

**Aus dem Institut für Kardiogenetik
der Universität zu Lübeck
Direktor: Prof. Dr. Jeanette Erdmann**

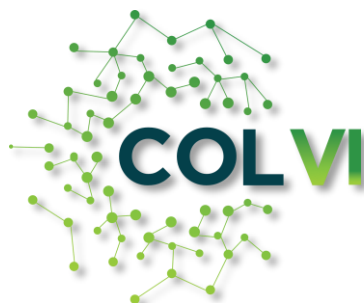
Allele-specific RNA-targeted Therapy as a Treatment Strategy for Collagen VI Congenital Muscular Dystrophy

Inauguraldissertation
zur
Erlangung der Doktorwürde
der Universität zu Lübeck
-Aus der Sektion Medizin-

vorgelegt von

Svenja Vishnolia
aus Rodalben

Lübeck 2021



1. Berichterstatter/Berichterstatterin: Prof. Dr. Jeanette Erdmann

2. Berichterstatter/Berichterstatterin:

Tag der mündlichen Prüfung:

Zum Druck genehmigt. Lübeck, den

Promotionskommission der Sektion Medizin

Table of Contents

1 Introduction	1
1.1 Collagen VI congenital muscular dystrophy	1
1.1.1 Clinical manifestation of Col6-CMD	2
1.2 The role of collagen VI in Col6-CMD	4
1.2.1 The collagen family	4
1.2.2 Collagen VI.....	6
1.2.3 Collagen VI in the pathogenesis of Col6-CMD	9
1.2.4 Diagnosis and therapeutic concepts of Col6-CMD.....	12
1.3 RNA-targeted therapy.....	13
1.3.1 Antisense oligonucleotides.....	13
1.3.2 RNA interference	15
1.3.3 Delivery of RNA targeted therapeutics	17
1.3.4. Clinical advancements in RNA-targeted therapies.....	19
2 Background and aim of this thesis.....	20
3 Materials and Methods	21
3.1 Materials	21
3.1.1 Chemicals and enzymes	21
3.1.2 Media, buffers and supplements	22
3.1.3 Cultured cells.....	23
3.1.4 Primers	23
3.1.5 Nucleic acids.....	24
3.1.6 Antibodies	24
3.1.7 Devices and consumables	25
3.1.8 Kits.....	26
3.1.9 Programs	26
3.2 Methods.....	27
3.2.1 Cell culture maintenance	27
3.2.2 Fibroblast collagen VI assay	28
3.2.3 Fibroblast immortalisation	29
3.2.4 Identification of SNPs on <i>COL6A2</i>	30
3.2.5 Design of siRNA and ASO.....	34
3.2.6 Quantification of mRNA knockdown by siRNA and ASO.....	35
3.2.7 RNA Sequencing	38

3.2.8 Human Cytokine Array	40
4 Results.....	41
4.1 Collagen VI production in primary fibroblasts	41
4.1.1 Effect of L-Ascorbic acid on collagen VI production	41
4.1.2 Effect of the mutation p.G283E on collagen VI production.....	42
4.2 Immortalized fibroblasts characterization.....	43
4.2.1 Immunofluorescence staining.....	43
4.2.2 Selection and analysis of markers expressed specifically in fibroblasts	44
4.3 siRNA and ASO design.....	46
4.3.1 SNP identification on <i>COL6A2</i>	46
4.3.2 Validation of the identified SNPs in Mut fibroblasts by nested PCR	46
4.3.3 Computational design	48
4.4. ASOs and siRNAs targeting at the mutation position	50
4.4.1 Allelic discrimination and target accessibility test.....	50
4.4.2 <i>COL6A2</i> mRNA knockdown using siCOL-1A	52
4.5 ASO and siRNA targeting SNPs on <i>COL6A2</i>	54
4.5.1 <i>COL6A2</i> mRNA knockdown	54
4.6 Collagen VI phenotype rescue	58
4.7 RNA sequencing	59
4.7.1 Differential gene expression (DGE) and gene enrichment analysis.....	59
4.7.2 Impact of <i>COL6A2</i> c.848G>A on network diffusion	62
4.7.3 Secretome analysis.....	63
4.7.4 Expression of circadian genes	65
4.8 Human Cytokine Array.....	66
5 Discussion	67
5.1 Functional effects of <i>COL6A2</i> c.848G>A	68
5.1.1 Effects on extracellular matrix of fibroblasts	68
5.1.2 Effects on different pathways and gene expression	70
5.1.3 Effects on inflammatory system	71
5.2 RNA-targeted therapy to correct the collagen VI phenotype <i>in vitro</i>	73
5.2.1. Conclusion and Outlook: Challenges and possibilities of gene therapy for Col6-CMD.....	76
Abstract	80
Zusammenfassung.....	81

Bibliography..... 82

List of Figures

Figure 1 Typical clinical features of BM.....	4
Figure 2 Supramolecular assemblies formed by collagens	6
Figure 3 Collagen VI domains.	8
Figure 4 Synthesis, secretion, and deposition of collagen VI.	9
Figure 5 Possible pathway how collagen VI deficiency causes muscle fibre death by altering mitochondrial function.....	11
Figure 6 ASO mechanisms of action to regulate gene expression.	14
Figure 7 RNAi mechanism.....	16
Figure 8 Structure of siRNA.	17
Figure 9 Oligonucleotide delivery systems.....	18
Figure 10 Molecular genetic analysis.	20
Figure 11 Effect of L-ascorbic acid on collagen VI ECM.....	41
Figure 12 Immunofluorescence staining of collagen VI in primary fibroblasts.....	42
Figure 13 Immunofluorescence staining of collagen VI in immortalized fibroblasts.....	43
Figure 14 Fibroblast specific marker gene expression.	45
Figure 15 Allele specific sequencing of the SNP positions in Mut fibroblasts.	47
Figure 16 Design of siRNAs and ASOs targeting c.848G>A on <i>COL6A2</i>	48
Figure 17 Design of siRNAs and ASOs targeting SNPs on <i>COL6A2</i>	49
Figure 18 Target accessibility test of the mutation site on <i>COL6A2</i>	50
Figure 19 Allelic discrimination of ASOs and siRNAs between wild type and mutated <i>COL6A2</i> allele.	51
Figure 20 Knockdown of <i>COL6A2</i> in Mut fibroblasts by siCOL-1A.	52
Figure 21 Knockdown of <i>COL6A2</i> in Mut fibroblasts by si-COL1A in extended dilution series.	53
Figure 22 Knockdown of <i>COL6A2</i> in immortalized fibroblasts Mut-imm. by siCOL-1A.	53
Figure 23 Target accessibility test of SNP1, SNP3 and SNP4 on <i>COL6A2</i>	54
Figure 24 Knockdown of <i>COL6A2</i> via SNP-targeting siRNAs in Mut-imm.	55
Figure 25 Knockdown of <i>COL6A2</i> via SNP-targeting ASOs in Mut-imm.	56
Figure 27 Phenotype rescue experiment with siCOL-1A.....	58
Figure 28 Network diffusion of mutated cells.....	62
Figure 29 Upregulated genes in secretome analysis.....	63
Figure 30 Downregulated genes in secretome analysis.....	64
Figure 31 Cell-specific expression pattern of circadian genes.	65
Figure 32 Results of the human cytokine array comparing wild type and mutated fibroblasts.....	66
Figure 33 Position of the pathogenic variant in <i>COL6A2</i> transcript and protein region.....	69

List of Tables

Table 1 Summarized are corresponding COL6 genes to each collagen VI chain, genetic position and protein size (adapted from Bönnemann et al., 2011).....	7
Table 2 Reagent mixture for primer test PCR.....	29
Table 3 Reagent mixture for the first reaction of the nested PCR.....	31
Table 4 Reagent mixture for the second reaction of the nested PCR.....	31
Table 5 Reagent mixture for the second reaction of the nested PCR.....	31
Table 6 PCR program steps 1 st run	32
Table 7 PCR program steps 2 nd run	32
Table 8 PCR program steps 2 nd run	33
Table 9 Gel electrophoresis samples: PCR products for extraction of bands	33
Table 10 Gel electrophoresis samples: PCR products for direct sequencing.....	33
Table 11 Reaction mix for Lipofectamine 2000 transfection	36
Table 12 Reaction mixture for reverse transcription	37
Table 13 Reaction mixture for real-time qPCR.....	37
Table 14 Programm steps for RT qPCR.....	37
Table 15 Content of each sample sent for RNA-sequencing.....	39
Table 16 Comparison scheme for RNA-sequencing analysis.....	40
Table 17 Identification of fibroblast specific marker genes.	44
Table 18 Results of variant calling on <i>COL6A2</i>	46
Table 19 Summary of results from siRNA and ASO tests.	57
Table 20 Differential gene expression analysis.	59
Table 21 Gene enrichment analysis in mutated fibroblasts, early passage.....	60
Table 22 Gene enrichment analysis in mutated fibroblasts, late passage.....	61

Abbreviations and Acronyms

AA	L-ascorbic acid
Ago2	Argonaute-2 protein
ASO	Antisense oligonucleotide
ATP	Adenosine triphosphate
BM	Bethlem Myopathy
Cas9	CRISPR associated protein 9
cDNA	Complementary DNA
Col6-CMD	Collagen VI congenital muscular dystrophy
CRISPR	Clustered regularly interspaced palindromic repeats
CRISPRi	CRISPR interference
crRNA	CRISPR RNA
DAPI	4',6-Diamidino-2-phenylindole
dCas9	Deactivated Cas9
DHA	Dehydroascorbic acid
DMD	Duchenne Muscular Dystrophy
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribose nucleoside triphosphate
DTT	Diethiothreitol
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic Acid
ETP	Endotrophin
FBS	Fetal bovine serum
GalNac	N-acetylgalactosamine
GAPDH	Glyceraldehyde 3-Phosphate Dehydrogenase
gRNA	Guide RNA
kDa	Kilo Dalton

KRAB	Krüppel-associated box
LNA	Locked nucleic acid
LNP	Lipid nanoparticle
M	Molar
miRNA	Micro ribonucleic acid
M-MLV-RT	Moloney Murine Leukemia Virus) Reverse Transcriptase
MO	2' O-methyl
MOE	2' O-methoxyethyl
mRNA	Messenger ribonucleic acid
Mut	Mutated
MW	Molecular weight
NFκB	Nuclear factor kappa B
nM	Nano molar
nm	Nano metre
OD	Optic density
PAA	Polyacrylamide
PBS	Phosphate-Buffered Saline
PCR	Polymerase chain reaction
PFA	Paraformaldehyde
PTO	Phosphorotioate oligonucleotide
PTP	Permeability transition pore
qPCR	Quantitative polymerase chain reaction
RES	Reticuloendothelial system
RISC	RNA induced silencing complex
RNA	Ribonucleic acid
RNAi	RNA interference
ROI	Reactive oxygen intermediates
siRNA	Small interfering ribonucleic acid
SNP	Single nucleotide polymorphism
SR	Sarcoplasmatic reticulum

TBE	Tris/Borate/EDTA-Buffer
TEMED	Tetramethylethylenediamine
TNA	Threoninol nucleic acid
tracrRNA	Trans-acting ribonucleic acid
TRBP	Transactivating response ribonucleic acid binding protein
UCMD	Ullrich Congenital Muscular Dystrophy
UNA	Unlocked nucleic acid
UTR	Untranslated region
WT	Wild type
α,β -CNA	α,β -constrained nucleic acid

1 Introduction

Collagen VI congenital muscular dystrophies (Col6-CMD) are rare hereditary muscle disorders caused by a genetic mutation in the genes encoding for collagen VI ¹. They encompass a broad clinical spectrum of muscular phenotypes related to dysfunctional collagen VI and, with a prevalence less than 10 out of 1 000 000 persons, are considered as rare disorders ^{2,3}. Range in severity varies from the so called Bethlem Myopathy (BM; OMIM: # 158810), the milder form, to the severe Ulrich Congenital Muscular Dystrophy (UCMD, OMIM: # 254090) ¹. Additionally, another phenotype, named myosclerosis, has also been associated with collagen VI ⁴.

The continuously increasing knowledge about molecular mechanism of gene expression and ambitious development of technologies allow decoding of the complete human genome in an efficient and precise way. This opened the door to new therapeutic strategies in modern medicine. Particularly rare diseases, which in the past suffered from deficit of scientific and medical knowledge, could be pinned down to their sources and new hope has arisen for so far “untreatable” conditions ^{5,6}. Statistics for so-called rare or “orphan” diseases, indicate that one out of 15 people is affected, thus, approximately 400 million people worldwide⁷. Scientific efforts must be stepped up to, at first, understand the diverse causes and, secondly, develop innovative ideas to advance therapeutic concepts. With revelation of basic underlying pathomechanisms, research focused on therapeutic approaches. Among those, a new and exciting strategy surfaced utilizing the latest scientific insights and technological progress to get down to the root of the disease and, theoretically, provide a cure. RNA-targeted oligonucleotides have been widely explored and used in basic scientific research, especially functional genomics, and in the last years discovered as a powerful tool to design new therapeutic strategies summarized by the term “RNA-targeted therapy” ⁸. In treatment of Col6-CMD RNA-targeted therapeutics figure prominently and most recent research efforts contributed to establishing them as promising treatment strategy and possible cure ^{9,10}.

1.1 Collagen VI congenital muscular dystrophy

Ullrich Congenital Muscular Dystrophy (UCMD) was first described in the 1930s by Otto Ullrich ⁹. Due to clinical features such as muscle weakness and joint hypermobility, which, in combination, appeared liked an atony in Ullrich’s original description and significant contractures imposing sclerotic, the condition was referred to as scleroatonic muscular dystrophy and treated as a distinct diagnostic entity ⁹. Bethlem Myopathy (BM), however, was identified in 1976 by Bethlem and van Wijngaarden in three families, in which 28 individuals showed symptoms of a myopathy with an autosomal dominant inheritance pattern ¹⁰. Several cases of the relatively benign, but still early-onset myopathy were then reported in the years afterwards ^{11–14} until in 1996 BM was linked the first time to mutations in collagen VI genes in families from Holland and USA ¹⁵. Finally, in 2002 Enrico Bertini and Mimma Pepe established the connection between both conditions, BM and

UCMD, because of the mutual clinical presentation and found the linkage of UCMD to collagen VI mutations, just like BM ¹. In the following years genetic analysis of patients presenting symptoms that comply with BM or UCMD as the causative disease were conducted and revealed dominant and recessive mutations in one of the three collagen VI genes *COL6A1*, *COL6A2* and *COL6A3* in the majority of patients ¹⁶. Since this discovery, BM and UCMD are summarized under the term collagen VI congenital muscular dystrophy (Col6-CMD). However, there is still a small number of cases, where no mutation could be detected, although the patients were showing distinctive clinical symptoms ^{17,18}. This circumstance indicates that there is a certain degree of genetic heterogeneity and a more complex aetiology than is known to date. Population studies performed in the northern England reported prevalence numbers of 0.13/100,000 for UCMD and 0.77/100,000 for BM classifying them both as rare diseases ³. Nevertheless, prevalence varies between populations and Col6-CMD is, according to studies carried out in Japan, one of the most common entities within the subcategory of congenital muscular dystrophy in Japanese population ¹⁹.

1.1.1 Clinical manifestation of Col6-CMD

Within the spectrum of Col6-CMD, covering Bethlem Myopathy and Ullrich Congenital Muscular Dystrophy as its two ends, along with a range of intermediately severe phenotypes, certain clinical symptoms are shared with varying degree of severity by all patients ^{1,20}. Particularly contractures and joint hypermobility among the mutual features and depending on severity defines to which part of the clinical spectrum a patient affiliate. However, the relative contribution and severity of these characteristics vary widely in individual patients even within one of the subgroups UCDM or BM ²⁰.

1.1.1.1 Ullrich Congenital Muscular Dystrophy

UCMD patients, who correspond to the severe end of the Col6-CMD spectrum, typically start showing significant symptoms already at birth or during the first year of life ⁹. These early-onset features include muscle hypotonia and weakness, impressing as a so called “floppy infant”, a striking hyperlaxity of distal joints associated with at the same time contractures and skeletal deformities in more proximal joints in approximately 50 % of the patients ^{9,16,21}. Muscle hypotonia sometimes presents already during pregnancy with reduced prenatal movements ²⁰. In distal extremities, such as hands, fingers and feet, joints are massively hyperlax and allow, for instance, the fingers to bend back onto the hand’s dorsum easily. Additionally, a prominent calcaneus is common in UCMD new-borns and remains as a characteristic sign of typical UCMD patients, although it can be seen in other congenital disorders associated with muscle hypotonia, too. Also, in half of the UCMD patients, a hip dislocation or dysplasia is found at birth ²⁰. On top of this, symptoms comprising torticollis, kyphoscoliosis and contractures of hips, knees and elbows can be existent at birth ¹. Giving a prognosis about the disease’s progress based on symptoms shown at birth is hardly possible due to very individual courses of disease. Severe contractures, for example, tend to improve from birth on over the first months of life, still,

new contractures manifest later on and can deteriorate the clinical picture again. As the patients grow older, usually, symptoms develop and progress in severity. The contracture aspect of UCMD more and more involves joints like elbows, hips and knees, as well as the spine, pectoralis muscle and long finger flexors. At the same time and independently from the progress of contractures, distal joint hyperlaxity persists and can be observed until very late stages of the disease in i.e. all interphalangeal joints²⁰. This combination of contracture and hyperlaxity leads to serious impairment of ambulation. The degree of impairment varies widely, and can, in most severe cases, never allow a child to achieve walking. The majority of UCMD patients, though, reach ambulatory ability at some point, but then, at the average of 10 years, lose it again due to progressive weakness and contractures especially in hips and knees. Scoliosis, developing out of kyphoscoliosis present at birth or independently during childhood and teenage years, also displays a serious problem in many UCMD patients and often calls for surgical intervention²². Since collagen VI is also a prominent component of skin tissue, the UCMD phenotype includes several dermatological symptoms²³. Extreme scar formation associated with large keloid formations occur in some patients and must be considered when planning surgical interventions. A rather purely diagnostic feature, since harmless but consistent in seriously affected UCMD patients, is the skin condition keratosis pilaris, which is mainly observed on the extensor side of the limbs²³.

Within the first 15 years of life, most of the UCMD patients develop a progressive respiratory insufficiency. The combination of chest wall stiffness and weakness of the diaphragm muscle and auxiliary respiratory muscles leads to restrictive lung disease with a strong measurable decline in forced vital capacity. Depending on the progress of respiratory insufficiency, night-time non-invasive ventilatory support becomes necessary for patients in second decade of life on average and detains respiratory aggravation for some years. In particularly severe cases, this supportive measure can be required already in early childhood, even before the ambulatory ability is lost. Timely initiation of ventilatory support is of life-crucial importance for respiratory insufficient patients, since otherwise respiratory failure leads to a patient's death in a short space of time²².

1.1.1.2 Bethlem Myopathy

Patients suffering from BM are affected to a much milder degree of severity in comparison to UCMD patients, still, the onset of BM can be congenital, too^{1,14-16}. Accordingly, symptoms like foot dorsiflexion and torticollis can be observed in early-onset BM, significant muscle hypotonia, however, has not been noted yet. During childhood patients may only display mild weakness combined with a certain degree of distal joint hyperlaxity. Then, as the children reach the end of the first decade of life, contractures increasingly set in, particularly on Achilles tendons and elbows (figure 1. A), long finger flexors (figure 1. B), interphalangeal joints and pectorales muscles. These contractures barely subside, on the contrary, they tend to progress, if not stay stable, and cause disabilities such as restricted hand function^{15,20}. In comparison to UCMD, spinal rigidity in BM is less severe. Muscle weakness, often predominantly affecting proximal muscles, remains mostly stable in childhood or even improves, however, in the third to fourth decade of life weakness slowly

starts to progress again. As a result, about two thirds of patients over the age of 60 years require walking aids.



Figure 1 Typical clinical features of BM. (A) Adult patient with BM and characteristic elbow and Achilles tendon contractures (B) finger flexor contractures (C) keloid formation (adapted from Bönemann et al., 2011)

In milder BM phenotypes the onset of symptoms can be delayed into childhood or young adulthood, in some cases families are not even aware of symptoms like mild weakness or mild contractures ¹⁴. Like UCMD, the progress of BM is potentially accompanied by restrictive pulmonary disease resulting in respiratory insufficiency and the need for night-time non-invasive ventilatory support. Still, respiratory symptoms typically develop to a much milder degree than in UCMD patients. Development of a skin phenotype, in particular keloid scar formation (figure 1, C), is similar to the one in UCMD ²⁴. In general, clinical features associated with BM vary widely in terms of severity, the contribution of either weakness or contractures to the overall clinical pattern and involvement of different anatomic regions. Some patients mainly suffer from proximal weakness and very few contractures presenting a clinical picture like a limb-girdle muscular dystrophy (LGMD), whereas others rather belong to a phenotype historically named myosclerosis, showing predominantly contractures with only mild weakness. Even within families carrying the same causative mutation, the clinical picture may be extremely diverse, which complicates and, consequently, often delays the diagnosis of Col6-CMD immensely ²⁰.

1.2 The role of collagen VI in Col6-CMD

1.2.1 The collagen family

Collagen VI ranks among the diverse family of collagens playing a substantive role in human's and animal's extracellular matrix, better known as connective tissues ²⁵. Collagens display a superfamily of structural proteins comprising 28 members to date ²⁶. With approximately 30% of total protein mass collagens are the most abundant proteins in mammals, present in a variety of tissues like ligaments, teeth or skin to provide mechanical stability and contribute to the molecular architecture and shape ^{26–28}. Members of this

superfamily are numbered with Roman numbers in vertebrates (I – XXVIII) and all share a common structural element, a triple helix domain, which constitutes a major (96 % in collagen I) or minor (less than 10 % in collagen XII) part of the respective collagen type and figures prominently into the structural and functional properties of collagens ^{25,26}. Nevertheless, apart from the common feature of a triple helix domain, there is no well-defined criteria if a protein counts to the collagen family or not. Deposited in the extracellular matrix, most of collagens undergo a supramolecular assembling process, which can differ even within a single collagen type and, combined with various molecular isoforms, alternative splicing or alternative promotor usage, therefore increases the level of biological diversity of collagen types ²⁶.

Collagens are composed of three polypeptide chains, called α chains, which come in variable number and forms per collagen type and vary widely in size from 662 in $\alpha 1(X)$ up to 3152 amino acids in $\alpha 3(VI)$. Accordingly, three α chains can either form homotrimeric monomers when identical (e.g. collagen II) or heterotrimeric monomers when different from each other (e.g. collagen VI) ^{26,28,29}. The primary structure of an α chain contains the repetitive motif Gly-X-Y, meaning the amino acid glycine is directly followed by two other amino acids X and Y, frequently proline and 4-hydroxyproline, respectively. Glycine residues are due to their small molecular size crucial amino acids to wind up triple helices and necessarily occur within this fixed pattern to ensure a proper helix formation ^{30,31}. Beside the triple helix domain representing a distinctive feature of all collagen types, collagens are multidomain proteins, with, depending on the type, various domains among which Fibronectin III, Kunitz, thrombospondin-1 and von Willebrand domains are found most abundant ^{26,32}. These additional domains participate in the structural assembling process and are frequently repeated within one collagen molecule ²⁶.

Based on their supramolecular assembling mechanism and shape, collagens can be subdivided into subgroups (figure 2), such as fibrils, beaded filaments, networks and anchoring fibrils. If fibrils contain more than one collagen type, which most of the fibrils do, they are called heterotypic fibrils. In the extracellular matrix, collagen fibrils align with other non-collagenous proteins or proteoglycans and especially fibronectin and integrins take part in the supramolecular organization of connective tissues ²⁶.

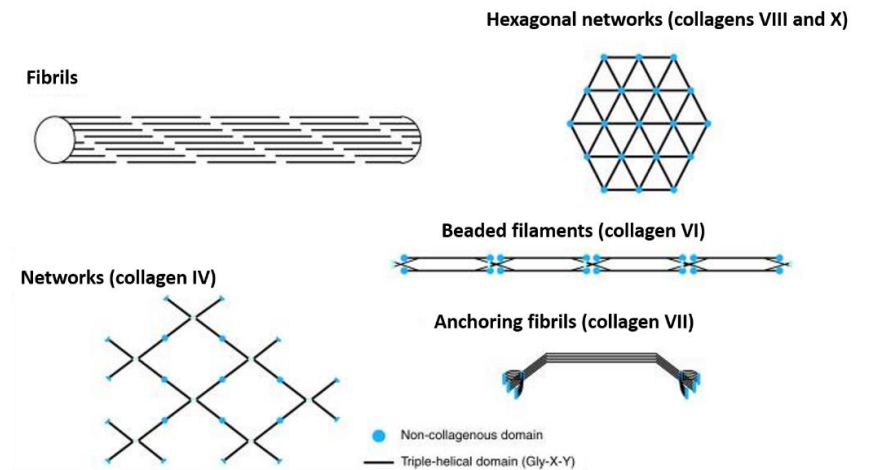


Figure 2 Supramolecular assemblies formed by collagens, such as fibrils (collagens I, II, III, V, XII, XXIV, XXVII), networks and hexagonal networks forming collagens (collagen IV and collagens VIII, X), beaded filaments (collagen VI) and anchoring fibrils (collagen VII; adapted from Ricard-Blum et al., 2011).

1.2.2 Collagen VI

Collagen VI is ubiquitously distributed in extracellular matrices of various tissues including skin, cartilage cornea and smooth as well as skeletal muscle. It is predominantly produced in skeletal muscle and deposited by interstitial fibroblasts. Collagen VI represents a prominent component of extracellular matrix acting as a link between cells and several constituents of the basal lamina, such as collagen I and IV, and the proteoglycans biglycan and decorin, and integrins (see figure 4) ^{33,34}. Additionally, absence of collagen VI has been proven to affect fibronectin deposition ³⁵. Apart from its anchoring mechanism, functions of collagen VI also include participation in cell differentiation, adhesion and migration and suppression of apoptosis ^{36–40}.

Collagen VI belongs to the subfamily of non-fibrillar collagens forming a network of beaded microfibrils in the ECM and undergoes a complex assembly process ⁴¹. Originally, there were three genetically different α chains identified (table 1), annotated as $\alpha 1(\text{VI})$, $\alpha 2(\text{VI})$ and $\alpha 3(\text{VI})$. Respectively, they are encoded by the *COL6A1*, *COL6A2* and *COL6A3* genes in humans, whereas *COL6A1* and *COL6A2* are located in tandem, likely arose from gene duplication on chromosome 21q22.3 and *COL6A3* maps to chromosome 2q37 ¹⁶.

Table 1 Summarized are corresponding COL6 genes to each collagen VI chain, genetic position and protein size (adapted from Bönemann et al., 2011).

α chains	size (kDa)	corresponding gene	gene position
$\alpha 1(VI)$	140	<i>COL6A1</i>	21q22.3
$\alpha 2(VI)$	140	<i>COL6A2</i>	21q22.3
$\alpha 3(VI)$	260-330*	<i>COL6A3</i>	2q37

**variable size depending on glycolysation state*

However, genomic database mining has recently enabled the identification of three additional genes, which encode for collagen VI α chains⁴². Due to the sequence homology and shared domain structure with $\alpha 3(VI)$, they were named $\alpha 4(VI)$, $\alpha 5(VI)$ and $\alpha 6(VI)$. The new chains are encoded by *COL6A4*, *COL6A5* and *COL6A6* at a single locus on chromosome 3q22.1. Although these additional chains double the collagen VI family and complex the role of collagen VI in the extracellular matrix, they do not participate in the heterotrimeric monomer construction of collagen VI. *COL6A4* in particular has been disrupted by a chromosome break in humans and is no longer functional, whereas $\alpha 5(VI)$ and $\alpha 6(VI)$, although genetically expressed in humans, do not assemble with $\alpha 1(VI)$ or $\alpha 2(VI)$ to form a heterotrimeric monomer^{42,43}.

The major three α chains, $\alpha 1(VI)$, $\alpha 2(VI)$ and $\alpha 3(VI)$, are multidomain proteins (Figure 3). As it is characteristic for collagen molecules, also collagen VI chains contain a triple-helix-domain. Specifically, in collagen VI they are relatively short with 335-336 amino acid residues, which are flanked by N- and C-terminal globular domains displaying similarity to the von Willebrand factor type A module (vWF-A). The $\alpha 1(VI)$ and $\alpha 2(VI)$ chains both comprise one N-terminal and two C-terminal vWF-A domains, N1, and C1 and C2, respectively⁴⁴. Two splice forms are generated of the *COL6A2* C2 domain additionally to the predominantly found full-length version, referred to as $\alpha 2C2$, $\alpha 2C2a$ and $\alpha 2C2a'$. Conversely, $\alpha 3(VI)$ is much bigger in size and contains a large N-terminal domain (N1-N10), mainly consisting of vWF-A modules. These domains are extensively subjected to alternative splicing, the regulation and physiological function of the resulting splice isoforms is unknown⁴⁵. C-terminal two vWF-A domains (C1 and C2) are located after the unique regions (C3-C5); where C3 domain contains a lysine and proline rich sequence, C4 is a fibronectin type III domain and C5 a Kunitz protease inhibitor motif. Within the triple helix domain of each α chain cysteine residues are found next to the N-terminal end and involved in higher-order assembly³⁴.



Figure 3 Collagen VI domains. Depicted are all to date known collagen VI chains and their structural domains (created with Biorender.com by J. Erdmann).

Synthesis of collagen VI molecules is a complex multi-step assembly process taking place inside and outside the cell. Initially, the basic structure of a collagen molecule, the triple-helix-monomer, is formed. Necessary to that end is the combination of all three primary α chains to heterotrimeric monomer (≈ 500 kDa) in a 1:1:1 stoichiometric ratio^{46–50}. Two of these monomers assemble into disulfide-bonded antiparallel dimers (≈ 1000 kDa), which subsequently associate to build large tetramers (≈ 2000 kDa), again stabilized by disulfide bonds¹⁶. Finally, these tetramers are secreted into extracellular matrix and align end-to-end through non-covalent bonds to form microfibrils, which appear in a characteristic beaded shape with a typical 105 nm periodicity and a diameter of 4,5 nm^{51–53}. These beaded collagen VI microfilaments are deposited into extracellular matrix, where they form an extended network and fulfil their function of bridging cells with the interstitial connective tissue. Accordingly, collagen VI displays a specifically pericellular distribution especially around tendon cells and a location related affinity to basement membranes. Thus, collagen VI is in direct vicinity of basal lamina constituents such as perlecan, laminin and collagen IV (see figure 4)^{20,25}.

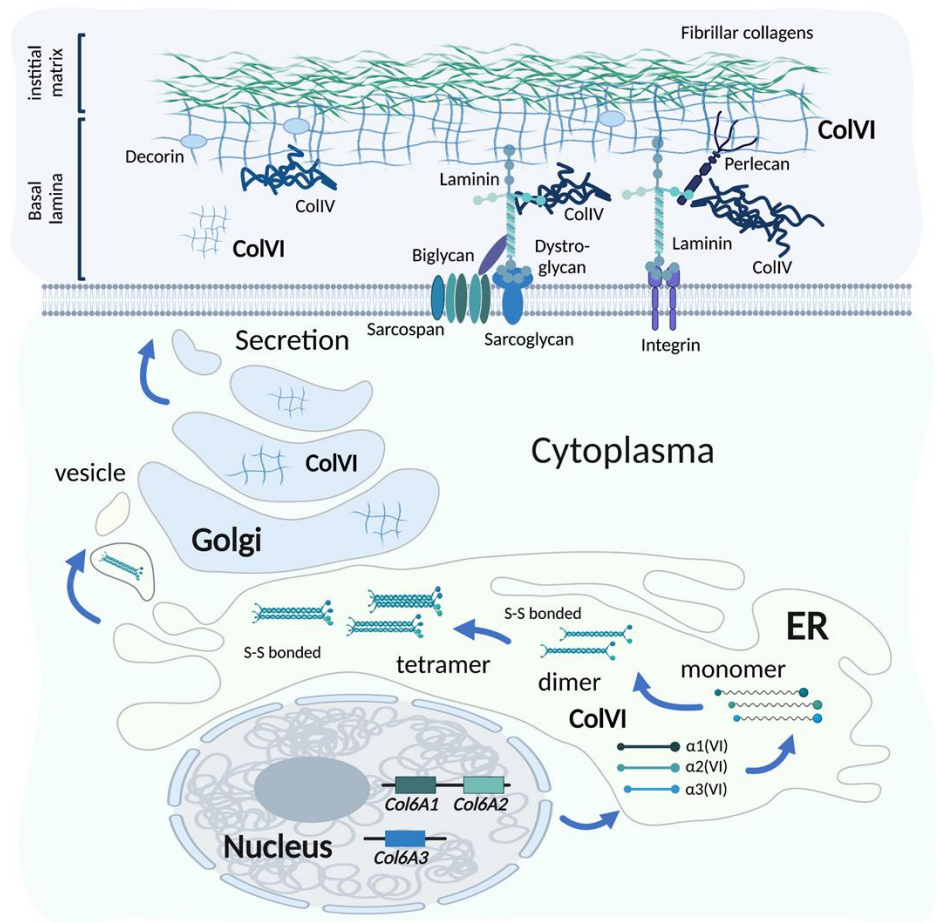


Figure 4 Synthesis, secretion, and deposition of collagen VI. The figure summarizes and visualizes the complex production process of collagen VI and its role in ECM. Each COL6 gene translates to its corresponding collagen VI chain. After assembly of these chains, collagen VI tetramers are secreted to ECM, where they align to beaded collagen VI microfilaments. Here collagen VI is in close vicinity to the basal lamina and provides a link between constituents of the basal lamina and the interstitial matrix (created with Biorender.com by J. Erdmann).

1.2.3 Collagen VI in the pathogenesis of Col6-CMD

All phenotypes within the group of Col6-CMD share a common cause: a mutation in one of the three genes encoding for the three collagen VI α chains. The mutational spectrum is broad and comprises recessive as well as dominant mutations ¹⁶. Based on knowledge about the functions of collagen VI, pathogenic defects and mechanisms are suggested, partly proved in experiments ^{39,54–56}, but in a large part still unknown.

1.2.3.1 Structural changes in muscle

First mechanical measurements on isolated muscle tissue were obtained from a mouse model of collagen VI deficiency through inactivation of *Col6a1* gene, showed that these *Col6a1*^{-/-} muscles develop less tension compared to wild type muscle tissue and present with a significant loss of contractile strength ³⁹. Skinned fibres are of irregular shape and look partially damaged in microscopic images, whereas this phenotype cannot be found in wild type muscle fibres ³⁹. In muscle biopsies of patients suffering from Col6-CMD, a range

of histological changes can be observed, varying in severity according to the clinical phenotype⁵⁴. The changes include variations in muscle fibre size affecting slow (type 1) as well as fast (type 2) fibres, an overall type 1 fibre predominance, increased endomysial connective and adipose tissue, increased amount of internal nuclei and mild necrotic processes in fibres⁵⁴. Electron microscopy on patient biopsies and experiments in cell culture were able to link mutations in collagen VI genes to a reduced connection between basement membrane and extracellular matrix, one of the main functions of collagen VI in interstitial matrix⁵⁵. Double immunostaining of collagen VI and collagen IV, a constituent of basement membranes, in muscle biopsies from UCMD patients revealed, that collagen VI is found in the interstitium, but not adjacent to the sarcolemma. This, “sarcolemma specific collagen VI deficiency” is most likely caused by an impaired connection between collagen VI microfibrils in the interstitial space and the basal lamina⁵⁶. Correspondingly, in Col6-CMD affected muscle tissue from biopsies, some extra space between muscle fibres and connective tissue can be found, where these structures normally are tightly attached displaying the anchoring function of collagen VI to be impaired^{55,56}.

