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DISSERTATION

Untersuchung der zellspezifischen In-situ-Effizienz des Nukleinsäuretransfers in das Herzgewebe

Testing the cell-specific in situ efficiency of nucleic acid transfer into heart tissue

zur Erlangung des akademischen Grades
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Contents

1. List of abbreviations

AAV: adeno-associated virus

calcein-AM: calcein-acetoxymethyl ester

CM: cardiomyocyte

cTNT: cardiac Troponin T

DPBS: Dulbecco's phosphate-buffered saline with magnesium and calcium

GFP: green fluorescent protein

HF: heart failure

LV: left ventricular

MTS: 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-

 sulfophenyl)-2H-tetrazolium

P/S: penicillin/streptomycin

RT: room temperature

αMHC: alpha-myosin heavy-chain

This dissertation summarizes the background, methods, results, potential clinical application, and the direction of future optimization based on the publication "Comparative analysis of adeno-associated virus serotypes for gene transfer in organotypic heart slices" published in the Journal of Translational Medicine (2020).

2. Abstracts

2.1 Abstract

Background and aims: The importance of nucleic acid-based methods ("gene therapy") for innovative concepts for the treatment of heart failure is growing steadily, but their clinical translation is only progressing slowly. A fundamental problem in the preclinical assessment of the transduction/ transfection efficiency is that human cardiomyocytes are only present in suspension or culture, while in vivo disease models are necessarily based on other species. In the present work, an organotypic heart slice culture model was established in order to directly examine the transduction efficiency of cells in the intact myocardium by different serotypes of adeno-associated viruses (AAVs).

Methods: Left ventricular tissue derived from 16±1-day-old homozygous alpha-myosin heavy-chain-mCherry mice was cut into 100–300 μm-thick slices by a vibrating microtome. The heart slices were cultured in an air-liquid interface culture system and their viability was assessed by calcein-acetoxymethyl ester staining, mCherry fluorescence intensity, and the tetrazolium assay. The cardiomyocytes were identified by immunofluorescence staining of cardiac Troponin T (cTNT). AAV vectors (AAV 1, 2, 6, 8) for constitutive expression of a GFP reporter gene were applied to the surface of the slices. The transduction efficiency, the number of GFP-positive cells, and the GFP-positive subpopulations were determined after 5 days.

Results: The mCherry fluorescence signal was specifically detected in cTNT-positive cells and thus indicates cardiomyocytes. Under normoxic culture, heart slices could maintain good viability for at least 5 days based on the mCherry fluorescence intensity and the calcein fluorescence area. Among the four serotypes, AAV6-mediated GFP expression resulted in the highest GFP-positive cell number in slices. AAV1- and 8-infected slices presented fewer GFP-positive cells, with almost no GFP signal was detected in AAV2-infected slices. AAV6 showed the best myocardial penetration. Almost 100% of the cardiomyocytes appeared GFP-positive, whereas the proportion of GFP-positive cardiac fibroblasts was only approximately 12%. None of the AAV serotypes induced any relevant cytotoxicity.

Conclusions: AAV-mediated gene transfer in cardiac cells can be induced and quantified in an organotypic murine heart slice culture system. AAV6 shows the highest in situ transduction efficiency. In the future, this model can be applied to pathologically altered human myocardium.

2.2 Zusammenfassung

Hintergrund und Ziele: Die Bedeutung Nukleinsäure-basierter Verfahren („Gentherapie") für innovative Konzepte zur Behandlung der Herzinsuffizienz wächst stetig, ihre klinische Translation schreitet jedoch nur langsam voran. Ein grundsätzliches Problem bei der präklinischen Beurteilung der Transduktions-/Transfektionseffizienz ist, dass humane Kardiomyozyten lediglich in Suspension bzw. Kultur vorliegen, während In-vivo-Krankheitsmodelle notwendigerweise auf anderen Spezies basieren. In der vorliegenden Arbeit wurde daher ein organotypisches Herzschnitt-Kulturmodell etabliert, um die Effizienz der Transduktion von Zellen im intaktem Myokard durch unterschiedliche Serotypen von Adeno-assoziierten Viren (AAV) direkt zu untersuchen.

Methoden: Linksventrikuläres Gewebe von 16±1 Tage alten homozygoten Alpha-Myosin-Heavy-Chain-mCherry-Mäusen wurde mit einem Vibrationsmikrotom in 100–300 µm dicke Scheiben geschnitten. Die Gewebescheiben wurden in einem Luft-Flüssigkeit-Grenzflächenkultursystem kultiviert, ihre Vitalität durch Calcein-Acetoxymethylester-Färbung, mCherry-Fluoreszenzintensität und Tetrazolium-Assay verfolgt und Kardiomyozyten durch Immunfluoreszenzfärbung von Troponin T (cTNT) identifiziert. AAV-Vektoren (AAV 1, 2, 6, 8) für die konstitutive Expression eines GFP-Reportergens wurden auf die Oberfläche des Gewebes appliziert. Die Transduktionseffizienz, die Anzahl GFP-positiver Zellen sowie die GFP-positiven Subpopulationen wurden nach 5 Tagen bestimmt.

