Project: Program and Plan
Project Title

Investigating the molecular mechanisms and underlying pathways of regenerative medicine approaches to the tissue-engineering and cell therapy of airways and lungs

Definition of Problems.

Lung diseases are the major reasons of morbidity and mortality all over the world since as much as 50 million individuals worldwide are living with end-stage lung disease. In 2020, out of 68 million deaths *worldwide*, 11.9 million will be caused by *lung diseases*. Despite several improvements in treating symptoms, allogeneic transplantation (Tx) remains the only curable therapeutic option. Unfortunately, there is an on-going shortage of donor organs and the long-term outcome of lung-Tx patients is still associated with high rates of complication due to chronic or acute rejection and negative side-effects of immunosuppressive. Beside this the costs for the health system and needed resources are immense. The paradigm of transplantation needs thus a change and therapeutic options should be pioneered. Our recent findings suggest that human lungs do repair or regenerate beyond the cellular level when promoted *via* cell therapy.[1] Mesenchymal stem cell (MSC) administration let to a significant impact on both clinical parameters and regeneration of lung tissue in a pulmonary hypertension model via several paracrine effects and alteration of protein expression in lung tissue (Figure 1, 2):

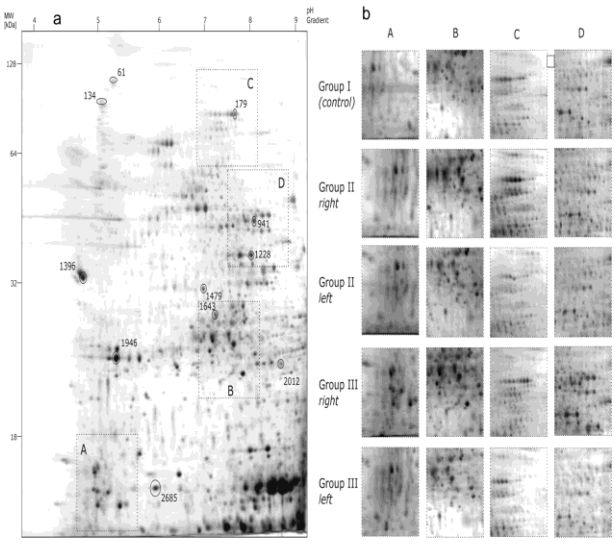


Figure 1 **a**. **Proteomic approach** Representative NEPHGE 2-D-SDS PAGE of proteins from *right lung* of Chronic Thrombembolic Pulmonary Hypertension (CTEPH)-induced and MSC-treated rats. Over all approximately 2000 most abundant proteins were detected after silver staining. Circles represent affected proteins singular detected for group III, identified by MS. Main variant regions in between different groups are assigned as dashed rectangles. **b Quantitative Analysis** Comparison of main variant regions (A-D) detected and analyzed in NEPHGE 2-D-SDS PAGE using Melanie 7.0. *Group I:* both lungs (matched protein profile) from thoracotomy-only-received animals; *Group II:* left and right lungs from left pulmonary artery legated with sham-treatment; *Group III:* left and right lungs from left pulmonary artery legated and MSC-treated animals.

Spot	Swiss-Prot	Proteine	Mr	pI	Score	Sequence
61	UPI000157F7A1	unnamed protein	216,384	5.68	170	24
134	P02563	Myosin-6, Myosin	223,371	5.58	230	26
179	Q9ER34	Aconitate	85,421	7.87	333	42
941	P09605	Creatine kinase S-	47,355	8.76	221	56
1228	P04797	Glyceraldehyde-3-	35,805	8.14	117	41
1369	Q91XN6	Tropomyosin	32,836	4.77	233	63
1479	P13803	Electron transfer	34,929	8.62	160	45
1643	P15999	ATP synthase	55,247	8.28	148	32
		ATP synthase,	59,717	9.22	139	29
1946	P16409	Myosin light chain	22,142	5.03	142	67
2012	UPI0001551E7C	Albumin, isoform	51,521	6.72	106	27
2685	P07483	Fatty acid-binding	14,879	5.90	145	22

