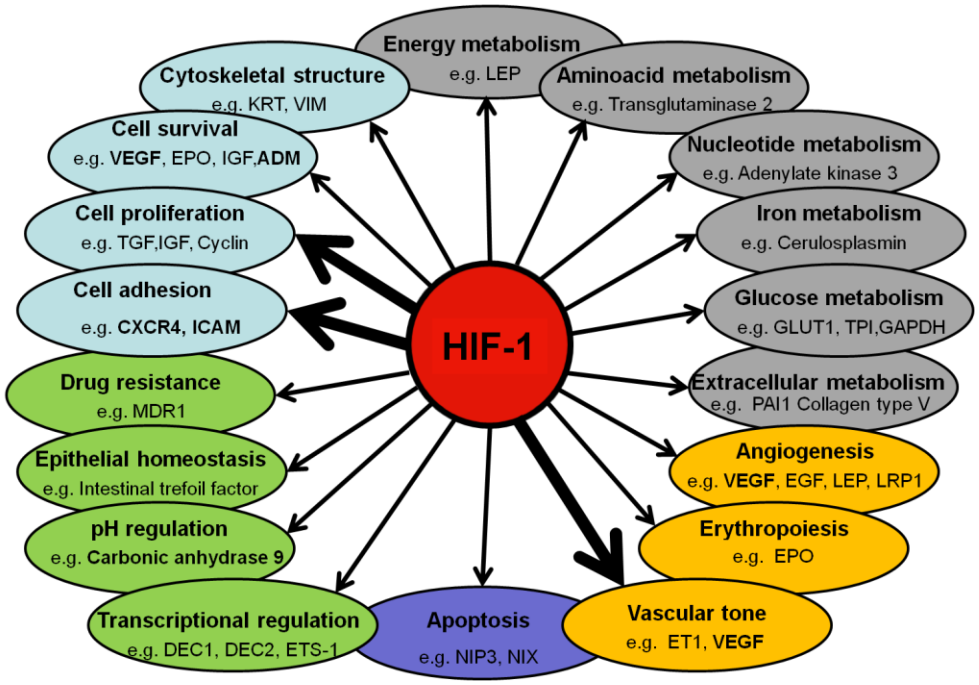

Figure 2: Gene products of HIF-1 and their function in the organism. The most important functions and genes for this work are bolt. (For abbreviations see appendix, created according to literature[170]).

HIF-1 is also shown to mediate the configurational change of endothelial cells, leading to gaps in the endothelial barrier.[171]

The inflammatory role of HIF-1 is clinically observed under extreme low oxygen conditions that high-altitude mountaineers are exposed to. This people often show lung and brain edema as well as the so called systemic inflammatory response syndrome.[47, 49, 172, 173] And as hypoxia leads to inflammation, inflammation itself is also causing hypoxia which ends in a downward circle concerning acute lung injury and lung transplantation.[174, 175]

To these inflammatory effects adds the increase of VEGF and therefore the high permeability of the tissue after the restoration of the blood flow which leads to early graft dysfunction.[176-180]

So it can be postulated, that, even if the effects of HIF-1 are supposed to prevent damage to cells and organs during hypoxia by effects like the amplification of glycolytic activity or the increase of hemoglobin levels, in the endothelium rich tissue especially in the transplanted lung, the sum of all effects leads to a damage of the tissue. This seems to be mostly an effect of the fast acting genes as VEGF and primary graft failure can be correlated with this. [67, 69, 169, 181]

1.5.1 Vascular Endothelial Growth Factor (VEGF)

Most important for this work is the effect of HIF-1 and its gene product, the Vascular Endothelial Growth Factor (VEGF), during lung damage. The members of the VEGF family are dimeric proteins with a molecular weight of approximately 45 kDa.[182] VEGF, one of the isoforms, is a major regulator for blood vessel formation and function, but it also has influence on permeability and inflammatory potential.[183, 184]

VEGF is examinated best in endothelial cells and highly expressed in lung epithelial cells of adults and cardiac myocites.[185] It is the most important angiogenic factor that can be induced *via* HIF-1 under hypoxia.[186]

Besides the benefits of the long acting effect of the gene product VEGF, which is important for the angiogenesis, it has a destructive effect on the lung through the short acting mechanisms. VEGF is a potent mediator of endothelial barrier dysfunction which is dramatic in the vulnerable system of the lung – the pulmonary vascular permeability is bluntly increased.[168] It is 50.000 times stronger than histamine in its potency to increase vascular permeability.[187] It is able to induce fenestrations in venular and capillary endothelium within 10 min and its effects may be correlated with organ failure.[67, 181, 188-193] VEGF, as one of the fast acting genes is known to be upregulated during ischemia in many organs and it is increased during ischemia in the lung.[194-197] Its receptors like the VEGF-receptor 1 (Flt-1), VEGF-receptor 2 (KDR) and Endothelin-1 (ET-1), play essential roles during ischemic damage. Also genes like VCAM-1 and ICAM-1 which regulate cell adhesion to the endothelium are important in this process.[69, 198, 199]

Even if VEGF is very specific for endothelial cells, it has also an effect on other cell types, which is most important for its malignant effect during transplantation, on inflammatory cells like monocytes and mast cells.[200] VEGF was also found to play a role upon neutrophil activation which are substantially involved in acute lung injury[201-203]

Although VEGF bears positive effects like neovascularization, this was described to be of less importance in the first critical hours of lung injury as neovascularization takes several days due to the duration of endothelial mitosis and migration whereas the effect on edema was shown to occur already after 24 h.[56, 204, 205]

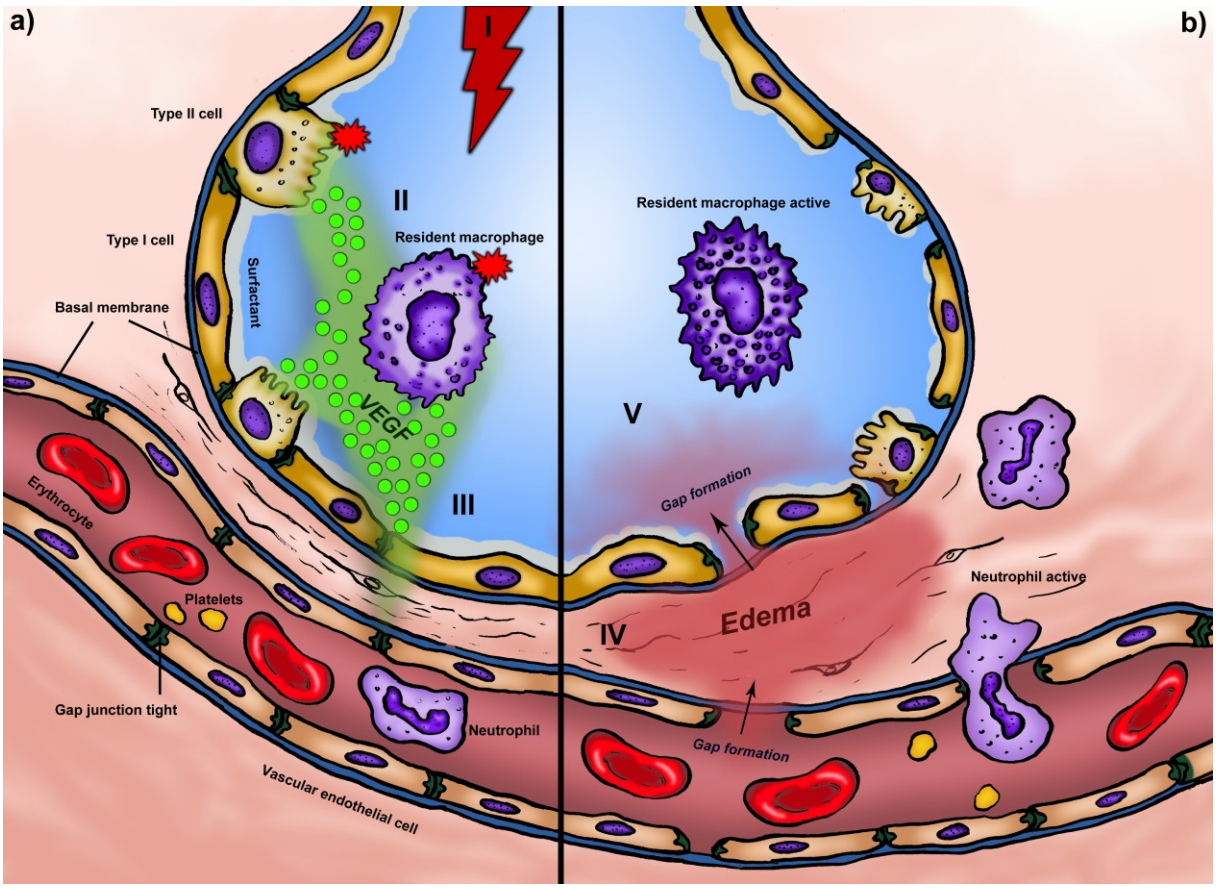

Figure 3: The overall hypothesized role of VEGF in acute lung injury. a) The alveoli are confronted with a *noxae* as hypoxia or stress (I). The release of VEGF is stimulated and huge amounts are secreted from type II cells and alveolar macrophages (II). The epithelial-endothelial barrier is now exposed to high concentrations of VEGF (III). The high amounts of VEGF lead to gap formation and vascular leakage which ends in interstitial edema. It also affects macrophages and neutrophils (IV). Due to the gap formation, liquid can enter the alveolar lumen and destroy the function of the surfactant and the gas-exchange (V). (Created according to literature[206, 207])

1.6 Deguelin

Deguelin is a rotenoid which belongs to the flavonoid family and is described most often in literature to exhibit antiangiogenic properties.[208, 209]

It was structurally first described in 1932 together with its derivate Tephrosin.[210] It is the second most important ingredient of the pesticide cubé resin besides Rotenone which was mentioned together with Deguelin already in 1902.[211, 212] Cubé resin is a root extract from different kinds of plants from the family of the fabacea, mostly *Lonchocarpus utilis* (Cubé), *L. urucu*, and *Mundulea sericea*. It was used as a botanically pesticide, especially against insects, but also for selection of fish in American sport-fishing.[212] It consists of different rotenoids - four major active ingredients, but studies could identify almost 29 minor rotenoids.[212] The most important ingredients are with the highest percentage Rotenone (44%)

and Deguelin (22%), followed by derivates of the two main ingredients, Rotenolon (6.7%) and Tephrosin (4.3%).[212]

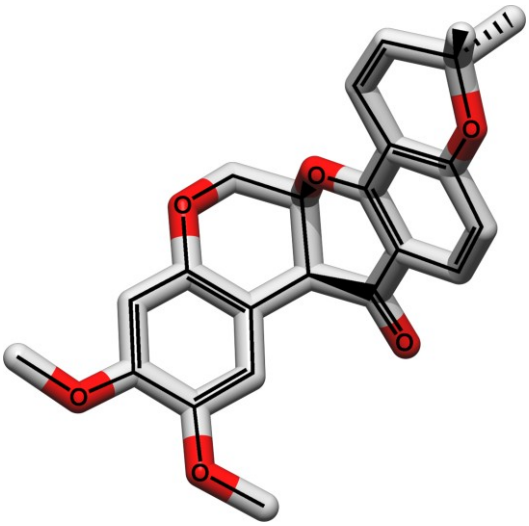

Figure 4: 3D model of Deguelin combined with the LEWIS-structure. White: carbon, red: oxygen, hydrogen left out for clarity.[210]

The ratio of Rotenon to Deguelin is 2.0 but varies due to the natural product and in different part of the plants.[213] As all rotenoids differ only in changes of substituents at the dihydrofuran or the pyran ring, respectively, almost all have a specificity in the inhibition of complex I (NADH:ubiquinone oxidoreductase) and the 12-O-tetradecarnoylphorbol-13-acetat induced ornithine decarboxylase (ODC).[209, 212] Both mechanisms are most important for their toxicity and also their chemopreventive activity. Due to their chemical similarity, the most effective way to obtain Deguelin with high purity is the chemical conversion of Rotenone to Deguelin.[210, 213]

Deguelin itself was examined especially in cancer-models. In contrast to Rotenon, it is optically inactive. It contains two hydrogen atoms that can be removed even under mild oxidative conditions which leads to a double bound instead and creates the so called Dehydrodeguelin. Hydroxilation of this double bound to a creates Hydroxydeguelin, also known as Tephrosin.[210]

Although Rotenone was the first identified substance in cubé resin and has the largest relative proportion, Deguelin shows some benefits for clinical use. It posesses the same characteristics as an inhibitor and is similar in its potency compared to Rotenone.[213] Recent data from 2011 show some significant differences between Rotenone and Deguelin in their pharmacological mechanism, even if their chemical structure seems to be so close. Deguelin does not show a pronounced inhibition of tubulin polymerization, which is an important factor in their different toxicity.[214]

In contrast to Rotenone, Deguelin is 2 x less toxic when injected subcutaneously to rats. LD50 levels in mice are 4.3 mg/kg and,thus, better than for Rotenone with 2.3 mg/kg. Measured brain levels showed a better outcome for Deguelin with 0.18 ppm after 2 h compared to Rotenone with 0.39 ppm.[213]

Deguelin showed also a good acceptance when administered over a long time to animals without systemic toxicity (**Table 1**).[215] The maximum tolerated dose of Deguelin in rats is

high (4 mg/kg) compared to the effective dose of Deguelin needed (100 nM – 1 μM).[215, 216] Also no cytotoxic effect on physiological cells could be demonstrated up to concentrations of 10 μM.[209]

Table 1: Pharmacokinetic properties of Deguelin. Modified after Udeani *et al*.[217]

Mean residence time	**Terminal half life**	**Total clear-ance**	**Elimination via 5 days (i.g.)**			
			feces		**urine**	
6.98 h	9.26 h	4.37 l/h per kg	eliminated	Unchanged	eliminated	Unchanged
			58.1 %	1.7 %	14.4%	0.4 %

Tissue distribution after i.v. injection > from left to right							
Heart	Fat	Mammary gland	colon	liver	kidney	brain	lung

Tissue distribution after i.g. administration > from left to right								
Perirenal fat	heart	Mammary gland	colon	kidney	liver	lung	brain	skin

It was shown that Deguelin has also an effect on tumor growth like it was demonstrated for Rotenone ealier.[208, 213] Deguelin could be used successfully as a chemopreventive agent in the therapy against various cancer types of lung, skin and intestine.[215, 216, 218-222]
It has an inhibiting effect on ODC, which is the catalyst for the decarboxylation of ornithine to yield putrescin that is converted into higher polyamines and has a crucial function for cellular proliferation.[209] The anti-proliferative effects were also demonstrated for the expression of cyclooxigenase-2 and the phoshatidylinositol-3-kinase (PI3K)-Akt mediated signaling.[215, 219-221]

Although the concrete mechanism of the inhibition still remains unclear, different studies showed that Deguelin has an inhibiting effect on HIF-1α and its transcription product VEGF.[209, 216] It could be demonstrated, that Deguelin does not affect HIF-1α through the inhibition of the ROS- and Akt-pathway.[209, 222] It leads to a degradation of HIF-1α protein whereas the inhibiton of the transcriptional activity of HIF-1α is visible at a dose of 10 μM of Deguelin treatment.[209, 216] Deguelin can also inhibit the *de novo* synthesis of HIF-1α and reduce the half-life of already synthesized protein.[216]
Besides that, it was suggested that Deguelin affects the heat shock protein 90 (Hsp90) that protects HIF-1α from oxygenation and therefore degradation, but recent studies showed no affinity to Hsp90.[214, 222] The same study showed that Deguelin has an inhibitory effect on the

respiratory chain in the mitochondria. [214] This seems to lead to more cytosolic O_2 and therefore to destabilization of HIF-1α. [223]

1.7 Lung Transplantation in Animals as Models to investigate post Transplantional Lung Injury

To get a better understanding of the complex mechanisms in transplantation, animal models are always the method of choice. They give the possibility to examinate not only the short-time reaction but also the long-term effects on the graft and the recipient's organism. Therefore, various experimental lung transplantation models have been used to develop a satisfying methodology. The first models were based on dogs.[224] Later on due to the immense cost in livestock for large animals and the advantage of inbred strains, rats became the preferred model[225]. On the one hand, the possibility to discriminate between immunologic and nonimmunologic events by using inbred rat strains was a valuable benefit but on the other hand, the small structure of the rat lung was a new great challenge for the surgeon.[226] Only the inventions in microsurgical equipment and techniques since 1963 made working on the small tissue structures possible and the common transplantation was refined in 1989 by Mizuta et al[227] by using small cuffs that could be stuck into the new structure and just tied instead of the complete suture that was used before. This was a significant improvement as an adaption *via* surgical suture technique takes long time and is due to leakage involved with a high risk of great blood loss or air leakage through the bronchus. This so called cuff-technique was modified over the years to minimize the foreign body reaction to the material of the cuff and the surgical method itself, but is still used as a well established animal model for lung transplantation.[228-232]

1.8 Physiology of the Rat Lung compared to Human Lungs

The lung of the rat bears some species-specific differences to other mammals and also the human lung on anatomically and physiologically level. Starting with the most obvious, the rat's lung lobes are segmented into five lobes: one left lobe and four on the right side. The so called postcaval lobe of the right lung lies in direct contact with the diaphragm and is notched to accommodate the *vena cava caudalis*, therefore called postcaval lobe (**Figure 5**).

Other than in humans, the removal of the right lung bears much more difficulties, than the left lung. The lung weight is related to the size of the body, which should be taken into considera-

tion when choosing donor and recipient animals. The important vessels also show some differences to humans – besides the fact, that rats only have one pulmonary artery and vein for each side, which is very good for the transplantation method. The pulmonary vein in the rat is also thicker than in most other mammals, due to the presence of striated muscle fibers that are contiguous to those of the heart.

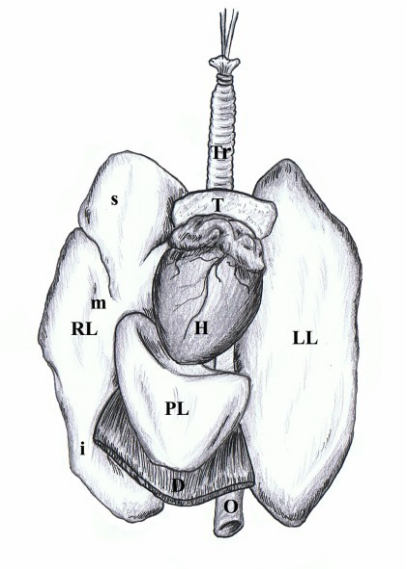

Figure 5: Schematical drawing of the heart-lung complex. RL = right lung lobe; s = superior part; m = median part; i = inferior part; PL = post caval lobe; LL = left lung lobe; D = rest of diaphragm; Tr = trachea; O = oesophagus (Illustration by Ockelmann®)

The pulmonary artery in the rat belongs to two types of elastic and muscular ones[233] The elastic artery type has lots of extracellular matrix in the media and smooth muscle cells that are connecting the neighboring elastic laminae. The muscular artery has only few extracellular matrix and the smooth muscle cells enclosing the lamina circumferentially.

The blood flow in the lungs is somehow centralized to the hilus-regions of the lung lobes in the conscious resting rat, whereas it is only less in the peripheral regions.[234]

The innervation of the lung is different from most other mammals as rats do not have adrenergic nerve supply to the bronchial muscle and therefore, the bronchoconstriction is controlled *via* vagal tone instead of sympathetic tone in humans and most other mammals.[235]

The lymphatics play a critical role, especially for clearing the lung fluid and therefore, have a special function during edema. One very big potential for edema-building in the rat is the position of the capillaries which are upstream from the veins that are supplied with venous sphincters[236] – this leads to a constricting potency that can control lung perfusion and therefore edema. Besides that, an increased number of lymphatics are positioned to this sphincters that help to moderate edema.[237]

The respiration itself is regulated *via* response to tissue CO_2 changes in the medullary respiratory center but beside this, the so called carotid 'glands' or bodies seem to play a role – they are located on each side of the neck in the bifurcation of the carotid artery. In the adult rat, a respiratory average of about 85 breaths/min is reached with a minute ventilation of about 100ml/min and an average tidal volume of 1.5 ml.[238, 239]

Regarding the histological structure of the lung, it contains 74% of alveolar tissue and 26% of nonalveolar tissue.[240] The typical alveolar cells are flat pneumocytes type I which are part of the barrier for the gas-exchange and round pneumocytes type II that are responsible for surfactant secretion. Both line the alveoli.[241]

Other cell types are goblet cells that have a function in producing mucin and glycoproteins[242] to react on airway insults – they work together with cilial cells that remove the produced mucus upwards the airway. Then, there exist septal cells in the alveolar interstitium that contain contractile filaments as actin, desmin and vimentin.[243] They can change the architecture of the air-blood barrier *via* contraction and therefore influencing the ventilation and perfusion ratio.

The alveolar walls consist of three layers. The epithelial layer lining the alveolar space, a basement membrane and an endothelial cell lining of the capillary lumen.[244, 245] All layers are relatively thin (about 1.5 µm). The alveoli are covered with the surfactant that has different functions as tension-lowering and maintaining the patency of conducting the airways.[246] Phospholipids and particulary phosphatidylcholines are essential components of it – whereas in most mammals they are monounsaturated, the rat has a high level of polyunsaturated phospholipids.[247]

1.9 Aims of the Study

The suppression or inhibition of the deleterious effects that result from transplanting an allogenic organ is of great interest in transplantation medicine, as it is the common donor situation. As the complete elimination of recognition of the donor organ as 'foreign' in the recipients body is still more an utopia than a realistic option, the focus has to be laid onto reduction of the inflammatory reaction.

As ischemia and hypoxia in the donor tissue are unavoidable facts during the transplantation process, with all the negative effects associated with them and the successive reperfusion are necessary in the situation during the transplantation process, a reduction of deleterious effects in this state are desirable. Due to many inventions and studies in the field of transplantation

medicine more and more factors, that have great influence during the surgical procedure, are revealed.

The role of HIF-1 which is described above seems to be of great importance in this process especially with a view on one of its transcription products, VEGF. Together with other proinflammatory gene products, induced *via* HIF-1, the suggestion rises up, that an inhibition of HIF-1 in the early acute phase of inflammatory reaction in the transplanted graft can lead to a benefit. Using a clinic-near animal model of orthotopic lung transplantation which had to be established and modified in the beginning, the investigation of the role of HIF-1 and its transcription products, especially VEGF, is the main interest in this work with the focus on following questions:

- What role does HIF-1 play in the lung during ischemia and in the transplanted lung?
- Which gene products of HIF-1 are involved during ischemia and in the transplanted lung?
- Is it possible to influence HIF-1 pharmacologically to get a benefit for the transplanted lung?
- What influence does HIF-1 have on the short-term survival after lung transplantation?

2 Materials and Methods

The following chapter comprises all methods that were applied in the presented work including new or modified ones. All used materials are listed in the addendum in 8.1.

2.1 Methods

2.1.1 Design of the Experiment

The experiment was divided into three parts:

The first part is set out to determine the appropriate Deguelin concentration for effective HIF-1 inhibition *via* a cell assay(2.1.2). In the second part, the functionality of Deguelin *in vivo* is tested in a pilot experiment on rat lungs (2.1.3). The establishment of the surgery method (3.1) and the observation of Deguelin *in vivo* during lung transplantation is the third part (2.1.4).

2.1.2 Cell Culture

Due to the data known about the rotenoids, it was essential to start the experiments in *in vitro* cultivated cell lines to ensure no great toxicity to the living cell. As we used commercially available cell lines (HMEC-1 and HTB-177), no isolation was necessary. Cells are normally cultivated and maintained in standardized condition at defined temperature in a appropriate gas mixture, normally 37 °C and 5% CO_2 for mammalian cells. The conditions vary from the different types of cells but should normally be done as described in the manufacturer's protocol for commercial available cell lines. Most commercially used cell lines are immortalized, which gives them great benefit as the limit of a cell passage is not as that dramatic as in non-immortalized cell lines which can often not be used more than seven times. The cell passage is a necessary step for the cultivation and normally 2-3 passages are done before cells are used for the experiment to let them recover from the frozen storage state.

2.1.2.1 *Cultivation*

Human Microvascular Endothelial Cells (HMEC-1)

Human microvascular endothelial cells (HMEC-1) were thawed and cultivated after manufacturers protocol in 250 cm² flasks with complete growth medium for HMEC-1 (**Table 2**) until they reached a confluence of 80%. Medium was changed 3 times per week. For passage, cells were washed with 10 ml sterile PBS and detached with 5ml Accutase at 37°C in an incubator with 2% O_2, 5% CO_2 until the cells were completely floating. The cell suspension was split 1:3 or maximum 1:5 and transferred with 15-20 ml new medium in a new flask.
For seeding in 6 cm petri dishes for experimental part, cells were harvested as described previously. After centrifugation at 300 x g for 5 minutes, the supernatant was discarded. The cell-pellet was resuspended in 5 ml new medium and the suspension was diluted 1:10 with Trypan-blue and counted using a Neubauer-counting chamber. Then 200,000 cells were seeded in each petri-dish and cultivated until they reached a confluence of 80%.

Table 2: Ingredients of used Growth Medium for HMEC-1 cells

HMEC-1 Complete Growth Medium		
Substance	**Concentration**	**volume**
MCDB 131 without L-Glutamin	500 ml	500 ml
Epidermal Growth Factor (EGF)	5µg/500ml	250 µl
Hydrocortison	500µg/ml	100 µl
Penicillin/Streptavidin solution	1%	5 ml

Human Lung Epithelial Cells (HTB-177)

Human immortalized lung epithelial cells (HTB-177) were thawed and cultivated after manufacturers protocol in 250 cm² flasks with complete growth medium for HTB-177 (**Table 3**) until they reached a confluence of not more than 80%. Medium was changed every second day and for passage cells were washed with 10 ml PBS and detached with 5 ml Trypsin + EDTA and incubated at 37°C with 2% O_2, 5% CO_2 until all cells were floating. Cells were centrifuged at 500 x g for 5 min and the supernatant was discarded. The pellet was resuspended in 5 ml medium and split 1: 3 or maximum 1:8 and put in 15-20 ml of new medium in a

new flask for passage. For the experiment cells were counted as described previously and 200.000 cells were seeded in 6 cm petri dishes.

Table 3: Ingredients for used Growth Medium for HTB-177 cells

HTB-177 Complete Growth Medium		
Substance	**Concentration**	**volume**
RPMI1640 Medium	500 ml	500 ml
Fetal calve serum (FCS)	10 %	50 ml

2.1.2.2 *Cell Culture Experiments*

Deguelin was solubilised in DMSO at an end-concentration of 4 mg/ml. From this stock, three stock dilutions were made for cell culture experiments: 10 mM, 100 µM and 10 µM.
Both cell lines were treated with different concentrations of Deguelin in DMSO (1 nM, 10 nM, 100 nM) for 12 h prior to hypoxia and the medium was equilibrated in the hypoxia chamber 24 h before experiment at 2% O_2, 5% CO_2 at 37°C. Then, the equilibrated medium with the Deguelin-concentrations was added under hypoxic conditions and cells were exposed to hypoxia for 6 h and then harvested. The best hypoxic time was detected *via* pre-experimental test in the hypoxic chamber by incubating cells under hypoxic conditions and harvesting after 2 h, 4 h, 6 h, 8 h, 12 h, 16 h and 24 h – the most effective inhibition was observed in cells incubated for 6 h under hypoxic conditions. Cells were grouped as shown in **Table 4**.