Ergebnisse: Das mCherry-Fluoreszenzsignal wurde spezifisch in cTNT-positiven Zellen detektiert und zeigt somit Kardiomyozyten an. In Normoxie konnte die Vitalität des Gewebes basierend auf der mCherry-Fluoreszenzintensität und der Calcein-Fluoreszenzfläche für mindestens 5 Tage aufrechterhalten werden. Unter den vier Serotypen führte die AAV6-vermittelte GFP-Expression zu der höchsten GFP-positiven Zellzahl in Schnitten. AAV1- und 8-infizierte Schnitte zeigten weniger GFP-positive Zellen, in AAV2-infizierten Schnitten wurde fast kein GFP-Signal detektiert. AAV6 zeigte die beste Durchdringung des Myokards. Fast 100% der Kardiomyozyten erschienen GFP-positiv, während der Anteil GFP-positiver kardialer Fibroblasten lediglich ca. 12% betrug. Keiner der AAV-Serotypen induzierte eine relevante Zytotoxizität.

Schlussfolgerungen: AAV-vermittelter Gentransfer in Herzmuskelzellen kann in einem organotypischen murinen Gewebekultursystem induziert und quantifiziert

werden. AAV6 zeigt die höchste In-situ-Transduktioneffizienz. In Zukunft soll dieses Modell auf pathologisch verändertes humanes Myokard übertragen werden.

3. Current state of research

3.1 Heart failure

Heart failure (HF) is associated with high morbidity and mortality worldwide. Over 26 million people worldwide suffer from HF, and the average annual mortality rate is approximately 33% [1, 2]. Heart failure is a complex multifactorial syndrome that occurs when the heart is unable to pump blood sufficiently to maintain the body's needs, for instance, in certain conditions of myocardial injury caused by myocardial infarction, cardiomyopathy, hemodynamic overload, and inflammation. The gold standard treatment for end-stage HF patients is heart transplantation, which faces huge challenges by the limited number of donor organs. Although drug therapy has achieved great progress in improving symptoms, the prognosis of HF still remains poor [2]. A potential solution to the problem would be the use of gene therapy, and related strategies aimed at HF are under investigation and have great prospects, such as correcting gene mutations, manipulating pathologic excitation-contraction coupling, targeting cytoprotective factors, or inducing endogenous heart tissue regeneration.

3.2 Cardiac regeneration and nucleic acid therapy

Cardiac regeneration after myocardial injury includes broad strategies aimed at repairing irreversibly damaged heart tissue, which includes (1) Induction of CM proliferation by forced reentry into the cell cycle; (2) Direct reprogramming of non-CMs into CMs; (3) Strengthen the ability of endogenous cardiac progenitor cells to proliferate and differentiate; and (4) Delivery of cardiac exogenous progenitor cells or their derivatives. Although decades of efforts, regenerative therapies that attempted to restore failing heart function using un- or minimally modified somatic cell products (e.g., bone marrow mesenchymal stem cells) present very limited or controversial effects in clinical trials [3], most likely because these cells generally fail to differentiate or mature into functional CMs. Accumulating evidence suggests that endogenous myocyte regeneration may be accomplished through the delivery of coding or non-coding nucleic acids that induce and modulate tissue regeneration by changing cellular phenotype and tissue composition. To stimulate the proliferation of cardiomyocytes (CMs), cell cycle-regulated genes were delivered into CMs, such as CDK1, CDK4, Cyclin D, Cyclin B, which can re-initiate the cell cycle of CMs [4]; to reprogramming cardiac fibroblasts into functional CMs, cardiac-specific transcript factor genes, such as Gata4, Mef2c, Tbx5, and Myocd, were delivered into cardiac fibroblasts in scar

areas, which could perform transdifferentiation of fibroblasts to contractile myocytes [5, 6]; to protect CMs from an injury like myocardial infarction, protective microRNAs, such as microRNA199a [7], were transferred to CMs, which could help CMs survival or stimulate cardiac repair. In addition to transient or stable overexpression of individual genes in target cells, delivery of nucleic acids may enable the repair of defective genes using CRISPR/Cas [8], the suppression of protein expression via small inhibiting RNAs or small hairpin RNAs, or the interference with intracellular processes via microRNAs. Therefore, myocardial regeneration induced through the transfer of specific nucleic acids into target cells may have promising prospects. A prerequisite to all the above processes is the efficient delivery of nucleic acids into the live cell cytoplasm and/or nucleus of a majority of target cells.

3.3 Viral vectors as therapeutic tools

Viral vector-mediated nucleic acid therapy is a promising tool for cardiac regeneration [9, 10], hence has great potential in dealing with millions of patients suffering from end-stage HF [6, 11]. Nowadays, with the application of a wide range of viral and non-viral vectors, transduction efficiency has been largely improved. Common viral vectors include Lentiviruses, Adenoviruses, Retroviruses, and Adeno-associated viruses (AAVs), and non-viral vectors include messenger RNAs, polyplexes, plasmid DNAs, and lipoplexes, are important tools in gene therapy, and many clinical trials of nucleic acid therapy are ongoing. Traditional integrating or non-integrating viral transduction systems, such as Lentiviruses or Adenoviruses, respectively, tend to have high transduction efficiency. Lentivirus vectors infect via inserting RNAs and integrate DNAs into their host cells' genome without requiring cell replication. Adenovirus vectors would remain epichromosomal upon infection and provide short time gene expression without a requirement for cell replication. However, these vectors have unspecific tropism and harbor potential safety risks. More and more evidence suggests that AAVs may be highly potent gene delivery vehicles. AAVs can infect both mitotic and non-mitotic cells and mostly don't integrate into the host genome for stable expression, thus it remaining episomal upon transduction. They allow the efficient delivery and lasting expression of therapeutic nucleic acids in heart tissue, while not being associated with a strong immune response or human pathogenicity. Thus, given their safety profile, they can be considered safe for clinical use [12-14].