1.2.3.2 Mitochondrial pathogenesis and defective autophagy

Further work with collagen VI deficient mice brought a new, unexpected pathomechanism to light. Ultrastructural examination of skeletal muscle obtained from these *Col6a1*^{-/-} mice compared to wild-type muscle samples revealed 20-30 % of these fibres having morphological abnormalities in mitochondria, which appeared swelled, partly fragmented and contained matrix inclusions³⁹. Myofibres affected by these mitochondrial alterations also presented with nuclear features of apoptosis, indicating a causative link between the organelle changes and increased apoptosis activity³⁹. The resting mitochondrial membrane potential remained unaffected by collagen VI deficiency but mitochondrial depolarization was observed after adding oligomycin, a F1F0 ATP synthase inhibitor and also after addition of rotenone, a respiratory chain complex I inhibitor⁵⁷. Membrane depolarization as a response to adding either of these substances is abnormal since inhibition of ATP synthesis by oligomycin should cause hyperpolarization and inhibition of respiratory chain should still enable the membrane potential to stay balanced and not depolarize. The cause of this mitochondrial behaviour is a dysfunction of the mitochondrial permeability transition pore (PTP), a protein located in the inner membrane of mitochondria, increasing mitochondrial membrane permeability³⁹. The dysfunctional protracted opening mechanism of PTP is mediated by cyclophilin D, a matrix protein sensitizing the pore to opening, and could be corrected by adding either wild-type collagen VI or ciclosporin A, an inhibitor of cyclophilin D³⁹. By creating collagen VI cyclophilin D double knock-out mice and comparing their phenotype to collagen VI null mice, the central role of cyclophilin D in this part of the disorder's pathogenesis could be reaffirmed. The experiments showed that the previously described correction of PTP by ciclosporin A and wild-type collagen VI, as well as the knock-out of cyclophilin D led to decreased apoptosis and were therefore able to rescue the mitochondrial phenotype⁵⁸. However, not only in mice, also in human cell culture or biopsy samples obtained from patients carrying collagen VI mutations, increased apoptosis due to dysfunctional PTP opening have been

demonstrated (figure 5), reinforcing an apoptotic mechanism as a significant contributor to skeletal muscle fibre death in Col6-CMD^{59,60}.

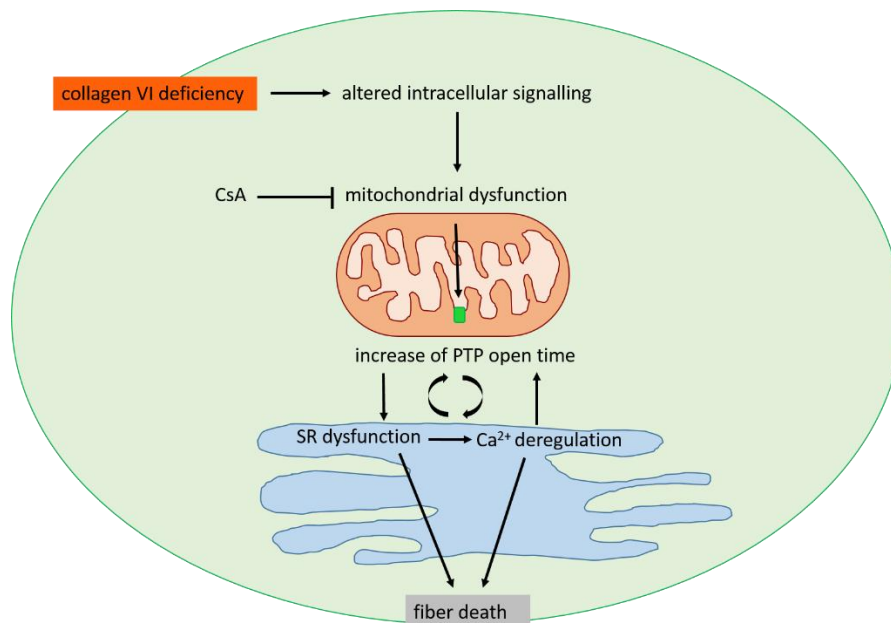


Figure 5 Possible pathway how collagen VI deficiency causes muscle fibre death by altering mitochondrial function. Collagen VI deficiency alters intracellular signalling pathways and influences mitochondria. This leads to increased transient opening time of PTP and abnormal Ca²⁺ regulation in the SR, which further increases PTP open time and, finally, leads to a vicious cycle between SR and mitochondria that eventually causes fibre death.

The detection of morphological abnormalities like swelling or fragmentation in the mitochondria and abnormal changes in the sarcoplasmic reticulum led in further studies to the examination of the autophagic system, which, if intact, should prevent the cell from accumulating defective organelles. Indeed, *Col6a1*^{-/-} muscle samples contain fewer amounts of autophagosomes in comparison to wild type muscle tissue⁶¹. This was proven to be a result of inefficient autophagy induction in *Col6a1*^{-/-} muscle as a response to a physiological autophagic stimulus by showing reduced levels of beclin-1 and Bnip3, two key proteins responsible for triggering autophagy⁶¹. As proof of principle, forced activation of autophagy by e.g., nutritional restriction led to increased formation of autophagosomes and the removal of abnormal organelles intracellularly. Additionally, autophagy reactivation ameliorated histological abnormalities in collagen VI deficient muscles such as leading to more consistently sized myofibres and, in long term, was able to improve muscle strength in *Col6a1*^{-/-} mice⁶¹. These findings unravelled the link between the previously described mitochondrial phenotype and muscle fibre apoptosis and revealed the mitochondrial dysfunction in combination with inefficient autophagy to play a key role in the pathogenesis of the condition's muscular phenotype.

1.2.4 Diagnosis and therapeutic concepts of Col6-CMD

1.2.4.1 Diagnosis

The first and, regarding the rarity of the disease, probably most challenging step in the establishment of a diagnosis is the recognition of the muscular phenotype pattern which leads to differential diagnoses that include Col6-CMD. Due to large phenotypical overlapping the most important differential diagnosis to consider among others are Emery–Dreifuss muscular dystrophies (EDMDs), which are caused by mutations in either emerin gene (*EMD*) for the X-linked form or lamin A/C gene (*LMNA*) for the autosomal form^{62,63}. Muscle imaging, for instance magnetic resonance imaging, is a possibly helpful method to distinguish between different patterns of (neuro-) muscular conditions and can unveil a typical picture with fatty and connective tissue replacement of muscle in patients affected by Col6-CM. However, this degenerative process does not appear in all patients or only temporarily in not yet too far progressed stages^{64–66}. In progressed stages muscle biopsies can be useful to establish the diagnosis as myofibre degeneration becomes more prominent with increasing age⁶⁷. When Col6-CMD is suspected, analysing dermal fibroblasts regarding their collagen VI production can provide strong hints pointing to collagen VI as being involved into the pathogenesis of the patient's condition⁶⁸. Dependent on the severity of the disease, collagen VI can be strongly reduced to completely absent in UCMD or rather slightly affected in BM^{67–69}. As a final and crucial step in diagnosis, sequencing all three collagen VI exons and mutational analysis can reveal causative mutations Col6-CMD. It is important to consider, that, since there are many not yet fully categorized polymorphisms in the *COL6* genes, the determination of a mutation to be disease associated or not may be hard. In these cases, mutational analysis of family members is highly useful to decide whether the genetic alteration may be disease causing or not⁷⁰.

1.2.4.2 Therapeutic concepts

To date, curative therapy for these diseases is not available and patients can only receive symptomatic treatment and supportive measures. Medical interventions during childhood usually address infections and respiratory failure and orthopaedic problems occurring due to contractures or spinal deformations²⁰. However, conservative and non-operative solutions are preferred over invasive and surgical interventions, since they, considering the continuous changes in a growing and developing child, better the long-term outcome when aimed at prevention. Consequent and regular stretching, for instance, can slow down the progression of contractures, although it can rarely completely be stopped. Surgically released contractures, on the other side, often tend to recur. Still, operative interventions, in the Achilles tendon to preserve ambulation in patients with BM, can be used to improve the quality of life. Notably, respiratory monitoring and the institution of respiratory support, which mostly includes non-invasive ventilatory support like bi level positive airway pressure, is of utmost importance as respiratory insufficiency progresses in patients with Col6-CMD and can be life threatening if not addressed in time^{20,22,71}.

1.3 RNA-targeted therapy

The term RNA-targeted therapy represents a variety of approaches in drug discovery using oligonucleotides to regulate the expression of a gene by targeting its messenger-RNA (mRNA). The purpose of these methods is to develop therapeutic agents, which transiently interfere in gene expression without the definite effect of a genomic correction on DNA level. First steps towards the utilization of RNA therapeutics have been taken 1978 ⁷², since the basic underlying mechanism, Watson-Crick hybridization, has been well understood and such drugs, at least in theory, promised to act more specific when compared to traditional small-molecule drugs. After more than 30 years of continuous development today's RNA therapeutics have reached not only clinical trials but also approval for commercial use, as will be explained in more detail below ⁸.

RNA therapeutics can affect the expression of a gene in many ways, whereas their usage to downregulate gene expression has been among the earliest and since then most important applications. For gene expression, a cell must undergo several steps starting from the chromosomal information coded as deoxyribonucleic acid (DNA) and ending in a functional protein consisting of amino acids. A crucial part in this process plays the transcription of double-stranded DNA to single-stranded mRNA, which subsequently leaves the cell nucleus and further gets translated to amino acids. This step is known to be carefully surveilled by the cellular machinery that checks transcribed mRNA molecules for defects in processing or folding and allows to degrade the respective mRNA. RNA degradation, as not only mRNA but all types of RNA can be degraded, comprises a plethora of different RNA degradation pathways which, despite their complexity, show substantial similarities between bacteria, archaea and eukaryotes proving their long-conserved and fundamental importance among all organisms ⁷³. RNA therapeutics have been developed to use this cellular surveillance machinery to sequence dependently target a specific mRNA and initiate its degradation. Since RNA degradation works in several pathways, RNA therapeutics too have been designed to interfere with different RNA degradation mechanisms. Two major accomplishments have been the development of antisense technology using antisense oligonucleotides (ASO) and RNA interference (RNAi) using small interfering RNA (siRNA). Both technologies allow to target and degrade a specific mRNA and therefore, interrupt and inhibit protein expression of a certain gene. For genetic diseases caused by defective proteins, RNA therapy can be a powerful tool to provide long term disease control and improve or even reverse clinical symptoms.

1.3.1 Antisense oligonucleotides

Antisense oligonucleotides (ASO) are single-stranded nucleic acids ranging from 18 to 30 base pairs in length and bind via Watson-Crick hybridization complementary to their target mRNA^{72,74}. Mechanisms to modify the processing of a specific mRNA (figure 6) depend on the ASO chemistry and the targeted mRNA sequence and include splice alteration, mRNA degradation by RNase H and inhibition or enhancement of translation ⁷⁴.

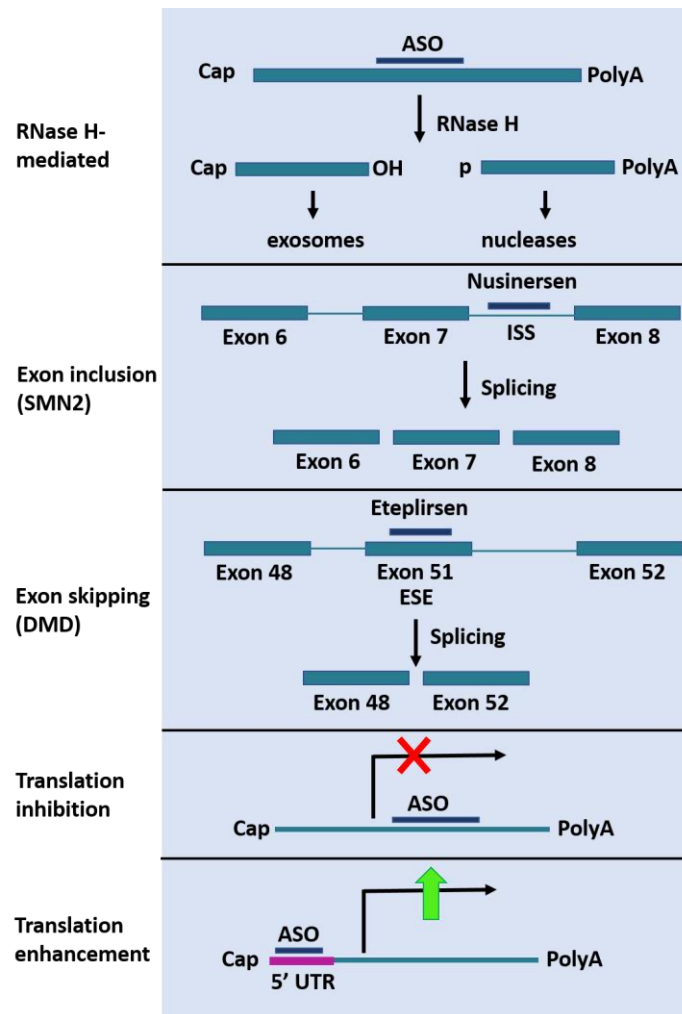


Figure 6 ASO mechanisms of action to regulate gene expression. DMD = Duchenne Muscular Dystrophy; ESE = exonic splicing enhancer; ISS = intronic splicing silencer; SMN = survival motor neuron (adapted from Scoles et al., 2019).

For gene silencing via RNase H- mediated mRNA degradation, the ASO binds to the local mRNA target sequence and activates RNase H, a ubiquitously existent cellular enzyme, that recognizes and cleaves DNA-RNA hybrids and subsequently leads to sequence specific depletion of the target mRNA (figure 6). Splice altering by ASOs targeting sequences in pre-mRNA may inhibit or enhance usage of splice sites. Nusinersen and Eteplirsen are two commercially used ASOs, which alter splicing for the treatment of Spinal Muscular Atrophy and Duchenne Muscular Dystrophy, respectively (figure 6). At last, translation modulation by ASOs either to inhibit translation by steric blocking or to enhance translation by targeting 5'UTR inhibitory elements (figure 6) that lead to recruitment of translation factors

75.

Natural nucleic acids are not suitable to use as *in vivo* drugs due to their susceptibility to nuclease degradation and therefore short half-life period in biological fluids such as blood, as well as weak protein binding that causes poor tissue uptake. To address these challenges, multiple modifications have been developed to improve their properties, which can be roughly categorized into three generations. First generation modifications address the alteration of the ASO backbone and include phosphorothioate modifications (PTOs),

which results in a higher resistance to nuclease degradation and increased protein binding while at the same time allowing RNase H cleavage. Concomitant with an increase of protein binding has been a higher unspecific binding affinity to proteins, which is why more modifications had to be developed. Second generation ASOs contain modified sugars and include the 2' O-methoxyethyl modification (MOE) which reduces unspecific protein binding and improves binding affinity to the target mRNA. Because MOE-ASOs failed to activate RNase H degradation, therefore gapmers were invented which provided a solution to this problem. Gapmers contain a tract of unmodified deoxyribonucleotides that support RNase H cleavage and is flanked by modified nucleotides to protect the ASO from nuclease degradation. ASOs of third generation contain unnatural bases such as so-called locked nucleic acids (LNA) that can also be incorporated into gapmers and both increase target specificity as well as decrease nuclease recognition while at the same time allowing RNase H activity^{74,76}. During development of therapeutic ASOs, a crucial step is maximization of mRNA target specificity and minimization of off-target effects. Accidental inhibition of gene expression can lead to numerous side effects and cytotoxicity. Quantitative polymerase chain reaction (qPCR) can assess expression of mRNA containing target sequences including mismatches to the ASOs and ensure specificity^{77,78}. Also, comparative transcriptome analysis of tissues before and after ASO treatment can mark differences in gene expression and reveal off-target effects⁷⁹.

1.3.2 RNA interference

RNA interference (RNAi) is a natural process to regulate gene expression based on the utilization of small double-stranded RNA to sequence dependently degrade a targeted RNA. Natural functions of this mechanism are most likely protection against viruses or transposons, whereas in eukaryotes they also facilitate developmental programs. Based on previous observation, that RNA can have antisense effects on genes, the RNAi mechanism has been discovered in 1998, when Fire and colleagues injected sense and antisense RNA at the same time into *C. elegans* and observed that due to double strand formation this combination of RNA led to a higher efficiency of selective gene silencing than when injected individually⁸⁰. The process of RNAi (figure 7) can be divided in two main steps. In the RNAi initiating step, a four domain endonuclease from the RNase III family named Dicer⁸¹ binds and cleaves a large double stranded RNA into RNA fragments of 21-25 nucleotides length with 2 nucleotides 3'-overhangs. These double stranded RNA fragments, referred as small interfering RNA (siRNA), consist of an antisense guide strand and a sense passenger strand. In the second step, the siRNA combines with Argonaute-2 (Ago2) protein, Dicer and Transactivating response RNA binding protein (TRBP) to form the RISC (RNA induced silencing complex), which catalyses the degradation of a complementary target mRNA⁸²

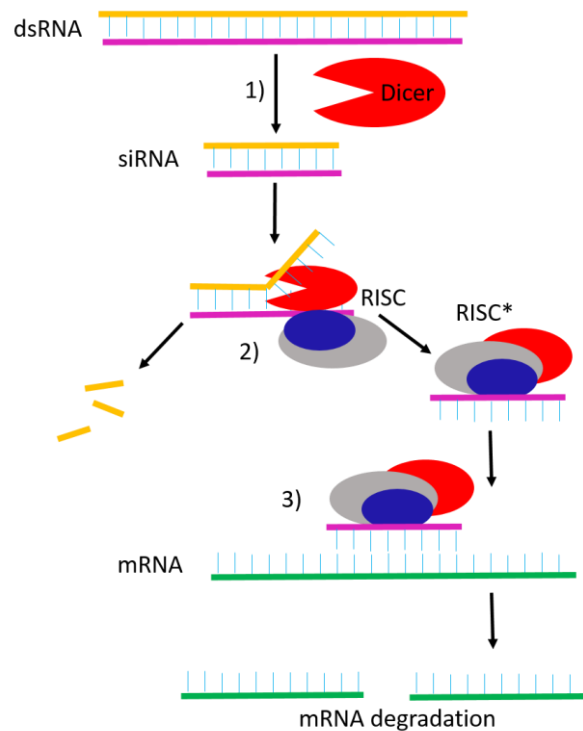


Figure 7 RNAi mechanism. 1) In the initiating step the RNase III type endonuclease Dicer cuts the dsRNA and leaves a 3' overhang of 2 nucleotides, resulting in the production of siRNA. 2) In the effector step, the double stranded siRNA forms RISC with Ago2, Dicer and TRBP. The interaction of Ago2, a four-domain protein with RNase activity, with siRNA induces cleavage of the siRNA passenger strand. Subsequently, the helicase domain of Dicer unwinds the siRNA duplex and the siRNA guide strand forms activated RISC*. 3) The activated RISC binds to the target mRNA with a complementary sequence and Ago2 induces cleavage of the mRNA, which undergoes degradation.

The sequence specific gene silencing effect of RNAi is considered as an essential tool in modern functional genomics and an appealing agent for gene-specific therapeutic approaches. However, efficiency and specificity of RNAi are influenced by many factors. Efficiency of RNAi is highly influenced by the mRNA accessibility, since secondary or tertiary structures on mRNA may block correct hybridization between the target sequence and RISC. Computational analysis of the targeted RNA structure effects on RNAi and development of siRNA selection tools led to the precise prediction of highly efficient siRNA^{83,84}. Extensive research has been invested in modifications of different parts of the siRNA guide strand to improve RNAi efficiency and diminish off-target effects⁸³ (figure 8).

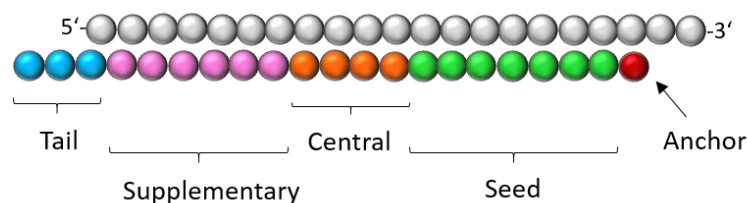


Figure 8 Structure of siRNA. Top strand of the siRNA is the passenger or sense, and bottom strand is the guide or antisense. During RISC formation and maturation, the passenger strand is degraded. The guide strand provides the complementary sequence to the target mRNA. SiRNA guide strand can be divided into anchor (red), seed (green), central (orange), supplementary (pink) and tail region (blue).

The stretch from position 2 to 8 (figure 8, green), referred to as the seed, is responsible for the initial base-pair formation during target recognition⁸⁵. 2'-O-methyl (MO) and 2'-O-methoxyethyl (MOE) modifications of the seed sequence resulted in less off-target effects due to the important impact of especially position 2 of the guide strand on target-recognition^{86,87}. Additionally, unlocked nucleic acids (UNA) modification locally destabilizes RNA-RNA hybridization and supports fully complementary target-binding⁸⁸. Base pairs of the central sequence (figure 8, green) lie within the catalytic centre of Ago2 and support cleavage of the targeted mRNA⁸⁹. Modifications including MO, MOE or 2'- fluoro modification are tolerated^{86,90–92}, others like the threoninol nucleic acid (TNA) modification leading to inhibition of silence activity or phosphorothioate substitution blocking passenger strand cleavage were proven to be disadvantageous^{92,93}. The tail part of the antisense strand spans nucleotides 19–21 (figure 8, blue) and contains a dinucleotide-unpaired structure, called overhang, which is critical for siRNA activity⁹⁴. Numerous overhang modifications in the tail region of the siRNA molecule (figure 8, blue) have been developed to protect the siRNA integrity from nuclease degradation and also show to improve long-term silencing effects^{95–99}.

1.3.3 Delivery of RNA targeted therapeutics

Key challenge and one of the biggest obstacles for RNA targeted therapeutics on their way to clinical application remains the development of reliable, specific, and safe drug delivery systems. Two main aspects must be considered: First, transport of the active oligonucleotide to their target tissue in a most specific way. Second, conveying of the drug to the intracellular structure of interest. The first aspect regards a variety of tissue barriers that affect an oligonucleotide's access to their target tissue site including vascular endothelial barriers such as blood tissue barrier, blood brain barrier and blood liver barrier¹⁰⁰. Additionally, mononuclear phagocytes and other parts of the reticuloendothelial system (RES) are able to inactivate free circulating oligonucleotides as well as clear the blood from oligonucleotides linked to nanoparticles¹⁰¹. The second challenge concerns the fate of an oligonucleotide once it reaches cell surface. Irrespective of being delivered as free oligonucleotide, as a conjugate or linked to nanocarriers, the molecules are uptaken by endocytosis and trafficked through various cell compartments in different endocytotic pathways. Most pathways finally lead to the oligonucleotide getting accumulated in late

endosomes or lysosomes, where they remain pharmacologically inert ^{102–104}. Thus, facilitating endosome escape is crucial for the success of oligonucleotide delivery.

Strategies for oligonucleotide delivery are subject of hundreds of publications every year, whereas the focus lies on two main strategies. One approach is to integrate the oligonucleotide into some sort of nanocarrier that regulates tissue distribution and cell interaction ^{105–107}. Using the second strategy oligonucleotides themselves are chemically modified, e.g. conjugation with a targeting ligand ^{108–110}. Main difference between both strategies concerns the size of the delivered drug which largely affects bio distribution and biological as well as pharmacological activity ¹⁰⁰.

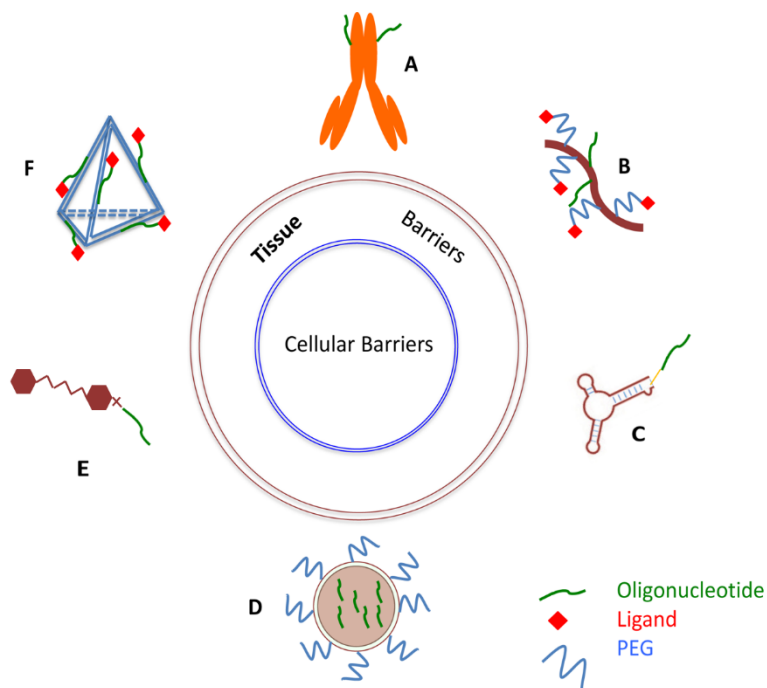


Figure 9 Oligonucleotide delivery systems. Various strategies for oligonucleotide delivery are depicted. (A) Antibody-oligonucleotide conjugate (B) Polymer-oligonucleotide conjugate with PEGylation and targeting ligand (C) Aptamer-oligonucleotide conjugate (D) Lipid nanoparticle with PEGylation (E) Glycoconjugate. (F) DNA nanostructure with oligonucleotide and targeting ligand incorporated. Images are not to scale (adapted from Juliano et al., 2016).

Monoclonal antibodies (figure 9, A) have a high potential to specifically determine targeting of oligonucleotides ¹¹¹. Investigations have been conducted especially with delivery of siRNA, where chemical linkage of siRNA to antibodies allows the oligonucleotide to enter its target cell via cell surface receptor. Remaining challenges include identification of adequate cell surface receptors, difficulties for antibody-siRNA conjugates to penetrate tissue due to their high molecular weight (approx. 180 000 MW), and large intracellular accumulation of conjugates in lysosomes ¹¹². Polymer- oligonucleotide conjugates with PEG linkage and targeting ligand (figure 9, B) came up as promising approach in siRNA delivery and offers a solution to endosome escape^{113,114}. Attachment of a membrane destabilizing peptide to the siRNA causes destabilization of endosome membrane once the conjugate gets accumulated in endosomes or lysosomes and subsequently releases the siRNA into cytosol ^{113,114}. Another strategy includes the utilization of aptamers (figure 9, C), nucleic acids forming 3 dimensional structures that can be designed to target any receptor on

nano- or picomolar level. Use of aptamer-oligonucleotide conjugates succeeded especially in tumor research ^{115–117}. Challenges of this technology include the conjugate's high susceptibility to nuclease degradation due to their size and molecular complexity as well as the identification of appropriate cell receptors that facilitate internalization into cells ¹⁰⁰. Lipid nanoparticles (LNPs, figure 9, D) play an important part in delivery of siRNA (siRNA LNPs), too ^{105,107}. To be used *in vivo* they include a polymer coating to reduce protein binding and phagocytosis by RES. Due to their comparably big size of 100–200 nm LNPs can only enter tissue with fenestrated endothelial barrier, which is why they are mainly used for oligonucleotides targeting liver tissue. Other disadvantages of LNPs, as well as of other developed technologies on nanoscale such as polymeric nanocarriers, concern toxicity upon chronic use due their high interaction with anionic biological molecules and cells. With the use of glycoconjugates (figure 9, E), e.g. oligonucleotides linked to N-acetylgalactosamine (GalNAc), it became possible to target the asialoglycoprotein receptor (ASGR) located on hepatocytes. Delivery to liver cells via ASGR ensures tissue specificity and shows fast internalization and turnover, as well as efficient knockdown of target gene expression¹¹⁸. Finally, oligonucleotides can be incorporated into 3 dimensional DNA nanostructures (figure 9, F) and combined with aptamers or small molecules as targeting ligands^{119,120}. Their small size (<20 nm) allows penetration through endothelial barriers and, therefore, access to non-hepatic tissue. However, to date there is little knowledge about their *in vivo* biodistribution and complex stability ¹⁰⁰.

1.3.4. Clinical advancements in RNA-targeted therapies

The field of RNA-targeted therapeutics has progressed tremendously with remarkable success over the last years and yielded first drug approvals as well as a numerous oligonucleotide-based late-stage clinical trials. To date, four antisense oligonucleotide therapeutics have been approved for commercial use and several ASO as well as siRNA therapies are investigated in phase 3 clinical trials. Commercialized ASO drugs are used to treat cytomegalovirus retinitis in AIDS patients (Fomivirsen) ^{121,122}, homozygous familial hypercholesterolemia (Mipomersen) ^{123–126}, spinal muscular atrophy (Nusinersen)^{127–130} and Duchenne muscular dystrophy (Eteplirsen) ^{131,132}. Advances in ASO and siRNA technology include phase 3 clinical trials addressing hereditary transthyretin amyloidosis^{133–135}, haemophilia A ^{136–138} and the inhibition of gene expression of proprotein convertase subtilisin/kexin type 9 (PCSK9) to reduce risk for coronary artery disease events and hypercholesterolemia ^{139,140}.

Future clinical applications depend on the resolution of major challenges including *in vivo* delivery, off-target effects, toxicity in long-term use and a relatively high-cost factor. However, nucleic acid-based technologies targeting gene expression on mRNA level like ASO-based therapy or RNAi offer immense potential for treatment of so far “undruggable” diseases, most notably genetic conditions.

2 Background and aim of this thesis

In a patient with an intermediate phenotype exome sequencing revealed a de novo mutation in a highly conserved region of codon 283 of *COL6A2* (p.G283E) gene at the age of 45¹⁴¹. First symptoms at that time for the unknown underlying condition include hip dislocation and overall muscle weakness in early childhood along with mild ambulatory impairment when compared to same age unaffected children. From the age of 28, nighttime ventilator support was necessary due to progressing respiratory insufficiency and hypoxemia. After many years of odyssey consulting numerous physicians to try to find a diagnosis, whole exome sequencing provided the key to the solution (figure 10). The find of a mutation in a collagen VI encoding gene completed the puzzle together with typical clinical features and, finally, the diagnosis of Col6-CMD with an intermediate phenotype was stated.

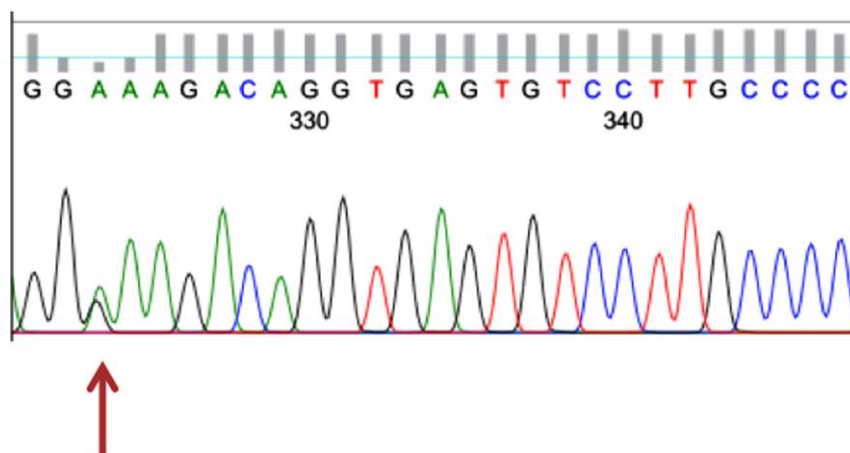


Figure 10 Molecular genetic analysis. Confirmation by conventional Sanger-sequencing of the heterozygous nucleotide substitution G>A at position +848 in *Col6A2* gene leading to an amino acid substitution at highly conserved position 283 (Gly>Glu)¹⁴¹.

Most mutations in of the three collagen VI genes have a dominant negative effect, with defects in collagen VI chain secretion and assembly¹⁴². As has been described before, large deletions in *COL6* genes leading to functional haploinsufficiency, conversely, are not causing clinical symptoms of Col6-CMD, even though they are proven to be associated with a reduction in collagen VI extracellular matrix (ECM) *in vitro*¹⁴³. Thus, RNA-targeting approaches aimed at preferentially inactivating the mutated *COL6A2* messenger may represent a possible therapeutic strategy¹⁴².

The aims of the current thesis are the following:

- 1) Establishment of a fibroblast cell assay with fibroblasts obtained by skin biopsy from the above-mentioned patient. Immunostaining for collagen VI to examine collagen VI morphology in ECM.
- 2) Messenger depletion targeting the p.G283E mutation in *COL6A2* gene by ASO and RNAi to rescue the phenotype *in vitro*.
- 3) Elucidation of Col6-CMD pathogenesis by RNA- sequencing and cytokine array analysis.

3 Materials and Methods

3.1 Materials

3.1.1 Chemicals and enzymes

Name	Manufacturer
100bp DNA Ladder plus (44ng/μl)	Peqlab/VWR
2-β-Mercaptoethanol	Merck KGaA
Accutase	Sigma-Aldrich Co. LLC.
APS (ammoniumperoxodisulfate)	Carl Roth GmbH & Co. KG
Boric acid	Sigma-Aldrich Co. LLC.
Bromphenol blue	Sigma-Aldrich Co. LLC.
BSA (bovine serum albumin)	Sigma-Aldrich Co. LLC.
Dako Fluorescence mounting medium	Agilent Technologies, Inc.
Dako Pen	Agilent Technologies, Inc.
DAPI (4',6-diamidino-2-phenylidone), stock solution in water: 1 mg/ml	Sigma-Aldrich Co. LLC.
Dextran blue	Sigma-Aldrich Co. LLC.
DMSO (Dimethylsulfoxide)	Sigma-Aldrich Co. LLC.
dNTP-mix, 10 mM each; working solution in water: 4 mM each	Bioline Reagents Limited
EDTA (Ethylenediaminetetraacetic acid disodium salt)	Sigma-Aldrich Co. LLC.
Ethanol (non-denatured)	J.T.Baker
Ficoll [®] 400	Sigma-Aldrich Co. LLC.
Formamide	Honeywell International Inc.
Cryo-SFM medium	PromoCell GmbH
Glycerin	Sigma-Aldrich Co. LLC.
Glycine	Sigma-Aldrich Co. LLC.
Hexanucleotide random primer mix	Carl Roth GmbH & Co. KG
LE agarose	Biozym Scientific GmbH
Lipofectamine [®] 2000 Reagent	Invitrogen [™] Thermo Fisher Scientific Inc.
Methanol	Carl Roth GmbH & Co. KG
M-MLV reverse transcriptase (200U/μl)	Thermo Fisher Scientific Inc.
MyTaq DNA polymerase, with 5x buffer	Bioline Reagents Limited
Nucleic Acid Sample Buffer, 5x conc.	Bio-Rad Laboratories, Inc.
La Taq DNA Polymerase, with 10x buffer w/o Mg ⁺ , with dNTPs	TaKaRa Bio Inc.
La Taq DNA Polymerase, with 2x GC buffers, with dNTPs	TaKaRa Bio Inc.
TEMED (Tetramethylethyldiamine)	Carl Roth GmbH & Co. KG
Para-formaldehyde, 4% in PBS, pH 7,4	Morphisto
PowerUp SYBR Green Master Mix	Thermo Fisher Scientific Inc.
RiboLock RNase Inhibitor (40U/μl)	Thermo Fisher Scientific Inc.
RNase free water	Thermo Fisher Scientific Inc.