Table 4: Scheme of cell culture experiment

Normoxia	No treatment, harvest at the endpoint of the experiment				
Hypoxia	SHAM	DMSO	Deguelin in DMSO (12h pretreatment)		
6h hypoxia + hypoxic medium	No treatment	1 µM	1 nM	10 nM	100 nM

2.1.3 Pilot experiment *in vivo*

Next, the effect of Deguelin on HIF-1α and the ischemic lung was tested *in vivo* after determination of a therapeutically effective dose of 100 nM from cell culture experiments.

After this, lungs of rats were harvested as described in the donor-procedure and left under ischemic conditions at 37°C for one hour. Three groups were build in which the effect of warm ischemia with no treatment, warm ischemia with previously 3 d treatment of Deguelin and a non-treated, non ischemic sham group was examined. To reduce the number of animals that were necessary, statistical significance was determined and reached at a number of 6 animals.

Table 5: Summary of groups in the pilot experiment.

Group Size	Treatment	Ischemic conditions
n=6	Deguelin for 3 d	warm ischemia for 1 h
n=6	untreated	warm ischemia for 1 h
n=6	untreated	no ischemia (sham)

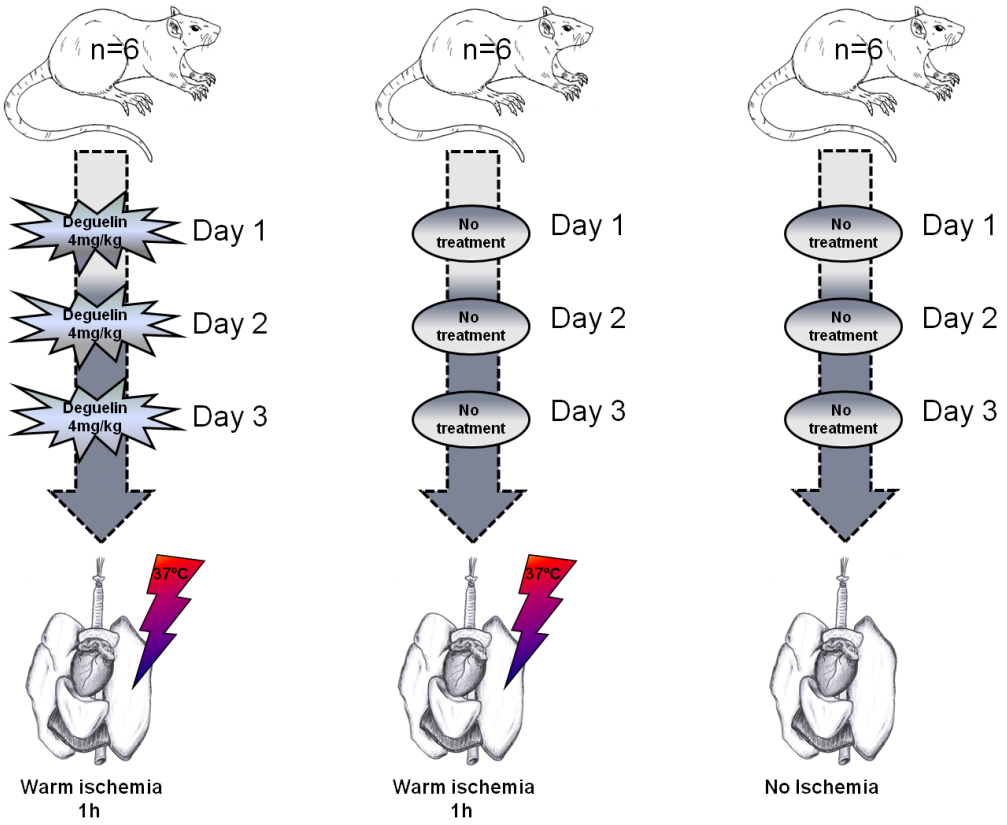

Figure 6: Schematical design of the Pilot experiment. The red bolts represent the ischemia at 37°C.

2.1.4 Main Experiment *in vivo*

The second step of the experiment was the examination of the inhibitory effect of Deguelin under the condition of a complete lung transplantation and the outcome after an observation period of 2 d post operation. Therefore, animals were divided in donor and recipients and the donor was treated 3 d before surgery with the described dose of Deguelin and recipients were also treated 2 d after surgery. Another group of donor and recipients was build in which also lungs were transplanted without any further medical treatment.

Table 6: Summary of groups in the main experiment.

Group Size	Treatment	Animal
n=6	Deguelin for 3 d preoperative	donor
	Deguelin for 3 d preoperative + 2 d postoperative	recipient
n=12	untreated	controls (donor + recipient)

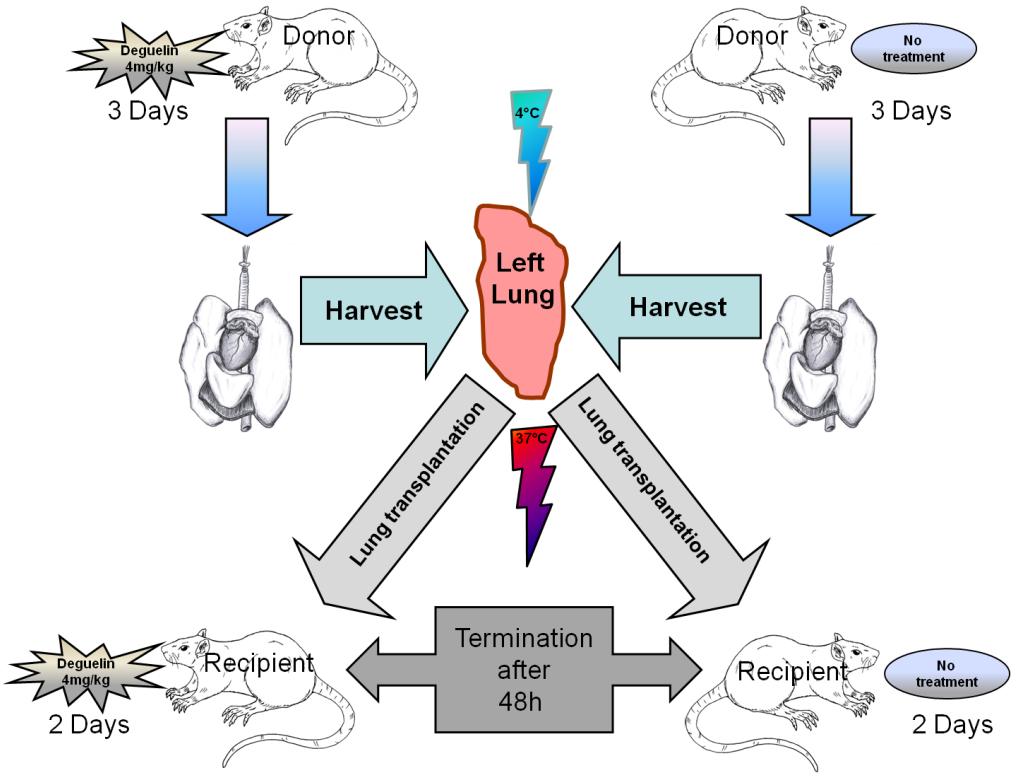

Figure 7: Schematical Design of the main experiment. The two bolts in red and blue symbolize the two ischemia-periods of cold and warm ischemia in the transplantation process.

2.1.5 Animals

The study was approved by the Animal Care and Use Committee of the state of Hesse (Regierungspräsidium Darmstadt), Darmstadt, Germany (V54-19c20/15-F91/56). Surgery and animal care were performed in accordance with the „Guide for the care and use of laboratory animals“ (National Institutes of Health, volume 25, no. 28, revised 1996), EU Directive 86/609 EEC and German Protection of Animals Act. Male Sprague Dawley rats (SD, Janvier, St. Berthevin, France) weighing 225-250 g were housed in the central research establishment of the Goethe-University Frankfurt. At the beginning of the experiment rats were randomized to form pairs of donor and recipient rats which were kept together in approved plastic cages (2 animals per cage), had water and food *ad libitum* and were housed in rooms equipped with a 12 h light cycle. Rats were individualized by a numeral code labeled onto the tail with water-resistant paint.

2.1.6 Deguelin Treatment of the Animals

For treatment with the HIF-1α inhibitor Deguelin, a stock solution in 100% DMSO was made from the crystalline form at 25 mg/ml and stored at – 20°C for further use. For the application of Deguelin to the animals, the stock solution was dissolved in corn oil at a final concentration of 10 mg/ml. Deguelin was applied *via* gavage at a dose of 4 mg/kg adapted to the individual bodyweight twice a day. Treatment started 3 d before lung transplantation in recipients and donors and ended 2 d after transplantation (recipients).

2.1.7 Orthotope left Lung Transplantation

To establish the method of the orthotope left lung transplantation, we first used a method, that is described earlier by Zhai *et al*[226] and leads back to the method of Mizuta *et al*[227] who invented the cuff-technique for the lung transplantation. The method of Zhai, which is used for our first group, is shortly described here and the modified method by our group is described in 3.1. The left lung lobe of the donor animal is harvested by removing the heart-lung package after perfusion over the *Vena cava caudalis* and the three important structures of artery, vein and bronchus are primed free from all surrounding connective tissue, so all structures are clearly visible. Cuffs are prepared after Zhai's method as shown in **Figure 13** and all three structures are fixed to a cuff without any tail. The single left lung lobe is stored at 4°C until the recipient is ready for transplantation. *Via* a left thoracotomy the recipient's left lung lobe

is moved out of the body and the three structures are clamped with microaneurysm clips. After the blood flow is arrested, the structures are incised and the structures of the donor lung are placed into the structures of the recipient by holding on to the cuff. Once in place, a thread is fastened around and the Cuff and the clamps are shortly removed to prove if the blood flow can be restored or if there is any obstruction left. After this, the clamps are removed completely and the connection to the recipients left lung is carefully cut. The lung is now removed back into the thoracic cavern and the thorax is closed.

For establishment of the method, some animals were transplanted as described above and then, transplanted with the modified method described in 3.1. The animals that had undergone this kind of surgery (n = 11) were compared with animals, that were operated by the modified method (n =11).

2.1.8 Wet-to-Dry Ratio

To analyse the dimension of lung edema between the different groups, the method of Wet-to-Dry ratio is used. A part from the collected lung sample is put into a previously weighted 2 ml Eppendorf tube of which the lid is perforated and the gained data is recorded. Then the tube is weighted again to get the weight of the 'wet' lung sample. To dry the sample, the tube is placed in an oven for 72 h at 60°C and the new weight of the tube is again recorded. By dividing the wet-weight by the dry-weight a ratio is calculated and compared. Based on this data, the ratio of previous and actual weight give an idea of the volume of water content in the tissue and therefore of the dimension of lung edema.

2.1.9 Histology

To examine structural changes in tissue, histology is the method of choice. For this method, thin slices of fixated tissue, normally about 3-5 µm, are microscope slides, heat fixated and stained and at last covered with a cover glass. The thus prepared tissue can now be examined under the microscope and, depending on the staining, evaluated. The advantage of this method is that all aspects of the tissue can be examined in the same appearance as they were in the organism. This gives the chance to not only to observe the mere structure of tissue, but also to mark different proteins or genes of interest by special immunological staining methods and examine them not only in their quantity but also in their location in the tissue.

2.1.9.1 *Fixation of the Samples*

From all harvested lungs, half of each lung lobe is fixated for histological use. The fixation of fresh harvested samples is essential for storage and further preparation processing; it stabilizes samples and avoids bacterial and fungal growth. Normally, aldehydes, mostly formaldehyde or its polymerized form paraformaldehyde, are used for fixation right after harvesting. Formaldehydes are so called cross linking fixatives which create covalent chemical bonds between the proteins and last long enough so that the fixation reagent can immerse deep enough inside the tissue samples. It should not be applied longer than necessary to avoid tissue damage. For the harvested rat lungs 8-12 h are used (rule-of-thumb: immersion speed of 1 mm per 1 h). Formaldehyde is used in a 4 % solution in PBS and afterwards, the samples are put in fresh PBS over 24 h. After this samples are dehydrated in different concentrations of alcohol, beginning in a low concetration and ending in pure alcohol. For the last dehydration step, methylbenzoat is used to make sure that the tissue is best prepared for the following contact with the paraffin. Before embedding the tissue, the samples are washed in three steps in liquid paraffin, that is heated and then left in the last bottle of paraffin in the oven at not more than 60 °C over 16 h (**Table 7**).

Table 7: Fixation and embedding scheme.

step	reagent	time
Fixation		
1	Formaldehyde 4% in PBS	8-12 h
Dehydration		
2	PBS (phosphate-buffered saline)	24 h
3	Ethanol 70%	1 h
4	Ethanol 80%	1 h
5	Ethanol 90%	1 h
6	Ethanol absolute	1 h
7	Methylbenzoat I	8 h
8	Methylbenzoat II	16 h
Paraffin treatment		
9	Liquid paraffin (56-58°C)	3x 5 min
10	Liquid paraffin (56-58°C)	16 h

2.1.9.2 *Embedding*

Tissue Samples are embedded after paraffination with the embedding machine in metal histology molds and quickly cooled down on ice until the paraffin completely hardened.

2.1.9.3 *Cutting*

For all Samples, 6 slides are cut, for immunostaining on Super Frost Plus Slides and on Süssefrost slides for H&E staining. A Leica microtome is used and all cuts had a thickness of 4 µm. The cuts are carefully shoved on the slide and the paraffin is smoothened in a waterbath with a temperature of 40 °C. Afterwards slides are drained and then, the tissue cuts are fixed on the slide *via* heat for 30 min at 60 °C in the oven.

2.1.9.4 *H&E staining*

The Hematoxylin – Eosin staining is a common dye in histology and gives a good overview over the tissue. The used Mayer's Hematoxyline belongs to the aluminium-hematoxylin-solutions that are also known as 'haemalum', which refers to the fact that an oxidative form of hematoxylin, the haematein, combines with the aluminium ions. Under alcoholic acidic conditions in its complex with aluminium, it is more soluble and appears in a dark red color and turns blue and less soluble under aqueous neutral or alkaline conditions. Aluminium-hematein is said to bind basophilic structures, which means in this case, that it binds to acidic moieties like phosphate groups (nucleid acid) of the DNA backbone. It is normally used to stain nuclei and it also stains some other materials as keratohyaline granules and calcified deposit.
Normally, after the staining of the tissue, a so called 'blueing' follows – in this step, the stained tissue is put in tap water to turn the color of the hematein into blue due to the more alkaline conditions of harder tap water than distilled water.
The Eosin-G (or Eosin-Y) is one of two derivates of fluorescein. It is used as a counterstain to hematoxylin and is acidic, so that stains basic structures such as cytoplasma in a more or less intense pink and red blood cells intensely red. To intense the dye, acetic acid can be added.
For the staining, the slides are hydrated in different alcohol concentrations and water. After removing excess water, slides are stained in Mayers hematoxylin for 3 min and afterwards shortly dunked in 0.1 % HCl-solution. Now follows the 'blueing' in running tab-water avoiding the water jet to get directly in contact with the tissue slides. The procedure should be continued until the wished intensity of blue is reached. After this, slides are shortly left in distilled water and again excess water is removed before staining with Eosin-Y for 3 min. Excess eosin is removed before the slides are rehydrated in alcohol again (**Table 8**). After the last dehydration step, the slides are fixated with DPX-glue under a cover glass and dried.

Table 8: Histological staining scheme.

Step	Reagent	Time
Rehydration/Deparrafination		
1	Xylol 100%	10 min
	Xylol 100%	10 min
2	Ethanol 100%	5 min
	Ethanol 100%	5 min
3	Ethanol 96%	10 min
4	Ethanol 70 %	10 min
5	Ethanol 50 %	10 min
6	Destilled water	5 min
Nucleus staining		
7	Mayers-hematoxylin	3 min
8	0.1 % HCl	2 sec
9	Blueing under running tab-water	5-15 min
10	Distilled water	1 min
Counterstaining		
11	Eosin-Y (+ concentrated acetic acid) 200 ml (+ 400 µl)	3 min
Dehydration		
12	Ethanol 70 %	5 min
13	Ethanol 96 %	5 min
14	Ethanol 100 %	5 min
	Ethanol 100 %	5 min
15	Xylol 100 %	5 min
	Xylol 100 %	5 min
Fixation with DPX under cover glass		

2.1.9.5 *Immunostaining*

As the name implies, immunohistochemistry works with a staining *via* immunoglobulin that bind to a specific structure in the tissue. Similar to the Westernblot, a primary and a secondary antibody is used. Whereas the primary antibody binds directly to the target, the secondary antibody normally bears an enzyme which is capable of catalyzing a reaction producing a colored product. This is normally a peroxidase or a alkaline phosphatase.

For our immunhistochemistry, Superfrost Plus slides are used, to avoid the sample slices to detach during the preparation steps. Dehydration is done as described for the H&E staining and after the slides are put in PBS or TBS for 5 minutes.

To expose the epitopes, slides are put in a heat-resistant cuvette and covered with unmasking solution. They are heated in the microwave at 600-700 W and evaporating liquid is filled up every 5 minutes so the slides are always covered. The time depends on the epitope and lies between 5-10 min.

After heating, the hot cuvette is taken out of the microwave and let cool down to room temperature.

If slides have cooled down, they are washed in TBS-T two times for 5 minutes.

A ‘wet-chamber’ is prepared for further steps:

Therefore a common ice box is used which is coated with two layers of paper towel and soaked with water to prepare the ‘wet chamber’.

Now several steps of washing and blocking are done, before the primary antibody can be added. If a biotinylated secondary antibody is used, all steps are performed, if a HRP labeled antibody is used, only steps 4-7 are performed.(**Table 11**)

One of the tissue slices has always to be free of primary antibody as a control for the selectivity of the secondary antibody and proof of background staining. The slices are surrounded with a hydrophobic barrier pen as shown in the scheme below. Then the barrier density is tested by covering the slice with TBS-T or PBS (**Figure 8**).

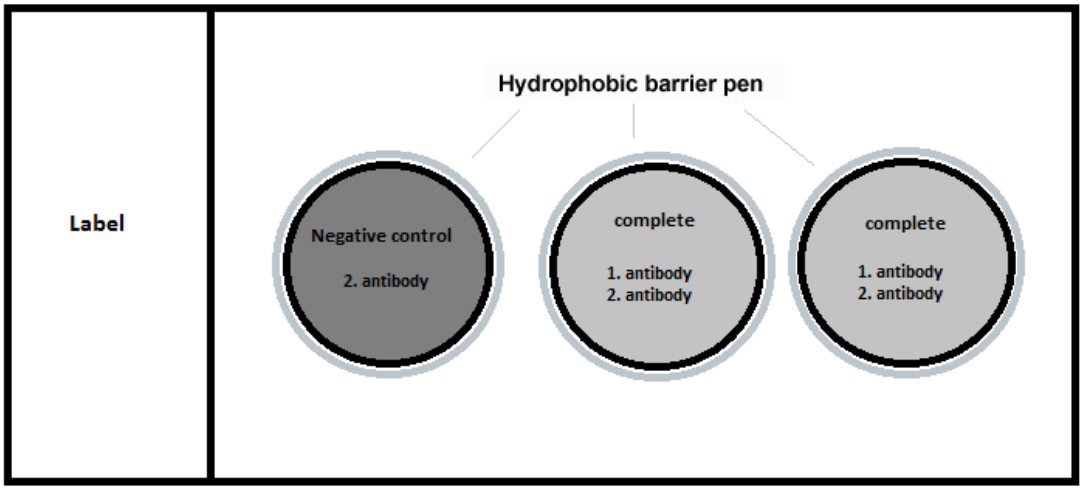

Figure 8: Schematical depiction of an immunohistochemical slide.

Buffer solutions:

Avidin blocking solution
1000 µl 0.1% TBS-T or PBS
100 µl serum from the species of the secondary antibody
4 drops Avidin blocking reagent from Avidin-Biotin blocking kit

Biotin blocking solution
1000 µl 0.1% TBS-T or PBS
100 µl serum from the species of the secondary antibody
4 drops Biotin blocking reagent from Avidin-Biotin blocking kit

Blocking buffer
1000 µl 0.1% TBS-T or PBS
100 µl serum from the species of the secondary antibody

Antibody solutions

The best concentration is tested before staining with one slide for every antibody used. All antibodies are diluted in blocking buffer. (
Table **9**)

Table 9: List of used primary antibodies

Primary Antibody					
Antigen	**Description**				**Dilution**
	Name	**Produced in clonality**	**Reactivity**	**Manufacturer**	
CD163	Mouse anti Rat CD 163 MCA342R	mouse monoclonal	rat	AbD Serotec	1:300
ICAM-1	Mouse anti Rat ICAM-1 LS-C45355	mouse monoclonal	rat	LSBio	1:300
CXCR4	Rabbit anti rat CXCR4 Ab2074	rabbit polyclonal	rat	abcam	1:300
CD 68	Mouse anti rat CD 68	mouse monoclonal	rat	CHEMICON International	1:300
Macrophage/Dendritic cells	Macrophage/Dendritic cells Antigen ABIN 289947	mouse monoclonal	rat	Antibodies – online GmbH	1:300

Secondary antibodies are used for biotinylated antibodies from the Elite ABC-Kits in following concentration:

1000 µl blocking buffer
10 µl secondary antibody from Kit

Following Kit is used for the detection of CXCR-4 antibody:

Vectastain Elite ABC Kit	Rabbit IgG

The following secondary antibody is used for the detection of CD 163, CD 68 and ICAM-1 (**Table 10**):

Table 10: List of used secondary antibodies

Secondary Antibody					
Antigen	**Description**				**dilution**
	Name	**Produced in** **clonality**	**reactivity**	**manufacturer**	
Anti Mouse	Goat anti mouse HRP labelled PO447	Goat 	mouse	DAKO	1:300

ABC-Solution:

For biotinylated antibodies, signals are enhanced by using the Vectastain-elite ABC kits. ABC-solution is prepared after manufacturers protocol.
ABC solution is prepared on the day of use and has to incubate 30 minutes before use. It is gently shaken during the 30 minutes.

DAB-solution:

Vectastain-DAB kit is used and DAB is prepared after manufacturer's protocol. It is prepared directly before use.

Table 11: Summary of immunohistochemical procedure.

Step	**Procedure**	**Time**
Day 1		
Avidin-Block		
1	Cover all slides with Avidin-blocking solution and incubate at room temperature	1h
Washing step		
2	Tip off the liquid and wash slides in fresh 0.1% TBS-T	5 min

Step	Procedure	Time
Biotin-Block		
3	Cover all slides with Biotin-blocking solution and incubate at room temperature	1h
Washing step		
4	Tip off the liquid and wash slides in fresh 0.1% TBS-T	5 min
Peroxidase-Block		
5	Cover all slides with freshly prepared 6% H_2O_2 and incubate at room temperature	30 min
Washing step		
6	Tip off the liquid and wash slides in fresh 0.1% TBS-T	5 min
Primary antibody		
7	Cover only two of three slides with the Primary antibody solution; incubate like it is said in the datasheet, the most save is over night at 4 °C	2 – 16 h (overnight)
Day 2		
Washing step		
8	Tip off the liquid and wash slides in fresh 0.1% TBS-T	5 min
Secondary antibody		
9	Cover all slides with secondary antibody solution and incubate at room temperature	1 h
Washing step		
10	Tip off the liquid and wash slides in fresh 0.1% TBS-T	5 min
ABC-solution		
11	ABC-solution should be prepared in the last 30 min of antibody solution and a small rest should be saved to test the ABC-DAB reaction. Cover all slides and incubate at room temperature	30 min
Washing step		
12	Tip off the liquid and wash slides in fresh 0.1% TBS-T	5 min
DAB-reaction		
13	Incubate with DAB at room temperature until the tone of brown is adequate – the negative control stays in a slight pink (depending on the DAB used) or colorless	5-7 min

Step	Procedure	Time
Stop of DAB reaction		
14	DAB reaction is stopped by putting the slides in fresh distilled water	5 min
Counterstaining with hematoxylin		
15	Hematoxylin is diluted with water 1:1 and directly put on the slices	10 min
Washing step and blueing		
16	slides were shortly washed in distilled water and then blued like described for the H&E stain and reaction is stopped when the adequate blue-tone is reached	10 min
Fixation under cover glass with Roti-Mount Aqua without dehydration or with DPX after dehydration as described for H&E stain		

2.1.9.6 *Cell Count for Immunohistochemical Staining*

To compare the slides of the stain and to reduce inaccuracy due to different intensities of the individual stains and tissue-non tissue ratio, the counting is done by a program code gratefully written for our group by Dr. Dipl. Phys. Scheller from the Clinics for Anesthesiology, Intensive care and Pain management.

Therefore, pictures are set in the three dimensional RGB (Red Green Blue) room with m x n pixels x 3 color layers. The benefit of this is that the matrix consists of the three layers for different colors that are divided into red, green and blue. By additive effects, the complex colors in a picture are presented and every pixel can have a value from 0-255.

This definition brings the benefit of not only being able to differentiate stained (brown) from unstained (blue) tissue, but also to measure the intensity of the brown and blue tissue and set it into relation. Furthermore, a miscalculation by adding the non – tissue area (white) can be avoided.

Pictures are loaded into Matlab with the Image Processing Toolbox as RGB color figures (8-bit depth, figure size 1944x2592 pixels). For each of the color matrices red, green and blue, pixel values are determined. Alveoli are determined, when the pixel value of the matrix coding for reed exceeded 0, the color brown is defined as a difference value between pixel values of blue and green not exceeding a value of 12 and, conditionally values in the matrix coding the color reed not exceeding a value of 80. All other combinations of pixel values are classified as tissue not stained in colors of brown. Results show the relative areas of areas stained in shadows of brown to the rest of the tissue with the area of alveoli subtracted.

2.1.10 Western Blot

2.1.10.1 *Homogenization and Preparation of Tissue Samples*

Samples are snap-frozen in liquid nitrogen and stored at -80°C until further use. To avoid thawing, they are kept in liquid nitrogen right before use and all steps are carried out on dry ice.

2.0 ml Eppendorf tubes are weighted and precooled on dry ice. A dry ice block is wrapped in clean aluminium foil and disinfected. The forceps to hold the sample is sterilized and put in 80% alcohol solution and a disposable scalpel is used. All instruments are cleaned after every sample, disinfected in alcohol and precooled on dry ice.

The frozen sample is carefully cut in small pieces and put in the 2.0 ml Eppendorf tube to be weight quickly so that it doesn't thaw. 1 ml of Frackelton-Lysis buffer (**Table 12**) is added to 50-100 mg of tissue. The tissue-buffer mixture is homogenized with an Ultraturrax until no big pieces are left and the mass has turned completely homogen.

The homogenized sample-buffer mixture is centrifuged at 15000 rpm for 10 min at 4°C. The supernatant is transferred to a new, precooled Eppendorf tube, without touching the pellet. To avoid freeze and thaw cycles, the supernatant is aliquoted in smaller Eppendorf tubes for further use.

Cell samples. To avoid degradation of HIF-1α, cells are directly harvested in the hypoxia chamber. The hypoxic medium is removed and the cell layer is briefly washed with 2 ml cold PBS. PBS is again removed and 100 µl of the Urease-buffer for HIF-1α (**Table 12**) is put on the cells. The cell layer is then removed with a disposal cell scraper and the buffer-cell mix is transferred into a 1.5 ml Eppendorf tube and put on ice.

The cell-buffer mixture is incubated on ice for 10 min and then sonificated with 5 pulses and centrifugated afterwards at 16 000 g for 20 min at 4 °C. The supernatant is transferred into a new Eppendorf-tube.