3.4 AAVs vectors for treating cardiac disease

AAVs could mediate energetic gene expression and target specific tissue. Over thirteen different AAV serotypes are known, of which, AAV1, 6, 8, and 9 are widely demonstrated to mediate efficient gene expression in CMs and proved cardiotropic in *in vivo* rodent animal models [12, 15-22], whereas, AAV2 only mediates low efficient gene expression [20]. In pig heart tissue, AAV6 is demonstrated to mediate a high gene transfer efficiency and superior to AAV8 and AAV9 [7]. In human embryonic stem cell- and induced pluripotent stem cell-derived CMs, AAV6 shows higher transfer efficiency over AAV1 and AAV9 [23]. In primary neonatal rat ventricular CMs and human induced pluripotent stem cell-derived CMs, AAV6 is superior to AAV1 and AAV9 [24]. However, some studies reported that AAV9 outperforms other serotypes in infecting CMs of mice or rats *in vivo* or *in vitro* [12, 25, 26], so the data were not uniform. Apart from studies with purified CM populations *in vitro*, a lot is already known about AAVs from *in vivo* experiments, such as tissue tropism, onset, and duration of gene expression kinetics for different serotypes, however, the cell-type tropism within heart tissue has not yet been investigated. For *in vivo* gene therapy, the serotypes with the most efficient transfer efficiency would be picked because a biologically relevant therapeutic effect can be achieved only if the maximum possible number of target cells are infected. The better the gene transfer efficiency is, the lesser viruses are required for treatment, which in turn has a beneficial impact on reducing cytotoxicity and minimizing side effects, thereby improving safety. In addition, serotypes with narrow cell tropism could minimize side effects when injected via intravascular. Thus, to successfully implement AAV-mediated gene therapy in clinics, more testing on the cell level to find an optimal AAV serotype for nucleic acid transfection as well as the development of suitable testing systems are urgently required [27].

3.5 Organotypic heart slice systems

Organotypic heart slices, represent mid-term systems between *in vitro* and *in vivo* models and are widely recognized as a powerful in situ platform for preclinical studies of gene therapy mediated regeneration as well as translational cardiovascular research. These multicellular slices retain native cardiac complex cellular and non-cellular structure, cardiac pathophysiology, and electrophysiology, making them excellent tools for studying heart cells. The slices can be generated from fresh heart tissue from a

wide range of sources, such as small and large animals, as well as diseased and healthy human biopsies [28].

It has been demonstrated that a slice thickness of 100–400 μm ensures the diffusion of sufficient oxygen, nutrients, and metabolic substrate when cultured in an air-liquid interface culture system. Organotypic heart slices could remain viable for up to 28 days [29], thus have an apparent advantage over ex vivo Langendorff perfused hearts, which could only preserve a few hours up to a day. This high throughput system of slices can apparently reduce the number of animals required for experiments because one heart can generate many slices, especially when the heart comes from a large mammal. Slices are superior to monolayer cell models because of their three-dimensional structure, and they are superior to animal models because they are independent of the complex circulating immune system. The absence of the circulating immune system is a double-edged sword, on the one hand, it could make experiment analysis simpler, and on the other hand, its presence would provide a more complete *in vivo* system. In addition, slices from human heart tissue can eliminate the effects of species differences.

In contrast to organotypic slice culture of brain and lung tissue, heart slice culture has rarely been used for gene therapy research although their utility has also been reported for small molecule screening. Thomas et al. [30] and Kang et al. [31] were the first to perform gene transfer experiments and found good transduction efficiency in cells of heart slices by adenoviruses with and without enzymatically degrading the extracellular matrix to allow for virus penetration. Further, Ou et al. [4] demonstrated that adenovirus-mediated cell cycle-related genes transfer into mouse organotypic heart slices could induce CM proliferation. Therefore, heart slices can be used to screen for suitable viral vectors and test the effects of viral vector-mediated transfer of different nucleic acids in heart regeneration therapy.

3.6 Hypothesis, aims, and the present study

For effective use of AAVs for treating cardiac disease, it is needed to know not only whether a given serotype infects cardiac tissue, but also to learn which cardiac cell types can be targeted to design customized therapies and to predict possible off-target transductions to determine the need for transcriptional and transduction targeting measures. This study aimed to establish a heart slice culture system, and to determine AAV1-, 2-, 6-, and 8-mediated gene transfer efficiency, cell-type tropism, and

cytotoxicity. The results will help to select suitable AAV serotypes for specific cardiac cell types under healthy conditions as well as under myocardial infarction-related changes and aid the clinical translation of novel therapies involving delivery of genes, microRNAs, lncRNAs, and circRNAs.

4. In-depth methods

In this part, the methods used in the present study are described in brief, for the detailed information of materials and explanation of methods, please refer to the methods section of the attached publication (see → *10. Publication "Comparative analysis of adeno-associated virus serotypes for gene transfer in organotypic heart slices"*).

4.1 Animals

Alpha-myosin heavy-chain αMHC-mCherry mice were ordered from Jackson Laboratory and were bred to homozygosity by the Research Facility for Experimental Medicine of the Charité – Universitätsmedizin Berlin. This homozygous transgenic mouse line has specific αMHC-driven mCherry expression in CMs, which allows to distinguish CM from other cardiac cells for further analysis. All animal-related procedures were carried out in accordance with the German Animal Welfare Act and the Charité Animal Welfare Guidelines.