Sodium chloride (NaCl)	Sigma-Aldrich Co. LLC.
Sodium dodecyl sulfate (SDS)	Carl Roth GmbH & Co. KG
SYBR-Green I	Molecular Probes™ Thermo Fisher Scientific Inc.
Triton-X100	Sigma-Aldrich Co. LLC.
Trizma® Base	Sigma-Aldrich Co. LLC.
Trypsin/EDTA 0.5%, diluted to 0.05% with PBS	Gibco™ Thermo Fisher Scientific Inc.
Urea	Carl Roth GmbH & Co. KG
Versene (EDTA) 0.02%	Biozym Scientific GmbH
Xylene cyanol	Sigma-Aldrich Co. LLC.

3.1.2 Media, buffers and supplements

Name	Manufacturer, Composition
Dulbecco's Modified Eagle medium (DMEM) high glucose, GlutaMAX™ supplement, sodium pyruvate	Gibco™ Thermo Fisher Scientific Inc.
Electrophoresis buffer for Western Blot	12.5 mM Trizma®Base, 9.6 mM Glycine, 1.75 mM SDS, pH 8.3
Fetal bovine serum (FBS) EU Professional	PAN-Biotech GmbH
Fetal bovine serum (FBS) Gold Plus	Bio& Sell GmbH
L-ascorbic acid, sodium salt	Sigma-Aldrich Co. LLC.
Loading buffer agarose gel	99% Formamide (v/v), 1% Dextran Blue (w/v), 80µl Bio-Rad 5x Nucleic Acid Sample Buffer
Loading buffer denaturing PAA gel	50% glycerine, 3 M urea, 0.1% bromphenol blue and 0.05% xylene cyanol (w/v) in 1x TBE
Loading buffer semi-denaturing PAA gel	25% Ficoll 400, 0.1% bromphenol blue and 0.05% xylene cyanol (w/v) in 1x TBE
Loading dye agarose gel	2/3 loading buffer agarose gel, 1/3 SYBR® Green I (1:200 dilution in DMSO)
McCoy's 5A (modified) medium	Gibco™ Thermo Fisher Scientific Inc.
Opti-MEM™ I Reduced Serum Medium	Gibco™ Thermo Fisher Scientific Inc.
Phosphate buffer saline (PBS) tablets	Thermo Fisher Scientific Inc.
Phosphate buffered saline (PBS) sterile, w/o calcium and magnesium	Lonza
TBE-Buffer	100mM Trizma®Base + 80mM Boric Acid + 1.3mM EDTA in RNase free water

3.1.3 Cultured cells

Cell type	Name	Manufacturer
Human osteosarcoma cell line	Saos-2	Sigma-Aldrich Co. LLC.
Human primary fibroblasts (<i>COL6A2</i> p.G283E)	Mut	Provided by: Institute for Neurogenetics, Lübeck
Human primary fibroblasts (<i>COL6A2</i> wildtype)	WT	Provided by: Institute for Neurogenetics, Lübeck
Human primary fibroblasts (<i>COL6A2</i> wildtype)	L6534	Provided by: Institute for Neurogenetics, Lübeck
Human primary fibroblasts (<i>COL6A2</i> wildtype)	L6699	Provided by: Institute for Neurogenetics, Lübeck
Human immortalized fibroblasts (from Mut)	Mut-imm.	InSCREENex
Human immortalized fibroblasts (from WT)	WT-imm.	InSCREENex

3.1.4 Primers

Gene	Sequence 5'-3'	Manufacturer
COL6A2_long_A1_F	GGA GAG CCT GGC CAG AAG GA	Eurofins Genomics
COL6A2_long_G1_F	GGA GAG CCT GGC CAG AAG GG	Eurofins Genomics
COL6A2_long_R	GTC CGT GAG GAA CAC GAA GGA C	Eurofins Genomics
COL6A2_short1_F	GGA CAG AAG GGC AAG CTG	Eurofins Genomics
COL6A2_short1_R	TCT CCC TTG GCT CCT TTG TT	Eurofins Genomics
COL6A2_short2_F	TGA CCT ACG TGA GGG AGA CC	Eurofins Genomics
COL6A2_short2_R	GAT GAA CTT CTC AGC GAC CAG	Eurofins Genomics
COL6A2_short3_F	CAC GAG AAG CAC GAG AGT GA	Eurofins Genomics
COL6A2_short3_R	AGG AAC ACG AAG GAC AGC TC	Eurofins Genomics
COL6A2_alleleG(WT)_F, qPCR	AGA GCC TGG CCA GAA GGG	Eurofins Genomics
COL6A2_alleleG(WT)_R, qPCR	CCC TGG ACT CCC TGC TTC	Eurofins Genomics
COL6A2_alleleT(mut)_F, qPCR	AGA TCG ACC AGG ACA CCA TC	Eurofins Genomics
COL6A2_alleleT(mut)_R, qPCR	CCG GGT CTC CCT GTC TTT	Eurofins Genomics
Saos2_3a(allele A)_F, qPCR	TCG CTA AGG ACC CCA AGT CC	Eurofins Genomics
Saos2_3a(allele A)_R, qPCR	TGA GGG TGT CCA GGT GCC A	Eurofins Genomics
Saos2_3g(allele G)_F, qPCR	TCG CTA AGG ACC CCA AGT CC	Eurofins Genomics
Saos2_3g(allele G)_R, qPCR	TGA GGG TGT CCA GGT GCC G	Eurofins Genomics
hFAP_F, qPCR	TAC GTT TCA TCA CTG GCC CT	Eurofins Genomics
hFAP_R, qPCR	CAT CTG CTG TTC CGT GGA TG	Eurofins Genomics
hCOL4A1_2_F, qPCR	AGC CAG ACC ATT CAG ATC CC	Eurofins Genomics
hCOL4A1_2_R, qPCR	CTC CTC TCT ATG GTG GCG AG	Eurofins Genomics
hVMAC_2_F, qPCR	TGA TGA CCT CAG AAG CCA CT	Eurofins Genomics
hVMAC_2_R, qPCR	CTC TTC CCC AGT GCT GTT GT	Eurofins Genomics
hFN1_F, qPCR	CCC CAT TCC AGG ACA CTT CT	Eurofins Genomics
hFN1_R, qPCR	TGC CTC CAC TAT GAC GTT GT	Eurofins Genomics
hTHY1_F, qPCR	AGT ACG AGT TCA GCC TGA CC	Eurofins Genomics
hTHY1_R, qPCR	TCT GAG CAC TGT GAC GTT CT	Eurofins Genomics
hITGB1_F, qPCR	TTC AAG GGC AAA CGT GTG AG	Eurofins Genomics
hITGB1_R, qPCR	GGA CAC AGG ATC AGG TTG GA	Eurofins Genomics

hCOL1A1_F, qPCR	GCT ACT ACC GGG CTG ATG AT	Eurofins Genomics
hCOL1A1_R, qPCR	ACC AGT CTC CAT GTT GCA GA	Eurofins Genomics
hCD248_F, qPCR	CAT TAT CCC AAC TGC CCA GC	Eurofins Genomics
hCD248_R, qPCR	CTG ATG GGT GAG GTC TGG TT	Eurofins Genomics
hS100A4_F, qPCR	ACC TTC CAC AAG TAC TCG GG	Eurofins Genomics
hS100A4_R, qPCR	ATG CAG GAC AGG AAG ACA CA	Eurofins Genomics
hPTPRC_F, qPCR	AGT ACA GAC GCC TCA CCT TC	Eurofins Genomics
hPTPRC_R, qPCR	TGG CTG TAC TCC TCT CTC CT	Eurofins Genomics
hSPN_F, qPCR	CCC TAC CTC CCT CAA CTT CC	Eurofins Genomics
hSPN_R, qPCR	CTG GTT GCA TGA GGG GTT TC	Eurofins Genomics
GAPDH_F, qPCR	TCA CCA CCA TGG AGA AGG C	Eurofins Genomics
GAPDH_R, qPCR	GCT AAG CAG TTG GTG GTG CA	Eurofins Genomics

3.1.5 Nucleic acids

Name	Sequence	Manufacturer
as1300-1	5' CCA TTG TTG CmCmT mTmGmA mTmAmC 3'	IBA Lifesciences
as2198-1	5' CCA CCT GCA AmTmC mCmAmC mTmCmG 3'	IBA Lifesciences
as2198-2	5' mAmGmG mTGC CAC CTG CAmA mTmCmC 3'	IBA Lifesciences
as2198-3	5' mTmGmT mCmCmA mGmGT GCC ACC TGC 3'	IBA Lifesciences
as2288-1	5' mGmTmG mATG ACT ACC GCmA mAmAmC 3'	IBA Lifesciences
asCOL-3G	5' mCmCmC mTGT CTT CCC TTmC mTmGmG 3'	IBA Lifesciences
asCOL-4G	5' mTmCmT mTCC CTT CTG GCmC mAmGmG 3'	IBA Lifesciences
BLOCK-iT™ Alexa Fluor™	undisclosed	Invitrogen™ Thermo Fisher Scientific
sc1570	5' CGG AGC GAT ACC GAG GGT 3'	IBA Lifesciences
si2198-1	5' UGG AUU GCA GGU GGC ACC UTT 3' 3' TTA CCU AAC GUC CAC CGU GGA 5'	IBA Lifesciences
si2201-1	5' AUU GCA GGU GGC ACC UGG ATT 3' 3' TTU AAC GUC CAC CGU GGA CCU 5'	IBA Lifesciences
si2288-1	5' UUU GCG GUA GUC AUC ACG GTT 3' 3' TTA AAC GCC AUC AGU AGU GCC 5'	IBA Lifesciences
siCOL-1A	5' CCA GAA GGA AAG ACA GGG ATT 3' 3' TT GGU CUU CCU UUC UGU CCC U 5'	IBA Lifesciences
siCOL-1G	5' CCA GAA GGG AAG ACA GGG ATT 3' 3' TT GGU CUU CCC UUC UGU CCC U 5'	IBA Lifesciences
si-scrambled	5' CGA ACU CAC UGG UCU GAC CTT 3' 3' TT GCU UGA GUG ACC AGA CUG G 5'	IBA Lifesciences

3.1.6 Antibodies

Name	Species	Manufacturer
anti-human collagen VI (MAB1944)	mouse	Merck KGaA
anti-human fibronectin (F3648)	rabbit	Sigma-Aldrich Co. LLC.
anti-mouse Alexa Fluor 568	donkey	Thermo Fisher Scientific Inc.
anti-rabbit Alexa Fluor 488	donkey	Thermo Fisher Scientific Inc.

3.1.7 Devices and consumables

Name	Manufacturer
384-well Multiply PCR plates	Sarstedt AG & Co.
48-well plates	Greiner bio-one
6-well plates	NUNCTM Thermo Fisher Scientific Inc.
7900 HT Fast Real-Time PCR	Thermo Fisher Scientific Inc.
8-well Tissue Culture Chambers on glass	Sarstedt AG & Co.
Biofuge pico	Heraeus Holding GmbH
Biosphere®Fil. Tip 1000 µl, blue	Sarstedt AG & Co.
Biosphere®Fil. Tip 20 µl, yellow	Sarstedt AG & Co.
Biosphere®Filter Tip 100 µl, neutral	Sarstedt AG & Co.
Centrifuge 5415 R	Eppendorf AG
Centrifuge 5702 R	Eppendorf AG
Centrifuge Rotanta 460R	Andreas Hettich GmbH & Co. KG
Chemidoc XRS documentation	Bio-Rad Laboratories, Inc.
CryoPure Tube 1.6ml white	Sarstedt AG & Co.
Electrophoresis chambers, trays maxi-mini	neoLab Migge GmbH
Elektrophoresis Power Supply	Micro-Bio-Tec Brand
Glass cover slips 24 x 60mm	Menzel-Gläser
Glass pasteur pipettes 230mm	Carl Roth GmbH & Co. KG
Incubation/Inactivation bath 1083	GFL GmbH
Incubator Heracell	Thermo Fisher Scientific Inc.
Magnetic stirrer MR3001K	Heidolph Instruments Labortechnik
Microscope BZ-9000 BioRevo	Keyence Corporation
Microscope Eclipse TS100	Nikon Corporation
Microscope IX70	Olympus Corporation
Mr. Frosty —Freezing Container	Thermo Fisher Scientific
NanoDrop 2000	Thermo Scientific
NucleoCounter NC-200	ChemoMetec A/S
PAA gel chamber	Biometra
Pipetboy acu 2	Integra Bioscience Corp
Pipette tip 1000 µL, blue	Sarstedt AG & Co.
Pipette tip 200 µL yellow	Sarstedt AG & Co.
Pipette tips 10 µL, neutral	Sarstedt AG & Co.
Pipette tips 20 µL, neutral	Sarstedt AG & Co.
Platform shaker Duomax 1030	Heidolph Instruments Labortechnik
Power Supply EPS 300	Pharmacia Corporation
PowerPac—Basic energy supply	Bio-Rad Laboratories, Inc.
Reaction tubes 0.2 ml	Greiner Bio-One GmbH
Reaction tubes 0.5 ml	Sarstedt AG & Co.
Reaction tubes 1.5 ml	Sarstedt AG & Co.
Reaction tubes 15 ml	Sarstedt AG & Co.
Reaction tubes 2.0 ml	Sarstedt AG & Co.
Reaction tubes 50ml	Sarstedt AG & Co.
Research pipettes (2.5µL, 1000µL)	Eppendorf AG
Research Plus pipettes (10µL, 100µL, 1000µL)	Eppendorf AG

Tissue Culture Flask, standard 175 cm ²	Sarstedt AG & Co.
Tissue Culture Flask, standard 75 cm ²	Sarstedt AG & Co.
SensoQuest labcycler Basic	SensoQuest GmbH
Serological pipette 10 mL	Sarstedt AG & Co.
Serological pipette 2 mL	Sarstedt AG & Co.
Serological pipette 25 mL	Sarstedt AG & Co.
Serological pipette 5 mL	Sarstedt AG & Co.
Sterile Workbench, HEPA-filtered	Clean Air Products

3.1.8 Kits

Name	Manufacturer
NucleoSpin Gel and PCR Clean-up Kit	Macherey-Nagel
Proteome Profiler Human Cytokine Array Kit	R&D Systems
RNeasy®Plus Mini Kit	QIAGEN GmbH, Hilden DE

3.1.9 Programs

Name	Source
Ensembl Version 93, Juli 2018	Open source http://www.ensembl.org/index.html
GraphPad Prism 7	GraphPad Software Inc
Microsoft Office 365	Microsoft
Primer 3 Version 0.4.0	Open source http://bioinfo.ut.ee/primer3-0.4.0/
Quantity One	Bio-Rad Laboratories Inc.
SDS 2.2.2	Thermo Fisher Scientific Inc.

3.2 Methods

3.2.1 Cell culture maintenance

For this thesis, Saos-2 cells, primary fibroblasts, and immortalized fibroblasts (section 3.2.2) were used. Primary fibroblasts were obtained from a skin biopsy of a woman diagnosed with an intermediate phenotype of Col6-CMD (see section 2: fibroblasts Mut, carriers of the mutation p.G283E in *COL6A2*), in addition to three healthy unaffected donors (fibroblasts WT, L6534, L6699, *COL6A2* wild type), which were used as a control. The fibroblasts were cultured in cell culture flasks with DMEM high glucose, GlutaMAX™ medium containing 10% FBS Gold Plus and incubated at 37 °C and 5% CO₂ in a humidified atmosphere. Saos-2 cells were cultured in cell culture flasks with McCoy's 5A medium, in which 15% FBS Gold Plus was added, and incubated at 37 °C and 5% CO₂ in a humidified atmosphere.

3.2.1.1 Thawing

Using the same method, the cell lines were brought into culture from cryopreservation in liquid nitrogen. The individual vials were quickly thawed in a pre-warmed 37 °C water bath and, using a sterile transfer pipette, added to 10 ml pre-warmed culture medium in a 15 ml tube. Cells were then pelleted via centrifugation at 200 g (900 rpm) for 3 min. The supernatant was removed, and the cell pellet was carefully re-suspended in 7 ml fresh pre-warmed culture medium. The cell suspension was then transferred to culture flasks with the appropriate volume of pre-warmed culture medium. Approximate seeding density for the fibroblasts was 7000 – 10000 cells per cm² (equals a 1:3-1:4 split ratio during subculture) and 30 000 cells per cm² (1:4 split ration during subculture) for the Saos-2 cell line.

3.2.1.2 Passaging

During subculture, medium was changed every second day with fresh culture medium. When cells reached 80 % confluency, the medium was removed, and the cells were incubated with Accutase (fibroblasts) or a 0.05% Trypsin/EDTA solution (Saos-2 cells) for 3-5 min at 37 °C until detached. The activity of the enzymes was stopped by adding 1:2 volume of culture medium into the cell suspension. This was then transferred to 15- or 50-ml tubes and centrifugation with 200 g for 3 min was performed. The supernatant was removed, and the cell pellet was resuspended carefully in culture medium. The cell suspension was transferred to new culture flasks in a split ratio of 1:3 or 1:4.

3.2.1.3 Cryopreservation

For long-term storage, cells were detached from the cell culture flasks as described in section 3.2.1.2 and, after centrifugation and removal of the culture medium, were resuspended in the appropriate volume of pre-cooled Cryo-SFM medium. The number of

cells per ml Cryo-SMF was equivalent to the number of cells needed to seed either a T175 or a T75 flask (see section 3.2.1.1). 1 ml of the cell suspension was then transferred to one cryo tube each and, using a pre-cooled isopropanol filled Mr. Frosty freezing container, cooled down at a speed of -1 °C/min to a temperature of -80 °C. On the next day, the cells were transferred to liquid nitrogen storage.

3.2.2 Fibroblast collagen VI assay

3.2.2.1 Preparation on chamber slides and transfection

To start the assay, the fibroblasts were first split as described in section 3.2.1.2, resuspended in culture medium, and transferred to 8-well chamber slides at a concentration of 0.2×10^5 cells per well in total volume of 0.5 ml per well. 24 hours afterwards, transfection of the tested siRNA was performed with Lipofectamine 2000 like the protocol used in section 3.2.6.1 at a siRNA concentration of 30 nM. Each experiment

3.2.2.2 Collagen VI stimulation with L-Ascorbic Acid

L-ascorbic acid was supplemented to the plated fibroblasts since it is an essential cofactor for collagen biosynthesis and can be used to stimulate collagen production ¹⁴⁴. For this, L-ascorbic acid at a molar concentration of 250 µM was added freshly prepared from a stock to fresh culture medium. The old medium then was removed from the wells and changed to fresh culture medium containing L-ascorbic acid. This stimulation procedure was repeated for 5 consecutive days with daily medium changes.

3.2.2.3 Fibroblast immunofluorescence staining

For the visualization of collagen VI and fibronectin within the cells and in the extracellular matrix, an immunofluorescence staining was used. To fix the beforehand prepared cells on the chamber slide, the medium was first removed, and the wells were washed with PBS. The cells were fixed on the slides in paraformaldehyde for 15 min and then washed again with PBS. The chambers were removed from the slides and the edges of each well were bordered with a Dako Pen. For permeabilization of the cell wall, 0.1% Triton-X (v/v) were added to the blocking solution already containing 2% BSA (w/v), 10% FBS (v/v, alternatively 5% donkey serum) in PBS, of which 75 µl were added to each well. The slides were incubated at room temperature for 45 min. Then, the blocking solution was removed and 75 µl of primary antibodies anti-collagen VI (1:900) and anti-fibronectin (1:400) diluted in PBS or in case of the control only with 75 µl PBS at 4 °C overnight was added and incubated at 4 °C overnight. On the next day, the slides were washed 3 times with PBS for 5 min each. Following this, the cells were treated 75 µl of the secondary antibodies Alexa Fluor 488 and Alexa Fluor 568 diluted in PBS (1:500 and 1:250, respectively) for 1 hour at room temperature and protected from light. Afterwards, the slides were washed three times with PBS for 15 min each. To visualize cell nuclei, DAPI nuclear staining was performed with DAPI diluted 1:1000 in PBS for 10 min at room temperature and protected from light. Then, the slides were carefully washed again with PBS and a glass cover slip was mounted on the

slides using Dako Fluorescence mounting medium. The slides were dried for at least 30 min at room temperature and then stored at 4 °C in the dark.

3.2.3 Fibroblast immortalisation

Primary fibroblasts were immortalized by InSCREENeX GmbH in Braunschweig, Germany. For this, three cryopreserved vials per fibroblast donor (wildtype and mutated) with 0.5×10^6 cells were provided to the company. InSCREENeX transduced the primary fibroblasts with their CI-SCREEN library, a set of genes involved in cell cycle pathways, via lentiviral transfer. After the establishment of a stable cell proliferation the integration pattern was analysed to identify the integrated genes from the CI-SCREEN gene library. The complete protocol remains undisclosed.

3.2.3.1 Fibroblast marker analysis

To characterize the immortalized fibroblasts and compare them to primary fibroblasts, we selected marker genes specific for fibroblasts (positive markers) and genes specifically not expressed in fibroblasts (negative markers) based on literature research. Primers for each gene were designed accordingly using the software Primer 3 Version 0.4.0 and tested for specific amplification of the targeted region before use. For this, PCR was performed. A reagent mix was prepared (table 2) using cDNA from Mut primary fibroblasts for the positive markers and, for the negative markers, cDNA from monocytes (cell line U937, day 0) and macrophages (cell line U937, day 3) since they are known to express these genes.

Table 2 Reagent mixture for primer test PCR.

Ingredient	Volume in μl
10X First Strand Buffer	1.5
dNTP mix (100mM each)	2
Water	7.85
LaTaq	0.15
cDNA	0.5
Primer forward (100 pmol/ μ l)	0.75
Primer reverse (100 pmol/ μ l)	0.75

The PCR products were run on agarose gel electrophoresis with 120 V for 60 min (see section 3.2.4.2) and fragments were visualized by UV illumination using ChemiDoc XRS imaging system. Then, RNA was isolated from immortalized fibroblast lines (see section 3.2.6.2) and converted to cDNA by RT-PCR (see section 3.2.6.3). Subsequently, qPCR with the primers for each fibroblast marker gene was performed on the previously gained cDNA (see section 3.2.6.4).

3.2.4 Identification of SNPs on *COL6A2*

3.2.4.1 Bioinformatic variant search

In order to find single nucleotide polymorphisms (SNPs) within the exon regions of which could be used as additional targets for siRNA and ASO, a variant search was kindly performed by our bioinformatics department using the exome sequencing data from the patient carrying the targeted p.G283E mutation in *COL6A2*. Total allele frequencies were searched on gnomAD v2.1.1 (reference: GRCh37).

3.2.4.2 Allele specific cDNA sequencing

3.2.4.2.1 Nested PCR

To validate the location of each SNP on the respective allele in mutant fibroblasts, allele specific DNA sequencing of *COL6A2* in fibroblasts was necessary and realized by nested PCR including two primer sets and two successive PCR runs.

First, two primer pairs (namely G1 and A1) for the amplification of a 2 kb fragment encompassing the area with all SNPs of interest were designed, using the open-source program Primer 3 (Version 0.4.0). For each primer pair, the forward primer was either specific for the G or A allele of the *COL6A2* p.G283E mutation. This was achieved by ending the respective forward primers with the corresponding complementary base.

PCR with these primers and the cDNA of the patient was performed with varying annealing temperatures and magnesium chloride concentrations to find the ideal conditions for the amplification of only the desired allele. After running the PCR products on a 0.9% agarose gel and visualization (as described in chapter 3.2.4.2.3), the amplified fragments were cut from the gel and extracted using the Gel and PCR Clean-Up Kit (as described in chapter 3.2.4.2.2)

Allele specific amplification was then assessed through sanger sequencing (performed by Microsynth, see section 3.2.4.2.4, sequencing results shown in section 4.3.2). The results showed that, only with the lowest Mg⁺ concentration and the highest annealing temperature used, 100 % allele specificity was achieved, the corresponding PCR reaction set up and program are shown in table 3 and 6, respectively.

For the second part of the nested PCR approach, three further primer pairs were designed for the subsequent amplification and sequencing of the individual SNPs within the longer PCR products generated with the A1/G1 primers. Primer pair short1 produced a 410 bp amplicon, containing SNP rs2839110; primer pair short2 produced a 611 bp amplicon with the SNPs rs1042917, rs13052956, rs13046639 and rs2839114 (see section 4.3.1); and primer pair short3 produced a 551 bp amplicon containing rs11554669. Each primer pair was tested for performance with different PCR conditions, the final conditions used for PCR are shown in table 4, 5, 7 and 8.

Table 3 Reagent mixture for the first reaction of the nested PCR: Primers A1 and G1 (used with TD69,2 min,35x)

Ingredient	Volume in μl
10X LaTaq-Buffer w/o Mg ⁺	1.5
dNTP mix (2.5 mM each)	1.2
MgCl ₂ (25 mM)	0.4
Water	9.75
LaTaq	0.15
cDNA (25 ng/ μ l)	0.5
Primer forward (10 pmol/ μ l)	0.75
Primer reverse (10 pmol/ μ l)	0.75

Table 4 Reagent mixture for the second reaction of the nested PCR: Primer short1 (used with TD65,30 s,35x)

Ingredient	Volume in μl
2X TaKaRa GCI-Buffer w/ Mg ⁺	7.5
dNTP mix (100 mM each)	1.2
Water	4.4
MyTaq	0.1
cDNA	0.3
Primer forward (10 pmol/ μ l)	0.75
Primer reverse (10 pmol/ μ l)	0.75

Table 5 Reagent mixture for the second reaction of the nested PCR: Primers short2 und short3 (used with TD61,30 s,35x)

Ingredient	Volume in μl
5X MyTaq-Buffer	3.0
Water	10.1
MyTaq	0.1
PCR 1:100	0.3
Primer forward (10 pmol/ μ l)	0.75
Primer reverse (10 pmol/ μ l)	0.75

Table 6 PCR program steps 1st run: TD69,2 min,35x

Step	[°C]	[min:sec]	Loop to step	Repeats
1	94	03:00		1
2	94	00:30		3
3	69	00:30		3
4	68	02:00	2	3
5	94	00:30		3
6	67	00:30		3
7	68	02:00	5	3
8	94	00:30		3
9	65	00:30		3
10	68	02:00	8	3
11	94	00:30		35
12	63	00:30		35
13	68	02:00	11	35
14	68	10:00		1
15	16	∞		1

Table 7 PCR program steps 2nd run: Primer short1 used with TD61,30 s,35x

Step	[°C]	[min:sec]	Loop to step	Repeats
1	94	03:00		1
2	94	00:30		3
3	61	00:30		3
4	72	00:30	2	3
5	94	00:30		3
6	59	00:30		3
7	72	00:30	5	3
8	94	00:30		3
9	57	00:30		3
10	72	00:30	8	3
11	94	00:30		35
12	55	00:30		35
13	72	00:30	11	35
14	72	10:00		1
15	16	∞		1

Table 8 PCR program steps 2nd run: Primer short2 and short3 used with TD65,30 s,35x

Step	[°C]	[min:sec]	Loop to step	Repeats
1	94	03:00		1
2	94	00:30		3
3	65	00:30		3
4	72	00:30	2	3
5	94	00:30		3
6	63	00:30		3
7	72	00:30	5	3
8	94	00:30		3
9	61	00:30		3
10	72	00:30	8	3
11	94	00:30		35
12	59	00:30		35
13	72	00:30	11	35
14	72	10:00		1
15	16	∞		1

3.2.4.2.2 Agarose gel electrophoresis

To analyse and visualize differences in length and possible contamination and to check for unspecific amplification products before using them as a template for the second PCR run and before sequencing, an agarose gel electrophoresis was performed. Therefore, agarose was melted in 1xTBE buffer with a final concentration of 0.9 % and the solution was filled in a gel casting tray to solidify. For the electrophoresis, the gel was placed in an electrophoresis chamber and submerged in 1xTBE buffer, the prepared samples (for composition see Table 9 and 10) and a 100 base pair ladder standard were loaded. The electrophoresis was performed at 120 V for 60 min. The fragments were visualized by UV illumination with the ChemiDoc XRS imaging system.

Table 9 Gel electrophoresis samples: PCR products for extraction of bands from agarose gel

Ingredient	Volume in µl
PCR product	15
loading dye	6

Table 10 Gel electrophoresis samples: PCR products for direct sequencing

Ingredient	Volume in µl
PCR product	5
Water	5
loading dye	4

3.2.4.2.3 Purification of PCR products

Before proceeding with the following steps, the PCR products were purified using the NucleoSpin Gel and PCR Clean-up Kit of Macherey-Nagel. The purification was performed as described in the manufacturer's protocol using either 500 µl undiluted NTI buffer for dissolving the gel pieces or using 200 µl 1:6 diluted NTI buffer for direct clean-up of the 10 µl of remaining PCR products. After the washing steps, 40 µl NE buffer were used for elution.

3.2.4.2.4 Sanger Sequencing and sequence analysis

The Sanger sequencing was performed by the company Microsynth Seqlab with the beforehand tested primers. The sample requirements for each sequence reaction were 22 ng purified PCR product per 100 bp amplicon length. Per reaction 3 µl of the 10 pmol/µl primer solution were added and the volume was filled to 15 µl with NE buffer from the PCR Clean-up Kit. The sequence data was analysed with the software Gene Runner.

3.2.5 Design of siRNA and ASO

3.2.5.1 Computational design

SiRNAs and ASOs which were used in this project were designed by the Institute of Molecular Medicine at University of Lübeck and synthesized by IBA Lifesciences. ASOs were delivered as all-bonds PTOs for higher resistance to nuclease degradation and increased protein binding (see also section 1.3.1) with MO-modifications (see section 3.1.5, modified bases are marked with an “m”) in solution of 0.1 nmol/µl. SiRNAs were delivered annealed in solution of 0.1 nmol/µl as 19-base-paired duplexes with two deoxythymidine (dT) nucleotide 3' overhangs at both ends for increased nuclease stability (see section 1.3.2).

3.2.5.2 Concentration and purity control

Photometrical measurement of the concentration and purity of the newly synthesized siRNA and ASO was performed with the NanoDrop. Absorption was determined at a wavelength of 260 nm and additionally the ratio of OD_{260nm} and OD_{280nm} was calculated. This can ratio be used as an indicator for the degree of nucleic acid purity, with pure RNA showing a 260/280 ratio of around 2.

Further validation of the purity of nucleic acid synthesis was conducted by polyacrylic (PAA) gel electrophoresis. The method allows for accurate depiction of small fragment size variations within the samples, which can be used as an indicator for non-optimal synthesis products or for premature degradation of the RNA.

3.2.5.2.1 Semi- denaturing PAA gel electrophoresis for siRNA

Quality control of siRNA purity of siRNA was performed by a semi-denaturing PAA gel electrophoresis for double strand oligonucleotides. For this, a gel solution containing 15 %

PAA, 4 M urea and 1x TBE was prepared and filled up with distilled water to a total volume of 20 ml, after which 200 µl of 10% (w/v) ammonium persulfate (APS) and 20 µl tetramethylethylenediamine (TEMED) were added to catalyse gel polymerization. Samples of siRNA were mixed with loading buffer for semi-denaturing gels (see section 3.1.2). After a pre-run of the empty gel for 20 min at 3 W and 4 °C external temperature, temperature of the gel was held stable at 20°C. Gel was loaded with the prepared siRNA samples and run at 3 W and an external temperature not exceeding 37 °C.

3.2.5.2.2 Denaturing PAA gel electrophoresis for ASO

In a similar way to the previous method, the synthesis quality of ASOs was examined. For single stranded oligonucleotides, such as ASOs, the gel contained 15 % PAA, 8 M urea and 1x TBE. Pre-run of the gel was carried out at 50 °C temperature. Then, 2 µl of ASO solution (stock concentration of 0.1 nmol/µl) were mixed with 10 µl loading buffer for denaturing gels (see section 3.1.2) for each sample. In addition, samples underwent a denaturation step. They were mixed with an additional 50 % (v/v) of formamide and denatured for 3 min at 95 °C, before being directly loaded to the gel. Gel electrophoresis was performed at 8 W and 50°C temperature.

Visualization of the ASOs and siRNAs on gel was kindly performed by the Institute of Molecular Medicine, Lübeck, following the manufacturer's protocol for Stains-all (Sigma-Aldrich Co. LLC.).

3.2.6 Quantification of mRNA knockdown by siRNA and ASO

To assess the knockdown potential of each siRNA and ASO, they were transfected at different concentrations ranging from 0.03 nM to 300 nM into either WT, Mut, WT-imm., Mut-imm. fibroblasts or Saos-2 cells depending on the specific approach, respectively. As control oligomers, fluorescence-labeled Block-It siRNA and a scrambled siRNA were used.

3.2.6.1 siRNA and ASO transfection

For transfection, cells were split as described in section 3.2.1.2 and transferred to 48-well plates at a concentration of 0.1×10^5 cells per well containing a culture medium volume of 1 ml per well. After a growing period of 24 hours, culture medium was removed and wells were washed with 1 ml Opti-MEM, which was removed again before adding the transfection mix. Oligomer-Lipofectamine 2000 complexes were prepared by mixing the appropriate amount of siRNA/ASO (volume depending on the desired end concentration, as shown on the left side of table 11) with Opti-MEM. A separate reaction tube with Opti-MEM and Lipofectamine 2000 (mixture shown on the right side of table 11) was prepared in parallel. After incubating these separately for 5 min at room temperature, the Lipofectamine 2000 and siRNA/ASO mixtures were combined and incubated for 20 min at room temperature. Of this transfection mix, 105 µl were added to each well. After 2 hours of incubation at 37 °C, 150 µl culture medium was added and the cells were incubated for a further 24 hours.

24 hours after transfection, cells transfected with Block-It were checked for successful transfection using fluorescence microscopy. Then, culture medium was removed, and the plated cells were frozen at -80 °C until RNA isolation.