Table 12: Buffers used for sample homogenization and preparation

Urea Lysis Buffer (100 ml)			**Frackelton Lysis Buffer (100 ml)**		
Volume	**Substance**	**Concentration**	**Volume**	**Substance**	**Concentration**
50 ml	Urea	6,65 M	0.121 g	Tris	10 mM
10 ml	Glycerol	10 %	1.338 g	Natriumpyro-phosphate	30 mM
5 ml	SDS (SDS 20%)	1 %	0.292 g	NaCl	50 mM
1 ml	Tris/HCl (1M) pH 6.8	10 mM	Set pH to 7.05		
Add immediately before use			1 ml	Triton X-100	1 %
	DTT (1M stock)	1mM	Add immediately before use		
40 µl/ml	Protease Inhibitor Cocktail 25x	1x	10 µl/ml	PMSF (100mM stock)	1 mM
			10 ml/ml	Natrium-vanadate (10mM stock)	100 µM
			40 µl/ml	Protease Inhibitor Cocktail 25x	1 x

2.1.10.2 *Protein Measure*

Protein mass is measured *via* colorimetric protein assay. Two different methods are used: The Bradford assay and the bicinchoninic acid protein assay (BCA). The last one has to be used for the samples that contained Urea buffer as it leads to an unwanted color change in the Bradford assay.

Bradford Assay. This assay works with the dye Coomassie Brilliant Blue G-250, which appears red and turns to blue depending on acidic conditions. When it binds to the amino acids of the protein in its red state, it donates its free electron pair to the ionizable functionalities of the protein, thereby disrupting the proteins native conformation. Thus, the hydrophobic pockets are exposed and can non-covalently interact *via* van der Waals forces with the non-polar regions of the dye This stabilizes the blue form, which can be measured *via* absorbance reading.

The Bradford assay is linear at low protein concentrations, normally between 0 µg/ml to 2000 µg/ml, therefore, the samples are diluted 1:5 (2 µl sample + 8 µl buffer) for the cell-samples and 1:25 (5 µl sample + 120 µl buffer) for the tissue-samples.
As a standard, different dilutions of BSA in water are prepared (0 – 1000 µg/ml) and 10 µl of each standard and sample are pipetted in a microplate in duplets. Then all slots are filled up to 200 µl with Bradford solution. The plate is covered with a lid or parafilm and incubated for 5 minutes. The absorbance is read out at 595 nm in a photospectrometer.

BCA Assay. For the BCA assay the BCA Kit from Pierce is used. This assay relies mainly on two interactions: in a temperature-dependent reaction, Cu^{2+} ions from the cupric sulphate in the BCA reagent are reduced to Cu^{1+} ions *via* the peptide bonds of proteins. Then two molecules of bicinchoninic acid chelate with one Cu^{1+} ion and the formally green solution turns purple. This purple product absorbs light strongly at a wavelength of 562 nm. For minimizing unequal amino acid composition, the assay is incubated at a temperature of 37°C.
All samples measured in BCA assay are diluted 1:10 and a BSA standard is used, equally to the one for Bradford assay. 10 µl of each sample and standard are pipetted in duplets in a microplate.
The reagents of the kit are mixed as discribed in the manufacturers protocol (1 ml reagent A + 20 µl reagent B) and 200 µl of the BCA solution is added to all samples and standard. Then the microplate is covered with a lid or parafilm and incubated at 37°C for 5-10 minutes. Measurement is done when the reaction turned purple. The absorbance is read out at 562 nm in a photospectrometer.

2.1.10.3 *Preparation for Blotting*

For tissue samples, 30 µg are loaded and 4 µl of loading buffer (**Table 13**) are added and all samples are filled up to 16 µl with water. For cell samples, 100 µg are loaded and 4 µl loading buffer are added. All samples are filled up to 16 µl with water.
Proteins are denaturized at 95°C for 5 min.

Table 13: Used Loading Buffer

Loading Buffer (10 ml)	
Volume	**substance**
6 ml	deionized water
1 ml	500 mM Tris-HCl pH 6,8
2 ml	Glycerol
1g	SDS
50 mg	Bromophenolblue (0,05%)
Add just before use	
1 ml	ß-Mercaptoethanol

SDS-PAGE. The Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) is a common method for separating denaturized proteins by molecular weight. Depending on the size of the proteins, we use 10% (HIF-1α) or 15% (VEGF) gels (**Table 14**,**Table 15**) for detection. First, separating or stacking buffer (**Table 16**) is mixed with water and the acrylamide. Polymerization is induced by adding ammoniumpersulfate (APS) and TEMED directly before use. The gels are polymerized in a gel caster starting with the separating gel which is polymerized for at least 30 min. To avoid drying and to get a smooth surface, isopropanol is put on top of the polymerizing gel. Then, the isopropanol is tipped and the stacking gel is filled right to the line of the gel chamber. The comb with 10 wells is carefully put into the still liquid gel and the gel is polymerized. It is then incubated overnight at 4°C in a wet chamber and used the next day.

Table 14: Used Separating Gel

Separating Gel (volume for two gels)					
Density	**30% Acrylamide**	**Separating Buffer**	**Distilled water**	**10% APS**	**TEMED**
10 %	6.65 ml	5.4 ml	7.95 ml	100 µl	80 µl
15 %	10.0 ml	5.4 ml	9.6 ml	100 µl	80 µl

Table 15: Stacking Gel

Stacking Gel (volume for two gels)					
Density	**30% Acrylamide**	**Loading Buffer**	**Distilled water**	**10% APS**	**TEMED**
5 %	2.5 ml	3.8 ml	8.7 ml	60 µl	20 µl

Table 16: Buffers used for SDS-PAGE

Separating Buffer (200 ml)			**Stacking Buffer (200 ml)**		
Volume	**Substance**	**Concentration**	**Volume**	**Substance**	**Concentra-tion**
45.4g	Sigma 7-9	1.875 M	15.1 g	Sigma 7-9	0.625 M
1 g	SDS	0.5 %	1 g	SDS	0.5 %
Fill up to 200 ml with deionised water			Fill up to 200 ml with deionised water		
Set to pH 8.8			Set to pH 6.8		

Running Buffer (1l)			**Transfer Buffer (1l)**		
Volume	**Substance**	**Concentra-tion**	**Volume**	**Substance**	**Concentra-tion**
3 g	Sigma 7.9	50 mM	3 g	Sigma 7-9	25 mM
14.4 g	Glycine	0.384 M	14.4g	Glycine	
1 g	SDS	0.1 %	800 ml	distilled water	
Fill up to 1l with deionised water			200 ml	Methanol	

For Detection of the protein, a sodium dodecyl sulphate (SDS) polyacrylamid gel electrophoresis (SDS-PAGE) is used. SDS-PAGE is a method that works with the denaturated variants of the protein and separates them by their molecular weight. To reach adequate denaturation,

a strong reducing agent – the loading buffer – is added to each sample and by short heating, this effect is enhanced. It removes tertiary and secondary structures, as for example disulfide bonds which are reduced to sulfhydryl groups. So prepared, the sample proteins become covered in the negative SDS and move to the positive charged electrode along the gel through the mesh of acrylamide by applied voltage on the gel that lets the protein run in different speed. Depending on the concentration of the mesh of acrylamide, smaller proteins can run through the gel better than bigger ones. Thus, high concentration of acrylamide are more suitable for detection of lower molecular weight proteins. High molecular weight proteins need low acrylamide concentrations. If bands are not adequately separated, or bands have run through the gel, the acrylamide concentration has to be adapted.

To get an overview over the progress of the running proteins, markers are used – a commercially available mixture of proteins – in this case, one which generates colored bands, and another, which is only visible under UV-light after luminol-treatment.

Using a biorad - MINI PROTEAN CELL 3 the earlier prepared gels were loaded with the samples and 5 µl SPECTRA Brood Range visible marker in the first slot and 5µl Magic Marker in the last slot. After filling the chamber with running buffer (**Table 16**) and prove if it does not leak, the gel runs at 80 volt for the first 30 minutes through the stacking gel and then for 60 minutes at 120 volt through the separating gel.

2.1.10.4 *Protein-Transfer and Detection*

For the detection of the proteins by antibodies, they first have to be transferred onto a membrane. This membrane normally is made of nitrocecullose or polyvilidene difluoride (PVDF). The membrane has to be placed on top of the gel and a varying number of filter papers and sponges are placed around it, depending on the manufacturer's protocol. Using a defined electrical potential, the proteins move from the gel to membrane and appear in the same bands as they were in the gel and are now on the surface of the membrane and ready for detection.

So after the run, the gel is carefully removed from the cassette and put on a prepared construction of one sponge and two filter papers which are soaked in transfer buffer (**Table 16**) and a nitrocellulose membrane is put on the gel. Then it is again covered with filter papers and a sponge and the transfer cassette is put in transfer buffer and the transfer is peformed at 4 °C for 1 ½ hours at 100 volt. After this, the success of the blot is proved by a short staining with Ponceau Red.

Table 17 Buffers for Protein Detection

PBS stock solution (10 x)			**TBS stock solution (10x)**		
Volume	**Substance**	**Concentration**	**Volume**	**Substance**	**Concentration**
80 g	NaCl	1.37 M	80 g	NaCl	1.37 M
11.5 g	Na_2HPO_4 (waterfree)	81 mM	12.1 g	Tris-base	100 mM Tris-HCl
2 g	KCl	27 mM	Ad 900 ml aqua bidest.		
2 g	KH_2PO_4 (waterfree)	14.7 mM	Set pH on 7.3 with HCl		
Ad 1 l aqua bidest.					
PBS (1x) 1 l			**TBS (1x) 1l**		
Volume	**Substance**		**volume**	**substance**	
100 ml	PBS-Stock solution (10x)		100 ml	TBS-stock solution (10x)	
900 ml	Aqua bidest.		900 ml	Aqua bidest.	
Blocking solution (BSA 5 %)			**Washing buffer (1l) (TBS-T 0.1 %)**		
100 ml	TBS - T (1x)		1 l	TBS (1x)	
5 g	BSA	5 %	1 ml	TWEEN	0.1 %

After washing off the Ponceau Red in 0.1 % TBS-T (**Table 17**) the Membrane is put into a solution of blocking solution for two hours to block non-specific binding. Then the primary antibody (**Table 18**) in a tested dilution in blocking solution (**Table 17**) is put on the membrane and incubated overnight at 4 °C. The next day, the membrane is washed in washing buffer shortly for 3 mins, then 2 x 5 mins, 1 x 10 mins and 1 x 30 mins – afterwards the secondary antibody (**Table 18**) in its tested dilution in blocking solution is put on and incubated at room temperature for one hour. Then again, the membrane is washed as described previously.

For imaging, the chemiluminescence-reaction of luminol with the horseradish-peroxidase (HRP) of the HRP-labeled second antibody, is used. Luminol is a chemiluminescent dye which must be activated first, to exhibit luminescence. This is normally reached by an oxidant as peroxides. The picture is taken with a Kodak Imager and analysed with ImageJ Software. As control, ß-Actin is detected.

Table 18: Used Antibodies for Westernblot

Primary antibodies					
Antigen	**Name**	**Produced in** **clonality**	**reactivity**	**manufacturer**	**dilution**
HIF-1 alpha	H1alpha67	Mouse monoclonal	Human, Mouse, Rat	abcam	1:500
HIF-1 alpha	HIF-1 alpha clone 54	Mouse monoclonal	Human	BD	1:500
VEGF	mAbcam 68334 ab68334	Mouse monoclonal	Human, mouse, rat	abcam	1:500
ß-Actin	ß-Actin (13E5)	rabbit monoclonal	Human, mouse rat	Cell signalling	1:1000
Secondary antibodies					
Antigen	**Name**	**Produced in** **clonality**	**reactivity**	**manufacturer**	**dilution**
Mouse-IgG	IgG-HRP Sc2005	goat polyclonal	mouse	Santa Cruz	1:2000
Rabbit-IgG	IgG-HRP (sc-2030)	goat polyclonal	rabbit	Santa Cruz	1:2000

2.1.11 PCR

2.1.11.1 *RNA Isolation*

For RNA extraction, the phenol-chloroform method is used, a special liquid-liquid extraction method for RNA, but also DNA and protein. For this, an aqueous sample is needed which is mixed with water saturated phenol and chloroform. By centrifugation, the phases are separated and split up, containing RNA and DNA in one, protein in another phase. As a last step, RNA is precipitated with 2-Propanol or ethanol.

RNA is isolated from tissue samples with TriReagent after manufacturers protocol. 50-100 mg of the tissue sample is homogenized after putting them in the adequate volume of TriReagent (1ml) and 0.2 ml Chloroform are added. Tubes are shaken vigorously for 15 s and

incubated at room temperature for 2-3 min. Samples are then centrifuged at 11000 x g for 15 minutes at 4°C. After centrifugation, the homogenized samples are splitted into three phases – a lower, red phenol-chloroform phase, an interphase and an aqueous upper phase. For RNA-isolation, only the aqueous phase is carefully removed and transferred into another Eppendorf-tube and 0.5 ml of isopropyl alcohol is added. Differing from the original protocol, samples are incubated overnight at -20°C to enhance precipitation. The next day, samples are centrifuged at 11 000 x g for 10 min at 4 °C. The supernatant is removed and the remaining pellet is washed two times with 1 ml 75% ethanol in DEPC-water by mixing the samples by vortexing and centrifugation at 7500 x g for 5 min at 4°C. After this, the pellet is air-dried for 10 mins and shortly dried at 55°C until the opaque pellet turned transparent. At the end of the procedure, the RNA is dissolved in 30 µl DEPC-water and incubated on a shaking heater for 5 minutes at 55 °C.

2.1.11.2 *cDNA Ttranscription*

As RNA can't be used for PCR, it is transcribed into cDNA using the enzyme reverse transcriptase. cDNA is the reverse transcribed product using iScript cDNA synthesis kit according to the manufacturers protocol. 1 µg of RNA is mixed with 1 µl transcriptase and 5 µl reaction mix, then the volume is filled up to 20 µl with DEPC-water. The samples are shortly centrifugated and reverse transcription is executed in a thermocycler and diluted 1:10 with PCR-water.

2.1.11.3 *Realtime PCR*

The polymerase chain reaction (PCR) is used to amplify DNA so few copies can be replicated into great numbers. It works with different phases of heating and cooling cycles. The heating process is needed for the DNA melting – to produce single strands where the polymerase can bind – and the enzymatic reaction. The polymerase which is used is heat-stable and the best known is the Taq-polymerase from the bacterium *Thermus aquaticus*. The polymerase can bind on the single strand DNA in 5' to 3' direction and uses nucleotides to build a new anti-sense strand. The selectivity of the PCR results from the primers, which are a sequence of nucleic acid that are complementary to selected regions of the targeted sequence.
The realtime polymerase chain reaction (realtime-PCR) is based on the PCR, with the additional feature that amplified DNA can be detected during the process, in 'realtime' different

from the normal PCR, where the detection is only at the end of the amplification. This is usually achieved by appropriately designed primer sets that generate a triggered fluorescence signal upon the hybridization/polymerization process. Thus, the DNA can be measured and either be quantified to an absolute number of copies or a relative amount, compared a so called housekeeping gene, in this case ß-Actin. Realtime PCR is performed, using the Step One Plus realtime PCR and the Power SYBR Green PCR Mix. mRNA levels are always normalized to ß-Actin. The following oligonucleotides are used:

rCXCR4 forw. CACCAACAGCCAGAGCGCGA, rev. TGCGCTTCTGGTGGCCCTTG; rVEGF forw. CCAGGCTGCACCCACGACAG, rev.:CGCACACCGCCATTAGGGGCA; rICAM-1 forw. CGCAGTCCTCGGCTTCTGCC, rev. CGCAGTCCTCGGCTTCTGCC; rACTB forw. CTTGCAGCTCCTCCGTCGCC, rev. CTTGCTCTGGGCCTCGTCGC; rVCAM-1 forw. GGTGGCTGCACAGGTTGGGG, rev. ACCCACAGGGCTCAGCGTCA; rCD 163 forw. TGGGATCGCCGTGACGCTTC, rev. CAGCGACTGCCTCCACCGAC; rIL-4 forw. GGCTTCCAGGGTGCTTCGCAA, rev. GTGGACTCATTCACGGTGCAGC

A mixture is prepared which contains the DNA-template, the two complementary primers, the nucleotides, the polymerase and special buffers. In this case we use the commercial Power SYBR Green PCR Mix and add the designed primers as mentioned above together with our sample DNA. The realtime PCR consists of different steps like initialization, denaturation, annealing and elongation. The initialization is needed to activate the polymerase. By disrupting the hydrogen bonds between the base pairs during the heating up in the denaturation step, the DNA is melted and the double strand splits into the single strands which is important for further steps as the polymerase can only bind on the single strand. In the annealing step, the temperature is lowered again to allow the primers to anneal to the single strand DNA molecules. A close match of the primers is of great importance for good binding. Once bound, the polymerase can attach to the primer-template hybrid and starts DNA synthesis. The temperature at the elongation steps depends on the optimum activity temperature of the used polymerase but is commonly 72°C. The polymerase synthesizes a new DNA strand in 5'-3' direction, using the oligonucleotids. The time that is needed depends on the polymerase but also on the length of the template. In this steps, the amount of DNA is doubled and leads to exponential amplification and huge amounts of DNA, even with only few template molecules of DNA in the beginning.

The optimal temperatures of the realtime PCR were evaluated in the beginning and 40 cycles were run. (**Table 19**)

Table 19: Parameters of the real time PCR cycles.

Status	Temperature	Time
Activation of TAQ-polymerase	95°C	10 minutes
Denaturation of cDNA	95°C	15 seconds
Annealing	60°C	10 seconds
Elongation	72°C	20 seconds
Heating up and measurement of fluorescence	84 °C	5 seconds
Melting curve	65-95° C in steps of 0.3° C	

2.1.12 Statistical Analysis

For stastistical analysis, Graph Pad Prism® 5.02 software is used and results are displayed as means +/- standard error of the mean (SEM). Statistical significance is calculated using Oneway ANOVA followed by Student's t-test. Statistical significance is set to $p < 0.05$.

3 Results

3.1 Establishment of the Orthotopic Lung Transplantation Model

The animal model of lung transplantation has many benefits for observation of a most similar situation to the clinic. But on the other hand, it also bears many difficulties compared to other used methods for simulation of lung transplantation, e.g. the trachea graft model. A complete thorax-surgery with a transplanted lung graft means big stress for the animal and requires precise control of animal health and pain management to ensure no unnecessary stress to the rats. Besides that, the surgery method itself has to be well established to guarantee comparable results and sources of error have to be found and erased by refining of the used method.

3.1.1 Anaesthesia

Preparation and Premedication:

All rats (donor and recipient) are pre-anesthetized with a body-adapted dose of Ketamine/Xylazine combination with a 75:10 ratio. 100 µl of this mixture are given per 100 g body weight intraperitoneally (i.p.). As preoperative pain-management 0.05 mg/kg Buprenorphine is given subcutaneous. A well anaesthesia is reached after 4-6 minutes.
Rats are shaved on a different table to avoid contamination of the surgery table with fur and the skin is disinfected with a standard skin-disinfection.
Donor-rats are shaved generously from the ventral and lateral abdomen to the jawbone and recipient-rats are shaved on the left lateral body-wall from the spine down to the sternum.

Intubation and maintenance of anaesthesia:

The pre-anaesthetized animals are rested on the back and the mouth was kept open with the tongue pulled out (**Figure 9** a). The opening of the trachea is lightened with the cold light source that is set on the trachea. The trachea presents itself as a small spot and is best hit with the tubus by sliding it along the ground of the lower jaw (**Figure 9** b). To prevent the trachea from swelling, a Lidocaine-spray soaked swap is used to carefully spot the area around the trachea. For intubation, a common 16G venous catheter is used of which the sharp end is cut and blunted. To prove if the tubus is lying in the trachea, the animal is ventilated shortly and if positive, the catheter is fixed with a loose surgical knot to the cheeks skin.

The rats are ventilated with 85 beats per minute and a maximum pressure of 25mbar, positive end-expiratory pressure (PEEP) against atmospheric pressure. Isoflurane is vaporized with 1.5% as maintenance of narcosis.

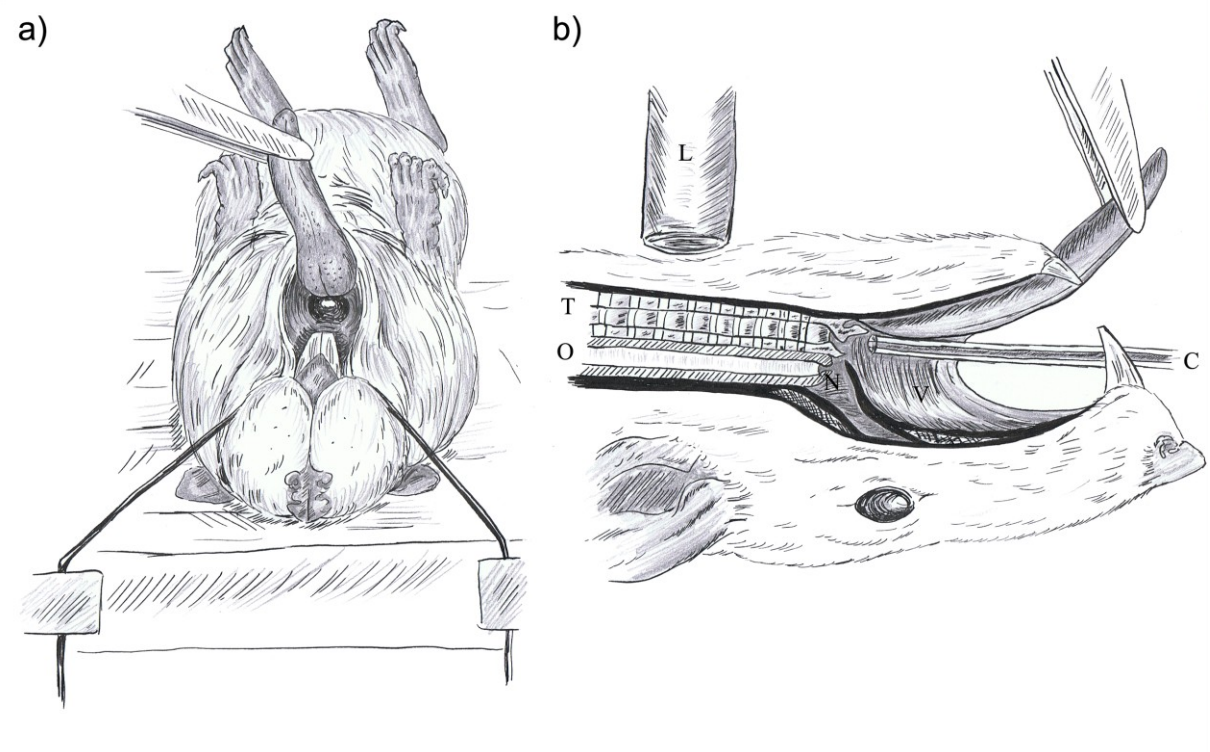

Figure 9: Schematical drawing of the intubation of a rat. a) Frontal view. The rat is positioned at the edge of the table and the upper jaw is fixated with a strong thread. The tongue is pulled out carefully and the light source is put in position. Now the opening of the trachea presents itself as a light spot. b) Cross section of the head. The tubus, made of a venous catheter, is best positioned by sliding it along the tongue. If reaching the trachea, a slight scraping can be felt which is caused by the cartilage rings of the trachea. Most animals show a reflective interruption of breathing when the trachea is touched, so the rat should be ventilated soon after. T = trachea; O = oesophagus; N = nasopharynx; V = oral cavern; C = venous catheter for intubation; L = cold light source. (Illustration by Ockelmann®)

3.1.2 Preparation of the Animals According to our Adapted Method

Preparation of the Donor Lung:

The donor-rat is embedded on the back and the skin is disinfected. Then the skin is cut in a line from the abdomen to the throat (**Figure 10** a)). The skin is dissected, avoiding opening the muscles, and then, the abdominal muscles are cut in the *Linea alba* until reaching the *Processus xyphoideus* of the sternum (**Figure 10** b)). Before opening the thorax, the donor is treated with 150 IU heparine by injection in the *Vena cava inferior* (**Figure 10** c)). The sternum is cut in the median of the *Vertebrae sterni* to the first rib. The ribcage opened to both

sides and it is spread with mosquito clamps on each side. The long lingual muscles were primed carefully, and the trachea, which lies in the median of the muscles, becomes visible (**Figure 11**).