4.2 Heart slice generation and culture

Mice aged 15-17 days were sacrificed by cervical dislocation after isoflurane anesthesia. After excising right ventricular, ventricular septal, and atrial tissue, left ventricular (LV) tissue was achieved and then fully flattened and embedded in a 4% agarose gel block on a tissue holder with endocardium up. A high-precision vibratome (HM 650 V, Microtome) equipped with a cooling unit (CU 65, Microm) was used to cut the LV tissue into organotypic heart slices at low temperature. In the cutting chamber, LV tissue was immersed in Tyrode cutting solution (140 mM NaCl, 6 mM KCl, 1 mM MgCl2, 1.8 mM CaCl2, 10 mM HEPES, 10 mM Glucose, 30 mM 2,3-Butanedione monoxime, pH 7.4) maintained at 4°C and saturated with 100% O_2. The cutting was parallel to the epicardial tangent plane, and parameters were set as trim 150 μm, cutting 100–300 μm thick, 0.08 mm/s advance speed, 1 mm vibration amplitude, 80 Hz frequency. After generation, slices were punched into uniform 4 mm discs by a biopsy punch and washed in Dulbecco's phosphate-buffered saline (DPBS) containing 3% penicillin/streptomycin (P/S). Finally, slices were transferred to porous transwell inserts placed in 6-well plates, and each well included 1.5 ml culture medium which consists of Medium 199, 1% P/S, and 1x insulin-transferrin-selenium. Slices were cultured in a

humidified incubator (37°C, 5% CO_2), and the O2 concentration was 20% in normoxia and 1% in hypoxia. Slices were washed on day 1 and the culture medium was changed on day 1 and day 3.

4.3 Analysis of CM viability

In the assessment of CM viability, slices were taken images by a fluorescence microscope, and the native mCherry fluorescence intensity was quantified by ImageJ. The viability of CMs was presented as a mean value of mCherry fluorescence intensity of the upper and lower slice surfaces and finally normalized to the value on day 0.

4.4 Calcein-acetoxymethyl ester staining of heart slices

The viability of cardiac cells of slices, including CMs, cardiac fibroblasts, and endothelial cells, was determined by a live-cell assay, which includes calcein-acetoxymethyl ester (calcein-AM) and Hoechst 33342. In brief, slices were transferred to a well of a 48-well plate with 200 µl culture medium containing 20 µM calcein-AM, after incubating for 15 min in a humidified incubator (37°C, 5% CO_2, 20% O_2), 20 µl Hoechst 33342 were added and mixed. After another 15 min of incubation, slices were washed with DPBS to remove the residual dye, then slices were observed and pictures were taken under a fluorescence microscope. The calcein fluorescence area was measured by the ImageJ software. The viability was assessed based on the percentage of the stained area to the entire slice area and finally normalized to the value on day 0.

4.5 Tetrazolium assay

In the study of the metabolic activity of freshly prepared or cultured organotypic heart slices, a tetrazolium compound and phenazine methosulfate were used. In detail, 20 ul of the freshly mixed solution of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) and the electron coupling reagent phenazine methosulfate (ratio, 20:1 v/v) were added to each well containing 100 µl culture medium and one slice in a 96-well plate. After incubating for 1.5 h in a humid incubator (37°C, 5% CO_2, 20% O_2), slices were respectively transferred to other wells with 30 µl dimethyl sulfoxide and incubated for 70 min for further extraction of formazan crystal. Finally, the two supernatants were mixed

thoroughly, and the intensity of formazan was measured based on the absorbance read at 490 nm by a plate reader.

4.6 AAV serotypes, infection of heart slices, and gene transfer analysis

Recombinant AAV vectors, including serotypes AAV1, AAV2, AAV6, and AAV8, directing green fluorescence protein (GFP) expression under the control of the cytomegalovirus early enhancer/chicken β actin (CAG) promoter were tested. These four hybrid AAV vectors were produced by cross-packaging AAV2 inverted terminal repeat genomes into capsid proteins of AAV serotypes 1, 2, 6, and 8. Three μl virus solution with a concentration of 1.33×10^{11} vector genomes per ml were added directly to slices. Afterward, plates with slices in inserts were transferred to a humidified incubator (37°C, 5% CO_2, 20% O_2). After 24 h, slices were washed in DPBS containing 1% P/S 3 times. The culture medium was changed on day 1 and day 3. On day 5, slices infected with AAV serotypes were observed from both the upper and lower slice sides and images were taken as mention above. For the test of AAV6-mediated GFP expression kinetics, slices incubated with either undiluted, 1:10, or 1:100 diluted virus solution were placed in the same Transwell insert, and images were taken on days 1, 3, and 5. GFP-positive cells on slices were manually counted.

4.7 Immunohistochemistry

Slices were fixed with 4% paraformaldehyde for 45 min and permeabilized and blocked in DPBS with 1% Triton X-100 and 1% bovine serum albumin for 120 min at room temperature (RT). Then, slices were incubated with 1:100 diluted mouse anti-cardiac Troponin T (cTNT) antibody or rabbit anti-vimentin antibody overnight at 4°C on a shaker. After washing, slices were incubated with 1:200 diluted Alexa Fluor 647-labeled donkey anti-mouse IgG or Alexa Fluor 647-labeled donkey anti-rabbit IgG for 2h at RT on a shaker, and aluminum foil was used to keep the incubation in the dark. The slices were washed and then stained with 300 nM DAPI for 30 min at RT on a shaker. Pictures of slices were taken by an Opera Phenix High-Content Screening System. The stack imaging procedure was produced with a setting parameter of 5 μm thickness per slice. In the quantification of cell-type-specific transduction, GFP-positive CMs or fibroblasts were counted manually while referred to the results of the upper and lower stack images. In the analysis of the mCherry expression in CMs, images from overlapping three consecutive images with a Z-stack thickness of 10 μm were used.

4.8 Statistical analysis

Data analysis was conducted by GraphPad Prism, and the one-way analysis of variance was used to study differences between groups. All data were presented as mean value ± standard deviation. A value of $p < 0.05$ was considered significant.