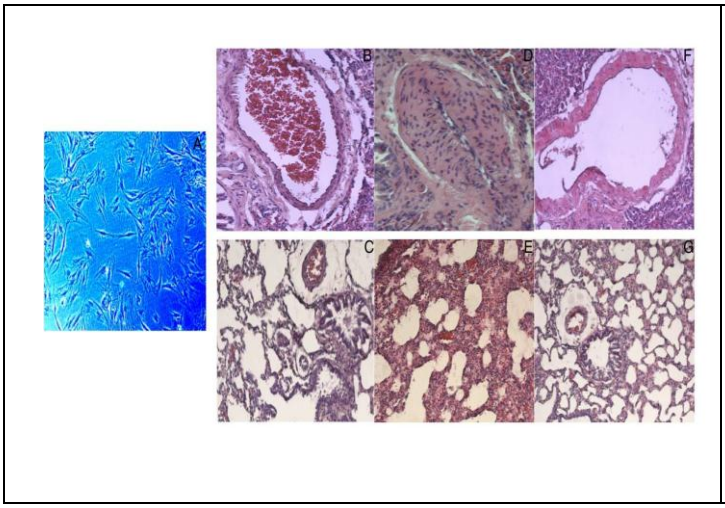
Figure 2: Highly expressed proteins of *right lungs* from left pulmonary artery legated and MSC treated animals (Group III) compared to other groups, identified in 2D-SDS-PAGE by MS.

One need to investigate the real impact of these non-lung-specific proteins on pulmonary hypertension course and its improvement. Currently we have different hypothesis how to explain: one might be that the non-specific proteins are expressed by the administrated MSCs promoted by systemically active substances/cytokines released by pressure-induced tissue damage in other organs

aside from the lungs. These proteins might than have a positive effect on these non-lung organs when circulating systemically.

Another hypothesis is that the detected proteins are released from their specific tissue and released into the systemic circulation and end-up in the high pressure exposed lung due to unknown homing mechanisms. And finally we hypothesize, that the protein are also derived from the administrated MSCs due to a mis-differentiation of the cells, but without any benefit for the progress of the pulmonary hypertension. Then one should discuss if specific growth factors are necessary when administer due lead to differentiation into the optimum direction. All need to be studied both in vitro and in vivo.

Histological findings showed a regeneration of the previously occurred pathological changes inside lung parenchyma. This demonstrates that inflammatory responses can be reduced with mesenchymal stem cell administration.[1] Several groups showed the immunomodulatory characteristics of this cell type, such as expressing anti-inflammatory mediators like Interleukin-10, -13,-1a, angiopoietin-1, keratinocyte growth factors or even inhibiting the production of pro-inflammatory cytokines, such as tumor necrosis factor-alpha, macrophage inflammatory protein 2 and many others.[2-4] We showed the anti-inflammatory effect of the administrated MSCs indirectly by the significant improvement of the lung injury score.[1] However, there is an immense potential of these cells in terms of immunomodulatory capacity that needs to be investigated and this will be one of the targets of this project.