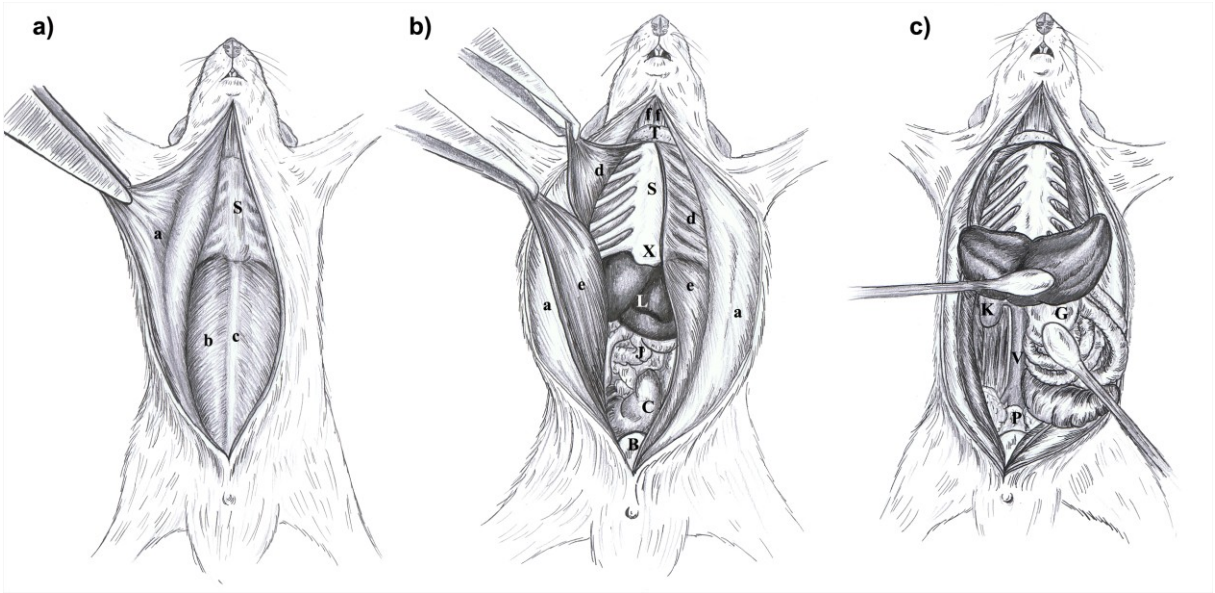

Figure 10: Schematical drawing of the opening of the rat. a) The skin is cut from the pubic bone to the jaw angle and carefully primed to avoid contamination with the unsterile outer side. Now, the abdominal muscles come to view, which are separated by the *Linea alba*, the aponeurosis of the abdominal muscles. The abdomen should be cut in the *Linea alba*, the pectoral muscles can be cut from the first rip to the *Processus xyphoideus*. b) View on the opened abdomen with the prominent liver and the convolute of the intestine. Once separated from the pectoral muscle, the ribs come clearly into view. c) By shoving the intestinal convolute to the right side and lifting the liver carefully with cotton buds, the *Vena cava caudalis* appears and the heparine can be injected directly in the prominent vessel – to avoid to much blood loss, the puncture should be compressed with a cotton bud afterwards for a few seconds. S = sternum; X = Processus xyphoideus; R = rip; T = thymus; H = heart; L = liver; V = Vena cava caudalis; G = stomach; K = kidney; P = preputial gland; a = skin with subcutis and cutaneus muscle; b = abdominal muscles; c = Linea alba; d =M..pectoralis; e = abdominal muscles completely cut; f = long hyoid muscles. (Illustration by Ockelmann®)

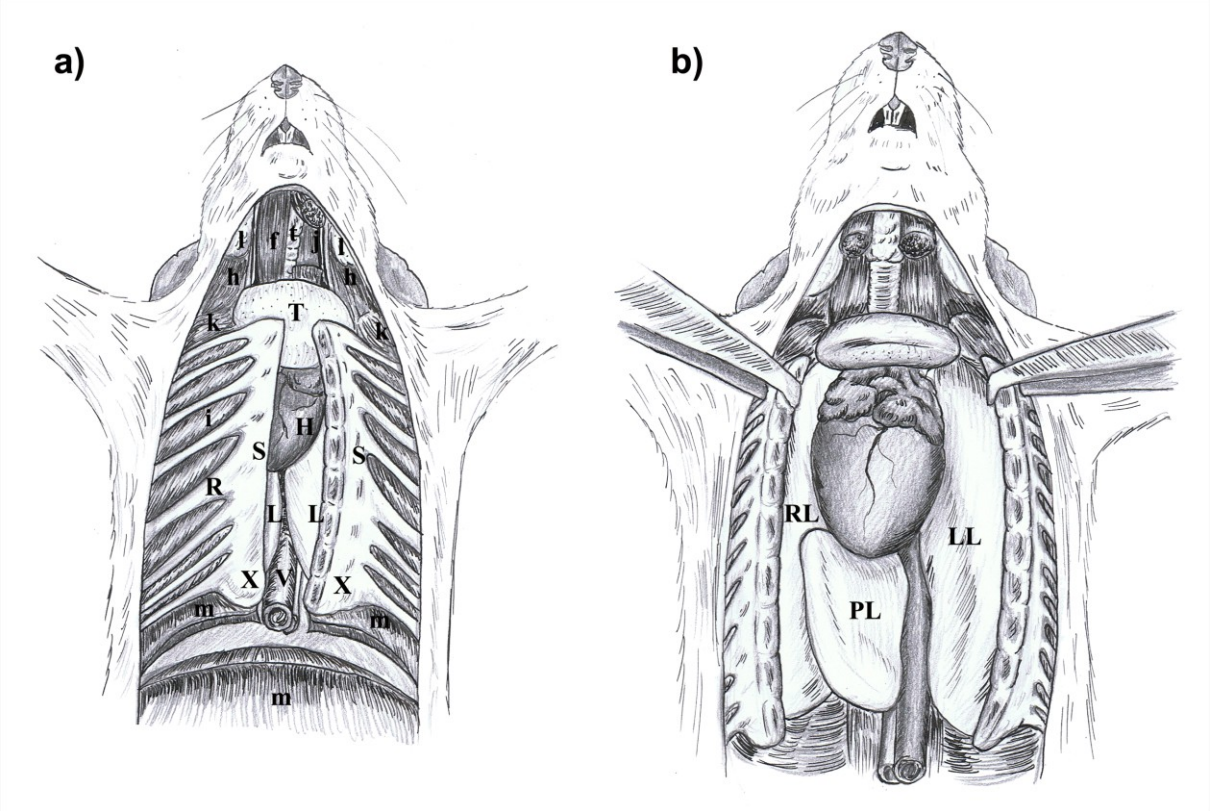

Figure 11: Schematical drawing of the opening of the thorax. a) With a strong preparation scissor, the sternum is cut in the median, from the *Processus xyphoideus* up to the first rip. If the ribcage is completely cut, it opens easily and can be fixated with e.g. mosquito forceps to widen it. If not already cut, now, with the lungs visible, the diaphragm is cut and in young animals, also the prominent thymus has to be dissected – therefore, only the apex should be cut, as the cutting of the big blood vessels in the basic leads to massive bleeding. S = sternum; X = Processus xyphoideus; R = rip; T = thymus; H = heart; LL= left lung; RL = right lung; PL = post caval lung lobeV = vena cava caudalis; f = long hyoidal muscles; h = M. omohyoideus; i = Mm. intercostalis; j = M. longus capitis; k = M. deltoideus; l = saliva glands; m = diaphragm; t = trachea. (Illustration by Ockelmann®)

Perfusion:

For perfusion, the heart must be free from remaining tissue like mediastinum or pericardium. In young rats, the prominent thymus, which lies still over the heart-base, has to be dissected. With a fine straight scissor, the right ventricle is cut in the middle deep enough to open the lumen. Soon after that, the left atrium is also incised.

A venous catheter (16G) which is connected to a tube containing 20 ml of cold perfusion solution (4°C) with Perfadex®- or Ringer®-solution with a few drops of heparine, is slid through the opening in the right ventricle and positioned in the '*Truncus pulmonalis'* (**Figure 12**).

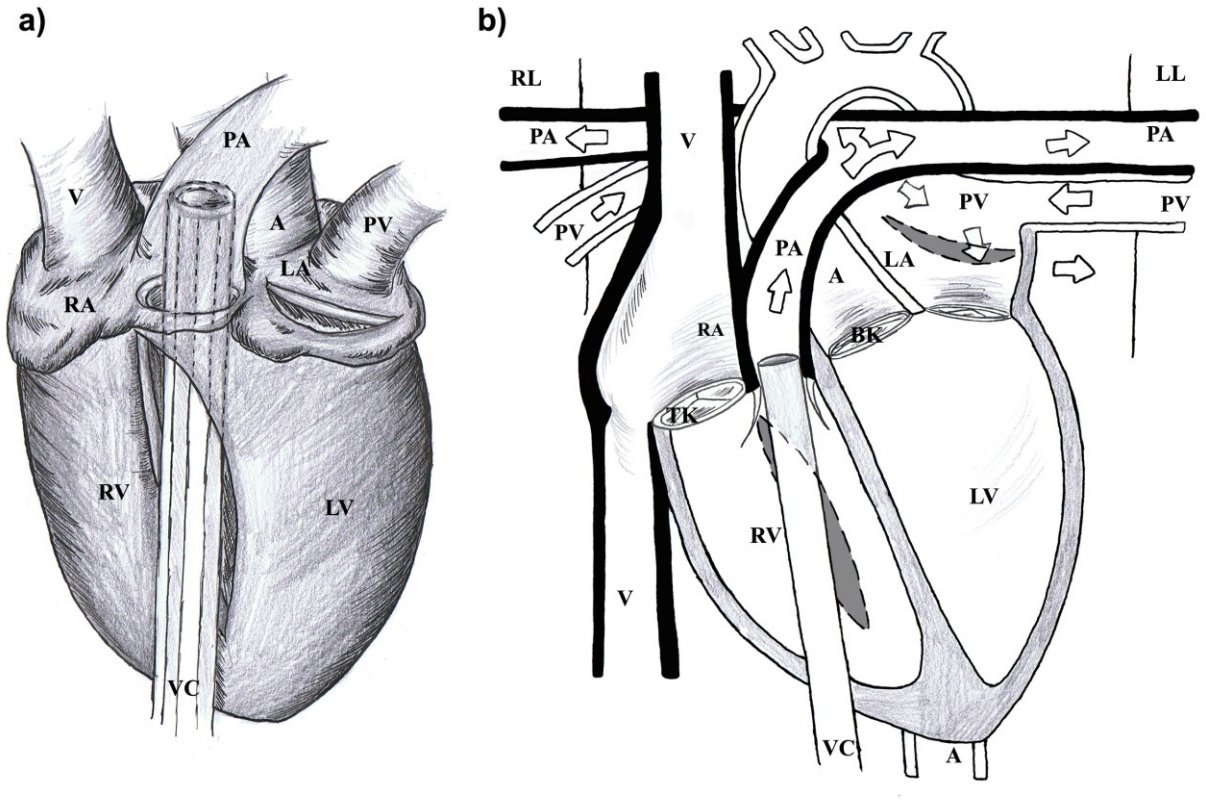

Figure 12: Schematical drawing of the perfusion method a) Schematical drawing of the cut into the ventricle with the already opened left atrium. The venous catheter lies in the pulmonary artery so that a direct flush of the lung is guaranteed. b) Scheme of the flow of the perfusion solution. A = Aorta; V = Vena cava; PA = Pulmonary artery; PV = Pulmonary; s = Superior lobe of right lung; m = Middle lobe of right lung; i = Inferior lobe of right lung vein; RV = Right ventricle; RA = Right atrium; LV = Left ventricle; LA = Left atrium; VC = Venous catheter for perfusion. (Illustration by Ockelmann®)

If the perfusion is effective, the lungs soon turn pale and become white. The flushing is stopped before air is reaching the circulation.

After that, the trachea is dissected by carefully cutting the connecting fascia between the long lingual muscles and then separated from the oesophagus by carefully pushing a closed scissor between them and then opening the blades.

Now a loose ligature is laid around the trachea and pulled tight in the phase of deepest inspiration, so that the lungs are filled with air. The trachea is then separated cranial to the ligature and the whole heart-lung package is removed, avoiding especially damage to the left lung.

A bended fine preparation scissor is used, with the bended side always in direction to the lung. The scissor should cut in a vertical direction with only little opened scissor blades and the tip always at the spine.

Storage of the Heart-Lung Package:

Directly after removing the heart-lung package, it is carefully wrapped into a moist gauze in a Petri - dish. Then the lung can be stored on ice or in a fridge at 4°C for the subsequent procedures.

Cuff-Sleeve-Technique:

Cuffs are made out of 17G or 16G venous catheter parts which are cut into pieces of 3-4 mm length. Each of the pieces is then cut vertically in the centre with a scalpel. The cut is about the half of the diameter. A second cut is made horizontal from the end to the first cut and the fragment removed, so that a strap remains. Any sharp edges were blunted to avoid damage to the tissue (**Figure 13**).

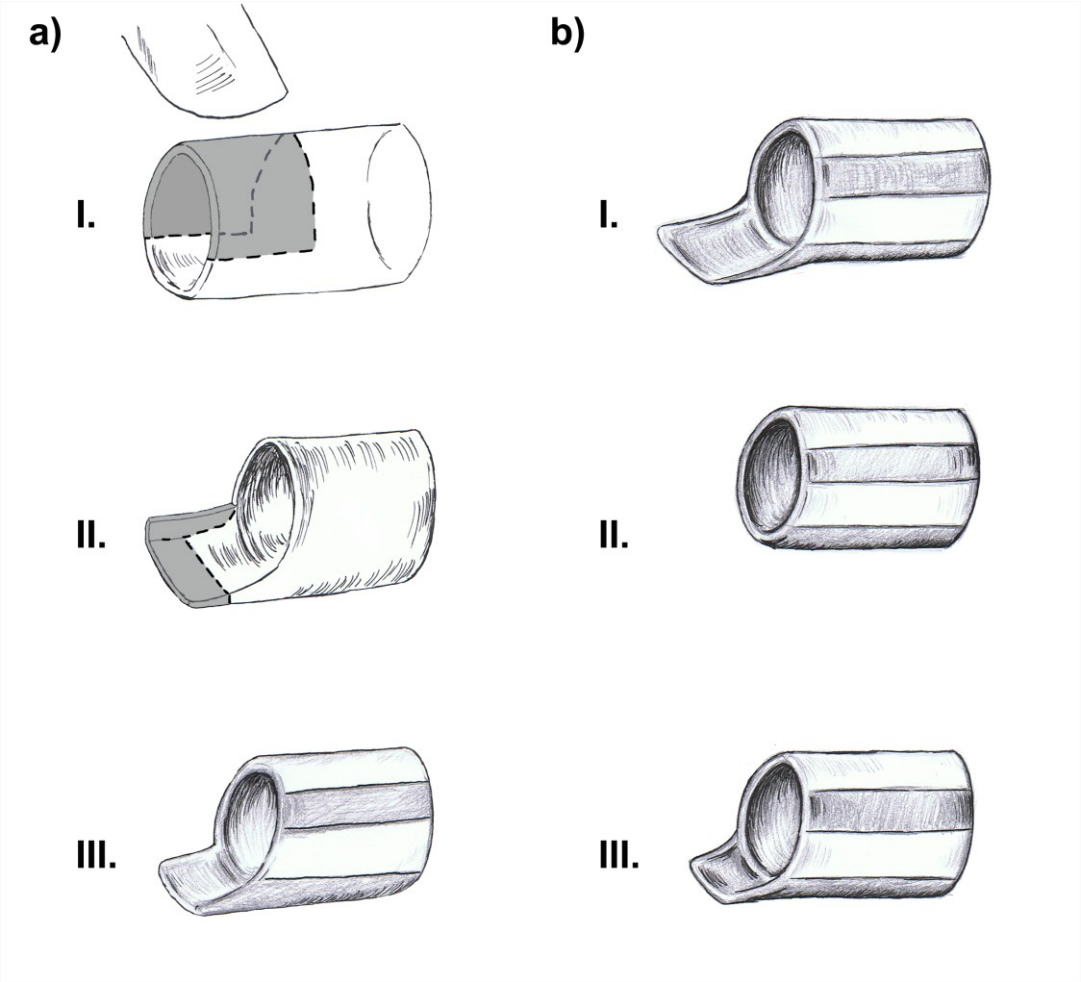

Figure 13: Schematical drawing illustrating the preparation of the cuff. a) The 3-4 mm piece of the venous catheter is first incised to the median in both directions (I.). The flap is cut as short as possible so it can still be hold with a microneedleholder but at least to the half. The remaining side walls are carefully cut, avoiding any sharp edges (II.). b) Sketches of the three possibilities of cuffs used for lung transplantation. Mizutas original cuff (I.), Zhais cuff without any flap (II.) Our cuff with a short flap (III.) (Illustration by Ockelmann®)

For the fixation to the cuff, ligature-loops (double surgical knot) of 6-0 Prolene thread are used. During preparation, the lung is still kept in the Petri - dish and kept cool and moist. The next working steps are accomplished under the microscope. For microsurgery two microneedleholders, one microscissor and one lockable microneedleholder are needed.

First the heart-lung package is positioned in the same way as *in situ* with the heart on top. The artery, vein, and bronchus are wrapped in connective and fat tissue that has to be removed very thoroughly. All structures are cut as long as possible. First, the vein is dissected. It is the structure most caudal and usually very short in the Sprague Dawley rats. Therefore it is first dissected from remaining tissue and then cut in the confluence of the left atrium. Next the artery is primed and at last, the bronchus (**Figure 14**).

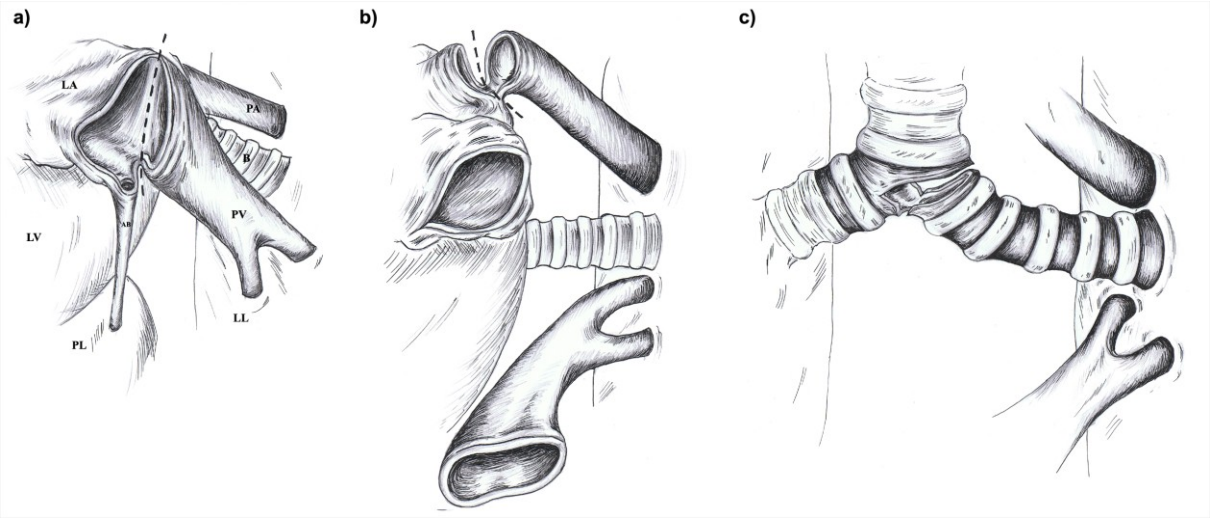

Figure 14: Schematical drawing of the preparation of the three important structures, surrounding tissue is already removed a) First the vein is cut in the confluence of the left atrium by cutting before the branch of the post caval lobe departs b) The artery is also cut as heart-near, as possible. c) The bronchus can only be primed safely after the vein is already shoved aside. It is cut in the *Pars membranacea* in the *Bifurcatio trachea*; LA = left atrium; LV = left ventricle; PA = pulmonary artery; PV = pulmonary vein; AB = accessory branch of the pulmonary vein; B = bronchus; LL = left lung. (Illustration by Ockelmann®)

For the vein, a 16G cuff is clamped with its strap in a lockable microneedleholder with the round open end showing upwards. The free end of the vein is carefully grabbed with the microneedleholder and pulled through the cuff. Once the end is slipped completely through, the vein is turned upside-down over the cuff and fixed with a 6-0 Prolene ligature. The strap is left free, so it can be hold. It has to be checked whether the lumen of the vein is completely continuous and the vessel is not twisted during the procedure.

The same procedure is performed for artery and bronchus, using a 17G cuff for the artery and a 16G cuff for the bronchus (**Figure 15**).

After this, the heart and the right lung are dissected and discarded. The prepared left lung is then again stored as described before until the recipient is prepared for transplantation.

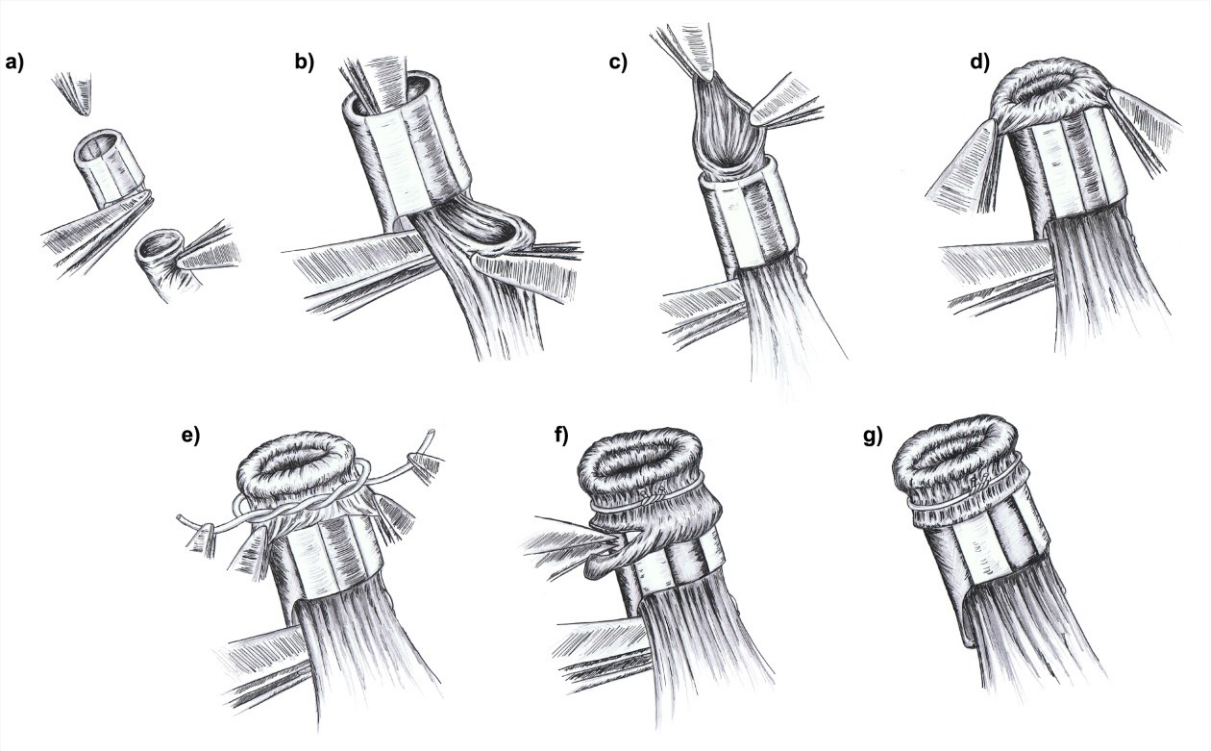

Figure 15: Schematical drawing of the cuff-technique a) The cuff is clamped. b) The structure is carefully shoved through and dragged with a microneedleholder c) Once completely shoved through, the walls are carefully grabbed with the microneedleholders. d) The structure is everted over the cuff. e) The so prepared vessel is hold in position by the surgeon while the assistant is fixating it with the prepared thread-loops. f) After ligation, overlapping tissue is cut with a microscissor. g) The cuff, ready for transplantation. (Illustration by Ockelmann®)

3.1.3 Recipient Preparation

Ligature-loops are made of 5-0 silk threat (double surgical knot). Meanwhile 2-3 sterile compresses are soaked in sterile NaCl-solution and precooled.

A syringe with a 1:100 diluted Heparin/NaCl-solution is prepared (Heparin 25,000 I.U.) and assembled with a blunted cannula. A syringe with atropine and another one with a 1:100 solution of adrenaline are also prepared.

Anesthesia, intubation, ventilation and shaving are performed as described earlier.

The rat is embedded on the right body side and the light source of the microscope is adjusted to the thorax. Before starting the surgery, a dose of 50 µl atropine is injected intramuscularly in the quadriceps against bradycardia.

The cutting line is between the 4.-5. intercostal space, which can be found by an imaginary line drawn from the straight spine to the shoulder and elbow in normal flexion (**Figure 16**).

The intercostal space can then be palpated. We found out, that the 5th intercostal space is the best choice in young animals, as the prominent thymus still covers the organs in the 4th intercostal space.

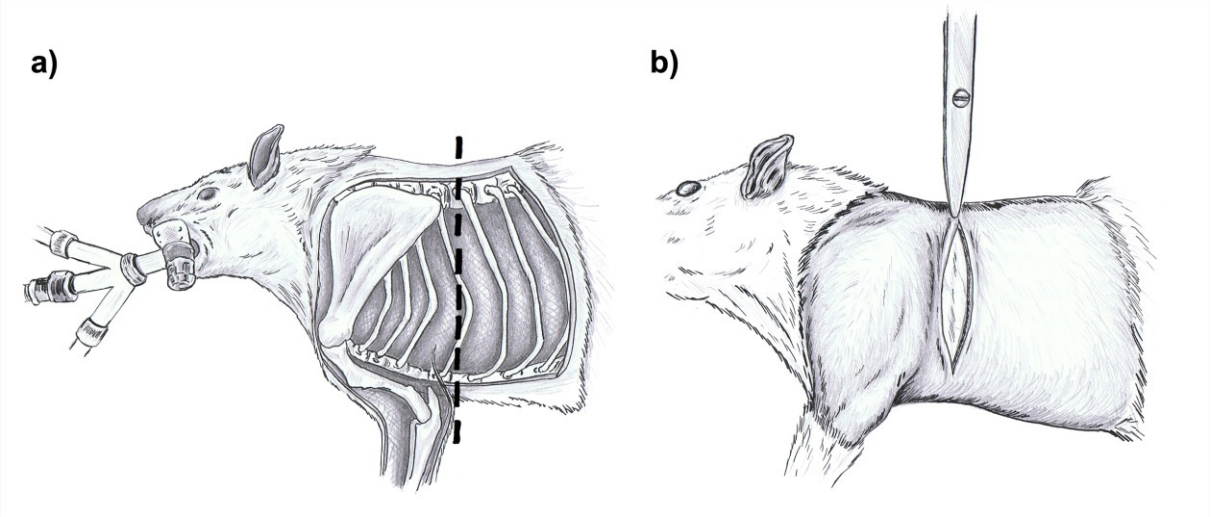

Figure 16: Schematical illustration of the cut. a) Transversal section of the thorax of a rat lying on the right body side b) First cut through the skin. (Illustration by Ockelmann®)

3.1.4 Transplantation Procedure

To establish the method of the orthotope left lung transplantation, we first used a method, that was described earlier by Zhai *et al*[226] and leads back to the method of Mizuta *et al*[227] who invented the cuff-technique for the lung transplantation. The method of Zhai, which was used for our first group, is not described here and only the modified method by our group is described below.

Surgery:

With a surgical scissor, the skin is cut straightly and the muscles are primed. The cut is started dorsal and elongated in ventral direction. Big blood vessels are cautered with a thermocauter or clamped with a mosquito forceps. After cutting the *M. latissimus dorsi* and *M. serratus ventralis*, the ribs and the *M. intercostalis*are visible. First a small incision is made in the determinded intercostal space with a straight small scissor, so that the thoracic cave is opened. To avoid cutting any big vessels, the cut should be made on the cranial part of the caudal rip belonging to the chosen intercostal space and it should be avoided to elongate the cut ventral as the mammary artery is located there. The final cut is about 1-1.5cm long (**Figure 17**).

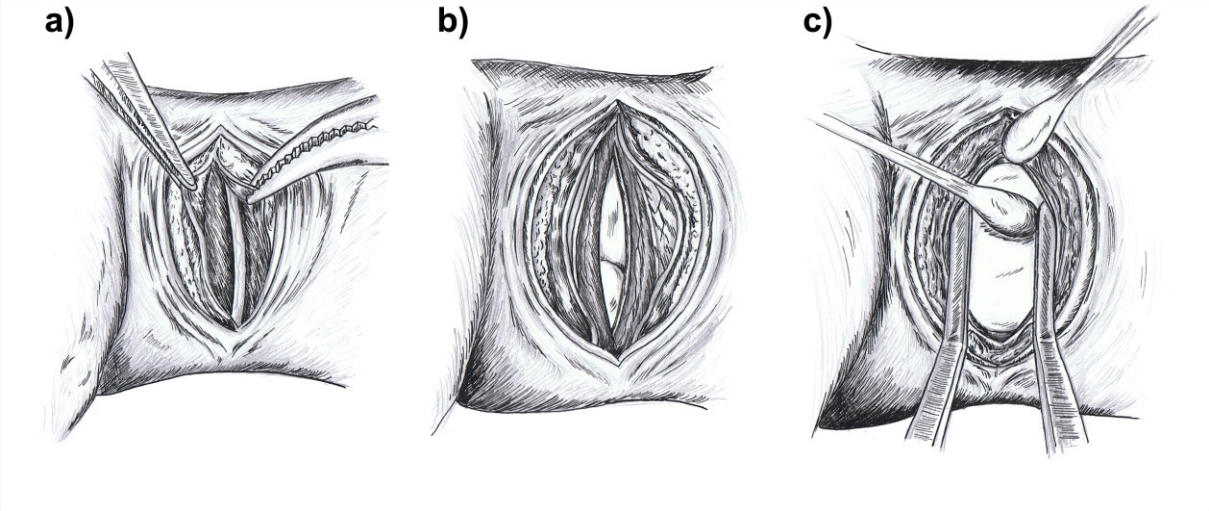

Figure 17: Schematical drawing of the thorax opening (transplantation). a) Subcutis and muscles are primed and bigger vessels clamped or thermocautered. The 5th rip comes into view b) By cutting along the cranial side of the 5th rip, cutting in the vessels and nerves is prevented as they are better protected on the cranial side than on the caudal side. In the right position, the left lung comes into view, together with caudal parts of the thymus c) The rips are spread with a rip-spreader. (Illustration by Ockelmann®)

Once opened, the heart and the left lung are visible. To spread the cut, a rip spreader is hooked in and the opening is widened carefully. The left lung is moved out of the body with two swabs, trying first to move the cranial part an then the longer caudal part (**Figure 18**).