Table 11 Reaction mix for Lipofectamine 2000 transfection (per well of a 48well plate)

Concentration	Opti-MEM	Oligomer (100 µM stock)		Opti-MEM	Lipofectamine 2000
0.03 nM	65.625 µl	0.0396 nl	+	65.625 µl	0.6575 µl
0.3 nM	65.625 µl	0.3957 nl	+	65.625 µl	0.6575 µl
1 nM	65.625 µl	1.3191 nl	+	65.625 µl	0.6575 µl
3 nM	65.625 µl	3.9573 nl	+	65.625 µl	0.6575µl
9 nM	65.625 µl	0.01187 µl	+	65.625 µl	0.6575µl
10 nM	65.625 µl	0.01319 µl	+	65.625 µl	0.6575µl
27 nM	65.625 µl	0.03562 µl	+	65.625 µl	0.6575 µl
30 nM	65.625 µl	0.03957 µl	+	65.625 µl	0.6575µl
50 nM	65.625 µl	0.06596 µl	+	65.625 µl	0.6575 µl
81 nM	65.625 µl	0.1068 µl	+	65.625 µl	0.6575 µl
100 nM	65.625 µl	0.1319 µl	+	65.625 µl	0.6575 µl
300 nM	65.625 µl	0.3957 µl	+	65.625 µl	0.6575 µl

3.2.6.2 RNA isolation

RNA from plated cells frozen at -80 °C was isolated with the Qiagen RNeasy Plus Mini Kit following the manufacturer's protocol. All steps were performed under a fume hood at room temperature. RNA was subsequently eluted with 30 µl of RNase-free water. Eluted RNA was transferred into smaller tubes (0.2 ml) for storage, aliquots were taken for measuring the concentration and samples were frozen at -80 °C. Concentration of RNA-samples was determined with the NanoDrop 2000 spectrophotometer.

3.2.6.3 Reverse transcription

For quantitative expression analysis, synthesis of cDNA is necessary, therefore reverse transcription of RNA into cDNA was performed. Up to 1 µg of total RNA per sample, filled up to a volume of 10 µl with RNase-free water, were used. First, RNA samples were denatured at 68 °C for 5 min in the PCR-cycler, then kept at -4 °C. The reaction mix (see table 4) was prepared on ice, adding the M-MLV-RT at the end, and mixed with the RNA samples; total reaction volume was 20 µl. Samples were incubated in the PCR-cycler for 1h at 37 °C, followed by an inactivation step for 5 min at 95 °C, and then immediately frozen at -20 °C until use.

Table 12 Reaction mixture for reverse transcription, per 10 µl of RNA

Ingredient	Volume in µl
5X First Strand Buffer	4
DTT (100mM)	2
Ribolock	1
dNTP mix (4mM each)	1
Random-Primer mix (30.6 pmol/µl)	1
M-MLV RT	1

3.2.6.4 Real-time quantitative PCR

A real-time qPCR was performed with the primer sets COL6A2_alleleG(WT) and COL6A2_alleleT(Mut) to analyse the expression of the gene *COL6A2* in fibroblasts, and subsequently the knock-down efficiency of the tested siRNAs and ASOs. Primers are allele specific due to the last primer base being complementary to the mutation position of the wild type or mutated allele, respectively. The expression of the housekeeping gene *GAPDH* (Glyceraldehyde-3-phosphate dehydrogenase) served as an internal control. The expression in the untreated fibroblasts was used for normalization. The analysis was performed in triplicates with three biological and three technical replications. The composition of the qPCR samples and the qPCR program are shown in Table 13 and 14.

Table 13 Reaction mixture for real-time qPCR, for a total volume of 7,5 µl

Ingredient	Volume in µl
2x PowerUp™ SYBR™ Green Master Mix	3.75
Primer forward (5 pmol/µl)	1.125
Primer reverse (5 pmol/µl)	1.125
cDNA (7,5 ng/µl, diluted in water)	1.5

Table 14 Programm steps for RT qPCR

Step	[°C]	[min:sec]	Loop to step	Repeats
1	50	03:00		1
2	95	10:00		1
3	95	00:15		40
4	60	01:00	3	40
5	95	00:15		1
6	60	00:15		1
7	95	00:15		1

Due to the intercalation of SYBR Green I into double-stranded DNA, every PCR cycle results in an increase of the recorded fluorescence signal in direct proportion to the accumulation of PCR product. Using the SDS 2.2.2 software, threshold cycles (ct) can be calculated for each reaction. These ct values represent the number of PCR cycles needed to reach a certain fluorescence signal threshold. Applying the $2^{-\Delta\Delta ct}$ method, they can be translated into expression fold changes and normalized to the expression in untreated cells.

The calculation using the $2^{-\Delta\Delta ct}$ method started with the determination of arithmetic means of the technical triplicates. The mean ct value of *GAPDH* for each sample was then subtracted from its respective mean ct value of *COL6A2* to calculate Δct (1).

$$(1) \quad \Delta ct = ct(COL6A2) - ct(GAPDH)$$

The mean of the biological replicates of the untreated cells' Δct values was calculated afterwards and subsequently the Δct values of the treated cells were normalized to the respective Δct mean value for the untreated cells determining the $\Delta\Delta ct$ value (2).

$$(2) \quad \Delta\Delta ct = \Delta ct(\text{treated}) - \Delta ct(\text{untreated})$$

From the $\Delta\Delta ct$ values, the relative expression level was then converted into the expression fold change (3). Afterwards, the arithmetic mean of the biological replicates of the treated samples was taken.

$$(3) \quad \text{expression fold change} = 2^{-\Delta\Delta ct}$$

3.2.6.5 Statistical analysis

To perform statistical analysis the software GraphPad Prism 8 was used. For determining the percentage change in expression levels, the expression fold changes of treated and untreated cells were compared in the following way (4):

$$(4) \quad \text{expression \%} = \frac{\text{expression fold change}(\text{treated})}{\text{expression fold change}(\text{untreated})}$$

After testing the normality of data, multiple t-test were applied to calculate significant difference among the tested groups (represented as mean $\pm$ standard deviation). Alpha p-values < 0.05 were considered significant. ROUT outlier test was performed to identify values which were out of range, these values were removed from further analysis.

3.2.7 RNA Sequencing

To elucidate cellular pathways influenced by the mutational status of *COL6A2*, RNA sequencing was performed with wild type and mutated primary fibroblasts in early and late passages as well as with and without L-ascorbic acid stimulation beforehand like in 3.2.2.2. After cultivation of the respective fibroblasts RNA was isolated following the protocol in 3.2.6.2. RNA integrity was analysed in an RNase-free agarose gel. For this, gel chambers were decontaminated with RNase AWAY and for the buffers RNase free water was used. 500 ng RNA and minimum 4 μ l of Loading Dye were added, samples were filled to a total

volume of 9 µl. As standard a 100bp-ladder (8 µl + 4 µl Loading Dye) was used. Samples were then pipetted into the agarose gel and electrophoresis was performed at 110 V for 60 min. 16S and 18S RNA bands were visualized with UV light. For shipping, RNA samples were transferred to 1.5 ml tubes and sent to Novogene Bioinformatics Technology Co. in Hong Kong for RNA sequencing (see Table 15). Then, RNA sequencing data was analysed by the Group for Medical Systems Biology at the University of Lübeck.

Table 15 Content of each sample sent for RNA-sequencing. Samples vary regarding *COL6A2* genotype (mutated versus wild type), culture passage (late versus early) and stimulation with L-ascorbic acid (with stimulation versus without stimulation). Per variant two biological replicates were sent for RNA-sequencing.

Sample	Fibroblast type	Passage	L- ascorbic acid stimulation
1	Mut	P9 (late)	with
2	Mut	P9 (late)	with
3	Mut	P9 (late)	w/o
4	Mut	P9 (late)	w/o
5	Mut	P4 (early)	with
6	Mut	P4 (early)	with
7	Mut	P4 (early)	w/o
8	Mut	P4 (early)	w/o
9	WT	P10 (late)	with
10	WT	P10 (late)	with
11	WT	P10 (late)	w/o
12	WT	P10 (late)	w/o
13	L6534 (wild type)	P11 (late)	with
14	L6534 (wild type)	P11 (late)	with
15	L6534 (wild type)	P11 (late)	w/o
16	L6534 (wild type)	P11 (late)	w/o
17	L6699 (wild type)	P5 (early)	with
18	L6699 (wild type)	P5 (early)	with
19	L6699 (wild type)	P5 (early)	w/o
20	L6699 (wild type)	P5 (early)	w/o

In the analysis, the following comparisons were investigated, resulting in an analysis scheme summarized in table 16:

- Treatment with or without L-ascorbic acid in culture process
- Late versus early passage within each cell type
- Patient versus control cell types

Table 16 Comparison scheme for RNA-sequencing analysis

Name	Compared samples
1	Patient Asc late vs Asc early
2	Patient late vs early
3	Control (L6699) Asc early vs Patient Asc early
4	Control (L6509+L6534) Asc late vs Patient Asc late
5	Control (L6509+L6534) late vs Patient late
6	Control (L6509+L6534) Asc late vs Control Asc early
7	Control (L6509+L6534) late vs Control early
8	Control (L6699) Asc early vs Patient Asc late
9	Control (L6699) early vs Patient late
10	Control (L6509+L6534) Asc late vs Patient Asc early
11	Control (L6509+L6534) late vs Patient early
12	Control (L6699) early vs Patient early

3.2.8 Human Cytokine Array

In order to further examine the impact of *COL6A2* p.G283E on its proteome environment, a proteome profiler human cytokine array was performed. Fibroblasts were split as described in section 3.2.1.2 and transferred to 6-well plates at a concentration of 0.4×10^5 cells per well and subsequently cultivated in 4 ml culture medium with or without L-ascorbic acid stimulation for 5 consecutive days as described in 3.2.2.2. Hereafter, medium of all wells was changed to 4 ml culture medium without L-ascorbic acid and cells were cultured for further 4 days without medium change. Then, 500 µl of cell culture supernatant was removed from each well, transferred to 1.5 ml tubes and centrifuged with 900 rpm for 3 minutes to precipitate cell debris and floating particles. The next steps were carried out following the manufacturer's protocol. The supernatant was collected, and the array was performed following the manufacturer's protocol. Briefly, after blocking of the membranes and preparation of the samples with 500 µl of the supernatant each, both were incubated together overnight at 4 °C on a rocking platform. On the next day, the membranes were washed and developed as described in the manual. Results were visualized and images were taken using the ChemiDoc XRS imaging system.

4 Results

4.1 Collagen VI production in primary fibroblasts

To assess collagen VI production and its localisation in primary fibroblasts we used immunofluorescence staining. For this purpose, wild type human skin fibroblasts denoted as WT, or mutated human skin fibroblasts denoted as Mut were cultured on chamber slides until 80-90% confluence. To enhance collagen production, we used L-ascorbic acid treatment. Staining against collagen VI and fibronectin, which is known to align closely to collagen VI in ECM¹⁴⁵ was performed (see section 3.2.2).

4.1.1 Effect of L-Ascorbic acid on collagen VI production

The effect of L-ascorbic acid supplementation during fibroblast culture on collagen VI biosynthesis was analysed in WT fibroblasts after stimulation with L-ascorbic acid (with). Cells without stimulation were used as controls (w/o). After 5 days of supplementation fibroblasts were stained with antibodies against collagen VI, fibronectin and DAPI.

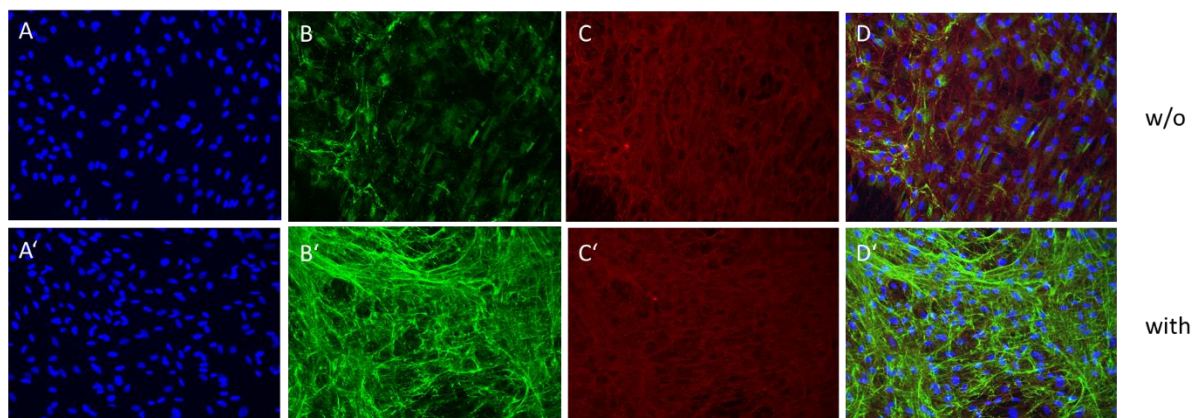


Figure 11 Effect of L-ascorbic acid on collagen VI ECM. Human WT primary fibroblasts without (A-D) and with (A'-D') L-ascorbic acid supplementation during culture. Displayed are fluorescence microscopy images (20x magnification) of WT extracellular matrix stained with DAPI in blue to visualize cell nuclei (A, A'), anti-collagen VI antibody in green (B, B'), anti-fibronectin antibody in red (C, C'), and an overlay picture (D, D'). The experiment comprised two technical replicates of fibroblasts without L-ascorbic acid supplementation and six technical replicates with supplementation.

The immunostaining pictures (figure 11) showed a strong increase in collagen VI fibres in supplemented fibroblasts (B') in comparison to non-supplemented fibroblasts (B). Thus, with this we confirmed a stimulating effect of L-ascorbic acid on collagen VI production of human fibroblasts, which was absent in without L-ascorbic (w/o) treated cells and only basal production of was observed.

4.1.2 Effect of the mutation p.G283E on collagen VI production

To analyse the effect of the mutation c.848G>A (p.G283E) in *COL6A2* on collagen VI production our established fibroblast ECM immunofluorescence assay was carried out with wild type and mutated human skin primary fibroblasts. In both experiments cells were supplemented with L-ascorbic acid to stimulate collagen VI synthesis (see section 3.2.2).

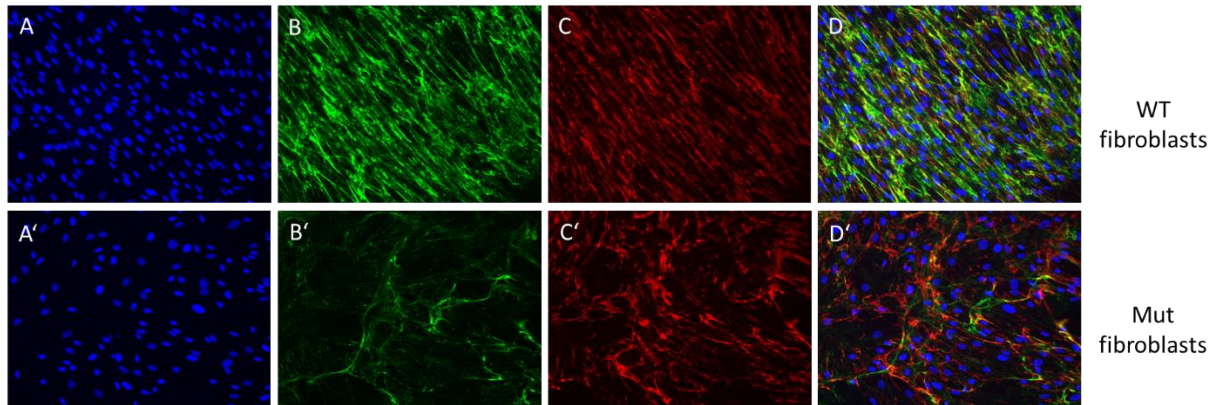


Figure 12 Immunofluorescence staining of collagen VI in primary fibroblasts. Fluorescence microscopy pictures (20x magnification) presented here are from human skin WT fibroblasts (homozygous wild type for *COL6A2*, A-D) and Mut fibroblasts (heterozygous carrier of the p.G283E mutation, A'-D'). For the ECM assay anti-collagen VI antibody (green, B, B') and anti-fibronectin antibody (red, C, C') were performed. DAPI was used as counterstaining to stain cell nuclei (blue, A, A'). Overlay pictures were represented in D and D'. For this assay three chamber slides with each eight wells per slide were used for each fibroblast type. The assay was carried out twice.

In comparison with homozygous WT fibroblasts (figure 12, B) collagen VI secreted by Mut fibroblasts (figure 12, B') was less homogeneously distributed in ECM and decreased in quantity. Furthermore, Mut fibroblasts fibre alignment appeared to be disturbed and resulted in kinked fibres which did not align to form ECM. Fibronectin also showed to be abnormally deposited in Mut fibroblast ECM (figure 12, C'), in comparison to fibronectin deposition in WT fibroblast ECM (figure 12, C). Proliferation rate of Mut fibroblasts was slower therefore they needed more time to reach the desired confluency and be passaged compared to WT fibroblasts.

4.2 Immortalized fibroblasts characterization

In literature, primary fibroblasts are known to show a strong senescence with higher passage numbers¹⁴⁶. From passage 9 onwards, WT and Mut fibroblasts proliferated at much slower rate, therefore sufficient cell density for experiments was difficult to reach in feasible time. To diminish or overcome the aging effects and get a stable cell model, WT and Mut fibroblasts were immortalized. Immortalization was carried out by InSCREENeX GmbH.

4.2.1 Immunofluorescence staining

The immortalized cells were first characterized for the morphological features of collagen VI extracellular matrix using immunofluorescence staining with L-ascorbic acid supplementation. As shown in figure 13, collagen VI distribution and alignment in the ECM of immortalized fibroblasts, denoted as WT-imm. and Mut-imm. significantly differs from ECM of primary fibroblasts (figure 12). Collagen VI ECM in WT-imm. (figure 13, B) appears cloudy and unevenly distributed with no clearly formed fibres. In Mut-imm. (figure 13, B') collagen VI assembles in patches rather than in fibres. This was also seen in fibronectin organization, where fibres appeared patchy (figure 13, C, C').

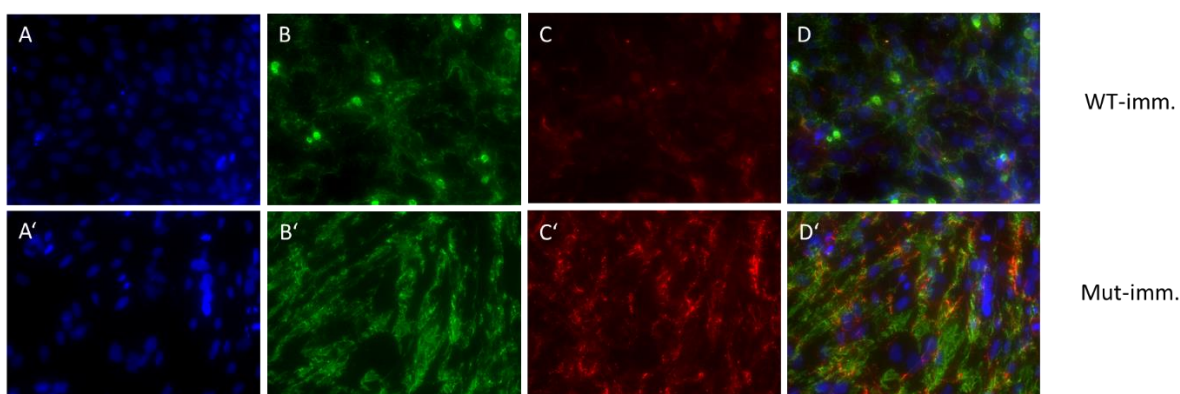


Figure 13 Immunofluorescence staining of collagen VI in immortalized fibroblasts. Displayed are fluorescence microscopy pictures (40x magnification) of immortalized fibroblasts WT-imm. (derived from WT primary fibroblasts, A-D) and Mut-imm. (derived from Mut primary fibroblasts, A'-D'). Shown is the extracellular matrix of fibroblasts cultured on chamber slides and stained with DAPI to visualize nuclei (blue, A, A'), anti-collagen VI antibody (green, B, B'), anti-fibronectin antibody (red, C, C'), and overlay pictures (D, D'). The assay comprised one chamber slide for each fibroblast type with six wells, respectively.

Due to these high differences between immortalized (figure 13) and primary fibroblasts (figure 12) in ECM appearance, further ECM immunostaining experiments were only carried out in primary fibroblasts to ensure comparability.

4.2.2 Selection and analysis of markers expressed specifically in fibroblasts

The immortalized fibroblasts could serve as a feasible model for quantitative knockdown experiments, as the artificial genetic manipulation of the immortalization process leads to indefinite proliferation and the possibility to culture these fibroblasts over many generations. We conducted literature search and selected a list of gene markers specifically expressed by fibroblasts, which are listed in table 17.

Table 17 Identification of fibroblast specific marker genes. Literature research led to a list of fibroblast markers which can be used to validate fibroblasts (green), as well as genes which are not expressed by fibroblasts serving as negative controls (red).

Protein	Gene	Expression in fibroblasts
Cluster of differentiation 44	<i>CD44</i>	yes ¹⁴⁷
Collagen I α 1	<i>COL1A1</i>	yes ¹⁴⁷
Collagen IV α 1	<i>COL4A1</i>	yes ¹⁴⁷
Endosialin	<i>CD248</i>	yes ¹⁴⁷
Fibroblast activation protein	<i>FAP</i>	yes ¹⁴⁷
Fibroblast growth factor receptor 4	<i>FGFR4</i>	yes ¹⁴⁸
Fibronectin	<i>FN1</i>	yes ¹⁴⁷
Integrin beta-1	<i>ITGB1</i>	yes ¹⁴⁷
S100 Calcium Binding Protein A4	<i>S100A4</i>	yes ¹⁴⁹
Thy-1 Cell Surface Antigen	<i>THY1</i>	yes ¹⁴⁷
Vimentin	<i>VMAC</i>	yes ¹⁵⁰
Protein Tyrosine Phosphatase Receptor Type C	<i>PTPRC</i>	no ¹⁴⁷
Sialophorin	<i>SPN</i>	no ¹⁴⁷

To analyse gene expression of the selected positive and negative fibroblast markers on the generated immortalized fibroblasts, we carried out qPCR analysis for these genes in primary fibroblasts, WT and Mut, and immortalized fibroblasts, WT-imm. and Mut-imm., respectively (see section 3.2.2.1).

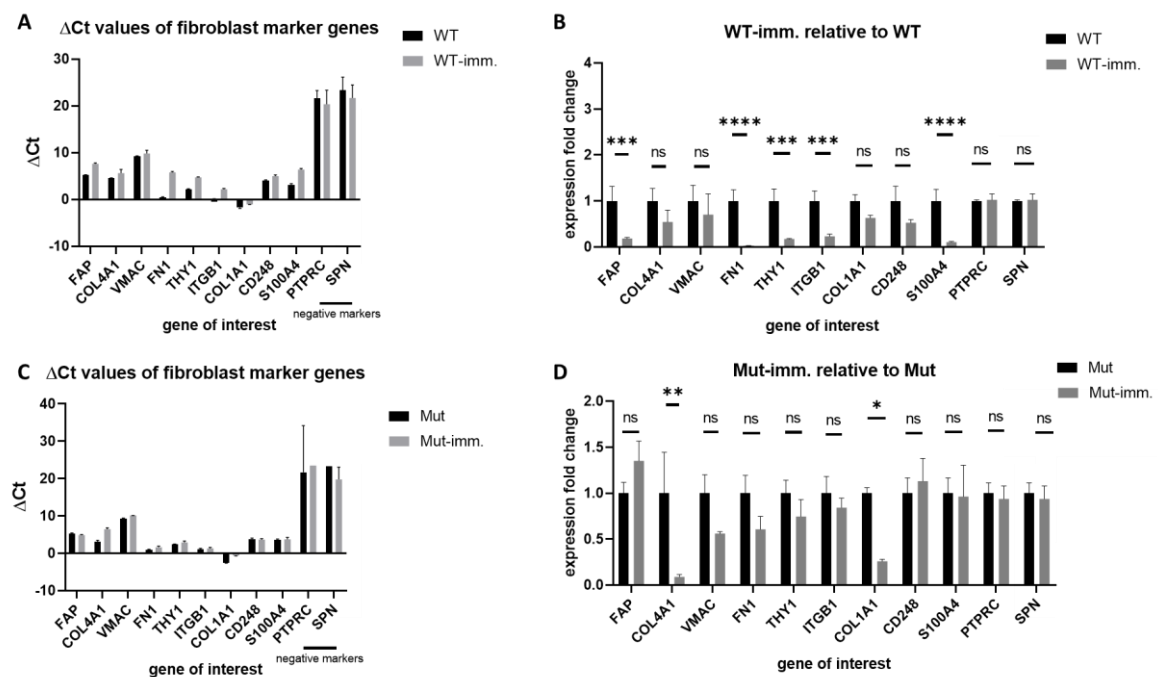


Figure 14 Fibroblast specific marker gene expression. Displayed are ΔC_t values on primary and immortalized wild type (A) and mutated (C) fibroblasts as well as corresponding expression fold changes (B, D). Expression fold changes of marker genes (gene of interest) tested in the immortalized cell lines WT-imm. (B) and Mut-imm. (D) are shown in relation to expression fold changes of their respective primary cell type WT and Mut. Statistical analysis was performed to evaluate differences in gene expression between immortalized and primary fibroblasts (B, D, for statistical analysis method see section 3.2.5.4.1). Three technical replicates were used for this experiment, for statistical analysis multiple t test was performed.

Here we tested 1) Whether the used markers are specific for fibroblasts and 2) whether there are differences on gene expression of the selected markers between immortalized and original primary fibroblasts.

As expected, qPCR analysis on fibroblasts showed a high expression for fibroblast specific markers and no relevant expression (high cycle thresholds and thus high ΔC_t between the gene of interest and the internal standard *GAPDH*) (Figure 14, A, C).

In wild type fibroblasts no significant difference was observed in the expression of 7 out of 11 fibroblast specific markers in WT-imm. fibroblasts compared to primary WT fibroblasts (Figure 14, B). In contrast significant difference in expression was observed for *FAP*, *FN1*, *THY1*, *ITGB1* and *S100A4*. In mutated fibroblasts, 9 out of 11 fibroblast specific markers had similar expression profile in immortalized cell line Mut-imm. compared to their primary counterpart Mut (Figure 14, D), whereas significant changes in expression were seen for *COL4A1* and *COL1A1*.

Based on these results we decided to use the immortalized cells WT-imm. and Mut-imm. as an alternative cell model to the slower proliferating primary fibroblasts WT and Mut, respectively, for gene knockdown experiments.

4.3 siRNA and ASO design

To perform *COL6A2* knockdown experiments siRNAs and ASOs were designed in cooperation with the Institute of Molecular Medicine at University of Lübeck. We designed siRNA and ASOs targeting the mutation *COL6A2* c.848G>A (p.G283E) and planned to design siRNAs and ASOs targeting common variants on *COL6A2*.

4.3.1 SNP identification on *COL6A2*

To identify SNPs in cis region of *COL6A2* c.848G>A, which could serve as potential targets for ASOs and siRNAs, variants were called in the exome analysis data of the patient carrying the mutation c.848G>A on *COL6A2*.

Table 18 Results of variant calling on *COL6A2*. Reference: Ensembl GRCh37 Feb 2014 (see section 3.2.3.1). For each variant the chromosomal location, common and variant base, reference SNP cluster ID, region (exon/intron), total allele frequency (source: gnomAD v2.1.1) and denotation used by us are displayed. Variants on exons are highlighted in yellow.

Chromosomal location	Common base	Variant base	Reference SNP cluster ID	Region	Total allele frequency	Denotation
chr21 47532544	C	T		intron		
chr21 47535832	G	A	rs886044088	exon	n/a	mutation p.G283E
chr21 47536546	C	T		intron		
chr21 47537882	G	A		intron		
chr21 47538960	G	A	rs2839110	exon	0.7803	SNP1
chr21 47539790	A	G		intron		
chr21 47542861	A	G		intron		
chr21 47544541	C	G		intron		
chr21 47545155	A	G		intron		
chr21 47545241	CGG	CG		intron		
chr21 47545768	G	A	rs1042917	exon	0.4875	SNP2
chr21 47545823	G	A	rs13052956	exon	0.4865	SNP3
chr21 47545826	C	T	rs13046639	exon	0.4865	SNP4
chr21 47545913	G	A	rs2839114	exon	0.3830	SNP5
chr21 47551833	C	T		intron		
chr21 47552103	G	T	rs11554669	exon	0.03303	SNP6

As demonstrated in table 18, six variants (SNP1-6) were identified in different exons of *COL6A2* to serve as potential siRNA and ASO targets along with the causative mutation c.848G>A.

4.3.2 Validation of the identified SNPs in Mut fibroblasts by nested PCR

To validate the identified SNPs in Mut fibroblasts and identify on which allele (mutated allele “A” or wild type allele “G”) each SNP is located, allele specific DNA sequencing of *COL6A2* in Mut fibroblasts was carried out via nested PCR (see section 3.2.3.2). Nested PCR consists of two subsequent PCR runs. The first run uses the cDNA which is to be sequenced and primers which are designed to differentiate between the mutated and wild type *COL6A2* allele. The second run uses the PCR product from the first run, so either amplicons of the wild type or mutated allele, and primers flanking the individual SNPs. These products

undergo Sanger sequencing. With this approach, it is known for each sequence from which allele it originates, and allele-specific sequencing is ensured. The sequencing results for Mut fibroblasts and Saos-2 cells are shown in figure 15.

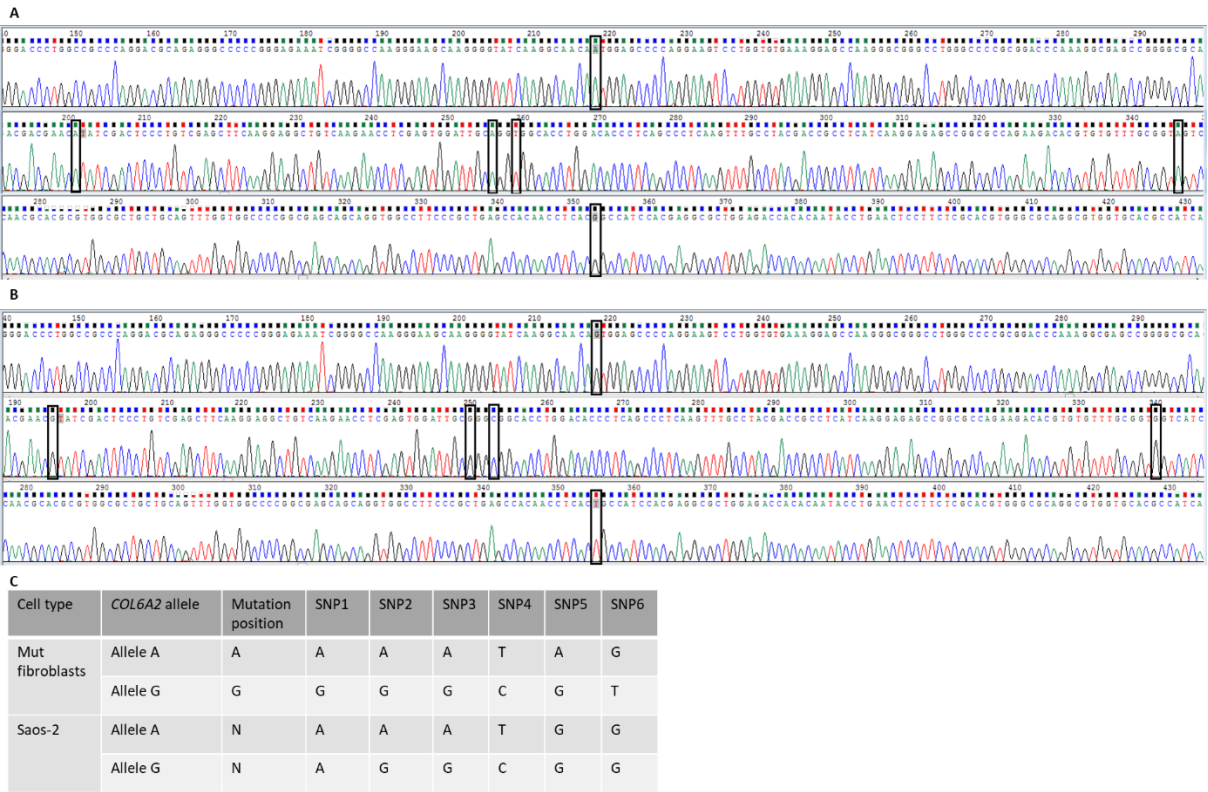


Figure 15 Allele specific sequencing of the SNP positions in Mut fibroblasts. Labeling according to IUPAC nucleotide code A: mutated *COL6A2* allele of Mut fibroblasts, highlighted are the SNP positions, chronologically SNP1-6. B: wildtype *COL6A2* allele, highlighted are the SNP positions, chronologically SNP1-6. C: Summary of allele specific sequencing results displaying how SNP1-6 are linked on each allele in Mut fibroblasts and Saos-2 cells.

Allele specific sequencing confirmed the heterozygous location of SNPs in *COL6A2* (figure 15, A, B) which were previously found based on whole exome data analysis (table 18). It also provided us with the SNP linkage pattern on both *COL6A2* alleles (figure 15, C) to design siRNAs and ASOs.

We investigated and found similar SNP linkage on Saos-2 cells. Due to their fast proliferation, robustness, and efficient transfection, they could be used for preliminary knockdown experiments. Therefore, sequencing of the corresponding regions in *COL6A2* was carried out (figure 15, C). The results showed, that in Saos-2 cells SNP2, SNP3 and SNP4 were heterozygous on *COL6A2* and showed a same linkage (in cis to c.848G>A) as in Mut fibroblasts. Therefore, these SNPs could be targeted in Saos-2 cells as well. SNP1, SNP5 and SNP6 were found homozygous on *COL6A2* which does not allow allele specific targeting by siRNAs and ASOs.

ASOs and siRNAs were designed in cooperation with the Institute of Molecular Medicine at University of Lübeck *in-silico*. Potential candidates were chosen according to different criteria such as target structure, dimerization, nucleic acid structure, melting temperature, GC-content, sequence motive. This filtering process led to a list of candidate ASOs and siRNAs (figure 16 and figure 17). According to the *in-silico* analysis SNP2 and SNP6 are localized in RNA structures which are unlikely to be accessible for RNAi or ASO-targeting. Therefore, we did not proceed to design siRNAs or ASOs targeting SNP2 or SNP6.