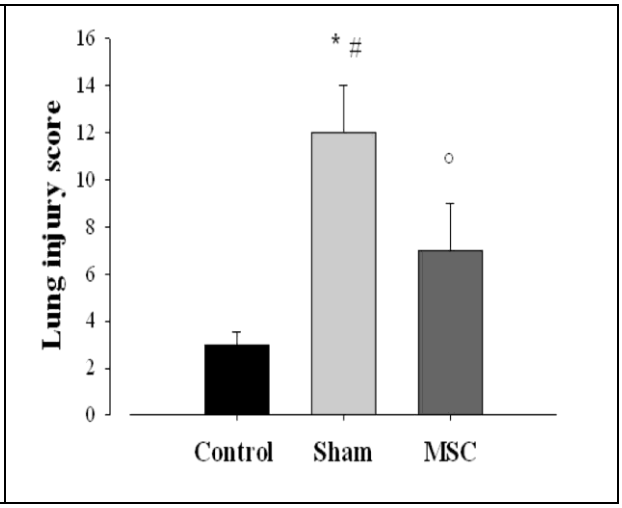


Figure 3: **Histological evidence of MSCs´ effect: A.** Bone marrow-derived mesenchymal stem cells (MSCs) with spindled, fibroblast appearance in MesenPro culture. Scale bar: 200 μm. **B, C** (group I) H&E staining of healthy lung parenchym; Scale bar (B): 200 μm, Scale bar (C): 100 μm. **D, E** (group II) showed CTEPH pulmonary vasculopathic abnormalities and broncho-pulmonary artery hypertrophy (**1**) and inflammatory signs such as hemorrhage, edema and granulocytes migration (**2**); Scale bar (D): 200 μm, Scale bar (E): 100 μm. **F,G** H&E staining of lung sections demonstrated improved lung injury in animals given MSCs; reduction in the degree of hemorrhage, edema, granulocytes migration and vascular remodeling. Scale bar (F): 200 μm, Scale bar (G): 100 μm.

Figure 4: **Lung injury score:** Lung injury score according to alveolar congestion, hemorrhage, infiltration or aggregation of neutrophils in airspace or vessel wall, and thickness of alveolar wall/hyaline membrane formation. Score ranges from a maximum of 16 (maximal damage) to a minimum of 0 (minimal damage). * $p<0.01$ vs. Control; # $p<0.025$ vs. MSC.

Notably, we detected the applied cells only in the high-pressure exposed lungs. One underlying mechanism of this cell-homing to damaged tissue is most likely related to the Stromal-derived factor-1 (SDF-1)/C-X-C chemokine receptor-4 (CXCR4) pathway but probably also associated to other so-far unknown pathways.