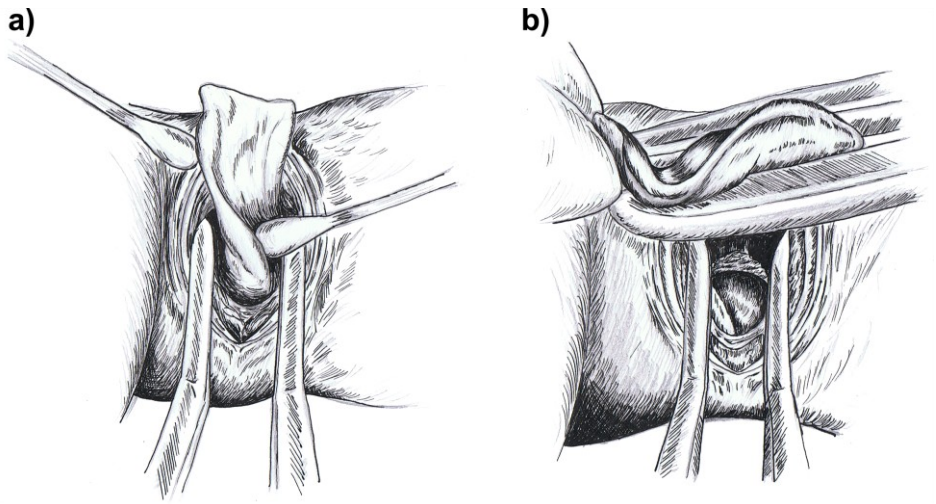

Figure 18: Schematical drawing of the retrieval of the left lung a) by gently rolling along the cranial parts of the lung with a sterile swab. The tip of the lung can be moved towards the wound opening and then fixed with another swab from the backside. b) Once completely put out of the wound opening, the lung is clamped with a special lung clamp and fixated. (Illustration by Ockelmann®)

All next steps are done under the microscope.

The vessels and the bronchus are carefully dissected from the surrounding fat and connective tissue and primed. Blood and other liquids are removed frequently using swabs (**Figure 19**).

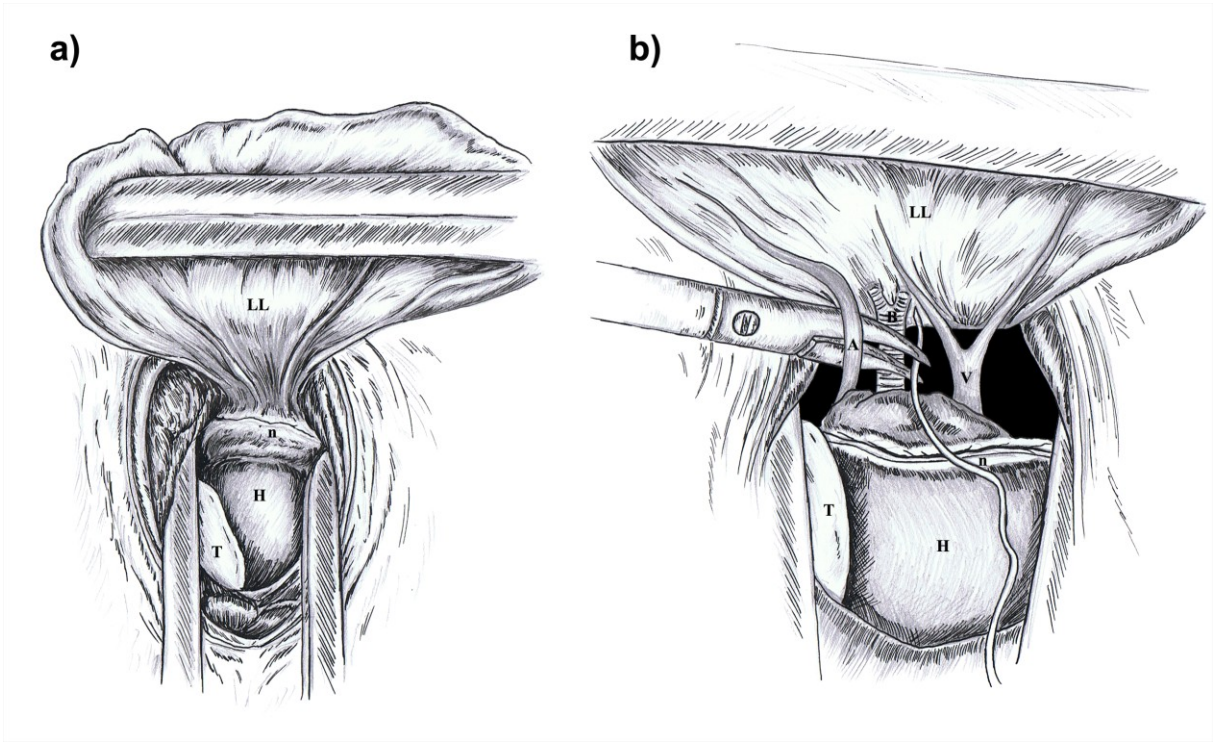

Figure 19: Schematical drawing of the fixated lung. a) If fixed in the right position the lung base comes into view, still covered in connective and fat tissue. b) After removal of surrounding tissue and preparation of the three structures, ligation threads are carefully layed around every structure. Touching of the heart and the *Nervus phrenicus* are avoided. LL = left lung; H = Heart; T = thymus; n = Nervus phrenicus. (Illustration by Ockelmann®)

All three structures are ligated with a 6-0 Prolene loop and fixed under light tension. A 2 mm cut is made with the microscissor in the front wall of artery and vein. For the vein, it is best, to cut in the arborisation on the dorsal end of the vein near the hilus. The bronchus should be cut later to avoid aspiration of liquid. Artery and vein are rinsed with the prepared 1:100 diluted Heparin-solution until they appear bloodless. Therefore, the cannula of the syringe must to be blunted. After clearing and swabbing the liquid out of the thorax, the bronchus is cut (**Figure 20**).

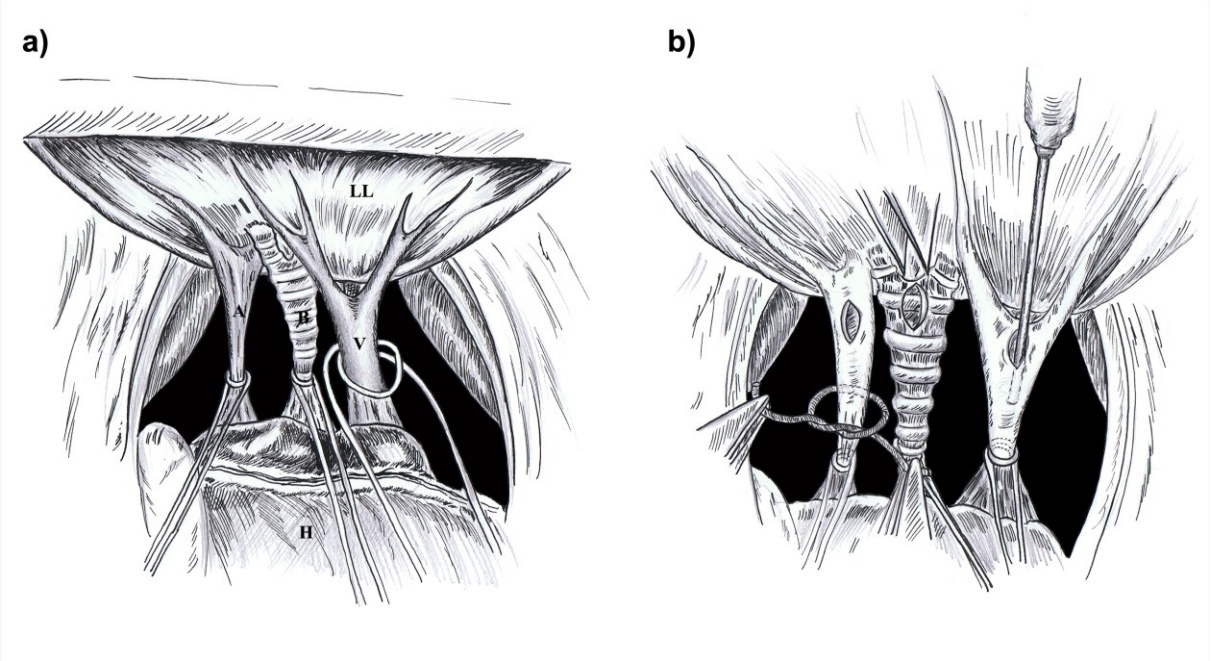

Figure 20: Schematical drawing of preparation of the recipient's vessels and bronchus. a) All three structures are reversible ligated with a single loop of a thread. The threads are fixated outside of the wound opening. b) The vessels are incised on the front wall and flushed with a solution of heparine (1:100) until they appear free of any left blood. The bronchus is incised after removal of every fluid. Loops of silk threads are laid around prepared structures. LL = left lung; A = artery; B = bronchus; V =vein; H = heart. (Illustration by Ockelmann®)

Finally a 5-0 silk thread is laid around the structures in a loose double-surgical knot. The donor lung is now fetched out of the can storage, wrapped into a cool, moist gauze and placed on top of the recipient's lung. The strap is fixed with a microneedleholder and the cuff is carefully slid into the opening of the recipient's vessels and bronchus as deep as reached. Again it has to be checked, whether the structures were not twisted. Once in the right position, the silk-knot is tightened around the cuff. In an optimal case, the silk ligature lies on the Prolene-ligature.

After all ligatures are tight enough, the vessel loops around the vessels and bronchus are removed, so that reperfusion and reoxygenation is started.

First the thread around the vein is cut, and watched if retrograde reperfusion occurs, ensuring the venous drainage, then the artery follows. If the lung appears to be filled with blood again, at last, the loop around the bronchus is removed.

Now the remaining recipients left lung lobe is carefully dissected *via* cutting the remaining connection to the tissue on the vessels and bronchus *via* microscissor (**Figure 21**).

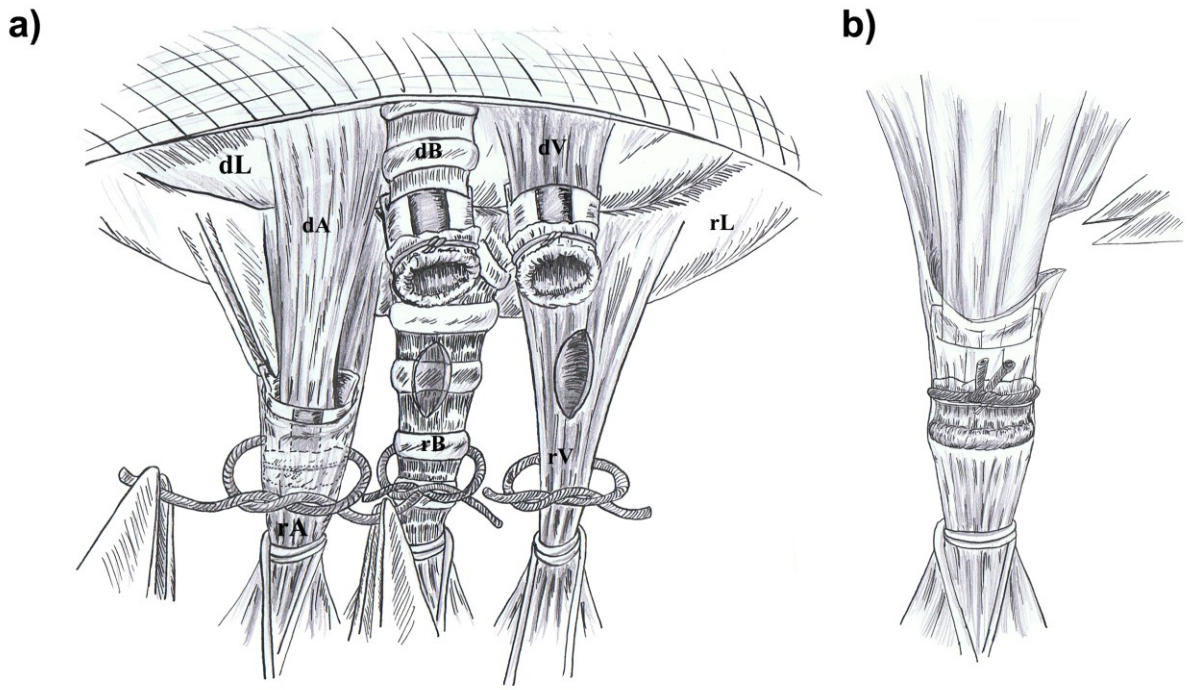

Figure 21: Schematical drawing of cuff-fixation. a) The silk loops are held in position by the assistant while the surgeon puts the cuff into the incised opening of the recipient's structure as deep as possible. b) The silk thread is pulled tight so the cuff can't slip. The silk ligature lies best directly on the Prolene-ligature of the cuff. dL = donor left lung; dA = donor pulmonary artery; dB = donor bronchus; dV = donor pulmonary vein; rA = recipients pulmonary artery; rB = recipients bronchus; rV = recipients pulmonary vein. (Illustration by Ockelmann®)

The transplanted lobe is now moved back into the thorax. For this, the ventilation has to be disconnected, so the lung collapses and can be shoved back through the opening. As soon as it is in position, ventilation is connected and the lung is shortly bloated, so the atelectatic areas are filled with air. After relocating the transplant, 3-4 pieces of threads are laid, so that both wound-ends can be connected adequately. A venous catheter is placed in the ventral corner between the two last threads, and the wound is closed with a double surgical knot. Then the remaining air and liquid is evacuated *via* the venous catheter removing it under constant motion. Then, muscles and skin are adapted to an anatomically correct position and constellation.

3.1.5 Pain Management

To reduce postsurgical pain, 600µl Ropivacain solution 0.2% is instilled around the wound instead of a presurgical pain-management with a aequivalent opoid. This instillation of a local anaesthesia serves as a intercostal nerval blockade and was used to avoid the systemical effect

of the opioid in the first hours after transplantation especially with the view on the respiratory depression. A body-adapted dose of enrofloxacin is given subcutaneously.

For further postoperative pain-management we used piritramid is used as a strong painkiller and administered *via* drinking water, containing glucose. Therefore, 3 ml piritramid (1.5 mg/ml) are dissolved in 230 ml water and 20 ml glucose 20% solution is added. The glucose is added to reduce the bitter taste of the piritramid.

The water bottles are weighed in the beginning and then everyday to guarantee an adequate supply with piritramid. The rats are observed twice a day and weighed every day.

3.1.6 Effects of Local Anaesthesia after Surgery on Animal Health

To confirm whether a local anaesthetic intercostal nerve blockade is an adequate analgesia, we analysed the animal health, including general condition, physiological status, explorative behaviour, motility, full loading of extremities and food and water intake on a scale from 0 to 10 in which 0 is worst and 10 best (**Table 20**).

Awaking animals that got a local anesthesia block show much better motility and full loading of the left body side compared to animals only treated with a presurgical single injection of buprenorphine. Animals that are treated with the ropivacain-block show an improved general condition and even active exploratory behaviour almost right after complete awakening (Figure 22).

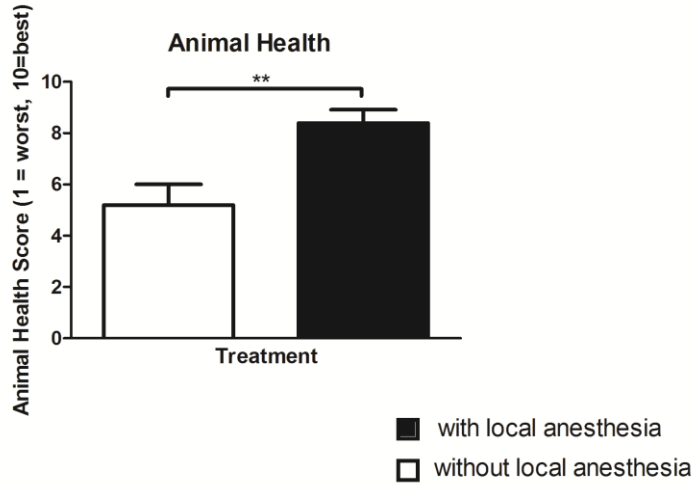

Figure 22: Comparison of the Animal health between the groups treated with local anesthesia block and those who were only treated with 'classical' injected opioid presurgical. n=6, **p<0.01.

Table 20: Parameters of the animal health score.

Category	**Points**		
	1	0.5	0
Recovering from anaesthesia	Awakening < 30 min Soon attempts to move	Awakening > 30 min No or only inadequate movement	**No awakening**
Exploration behaviour	No discrepancy to natural condition	*Hesitant, interest in strong stimuli (food, water, other rat)*	No attempts
Loading of extremities	No discrepancy to natural condition	*Relief of anterior body part, but movement of all extremities*	Hesitant setting of extremities, only moving if necessary, much resting
Breathing	No discrepancy to natural condition	*Careful breathing and movement of thorax, slight respiratory sound*	Gasping for air with open mouth, strong respiratory sound
Pilierection	No discrepancy to natural condition	Slightly, over the first day	Strong, lasting over the second day
Social grooming	No discrepancy to natural condition	Restricted social grooming	No social grooming
Food and water intake	No discrepancy to natural condition, gaining weight	*Acceptable intake, loss of bodyweight < 10%*	Inacceptable or no intake, loss of bodyweight > 10%
Pain signals	No discrepancy to natural condition	Slight signs of unwell feeling over the first hours after surgery	**Signs of pain, apathy**
Individual body care	No discrepancy to natural condition	Restricted body care (Hadersches secret around the eyes, scruffy fur)	No body care
Interest in the enviroment	No discrepancy to natural condition	*Interest only to strong stimuli*	No interest

more than 2 fields that are highlighted in grey	**1 field highlighted in grey and bold letters**	**more than 2 fields in italic letters**
termination of experiment and humanely killing of the rat	termination of experiment and humanely killing of the rat	prove of pain management

3.1.7 Surgical Complications

To compare the method of Zhai *et al* [226] with our modified transplantation procedure, three categories of cause of death are taken into consideration (**Figure 23**, **Figure 24**):

The first category includes animals that are included in the end of the experiment, have survived the transplantation and the 48 h postsurgical observation time. Another category describes animals that have died due to surgical complications which correlate to problems that are linked to the surgical procedure itself as damage to important structures by instruments or unstoppable bleedings due to loosening of clamps or ligatures. Finally, a last category comprises animals that have died due to other causes including unpredictable problems that lead to exclusion from the experiment like heart arrest or pathological conditions that were unassigned to the experiment.

Taken together these data indicate that, compared to the method of Zhai *et al*[226], the modified method is less sensible to surgical complications that ended in exclusion of the animals.

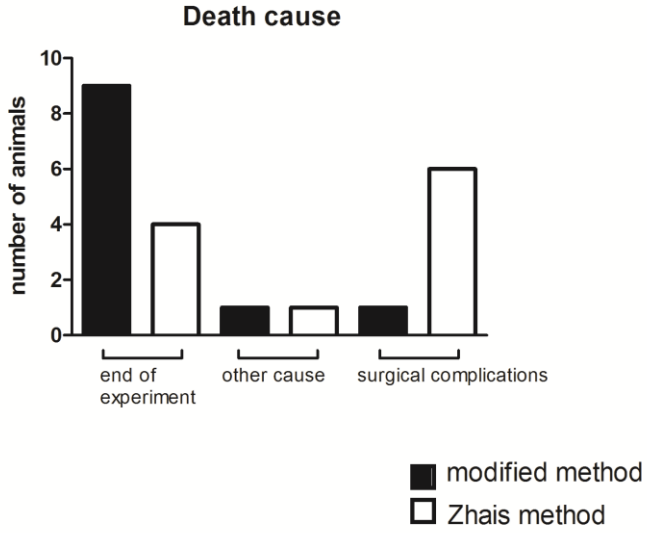

Figure 23: Cause of death according to the surgery method. n=11

Figure 24: Cause of death in a group score that shows a significant difference in surgical complications regarding the two methods. n=11, *p< 0.05.

3.1.8 Time Cost of Surgery

Moreover, the modification of the surgical procedure is a benefit for the time needed to completely transplant the lung. The graph shows the time difference for the surgical procedures, which is defined as the time from the start of the warm ischemia of the lung when the lung is taken from the cold storage until the moment of the complete adaption of the transplant and prove of the function. Our method is significantly faster than the one described by Zhai *et al* [226](**Figure 25**).

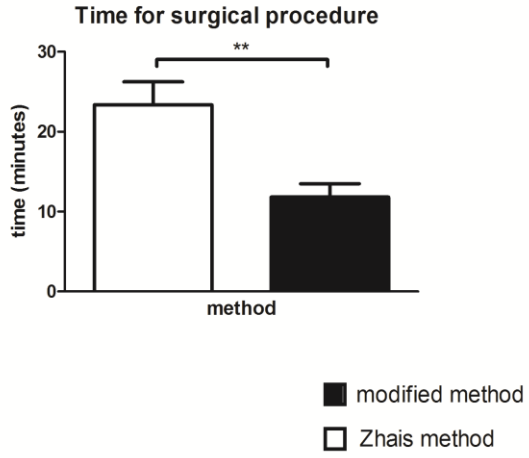

Figure 25: Comparison of the time needed for surgical procedure with the modified and Zhais method. n=11, **p<0.01.

3.1.9 Comparison of the Survival Rate

Comparing the survival rates of all animals in the both groups, we have found that our modified method results in a much better outcome. The graph shows the days of survival in %, with the complete time period of 48h as 100%. In the group that is treated according to the method of Zhai[226], a mean survival of 40% is reached, whereas our modified method group reached a mean survival of 80% (**Figure 26**).

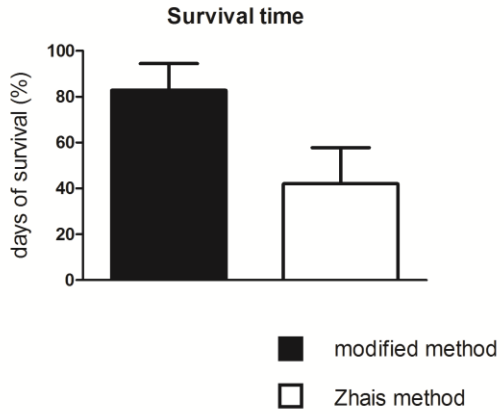

Figure 26: Survival rate comparing both surgical methods. n=11

3.2 Pilot Experiment

3.2.1 Cell culture Experiments

3.2.1.1 *Dose dependent Effects of Deguelin on HIF-1 α in vitro*

To prove the anti HIF-1 effects of Deguelin, we first determined its inhibitory capability in a cell culture experiments. As described previously, Deguelin was tested at increased concentrations under hypoxia as well in HMECs as in HTB-177.

With a working concentration of 100 nM we showed that after 6 h of hypoxia Deguelin effectively reduces HIF-1α protein expression in HMECs and HTB-177 compared to normoxia-controls. DMSO (solvent) alone has no suppressive effect and hypoxia alone stabilizes HIF-1α as expected. (**Figure 27** a)

Deguelin also inhibits exemplary downstream-genes of HIF-1α in a concentration dependent manner. The mRNA of CAIX, VEGF, LOX, ANGPTL4, EGLN3 and ADM are significantly downregulated compared to untreated hypoxic cells. To verify that Deguelin is not a general transcription inhibitor, EGLN2 is used as negative control (**Figure 27** b).

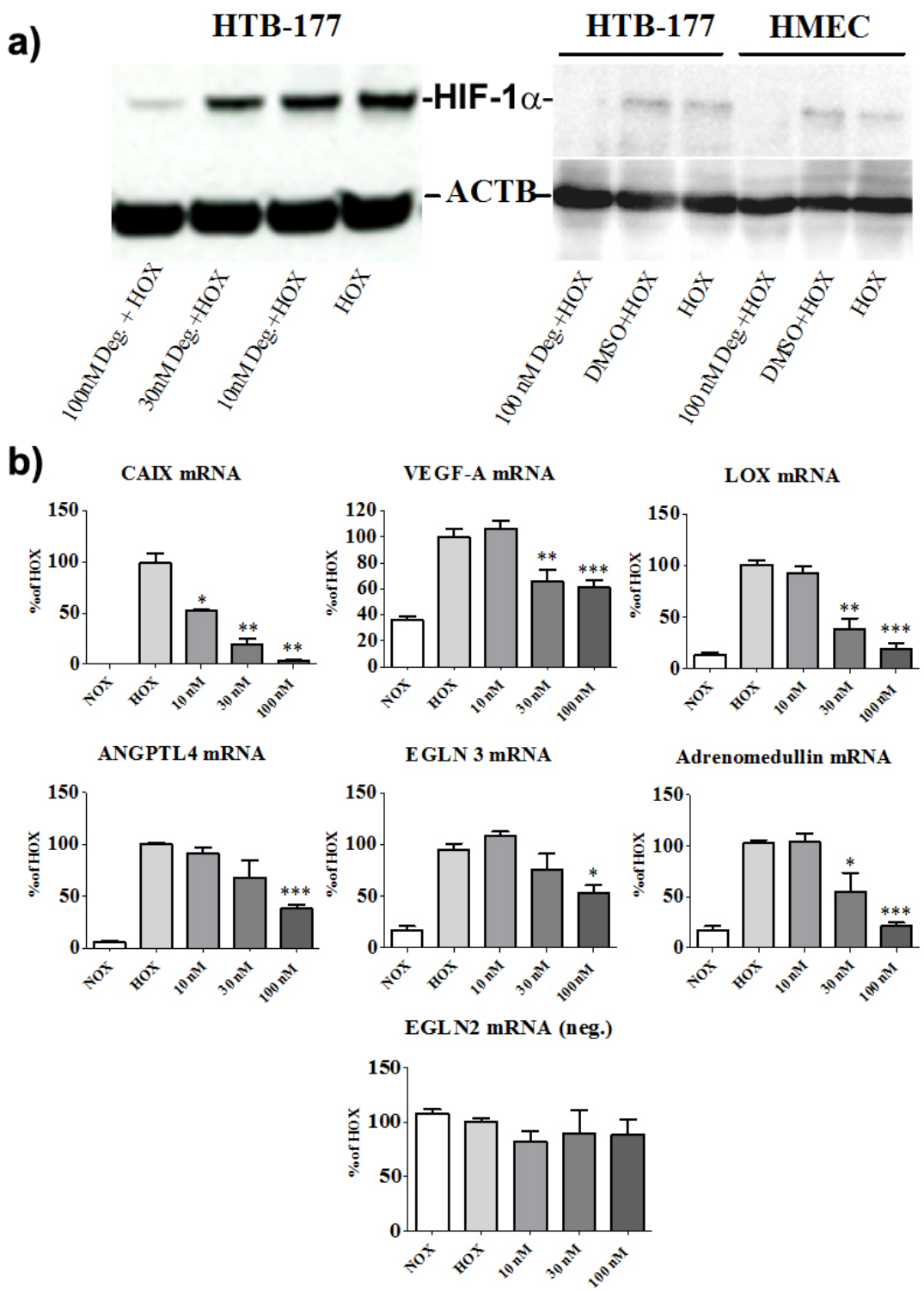

Figure 27: Effects of Deguelin *in vitro*. a) Western analysis of HTB-177 and HMEC cells following treatment with Deguelin under normoxia and hypoxia (6h). b) mRNA levels of HIF-1α target genes under influence of normoxia, hypoxia and Deguelin treatment in combination with hypoxia. HIF-1α protein is decreased at a Deguelin concentration of 100nM and 6h hypoxia. Deguelin also suppresses down-stream genes concentration dependent. n=3, *p< 0.05, **p<0.01 ***p<0.001.