A

	940	950	960	970
WT	5'-GGGAGAGCCTGGCCAGAAGG	G AAGACAGGGAGACCCGGGCATCGAAGGC		
pCOL-1G	5'-CCAGAAGGG	G AAGACAGGGAtt		
gCOL-1G	ttGGUCUUCC	C UUCUGUCCCU-5'		

B

	940	950	960	970
Mut	5'-GGGAGAGCCTGGCCAGAAGG	A AAGACAGGGAGACCCGGGCATCGAAGGC		
pCOL-1A	5'-CCAGAAGGA	A AAGACAGGGAtt		
gCOL-1A	ttGGUCUUCC	U UUCUGUCCCU-5'		

C

	940	950	960	970
WT	5'-GGGAGAGCCTGGCCAGAAGG	G AAGACAGGGAGACCCGGGCATCGAAGGC		
Mut	5'-GGGAGAGCCTGGCCAGAAGG	A AAGACAGGGAGACCCGGGCATCGAAGGC		
asCOL-3G		GGTCTTCC	C TTCTGT	TCCC-5'
asCOL-4G		GGACCGGTCTTCC	C TTCT	-5'

To target the c.848G>A mutation position on *COL6A2* computational design provided two siRNAs (figure 16 A, B) and two ASOs (figure 16, both in C). The siRNA siCOL-1G (figure 16, A, pCOL-1G is passenger and gCOL-1G guide strand) targets the wild type allele “G” at the mutation site, siCOL-1A (Figure 16, B, pCOL1-A and gCOL-1A) targets the mutated allele “A”. The two ASOs asCOL-3G and asCOL-4G (Figure 16, C) both target allele “G” and mainly differ in their MO-modification sequences.

4.3.3.2 Design of siRNAs and ASOs targeting SNPs on *COL6A2*

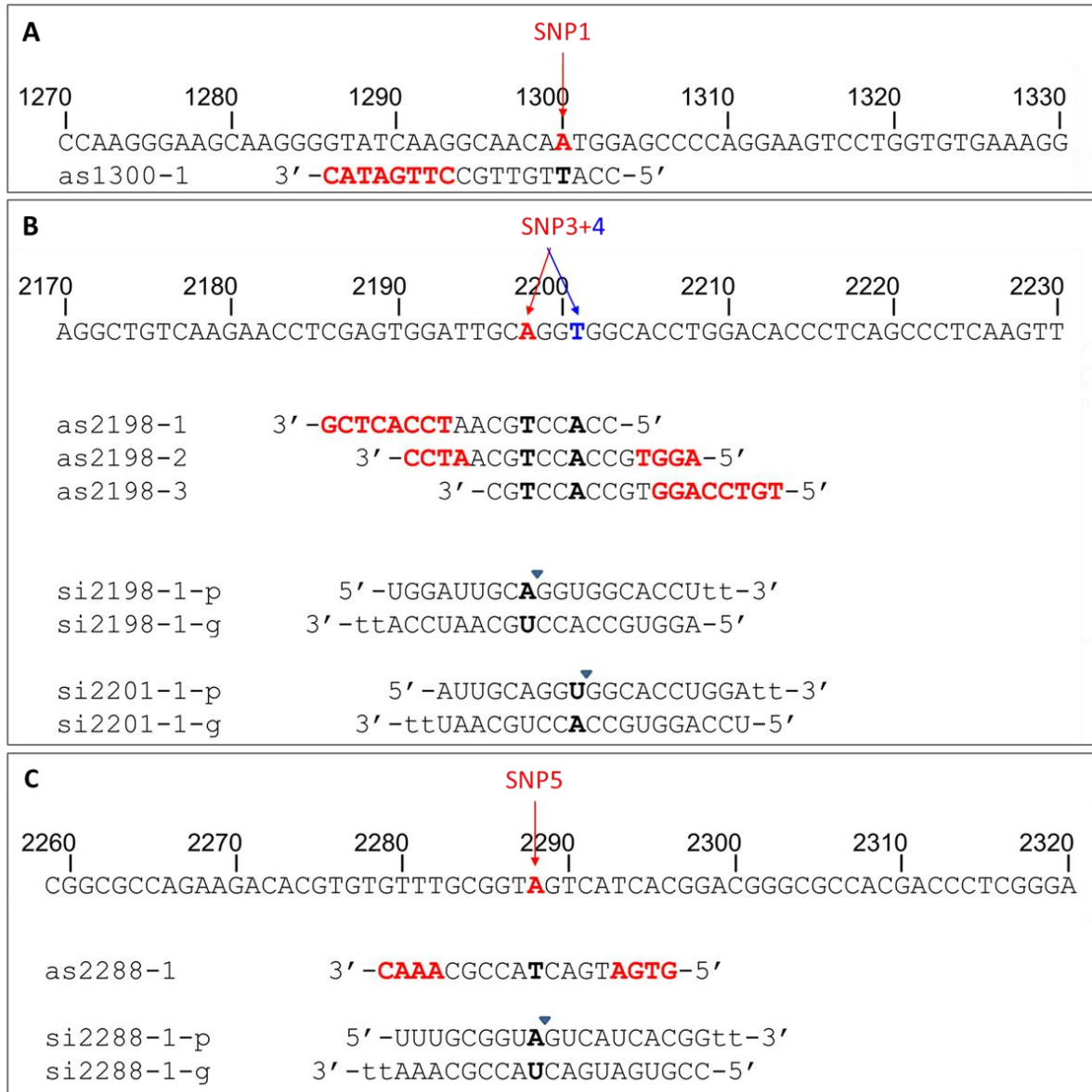


Figure 17 Design of siRNAs and ASOs targeting SNPs on *COL6A2*. Three siRNAs and five ASOs targeting the SNPs on the mutated allele in *COL6A2* in the patient's fibroblasts were designed. Displayed are the mutant allele sequences of *COL6A2* (numbering of *COL6A2* reference sequence: Ensembl ENST00000300527 (*COL6A2*-201)) with the respective SNP highlighted in red and blue and, in alignment to it, the nucleic acid sequences. ASOs: all-bonds PTO; highlighted in red: MO-modification. siRNAs: cleavage position marked with an arrow.

To target the identified common variants on *COL6A2* in section 4.3.2, we designed five ASOs and three siRNAs, as shown in figure 17. SNP1 is targeted by the ASO as1300-1 (figure 17, A). SNP3 and SNP4 can be targeted by a single ASO due to their proximity. The three ASO as2198-1, as2198-2 and as2198-3 target SNP3 and SNP4 together (figure 17, B). In addition, SNP3 is targeted by the siRNA si2198-1 (comprised of the passenger strand si2198-1-p and the guide strand si2198-1-g, figure 17, B) and SNP4 is targeted by the siRNA si2201-1 (comprised of si2201-1-p and si2201-1-g, figure 17, B) separately. To target SNP5 we designed the ASO as2288-1 and the siRNA si2288-1 (comprised of si2288-1-p and si2288-1-g, figure 17, C).

4.4. ASOs and siRNAs targeting at the mutation position

4.4.1 Allelic discrimination and target accessibility test

To test target accessibility at the mutation site c.848G>A on *COL6A2* the expression levels of *COL6A2* wild type allele “G” messenger were measured after treatment with ASOs and siRNAs (see section 3.2.6). The experiment was carried out on cDNA from Saos-2 cells (homozygous wild type for *COL6A2*). Primers which were proven to be able to differentiate between the wild type and mutated allele were used (*COL6A2* wild type forward and reverse, *COL6A2* mutated forward and reverse, see section 3.1.4). These primers contain single nucleotide exchanges at their last nucleotide position which are designed to fit complementary to the mutation position and according to the targeted allele.

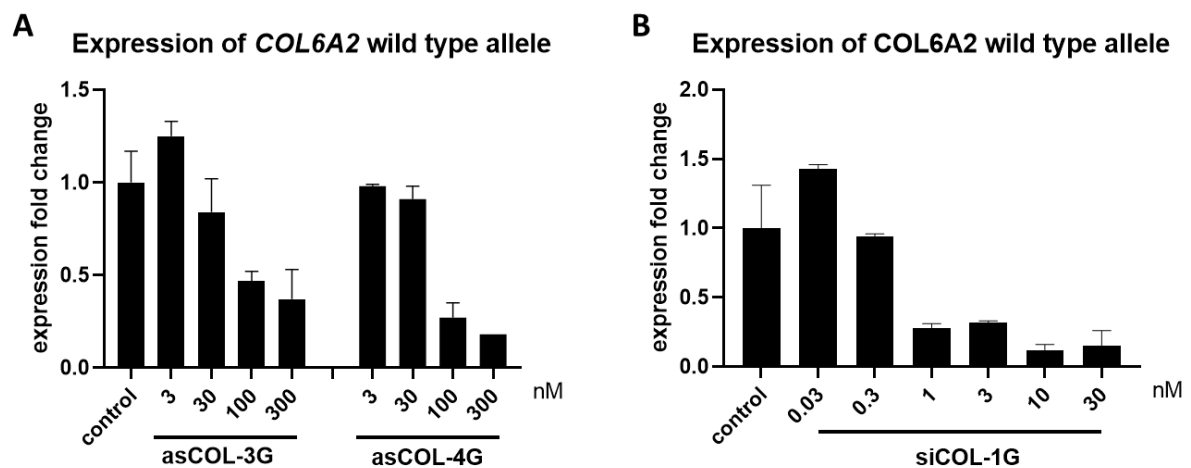


Figure 18 Target accessibility test of the mutation site on *COL6A2*. A: Displayed are expression fold changes of the WT *COL6A2* allele “G” after treatment with asCOL-3G or asCOL-4G at concentrations of 3, 30, 100 and 300 nM. The scrambled ASO sc1570 served as untreated control to which expression fold changes of treated cells were normalized. B: Shown are expression fold changes of *COL6A2* allele “G” after treatment with siCOL-1G at concentrations of 0,03, 0,3, 1, 3, 10 and 30 nM. The scrambled siRNA si-scrambled was used as a untreated control, treated samples were normalized to it. The experiment contained two biological and three technical replicates per variant.

First, we tested the knockdown effect of *COL6A2* after treatment with ASOs (asCOL) and siRNAs (siCOL) in WT cells carrying the G allele. The qPCR results represented in figure 18 show a dilution-dependent knockdown of *COL6A2* mRNA in cells treated with asCOL-3G and asCOL-4G (figure 18, A) as well as siCOL-1G (figure 18, B). This indicated a sufficient accessibility of the mutation site on *COL6A2* for ASO and siRNA targeting.

Next, we analysed the discrimination of ASOs and siRNAs between the wild type allele “G” and mutated allele “A” of *COL6A2*. For this, transfection of asCOL-3G, asCOL-4G at a concentration of 100 nM and siCOL-1G at a concentration of 50 nM was carried out in the primary Mut fibroblasts (heterozygous for c.848G>A on *COL6A2*). Then, expression fold changes of *COL6A2* allele “G” and allele “A” mRNA after with ASO or siRNA were examined.

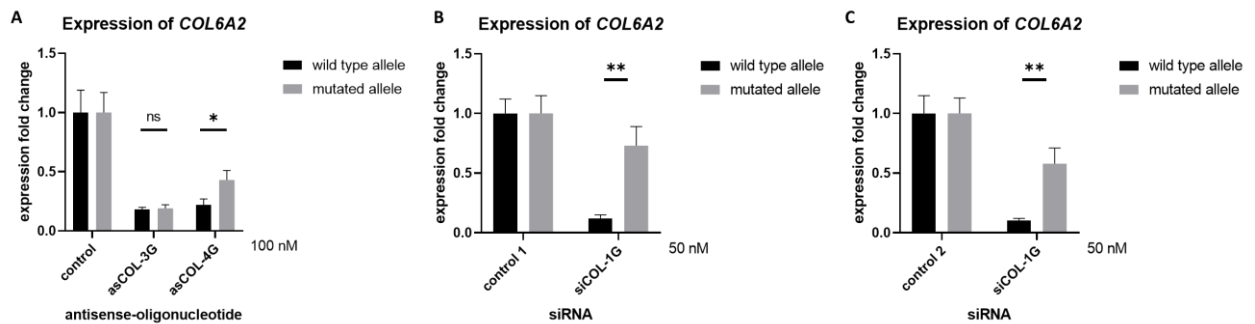


Figure 19 Allelic discrimination of ASOs and siRNAs between wild type and mutated *COL6A2* allele. A: Displayed are expression fold changes of *COL6A2* wild type “G” and mutated “A” allele after treatment with asCOL-3G and asCOL-4G. As a control the scrambled antisense oligonucleotide sc1570 was used, expression fold changes of asCOL-3G and -4G are calculated in relation to control. B: Shown are expression fold changes of *COL6A2* mutated and wild type allele after treatment with siCOL-1G. As a control a scrambled siRNA was used (control 1). C: Expression fold changes of *COL6A2* mutated and wild type allele after treatment with siCOL-1G. As a control Block-It was used (control 2). Since the commercially available Block-It does not provide sequence data, we used two different controls in case we observe an effect of the control on *COL6A2* expression. The experiment was carried out with three biological and three technical replicates per variant. Statistical analysis was performed using multiple t testing.

Like shown in figure 19 (A), treatment with asCOL-3G leads to a mRNA knockdown of 82 % and 81 % of wild type and mutated *COL6A2* allele, respectively. Therefore, treatment with asCOL-3G did not achieve a significant allelic discrimination for *COL6A2*. Treatment with asCOL-4G led to a significant allelic discrimination with a knockdown of 78 % knockdown for wild type allele and 57 % knockdown of mutated allele compared to control (100 %). Although allelic discrimination was significant, off-target knockdown was too high (57 %) to consider asCOL-4G as a promising candidate for further experiments.

Transfection of siCOL-1G (Figure 19, B) led to a highly significant knockdown of *COL6A2* wild type allele in comparison to the expression of the mutated allele. The wild type allele expression was reduced by 88 % (control 1), the mutated allele by 27 % (control 1). Using control 2 for normalization of the qPCR data (figure 19, C), we calculated a reduction of wild type allele expression by 90 % and a knockdown of mutated allele of 42 %. On average, siCOL-1G at a concentration of 50 nM achieved a target allele knockdown of 89 % (+/- 1 %) and an off-target allele knockdown of 34 % (+/- 10 %). Due to this successful and sufficient allelic discrimination of siCOL-1G, we proceeded to start experiments with siCOL-1A targeting the mutated allele (allele “A”) in primary and mutated fibroblasts.

4.4.2 *COL6A2* mRNA knockdown using siCOL-1A

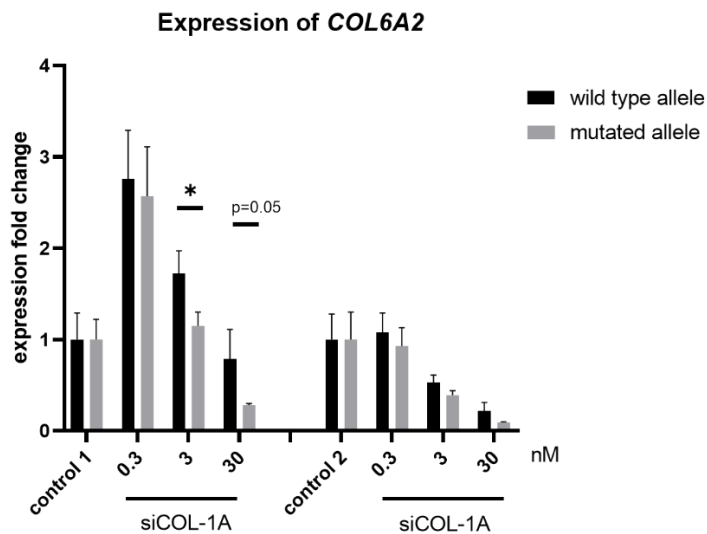


Figure 20 Knockdown of *COL6A2* in Mut fibroblasts by siCOL-1A. Shown are expression fold changes of *COL6A2* wild type and mutated allele after treatment of Mut fibroblasts with siCOL-1A targeting the mutated allele (allele “A”). Two controls were used for calculation of relative expression: control 1: si-scrambled, control 2: Block-It. The experiment was performed with three biological and three technical replicates per variant. For statistical analysis multiple t testing was used.

Treatment with siCOL-1A leads to a dilution-dependent knockdown of *COL6A2* on mRNA level with which can be seen in figure 20. At a concentration of 30 nM, allelic discrimination is visible and near-to-significant. To further investigate the siRNA’s effect on *COL6A2*, dilution series was expanded in the following transfection (figure 21).

Normalization to si-scrambled (control 1) led to the impression of a strong *COL6A2* overexpression in low siRNA concentrations (figure 20). This observation indicated that there might be an effect of si-scrambled itself on *COL6A2* levels. Due to this, we did not use si-scrambled (control 1) in further experiments. For all further experiments Block-It was used as control.

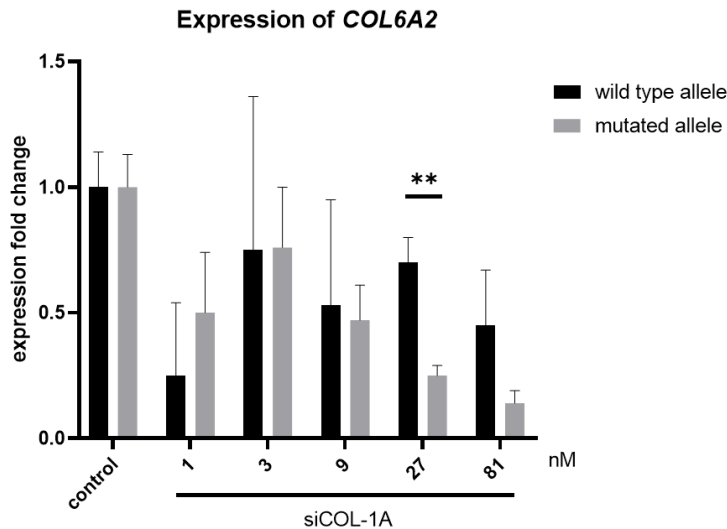


Figure 21 Knockdown of *COL6A2* in Mut fibroblasts by si-COL1A in extended dilution series. Displayed are expression fold changes of *COL6A2* in Mut fibroblasts after treatment with siCOL-1A in an extended dilution series. Block-It was used as control.

We repeated the previous experiment (figure 20) factor-3-dilution series of siCOL-1A starting from a concentration of 1 nM. As visible in figure 21, except for the lowest concentration of 1 nM, a dilution-dependent *COL6A2* knockdown by siCOL-1A was confirmed. At a siCOL-1A concentration of 27 nM a highly significant allelic discrimination with a knockdown of the mutated allele by 75% and a knockdown of the wild type allele by only 30% was observed.

To test if we can see the same results in another in our immortalized fibroblasts setting this experiment was repeated using Mut-imm. (figure 22).

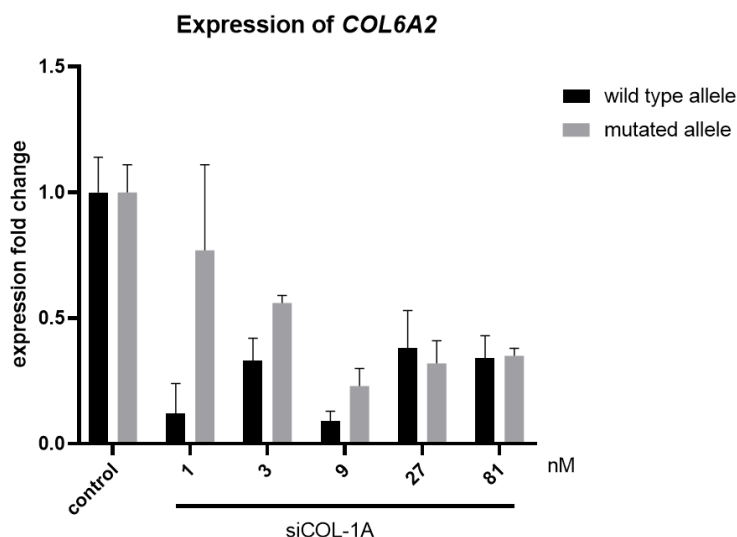


Figure 22 Knockdown of *COL6A2* in immortalized fibroblasts Mut-imm. by siCOL-1A. Shown are expression fold changes of *COL6A2* mutated and wild type allele after treatment of immortalized fibroblasts Mut-imm. with siCOL-1A in dilution series. Control 2 was used, expression fold changes of samples treated with siCOL-1A were calculated in relation to control.

Represented in figure 22, the knockdown of *COL6A2* after treatment with siCOL-1A could be confirmed in mutated immortalized fibroblasts Mut-imm. However, off-target knockdown of the wild type allele was observed to be stronger in immortalized fibroblasts, especially in lower siRNA concentrations. Therefore siCOL-1A did not show significant allelic discrimination in transfection of immortalized fibroblasts.

4.5 ASO and siRNA targeting SNPs on *COL6A2*

4.5.1 *COL6A2* mRNA knockdown

We tested whether SNPs on *COL6A2* are accessible for siRNA- and ASO-application. The high number of cells needed for this experiment could not be reached by primary fibroblasts culture due to senescence and lack of cells at low passage for this line. The previously identified SNP linkage pattern on *COL6A2* in Saos-2 cells allowed us to carry out *COL6A2* knockdown experiments. However, only SNP1, SNP3 and SNP4 were targeted using Saos-2 cells as a model with the respective ASOs and siRNAs (as13001-1, as2288-1, as2198-2, as2198-3, si2198-1 and si2201-1). For SNP5, Saos-2 cells are homozygous for the allele “G” in Mut fibroblasts (figure 15), therefore cannot be targeted by as2288-1 and si2288-1.

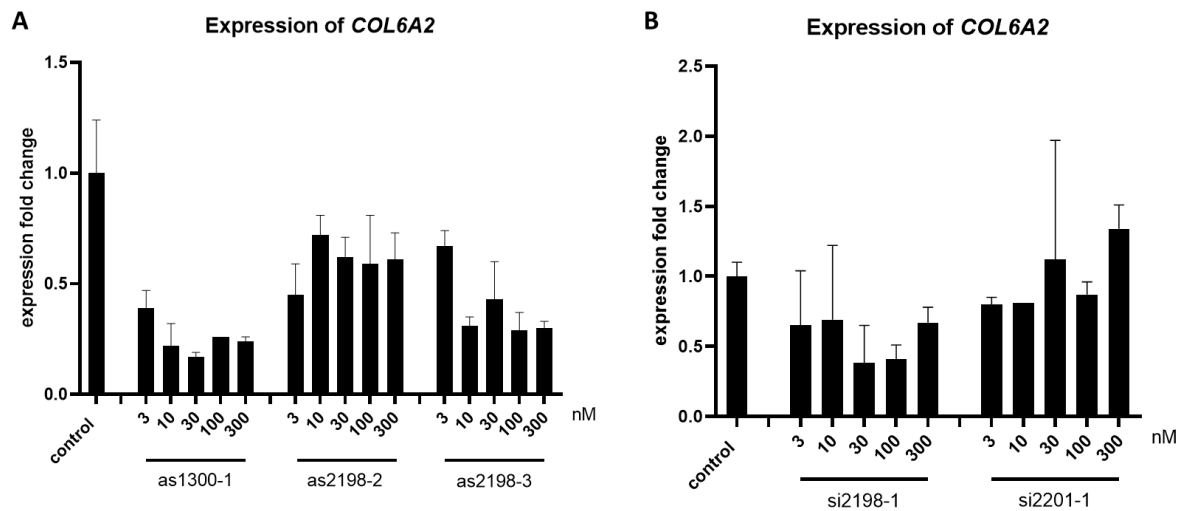


Figure 23 Target accessibility test of SNP1, SNP3 and SNP4 on *COL6A2*. Displayed are expression fold changes of *COL6A2* (allele “A”) after treatment of Saos-2 cells with ASOs (A) and siRNAs (B) targeting SNPs on *COL6A2*. As control for ASOs sc1570 was used (A), and for siRNAs Block-It was used (B). Expression fold changes were calculated in relation to the respective control. A: SNP1 was targeted by as1300-1; as2198-2, as2198-3 targeted both SNP3 and SNP4; si2198-1 targeted SNP3; si2201-1 targeted SNP4. For the experiment two biological and three technical replicates were used.

The qPCR results from figure 23 showed partially a knockdown of *COL6A2* mRNA in cells treated with ASOs and siRNAs. SNP1 on *COL6A2* shows accessibility when targeted by as1300-1 (figure 23, A). Until an as1300-1 concentration of 30 nM there is a dilution-dependent *COL6A2* knockdown up to 83 %, however, *COL6A2* levels do not correlate with higher concentrations of as1300-1. SNP3+4, too, showed accessibility for ASO-targeting.

Treatment with as2198-2 and as2198-3 led to a *COL6A2* knockdown of up to 55 % for as2198-2 at 3 nM and 71 % for as2198-3 at 100 nM. There was no clear correlation between concentration of ASOs and *COL6A2* expression fold change. SNP3 was successfully accessed by si2198-1 (figure 23, B). Treatment of Saos-2 cells with si2198-1 led to a maximum *COL6A2* knockdown of 62 %. However, dilution dependent *COL6A2* knockdown was not clear especially in siRNA concentrations higher than 100 nM. SNP4 did not show sufficient accessibility for si2201-1 (Figure 23, B), as only maximum *COL6A2* knockdown of 20 % was reached and expression fold changes did not correlate with concentration of si2201-1.

To analyse *COL6A2* knockdown and allelic discrimination of these ASOs and siRNAs, they were transfected into the immortalized mutated fibroblasts Mut-imm. in dilution series.

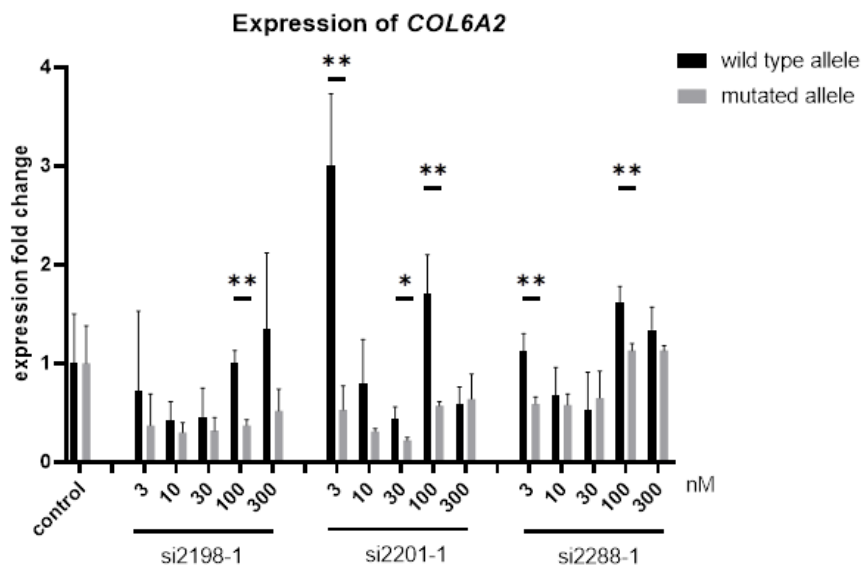


Figure 24 Knockdown of *COL6A2* via SNP-targeting siRNAs in Mut-imm. Displayed are expression fold changes of *COL6A2* mutated and wild type allele after treatment with siRNAs targeting SNPs on *COL6A2*. As control Control 2 was used, and expression fold changes were calculated relative to it. si2198-1 targeted SNP3; si2201-1 targeted SNP4; si2288-1 targeted SNP5 on *COL6A2*. For the experiment three biological and three technical replicates were used per variant, statistical analysis was performed with multiple t tests.

Treatment with si2198-1 (SNP3) led to a maximum *COL6A2* mutated allele knockdown of 70% at a concentration of 10 and 30 nM and minimum knockdown of 48 % at 100 nM (figure 24). Allelic discrimination was significant at a concentration of 100 nM. A dilution dependent knockdown was not observed. Targeting SNP4 with si2201-1 showed a maximum *COL6A2* mutated allele knockdown of 78 % at a concentration of 30 nM with significant allelic discrimination at 100 nM. However, there was again no correlation between concentration of si2201-1 and *COL6A2* expression fold changes visible. Treatment with si2288-1 (SNP5) did only achieve a maximum *COL6A2* knockdown of 42 % at a concentration of 10 nM.

Due to the lack of dilution dependent knockdown of *COL6A2* mutated allele and strong off-target knockdown of the *COL6A2* wild type allele, si2198-1, si2201-1 and si2288-1 did not appear as promising candidates for the planning of a phenotype rescue experiment.

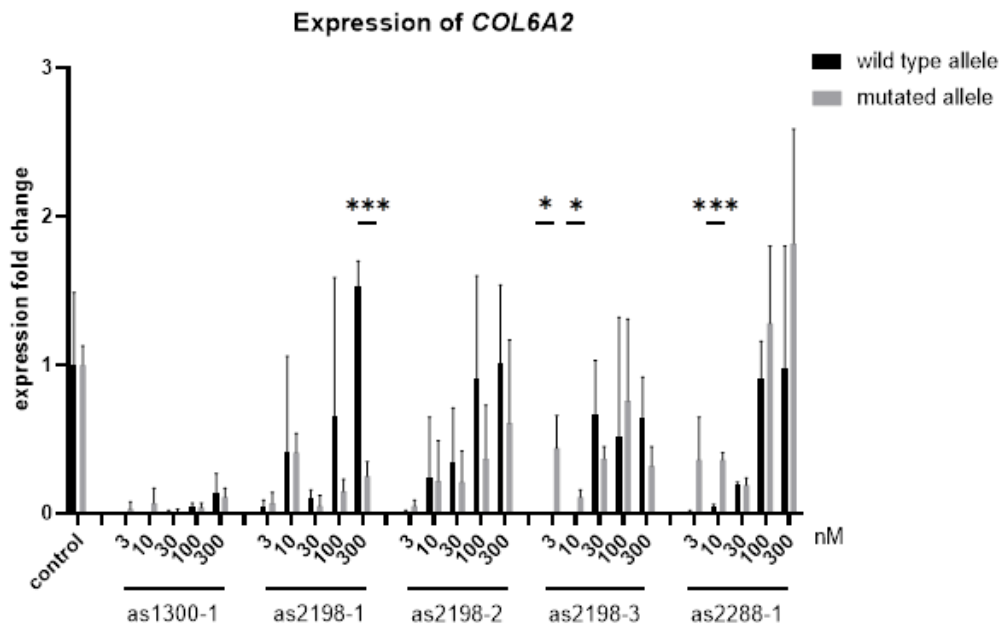


Figure 25 Knockdown of *COL6A2* via SNP-targeting ASOs in Mut-imm. Shown are expression fold changes of *COL6A2* mutated and wild type allele after treatment with ASOs targeting SNPs on *COL6A2*. As control sc1570 was used, and expression fold changes were calculated relative to it. As1300-1 targeted SNP1; as2198-1, as-2198-2 and as2198-3 targeted SNP3 and SNP4; as2288-1 targeted SNP5 on *COL6A2*. The experiment comprised three biological and three technical replicates per variant, statistical analysis was carried out using multiple t tests.

Treatment with as1300-1 targeting SNP1 showed a strong knockdown (up to 99 %) of *COL6A2* in all concentrations, however, there was no significant allelic discrimination (Figure 25). To target SNP3 and SNP4 as2198-1, -2 and -3 were used. All three ASOs led to a *COL6A2* knockdown with partially significant allelic discrimination, but a dilution-dependent knockdown of the mutated allele of *COL6A2* could not be observed. A similar result is shown by as2288-1 targeting SNP5 with a maximum *COL6A2* mutated allele knockdown of 81 % at 30 nM, but very strong off-target knockdown of *COL6A2* wild type allele (significant at 10 nM). None of the ASOs which were tested here showed a sufficient allelic discrimination or dilution-dependent knockdown of mutated *COL6A2*.

Due to these results which are summarized in table 19, we decided for siCOL-1A as the most promising candidate to achieve sufficient dilution-dependent *COL6A2* mutated allele knockdown and allelic discrimination and, therefore, rescue the collagen VI phenotype.

Table 19 Summary of results from siRNA and ASO tests. Here, our results from qPCR analysis are summarized. Important for the evaluation of a siRNA or an ASO are especially a dilution-dependent knockdown (KD), allelic discrimination, which are evaluated in this table, and a sufficient KD potential for the mutated COL6A2 allele, summarized by the maximum and minimum KD of each allele and the siRNA or ASO concentration at which this KD was reached.

Target	Name	Dilution-dependent knockdown	Allele discrimination	Maximum knockdown		Minimum knockdown	
				WT allele	Mut allele	WT allele	Mut allele
Mutation	siCOL-1A	yes	partially	78% (30 nM)	91% (30 nM)	0% (0,3 nM)	7% (0,3 nM)
SNP3	si2198-1	no	partially	58% (10 nM)	70% (10 nM)	0% (100 and 300 nM)	48% (300 nM)
SNP4	si2201-1	no	partially	56% (30 nM)	78% (30 nM)	0% (3 and 100 nM)	36% (300 nM)
SNP5	si2288-1	no	partially	47% (30 nM)	42% (10 nM)	0% (100 and 300 nM)	0% (100 and 300 nM)
SNP1	as1300-1	no	no	100% (10 nM)	99% (30 nM)	86% (300 nM)	89% (300 nM)
SNP3+4	as2198-1	no	partially	95% (3 nM)	95% (30 nM)	0% (300 nM)	59% (10 nM)
SNP3+4	as2198-2	no	no	99% (3 nM)	95% (3 nM)	0% (300 nM)	39% (300 nM)
SNP3+4	as2198-3	no	partially	100% (3 and 10 nM)	89% (10 nM)	20% (100 nM)	24% (100 nM)
SNP5	as2288-1	no	partially	99% (3 nM)	81% (30 nM)	2% (300 nM)	0% (100 and 300 nM)

4.6 Collagen VI phenotype rescue

To test, if siCOL-1A was able to restore collagen VI production and organisation in fibroblast ECM, an in vitro phenotype rescue approach was performed. For this, Mut fibroblasts were cultured on chamber slides, transfected with siCOL-1A (and siCOL-1G and Block-It), stimulated with L-ascorbic acid and then underwent immunofluorescence staining (section 3.2.2.3). The representative immunofluorescence pictures are displayed in figure 27.