5. Essential results

In this part, the results of the present study are described in brief, for detailed information, please refer to the results section of the attached publication (see → *10. Publication "Comparative analysis of adeno-associated virus serotypes for gene transfer in organotypic heart slices"*).

5.1 Establishment of an organotypic heart slice culture model

Organotypic heart slices were prepared by a vibratome cutting machine, which can precisely cut LV tissue into slices with certain thicknesses. During the cutting process, maintaining a slow advancement speed of the blade can reduce the heart tissue tearing and displacement caused by the pushing of the blade, thereby generating smooth slices with a uniform thickness. However, excessively slowing down the speed would obviously increase the cutting time, which may weaken the viability of CMs. An advanced speed of approximately 0.08 mm/s was a relatively ideal cutting velocity for heart tissue. The endocardial layer was removed during the trimming process, and the epicardial layer was stuck and cannot detach with agarose block, therefore the heart slices consisted of the myocardial layer. Slices with glue residue on the surface were discarded because the glue on slices would prevent the penetration of nutrition and oxygen, which has an adverse effect on slices viability during culture. Generally, LV tissue from a mouse with an age of approximately 2 weeks could generate about two 300-µm-thin slices, or three 200-µm-thin slices, or six 100-µm-thin slices. Punching the slices in the center area with a 4-mm-diameter biopsy punch could remove incomplete edges and make all slices uniform to facilitate comparison in experiments. Finally, slices were cultured in an air-liquid interface culture system in a humid incubator. In general, heart slices from ventricular tissue of mice were feasible to prepare.

5.2 Expression of mCherry in CMs

CMs of slices generated above should have specific mCherry expression driven by αMHC promoter-enhancer because they derived from αMHC-mCherry transgenic mice. To validate it, I stained 300-µm-thin slices cultured 5 days for cTNT, a specific CM marker, and the three-layer reconstructed image showed that mCherry fluorescence exclusively presented in cTNT-positive cells, which indicated that mCherry specifically expressed in CMs and can indicate CMs.

5.3 mCherry fluorescence intensity and organotypic heart slice thickness

In order to determine whether mCherry fluorescence intensity linearly increases with the number of live CMs, I analyzed freshly prepared slices with thicknesses of 100, 200, and 300 μm. A significant increase in mCherry fluorescence intensity was observed with the increase of slice thicknesses from 100 μm to 300 μm. The 300-μm-thick slices exhibited a much higher value of mCherry fluorescence intensity than slices with thicknesses of 200 μm and 100 μm, which was because the thicker slices contained more CMs and therefore stronger fluorescence intensity was accumulated. In addition, this result was also consistent with the gradual increase of the formazan absorbance value of the MTS assay from thin to thick slices.

5.4 Viability of cultured organotypic heart slices

The viability of cultured heart slices was tested because good slice vitality is the prerequisite for subsequent experiments. Viable CMs present bright mCherry fluorescence because of the specific αMHC-mCherry transgene in CMs, and after staining with calcein-AM, only living cells present green calcein fluorescence. The area of calcein fluorescence of slices under normoxic conditions was reduced by approximately 13% on day 1, thereafter, there was almost no significant decrease until day 5. To test how O_2 influences the viability of slices, I studied slices cultured under hypoxic conditions, and the result showed the calcein fluorescence area of slices was reduced by almost 73% on day 1, and even no signal was detected on day 3. The value of mCherry fluorescence intensity of slices under normoxic conditions diminished approximately 27% on day 1, and remained stable in the following 4 days, whereas that of slices under hypoxic conditions diminished to approximately 31% on day 1, but the mCherry signal was not completely disappeared on day 3, which may be because of a strong background. Slices were dying fast when cultured under hypoxia thus can be a distinguished negative control. In sum, slices are highly viable for at least 5 days under normoxic culture based on calcein assays and native mCherry fluorescence intensity, therefore, they could be promising models to study AAV-mediated gene transfer into cardiac cells.

5.5 AAV1, 2, 6, and 8 infect organotypic heart slices

Slices were infected with AAV 1, 2, 6, and 8 on day 0, and GFP-positive cell numbers in slices were calculated on day 5 for the comparison of gene transfer efficiency of different serotypes. AAV9 was not tested because of non-availability during the period. Overall, GFP-positive cells were more on the upper slice surface. Heart slices infected with AAV6 showed the most GFP-positive cells number on both the upper and lower slice surfaces, whereas those infected with AAV1 and AAV8 showed less, and almost no GFP signal was observed in slices infected with AAV2. In addition, there were no significant differences between slices infected with different AAV serotypes and PBS in mCherry fluorescence intensity and formazan absorbance value of MTS assay (data not shown), therefore, these four AAV serotypes were nontoxic to CMs or total cells within the used dose range.

5.6 AAV6-mediated GFP expression kinetics in organotypic heart slices

Heart slices were respectively infected with three different AAV6 concentrations, undiluted, 1:10, or 1:100 diluted, and GFP-positive cell number was counted on days 1, 3, and 5. AAV6-mediated GFP expression was dose- and time-dependent. The number of GFP-positive cells was large in slices infected with the undiluted virus solution, whereas the number progressively decreased with increasing virus solution dilutions. GFP-positive cells were only observed on the upper surface of slices infected with the undiluted virus solution on day 1 and apparently increased on day 3 and day 5. Slices infected with diluted virus solutions presented much fewer GFP-positive cells, especially, on the lower slice surface. In addition, almost no GFP-positive cell was found on the lower surface of slices that received the 1:100 dilution.