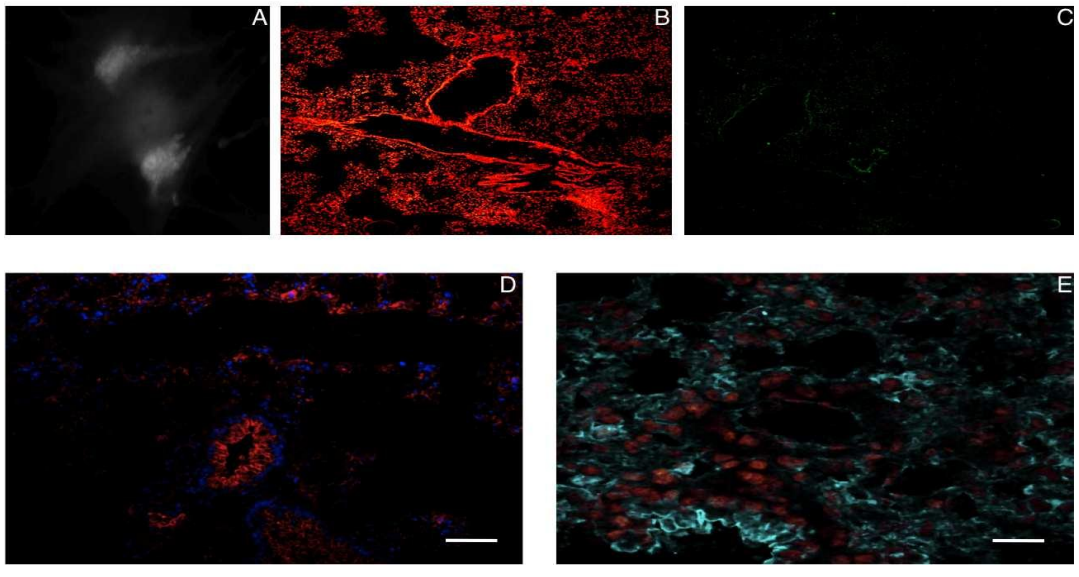


Figure 5: **Recruitment of MSCs via SDF-1:** MSCs were incubated with Cell-carboxy-fluorescein succinimidyl ester (CFSE) and intratracheal administrated. MSCs were only detected by confocal microscopy in non-ligated right lung, primarily at the lung parenchyma; Scale bar: 400 μm (**A**), and via fluorescence microscopy bromodeoxyuridine-labeled (**B**) MSCs (Alexa-488, green) only detected in right lung next to resident cells (red) (**C**); Scale bars: 100μm. (**D**) right lung stained for anti-SDF-1 (stained red), nuclei (stained blue) were counterstained with TOPRO3, fluorescence microscopy view (Scale bar:100 μm). **E** confocal microscopy view, Nuclei (blue) and anti-SDF-1 (green) (Scale bar: 200 μm).

Even though these initial findings are very promising, underlying mechanism have to be investigated more accurately before transferring to the clinic. It has been shown that the level of cell engraftment is very low and effects are mostly related to the paracrine capacity of the administrated cells.[5] One should evaluate novel techniques to enhance cell engraftment and thus effect of applied cells. This might improve regenerative processes inside lung tissue. Beside this the viability of cells needs to be increased by distinct methods.

Another potential approach to replace diseased lung tissue is the method of tissue engineering.

The achievement of our group with tissue-engineered airway transplantation, using an acellular graft seeded with autologous stem and respiratory cells provides evidence about the clinical potential of tissue engineering.[6-9]

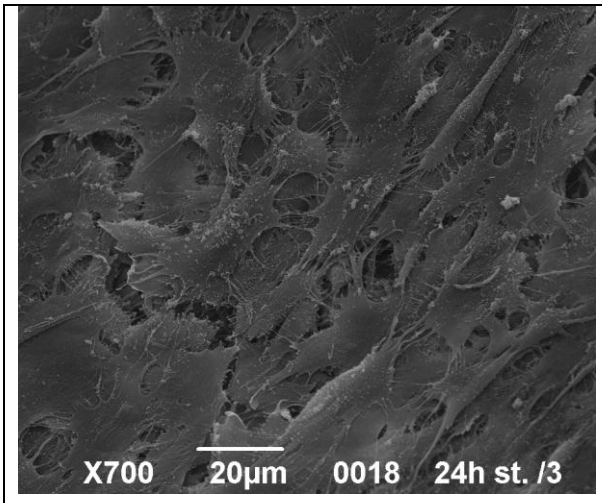


Figure 6: SEM of seeded trachea graft

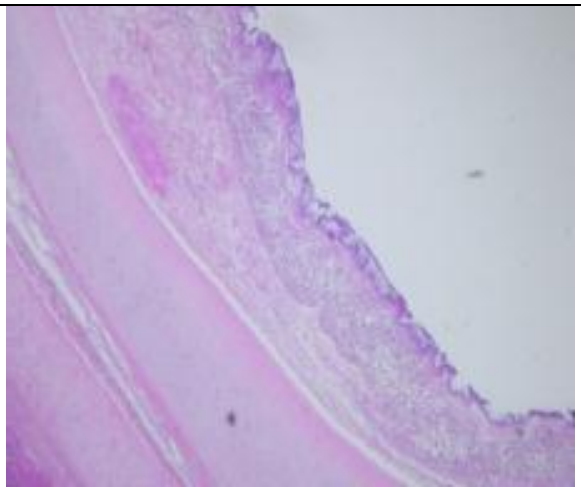


Figure 7: Histological View on engineered trachea, Cytokeratine 5,8 and Collagen II stained

Results from the use of polymers, and natural materials such as collagen or Matrigel demonstrate the importance of preserving the extracellular matrix's role (ECM).[10] The ECM does not only define the lung's architecture and contain physical properties but also influence the direction of pulmonary cell differentiation.