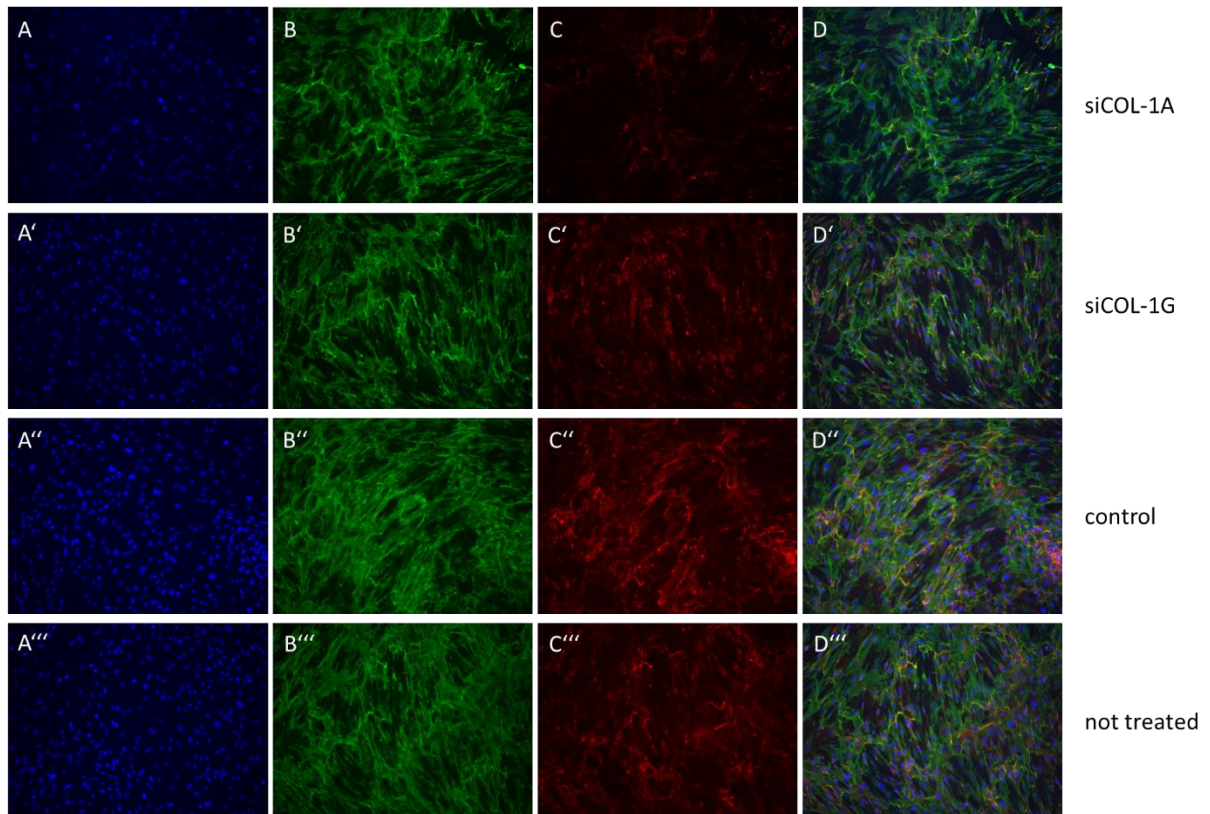


Figure 26 Phenotype rescue experiment with siCOL-1A. Displayed are fluorescence microscopy pictures of Mut fibroblasts (heterozygous carriers of c.848G>A on *COL6A2*) treated with siCOL-1A at a concentration of 27 nM per well (A-D) to rescue the collagen phenotype. For comparison, siCOL-1G targeting the wild type allele at the same position was transfected (A'-D'), Block-It as a control siRNA (A''-D'') and untreated fibroblasts (A'''-D''') were analyzed, too. Immunofluorescence staining included DAPI to visualize nuclei (blue, A-A'''), anti-collagen VI antibody (green, B-B''') and anti-fibronectin antibody (red, C-C'''). Additionally, an overlay picture is displayed (D-D'''). The assay was performed twice. The first experiment comprised three chamber slides with two wells per variant (siCOL-1A, control, not treated). For the second repetition two chamber slides with two wells per variant (siCOL-1A, siCOL-1G, control, not treated) were used.

Immunofluorescence staining showed a similar collagen VI ECM quantity and organisation in fibroblasts treated with 27 nM siCOL-1A (figure 27, B) in comparison to fibroblasts treated with siCOL-1G (figure 27, B'). In comparison to fibroblasts treated with control siRNA (figure 27, B'') or not treated (figure 27, B'''), collagen VI appeared even less in quantity. Therefore, treatment with siCOL-1A at 27 nM did not improve collagen VI ECM. The effect of siCOL-1A on *COL6A2* on mRNA level (see section 4.4.2) could not be translated to rescue the phenotype on protein level.

4.7 RNA sequencing

To better understand the effect of *COL6A2* c.848G>A, the effect of aging and L-ascorbic acid stimulation in fibroblasts, RNA sequencing and analysis was carried out in cooperation with the Group for Medical Systems Biology at the University of Lübeck (see section 3.2.7).

4.7.1 Differential gene expression (DGE) and gene enrichment analysis

To identify differences in gene expression between different types of fibroblasts, differential gene expression analysis was performed. In this, in total 12 comparisons were made depending on *COL6A2* genotype, fibroblast age and L-ascorbic acid stimulation (table 20).

Table 20 Differential gene expression analysis. Displayed are the numbers of significantly differentially expressed genes, defined by q values from likelihood ratio tests (lrt), calculated by SLEUTH. Comparisons (1-12) were made considering *COL6A2* genotype: mutated (Mut) (heterozygous carrier of c.848G>A) and wild type (WT) fibroblasts (three different fibroblasts donors L6509, L6534, L6699 all three homozygous wild type for *COL6A2*); fibroblast passage: early passage (passages 4 or 5) versus late passage (passages 9, 10 or 11); L-ascorbic acid stimulation: with or without (w/o) L-ascorbic acid (AA) stimulation for 5 days during culture.

Comparison No.	q lrt < 0.05	Condition	AA-stimulation
1	276	Mut late vs early	with
2	0		w/o
3	2709	WT early vs Mut early	with
12	1506		w/o
4	1398	WT late vs Mut late	with
5	984		w/o
6	533	WT late vs early	with
7	730		w/o
8	4950	WT early vs Mut late	with
9	3138		w/o
10	1017	WT late vs Mut early	with
11	1005		w/o

We observed from the analysis summarized in table 20 that fibroblasts stimulated with L-ascorbic acid generally showed stronger differences in gene expression when comparing mutated to wild type fibroblasts (exception: WT late vs early).

We further investigated, which gene sets show an upregulation in Mut fibroblasts of early passage. Table 21 below summarizes gene sets which are upregulated in both, L-ascorbic acid supplemented and not supplemented Mut fibroblasts.

Table 21 Gene enrichment analysis in mutated fibroblasts, early passage. Displayed are upregulated gene sets in mutated cells of early passage in comparison to wild type fibroblasts of early passage based on gene enrichment analysis using the REACTOME database and Gene Ontology (GO) terms. It was differentiated between cells with and without L-ascorbic acid stimulation. Green indicates an upregulated gene set in the mutated fibroblasts, grey indicates no difference between mutated and wild type cells.

REACTOME			GO terms		
Gene set	with AA	w/o AA	Gene set	with AA	w/o AA
RNA and protein pathways			Tumor response pathways		
Cell cycle			Mitochondrial pathways		
Notch-1 signaling			Mitochondrial translation		
Electron transport			Translation		
Fibrin clotting			Metabolic processes		
Wnt signaling			ncRNA processes		
NFkB			Glycosyl processes		
Apoptosis					

The results of the gene enrichment analysis are summarized in table 21. According to REACTOME database, mutated fibroblasts in comparison to wild type fibroblasts show an upregulation of RNA and protein pathways, as well as pathways involved in cell cycle and Wnt signaling. In GO terms an upregulation of tumor response pathways, protein translation and metabolic processes were observed.

In REACTOME it was seen that fibroblasts stimulated with L-ascorbic acid showed upregulation of Notch-1-signaling and fibrin clotting. In contrast, lack of L-ascorbic acid stimulation led to an upregulation of apoptosis and NFkB pathway. Results of GO terms showed that cells stimulated with L-ascorbic acid showed an upregulation of mitochondrial translation and, on the other hand, cells without stimulation had upregulated gene sets regarding mitochondrial pathways, non-coding RNA (ncRNA) processes and glycosyl processes.

The same analysis was carried out for fibroblasts of late passage where we found a partly different set of upregulated genes, as it is summarized in table 22.

Table 22 Gene enrichment analysis in mutated fibroblasts, late passage. Displayed are upregulated gene sets in mutated cells of late passage in comparison to wild type fibroblasts of late passage based on gene enrichment analysis using the REACTOME database and Gene Ontology (GO) terms. Fibroblasts with and without L-ascorbic acid stimulation were investigated separately. Green indicates an upregulated gene set in the mutated fibroblasts, grey indicates no difference between mutated and wild type cells.

REACTOME			GO terms		
Gene set	with AA	w/o AA	Gene set	with AA	w/o AA
Notch3 signaling			Developmental pathways		
Notch1 signaling			Disassembly of extracellular matrix		
Adenylate inhibitor pathway			Mitochondrial membrane		
RNA/Protein pathways			Hydrogen transport		
Glycosylation of mucins					
Extrinsic apoptosis pathway					

These results from REACTOME database demonstrated that mutated fibroblasts stimulated with L-ascorbic acid showed in comparison to wild type cells an upregulation of Notch-3-signaling, adenylate inhibitor pathway and extrinsic apoptosis pathway. In contrast, lack of L-ascorbic acid stimulation led to an upregulation of Notch-1-signaling, RNA and protein pathways and glycosylation of mucins. According to GO terms database mutated cells stimulated with L-ascorbic acid in comparison to wild type fibroblasts showed an upregulation of developmental pathways and disassembly of extracellular matrix. On the other hand, cells without stimulation had upregulated gene sets regarding mitochondrial membrane and hydrogen transport.

4.7.2 Impact of *COL6A2* c.848G>A on network diffusion

To examine how the *COL6A2* c.848G>A mutation affects other genes, network diffusion was analysed.

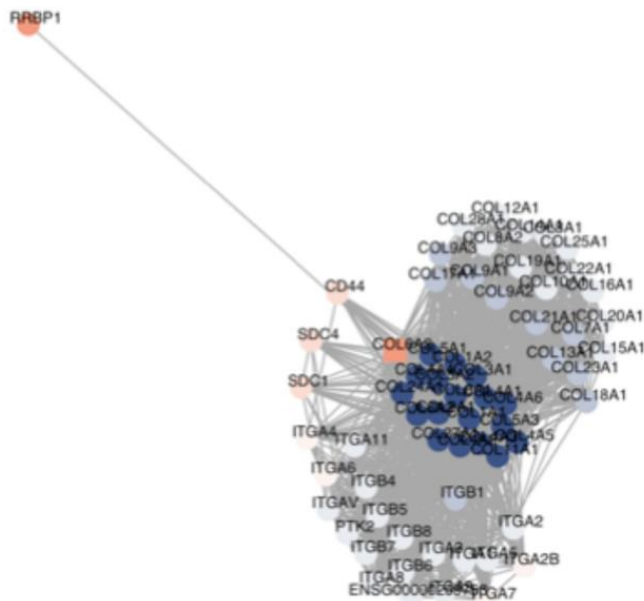


Figure 27 Network diffusion of mutated cells. Displayed is a network diffusion of mutated fibroblasts. Genes displayed close to *COL6A2* (marked in orange) lie in functional proximity to *COL6A2* in gene network.

The results displayed in figure 28 show that majorly genes from the integrin family (e.g., *ITGA6*, *ITGA11*, *ITGB4*) and other collagens (e.g., *COL12*, *COL18*, *COL9*) were affected by the mutation on *COL6A2*. These genes, and others like *SDC4* (Syndecan 4) or *SDC1* (Syndecan 1), lie functionally near *COL6A2*, as it is pictured in figure 28. The mutation on *COL6A2* does not seem to have many effects on distal genes. The most distal gene affected by *COL6A2* c.848G>A is *RRBP1* (Ribosome-Binding Protein 1).

4.7.3 Secretome analysis

As collagen VI is a protein secreted by fibroblasts into ECM, we anticipated to find an impact of the *COL6A2* c.848G>A mutation on other genes encoding for secretory proteins. To investigate this, secretome analysis from our RNA-sequencing data was performed and results visualized in network plots (figure 29 and figure 30).

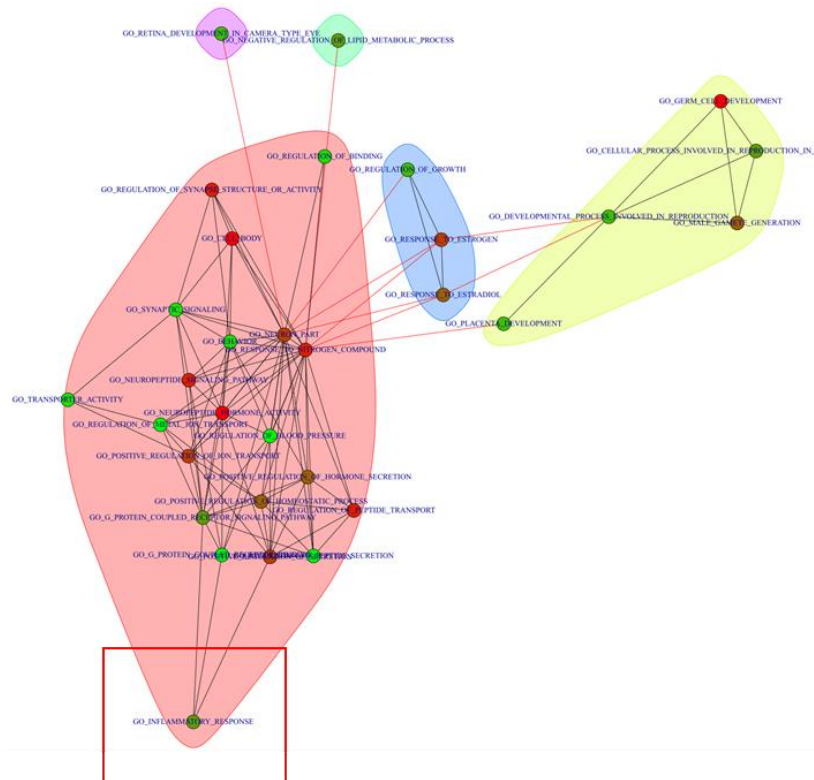


Figure 28 Upregulated genes in secretome analysis. Depicted is a network plot of upregulated secretome gene sets based on gene set enrichment analysis using GO terms. The red box highlights the upregulation of an inflammatory response associated gene set.

4.7.4 Expression of circadian genes

As part of the transcriptomic analysis, circadian genes were examined in all cell variants. The results are shown as an expression pattern in figure 31.

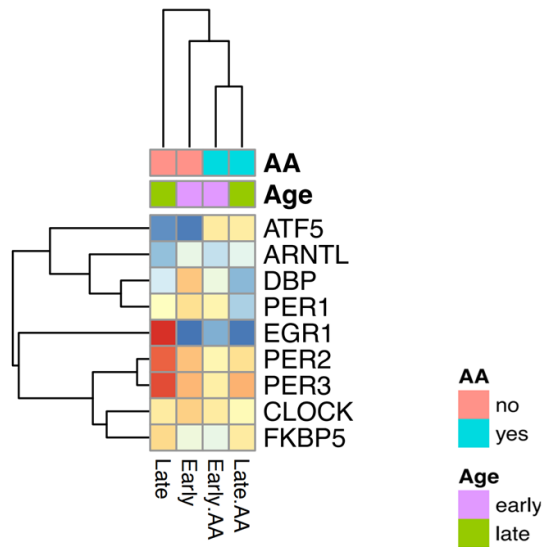


Figure 30 Cell-specific expression pattern of circadian genes. AA = with (yes) or without (no) L-ascorbic acid, Age = early or late passage, red values = upregulated in control cells, blue values = upregulated in mutated cells, orange and yellow values = in between up- and downregulation.

As depicted in figure 31, strongest upregulation in mutated fibroblasts was observed for EGR1 (Early Growth Response 1) except in late passage without L-ascorbic acid stimulation. Upregulation was also seen in ATF5 (Activating Transcription Factor 5) only without L-ascorbic acid stimulation, and slightly in ARNTL (Aryl Hydrocarbon Receptor Nuclear Translocator Like) and DBP (D-Box Binding PAR BZIP Transcription Factor) expression. Strongest downregulation was observed in PER3 (Period Circadian Regulator 3) and PER2 (Period Circadian Regulator 2) expression. Neither strongly up- or downregulated were CLOCK (Clock Circadian Regulator), FKBP5 (FKBP Prolyl Isomerase 5) and PER1 (Period Circadian Regulator 1). In summary circadian genes only showed small differences in expression between mutated and wild type cells.

4.8 Human Cytokine Array

Based on RNA-Seq data we aimed to analyse the role of *COL6A2* mutation in inflammation. For this we analysed effects of the *COL6A2* mutation c.848G>A and L-ascorbic acid stimulation in fibroblasts on secretion of cytokines. We performed a human cytokine array with mutated and wild type fibroblasts with or without L-ascorbic acid stimulation.

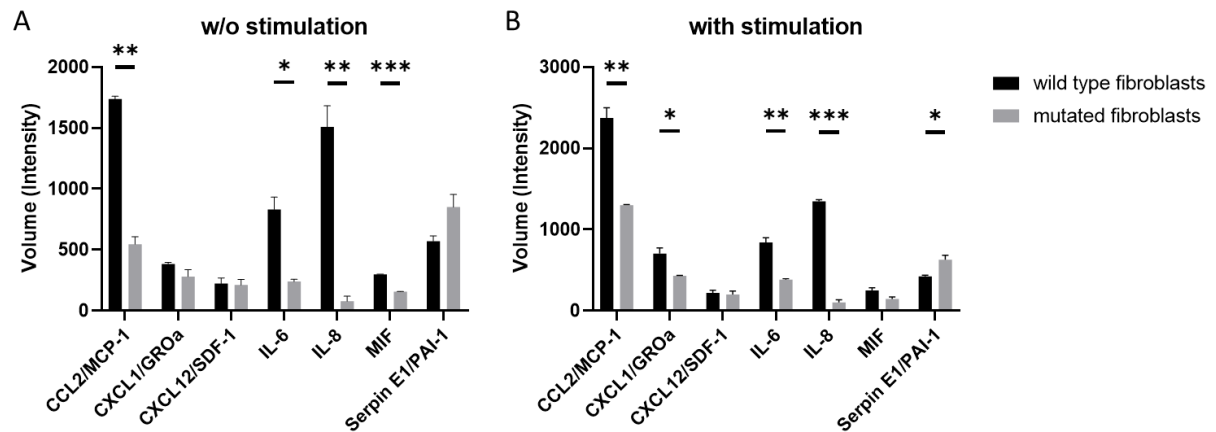


Figure 31 Results of the human cytokine array comparing wild type and mutated fibroblasts. The array was carried out in mutated and wild type cells stimulated with L-ascorbic acid (A) or non-stimulated (B). The array comprised following cytokines: CCL2/MCP-1: chemokine (C-C motif) ligand 2 or monocyte chemoattractant protein 1, CXCL1/GROα: chemokine (C-X-C motif) ligand 1 or growth-regulated oncogene-alpha, CXCL12/SDF-1: chemokine (C-X-C motif) ligand 12 or stromal cell-derived factor 1, IL-6: interleukin 6, IL-8: interleukin 8, MIF: macrophage migration inhibitory factor, Serpin E1/PAI-1: serpin peptidase inhibitor (clade E (nexin, plasminogen activator inhibitor type 1), member 1). The array was performed with two technical replicates for each variant, for statistical analysis multiple t tests were used.

Like visible in figure 32, stimulation with L-ascorbic acid resulted over all in a lower cytokine production of both fibroblast types. Also, except Serpin E1/PAI-1, wild type fibroblasts produced generally higher amounts of cytokines. The differences in both, stimulated and non-stimulated cells, were most significant in CCL2/MCP-1, IL-6 and IL-8.

5 Discussion

Collagen VI congenital muscular dystrophies (Col6-CMD) encompass a clinical continuum of rare muscular dystrophies that range from Ullrich Congenital Muscular Dystrophy (UCMD, OMIM: 254090, severe symptoms with early onset) and intermediate phenotypes to Bethlem Muscular Dystrophy (Bethlem-MD, OMIM: 158810, milder symptoms with late onset). Symptoms of Col6-CMD typically include progressive muscle hypotonia, distal joint hyperlaxity, contractures, rigid spine or other spine deformities, skin abnormalities and respiratory insufficiency. To date there is no curative therapy for this disorder, treatment remains purely supportive and focused on individual symptoms^{1,20}.

Known causes of Col6-CMD are pathogenic variants in one of three genes encoding for collagen VI (*COL6A1*, *COL6A2*, *COL6A3*). The disorder follows autosomal dominant or, less commonly, recessive inheritance pattern or occurs *de novo*. Collagen VI is a protein localized in the extracellular matrix (ECM) of many connective tissues, most importantly in skeletal muscle, where it is produced and secreted by interstitial fibroblasts. The protein is assembled from three distinct chains $\alpha 1$, $\alpha 2$, $\alpha 3$, each one encoded by one of the three genes *COL6A1*, *COL6A2*, *COL6A3*, respectively. Collagen VI assembly is a complex process that comprises first the formation of a triple helix monomer, then alignment of these monomers to tetramers, which are then secreted into extracellular space where they align to beaded microfilaments to form collagen VI. Mutations in one of the three genes show a dominant negative effect, with defects in collagen VI secretion and assembly¹⁶. It is known, that, reversely, collagen VI haploinsufficiency has no pathological consequences and do not cause Col6-CMD^{20,41}. Thus, RNA-targeting approaches aimed at inactivating the mutated COL6 messenger RNA may represent a possible therapeutic strategy.

In a patient with an intermediate clinical phenotype, exome sequencing and segregation analysis revealed a *de novo* pathogenic variant in a highly conserved region of *COL6A2* (c.848G>A, p.G283E)¹⁴¹.

Two main goals were followed through in this thesis, a) to elucidate the functional effect of the identified *COL6A2* mutation by establishment of a cell culture assay with fibroblasts obtained by skin biopsy from the patient, RNA-sequencing and a cytokine array, and b) to develop an RNA-targeted therapy based on RNA interference and antisense mechanism *in vitro*.

5.1 Functional effects of *COL6A2* c.848G>A

Interstitial fibroblasts of skeletal muscle tissue are the main contributors to collagen VI production overall. Thus, fibroblasts obtained from skin biopsy can be used to analyse the effect of a pathogenic variant in one of the *COL6* genes on structure and organization of secreted collagen VI *in vitro*, which has been demonstrated by several research groups before^{16,41,142}.

In this thesis, an immunofluorescence staining assay with dermal fibroblasts carrying the *COL6A2* c.848G>A mutation was established to analyse the effect of this disease-causing mutation *in vitro*. In addition, RNA-sequencing was carried out to unravel other, more distal effects of *COL6A2* c.848G>A and help to understand the wide range of symptoms associated with Intermediate Col6-CMD. Also, since chronic inflammation plays a key role in congenital muscular dystrophies¹⁵¹ and our RNA-sequencing results suggested a closer look into immunological pathways we investigated the mutation's effect on the production of cytokines by array analysis.

5.1.1 Effects on extracellular matrix of fibroblasts

As a first step in assay establishment, we analysed the effect of L-ascorbic acid on collagen VI secretion. L-ascorbic acid is known to be essential for successful collagen production as it is required for the synthesis of hydroxyproline and -lysine, both amino acids needed for stable collagen formation¹⁵². In our hands, the supplementation of L-ascorbic amino acid to cultured fibroblasts led to an increase of secreted collagen VI in ECM. To be able to evaluate the effect of the pathogenic variant *COL6A2* c.848G>A, it is necessary to provide optimal conditions for the fibroblasts to produce collagen VI. Therefore, we carried out all following fibroblast immunostaining assays with L-ascorbic acid stimulation during culture.

A cell culture assay with fibroblasts from the patient's skin biopsy carrying the *COL6A2* c.848G>A, p.G283E mutation was established to compare them to wild type fibroblasts and investigate differences in collagen VI ECM quality and quantity. In our hands we have identified a significant decrease in collagen VI matrix and speckled microfibrils from which we concluded the mutation's severe impact on ECM production. These results are consistent to what has been shown for various patients with a Col6-RD phenotype and pathogenic variants in *COL6A1*, *COL6A2* and *COL6A3*^{16,41,142,153}. Interestingly, glycine substitutions, such as p.G283E, in the triple helical domain of *COL6* genes account for almost 1/3 of pathogenic missense variants¹⁵⁴, which can be seen in figure 1.

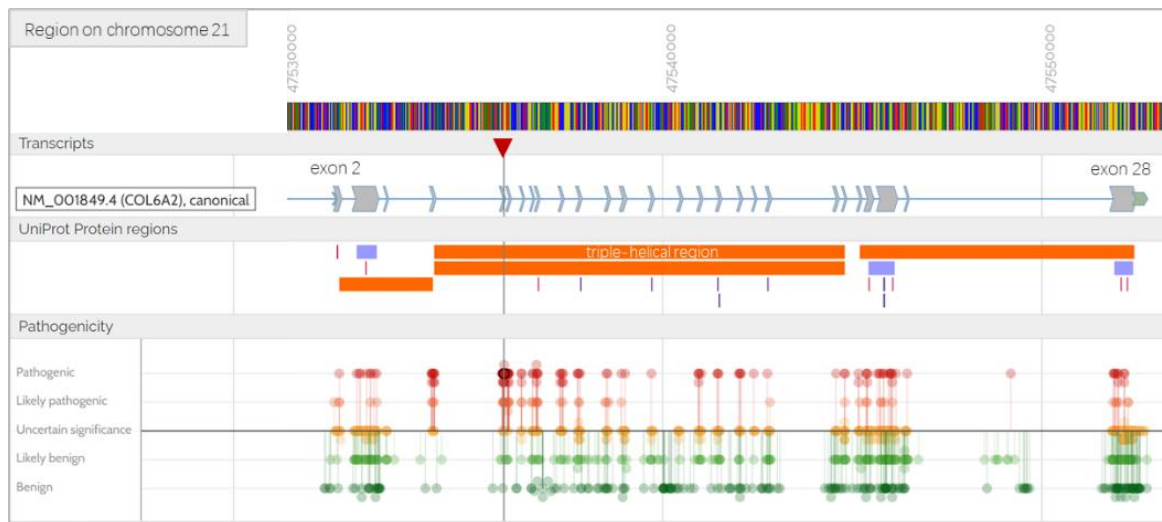


Figure 32 Position of the pathogenic variant in *COL6A2* transcript and protein region (reference transcript: NM_001849.4 *COL6A2*), protein regions source: UniProt). Location of the in this thesis analysed pathogenic variant: chr21-47535832-G-A *COL6A2*:p.G283E (highlighted with an arrow). Depicted are exons 2 to 28. The mutation lies within the triple-helical region (orange box). In this protein region pathogenic variants (in red) are clustered. Figure adapted from schematic view of Varsome.com browser.

The position of the mutation *COL6A2* c.848G>A in the triple helical domain of the collagen VI protein (see figure 33) and the substitution of glycine, the smallest of all amino acids (molecular weight: 75.07 g/mol) and crucial for triple helix formation, to the larger (molecular weight: 147.13 g/mol) and electrically charged glutamic acid may contribute in large parts to the molecular alterations which ultimately lead to defects in the complex molecular assembly process of collagen VI. The dominant negative mechanism in Col6-CMD reflects that mutated chains can be incorporated into the protein assembly but disturb microfibre formation^{16,154}. This is most likely the pathomechanism underlying the observations we made in our immunostaining assay, where collagen VI microfibrils of mutated fibroblasts are still produced, although in lower quantity, but most strikingly look disordered.

We also observed an alteration the fibronectin formation in ECM secreted by mutated fibroblasts. Fibronectin, as an important component of ECM, interferes with various ECM proteins and collagen VI among them¹⁴⁵. Fibronectin binds to integrin receptors located at the cell surface and induces pathways associated with cytoskeleton reorganization, cell cycle progression and differentiation¹⁵⁵. It has been demonstrated, that the organization of fibronectin in ECM is mediated by secreted collagen VI via direct connection¹⁴⁵ which explains the observations we made in our fibroblasts immunostaining assay. The pathological impact of defected collagen VI on fibronectin and its connected pathways might contribute to the myopathic phenotype of Col6-CMD, in the end.

5.1.2 Effects on different pathways and gene expression

RNA-Sequencing of wild type and mutated fibroblasts gave us a closer insight into the pathogenesis of Col6-CMD caused by the pathogenic variant *COL6A2* c.848G>A. We anticipated that important pathways involved in the regulation of connective tissue and inflammatory phenotypes can be unraveled with this approach.

As a first observation we saw that fibroblasts supplemented with L-ascorbic acid in general showed higher differences in gene expression between Mut and WT fibroblasts. Since ascorbic acid is an important cofactor for collagen VI synthesis and was used in our fibroblast assay make the difference of collagen VI in ECM between Mut and WT fibroblasts visible, this observation did not come as a surprise but, more importantly, suggests that ascorbic acid modulates not only the synthesis of collagen VI but might also have an impact on associated pathways directly or indirectly through collagen VI.

One of the main observations made was the result of secretome analysis (section 4.7.4). In Mut fibroblasts, pathways involved into the cellular immune response were upregulated. This indicated that collagen VI deficiency leads to an activation of immune response pathways. Macrophages have been suggested as key inflammatory components in Col6-RD patients ¹⁵⁶. Macrophage infiltration into muscle has been proven to contribute to the severity of many muscular dystrophies, for instance to the muscular wasting in Duchenne muscular dystrophy ¹⁵⁷. Furthermore, activation of complement system might play a role in Col6-CMD pathogenesis, too. It has been demonstrated that levels of C3, a main component of the complement system, are increased in Col6-CMD patients ¹⁵⁶. Our findings fall in line with these previous reports and underpin the role of inflammation in the Col6-CMD pathogenesis. However, recent transcriptome analysis of Col6-CMD muscle biopsies could not confirm this immune response activation ¹⁵⁸. This discrepancy could be explained by the larger cohort of analysed samples they investigated which comprised various disease severities and underlying mutations in contrast to our focus specifically on the *COL6A2* c.848G>A mutation. Anti-inflammatory drugs such as corticosteroids are to date not part of the treatment strategies for patients suffering from Col6-CMD. Considering the key role inflammation plays at least in some cases of these disorders, anti-inflammatory drugs might attenuate the Col6-CMD phenotype and slow the disease progression for some patients.

Secretome analysis also revealed a downregulation of cell adhesion pathways in mutated fibroblasts. From what we know about key roles of collagen VI, we hypothesize that this effect is directly linked to collagen VI failing to provide a connection between basement membranes and extracellular matrix (ECM). This main function of collagen VI as an anchor between these critical structures in muscle tissue has been demonstrated especially by research groups using electron microscopy^{33,55}. In immunostaining experiments in muscle biopsies of UCMD patients this effect was observed as expected, where an impaired connection between collagen VI fibres and basal lamina due to collagen VI defects was suggested ⁵⁶. Adhesion in muscle tissue is of utmost importance for muscle integrity and proper functioning as the transformation of force into movement requires a strong attachment of myofibres to its surrounding structures. It also plays an important role in the muscle's ability to regenerate after injury ¹⁵⁹. Therefore, impaired cell adhesion as a direct effect of defects in collagen VI explain typical symptoms of Col6-CMD such as muscle

weakness and tendon contractures and contribute to the disorder's progressive character as injuries might accumulate over time and full muscle regeneration is disturbed.

Additionally, our observations from network diffusion (section 4.7.2) suggested an indirect effect of collagen VI to muscle tissue integrity by affecting other ECM proteins, most importantly various types of collagens and proteins from the integrin family. Integrins serve, by name, as integrators of cytoskeleton and ECM and consist of two subunits, α and β which are encoded by *ITGA* and *ITGB* genes, respectively ^{160,161}. The $\alpha7\beta1$ -integrin is highly expressed in skeletal muscle tissue where it connects laminin in the ECM with actin in skeletal muscle fibers ¹⁶². Pathogenic variants of the corresponding *ITGA7* gene have been associated with congenital muscular dystrophy (OMIM: 613204) ^{162,163}. This disorder shows a considerable phenotype overlap with Col6-CMD such as delayed motor milestones, impaired ambulation, progressive muscle weakness and scoliosis ¹⁶⁴. Our network diffusion analysis identified a close functional connection between *COL6A2* and *ITGA7*. On the other hand, there were no identified connections between *COL6A2* and the genes encoding for laminin or actin, *LAM* and *ACT* genes, respectively, which could function as mediators between *COL6A2* and *ITGA7*. However, direct molecular interactions between collagen VI and $\alpha7\beta1$ -integrin have not been described to date but might be an interesting aspect of Col6-CMD pathogenesis or, thinking further, of the pathomechanisms underlying congenital muscular dystrophy due to *ITGA7* deficiency.

Analysis of circadian gene expression showed overall rather small alterations in mutated cells compared to wild type cells. Dysregulation of circadian genes in cells with a muscle dystrophy phenotype link skeletal muscle ECM to the human intrinsic molecular clock. First hints towards this connection were given by gene expression data which demonstrated that physical activity regulates circadian genes in skeletal muscle ¹⁶⁵. In addition, circadian genes are involved crucially in immune function, e.g., the *PER* genes which were slightly downregulated in mutated fibroblasts, are expressed in mononuclear cells and regulate the immune system in a rhythmic manner ¹⁶⁶. Previous research groups investigating circadian genes in Col6-CMD via RNA profiling reported profound differences. Contradicting to our data, a reported an upregulation of *CLOCK* (Clock Circadian Regulator) which they confirmed on protein level *Col6a1* deficient mice, whereas we found *CLOCK* not to be upregulated in mutated fibroblasts ¹⁶⁷. However, it needs to be considered that comparison of RNA profiles of fibroblasts and tissue from a mouse most probably will show a variety of expression differences due to the mouse being a much more complex organism than *in vitro* cells. Also, circadian genes are known to be expressed tissue-specifically and expression varies widely between different tissues ¹⁶⁶.

5.1.3 Effects on inflammatory system

The results of our human cytokine array specified the involvement of collagen VI in inflammatory system by influencing certain cytokine levels.

Our results showed that fibroblasts supplemented with L-ascorbic acid during culture, secreted an overall lower level of cytokines in comparison to non-supplemented fibroblasts. L-ascorbic acid is a well-known physiological antioxidant which conserves

immune homeostasis by reversing the harmful effects of reactive oxygen intermediates (ROI) on the immune system ¹⁶⁸. Previous research could prove a modulating effect of L-ascorbic acid on certain cytokines, for instance a reduction of IL-6 levels was associated with L-ascorbic acid treatment ^{168,169}. Our results fall in line with those research results and support the role of L-ascorbic acid as a modulator of immune system by influencing and, in our hands reducing, cytokine levels. Interestingly, L-ascorbic acid interferes with inflammation via nuclear factor kappa B (NFκB) signaling. ROI are known to act pro-inflammatory by activating the NFκB pathway, L-ascorbic acid firstly counteracts ROI and secondly, in form of its oxidized derivate dehydroascorbic acid (DHA), deactivates inhibitors of nuclear factor kappa-B kinase subunit alpha and beta (IKKα and IKKβ, respectively) which ultimately inhibits NFκB signaling and therefore downregulates inflammatory processes ¹⁷⁰. In our gene enrichment analysis (see section 4.7.1) from our RNA-sequencing data we observed an activation of NFκB signaling in Mut fibroblasts that were not supplemented with L-ascorbic acid in comparison to no activation of NFκB signaling in Mut fibroblasts that were supplemented with L-ascorbic acid during culture. This observation can be explained by the direct and indirect modulatory effect of L-ascorbic acid on NFκB signaling.

We observed that levels of chemokine (C-C motif) ligand 2 or also called monocyte chemoattractant protein 1 (CCL2/MCP-1) are significantly decreased by 2-3-fold in Mut fibroblasts compared to WT fibroblasts. In both fibroblast types, stimulation with L-ascorbic acid led to higher levels of CCL2/MCP-1. This chemokine regulates migration and infiltration of monocytes and macrophages and is involved in the pathogenesis of various diseases ¹⁷¹. A group investigating the role of collagen VI in adipose tissue inflammation and insulin resistance found an almost complete loss of CCL2/MCP-1 expression in *COL6A3* deficient adipocytes ¹⁷². Considering the dominant negative effect of mutations in *COL6* genes and their consequence for the whole collagen VI formation, it appears likely that the *COL6A2* p.G283E mutation changes CCL2/MCP-1 levels following a similar, if not the same, pathway as it happens for *COL6A3*. This hypothesis is backed up by our observation, that L-ascorbic acid, which stimulates collagen VI synthesis, also confers increased levels of CCL2/MCP-1 regardless of the *COL6A2* genotype. This finding also underpins the role of macrophage infiltration in Col6-CMD, as described in section 5.1.2.