5.7 AAV6 infects CMs and cardiac fibroblasts

I detected the cell tropism of AAV6 by comparing its transfer efficiency into CMs and cardiac fibroblasts because these two cell types accounted for the majority of cardiac cells. Cardiac fibroblasts in slices were identified by immunofluorescence staining of vimentin. Most of the GFP-positive cells were CMs, whereas only very few were fibroblasts. Cell type-specific quantification revealed that nearly 100% of analyzed CMs were GFP-positive, whereas only 12% of analyzed fibroblasts were GFP-positive. Thus, the AAV serotype 6 had a strong preference to transfer genes into CMs. Further, I studied the characteristics of AAV6-mediated GFP expression in fibroblasts by

comparing GFP-positive fibroblasts in CM dense and sparse areas. Although fibroblasts are much denser in CM sparse areas, however, no significant difference was found in the number of GFP-positive fibroblasts between these two areas. Therefore, the local heart tissue microenvironment does not affect AAV6-mediated poor gene transfer efficiency in fibroblasts.

6. Clinical application and outlook

6.1 Organotypic heart slices and clinical translation

Gene therapy needs more and better *in vitro* platforms to screen suitable gene delivery systems and test their nucleic acid transfer efficiency as well as therapeutic effects to accelerate its clinical translation. Of *in vitro* models, primary CMs isolated from adults are difficult to culture after isolation, and stem cell-derived CMs are not able to recapitulate cardiac cellular behavior in presence of the extracellular matrix environment and cannot reflect cellular behavior in chronic ischemic tissue, whereas human organotypic heart slices don't have above limitations and have an unprecedented research advantage in clinical translation, but related studies are progressing slowly, with most studies struggling to maximally maintain both viability and function [32, 33]. An important reason is heart tissue is very precious and not readily available. In addition, CMs are fragile and difficult to preserve, and complex structural and functional features, such as contractility and electrophysiological features, are difficult to maintain during long-term culture. A complete LV tissue biopsy can only be stored for an extremely short time, so repeated experiments need to continuously obtain fresh heart tissue and cut it into slices immediately. Since human myocardial tissue from large numbers of different individuals is required to build heart slices as a stable experimental model and to further optimize subsequent experiments, it is more common to first use a mouse heart slice culture model.

In this study, heart slices generated were viable for at least 5 days under normoxic culture conditions, although an initial drop in viability between days 0 and 1 was observed, and after this time point viability was maintained well. The viability of slices under hypoxic conditions was lost rapidly based on calcein staining and mCherry expression. The slices present good viability after culturing in the absence of electromechanically stimulating in this study is consistent with other studies [28, 31]. In the future, via optimizing the time of hypoxic culture and combining with glucose-deprived medium, slices under hypoxic culture can mimic an acute myocardial ischemia injury and thus could be an ideal platform to test the cardiac protective effect of transferred protein-encoding nucleic acids, microRNAs, and extracellular vesicles like exosomes. The formation of CM low-density area was because of damage during the preparation of slices, where cardiac fibroblasts apparently proliferated during culture, thus it may be a suitable model to test the anti-fibrosis effect of small molecule drugs, nuclear acids, proteins, and extracellular vesicles.

A limitation of this study is the lack of inclusion of mechanical loading and electrical stimulation in this culture model. These two factors are important for contractility and electrophysiology function maintenance and could apparently inhibit fibrosis of slices [32-34]. However, due to the technical difficulties involved in electromechanically stimulating, the hearts of young mice that are too small to fit into a standard device, and a large number of viral vectors are needed, it is currently not possible to test viral vectors by using a model that includes mechanical loading and electrical stimulation. To overcome this limitation, a custom device is required [4], which was not available at the time of research.

6.2 AAV6 and clinical translation

This study focused on screening AAV serotypes because vector-mediated nucleic acid therapy presents promising prospects in tissue regeneration. AAV6 presented the best tissue penetration ability on heart slices of the tested serotypes, and application of high concentrations of AAV directly to infect tissue could precisely model the way of injecting viruses into myocardium *in vivo* and observe the uptake of the viruses by cardiac cells. In addition, its penetration doesn't require enzymatically degraded extracellular matrix, in contrast to a study by Kang et al.[31]. However, its tissue penetration throughout an entire heart is still uncertain because the mean thickness of human LV is over one centimeter compared to approximately 1 mm of a murine heart slice [31]. Thus, the penetration test on slices is not enough to reflect the actual clinical situation. In addition, a concentration dependency GFP fluorescence signal decrease was observed on gene transfer with un-diluted, 1:10, and 1:100 dilutions, and no GFP signal was observed on the lower surface of slices infected with 1:100 dilution. The viruses were not considered cytotoxic based on mCherry fluorescence intensity and MTS assay. Consistent with a monolayer cell study [24], the serotype doses used in this project were not toxic to CMs or total cardiac cells of slices. Therefore, the four recombinant AAV serotypes constructed as a biosafety level 1 agent have scarcely potential toxicity, which will benefit future clinical application.