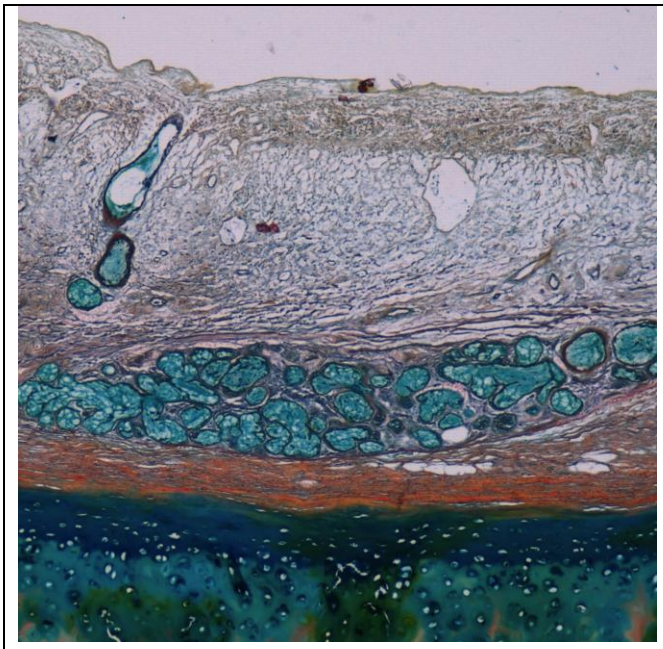


Figure 8: Decellularized trachea stained via MOVAT method to detect components of Extracellular Matrix.

Most important for the success of engineered organs and tissue is a sufficient vascularization of the graft. We detected pro-angiogenic factors inside our decellularized matrix (such as beta-fibroblast growth factor; FGF) and demonstrated scaffold´s capacity to promote *in vivo* vessel formation.

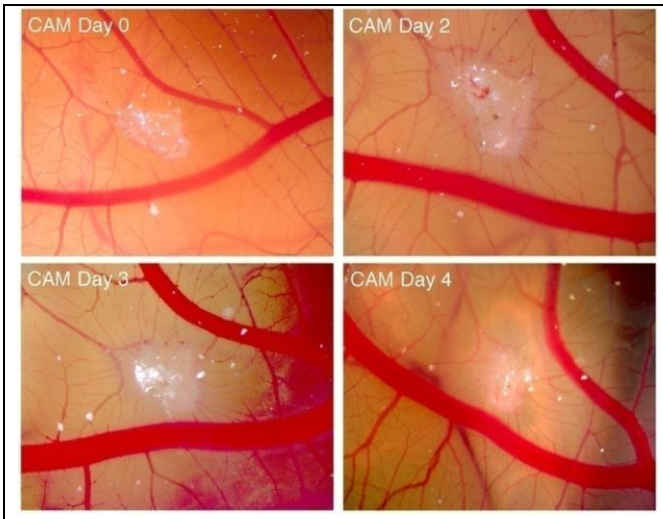


Figure 8: *In vivo* neoangiogenesis; CAM (chorioallantoic membrane) and were

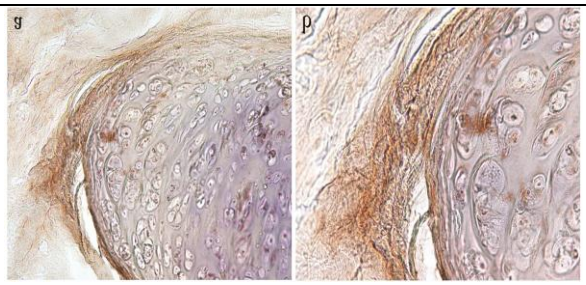


Figure 9: anti beta-FGF (fibroblast growth factor) staining of the

totally enveloped by newly formed blood vessels promoted by decellularized matrix.	decellularized graft.