Also, levels of interleukin 6 and interleukin 8 (IL-6 and IL-8) were significantly decreased in mutated fibroblasts compared to wild type. For IL-6, this difference became even stronger after stimulation with L-ascorbic acid. Previous work has demonstrated that collagen VI enhances expression of transcription factors, such as IL-8 ¹⁷³. For IL-6 it has been reported that the cleaved C5 domain of collagen VI α3 chain, defined as endotrophin (ETP), upregulates production of inflammatory factors like IL-6 and increases macrophage recruitment ¹⁷⁴. These reports have identified collagen VI as a factor in tumor development and metastasis by modulation of immune system. Moreover, the fact that a mutation on *COL6A2* which as a most direct consequence leads to alterations of the α2 chain, results in lower IL-6 levels, reflects once again the distal impact of a dominant negative mechanism, as a disturbed α2 chain obviously affects functions of the α3 chain and the whole collagen VI molecule.

Further, we saw decreased levels of chemokine (C-X-C motif) ligand 1 also known as growth-regulated oncogene-alpha (CXCL1/GRO α) in mutated fibroblasts compared to wild type fibroblasts. In fibroblasts stimulated with L-ascorbic acid this difference became statistically significant. CXCL1/GRO α is a member of the CXC chemokine family and involved in atherosclerosis, angiogenesis and various other inflammatory conditions¹⁷⁵. Our findings are unlike with what has been reported recently. In comparative proteome analysis of muscle biopsies from Col6-CMD patients, chemokines of the GRO family (CXCL1, CXCL2, CXCL3) were elevated in comparison to control muscle tissue¹⁵¹. However, it is important to highlight the much more complex molecular environment in muscle tissue obtained from biopsies compared to *in vitro* cultured fibroblasts. This, and the fact that the group analysed the GRO family instead of CXCL1 alone, like in our experiment, might be reasons for the at a first glance contradictory result.

5.2 RNA-targeted therapy to correct the collagen VI phenotype *in vitro*

RNA-targeted therapy systems such as antisense oligonucleotides or RNA interference appear to be a promising approach and has been proven successful for the correction of other *COL6* mutations^{41,142}. For this reason, we aimed to rescue the Col6-CMD phenotype in our fibroblast immunostaining assay using this approach.

We tested a set of siRNAs and ASOs targeting both, the mutation c.848G>A as well as SNPs *in cis* to the mutation on *COL6A2*, to knockdown the mutated *COL6A2* allele.

To assess our first siRNA, which targeted the mutation site, we first analysed whether the target mRNA structure is accessible for RNAi mediated knockdown. For this, we designed siCOL-1G, targeting the *COL6A2* wild type allele. After we demonstrated successful knockdown, we proceeded to design siCOL-1A, a siRNA which is different to siCOL-1G only in one nucleotide, at the mutation position. Also, siCOL-1A showed high *COL6A2* knockdown potential, however, it also partly inhibited the wild type allele as off-target effect (see sections 4.4.1 and 4.4.2).

After this first successful experiment we designed siRNAs targeting different common SNPs on *COL6A2*. The benefit of targeting variants which are much more frequent in population than the mutation c.848G>A is that a future therapy which might arise from this will be available for more patients. With this, theoretically all patients with mutations in *COL6A2* who carry one of these common variants could be treated with the respective siRNA. With a siRNA targeting the rare c.848G>A mutation, the treatment would be limited to the much smaller amount of this rare mutation carriers. This multi-target approach aimed at increasing the probability to find an accessible target and, in long-term, make a therapeutic use applicable for more Col6-CMD patients. Unfortunately, though, our qPCR results did not show a promising effect of these siRNAs (see sections 4.5). For these siRNAs not primarily off-target inhibition was problematic, but the lack of a reliable dose-dependent mRNA knockdown of the targeted allele. Target site access is one of the main challenges in siRNA design and owes largely to the intrinsic mRNA secondary structures, where any base

pair variations of the target site will influence the siRNA's effectiveness and the location of unpaired nucleotides in the mRNA molecule has a considerable influence on siRNA efficiency^{176,177}.

Another parameter that can influence siRNA efficiency is percentage GC content of the siRNA molecule. Low percentage GC content may lead to unspecific and weak siRNA-mRNA binding and, on the contrary, high percentage GC content could hinder siRNA unwinding by helicase and RISC complex, so that the recommended percentage GC content for siRNA guide strands lies between 36–52 %¹⁷⁶. However, percentage GC content of our siRNAs were kept on similar levels between 47 % and 52 % and therefore, this may not be the key reason for the differences in knockdown efficiency of our siRNAs. It is more likely, that differences in accessibility of the targeted mRNA structure led to the observed results.

Our ASOs, which targeted the mutation position on the wild type allele also achieved an efficient dilution-dependent knockdown. The ASOs we used all contained 2'-O-methyl modifications to improve stability and increase target affinity. However, these modifications hinder RNase H cleavage which is a crucial part of the antisense mechanism. The invention of gapmers addressed this problem, by design of a central 'gap' of unmodified nucleotides flanked by nucleotides with 2'-O-methyl modifications¹⁷⁸. All of the ASOs used in our project were designed as gapmers, most of them with a central gap and symmetric 2'-O-methyl modification on each end. As1300-1, as2198-1 and as2198-3 are considered asymmetric gapmers, since the high-affinity 2'-O-methyl modification is on only one end.

Unfortunately, the allelic discrimination between wild type and mutated allele was not sufficient in our experiments. For the allele selective application of ASOs several chemical modifications for gapmers have been developed and can be used in various combinations (see also section 1.3.1). Examples for these modifications are the introduction of 3'-fluorohexitol nucleic acid (FHNA), and α,β -constrained nucleic acid (α,β -CNA), in which the phosphate is built in a ring structure, and can be combined with shorter gaps¹⁷⁸. These modified gapmers show improved allele selectivity since the region available for RNase H cleavage is minimized and single nucleotide mismatches in the cleavage site (i.e. *COL6A2* wild type allele) lead to a major reduction of cleavage activity¹⁷⁹. Alternative ASO design like this could be used to target the mutated *COL6A2* allele more specifically and diminish off-target wild type allele depletion.

Similarly, unreliable *COL6A2* knockdown was observed for our ASO targeting the common SNPs. For successful ASO design factors like secondary mRNA structure and GC content play key roles, too. For instance, local structure accessible for ASO hybridization are usually at the terminal end, within loops or hairpins. Other important parameters are the thermodynamic properties of ASO and mRNA which need to be considered to calculate the binding energy necessary for ASO/ASO or ASO/mRNA binding¹⁸⁰. However, during the design of all our siRNAs and ASO these factors like GC content, melting temperature, dimerization and specific sequence motifs and thermodynamic energy have been considered. Recommended percentage GC content for ASOs lies between 40 and 60%¹⁸¹. GC content of our ASOs ranged from 44 % to 61 %, only as2198-1 contained 66 % GC. As

this parameter is too high for as2198-1, it might contribute to the poor knockdown performance of this ASO.

But overall, the fact that both siRNAs and ASOs targeting the mutation site achieved dilution dependent *COL6A2* knockdown and, on the other hand, both siRNAs and ASOs targeting other target sites failed to efficiently inhibit the mRNA, indicates that the most likely cause for this observation indeed is target accessibility of the respective mRNA region itself.

After all the beforehand testing of different siRNAs and ASOs we decided to further test our most promising candidate siCOL-1A in our cell culture model in a phenotype rescue experiment. The results of our in vitro phenotype rescue (section 4.6) demonstrated that treatment with the *COL6A2* c.848G>A targeting siRNA siCOL-1A unfortunately has no visible beneficial effect on collagen VI ECM in cultured fibroblasts. In our assay siCOL-1A was not able to rescue the collagen VI phenotype, although on mRNA level it led to a significant and allele-specific knockdown of the *COL6A2* mutated allele. There are two main scenarios which might cause these contradicting results.

First, it is possible that siCOL-1A indeed has a beneficial effect on the collagen VI phenotype, however, our assay was not appropriate to show the true effect. A plausible reason for this might be the short gene silencing half-time of siRNAs standing against the long half-time of a matrix protein like collagen VI. Research on siRNA kinetics showed that gene silencing duration varies between approximately 7 days for rapidly dividing cells up to 21 days for non-dividing cells, indicating that dilution due to cell division is the main factor controlling the duration of siRNA mediated gene silencing ¹⁸². For this assay used primary fibroblasts were dividing at rapid pace, so the duration of siCOL-1A mediated *COL6A2* silencing can be estimated up to 7 days. To measure the effect of siCOL-1A cells were harvested one day after transfection, so according to literature it is likely that at this time point we measured the effect of siCOL-1A on *COL6A2* mRNA at its peak. For the phenotype rescue assay, between transfection of siCOL-1A and immunofluorescence staining was a 5-day period of stimulation with L-ascorbic acid in fibroblast culture. Thus, at the time of EMC-staining the effect of siCOL-1A could have been decreased to some degree already. However, transfection of fibroblasts after L-ascorbic-acid stimulation is not a promising approach too since collagens are of very long half-time in extracellular matrix. Depending on the type of collagen the fibres show half-lives of 70 days up to several years ^{183–185}, therefore introducing siRNAs to the fibroblasts after being stimulated to produce collagen VI, will only have an impact on a very small part of the collagen VI matrix. Repetitive transfection of siRNA before, during and shortly after L-ascorbic acid stimulation might offer a solution to this challenge. It has been demonstrated before, that repetitive transfection and replating of fibroblasts after each transfection could achieve successful phenotype rescue ⁴¹. The cell replating ensures that the collagen ECM, which is stained later, has been produced under the influence of RNAi as it eliminates collagen VI produced before siRNA transfection. However, multiple transfections in one batch of fibroblasts also cause a lot of cell stress. In our hands, double transfection with a gap of 48 hours in between each already led to quick cell death and did not allow us to follow this approach further. The try of alternative transfection reagents as well as electroporation-based

transfection might contribute to a gentle and effective fibroblast transfection and allow multiple transfections without loss of cells.

A second possible reason for the failure of our phenotype rescue might be a too unspecific allelic discrimination of siCOL-1A. As we observed from qPCR with allele specific primers (see section 4.4.2), the *COL6A2* wild type allele was depleted by siCOL-1A to a lower degree as the mutated allele, but still was affected from siRNA treatment. As a result, the whole *COL6A2* gene was knocked down partially and collagen VI production was reduced in treated fibroblasts. This hypothesis is underpinned by the comparison of ECM between fibroblasts treated with siCOL-1A and fibroblasts treated with siCOL-1G targeting the wild type allele of *COL6A2*. As expected, siCOL-1G treatment led to a decrease in collagen VI quantity when compared to controls. However, even treatment with siCOL-1A showed this effect, ECM of siCOL-1A treated fibroblasts appeared very similar to ECM of siCOL-1G treated fibroblasts with a lower collagen VI quantity in ECM. To resolve this, modification of siCOL-1A could be considered to increase allelic discrimination. Previous studies suggest the introduction of additional single-nucleotide mismatches in siRNAs to enhance allele specificity without affecting the knockdown potency^{186,187}. In our experiments siCOL-1A perfectly matches the target mutated allele but does not correspond with the wild type allele due to one mismatched base pairing, the mutation site. The introduction of another single-nucleotide mismatch will generate a mismatch against the target mutated allele, but, more importantly, two nucleotide mismatches against the wild type allele, which are the original variation at the mutation site and the newly and introduced mismatch. Especially additional mismatches in the central and seed region of the siRNA guide strand have been proven to increase allelic discrimination without having a strong influence on siRNA knockdown potency of the mutated allele¹⁸⁸. Due to most likely thermodynamic properties of such modified siRNAs, different base pair mismatches confer different levels of allelic discrimination and knockdown magnitude¹⁸⁸. Therefore, to improve the allelic discrimination of siCOL-1A and at the same time ensure sufficient inhibition of the target mutated allele, it is necessary to design a variety of modified siCOL-1A with different base pair mismatches at different positions within the central and seed region. For Col6-CMD with *COL6A1* defect this approach has already been taken on and resulted in successful allele specific gene silencing and *in vitro* phenotype rescue¹⁸⁹. In a similar way it could be attempted for our mutation in *COL6A2*.

5.2.1. Conclusion and Outlook: Challenges and possibilities of gene therapy for Col6-CMD

Rare genetic diseases, like Col6-CMD, have remained untreatable and incurable for many decades. The improved understanding of pathological mechanism and technological progress, especially with the development of gene therapies brought a silver lining on the horizon and a cure for these diseases has moved within reach. RNA-targeted therapies like RNA interference and antisense oligonucleotides are powerful tools for gene knockdown and emerged as a potential cure for monogenic diseases.

The results gained during this thesis lay the foundation for further development and investigation of RNAi and ASO-mechanism to treat and possibly cure Col6-CMD. We were able to prove that a siRNA targeting the pathogenic variant *COL6A2* c.848G>A, siCOL-1A, can lead to an allele-selective gene knockdown. Based on these results, further work should focus on the improvement of siCOL-1A by, for instance, modification of its sequence to increase allele specificity and, in parallel, further optimize the collagen VI fibroblast assay. Once these challenges have been solved, siCOL-1A may be able to rescue the collagen VI phenotype *in vitro* and next steps towards treatment application need to be considered.

A main concern in the utilization of RNAi are off-target effects in the human genome. Although siRNAs in general and siCOL-1A, too, perfectly match their target sequence, erroneous gene silencing can occur and must be evaluated regarding its biological consequences in humans. Due to chemical modifications in siRNAs (see also section 1.3.2) and perfect sequence complementarity off-target effects based on sequence similarity can be reduced. However, another mechanism can cause off-target gene silencing, namely miRNA-like off-target repression. MicroRNAs (miRNA) are small endogenous RNAs involved in the regulation of various biological functions. In their procession, endogenous miRNAs and exogenous siRNAs share the same effector protein, Ago2, which induces mRNA cleavage and, therefore, mediates gene silencing but cannot discriminate between miRNA and siRNA. In contrast to siRNAs, miRNAs do not necessarily match their target sequence perfectly, they potentially pair with and subsequently repress several hundreds of targets. Therefore it is inevitable that exogenously administered siRNAs partly function as miRNAs and lead to widespread miRNA-like off-target effects¹⁹⁰. Transcriptome analysis such as microarray gene expression profiling or RNA-sequencing must be used to identify downregulation of off-target transcripts and prevent unwanted and unexpected side effects and toxicity¹⁹¹.

To investigate the biodistribution and effect of siRNAs in a physiological environment, an animal model must be established. Previous research groups created mouse models as well as zebrafish with phenotypical features of Col6-CMD^{192,193}. In *Col6a1*-deficient mice histological alterations such as muscle necrosis were observed, however, the overall phenotype was rather mild and not clearly visible by pure inspection of the mice¹⁹². Zebrafish deficient of *col6a1* showed abnormalities like shortness, hypoplastic midbodies and a bent tail¹⁹³. To study the phenotypical effect of the *COL6A2* c.848G>A mutation, it first is necessary to insert this human mutation into the animal's genome by gene editing techniques like clustered regularly interspaced palindromic repeats/CRISPR-associated protein 9 (CRISPR/Cas9). Then, the phenotype can be investigated similarly to how it is investigated for *COL6*-knockout zebrafish or mice by inspection and histological analysis. Provided, that a Col6-CMD-like phenotype can be observed, a rescue experiment can be conducted using RNAi. A similar approach with exonskipping ASOs has been successfully performed for a gene-edited mouse model of limb-girdle muscular dystrophy 2C and could help to analyse the effect of siRNAs in an *in vivo* organism¹⁹⁴. However, a major obstacle remains with the systemic administration of siRNAs (see also section 1.3.3). Since collagen VI is a protein produced by many cell types with various pleiotropic effects, a delivery system with access to different cells, first and foremost interstitial fibroblasts, must be used

to convey the siRNA. A possible carrier could be 3 dimensional DNA nanostructures that can penetrate various tissues due to their small size and are not, unlike several alternative carrier systems, bound to hepatic tissue¹⁰⁰. Since RNAi can only transiently silence target genes, repetitive systemic drug administration is necessary to achieve a long-term therapeutic effect.

An alternative strategy to RNAi or antisense-mediated gene silencing is the recently emerged clustered regularly interspaced palindromic repeats interference (CRISPRi). The CRISPRi system has its origin in the *Streptococcus pyogenes* CRISPR machinery and utilizes small RNAs to recognize and cleave sequence-specific DNA elements and confers genetic resistance to foreign DNA, such as viral DNA, as part of a nucleic-acid-based adaptive immune system in bacteria and archae^{195,196}. In *Streptococcus pyogenes* the CRISPR system comprises an endonuclease, the CRISPR-associated protein 9 (Cas 9), and two types of RNA, a CRISPR RNA (crRNA) and trans-acting RNA (tracrRNA). Instead of these two endogenous RNAs used by the bacterium, engineered so called guide RNAs (gRNA) can be used to determine their target DNA by complementary base pairing. Once the gRNA-Cas 9 complex identifies and binds to their target sequence, Cas 9 induces a double-strand break within the target region¹⁹⁶. It has been demonstrated that Cas 9 blocks transcription when its endonuclease activity is inactivated. This mechanism, known as CRISPRi, can be used to regulated gene expression on transcript level and is highly specific without significant off-target effects¹⁹⁷. The original CRISPR technology is currently used in 52 clinical studies, however CRISPRi could not advance to this stage so far (source: clinicaltrials.gov, search date: 14th November 2021). Nevertheless, pre-clinical studies successfully used CRISPRi for gene silencing both *in vivo* and *in ex vivo*^{198,199}.

In comparison to CRISPRi, RNAi requires only the design and delivery of a short double stranded RNA and therefore, is an arguably simpler technology. However, CRISPRi has a wider access to potential target as it binds to DNA and reduces transcription, whereas RNAi inhibits translation and is limited to the transcribed part of the genome. Therefore, CRISPRi, but not RNAi, can be used to target untranscribed regions such as introns, promoters or distal regulatory elements. Furthermore, CRISPRi and RNAi have comparable knockdown efficiencies but off-target effects are seen much more commonly with RNAi^{198,200}. Therefore, CRISPRi, although so far less well characterized and clinically tested in comparison to RNAi, is a promising alternative to RNAi and should be evaluated further as treatment strategy for genetic diseases like Col6-CMD. To use CRISPRi as a treatment strategy for Col6-CMD, allele-specific gRNAs for e.g., *COL6A2* must be designed and cloned into different vectors containing Cas9 with a non-functional nuclease domain (dCas9). On top, dCas9 can be fused to transcriptional repressors like Krüppel-associated box (KRAB) to enhance transcriptional interference¹⁹⁹. After transfection of this complex into mutation-carrying fibroblasts, qPCR must be used to determine knockdown potential and allele specificity for each gRNA. The most effective and promising gRNA can then be further analysed in a phenotype rescue experiment using immunofluorescence staining.

Most recently, in 2021, a new CRISPR method called CRISPROff has been developed. This method utilizes an adapted dCas9 which is coupled not only to KRAB but also to two methyltransferase domains. This complex works as an epigenomic editor that can be

designed to target regulatory regions of the target gene, e.g., *COL6A2*, and generates a heritable knockdown that gets passed through cell divisions. CRISPROff-created methylations can be rapidly reversed by a construct called TETv4 or CRISPRon. With this gene expression can be programmed genome-wide by writing and erasing epigenetic memories ²⁰¹.

New technologies, such as CRISPRi and CRISPROff, and increasing research on RNAi and antisense-mediated gene silencing like what has been investigated in the present dissertation, give reason to hope for a genetic therapy and even cure for many people who suffer from rare diseases such as Col6-CMD.

Abstract

Collagen VI congenital muscular dystrophies (Col6-CMD) encompass a broad clinical spectrum from the more severe Ullrich Congenital Muscular Dystrophy (UCMD, OMIM: 254090) and intermediate phenotypes to the milder form Bethlem Muscular Dystrophy (Bethlem-MD, OMIM: 158810). These rare muscular dystrophies are typically caused by single nucleotide mutations in one of three genes encoding for collagen VI (*COL6A1*, *COL6A2*, *COL6A3*). Collagen VI is a major component of extracellular matrix and functional defects in this protein affect foremost connective tissue and skeletal muscle. To date, Col6-CMD cannot be cured, patients can only receive symptomatic treatment and supportive measures. Most disease-causing mutations have a dominant negative effect with defects in collagen VI chain assembly and secretion. It is generally accepted that, reversely, *COL6* haploinsufficiency has no pathological consequences. Thus, RNA-targeting therapeutics aimed at specifically inactivating the mutated *COL6* messenger RNA represent a promising treatment strategy. Exome sequencing revealed a *de novo* pathogenic variant in a highly conserved region of codon 283 of *COL6A2* (c.848G>A, p.G283E) in a patient with an intermediate Col6-CMD phenotype. A cell culture assay with mutant fibroblasts obtained from patient skin biopsy was established in order to compare them to wild type fibroblasts and analyse differences in ECM quality and quantity. We have identified a significant decrease in collagen VI matrix and non-consistent microfibrils from which we concluded the mutation's serious impact on ECM.

To elucidate further effects of the mutation *COL6A2* c.848G>A and identify pathogenic pathways in Col6-CMD, we performed RNA sequencing with mutated fibroblasts and compared them to wild type fibroblasts. Here we found indications that collagen VI deficiency leads to a downregulation of cell adhesion pathways, affects ECM proteins like integrins and other collagen types, and activates immune response pathways. To investigate the role of collagen VI in immune and inflammatory response further, we performed a cytokine array. Based on these results alterations in macrophage infiltration processes and influences on specific interleukine production were suggested as possible mechanisms that play a role in the pathogenesis of Col6-CMD.

For the development of a treatment for Col6-CMD based on RNA-targeted therapy, we designed antisense oligo nucleotides (ASOs) and small interfering RNA (siRNAs) targeting the *COL6A2* c.848G>A mutation and more beforehand identified single nucleotide polymorphisms (SNPs) *in cis* to this mutation, respectively. Transfection of these ASOs and siRNAs was carried out in patient-derived fibroblasts to define target accessibility, allelic discrimination, and knockdown potential, quantified by quantitative PCR. We found that a siRNA targeting the *COL6A2* c.848G>A mutation (siCOL-1A) showed significant and allele-specific *COL6A2* depletion on mRNA level. We tested its effect on collagen VI ECM in our fibroblast assay but could, unfortunately, not validate a rescue of the collagen VI phenotype by this siRNA. However, the results of this thesis are an important building brick in the concept and use of RNA-targeted therapy to find a cure for Col6-CMD.

Zusammenfassung

Die kongenitalen Kollagen-VI-Muskeldystrophien (Col6-CMD) umfassen ein breites Spektrum, das von der schweren kongenitalen Ullrich-Muskeldystrophie (UCMD) über intermediäre Ausprägungen bis zur milderen Form der Bethlem-Muskeldystrophie (Bethlem-MD) reicht. Diese seltenen Erkrankungen werden in der Regel durch Mutationen in einem von drei Genen verursacht, die für Kollagen VI (*COL6A1*, *COL6A2*, *COL6A3*) kodieren. Kollagen VI ist ein Hauptbestandteil der extrazellulären Matrix, Störungen betreffen vor allem das Bindegewebe und die Skelettmuskulatur. Bislang gibt es keine Heilung für Col6-CMD, Patienten können nur symptomatisch behandelt werden. Viele krankheitsursächlichen Mutationen haben einen dominant-negativen Effekt, da sie den Aufbau und Sekretion der Kollagen-VI-Fasern stören. Eine *COL6*-Haploinsuffizienz verursacht keine Symptome. Daher stellen RNA-gerichtete Therapeutika, die die mutierte *COL6*-Messenger-RNA spezifisch inaktivieren, eine vielversprechende Behandlungsstrategie dar. Durch Exom-Analyse wurde bei einer Patientin mit einem intermediären Phänotyp eine *de novo*-Mutation in einer konservierten Region in *COL6A2* (c.848G>A, p.G283E) entdeckt. Es wurde ein Zellkultur-Assay mit mutationstragenden Fibroblasten aus der Hautbiopsie der Patientin etabliert, um diese mit Wildtyp-Fibroblasten zu vergleichen und Unterschiede der ECM zu analysieren. Wir haben eine signifikante Abnahme der Kollagen-VI-Matrix und abnorme Kollagen-VI-Fasern festgestellt, woraus wir auf den gravierenden Einfluss der Mutation auf die ECM schließen.

Um weitere Auswirkungen der Mutation aufzuklären und weitere mögliche pathogene Signalwege zu identifizieren, führten wir eine RNA-Sequenzierung mit mutierten Fibroblasten durch und verglichen sie mit Wildtyp-Fibroblasten. Dabei fanden wir Hinweise darauf, dass ein Kollagen-VI-Mangel zu einer Herunterregulierung von Zelladhäsionswegen führt, ECM-Proteine wie Integrine und andere Kollagenarten beeinflusst und Prozesse der Immunantwort aktiviert. Um die Rolle von Kollagen VI bei der Immun- und Entzündungsreaktion weiter zu untersuchen, haben wir einen Zytokin-Array durchgeführt. Auf der Grundlage dieser Ergebnisse schlagen wir Veränderungen bei Infiltration von Makrophagen und Einflüsse auf die Produktion spezifischer Interleukine als mögliche Mechanismen vor, die bei der Pathogenese von Col6-CMD eine Rolle spielen könnten.

Zur Entwicklung einer RNA-gerichteten Therapie für Col6-CMD haben wir Antisense-Oligonukleotide (ASOs) und small interfering RNAs (siRNAs) designt, die auf die *COL6A2* c.848G>A-Mutation bzw. auf zuvor identifizierte Einzelnukleotid-Polymorphismen (SNPs) *in cis* zu dieser Mutation gerichtet sind. Die Transfektion dieser ASOs und siRNAs wurde in von Patienten stammenden Fibroblasten durchgeführt, um die Zugänglichkeit der Ziel-mRNA, die allelische Diskrimination und das Knockdown-Potenzial zu bestimmen, was mittels quantitativer PCR quantifiziert wurde. Wir fanden heraus, dass eine siRNA, die gegen die *COL6A2* c.848G>A-Mutation gerichtet ist (siCOL-1A), eine signifikante und allelspezifische *COL6A2*-Depletion auf mRNA-Ebene erreichen konnte. Wir testeten die Wirkung auf die Kollagen-VI-ECM in unserem Fibroblasten-Assay, konnten aber leider keine Rettung des Kollagen-VI-Phänotyps durch diese siRNA nachweisen. Die Ergebnisse dieser Arbeit sind jedoch ein wichtiger Baustein für das Konzept und die Anwendung einer RNA-gerichteten Therapie zur Heilung von Col6-CMD.

Bibliography

1. Bertini, E. & Pepe, G. Collagen type VI and related disorders: Bethlem myopathy and Ullrich scleroatonic muscular dystrophy. *Eur. J. Paediatr. Neurol. EJPN Off. J. Eur. Paediatr. Neurol. Soc.* **6**, 193–198 (2002).
2. Richter, T. *et al.* Rare Disease Terminology and Definitions—A Systematic Global Review: Report of the ISPOR Rare Disease Special Interest Group. *Value Health* **18**, 906–914 (2015).
3. Norwood, F. L. M. *et al.* Prevalence of genetic muscle disease in Northern England: in-depth analysis of a muscle clinic population. *Brain J. Neurol.* **132**, 3175–3186 (2009).
4. Bradley, W. G., Hudgson, P., Gardner-Medwin, D. & Walton, J. N. The syndrome of myosclerosis1. *J. Neurol. Neurosurg. Psychiatry* **36**, 651–660 (1973).
5. Dawkins, H. J. S. *et al.* Progress in Rare Diseases Research 2010–2016: An IRDiRC Perspective. *Clin. Transl. Sci.* **11**, 11–20 (2018).
6. Tambuyzer, E. *et al.* Therapies for rare diseases: therapeutic modalities, progress and challenges ahead. *Nat. Rev. Drug Discov.* **19**, 93–111 (2020).
7. Priority Medicines for Europe and the World 2013 Update, Chapter 6.19 Rare diseases. *WHO* http://www.who.int/medicines/areas/priority_medicines/Ch6_19Rare.pdf.
8. Crooke, S. T., Witztum, J. L., Bennett, C. F. & Baker, B. F. RNA-Targeted Therapeutics. *Cell Metab.* **27**, 714–739 (2018).
9. Ullrich, O. Kongenitale, atonisch-sklerotische Muskeldystrophie, ein weiterer Typus der heredodegenerativen Erkrankungen des neuromuskulären Systems. *Z. Für Gesamte Neurol. Psychiatr.* **126**, 171–201 (1930).
10. Bethlem, J. & Wijngaarden, G. K. Benign myopathy, with autosomal dominant inheritance. A report on three pedigrees. *Brain J. Neurol.* **99**, 91–100 (1976).

11. Arts, W. F., Bethlem, J. & Volkers, W. S. Further investigations on benign myopathy with autosomal dominant inheritance. *J. Neurol.* **217**, 201–206 (1978).
12. Schmalbruch, H., Kamieniecka, Z., Fuglsang-Frederiksen, A. & Trojaborg, W. Benign congenital muscular dystrophy with autosomal dominant heredity: problems of classification. *J. Neurol.* **234**, 146–151 (1987).
13. Leyten, Q. H. *et al.* An autosomal dominant type of congenital muscular dystrophy. *Brain Dev.* **8**, 533–537 (1986).
14. Merlini, L., Morandi, L., Granata, C. & Ballestrazzi, A. Bethlem myopathy: early-onset benign autosomal dominant myopathy with contractures. Description of two new families. *Neuromuscul. Disord. NMD* **4**, 503–511 (1994).
15. Jöbsis, G. J. *et al.* Type VI collagen mutations in Bethlem myopathy, an autosomal dominant myopathy with contractures. *Nat. Genet.* **14**, 113–115 (1996).
16. Lampe, A. K. & Bushby, K. M. D. Collagen VI related muscle disorders. *J. Med. Genet.* **42**, 673–685 (2005).
17. Tétreault, M. *et al.* A new form of congenital muscular dystrophy with joint hyperlaxity maps to 3p23-21. *Brain J. Neurol.* **129**, 2077–2084 (2006).
18. Petrini, S. *et al.* Ullrich myopathy phenotype with secondary ColVI defect identified by confocal imaging and electron microscopy analysis. *Neuromuscul. Disord.* **17**, 587–596 (2007).
19. Okada, M. *et al.* Primary collagen VI deficiency is the second most common congenital muscular dystrophy in Japan. *Neurology* **69**, 1035–1042 (2007).
20. Bönnemann, C. G. The collagen VI-related myopathies Ullrich congenital muscular dystrophy and Bethlem myopathy. *Handb. Clin. Neurol.* **101**, 81–96 (2011).
21. Voit, T. Congenital muscular dystrophies: 1997 update. *Brain Dev.* **20**, 65–74 (1998).

22. Nadeau, A. *et al.* Natural history of Ullrich congenital muscular dystrophy. *Neurology* **73**, 25–31 (2009).
23. Kirschner, J. *et al.* Ullrich congenital muscular dystrophy: connective tissue abnormalities in the skin support overlap with Ehlers-Danlos syndromes. *Am. J. Med. Genet. A*. **132A**, 296–301 (2005).
24. Nadeau, A. & Muntoni, F. Skin changes in Ullrich congenital muscular dystrophy. *Neuromuscul. Disord. NMD* **18**, 982 (2008).
25. Cescon, M., Gattazzo, F., Chen, P. & Bonaldo, P. Collagen VI at a glance. *J Cell Sci* **128**, 3525–3531 (2015).
26. Ricard-Blum, S. The Collagen Family. *Cold Spring Harb. Perspect. Biol.* **3**, (2011).
27. Kadler, K. Extracellular matrix 1: Fibril-forming collagens. *Protein Profile* **2**, 491–619 (1995).
28. *Unconventional Collagens: Types VI, VII, VIII, IX, X, XII, XIV, XVI and XIX.* (Oxford University Press, 2000).
29. Gordon, M. K. & Hahn, R. A. Collagens. *Cell Tissue Res.* **339**, 247–257 (2010).
30. Persikov, A. V., Ramshaw, J. A. M., Kirkpatrick, A. & Brodsky, B. Electrostatic interactions involving lysine make major contributions to collagen triple-helix stability. *Biochemistry* **44**, 1414–1422 (2005).
31. Weis, M. A. *et al.* Location of 3-Hydroxyproline Residues in Collagen Types I, II, III, and V/XI Implies a Role in Fibril Supramolecular Assembly. *J. Biol. Chem.* **285**, 2580–2590 (2010).
32. Shaw, L. M. & Olsen, B. R. FACIT collagens: diverse molecular bridges in extracellular matrices. *Trends Biochem. Sci.* **16**, 191–194 (1991).

33. Kuo, H. J., Maslen, C. L., Keene, D. R. & Glanville, R. W. Type VI collagen anchors endothelial basement membranes by interacting with type IV collagen. *J. Biol. Chem.* **272**, 26522–26529 (1997).
34. Bonaldo, P., Russo, V., Bucciotti, F., Doliana, R. & Colombatti, A. Structural and functional features of the alpha 3 chain indicate a bridging role for chicken collagen VI in connective tissues. *Biochemistry* **29**, 1245–1254 (1990).
35. Sabatelli, P. *et al.* Expression of the collagen VI $\alpha 5$ and $\alpha 6$ chains in normal human skin and in skin of patients with collagen VI-related myopathies. *J. Invest. Dermatol.* **131**, 99–107 (2011).
36. Perris, R., Kuo, H. J., Glanville, R. W. & Bronner-Fraser, M. Collagen type VI in neural crest development: distribution in situ and interaction with cells in vitro. *Dev. Dyn. Off. Publ. Am. Assoc. Anat.* **198**, 135–149 (1993).
37. Klein, G., Müller, C. A., Tillet, E., Chu, M. L. & Timpl, R. Collagen type VI in the human bone marrow microenvironment: a strong cytoadhesive component. *Blood* **86**, 1740–1748 (1995).
38. Kawahara, G. *et al.* Reduced cell anchorage may cause sarcolemma-specific collagen VI deficiency in Ullrich disease. *Neurology* **69**, 1043–1049 (2007).
39. Irwin, W. A. *et al.* Mitochondrial dysfunction and apoptosis in myopathic mice with collagen VI deficiency. *Nat. Genet.* **35**, 367–371 (2003).
40. Rühl, M. *et al.* Soluble collagen VI drives serum-starved fibroblasts through S phase and prevents apoptosis via down-regulation of Bax. *J. Biol. Chem.* **274**, 34361–34368 (1999).
41. Bolduc, V., Zou, Y., Ko, D. & Bönnemann, C. G. siRNA-mediated Allele-specific Silencing of a COL6A3 Mutation in a Cellular Model of Dominant Ullrich Muscular Dystrophy. *Mol. Ther. - Nucleic Acids* **3**, e147 (2014).