Of the four AAV serotypes, AAV6 presented the highest transfer efficiency in infecting heart slices. Although AAV1 and AAV6 can bind to the same primary receptor named N-linked sialic acid on the cell surface for cellular recognition [35], AAV6 outperformed AAV1 in infecting heart slice cells. These observations are consistent with Ambrosi et al. [24] and may be attributed to their difference in the secondary receptor, for instance,

the epidermal growth factor receptor is the secondary receptor of AAV6, but not AAV1. The low gene expression mediated by AAV2 was consistent with Palomeque et al. [20], possibly due to insufficient primary receptors on the surface of rodent CMs, such as heparan sulfate proteoglycans [36]. In addition, terminal N-linked galactose on the cell surface is the primary receptor of AAV9 [37], whereas the 37/67-kDa laminin receptor is an important receptor of AAV8 [38]. The difference in primary receptors on the cell surface may be the main reason for transfer efficiency variation among AAV serotypes. Therefore, the modification of the AAV serotypes receptor could improve the efficiency of targeting specific tissue to a certain extent. In addition, AAV6 can transfer genes into both CMs and cardiac fibroblasts but goes so preferentially into CMs indicated by the stronger GFP fluorescence in CM dense regions. Regions with low CMs are characteristics of ischemic injuries, and AAV6 transduction resulted in low GFP signal in these areas because CMs were dead. AVV6 would therefore be suitable to target CM to treat an acute ischemic injury but would be less suitable to chronic scenarios, where CMs are permanently lost and no CMs may be remained to be targeted by gene therapy. Then, another serotype targeting cardiac fibroblasts is required to reprogram scar fibroblasts into CM in chronic ischemic settings.

6.3 Future optimization and application of AAV6

Although AAV6 was a strong candidate for further evaluation as a vector for gene therapy in cardiac indications, lots of efforts in modifying promoters or/and capsid of AAV6 are still needed to limit its infection to CMs because there also other cells are infected, albeit with lower efficiency, and eliminate potential negative off-target effects [13, 17, 39]. In addition, AAV6 has only a small packaging capacity of approximately 4.7kb fragments, which should include the GFP gene, AAV's own inverted terminal repeat, promoter region, and RNA tailing signal. Therefore, the length of the therapeutic gene should be relatively small, and its future use may be limited to a single small gene, and also microRNAs. One possibility to circumvent this limitation would be to use a cocktail of AAV, with each AAV carrying a different gene, thereby allowing for co-transduction of multiple nucleic acids.

In the future, cardiac protective or regenerative related genes, microRNAs, lncRNAs, and circRNAs can be delivered via AAV6, testing their therapeutic effect on healthy or diseased organotypic slices. It is quite meaningful to establish disease-specific human organotypic heart slice culture models by using viable tissue from patients with HF and

other cardiovascular diseases, and test the feasibility of AAV6-mediated gene transfer into myocardial within an impaired tissue architecture, and observe changes in cell behavior and extracellular matrix composition. By constantly improving the therapeutic effect of AAV6-mediated nucleic acid transfer on human organotypic heart slices with different disease conditions, it is possible to significantly accelerate the clinical translation of regeneration strategies involving nucleic acid delivery.

In summary, I established a heart slice experimental model, which is superior to cell culture experiments as it allows to analyze the impact of different cell types, extracellular matrix, and tissue architectures. This model can help to identify a suitable vector for gene therapy *in vivo* for cardiac disease and can be used to finesse these vectors for future *in vivo* studies. I identified AAV6 as a potential vector for gene therapy of cardiac indications, which was superior to AAV 1, 2, and 8 in transferring genes to CMs in terms of overall expression and in terms of tissue penetration. In the future, it is possible to obviously accelerate clinical translation via testing the therapeutic effect of AAV6-mediated nucleic acids on slices derived from different diseased human heart tissue.

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8. Statutory Declaration

"I, Zihou Liu, by personally signing this document in lieu of an oath, hereby affirm that I prepared the submitted dissertation on the topic: "Testing the cell-specific in situ efficiency of nucleic acid transfer into heart tissue"/ "Untersuchung der zellspezifischen In-situ-Effizienz des Nukleinsäuretransfers in das Herzgewebe", independently and without the support of third parties, and that I used no other sources and aids than those stated.
All parts which are based on the publications or presentations of other authors, either in letter or in spirit, are specified as such in accordance with the citing guidelines. The sections on methodology (in particular regarding practical work, laboratory regulations, statistical processing) and results (in particular regarding figures, charts and tables) are exclusively my responsibility.

Furthermore, I declare that I have correctly marked all of the data, the analyses, and the conclusions generated from data obtained in collaboration with other persons, and that I have correctly marked my own contribution and the contributions of other persons (cf. declaration of contribution). I have correctly marked all texts or parts of texts that were generated in collaboration with other persons.

My contributions to any publications to this dissertation correspond to those stated in the below joint declaration made together with the supervisor. All publications created within the scope of the dissertation comply with the guidelines of the ICMJE (International Committee of Medical Journal Editors; www.icmje.org) on authorship. In addition, I declare that I shall comply with the regulations of Charité – Universitätsmedizin Berlin on ensuring good scientific practice.

I declare that I have not yet submitted this dissertation in identical or similar form to another Faculty.

The significance of this statutory declaration and the consequences of a false statutory declaration under criminal law (Sections 156, 161 of the German Criminal Code) are known to me."

Date   Signature

Declaration of your own contribution to the publications

Zihou Liu contributed the following to the below listed publication:

Publication 1: Zihou Liu, Kristin Klose, Sebastian Neuber, Meng Jiang, Manfred Gossen, Christof Stamm, Comparative analysis of adeno-associated virus serotypes for gene transfer in organotypic heart slices. Journal of translational medicine, 2020 Nov 18;18(1):437.

Contribution: I, Zihou Liu, contributed to the conception and design of this original research, most of the operation of experiments, most of the data acquisition, and all statistical analysis, including Figure 1, Figure 2a-c, Figure 3a-c, Figure 4a-b, Figure 5a-d, and Supplementary Figure 1 and Figure 2a-c. In addition, I independently drafted the manuscript and critically revised the manuscript. Kristin Klose helped to generate Figure 5a and Supplementary Figure 1, and she contributed to the design of the study and critical revision of the manuscript. Sebastian Neuber helped to generate Figure 1, and he contributed to the design of the study and critical revision of the manuscript. Meng Jiang helped to generate figure 5d, and she contributed to the design of the study and critical revision of the manuscript. Manfred Gossen contributed to the design of the study, supervising the project, and critical revision of the manuscript. Christof Stamm contributed to the conception and design of this study, supervising the project, providing funding, and critical revision of the manuscript.