Ott H.C. and Petersen T.H. confirmed experimentally our approach even for the higher complexity and enlarged architecture of small animal lung tissue.[11,12] The so far engineered lung tissue that can be ventilated and perfused through a patient's airway and vasculature has been limited by the inability to generate a biodegradable, highly elastic lung scaffold that reproduces the lung's complex airway, alveolar, and vascular architecture that can support gas exchange over a large surface area and therefore findings are hitherto fairly sobering. An engineered lung should have the following characteristics: (a) contain lung-specific cells; (b) display the branching geometry of the airways and contain a perfusing microvasculature; (c) provide barrier function to separate blood from air, and (d) have mechanical properties that allow ventilation at physiological pressures. The recent result showed that lung scaffold could be seeded with several cell types expressing side-specific surface markers. Developing functional characteristics of the lung and evaluating engineered lungs in large animal studies are the challenge for ongoing research.

Even though lung tissue transplantation in a clinic scenario is still far away, yet potential approaches have been developed and are available to test their capability**.**

Cell therapy and bioengineered organs would bypass the mentioned problems of donor organs and immunosuppression and could therefore represent a very promising therapeutic option.

1. Project Goals

The regenerative medicine is a fast growing and high potential field in medical research. In order to enhance the knowledge in this area and tranfer the findings to the clinic we need to build up core facilities that allow for high level and advanced research. Such a facility should provide a place to bring expertise and knowledge

from experts together and support young academics and researchers to get into this field. We have already demonstrated the potency of bioengineered tissue for the clinic application.

The aim of this project is to initiate a facility able to investigate and study mechanism and pathways of cell therapy and tissue engineering in the field of lung tissue. Furhter, we purpose to transfer our already clinically applied *in vivo* bioengineering tracheal methodology and the experimentally applied cell therapy to the lung and to develop organs able to be used in clinical conditions. Therefore we will perform a 4-phase project within the next three years.

First stage. Experimental settings will be established and the following laboratory resources installed:

- Work benches
- Clean rooms, cell culture room
- Consumables
- Small animal surgery working places
- Decellualrization and testing devices
- Incubator, refrigerator, fridges
- Facilities for histological and immunhistochemical evaluation, different microscopes
- PCR (polymerase chain reaction), real time-PCR, Western blot devices

Second stage. An intensive *in vitro* phase will be performed and variable evaluation processes conducted. Proteomics will investigate protein expression level both on acellular scaffolds and engineered lungs. *In vitro* studies will be performed on cells and gene expression profile within culture, differentiation and post-seeding.

The aim of these *in vitro* studies is to standardize and verify initial findings of our previously performed studies of cell therapy in pulmonary hypertension and the cell application for tracheal tissue engineering. Underlying pathways should be elucidated to enhance the knowledge of stem cell biology. Fundamental in vitro knowledge about stem cell behaviour is absolutely necessary to understand in vivo

processes and interactions. Proteomics and RNA studies will be performed to detect expression level of target genes within the differentiation process of stem cells. Likewise regulator genes will be elucidated via mi-RNA.[13]

Total RNA will be labeled and hybridized to the array-system miCHIP based on Tm- normalized cap- ture probes (miRCURY). Array images will be generated by using a laser scanner, miCHIP arrays scanned in batches using the auto Photo Multiplayer algorithm, with pixel saturation tolerance set to 0.2%. Tiff images generated by the laser scanner will be processed by a microarray analysis software and Gene array analysis will be performed as previously described[13]. These methods can be performed either by our collaborator partners from abroad or by local partner units using the same technologies. However, one of the goals of the project will be to establish these methods in the near future at the new facility.