3.2.2 Animal Experiments

3.2.2.1 *Toleration of Deguelin Gavage*

To reach adequate tissue levels of Deguelin in the animals, we chose to feed *via* intragastrical gavage. This method can be executed without anesthesia. Animals are treated with Deguelin dissolved in DMSO at 4 mg/kg bodyweight with an end volume of 200 µl. The rats receive the Deguelin intragastrically *via* gavage twice a day at a volume of 100 µl. All animals in the experiment tolerated the gavage very well and all of them gain weight (297.8 ±9.805 g and 335.6 ±2.064 g vs. 257.1 ±4.389 g, P=0.0009, P<0.0001 resp.) compared to a group only treated with solvent. Nevertheless, animals treated only with solvent were significantly heavier than those treated with Deguelin (335.6 ±2.064 vs. 297.8±9.805, †P=0.0196), but no harmful effects of the treatment are observed in both groups (**Figure 28**)

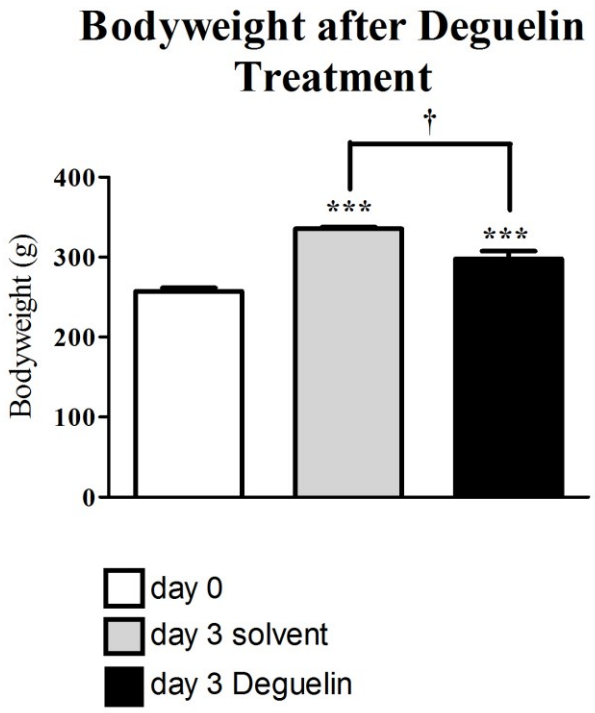

Figure 28: Bodyweight after treatment with Deguelin. n=6, ***p<0.001, †p<0.05.

3.2.2.2 *In vivo Effects of Deguelin on HIF-1α*

To examine the *in vivo* effects of Deguelin, lungs of rats treated with the inhibitor, were exposed to warm ischemia (37°) for 1 h. For controls, lungs of non-treated rats are harvested and immediately snap-frozen in liquid nitrogen.

Realtime-PCR measurements reveal that there are detectable basal mRNA levels under basal control (SHAM) for VEGF (100.0±12.28), CXCR4 (100.0±19.97) and ICAM-1 (99.9±12.28). Under warm ischemia, upregulations of VEGF (217.8±44.98; P=0.0338) and CXCR4 (272.0±60.82; P=0.0291) are significant compared to the SHAM-group, whereas the changes in ICAM-1 (136.5±18.93; n.s.) show no significant regulation. A inhibition of HIF-1 by Deguelin showed significant downregulation compared to the warm ischemia group foVEGF (217.8±44.98; P=0.0338), CXCR4 (272.0±60.82; P=0.0291) and ICAM-1 (42.4±7.36; P<0.0001). The gene expression of the corresponding negative control β-Actin (ACTB) remained unchanged.

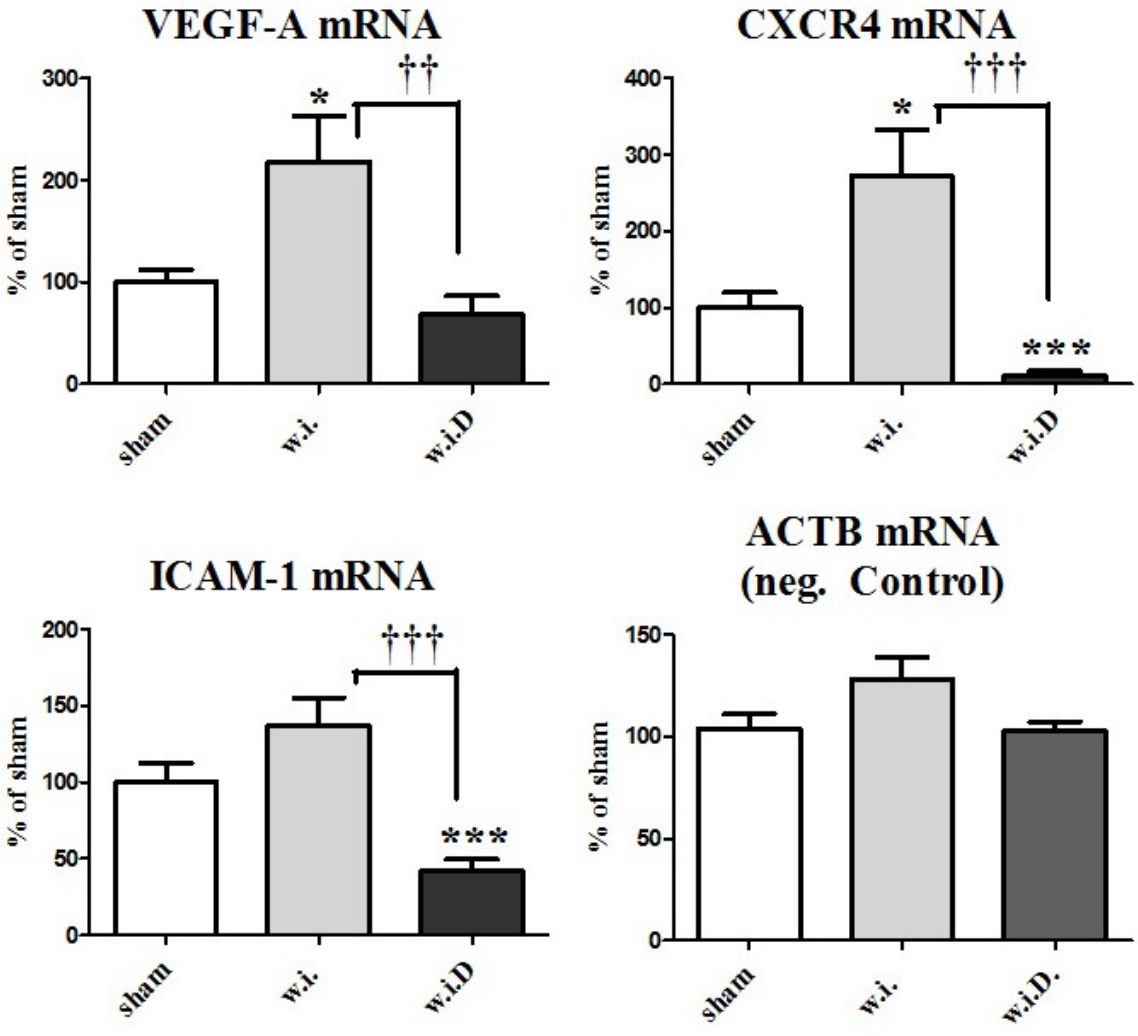

Figure 29: mRNA level of various HIF-1 target genes in sham lungs and lungs that were exposed to warm ischemia with or without treatment with Deguelin. n=6, *p< 0.05, ***p<0.001, †p<0.05, ††p<0.01, †††p<0.001

3.2.2.3 *Effects of HIF-1 Blockade on the Lung Microstructure*

Besides the molecular changes, we also examined the microstructure of the lung tissue. As the destruction of the alveolar integrity by flooding with extravascular liquid and the resulting lung edema is one of the most critical points in the maintenance of lung function, this change of lung structure has to be investigated carefully (**Figure 30**). Therefore, micrographs of the lung samples are taken at 10x and 40x magnification. 3 randomly taken pictures are made from every slice and the tissue area (blue) is compared to free space area (white) (Figure 31). Tissue edema and damage seen directly correlates with the increase in area compared to free space. This data demonstrates that the rates differ significantly between the compared groups. Lungs from the Deguelin group have significant lower edematous changes shown in massive thickening of the thin cell-layer and loss of free air-filled space between the alveoli (36.5±2.88 % tissue / area) than animals from the warm ischemia group (55.2±2.73 % tissue / area). This values are even higher than the ones for the SHAM animals (50.5±1.34 % tissue / area). In the lungs of the animals, that have received the Deguelin therapy, the microstructure of the lung is preserved best whereas the lung structure from lungs of the warm ischemia group show massive thickening of the tissue (**Figure 32**).Interestingly, the lungs from the SHAM animal group show almost the same tissue changes like the lungs exposed to warm ischemia.

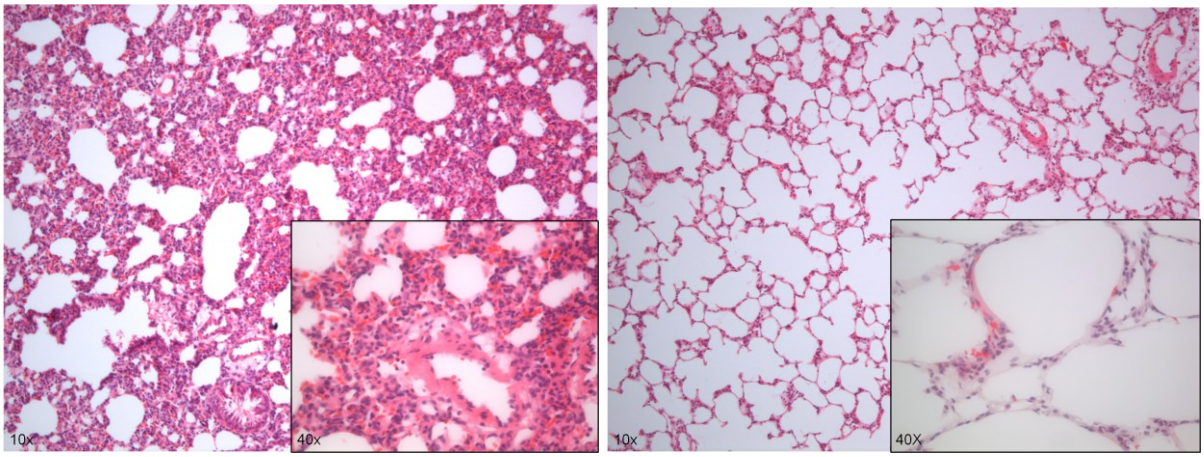

Figure 30: Tissue of a lung graft exposed to ischemia (37°C) for 1h. The thin tissue structure of the alveoli has thickened rapidly (left picture). For comparison, the normal tissue of the lung with the thin endothelial layer of the alveoli (right picture).

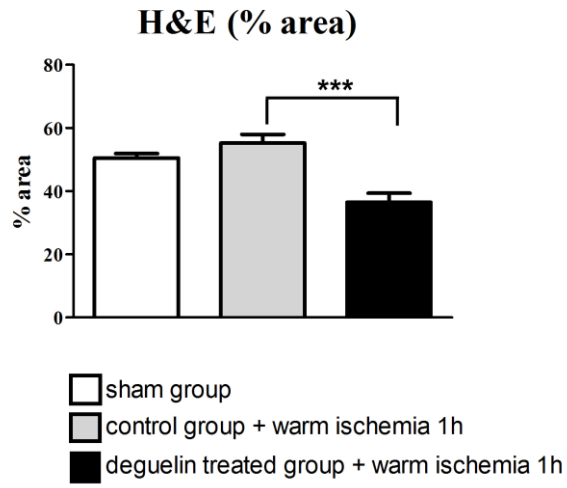

Figure 31: Comparison of tissue area vs. non tissue area of Deguelin treated lungs and those without treatment exposed to warm ischemia. **n=6, ***p<0.001.**

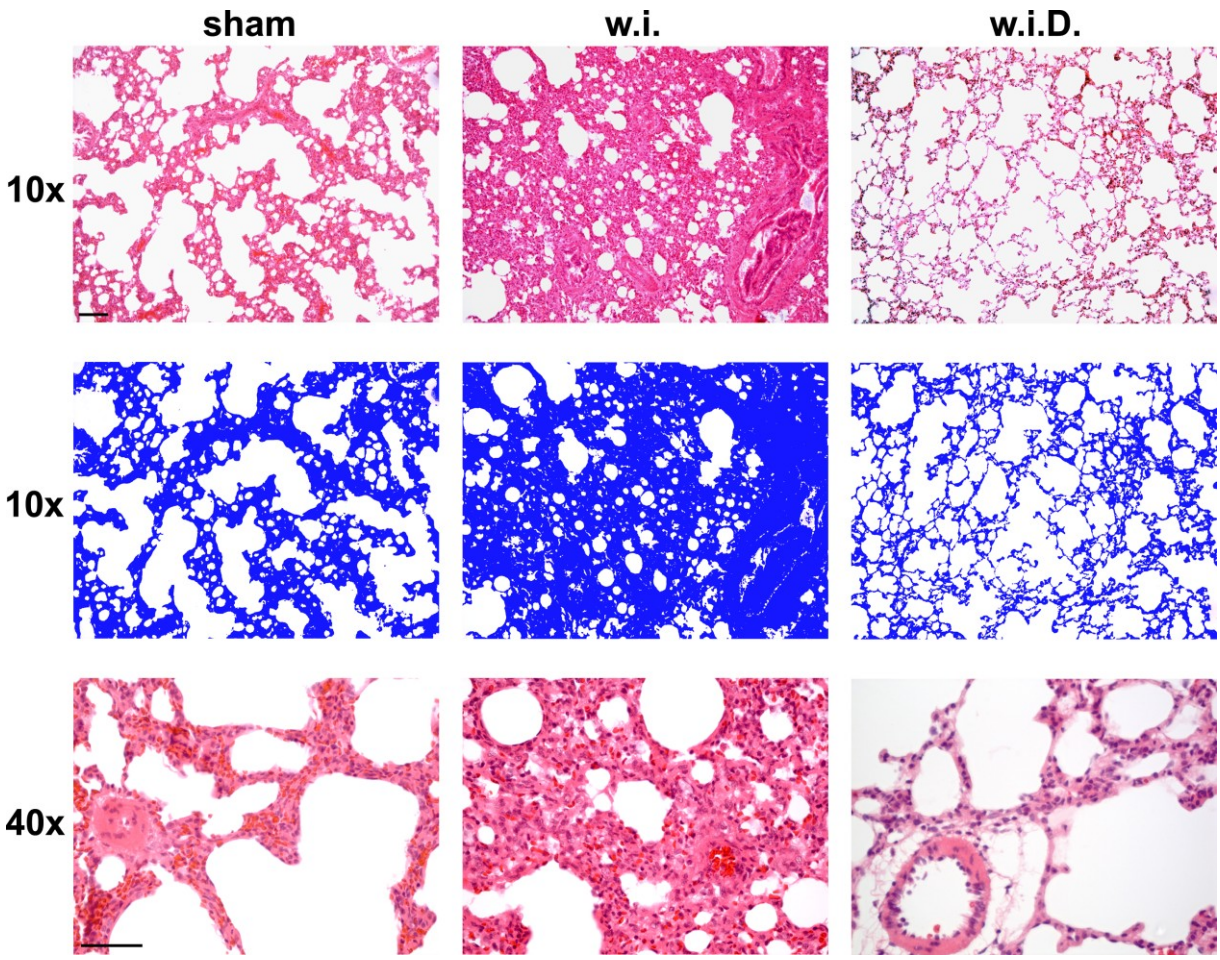

Figure 32: Micrographs of tissue slides from lungs of sham-group, warm-ischemia without treatment and Deguelin treated animals. The 10x magnification (first row) is evaluated *via* measuring the tissue/non tissue area (second row). The swelling of the tissue is presented more closely in the 40X magnification (last row). Deguelin also shows a protective effect from microstructural edema in the ischemic lung. w.i. = warm ischemia, w.i.D = warm ischemia + Deguelin.

3.3 Effects of HIF-1 Inhibition on LTX Outcome

3.3.1 Effects on VEGF Protein Expression

To prove that Deguelin is effective in suppressing a reperfusion edema, a wet-to-dry ratio for the lungs of the transplanted animals are determined to evaluate edema formation. Animals from the Deguelin-treated group have significant less tissue edema than animals without treatment (Figure 33 a). Corresponding micrographs show that lungs with high degrees of edema posess similar structural changes than lungs from warm ischemia group of the preliminary experiment (Figure 33 b). To determine the role of VEGF in edema formation further, western-blot and realtime-PCR analysis are made and show significantly lower levels of VEGF protein and mRNA in Deguelin treated animals. These data suggest, that HIF-1α directly induces tissue edema *via* VEGF upregulation.

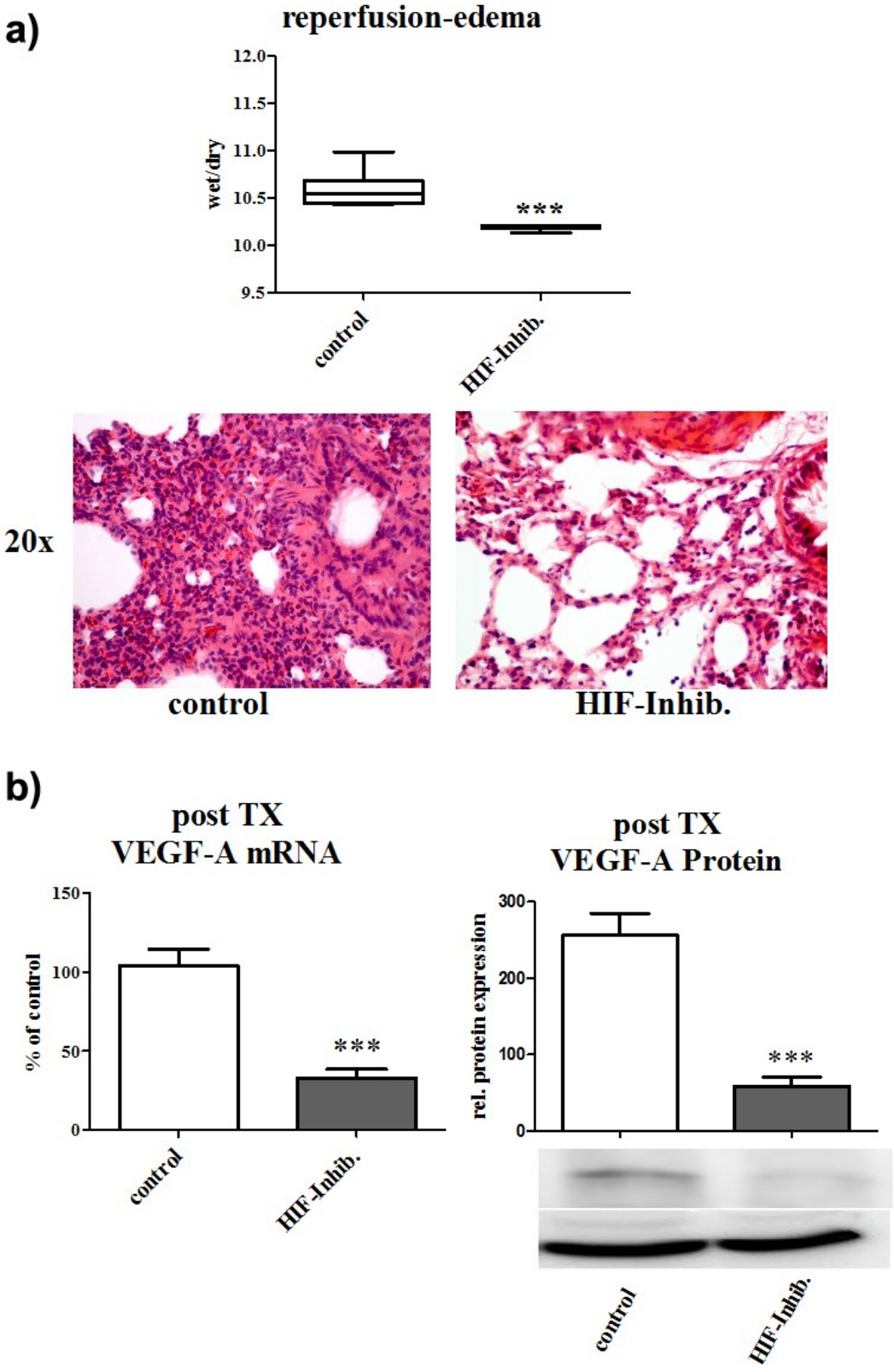

Figure 33: Deguelin effectively suppresses VEGF in lung tissue. a) Results of the wet-to-dry ratio between control and Deguelin treated animals showing a significant difference between both. b) mRNA and protein levels of VEGF after transplantation, comparing animals of the untreated control group with the Deguelin group (left). VEGF protein analysis shows also significant downregulation (right) in the Deguelin group. Upper band = VEGF, lower band = ß-Actin. n=6, ***p<0.001.

3.3.2 Effects of HIF-1 Blockade on Short Term Survival

Besides the decrease of the tissue edema, the Deguelin treated animals have a significantly improved short-term survival rate compared to the non-treated group. 100 % of the animals from the Deguelin-treated group have reached the endpoint of 48 h post transplantation. A significant difference is also seen in the mere view of the harvested lungs after 48h (**Figure 34**). In the non-treated group there is a mean survival of only 17.42 ± 9.67 hours. The plenteous findings directly correlate with the better outcome of survival and seem to be correlated with the reduced VEGF expression (Figure 35).

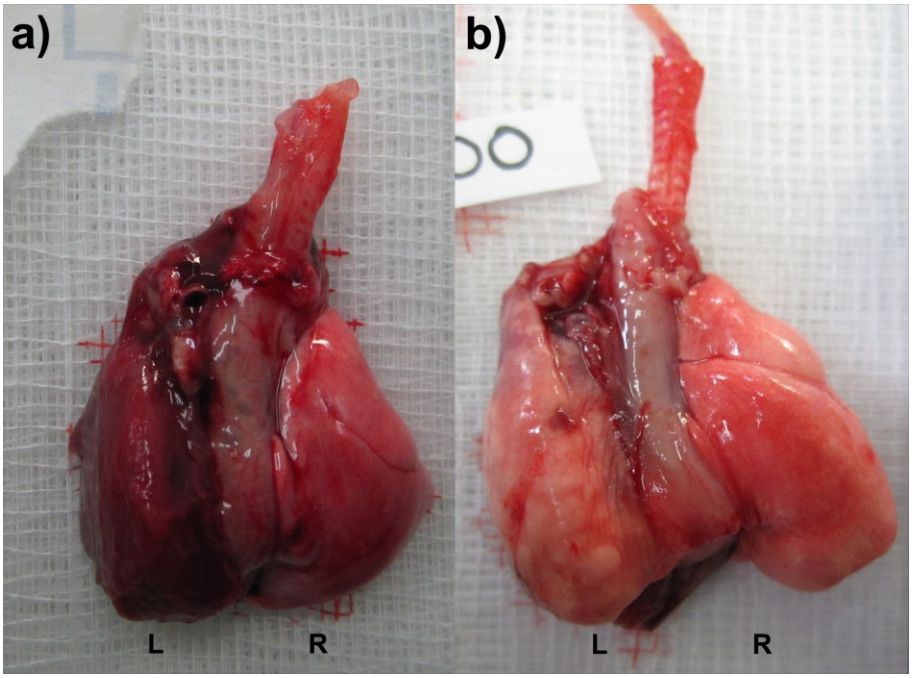

Figure 34: Harvested heart-lung packages from transplanted animals after 48 h. a) Control, b) Deguelin treatment. L = left lung, R = right lung.

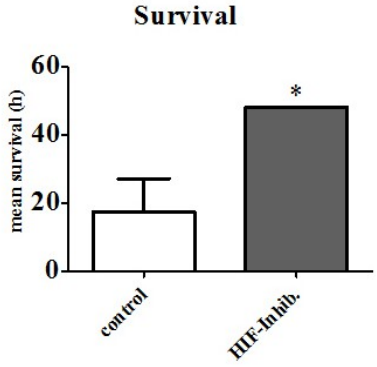

Figure 35: Mean survival of the transplanted animals, comparing the control group to the HIF-1 inhibition group in hours (h). n=6, *p< 0.05.

3.3.3 Effects of Deguelin on the Immune Response

As described in 1.3 the immune system plays an important role in the reaction to the graft, because it can induce negative reactions like the destruction of tissue and the initialization of subsequent processes like PGD or rejection (Figure 36, Figure 37). The investigation of the histological slides, shows that a huge amount of macrophages can be observed in the Deguelin treated group, that are only found in the lungs of surviving animals with a good clinical appearance. Due to the normal inflammatory role of lung-macrophages this seems to stand in contradiction to the significantly improved survival rate and health of the animals from the Deguelin group. This may be an indicator that these macrophages might play a role in the healing process of the lung tissue. This hypothesis is supported by the detection of markers for anti-inflammatory macrophages as CD 68 + and CD 163 *via* immunostaining. These markers show a much higher abundance of macrophages like M2-macrophages in the Deguelin-treated animals. These cells are known to exhibit an anti-inflammatory role. CD 163+ (1262.0 ± 266.6 vs. 100.0 ± 18.5; P=0.0008) and CD 68+ (8.500 ± 2.225 vs. 37.81 ± 5.499 ; P= < 0.0001) are significantly upregulated in HIF-1 blocked lungs. It is known that usually there are only macrophages of the CD 68+ type stay in the lung. Thus, these findings give rise to the assumption that the macrophages of the CD 163 type are from a systemic source (Figure 38).

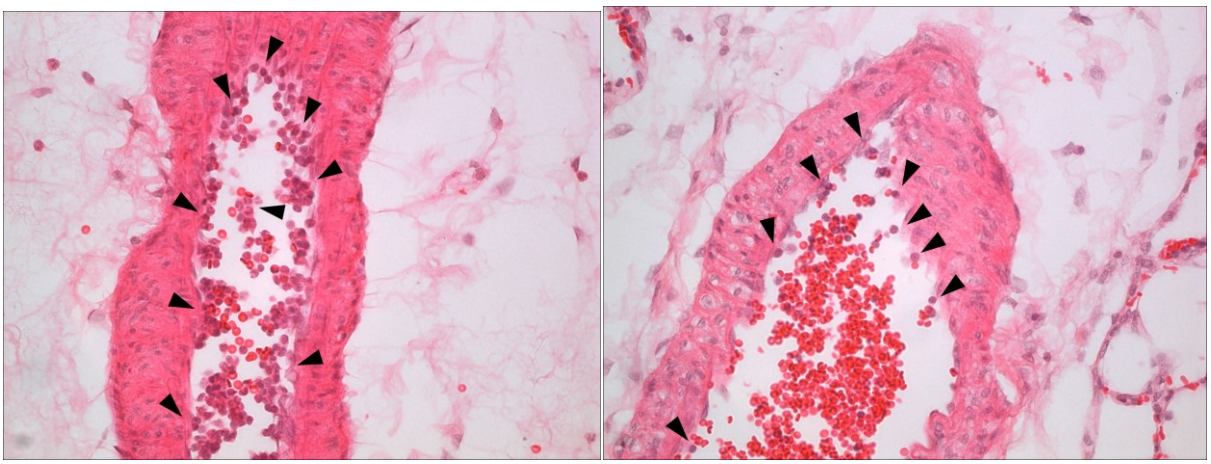

Figure 36: H&E staining of a transplanted left lung of an animal that died early on severe PGD. The blood filled vessel shows a massive appearance of white blood cells (arrows), that show typical 'rolling' and migration on the intima of the vessel.