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Signature, date and stamp of first supervising university professor / lecturer

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Signature of doctoral candidate

9. Journal Summary list "Medicine, Research and Experimental"

Journal Data Filtered By: **Selected JCR Year: 2018** Selected Editions: SCIE,SSCI
Selected Categories: **"MEDICINE, RESEARCH and EXPERIMENTAL"**
Selected Category Scheme: WoS
Gesamtanzahl: 136 Journale

Rank	Full Journal Title	Total Cites	Journal Impact Factor	Eigenfactor Score
1	NATURE MEDICINE	79,243	30.641	0.162840
2	Science Translational Medicine	30,485	17.161	0.121980
3	JOURNAL OF CLINICAL INVESTIGATION	108,879	12.282	0.139970
4	TRENDS IN MOLECULAR MEDICINE	9,946	11.028	0.018900
5	JOURNAL OF EXPERIMENTAL MEDICINE	63,983	10.892	0.071790
6	EMBO Molecular Medicine	7,507	10.624	0.025980
7	Annual Review of Medicine	6,068	10.091	0.009030
8	MOLECULAR THERAPY	16,991	8.402	0.030050
9	MOLECULAR ASPECTS OF MEDICINE	5,568	8.313	0.009020
10	Theranostics	8,769	8.063	0.020270
11	EBioMedicine	5,401	6.680	0.022310
12	ALTEX-Alternatives to Animal Experimentation	1,361	6.183	0.001920
13	Wiley Interdisciplinary Reviews-Nanomedicine and Nanobiotechnology	2,345	6.140	0.004130
14	JCI Insight	4,351	6.014	0.020440
15	Molecular Therapy-Nucleic Acids	3,189	5.919	0.010410
16	Molecular Therapy-Oncolytics	486	5.710	0.001990
17	Nanomedicine-Nanotechnology Biology and Medicine	10,131	5.570	0.014480
18	Cold Spring Harbor Perspectives in Medicine	6,223	5.564	0.016730
19	CLINICAL SCIENCE	10,951	5.237	0.014190
20	JOURNAL OF BIOMEDICAL SCIENCE	4,083	5.203	0.006300

Rank	Full Journal Title	Total Cites	Journal Impact Factor	Eigenfactor Score
21	npj Vaccines	282	5.020	0.001120
22	AMYLOID-JOURNAL OF PROTEIN FOLDING DISORDERS	1,335	4.919	0.003270
23	Translational Research	3,669	4.915	0.008530
24	Molecular Therapy-Methods & Clinical Development	1,078	4.875	0.004020
25	Vaccines	1,077	4.760	0.003910
26	JOURNAL OF MOLECULAR MEDICINE-JMM	7,195	4.746	0.010880
27	EXPERIMENTAL AND MOLECULAR MEDICINE	4,046	4.743	0.007380
28	Stem Cell Reviews and Reports	2,436	4.697	0.004690
29	CANCER GENE THERAPY	2,842	4.681	0.003200
30	EPMA Journal	815	4.661	0.001320
31	JOURNAL OF CELLULAR AND MOLECULAR MEDICINE	12,391	4.658	0.015760
32	Stem Cell Research & Therapy	6,132	4.627	0.015810
33	Cancer Biology & Medicine	1,043	4.467	0.003040
34	EXPERT REVIEWS IN MOLECULAR MEDICINE	1,758	4.407	0.001450
35	mAbs	4,415	4.405	0.011150
36	MOLECULAR PHARMACEUTICS	16,792	4.396	0.028020
37	CYTOTHERAPY	5,969	4.297	0.009690
38	JOURNAL OF INHERITED METABOLIC DISEASE	5,868	4.287	0.008410
39	PPAR Research	1,434	4.186	0.001600
40	ARCHIVES OF PATHOLOGY & LABORATORY MEDICINE	10,039	4.151	0.012620
41	Journal of Translational Medicine	10,831	4.098	0.022910
42	CTS-Clinical and Translational Science	1,351	3.989	0.003190

Selected JCR Year: 2018; Selected Categories: "MEDICINE, RESEARCH and EXPERIMENTAL"

10. Publication "Comparative analysis of adeno-associated virus serotypes for gene transfer in organotypic heart slices"

Liu et al. J Transl Med 2020, 18:437. https://doi.org/10.1186/s12967-020-02605-4

11. Curriculum Vitae

My curriculum vitae does not appear in the electronic version of my paper for reasons of data protection.

12. Complete list of publications

1. **Zihou Liu**, Kristin Klose, Sebastian Neuber, Meng Jiang, Manfred Gossen, Christof Stamm. Comparative analysis of adeno-associated virus serotypes for gene transfer in organotypic heart slices. Journal of transnational medicine. 2020 Nov 18;18(1):437. (Impact Factor 2019: 4.124)

2. **Zihou Liu**, Shunjun Wang, Yongqiang Wang, Ningbo Zhou, Jie Shu, Christof Stamm, Meng Jiang, Fanyan Luo. Association of epicardial adipose tissue attenuation with coronary atherosclerosis in patients with a high risk of coronary artery disease. Atherosclerosis. 2019 May; 284:230-236. (Impact Factor 2018: 4.255)

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