Scaffold will be evaluated to understand and probably improve cells engraftment due to specific surface molecules. Several cell culture and differentiation protocols will be evaluated to improve and setect optimum methods. Differentiated cell will be characterized during culture and differentation in plane dished and compared to 3-dimensional matrices to detect alteration of their phenotypes related to different enviroment. One challenge will be the evaluation of the optimum cell source to engineer respiratory and other lung specific cells. Several cell types are potential candidates for this issue such iPSCs (induced pluripotent stem cells), mesenchymal stem cells (MSCs), hematopoietic stem cells (HSCs) , human umbilical cord endothelial cells (HUVECs) amniotic derived stem cells, however, so-far obtained data are not ultimately satisfying. One has demonstrated for instance that there is no evidence for the until recently assumed hypothesis of lung alveolar epithelial cell reconstitution by unfractionated bone marrow cells or purified hematopoietic stem cells when administrated systemically.[14] We showed the benefit and improved remodeling when applying MSCs via the trachea into an animal model of pulmonary hypertension. The engraftment of our cells was likewise low and thus differentiation potentially marginal. Therefore MSCs can be used for therapeutical application such as cell therapy but probably not for engineering lung

tissue. Further investigation of cell behavior in 3-D scaffolds (mimicing physiological enviroment) and transplanted in vivo into genetically modified mouse lines are necessary and will be performed.

We will investigate the presence of lung tissue specific cell markers (such as the type II pneumocyte–specific marker SP-C, the nonspecific epithelial and endothelial marker caveolin-1 and the pan-epithelial cell marker CK-18) when re-cellularizing acellular scaffolds with various cell types such as MSCs, iPCSs, HSCs and HUVECs. Likewise the expression of surfactant proteins A and C, thyroid transcription factor-1 (Ttf1, also known as Nkx2-1), type I pneumocyte marker T1α and vimentin will be studied.

One is able to differentiate stem cells into lung specific surface marker expressing cells but their functionality is poor or surface marker disappear during long lasting culture. One other target will be the tracking of seeded or adminstrated stem cells to observe their potential *in vivo* differentiation or engraftment. If so, we will evaluate the level of cell engraftment and try to enhance this level. The effect of boosting therapy will be monitored and investigate by taking blood samples and isolating progenitor and peripheral stem cells. Cell genomic and epigenetic profile will be evaluate to test the validity of the findings and thus the possibility to apply this method to a wide range of patients.

As mentioned underlying pathways of MSC homing are poorly investigated. Therefore one of the aims of the project will be to study specific homing pathways of stem cells. Multiplex Cytokine Analysis can help to provide essential cytokines to understand the migration processes of MSCs to damaged tissue side.

Devised that are necessary for these studies are: microscopes, Fluorescence-activated cell sorting (FACS), PCR-devices, Western blot. Some specifc divices and knowledge (such as epigenetic or proteomics) will be provided from collaborations (Karolinska Institutet, Sweden; University Aachen,Germany; Robert Koch Institute Berlin, Germany; Bioairlab Florenz, Italy; University Burlington, Vermont) and stepwise established into the new facility.

Third stage. *In vitro* findings will be evaluated in several *in vivo* animal models.

In vitro results and data are essential to understand and detect underlying pathways and mechanisms. However, only in vivo studies can provide profound data for potential clinical transfer. The main target in this stage is to investigate and validate all the in vitro obtained data by tranfering them into in vivo animal models. After selecting the right cell source and culture/differentiation protocol we will conduct multiple animal models.

To investigate the effects of cell therapy and its mechanism in different lung diseases condition both rat and mouse animal models will be used. For this purpose we will use surgical induced, toxic reagents induced (MCT), and hypoxia-induced models of pulmonary hypertension. To detect the significance of inflammatory response to the development and progress of this disease, genetically modified animals will be used (Cre-Lox recombination for specific inflammatory realted cell types). Administrated cells will be followed and detected by previously applied tracking methods. Cells´ differentiation and engraftment evaluated and expressed inhibited cytokines measured. Also the *in vitro* applied methods of gene and protein expression will be repeated.