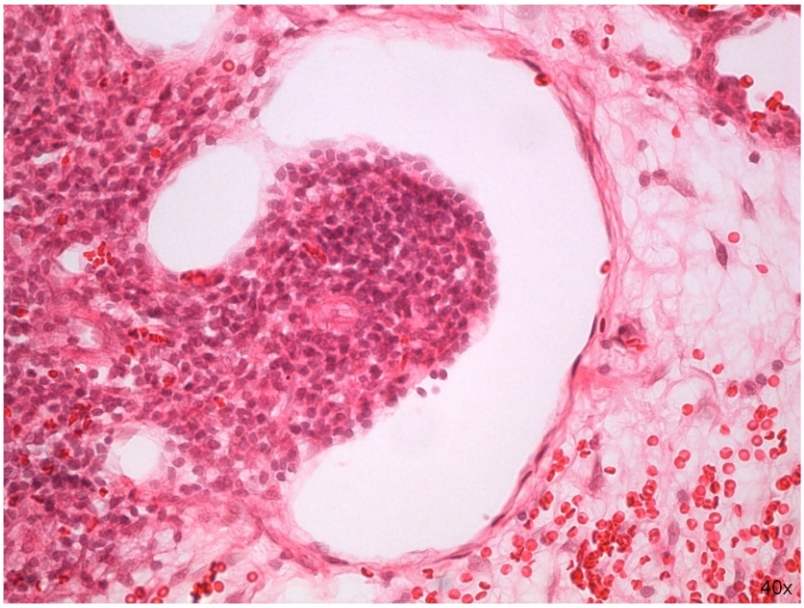

Figure 37: Massive infiltration of lung tissue *via* leukocytes in a transplanted lung.

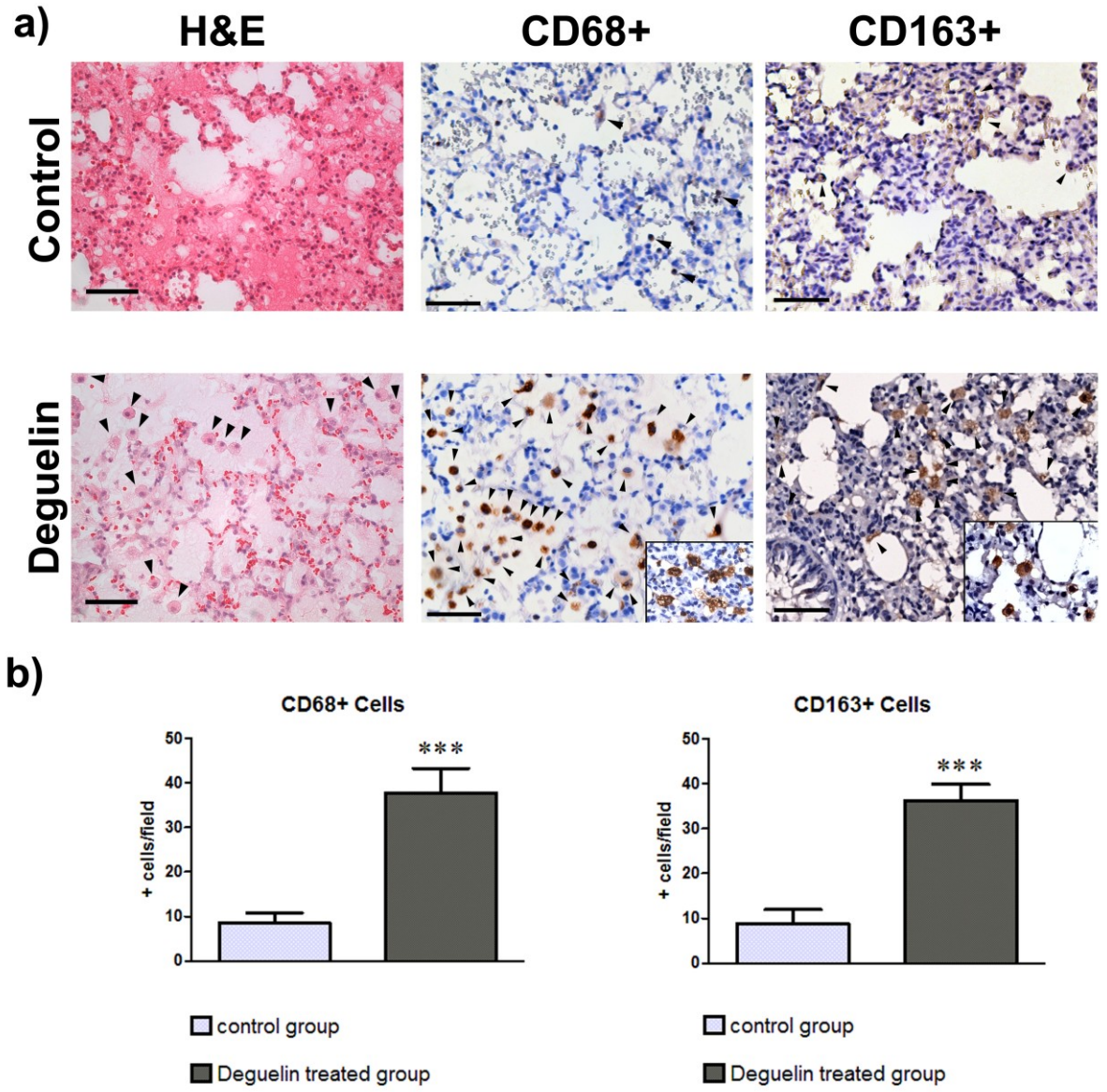

Figure 38: Histological pictures of slides of transplanted lungs. a) Histological stains of control and Deguelin treated animals in H&E stains, no macrophages are found in the control, but in the Deguelin group (arrows). b) statistical analysis of the findings in the staining of CD 68+ and CD 163+ cells comparing control and Deguelin group. Significantly higher levels of cells can be found in Deguelin treated animals. n=6, ***p<0.001.

Besides the recruitment of M2 macrophages, the HIF-1 inhibition also leads to the downregulation of pro-inflammatory proteins as ICAM-1 and CXCR4. There is also an upregulation of IL-4 (153.1 ± 14.5 vs. 100.0 ± 17.2; P=0.0357) which is known to have immunomodulatory influence. This is interesting especially considering findings that the levels of CD 163 are also increased, which are indeed, amongst others, induced by IL-4.[74, 75] Furthermore, ICAM-1 (26.5 ± 5.3 vs. 127.8 ± 49.6; P=0.0188) and CXCR4 (58.9 ± 8.7 vs. 107.7

± 20.6; P=0.0274) gene expression was significantly downregulated in HIF-1 blocked animals compared to controls (Figure 39, Figure 40).

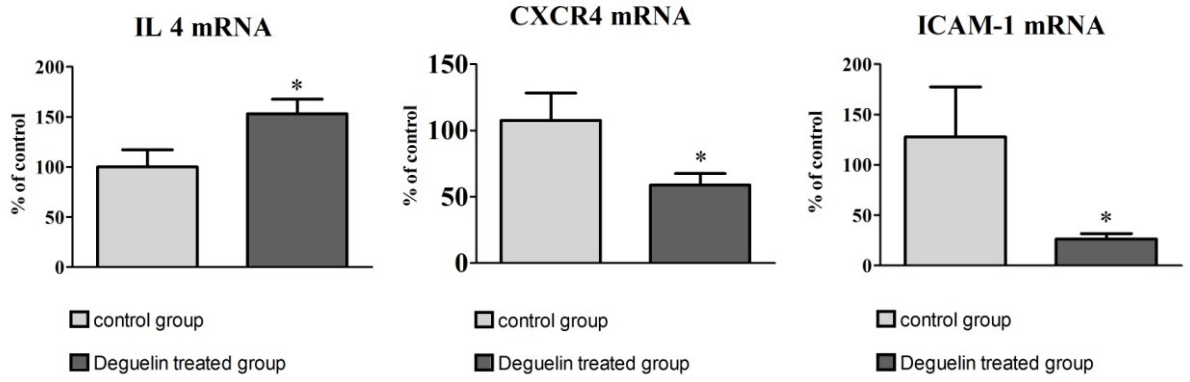

Figure 39: Results of mRNA analysis of relevant HIF-1 gene products. The control and the Deguelin treated group are compared. It shows an upregulation of CD163 and IL-4 in the Deguelin group, whereas CXCR4 and ICAM-1 are downregulated, showing that HIF-1 blockade leads to a systemic anti-inflammatory and immunomodulatory phenotype. n=6, *p< 0.05.

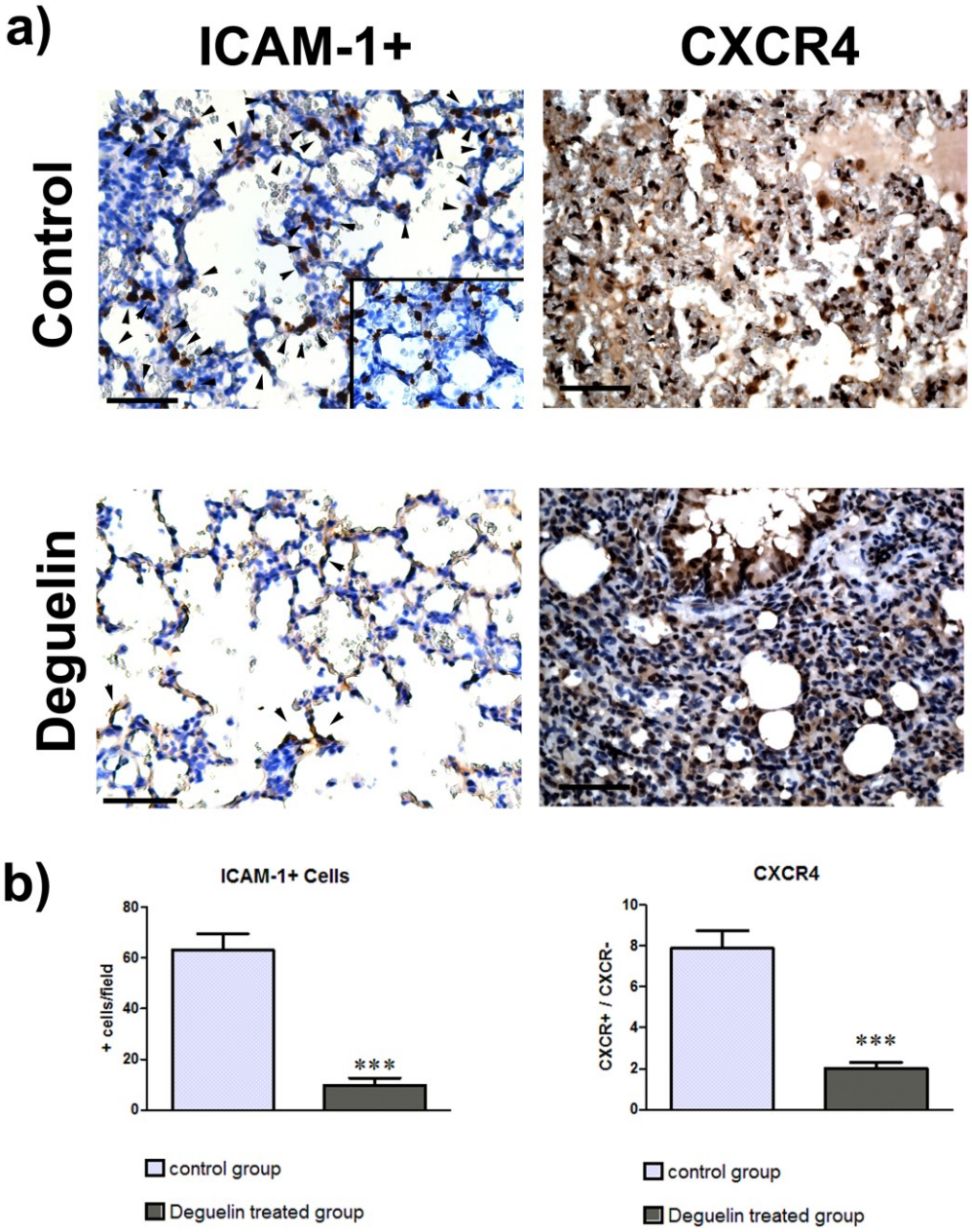

Figure 40: Immunostaining for proinflammatory markers a) Immunehistochemical pictures of the control and Deguelin group, showing the proinflammatory markers ICAM-1 and CXCR4. b) Statistical analysis of ICAM-1 and CXCR4 in both groups showing significant downregulation in the Deguelin group. n=6, $p<0.01$ ***.

4 Discussion

Although a lot of studies have been published that investigate the function of HIF-1 during cancer development, its role during hypoxic conditions in tissue of grafts and its effect on the immunological reaction have been subject of latest studies.[69, 187, 194, 198] The role of HIF-1 seems to be strongly tissue dependent and leads to somehow contradictory results from benefits in HIF-1 upregulation to great disadvantages caused by HIF-1 translated gene products.[168, 248]

Effects in the lung tissue such as accumulation of HIF-1 leads to tissue damage due to upregulation of genes as VEGF, or inflammatory genes such as ICAM-1 or CXCR4.[69, 187, 194, 198] The proinflammatory response is a major problem in primary graft dysfunction. Therefore, the inhibition of factors that are responsible for the initiation of these pathologic states seems to be an effective therapeutic concept. As shown earlier, chronic hypoxia itself may obviously lead to inflammatory tissue damage and finally to apoptosis.[173] However, during transplantation, ischemia of the tissue and the resulting hypoxic condition are inevitable situations. Therefore, inhibition of HIF-1, the major hypoxic factor, is beneficial as it shows not only great responsibility in regulation during hypoxia, but also presents itself as capable of strongly initiating on inflammatory reaction in the lung tissue.[69]

Deguelin as a candidate for HIF-1 inhibition was already presented in many studies, although it has also been reported to target also other pathways leading all to a downregulation of HIF-1 protein expression. However the complete mechanism and the concrete way of binding of Deguelin still remains unclear. Nevertheless, recent studies mark its function in inhibiting the complex I in the mitochondria which suggests that Deguelin acts on a very early point at the respiratory chain.[214] Furthermore, it was also demonstrated that Deguelin can inhibit HIF-1 without affecting the ROS and Akt-pathway.[209, 222]

Most of the studies described the effects of Deguelin in cancer cell lines or cancer animal models. The reaction of healthy, not altered tissue to Deguelin was tested only in a few models.[209, 221] The same is true for its toxicity to the organism.[221] It was never used as a protecting agent for healthy tissue during LTX.

To ensure that Deguelin has an inhibitory effect in the used concentration, two cell lines of immortalized human microvascular endothelial cells (HMEC) and an immortalized human epithelial lung cell line (HTB-177) are used in the presented work. These two cell-types are

very important for functionality of the lung tissue. In this trial we show a concentration dependent inhibitory effect at 100nM Deguelin in the two cell lines, without visible cytotoxic effects. No individual effect is observed for the solvent DMSO.

As the i.g. application of Deguelin shows better basic levels of Deguelin in the lung compared with i.v. and s.c. application, we decided to feed the rats with a dose of 4 mg/kg BW twice a day over three days to reach adequate levels in the lung. [217] This treatment shows no harmful effect to the animals and all gained weight. Also the i.g. gavage is well tolerated using a hand trained rat strain, which allows for omittance of the anesthesia.

In comparison to the SHAM group, a control group of warm ischemia (1h at 37°C) and animals treated with Deguelin are investigated. The lungs from the control group show macroscopical signs of lung edema: like a clear change of their color to dark red and a liver-like consistence. Furthermore, the microstructure changes significantly by massive thickening of the tissue and a great to almost complete loss of air volume compared to the Deguelin group. The lungs from the Deguelin group have a much less dramatic appearance in the first view, but they also show significantly less thickening in the tissue. Additionally, the thin alveolar structure is preserved much better even in comparison to the SHAM group.

Deguelin shows a concentration dependent reduction of HIF-1α protein expression, which is in accordance with previous studies.[209, 216] Moreover Deguelin also reduces mRNA levels of HIF-1α and HIF-1 dependent downstream-genes as VEGF, CAIX, LOX, ANGPTL4, EGLN3 and ADM.

Especially VEGF is of great interest, as it is already known that it acts as major inducer of lung edema development. It is shown in earlier studies, that VEGF not only leads to an increase of vascular permeability in an I/R model in rats, but also causes massive pulmonary edema. An inhibition of VEGF cell signalling on the other hand can reduce this damage to the endothelium.[249, 250] Its inhibition can lead to prevention of ischemia and reperfusion injury. This findings lead to the primary idea of an inhibition of VEGF before its activation. Deguelin seems to be perfect pharmacological agent for this purpose, as it has already been demonstrated to have an influence on HIF-1 and VEGF expression.[216] Contrary to this idea of an inhibition of VEGF, some researchers uphold the opinion that HIF-1 and VEGF upregulation results in a benefit for the tissue in graft processes, due to its angiogenetic activity.[251] Although this hypothesize can be supported by model experiments, using subcutaneous trachea transplants, our data suggest that in the case of a fully functional lung transplant, this approach of VEGF upregulation will not lead to the desired improvements. Several reasons can be pointed out for this assumption: First, the angiogenetic activity of VEGF can never be a

fast acting effect, which prevents the desired effects from manifesting itself during the early phase of reperfusion. Additionally the mere generation of new vessels is not in every situation a benefit for the tissue, especially in tissue, that will be disturbed in its function as e.g. the retina.[209] Angiogenesis does not seem to be the most important process in the early phase of reperfusion of the lung graft. In this phase, HIF-1 is more likely to be a potent mediator of lung edema due to its ability to increase vascular permeability *via* VEGF during the first hours.[252] Of course, vessel migration can be positive for adaption to blood circulation when there is no primary adaption, but a transplanted lung – different than a skin flap or trachea graft under the skin – does not need new vessels, as it is adapted directly to the big blood vessels of the recipient. Furthermore, a disturbance of the thin layer of the lung tissue, which is responsible for gas-exchange, leads to critical function loss.

Our data clearly shows the correlation of VEGF mRNA and protein to the development of edema in the lung. We also demonstrate the downregulation of both observevables in the Deguelin group compared to the control in the pilot experiment and after LTX.[249, 250] As lung edema is a very critical situation for the sensible lung structure and complicates or even completely inhibits the gas exchange, a prevention of edema is essential for the functionality of the graft.[252]

Moreover realtime PCR results show a downregulation of, CXCR4 and ICAM-1while both are known to mediate inflammatory processes due to stimulation of leukocyte migration through chemotaxis.[17, 253]

To underline the preliminary data and to prove, whether these findings are translationable to the setting of LTX, we apply a HIF-1 inhibitor during orthotope lung transplantation in the rat. As a more intense reaction and changing of the tissue is expected after 48h compared to the 1h in the pilot experiment, a direct comparison of the thickening of the tissue is not trivial to determine histology. Besides the longer duration of the experiment, the edema in the lung graft is also a result of the additionally necessary reperfusion. To examine the edema, we therefore use a common technique.[181] The Wet-to Dry ratio as an estimation of tissue water, is a commonly used method to determine edematous conditions and shows a difference in the water content of the lungs between the untreated group and the group that was treated with Deguelin. This leads to the conclusion, that the reperfusion edema has a much stronger impact on the untreated group compared to Deguelin treated animals.

Upon harvest, the left lung of the control group presents itself as almost of liver-like consistence whereas the Deguelin graft is of slight rose color and shows much more lung-like appearance (**Figure 34**).

The benefit of the HIF-1 inhibition also manifests in the survival of the animals. Whereas 100% of the Deguelin treated animals reach the endpoint of the experiment at 48h, the control group has only a mean survival time of 17.42 ± 9.67 hours and many of them died already on acute lung edema in the first hours after transplantation. This emphasizes the role of the immunogenic reaction of the recipient's body to the foreign graft in the control group, which negates the theory, that the animals do not have a strong immune reaction at all. In what way there is an influence of the close blood relationship in most bred strains on the fact that some of the untreated animals reached the 48h is not clear. Although, the used Sprague Dawley strain is an outbred strain that should show a wide spectrum of individual antigenes. Realtime- PCR analysis of the proinflammatory cytokines ICAM-1 and CXCR4 shows a downregulation of mRNA in the Deguelin group compared to the control, whereas anti-inflammatory markers as IL-4 are upregulated. These findings were also seen in immunhistochemistry. Taken together this data support the assumption that an inhibition of HIF-1 leads to a positive effect on the graft due a lower inflammatory and also some anti-inflammatory gene regulation.

Interestingly, screening of histology slides of the Deguelin group shows many big macrophages, which cannot be found in this mass in the control. Nevertheless, a macrophage demolition of the tissue is not observed. As PCR-data confirm a high upregulation of CD-163, a marker for anti-inflammatory macrophages, the addressed cells might be of an anti-inflammatory M2-kind.[254] This is also supported by the immunhistochemical staining that shows many of the CD-163 labeled macrophages in the Deguelin, but not the control group. Counting them displays a massive abundance in the Deguelin treated group, but none in the control. Fascinatingly, this findings do not match with an upregulation of IL-10 that is known to be often upregulated upon this anti-inflammatory processes,[73, 255] whereas IL-4 is indeed upregulated. IL-4 has a two-sided role in transplantation processes – on the one hand it is known to play a role in anti-inflammatory immunemodulation[73] and on the other hand it also modulates the Type 2 immune response in the process of chronic rejection[140] which may lead to *bronchiolitis obliterans*. This fact is not uncommon, as the role of pro- and anti-inflammatory interleukins can be different in several situations – depending on the fact, that higher IL-4 levels are correlated with anti-inflammatory macrophages of the M2-type let suggest, that it has more the role as a factor of repair than destruction.[74, 75, 119]

Despite this, we modified the method of lung transplantation we used. There are some points in the protocol of Zhai that showed difficulties in execution of the method. We have changed

the perfusion method over the *Vena cava inferior* to the directive perfusion over the heart (Figure 12) as this leads to less resistance of valves and directly flushes the heart-lung system by bypassing the body-circulation. Then, we have left a small part of the cuff tail because this guarantees a better manipulation of the cuff, without touching the cuff body that has to lie almost completely in the recipient's vessel during transplantation (Figure 13 b). The last important change is the use of Prolene 6-0 threads for the ligation of the recipients structures instead of microaneurysm clamps (Figure 20). The most critical step is the unilateral damage by clamps in the small wound opening and the proximity of very vulnerable structures like the beating heart and the *Nervus phrenicus*. This brings the benefit of less danger of very big objects in the surgery field and faster removal of the threads which are just cut after all is finished.

Via this modifications, we are able to decrease surgical irregularities that often end with the death of the recipient or suspension from the experiment.

Our modified method shows increased survival over the observative time of 48 h. There is no change in the other death cause category, while the method by Zhai shows much higher mortality due to surgical complications (Figure 23,Figure 24).

The described technique of Zhai *et al* [226] shows some disadvantage especially for surgeons who are new in the field of microsurgery and not highly experienced in surgical techniques. Our two person surgery approach makes situations of loosing eye-contact to the microscopy field as for example for searching surgery tools or material, less necessary. Also the use of threads instead of clamps makes working in the small, always moving surgery field, much less complicated. The clamps present the risk of a big nonelastic body in direct contact to very sensible structures as the heart, the *Nervus phrenicus* or the structures of the vessels and the bronchus. During the surgery, three of these clamps would be necessary, while fixing and loosing the clamps is a skill of microsurgery most surgeons won't be experienced with. Here, a high risk of generating damage by uncontrolled bouncing clamps is eliminated by our procedure.

These changes lead to a much better outcome in animal survival. But not only the surgical complications, also the time saving is significant in the modified method. While the complete procedure of transplantation (time from the moment of end of cold ischemia until complete reperfusion) takes 23.36 ± 2.9 minutes according to the method of Zhai, only 11.82 ± 1.7 minutes are needed now. This is of great interest, as the time of warm ischemia is a critical moment for damage to the graft and should be as short as possible. Compared to Zhais method, it is almost the same time span, but this might be due to the great knowledge in the

field of microsurgery and therefore the experienced skills of the surgeons as referring to their own information of their experience. This shows that at least, for surgeons who are new on the field of microsurgery, our modifications could be of interest to diminish failures that result from surgical complications.

Table 21: Changes to Zhai's technique compared to our method

Zhai´s Method	Modification	Benefits
Perfusion over the *Vena cava inferior*	Perfusion over the right ventricle	Bypass of the body circulation and direct perfusion of the heart-lung circulation
No cuff tail	Short, but still remaining cuff tail	Better control over the cuff body
Using the same size of cuffs for all structures	Using 17-G cuff for artery	No piling up of blood in the lung by lower flow to the lung and faster flow away from it
Using microaneurysm clips for ligation during surgery	Using Prolene 6-0 thread loops	More space in the surgery field, time saving and less risk of damage

Another interesting result is the anaesthesia of the surgical wound. The lung transplantation has special requirements on anaesthesia, due to the importance of good ventilation of the graft and therefore, a good breathing of the recipient. The use of strong analgesia, which is needed for a painful intervention as this, is necessary and usually requires the use of opioidanageltica, that are unfortunately known for their respiratory repressive effect. A willingly used analgetica is therefore buprenorphine, which has an acceptable effect endurance and shows no strong respiratory depression in most species – but it has a special effect on the rat, that makes its use not feasable. Different from the mouse, the rat shows a strong allophagic effect after buprenorphine injection[235] which is so strong, that the animals almost kill themselves by eating every kind of bedding in their cage that can lead to rupture of the stomach. This effect is assumed to be a signal of a very acute nausea, which presents itself in this allophagia, common for most rodents.[235] The alternative opioide piritramide makes the rats lethargic over a long time after anaesthesia and shows respiratory depression. To omit a presurgical injection of an opioide but to set a block of the local anesthesia ropivacain instead, results in a significantly improved animal health score. The animals show almost no lethargic behaviour and therefore complete uninterrupted general condition and loading of the left side. Whereas the animals of the first, presurgical opioide treated group show clear signs of breathing problems,

probably due to the painful movement of the thorax, the animals with the local anesthesia block do not show such signs at all.

5 Conclusion and Outlook

The model of orthotopic lung transplantation in the rat is a very effective way to investigate the diverse problems that occur during this process. It shows many advantages compared to other accepted models as for example the trachea graft, which has not the same validity for the fact, that the trachea is not part of the gas exchanging tissue. A difficulty is the surgery model itself as it needs a skilled surgeon with experience in microsurgery. Therefore, our changes to the old model may help to simplify this method and to make it more attractive even for more unexperienced surgeons on this field.

We found that Deguelin is a potent drug to inhibit some deleterious effects responsible for early graft dysfunction. Furthermore, it has no acute toxic effects, neither on cell nor on the animals even after long time of intake. This leads to the conclusion, that the effects of this chemical compound could be interesting not only in cancer research but also in the very wide field of transplantation medicine.

Futhermore, we found interesting effects on the immunomodulation *via* Deguelin treatment that could help preventing deleterious inflammation processes by activating the proinflammatory macrophages. But is has still to be proven, if the long-time effects of this activation - especially under the influence of IL-4 –also leads to positive results in a long-term survival model.

The role of the macrophages we found and their appearance in the lung is also an aspect that could be investigated further. To find out the source of the immigrated macrophages is of great pharmacological interest as their drug dependent recruitment may find application in a wide spectrum of inflammatory conditions.