42. Fitzgerald, J., Rich, C., Zhou, F. H. & Hansen, U. Three Novel Collagen VI Chains, $\alpha 4(VI)$, $\alpha 5(VI)$, and $\alpha 6(VI)$. *J. Biol. Chem.* **283**, 20170–20180 (2008).
43. Gara, S. K. *et al.* Three Novel Collagen VI Chains with High Homology to the $\alpha 3$ Chain. *J. Biol. Chem.* **283**, 10658–10670 (2008).
44. Bonaldo, P., Russo, V., Buccioti, F., Bressan, G. M. & Colombatti, A. Alpha 1 chain of chick type VI collagen. The complete cDNA sequence reveals a hybrid molecule made of one short collagen and three von Willebrand factor type A-like domains. *J. Biol. Chem.* **264**, 5575–5580 (1989).
45. Doliana, R. & Bonaldo, P. Multiple Forms of Chicken $\alpha 3(VI)$ Collagen Chain Generated by Alternative Splicing in Type A Repeated Domains. *J. Cell Biol.* **111**, 10 (1990).
46. Engel, J. *et al.* Structure and macromolecular organization of type VI collagen. *Ann. N. Y. Acad. Sci.* **460**, 25–37 (1985).
47. Colombatti, A., Bonaldo, P., Ainger, K., Bressan, G. M. & Volpin, D. Biosynthesis of chick type VI collagen. I. Intracellular assembly and molecular structure. *J. Biol. Chem.* **262**, 14454–14460 (1987).
48. Colombatti, A. & Bonaldo, P. Biosynthesis of chick type VI collagen. II. Processing and secretion in fibroblasts and smooth muscle cells. *J. Biol. Chem.* **262**, 14461–14466 (1987).
49. Colombatti, A., Mucignat, M. T. & Bonaldo, P. Secretion and Matrix Assembly of Recombinant Type VI Collagen. *J. Biol. Chem.* **270**, 13105–13111 (1995).
50. Chu, M. L. *et al.* The structure of type VI collagen. *Ann. N. Y. Acad. Sci.* **580**, 55–63 (1990).

51. Furthmayr, H., Wiedemann, H., Timpl, R., Odermatt, E. & Engel, J. Electron-microscopical approach to a structural model of intima collagen. *Biochem. J.* **211**, 303–311 (1983).
52. Knupp, C. *et al.* Reprint of ‘Structural correlation between collagen VI microfibrils and collagen VI banded aggregates’ [J. Struct. Biol. 154 (2006) 312–326]. *J. Struct. Biol.* **155**, 379–393 (2006).
53. Bernardi, P. & Bonaldo, P. Dysfunction of Mitochondria and Sarcoplasmic Reticulum in the Pathogenesis of Collagen VI Muscular Dystrophies. *Ann. N. Y. Acad. Sci.* **1147**, 303–311 (2008).
54. Mercuri, E. *et al.* Collagen VI involvement in Ullrich syndrome: a clinical, genetic, and immunohistochemical study. *Neurology* **58**, 1354–1359 (2002).
55. Ishikawa, H. *et al.* Ullrich disease: Collagen VI deficiency: EM suggests a new basis for muscular weakness. *Neurology* **59**, 920–3 (2002).
56. Ishikawa, H. *et al.* Ullrich disease due to deficiency of collagen VI in the sarcolemma. *Neurology* **62**, 620–623 (2004).
57. Bernardi, P. & Bonaldo, P. Mitochondrial Dysfunction and Defective Autophagy in the Pathogenesis of Collagen VI Muscular Dystrophies. *Cold Spring Harb. Perspect. Biol.* **5**, (2013).
58. Palma, E. *et al.* Genetic ablation of cyclophilin D rescues mitochondrial defects and prevents muscle apoptosis in collagen VI myopathic mice. *Hum. Mol. Genet.* **18**, 2024–2031 (2009).
59. Angelin, A. *et al.* Mitochondrial dysfunction in the pathogenesis of Ullrich congenital muscular dystrophy and prospective therapy with cyclosporins. *Proc. Natl. Acad. Sci. U. S. A.* **104**, 991–996 (2007).

60. Merlini, L. *et al.* Cyclosporin A corrects mitochondrial dysfunction and muscle apoptosis in patients with collagen VI myopathies. *Proc. Natl. Acad. Sci. U. S. A.* **105**, 5225–5229 (2008).
61. Grumati, P. *et al.* Autophagy is defective in collagen VI muscular dystrophies, and its reactivation rescues myofiber degeneration. *Nat. Med.* **16**, 1313–1320 (2010).
62. Bonne, G. *et al.* Clinical and molecular genetic spectrum of autosomal dominant Emery-Dreifuss muscular dystrophy due to mutations of the lamin A/C gene. *Ann. Neurol.* **48**, 170–180 (2000).
63. Bonne, G. *et al.* Mutations in the gene encoding lamin A/C cause autosomal dominant Emery-Dreifuss muscular dystrophy. *Nat. Genet.* **21**, 285–288 (1999).
64. Deconinck, N. *et al.* Differentiating Emery-Dreifuss muscular dystrophy and collagen VI-related myopathies using a specific CT scanner pattern. *Neuromuscul. Disord. NMD* **20**, 517–523 (2010).
65. Mercuri, E. *et al.* Muscle magnetic resonance imaging involvement in muscular dystrophies with rigidity of the spine. *Ann. Neurol.* **67**, 201–208 (2010).
66. Mercuri, E. *et al.* Muscle MRI in Ullrich congenital muscular dystrophy and Bethlem myopathy. *Neuromuscul. Disord. NMD* **15**, 303–310 (2005).
67. Schessl, J. *et al.* Predominant fiber atrophy and fiber type disproportion in early ullrich disease. *Muscle Nerve* **38**, 1184–1191 (2008).
68. Hicks, D. *et al.* A refined diagnostic algorithm for Bethlem myopathy. *Neurology* **70**, 1192–1199 (2008).
69. Jimenez-Mallebrera, C. *et al.* A comparative analysis of collagen VI production in muscle, skin and fibroblasts from 14 Ullrich congenital muscular dystrophy patients

- with dominant and recessive COL6A mutations. *Neuromuscul. Disord. NMD* **16**, 571–582 (2006).
70. Lampe, A. K. *et al.* Automated genomic sequence analysis of the three collagen VI genes: applications to Ullrich congenital muscular dystrophy and Bethlem myopathy. *J. Med. Genet.* **42**, 108–120 (2005).
71. Wallgren-Pettersson, C., Bushby, K., Mellies, U., Simonds, A., & ENMC. 117th ENMC workshop: ventilatory support in congenital neuromuscular disorders -- congenital myopathies, congenital muscular dystrophies, congenital myotonic dystrophy and SMA (II) 4-6 April 2003, Naarden, The Netherlands. *Neuromuscul. Disord. NMD* **14**, 56–69 (2004).
72. Stephenson, M. L. & Zamecnik, P. C. Inhibition of Rous sarcoma viral RNA translation by a specific oligodeoxyribonucleotide. *Proc. Natl. Acad. Sci.* **75**, 285–288 (1978).
73. Houseley, J. & Tollervey, D. The Many Pathways of RNA Degradation. *Cell* **136**, 763–776 (2009).
74. Scoles, D. R., Minikel, E. V. & Pulst, S. M. Antisense oligonucleotides. *Neurol. Genet.* **5**, (2019).
75. Liang, X. *et al.* Antisense oligonucleotides targeting translation inhibitory elements in 5' UTRs can selectively increase protein levels. *Nucleic Acids Res.* **45**, 9528–9546 (2017).
76. Mansoor, M. & Melendez, A. J. Advances in Antisense Oligonucleotide Development for Target Identification, Validation, and as Novel Therapeutics. *Gene Regul. Syst. Biol.* **2**, 275–295 (2008).
77. Kamola, P. J. *et al.* In silico and in vitro evaluation of exonic and intronic off-target effects form a critical element of therapeutic ASO gapmer optimization. *Nucleic Acids Res.* **43**, 8638–8650 (2015).

78. Hagedorn, P. H. *et al.* Identifying and avoiding off-target effects of RNase H-dependent antisense oligonucleotides in mice. *Nucleic Acids Res.* **46**, 5366–5380 (2018).
79. Domenger, C. *et al.* RNA-Seq Analysis of an Antisense Sequence Optimized for Exon Skipping in Duchenne Patients Reveals No Off-Target Effect. *Mol. Ther. Nucleic Acids* **10**, 277–291 (2018).
80. Fire, A. *et al.* Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature* **391**, 806–811 (1998).
81. Ketting, R. F. *et al.* Dicer functions in RNA interference and in synthesis of small RNA involved in developmental timing in *C. elegans*. *Genes Dev.* **15**, 2654–2659 (2001).
82. Nikam, R. R. & Gore, K. R. Journey of siRNA: Clinical Developments and Targeted Delivery. *Nucleic Acid Ther.* **28**, 209–224 (2018).
83. Alagia, A. & Eritja, R. siRNA and RNAi optimization: siRNA and RNAi optimization. *Wiley Interdiscip. Rev. RNA* **7**, 316–329 (2016).
84. Tafer, H. *et al.* The impact of target site accessibility on the design of effective siRNAs. *Nat. Biotechnol.* **26**, 578–583 (2008).
85. Wang, Y. *et al.* Nucleation, propagation and cleavage of target RNAs in argonaute silencing complexes. *Nature* **461**, 754–761 (2009).
86. Lima, W. F. *et al.* Binding and Cleavage Specificities of Human Argonaute2. *J. Biol. Chem.* **284**, 26017–26028 (2009).
87. Jackson, A. L. *et al.* Position-specific chemical modification of siRNAs reduces ‘off-target’ transcript silencing. *RNA N. Y. N* **12**, 1197–1205 (2006).
88. Bramsen, J. B. *et al.* A screen of chemical modifications identifies position-specific modification by UNA to most potently reduce siRNA off-target effects. *Nucleic Acids Res.* **38**, 5761–5773 (2010).

89. Chak, L.-L. & Okamura, K. Argonaute-dependent small RNAs derived from single-stranded, non-structured precursors. *Front. Genet.* **5**, 172 (2014).
90. Zheng, J. *et al.* Single modification at position 14 of siRNA strand abolishes its gene-silencing activity by decreasing both RISC loading and target degradation. *FASEB J. Off. Publ. Fed. Am. Soc. Exp. Biol.* **27**, 4017–4026 (2013).
91. Prakash, T. P. *et al.* Positional effect of chemical modifications on short interference RNA activity in mammalian cells. *J. Med. Chem.* **48**, 4247–4253 (2005).
92. Sipa, K. *et al.* Effect of base modifications on structure, thermodynamic stability, and gene silencing activity of short interfering RNA. *RNA* **13**, 1301–1316 (2007).
93. Alagia, A., Terrazas, M. & Eritja, R. Modulation of the RNA Interference Activity Using Central Mismatched siRNAs and Acyclic Threoninol Nucleic Acids (aTNA) Units. *Mol. Basel Switz.* **20**, 7602–7619 (2015).
94. Elbashir, S. M., Martinez, J., Patkaniowska, A., Lendeckel, W. & Tuschl, T. Functional anatomy of siRNAs for mediating efficient RNAi in *Drosophila melanogaster* embryo lysate. *EMBO J.* **20**, 6877–6888 (2001).
95. Alagia, A., Terrazas, M. & Eritja, R. RNA/aTNA Chimeras: RNAi Effects and Nucleases Resistance of Single and Double Stranded RNAs. *Molecules* **19**, 17872–17896 (2014).
96. Potenza, N. *et al.* RNA Interference in Mammalia Cells by RNA-3'-PNA Chimeras. *Int. J. Mol. Sci.* **9**, 299–315 (2008).
97. Kamiya, Y. *et al.* Enhancement of stability and activity of siRNA by terminal substitution with serinol nucleic acid (SNA). *Chembiochem Eur. J. Chem. Biol.* **15**, 2549–2555 (2014).
98. Terrazas, M., Ocampo, S. M., Perales, J. C., Marquez, V. E. & Eritja, R. Effect of North Bicyclo[3.1.0]hexane 2'-Deoxy-pseudosugars on RNA Interference: A Novel Class of siRNA Modification. *ChemBioChem* **12**, 1056–1065 (2011).

99. Terrazas, M., Alagia, A., Faustino, I., Orozco, M. & Eritja, R. Functionalization of the 3'-ends of DNA and RNA strands with N-ethyl-N-coupled nucleosides: a promising approach to avoid 3'-exonuclease-catalyzed hydrolysis of therapeutic oligonucleotides. *Chembiochem Eur. J. Chem. Biol.* **14**, 510–520 (2013).
100. Juliano, R. L. The delivery of therapeutic oligonucleotides. *Nucleic Acids Res.* **44**, 6518–6548 (2016).
101. Gordon, S. & Taylor, P. R. Monocyte and macrophage heterogeneity. *Nat. Rev. Immunol.* **5**, 953–964 (2005).
102. Huotari, J. & Helenius, A. Endosome maturation. *EMBO J.* **30**, 3481–3500 (2011).
103. Alam, Md. R. *et al.* The Biological Effect of an Antisense Oligonucleotide Depends on Its Route of Endocytosis and Trafficking. *Oligonucleotides* **20**, 103–109 (2010).
104. Koller, E. *et al.* Mechanisms of single-stranded phosphorothioate modified antisense oligonucleotide accumulation in hepatocytes. *Nucleic Acids Res.* **39**, 4795–4807 (2011).
105. Huang, L. & Liu, Y. In vivo delivery of RNAi with lipid-based nanoparticles. *Annu. Rev. Biomed. Eng.* **13**, 507–530 (2011).
106. Li, J., Wang, Y., Zhu, Y. & Oupický, D. Recent advances in delivery of drug-nucleic acid combinations for cancer treatment. *J. Control. Release Off. J. Control. Release Soc.* **172**, 589–600 (2013).
107. Leung, A. K. K., Tam, Y. Y. C. & Cullis, P. R. Lipid nanoparticles for short interfering RNA delivery. *Adv. Genet.* **88**, 71–110 (2014).
108. Juliano, R. L., Ming, X. & Nakagawa, O. The Chemistry and Biology of Oligonucleotide Conjugates. *Acc. Chem. Res.* **45**, 1067–1076 (2012).

109. Lönnberg, H. Solid-phase synthesis of oligonucleotide conjugates useful for delivery and targeting of potential nucleic acid therapeutics. *Bioconjug. Chem.* **20**, 1065–1094 (2009).
110. Winkler, J. Oligonucleotide conjugates for therapeutic applications. *Ther. Deliv.* **4**, 791–809 (2013).
111. Song, E. *et al.* Antibody mediated in vivo delivery of small interfering RNAs via cell-surface receptors. *Nat. Biotechnol.* **23**, 709–717 (2005).
112. Cuellar, T. L. *et al.* Systematic evaluation of antibody-mediated siRNA delivery using an industrial platform of THIOMAB-siRNA conjugates. *Nucleic Acids Res.* **43**, 1189–1203 (2015).
113. Rozema, D. B. *et al.* Dynamic PolyConjugates for targeted in vivo delivery of siRNA to hepatocytes. *Proc. Natl. Acad. Sci. U. S. A.* **104**, 12982–12987 (2007).
114. Rozema, D. B. *et al.* Protease-triggered siRNA delivery vehicles. *J. Control. Release Off. J. Control. Release Soc.* **209**, 57–66 (2015).
115. Dassie, J. P. & Giangrande, P. H. Current progress on aptamer-targeted oligonucleotide therapeutics. *Ther. Deliv.* **4**, 1527–1546 (2013).
116. Nimjee, S. M., Rusconi, C. P. & Sullenger, B. A. Aptamers: an emerging class of therapeutics. *Annu. Rev. Med.* **56**, 555–583 (2005).
117. McNamara, J. O. *et al.* Cell type-specific delivery of siRNAs with aptamer-siRNA chimeras. *Nat. Biotechnol.* **24**, 1005–1015 (2006).
118. Nair, J. K. *et al.* Multivalent N-acetylgalactosamine-conjugated siRNA localizes in hepatocytes and elicits robust RNAi-mediated gene silencing. *J. Am. Chem. Soc.* **136**, 16958–16961 (2014).

119. Charoenphol, P. & Bermudez, H. Aptamer-targeted DNA nanostructures for therapeutic delivery. *Mol. Pharm.* **11**, 1721–1725 (2014).
120. Zhang, H. *et al.* A controllable aptamer-based self-assembled DNA dendrimer for high affinity targeting, bioimaging and drug delivery. *Sci. Rep.* **5**, 10099 (2015).
121. Vitravene Study Group. Safety of intravitreal fomivirsen for treatment of cytomegalovirus retinitis in patients with AIDS. *Am. J. Ophthalmol.* **133**, 484–498 (2002).
122. Vitravene Study Group. Randomized dose-comparison studies of intravitreal fomivirsen for treatment of cytomegalovirus retinitis that has reactivated or is persistently active despite other therapies in patients with AIDS. *Am. J. Ophthalmol.* **133**, 475–483 (2002).
123. Duell, P. B. *et al.* Long-term mipomersen treatment is associated with a reduction in cardiovascular events in patients with familial hypercholesterolemia. *J. Clin. Lipidol.* **10**, 1011–1021 (2016).
124. Raal, F. J. *et al.* Mipomersen, an apolipoprotein B synthesis inhibitor, for lowering of LDL cholesterol concentrations in patients with homozygous familial hypercholesterolaemia: a randomised, double-blind, placebo-controlled trial. *Lancet Lond. Engl.* **375**, 998–1006 (2010).
125. Raal, F. J., Braamskamp, M. J., Selvey, S. L., Sensinger, C. H. & Kastelein, J. J. Pediatric experience with mipomersen as adjunctive therapy for homozygous familial hypercholesterolemia. *J. Clin. Lipidol.* **10**, 860–869 (2016).
126. Santos, R. D. *et al.* Mipomersen, an antisense oligonucleotide to apolipoprotein B-100, reduces lipoprotein(a) in various populations with hypercholesterolemia: results of 4 phase III trials. *Arterioscler. Thromb. Vasc. Biol.* **35**, 689–699 (2015).

127. Chiriboga, C. A. *et al.* Results from a phase 1 study of nusinersen (ISIS-SMN(Rx)) in children with spinal muscular atrophy. *Neurology* **86**, 890–897 (2016).
128. Finkel, R. S. *et al.* Treatment of infantile-onset spinal muscular atrophy with nusinersen: a phase 2, open-label, dose-escalation study. *Lancet Lond. Engl.* **388**, 3017–3026 (2016).
129. Finkel, R. S. *et al.* Nusinersen versus Sham Control in Infantile-Onset Spinal Muscular Atrophy. *N. Engl. J. Med.* **377**, 1723–1732 (2017).
130. Mercuri, E. *et al.* Nusinersen versus Sham Control in Later-Onset Spinal Muscular Atrophy. *N. Engl. J. Med.* **378**, 625–635 (2018).
131. Mendell, J. R. *et al.* Eteplirsen for the treatment of Duchenne muscular dystrophy. *Ann. Neurol.* **74**, 637–647 (2013).
132. Cirak, S. *et al.* Exon skipping and dystrophin restoration in patients with Duchenne muscular dystrophy after systemic phosphorodiamidate morpholino oligomer treatment: an open-label, phase 2, dose-escalation study. *Lancet Lond. Engl.* **378**, 595–605 (2011).
133. Ackermann, E. J. *et al.* Suppressing transthyretin production in mice, monkeys and humans using 2nd-Generation antisense oligonucleotides. *Amyloid Int. J. Exp. Clin. Investig. Off. J. Int. Soc. Amyloidosis* **23**, 148–157 (2016).
134. Adams, D. *et al.* Phase 2 Open-Label Extension (OLE) Study of Patisiran, an Investigational RNAi Therapeutic for the Treatment of Polyneuropathy due to Hereditary ATTR (hATTR) Amyloidosis: Final 24-month data. 20.
135. Coelho, T. *et al.* Safety and efficacy of RNAi therapy for transthyretin amyloidosis. *N. Engl. J. Med.* **369**, 819–829 (2013).

136. Pasi, K. J. *et al.* Targeting of Antithrombin in Hemophilia A or B with RNAi Therapy. *N. Engl. J. Med.* **377**, 819–828 (2017).
137. Pasi, K. J. *et al.* Fitusiran, an Investigational RNAi Therapeutic Targeting Antithrombin for the Treatment of Hemophilia: Interim Results from a Phase 2 Extension Study in Patients with Hemophilia A or B with and Without Inhibitors. 16.
138. Ragni, M. V. *et al.* Fitusiran, an Investigational RNAi Therapeutic Targeting Antithrombin for the Treatment of Hemophilia: Results from Phase 1 and Phase 2 Extension Studies in Patients without Inhibitors. 13.
139. Ray, K. K. *et al.* Inclisiran in Patients at High Cardiovascular Risk with Elevated LDL Cholesterol. *N. Engl. J. Med.* **376**, 1430–1440 (2017).
140. Fitzgerald, K. *et al.* A Highly Durable RNAi Therapeutic Inhibitor of PCSK9. *N. Engl. J. Med.* **376**, 41–51 (2017).
141. Erdmann, J. & Schunkert, H. Forty-five years to diagnosis. *Neuromuscul. Disord.* **NMD 23**, 503–505 (2013).
142. Gualandi, F. *et al.* Antisense-Induced Messenger Depletion Corrects a COL6A2 Dominant Mutation in Ullrich Myopathy. *Hum. Gene Ther.* **23**, 1313–1318 (2012).
143. Foley, A. R. *et al.* Large Genomic Deletions: a Novel Cause of Ullrich Congenital Muscular Dystrophy. *Ann. Neurol.* **69**, 206–211 (2011).
144. Pinnell, S. R. Induction of Collagen Synthesis by Ascorbic Acid: A Possible Mechanism. *Arch. Dermatol.* **123**, 1684 (1987).
145. Sabatelli, P. *et al.* Collagen VI deficiency affects the organization of fibronectin in the extracellular matrix of cultured fibroblasts. *Matrix Biol.* **20**, 475–486 (2001).
146. Cristofalo, V. J. & Pignolo, R. J. Replicative senescence of human fibroblast-like cells in culture. *Physiol. Rev.* **73**, 617–638 (1993).

147. Pilling, D., Fan, T., Huang, D., Kaul, B. & Gomer, R. H. Identification of Markers that Distinguish Monocyte-Derived Fibrocytes from Monocytes, Macrophages, and Fibroblasts. *PLOS ONE* **4**, e7475 (2009).
148. Goodpaster, T. *et al.* An immunohistochemical method for identifying fibroblasts in formalin-fixed, paraffin-embedded tissue. *J. Histochem. Cytochem. Off. J. Histochem. Soc.* **56**, 347–358 (2008).
149. Österreicher, C. H. *et al.* Fibroblast-specific protein 1 identifies an inflammatory subpopulation of macrophages in the liver. *Proc. Natl. Acad. Sci. U. S. A.* **108**, 308–313 (2011).
150. Xiang, Y. *et al.* Establishment, characterization and immortalization of a fibroblast cell line from the Chinese red belly toad *Bombina maxima* skin. *Cytotechnology* **64**, 95–105 (2012).
151. Alexeev, V., Olavarria, J., Bonaldo, P., Merlini, L. & Igoucheva, O. Congenital muscular dystrophy-associated inflammatory chemokines provide axes for effective recruitment of therapeutic adult stem cell into muscles. *Stem Cell Res. Ther.* **11**, 463 (2020).
152. Murad, S. *et al.* Regulation of collagen synthesis by ascorbic acid. *Proc. Natl. Acad. Sci. U. S. A.* **78**, 2879–2882 (1981).
153. Pan, T.-C. *et al.* New Molecular Mechanism for Ullrich Congenital Muscular Dystrophy: A Heterozygous In-Frame Deletion in the COL6A1 Gene Causes a Severe Phenotype. *Am. J. Hum. Genet.* **73**, 355–369 (2003).
154. Butterfield, R. J. *et al.* Position of glycine substitutions in the triple helix of COL6A1, COL6A2, and COL6A3 is correlated with severity and mode of inheritance in collagen VI myopathies. *Hum. Mutat.* **34**, 1558–1567 (2013).

155. Schwarzbauer, J. E. & Sechler, J. L. Fibronectin fibrillogenesis: a paradigm for extracellular matrix assembly. *Curr. Opin. Cell Biol.* **11**, 622–627 (1999).
156. Paco, S. *et al.* Gene expression profiling identifies molecular pathways associated with collagen VI deficiency and provides novel therapeutic targets. *PloS One* **8**, e77430 (2013).
157. Tidball, J. G. & Wehling-Henricks, M. Damage and inflammation in muscular dystrophy: potential implications and relationships with autoimmune myositis: *Curr. Opin. Rheumatol.* **17**, 707–713 (2005).
158. Guadagnin, E. *et al.* Transcriptome analysis of collagen VI-related muscular dystrophy muscle biopsies. *Ann. Clin. Transl. Neurol.* **8**, 2184–2198 (2021).
159. Hurme, T. & Kalimo, H. Adhesion in skeletal muscle during regeneration. *Muscle Nerve* **15**, 482–489 (1992).
160. Alberts, B. *et al.* Integrins. *Mol. Biol. Cell 4th Ed.* (2002).
161. Giancotti, F. G. & Ruoslahti, E. Integrin Signaling. *Science* **285**, 1028–1033 (1999).
162. Boppart, M. D. & Mahmassani, Z. S. Integrin signaling: linking mechanical stimulation to skeletal muscle hypertrophy. *Am. J. Physiol. Cell Physiol.* **317**, C629–C641 (2019).
163. Hayashi, Y. K. *et al.* Mutations in the integrin $\alpha 7$ gene cause congenital myopathy. *Nat. Genet.* **19**, 94–97 (1998).
164. Nakashima, H., Kibe, T. & Yokochi, K. ‘Congenital muscular dystrophy caused by integrin alpha7 deficiency’. *Dev. Med. Child Neurol.* **51**, 245 (2009).
165. Wolff, G. & Esser, K. A. Scheduled Exercise Phase Shifts the Circadian Clock in Skeletal Muscle. *Med. Sci. Sports Exerc.* **44**, 1663–1670 (2012).

166. Esquifino, A. I., Cano, P., Jiménez-Ortega, V., Fernández-Mateos, P. & Cardinali, D. P. Neuroendocrine-immune correlates of circadian physiology: studies in experimental models of arthritis, ethanol feeding, aging, social isolation, and calorie restriction. *Endocrine* **32**, 1–19 (2007).
167. Scotton, C. *et al.* Deep RNA profiling identified CLOCK and molecular clock genes as pathophysiological signatures in collagen VI myopathy. *J. Cell Sci.* **129**, 1671–1684 (2016).
168. de Oliveira, B. F. *et al.* Ascorbic acid, alpha-tocopherol, and beta-carotene reduce oxidative stress and proinflammatory cytokines in mononuclear cells of Alzheimer's disease patients. *Nutr. Neurosci.* **15**, 244–251 (2012).
169. Kazemi, M. *et al.* Evaluation of the Effect of Ascorbic Acid Administration on Gene Expression Level of IL-6 and TNF- α Cytokines in Deceased Donors. *Iran. J. Allergy Asthma Immunol.* **14**, 149–157 (2015).
170. Mandl, J., Szarka, A. & Bánhegyi, G. Vitamin C: update on physiology and pharmacology. *Br. J. Pharmacol.* **157**, 1097–1110 (2009).
171. Deshmane, S. L., Kremlev, S., Amini, S. & Sawaya, B. E. Monocyte Chemoattractant Protein-1 (MCP-1): An Overview. *J. Interferon Cytokine Res.* **29**, 313–326 (2009).
172. Gesta, S. *et al.* Reduced expression of collagen VI alpha 3 (COL6A3) confers resistance to inflammation-induced MCP1 expression in adipocytes: COL6A3 Gene Silencing and Adipocyte Inflammation. *Obesity* **24**, 1695–1703 (2016).
173. Iyengar, P. *et al.* Adipocyte-derived collagen VI affects early mammary tumor progression in vivo, demonstrating a critical interaction in the tumor/stroma microenvironment. *J. Clin. Invest.* **115**, 1163–1176 (2005).

174. Chen, P., Cescon, M. & Bonaldo, P. Collagen VI in cancer and its biological mechanisms. *Trends Mol. Med.* **19**, 410–417 (2013).
175. Bechara, C., Chai, H., Lin, P. H. & Chen, C. Growth related oncogene-alpha (GRO- α): Roles in atherosclerosis, angiogenesis and other inflammatory conditions. *Med. Sci. Monit.* **13**, RA87–RA90 (2007).
176. Fakhr, E., Zare, F. & Teimoori-Toolabi, L. Precise and efficient siRNA design: a key point in competent gene silencing. *Cancer Gene Ther.* **23**, 73–82 (2016).
177. Gredell, J. A., Berger, A. K. & Walton, S. P. Impact of target mRNA structure on siRNA silencing efficiency: a large-scale study. *Biotechnol. Bioeng.* **100**, 744–755 (2008).
178. Khvorova, A. & Watts, J. K. The chemical evolution of oligonucleotide therapies of clinical utility. *Nat. Biotechnol.* **35**, 238–248 (2017).
179. Østergaard, M. E. *et al.* Rational design of antisense oligonucleotides targeting single nucleotide polymorphisms for potent and allele selective suppression of mutant Huntingtin in the CNS. *Nucleic Acids Res.* **41**, 9634–9650 (2013).
180. Chan, J. H., Lim, S. & Wong, W. F. ANTISENSE OLIGONUCLEOTIDES: FROM DESIGN TO THERAPEUTIC APPLICATION. *Clin. Exp. Pharmacol. Physiol.* **33**, 533–540 (2006).
181. Garanto, A., Duijkers, L., Tomkiewicz, T. Z. & Collin, R. W. J. Antisense Oligonucleotide Screening to Optimize the Rescue of the Splicing Defect Caused by the Recurrent Deep-Intronic ABCA4 Variant c.4539+2001G>A in Stargardt Disease. *Genes* **10**, 452 (2019).
182. Bartlett, D. W. & Davis, M. E. Insights into the kinetics of siRNA-mediated gene silencing from live-cell and live-animal bioluminescent imaging. *Nucleic Acids Res.* **34**, 322–333 (2006).

183. Wong, H. H., Seet, S. H., Bascom, C. C., Isfort, R. J. & Bard, F. Red-COLA1: a human fibroblast reporter cell line for type I collagen transcription. *Sci. Rep.* **10**, 19723 (2020).
184. Sun, B. The mechanics of fibrillar collagen extracellular matrix. *Cell Rep. Phys. Sci.* **2**, 100515 (2021).
185. Birch, H. L. Extracellular Matrix and Ageing. *Subcell. Biochem.* **90**, 169–190 (2018).
186. Du, Q., Thonberg, H., Wang, J., Wahlestedt, C. & Liang, Z. A systematic analysis of the silencing effects of an active siRNA at all single-nucleotide mismatched target sites. *Nucleic Acids Res.* **33**, 1671–1677 (2005).
187. Schwarz, D. S. *et al.* Designing siRNA that distinguish between genes that differ by a single nucleotide. *PLoS Genet.* **2**, e140 (2006).
188. Ohnishi, Y., Tamura, Y., Yoshida, M., Tokunaga, K. & Hohjoh, H. Enhancement of Allele Discrimination by Introduction of Nucleotide Mismatches into siRNA in Allele-Specific Gene Silencing by RNAi. *PLoS ONE* **3**, e2248 (2008).
189. Noguchi, S., Ogawa, M., Kawahara, G., Malicdan, M. C. & Nishino, I. Allele-specific Gene Silencing of Mutant mRNA Restores Cellular Function in Ullrich Congenital Muscular Dystrophy Fibroblasts. *Mol. Ther. Nucleic Acids* **3**, e171 (2014).
190. Seok, H., Lee, H., Jang, E.-S. & Chi, S. W. Evaluation and control of miRNA-like off-target repression for RNA interference. *Cell. Mol. Life Sci.* **75**, 797–814 (2018).
191. Jackson, A. L. *et al.* Expression profiling reveals off-target gene regulation by RNAi. *Nat. Biotechnol.* **21**, 635–637 (2003).
192. Bonaldo, P. Collagen VI deficiency induces early onset myopathy in the mouse: an animal model for Bethlem myopathy. *Hum. Mol. Genet.* **7**, 2135–2140 (1998).
193. Telfer, W. R., Busta, A. S., Bonnemann, C. G., Feldman, E. L. & Dowling, J. J. Zebrafish models of collagen VI-related myopathies. *Hum. Mol. Genet.* **19**, 2433–2444 (2010).

194. Demonbreun, A. R. *et al.* A gene-edited mouse model of limb-girdle muscular dystrophy 2C for testing exon skipping. *Dis. Model. Mech.* **13**, (2019).
195. Wiedenheft, B., Sternberg, S. H. & Doudna, J. A. RNA-guided genetic silencing systems in bacteria and archaea. *Nature* **482**, 331–338 (2012).
196. Larson, M. H. *et al.* CRISPR interference (CRISPRi) for sequence-specific control of gene expression. *Nat. Protoc.* **8**, 2180–2196 (2013).
197. Qi, L. S. *et al.* Repurposing CRISPR as an RNA-guided platform for sequence-specific control of gene expression. *Cell* **152**, 1173–1183 (2013).
198. Peddle, C. F., Fry, L. E., McClements, M. E. & MacLaren, R. E. CRISPR Interference—Potential Application in Retinal Disease. *Int. J. Mol. Sci.* **21**, 2329 (2020).
199. Mandegar, M. A. *et al.* CRISPR Interference Efficiently Induces Specific and Reversible Gene Silencing in Human iPSCs. *Cell Stem Cell* **18**, 541–553 (2016).
200. Smith, I. *et al.* Evaluation of RNAi and CRISPR technologies by large-scale gene expression profiling in the Connectivity Map. *PLoS Biol.* **15**, e2003213 (2017).
201. Nuñez, J. K. *et al.* Genome-wide programmable transcriptional memory by CRISPR-based epigenome editing. *Cell* **184**, 2503-2519.e17 (2021).

Danksagung und Widmung

Zunächst möchte ich mich bei Frau Prof. Dr. Jeanette Erdmann für die Überlassung eines besonderen und persönlichen Themas sowie die hervorragende Betreuung bedanken.

Ich danke besonders Herrn Dr. Zouhair Aherrahrou als mein Ansprechpartner und Betreuer während dieser Arbeit.

Außerdem möchte ich Frau Sandra Wrobel für die Anleitung und Einführung in die technische Laborarbeit danken.

Darüber hinaus gilt mein Dank allen Mitgliedern des Institutes für Kardiogenetik für das freundliche und persönliche Arbeitsklima.

Es ist mir ein besonderes Anliegen, meinem Ehemann, meinen Eltern und meinem Bruder für ihre Unterstützung und ihren Glauben an mich zu danken.

Ich widme diese Arbeit meinen Großeltern, die leider nicht mehr hier sind, aber heute sehr stolz gewesen wären.