One challenge that has so-far not been discovered sufficiently is the detection of circulating mesenchymals from the peripheral blood. We could show in recent animal models and clinical trials that the level of CD34 positive cell in the peripheral blood after boosting therapy correlates with the number of in vitro cultured endothelian progenitor (EP) and mesenchymal stem cells (MSCs). However the definitive characterization or real-time sorting of these progenitor cells in the peripheral blood as a response of mobilization has yet not been demosntrated. It is hypothesized that this mobilization of these cells is only a response of the organism to an inflammaotry event. We aim to investigate novel detection methods via FACS analysis to give definitive answers to these issues. Furthermore we purpose to study the functionality of these how-ever-mobilized progenitor cells and their in vivo behavior.

This stage should bring out profound data for a potential clinical trial of cell therapy in patients suffering from severe pulmonary hypertension and non-response to conventional treament.

Concerning the tissue engineering studies: experiments will be first conducted in rats. Previously performed studies will be validated and improved. In this stage Bioreactors will have an essential part to decellualrize and re-cellularize engineered scaffolds. In vivo function will be widely evaluated and monitored. Seeded cells will be studied after in vivo transplantation and tested for phenotype and gene expression level. Lung function and clincial outcome of all animals receiving engineered organs will studied and evaluated. If the data are astisfactory the potential transfer to non-human primates will be discussed. Pigs can be used to validate the mechanical propertis of the engineered organs. However, the knowledge of their stem cell biology is to weak to transfer obtained data directly to the clinic.

If the data are sufficient to transfer to non-human primates we purpose to engineer lung tissue (whole lung or lobes) able to be trsnplanted into patients with end-stage lung diseases.

Fourth stage. After sufficient *in vitro* and pre-clinic studies we will potentially transfer the concept of cell therapy and tissue engineering into the clinic. Concering the cell therapy: some selected patients with severe progressive pulmonry hypertensiona and non-respondent to conventional treament will be treated with our investigated stem cell therapy. Concerning the engineered lung tissue: some selected patients with end-stage lung diseases and no chance to obtain a donor lung within reasonable time will be treated with the engineered lung tissue.

During the whole project several workshops, courses and communication will take place to provide and improve teaching for a wide range of young academician.

Conclusion. The main goal of this project is to establish one of the best research, educative and clinical center in the world in the field of regenerative medicine, which will be internationally recognized through publications in the top

level journals and presentations on large international events. The main aim in the basic scientific aspect is the evaluating the molecular mechanisms and underlying pathways of tissue-engineering and cell therapy for regenerating airways and lung tissue. The biomedical goal is using revolutionary science advances to provide translational studies for prevention and effective treatment of wide range of diseases, and create new methods of organ's reparation.

Schedule

Stage Number	***Stage goal***	***Stage result***	***Duration***	***Budget MRub.***
1	Experimental settings and laboratory. Purchase of the required equipment	1. Laboratory for specific projects will be set up and established. In vitro and in vivo facilities will be arranged. Bioreactors, cell culture rooms, clean areas work places organized. 2. Stuff will be trained and instructed for distinct purposes 3. Working plans and structures establish 4. Cooperation with foreign and Russian research groups will be initiated	10/2011 - 03-05/ 2012	40
2	*In vitro* studies	1. a wide range of *in vitro* studies will be designed and performed such as: cell isolation (different protocols and species), culture, differentiation, proteomics, gene expression, proliferation, seeding on different scaffolds (both natural and synthetic), immunochemistry, morphological and mechanical analysis 2. preparation of ethical proposals for *in vivo* studies	04/2012- ongoing	70
3	*In vivo* studies	1. several in vivo animal studies will be established and performed (including mice, rats, pigs and non-human primates) 2. detailed evaluation of the in vivo studies	09.2012- 06.2013	25

4	Starting transfer to the clinic	1. after successful in vitro and pre-clinic studies we can hopefully transfer the approach of the cell therapy and/or tissue engineering to the clinic in selected cohort of patients	2013	15
	Teaching/ Education	- Workshops and courses for young academics to train in the field of regenerative medicine will be held - Scientific conferences on the different research aspects will be organized	10.2011-ongoing	

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