We also noticed a tendency for bleeding in the treated animals which might be due to the influence of HIF-1 on different factors like PAI-1 or other entities in the coagulation cascade.

6 Summary

6.1 Summary

Hypoxia is a dangerous condition for every organ and often cannot be completely avoided during surgical intervention. This is a result of ischemia which occurs when blood flow and, therefore, supply with the needed oxygen has to be interrupted for some time. During a transplantation process, the ischemia is very severe as the organ has to completely removed from all supplying blood vessels and stored for hours in this case until the recipient is made ready. Due to this, ischemic damage and hypoxic condition is one of the important factors that influence the graft and can lead to primary graft dysfunction (PGD) which might end in the loss of function (*bronchiolitis obliterans*) or even the complete rejection of the organ. The loss of function is even more dramatic if the transplanted organ has to supply the complete organism with oxygen as the lung. Therefore, the reaction of a lung graft to the non-avoidable ischemic conditions and the role of hypoxia responding genes is crucial to refine the transplantation process.

In this work we concentrate on the role of the hypoxia inducible factor 1 (HIF-1) and its gene product the vascular endothelial growth factor (VEGF) in the lung transplantation. It was already described earlier, that upregulation of VEGF showed a responsibility for the development of edema in the lung and other pathological changes. Under this aspect an inhibition of HIF-1 as the main regulator of VEGF seems an interesting approach for the reduction of graft damage. Therefore the known rotenoid Deguelin, which was already described in its inhibitory effects on HIF-1 concerning its vascular growth abilities in cancer research, was used. To investigate the effects under the most concrete clinical situation, our group established and refined an orthotope left lung lobe transplantation in the rat which was modified after an already known cuff-technique method of lung transplantation. This method bears the benefit that the lung is completely connected to all structures of the recipient and functions as a real lung lobe transplant over the observation time, different from other methods where only the contact to the foreign organism is guaranteed but not the ventilation or complete perfusion. Our modification on the microsurgial procedure in the rat bears furthermore some advantages especially for scientists that are new on the field of microsurgery. We could demonstrate that these modifications bear fewer risks of complications and, thus, fewer cases of early terminated animals during the experiment. Also the needed time for the transplantation process, which

is essential to keep warm ischemia as short as possible was equal compared to the other method. The pain management we used in our method avoided a presurgical injection of an opioid which always made a respiratory depression that was dangerous for the recovery during the first hours after transplantation. We could demonstrate that our intercostals block with a local anesthetic resulted in much better animal health compared to a classical presurgical opioid-injection.

Concerning the effects of Deguelin, we could show that Deguelin significantly inhibited HIF-1 reporter genes as VEGF, Carbonic anhydrase IX (CAIX), lysyl oxidase (LOX), Angiopoietin related protein 4 (ANGPTL4), Egl nine homolog 3 (EGLN3) and Adrenomedullin (ADM) in a concentration dependent manner *in vitro*. It also suppressed mRNA levels of VEGF, chemokine receptor type 4 (CXCR4) and intercellular adhesion molecule 1 (ICAM-1) in the ischemic lung. Furthermore, the VEGF modulated reperfusion edema was significantly less pronounced in lungs and a much higher survival rate was observed for Deguelin treated animals. In addition, under Deguelin treatment, proinflammatory proteins such as ICAM-1 and CXCR4 were down-regulated and anti-inflammatory monocytes (CD 163, CD 68) were recruited to the transplanted organ.

These findings were underlined by the significant higher survival rate of Deguelin treated animals compared to the untreated ones. This let us suggest that HIF-1 inhibition can improve the condition of the lung graft.

6.2 Zusammenfassung

Für alle Organe ist Hypoxie ein gefährlicher Zustand, der sich allerdings gerade während chirurgischen Eingriffen oft nicht vermeiden lässt. Die bei Eingriffen oft durch Unterbrechung der Blutversorgung auftretende Ischämie führt durch den versiegenden Zustrom von frischem, sauerstoffreichem Blut zur Hypoxie. Besonders Transplantate sind aufgrund der Entfernung aus dem Spender und der oft längeren Lagerung bis zur Vorbereitung des Spenders, von einer extremen Ischämie betroffen. Diese unvermeidliche Ischämie ist auch oft der Grund für die Schäden durch Hypoxie und Ischämie die sich bei der Reperfusion des Organs im Spender präsentieren. Diese Ischämie- und Reperfusionsschäden führen häufig zu Folgeschäden wie der Primären Transplantat Dysfunktion (Primary Graft Dysfunction PGD) die einen Funktionsverlust zur Folge hat (z.B. die *Bronchiolitis obliterans* in der Lunge) und auch im kompletten Transplantatverlust gipfeln kann. Dieser Funktionsverlust ist besonders dramatisch wenn das transplantierte Organ eine Versorgerfunktion für den gesamten Organismus innehat, wie es bei der Lunge der Fall ist. Daher ist die genaue Reaktion des Transplantats auf die

nicht zu vermeidende Ischämie und die Rolle der auf Hypoxie reagierenden Gene von besonderem Interesse um Folgeschäden zu vermindern und den Transplantationsprozess stetig zu verbessern.

Diese Arbeit beschäftigt sich daher mit der Rolle des Hypoxia Inducible Faktors-1 (HIF-1) und seines Genprodukts, dem Vascular Endothelial Growth Factor (VEGF) in der Lungentransplantation. Es wurde bereits beschrieben dass die Raufregulation von VEGF mit der Ödembildung und anderen pathologischen Veränderungen in der Lunge zusammenhängt. Unter Anbetracht dieser Tatsache scheint eine Inhibition von HIF-1 als Hauptregulator von VEGF ein interessanter Ansatz bei der Verminderung von Transplantatschäden in der Lunge zu sein. Als Inhibitor wurde ein bereits in der Krebsforschung als HIF-1 Inhibitor beschriebendes Rotenoid namens Deguelin verwendet, das in den Studien vor allem eine Gefäßwachstum hemmende Wirkung in Bezug auf HIF-1 zeigte.

Um die Auswirkung einer Hemmung unter möglichst kliniknahen Bedingungen zu untersuchen wurde von unserer Arbeitsgruppe ein Transplantationsmodell an der Ratte etabliert und modifiziert, bei dem der linke Lungenflügel orthotop transplantiert wurde. Dieses Modell hat den Vorteil einer sehr realen Transplantationsbeobachtungen, bei der das Transplantat komplett an den Kreislauf des Empfängers angeschlossen und somit funktionstüchtig ist, anders als bei vielen anderen Modellen, bei denen nur sehr geringer Kontakt zum Empfänger besteht und oft keine Ventilation und komplette Perfusion der transplantierten Lunge gegeben ist. Die Transplantationsmethode wurde nach einer früher beschriebenen Cuff-Methode modifiziert, um einige auftretende Komplikationen zu verbessern. Die Veränderungen zeigten eine deutliche Verbesserung in Bezug auf chirurgische Komplikationen und somit weniger Tiere, die aufgrund dieser vom Versuch ausgeschlossen werden mussten. Die dafür benötigte Zeit für die Transplantation, die aufgrund der eintretenden warmen Ischämie, entscheidend ist, unterschied sich nicht von der Methode die unserer zugrunde lag. Die Schmerztherapie die wir verwendeten vermied eine präoperative Opioidinjektion um die darauf folgende Atemdepression zu vermeiden die besonders in den ersten Stunden nach Transplantation gefährlich war. Wir konnten zeigen das ein Interkostaler Nervenblick mit einem Lokalanästhetikum zu einer Verbesserung des Animal-Health Scores führte verglichen mit einer präoperativen Opoidinjektion.

Bezüglich der Effekte von Deguelin auf die untersuchten Tiere konnten wir zeigen, das eine *in vitro* Deguelingabe zu einer signifikanten konzentrationsabhängigen Inhibition von HIF-1 Reportergenen wie VEGF, Carboanhydrase IX (CAIX), Lysyloxidase (LOX), Angiopoietin related protein 4 (ANGPTL4), Egl nine homolog 3 (EGLN3) und Adrenomedullin (ADM)

führt. Außerdem konnten wir eine mit der Deguelingabe zusammenhängende Runterregulation von mRNA von VEGF, Chemokine Receptor Type 4 (CXCR4) und Intercellular Adhesion Molecule 1 (ICAM-1) in der ischämischen Lunge feststellen. Desweiteren zeigte sich eine deutliche Verminderung des VEGF modulierten Reperfusionsödems bei den mit Deguelin behandelten Tieren. Unter Deguelingabe zeigte sich auch eine Runterregulation von proinflammatorischen Proteinen wie ICAM-1 und CXCR4 feststellen, während antiinflammatorische Monozyten (CD 163, CD 68) scheinbar unter Deguelingabe in das transplantierte Organ rekrutiert wurden.

Diese Erkenntnisse in Verbindung mit der deutliche besseren Überlebensrate der mit Deguelin behandelten Tiere, lassen den Schluss zu, das eine Inhibition von HIF-1 eine Verbesserung für das Lungentransplantat darstellt und Schäden vermindern kann.

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8 Addendum

8.1 Materials

Name	Manufacturer	Location
	LTX	
Adrenalin 1:100	Pharmacy of the JWG-University	Frankfurt am Main
Alm rectractor 5.5 mm	FST	Heidelberg, Germany
Animal clipper (Aesculap Action Ex-acta)	Aesculap	Tuttlingen, Germany
Atropin	Braun Melsungen	Melsungen, Germany
Buprenorphine	Reckitt Benckiser	Mannheim, Germany
Cold light source CL-200	Schott AG	Mainz, Germany
Corn oil (Mazola)	Unilever	Hamburg, Germany
Crile-Wood Needleholder 13mm; 1.5 mm	FST	Heidelberg, Germany
Deguelin	Sigma GmbH/Enzo Life Science	Steinheim/Lörrach, Germany
Enrofloxacin (Baytril)	Bayer	Leverkusen, Germany
Extra fine Graefe Forceps 0.5 mm	FST	Heidelberg, Germany
Halsted Mosquito Forceps	Eickemeyer	Tuttlingen, Germany
Heparine 25.000 I.U.	Ratiopharm GmbH	Ulm, Germany
Isoflurane evaporator ‘ Vapor 19.3’	Dräger	Lübeck, Germany
Ketamin	Pfizer	Berlin, Germany
Mayo Scissor	FST	Heidelberg, Germany
Metzenbaum Baby Scissor	FST	Heidelberg, Germany
Metzenbaum Scissor	FST	Heidelberg, Germany
Moria Iris Forceps 0.5 mm	FST	Heidelberg, Germany
Moria Iris Scissor	FST	Heidelberg, Germany
Narrow pattern forceps 14.5 cm	FST	Heidelberg, Germany
Perfadex (R)	vitrolife	Göteborg, Sweden

Piritramid (Dipidolor)	Jannsen	Pewaukee, USA
Polidon-Jod (Braunol)	Braun Melsungen	Melsungen, Germany
Ringer-Infusion Solution	Braun-Melsungen	Melsungen, Germany
Ropivacain (Naropin)	Astra Zeneca	Wedel, Germany
Small animal ventilator RUS-13	Föhr Medical Instruments	Pohlheim, Germany
Spencer ligature Scissor	FST	Heidelberg, Germany
Standard Scissor blunt	FST	Heidelberg, Germany
Sterile gauze compresse	Beesana	Barsbüttel, Germany
Strong forceps 1x2 teeth, curved 14 cm	FST	Heidelberg, Germany
Tissue Forceps 1x2 Teeth 14.5 cm	FST	Heidelberg, Germany
Vannas Spring Scissor 2mm cutting edge	FST	Heidelberg, Germany
Xylazin	Bayer Healthcare	Leverkusen, Germany

Histology		
Acetic acid concentrated	J.T. Baker	Griesheim, Germany
Avidin-Biotin-Blocking Kit	Vector labs	Burlingame, USA
Cover glass	Menzel GmbH	Braunschweig, Germany
DAB-Substrate Kit for Peroxidase	Vector labs	Burlingame, USA
DPX (R)	Merck	Darmstadt, Germany
Embedding cassettes	Sanowa	Leimen, Germany
Embedding machine	Medax	Salt Lake City, USA
Eosin –Y	Carl Roth	Karlsruhe, Germany
Ethanol absolute	Sigma-Aldrich	Seelze, Germany
Formaldehyde 36.5 %	Carl Roth	Karlsruhe, Germany
Goat Serum	Vector labs	Burlingame, USA
Goat anti mouse secondary antibody HRP-labeled (PO447)	Dako(biozol diagnostica)	Eching, Germany
Heating Plate	Medax	Salt Lake City, USA
Histology mold	Medite online	Burgdorf, Germany

Hydrochloric acid	AppliChem	Darmstadt, Germany
Hydrogen peroxide 30%	AppliChem	Darmstadt, Germany
Imm Edge Hydrophobic Barrier Pen	Vector labs	Burlingame, USA
Macrophage/Dendritic cells Antigen ABIN 289947	Antibodies – online GmbH	Aachen, Germany
Mayers hematoxylin	AppliChem	Darmstadt, Deutschland
Methylbenzoat	Carl Roth	Karlsruhe, Germany
Microtome (Leica RM 2125RT)	Leica	Nussloch, Deutschland
Microtome Blades R 35	FEATHER	Osaka, Japan
Mouse anti rat CD163 monoclonal (MCA342R) primary antibody	AbD Serotec	Düsseldorf, Germany
Mouse anti rat CD 68 monoclonal (MAB1435) primary antibody	Chemicon International (Millipore)	Schwalbach, Germany
Mouse anti rat ICAM-1 monoclonal (LS-C45355) primary antibody	LSBioscience	Seattle, USA
Rabbit anti rat CXCR4 polyclonal (ab2074) primary antibody	abcam	Cambridge, UK
Rabbit Serum	Vector labs	Burlingame, USA
RotiClear ®	Roth	Karlsruhe, Deutschland
Roti-Mount Aqua	Roth	Karlsruhe, Deutschland
Slides Super Frost Plus ®	Menzel GmbH	Braunschweig, Deutschland
Slides Süsse Frost ®	Süsse	Gudenberg, Deutschland
Unmasking Solution	Vector labs	Burlingame, USA
Vectastain ABC-Peroxidase Kit	Vector labs	Burlingame, USA
Vectastain Elite ABC Kit Rabbit IgG (Pk-6102)	Vector labs	Burlingame, USA
Waterbath	Daeglef Patz KG	Wankendorf, Germany
Xylol	Carl Roth	Karlsruhe, Germany

Western Blot		
Acrylamide 30%	Applichem	Darmstadt, Germany
Ammoniumpersulfate for electrophoresis (APS)	Sigma-Aldrich	Seelze, Germany
ß-Actin antibody N21 rabbit poly-clonal	Santa Cruz	Heidelberg, Germany
BCA-Kit	Pierce/Thermo-Fischer Scientific	Bonn, Germany
BSA	Carl Roth	Karlsruhe, Germany
Bradford Solution (RotiQuant)	Carl Roth	Karlsruhe, Germany
ß-Mercaptoethanol	Carl Roth	Karlsruhe, Germany
Bromophenolblue	Sigma-Aldrich	Seelze, Germany
Dithiothreitol (DTT)	Applichem	Darmstadt, Germany
GAPDH FL 335 antibody rabbit polyclonal	Santa Cruz	Heidelberg, Germany
Glycerol	Carl Roth	Karlsruhe, Germany
HIF-1 alpha 67 antibody	abcam	Cambridge, UK
HIF-1 alpha clone 54 antibody	BD bioscience	Heidelberg, Germany
IgG-HRP Sc2005 anti mouse	Santa Cruz	Heidelberg, Germany
IgG-HRP Sc 2004 goat anti rabbit	Santa Cruz	Heidelberg, Germany
Implen NanoPhotometer	Implen	München, Germany
Kaliumchloride	Carl Roth	Karlsruhe, Germany
Kaliumhydrogenphosphate	Carl Roth	Karlsruhe, Germany
Westernblot Luminol Reagent	Santa Cruz	Heidelberg, Germany
mAbcam 68334 VEGF antibody	abcam	Cambridge, UK
Magic Mark XP WB standard	Invitrogen	Darmstadt, Germany
Methanol	Carl Roth	Karlsruhe, Germany
MINI PROTEAN CELL 3	Biorad	München, Germany
Natriumchloride	Carl Roth	Karlsruhe, Germany
Natrumdihydrogenphosphate	Carl Roth	Karlsruhe, Germany

Natriumpyrophosphate	Carl Roth	Karlsruhe, Germany
Natriumvanadate	Carl Roth	Karlsruhe, Germany
Nitrocellulosemembrane Hypond C Extra	Amersham	Freiburg, Germany
PBS-tablets	Sigma-Aldrich	Seelze, Germany
Phenylmethylsulfonylfluoride (PMSF)	Sigma-Aldrich	Seelze, Germany
Ponceau-Red	Sigma-Aldrich	Seelze, Germany
Protease Inhibitor Cocktail Tablets	Roche	Mannheim, Germany
Restore Westernblot Stripping Buffer	Pierce/Thermo-Fischer Scientific	Bonn, Germany
Sodiumdodecylsulfate (SDS 20%)	Sigma-Aldrich	Seelze, Germany
Sigma 7-9	Sigma-Aldrich	Seelze, Germany
Spectra Brood Range Marker	Fermentas	St. Leon-Rot, Germany
Spectrophotometer µQuant	Biotech Germany	Bad Friedrichshall, Germany
TEMED	Carl Roth	Karlsruhe, Germany
TWEEN-20	Sigma Aldrich	Seelze, Germany
Tris	Carl Roth	Karlsruhe, Germany
Triton X-100	Applichem	Darmstadt, Germany
Urea	Sigma Aldrich	Seelze, Germany
Cell culture		
Cell flask 175 cm²	Greiner Bio One	Frickenhausen, Germany
Disposable cell scraper	Greiner Bio One	Frickenhausen, Germany
DMSO for cell culture	Sigma-Aldrich	Seelze, Germany
Epidermal Growth Factor (EGF)	BD bioscience	Heidelberg, Germany
Fetal bovine calve serum gold	PAA	Pasching, Austria
HERA bench	Thermo Scientific	
HMEC	ATCC	Manassas, USA

HTB-177 human lung epithelial cells	ATCC	Manassas, USA
Hydrocortison		
Hypoxia chamber INVIVO2 400 Hypoxia Workstation	Ruskinn Technology LTD	Pencoed, UK
MCDB 131 without L-Glutamin	Gibco	Darmstadt, Germany
Penicillin/Streptavidin solution	Sigma Aldrich	Seelze, Germany
Petri dish (6 cm)	Greiner Bio One	Frickenhausen, Germany
RPMI 1640 Medium	PAA Laboratories	Pasching, Austria
PBS	PAA Laboratories	Pasching, Austria
Trypsin-EDTA	Lonza	Basel, Switzerland
PCR		
Chloroform	Sigma-Aldrich Biochemistry	Hamburg, Deutschland
DEPC-treated water	Fermentas	St. Leon-Rot, Germany
Isopropyl alcohol	Carl Roth	Karlsruhe, Germany
i-Script cDNA synthesis kit	Biorad	München, Germany
Oligonucleotide primers	Sigma Aldrich	Hamburg, Deutschland
PCR-water	Eppendorf	Wesseling-Berzdorf, Germany
PowerSYBR Green PCR Master Mix	Applied Biosystems	Carlsbad, California, USA
PCR-Realttime Step-One Plus	Applied Biosystems	Carlsbad, California, USA
Thermocylcer Veriti 96 well fast	Applied Biosystems	Carlsbad, California, USA
TriReagent	Sigma-Aldrich Biochemistry	Hamburg, Deutschland
Consumable supplies		
Eppendorf tubes	Sarstedt	Nümbrecht, Germany

Falcon tubes 15/50 ml	Greiner Bio-one	Frickenhausen, Germany
Gloves non sterile nitrile/latex	Ansell	Brüssel, Belgium
Parafilm 'M'	Pechiney plastic bagging	Chicago, USA
Pipette tips	Starlab	Ahrensburg, Germany
Stripette	Sigma-Aldrich	Steinheim, Germany

Statistical analysis		
Graph Pad Prism (R) 5.02 software	Graph Pad Software Inc.	La Jolla, USA

Computer software		
Adobe Photoshop CS5.1	Adobe	München, Germany
Endnote	Adept Science	Frankfurt, Germany
Graph Pad Prism (R) 5.02	Graph Pad Software Inc.	La Jolla, USA
Image J Software (Freeware)		
Leica Application Software V 3.8	Leica	Wetzlar, Germany
Matlab	The Mathworks	Nattick, USA
Step One Software v.2.1.	Applied Biosystems	Carlsbad, California, USA
Windows Excel	Microsoft	München, Germany
Windows Paint	Microsoft	München, Germany
Windows Word	Microsoft	München, Germany

Equipment		
Centrifuge 5702	Eppendorf	Wesseling-Berzdorf, Germany
Centrifuge 5417R	Eppendorf	Wesseling-Berzdorf, Germany
Centrifuge Multifuge 1S-R	Thermo-Scientific	Bonn, Germany
Centrifuge 'The butterfly rotor'	Carl Roth	Karlsruhe, Germany
Fine scale	Sartorius	Goettingen, Germany
Heating Thermomixer MHR 23	HLC (DITABIS AG)	Pforzheim, Germany

HERA cell 15 0i CO2 incubator	Thermo Scientific	Bonn, Germany
HERA bench	Thermo-Scientific	Bonn, Germany
Kodak Image Station 4000 MM Pro	Carestream	New York, USA
Incubator B15	Thermo Scientific	Bonn, Germany
Isoflurane Vaporator	Drägerwerk	Lübeck, Germany
PCR-Realttime Step-One Plus	Applied Biosystems	Carlsbad, California, USA
Photometer		
Pipette 1000/200/100/10 µl	Eppendorf	Wesseling-Berzdorf, Germany
Pipetus – akku	Hirschmann Laborgeräte	Eberstadt, Germany
Microscope Module CTR 5000	Leica	Wetzlar, Germany
Microscope DM 5000 B	Leica	Wetzlar, Germany
Microscope surgery	Zeiss	München, Germany
MINI Protean cell for Westernblot	BioRad	München, Germany
Roller mixer SRT9D	Stuart	Dublin, Ireland
Shaking platform ST5	CAT	Staufen, Germany
Small animal ventilator	Drägerwerk	Lübeck, Germany
SonoPlus Sonificator	Bandelin	Berlin, Germany
Spectrophotometer µQuant Microplate	Bio-Tek Instruments Inc.	Bad Friedrichshall, Germany
Tank transfer system for blotting	BioRad	München, Germany
Thermocylcer Veriti 96 well fast	Applied Biosystems	Carlsbad, California, USA
Vacuum safety pump system AA 02	HLC (DITABIS AG)	Pforzheim, Germany
Vortexer	VWR International	Darmstadt, Germany
Ultraturrax T10 Basic	IKA Laboratory Equipment	Staufen, Germany

8.2 Abbreviations

Abbreviation	Name
AAMR	Acute antibody mediated rejection
ACTB	Gene name of ß-Actin
ADM	Adrenomedullin
ANGPTL4	Angiopoietin-related protein 4
APS	Ammoniumpersulate
APC	Antigen presenting cells
ARNT	Aryl hydrocarbon receptor nuclear translocator
ATP	Adenosinetriphosphate
BCA	Bicinchoninic acid protein assay
BOS	Bronchiolitis obliterans syndrome
bHLH	Basic HELIX-LOOP-HELIX
BSA	Bovine serum albumine
BW	Bodyweight
°C	Degree in celsius
CAIX	Carboanhydrase 9
CD	Cluster of differentiation
cDNA	Complementary DNA
CO_2	Carbondioxide
CXCR-4	Chemokin receptor type 4
d	Days
DAB	3,3'-diaminobenzidin
DEC	Deleted in colorectal cancer gene
DEPC	Diethylpyrocarbonat
DMSO	Dimethyl sulfoxide
DNA	Desoxyribonucleic acid
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
EGLN3	Egl nine homolog 3 gene

EIP	Etoposide induced protein
EPO	Erythropoietin
ET1	Endothelin-1
FCS	Fetal calve serum
G	Gauge
g	Gram
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GLUT1	Glucose transporter 1
h	Hours
HCL	Hydrochloric acid
HIF-1	Hypoxia inducible factor 1
H&E	Hematoxlyin&Eosin
HLA	Human leukocyte antigen
HMEC	Human microvascular endothelial cells
HRE	Hypoxia responsible element
HRP	Horseradish peroxidase
ICAM -1	Intercellular adhesion molecule-1
ICU	Intensive care unit
IFN	Interferon
IGF	Insuline growth factor
Ig	Immuneglobuline
i.g.	Intragastrical
IL	Interleukin
IRI	Ischemia and reperfusion injury
I/R	Ischemia/reperfusion
IU	International units
K^+	Kalium ionised
KCL	Kaliumchloride
kDa	KiloDalton
kg	Kilogram
KH_2PO_4	Kaliumhydrogenphosphate
KRT	keratin
i.v.	intravenous

LD50	Lethal dose 50
LEP	Leptin
LOX	Lysyl oxidase
LRP1	LDL-receptor related protein
LTX	Lung transplantation
M.	Musculus
M	Molar
Mm.	Musculi
mm	Millimeter
MAPK	P38 mitogen activated kinase
MDR1	Multidrug resistance receptor 1
MHC	Major histocompatibility complex
min	Minutes
MCP-1	Monocyte chemotactic protein-1
ml	Milliliter
mRNA	Messenger RNA
n	Number
N.	Nervus
Na^+	Natrium ionised
Na_2PO_4	Natriumdiphosphate
NaCl	Natriumchloride
NADPH	Nicotinamidadenindinukleotidphosphat
NF-κB	Nuclear factor 'kappa-light-chain-enhancer'
nM	nanomolar
NOS	Nitrogen monoxide synthase
O_2	Oxygen
OB	Obliterative bronchiolitis
ODD	Oxygen dependent degradation domain
ODC	12-O-tetradecarnoylphorbol-13-acetat induced ornithine decarboxylase
OPTN	Organ procurement and transplantation network
PAI-1	Plasminogen activator inhibitor -1

PCR	Polymerase chain reaction
PBS	Phosphate buffered saline
PEEP	Positive end expiratory pressure
PER	Periodic circadian protein
PGD	Primary graft dysfunction
PI3K	Phosphositide-3-Kinase
PMSF	Phenylmethylsulfonylfluoride
ppm	Parts per million
RANTES	Regulated upon Activation, Normal T-Cell Expressed and Secreted
RNA	Ribonucleidacid
ROS	Reactive oxygen species
rpm	Rounds per minute
s.c.	Subcutaneous
SDS	Sodiumdodecylsulfate
PAGE	Polyacrylamide gel electrophoresis
SHAM	Control group
SIM	Single-minded protein
TBS	Tris-buffered saline
TBS-T	Tris-buffered saline with TWEEN
TGF	Transforming growth factor
TLR	Toll-like receptor
TPI	Triosephosphate isomerase
TNF-α	Tumor necrosis factor α
VCAM-1	Vascular cell adhesion molecule-1
VEGF	Vascular endothelial growth factor
VHL	Von Hippel-Lindau tumor suppressor gene
pVHL	Product of VHL
VIM	Vimentin
µ	Micro

8.3 Legends

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9 Declaration

I declare that I have completed this dissertation without the unauthorized help of a second party and only with the assistance acknowledged therein. I have appropriately acknowledged and referenced all text passages that are derived literally from or are based on the content of published or unpublished work of others, and all information that relates to verbal communications. I have abided by the principle of good scientific conduct laid down in the charter of the Justus Liebig University of Giessen in carrying out the investigations described in the dissertation.

Giessen,

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