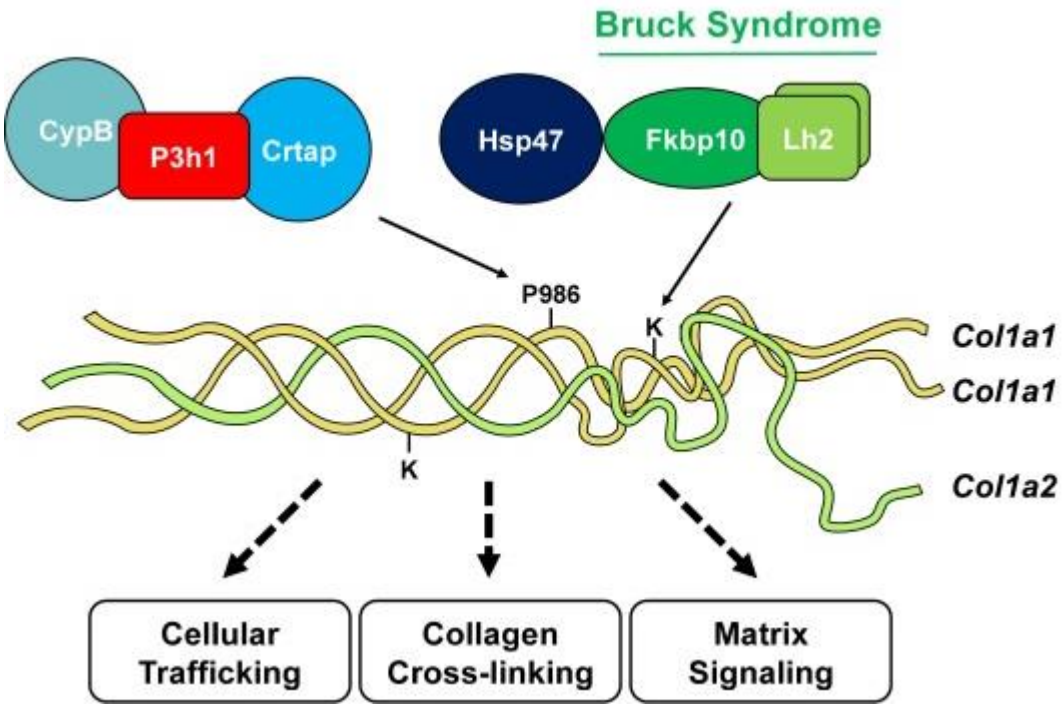
The future of Bruck Syndrome management: implementation of existing cell- and gene-targeted treatment options



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Source image title page: Lim J, Grafe I, Alexander S, Lee B. Genetic causes and mechanisms of Osteogenesis Imperfecta. *Bone*. 2017 Sep;102: 40-49. Figure 1, Genes involved in the post-translational modification of type I collagen; p.42.

Foreword

First and foremost, I want to thank prof. dr. Bank that he offered me a chance to look into future treatment options of a rare genetic disorder, since I am one of the few persons that has the chance to do so. I want to thank him for his guidance during the writing of this thesis.

This thesis is written for the completion of my pre-master biology, extending my knowledge about epigenetic editing tools as learned previously in the bachelor research project. As mentioned, I am honoured to make a (small) contribution, and hopefully, give insights for researchers that get to work on future treatment options for this rare genetic disease, about which little is known.

Summary

Bruck Syndrome (BRKS) is a rare autosomal recessive genetic disorder that affects bone development, resulting in joint contractures and bone fragility that start at infancy or early childhood. Mutations in the *FKBP10* and *PLOD2* genes result in the creation of abnormal bone formation. The current treatment is a combination of alleviation of symptoms and increase of bone formation by biphosphonates. These treatments are falling short of alleviating or resolving symptoms over a mid- to long term period. Therefore, implementation of new treatments are needed to tackle the problem of current treatments having too little impact. Existing methods like stem cell transplantation (SCT) and gene-editing techniques like CRIPSR/Cas and siRNA have been proven to have therapeutical value in various diseases, including rare genetic disorders. Mesenchymal stem cells (MSCs) can be used for allogenic transplantation *in utero* and postnatally, which provides a right functioning *FKBP10* gene. CRISPR/Cas9 is potentially able to knockout a mutated *FKBP10* gene, and knock-in a correctly functioning gene. siRNA is able to degrade the target sequence *FKBP10*, to stop the production produced by the mutated gene. These gene-targeted therapies can also assist in increasing the efficiency of MSC transplantation. The main limitation is the lack of *in vivo* studies on all fronts, and the main challenges of implementing these techniques are transfection of designed RNA into the nucleus and the risk of using toxic of immunogenic components. In conclusion, although these techniques remain highly experimental and have complicated challenges, especially regarding bone-related diseases like BRKS, current evidence shows enormous potential for future implementation of these techniques into the clinic.

Contents

Introduction

Bruck syndrome

Bruck Syndrome (BRKS) is a connective tissue disorder first described in 1897 by Alfred Bruck. It is a very rare disorder that is affects less than 1000 people in the US. It is also known as autosomal recessive osteogenesis imperfecta (OI) or OI type XI, and characterized by abnormal skeletal formation, congenital contractures, brittle bones, pterygia (blue colouring of the eyes) and joint contractures. The symptoms of BRKS often develop at a very young age. At birth, length is usually normal, but deformities are responsible for the short stature that is often discernible [1,2]. There are two forms of BRKS: BRKS1 and BRKS2. Although the two types are phenotypically indistinguishable, the biochemical processes are not. These two forms reflect autosomal recessive inheritance of *FKBP10* and *PLOD2* loss-of-function mutations, genes that are located on the *COL1A1/COL1A2* genes. BRKS1 is linked to the former: the *FKBP10* gene that encodes for the FKBP65 protein. BRKS2 is caused by mutations of specific loci on the *PLOD2* gene, that affects the production of lysl hydroxylase 2 (LH2) (Figure 1) [2].

FKBP10 gene functionality

Some patients that have OI symptoms have a mutation in the *FKBP10* gene, while having normal *PLOD2* function (BRKS1) [3]. The *FKBP10* gene is located on chromosome 17q21.2. The protein that is encoded by this gene is known as FKBP prolyl isomerase 10 or FKBP65 protein (FK506 binding protein65), which belongs to the FKBP-type peptidyl-prolyl cis/trans isomerase (PPIase) family of genes. FKBP65 can be found in the endo plasmatic reticulum (ER) [4]. The protein functions as a molecular chaperone, that has a critical role in the folding of type 1 procollagen [2]. Type 1 procollagen is a heterotrimer that is composed of two α1(I) chains and one α2(I) chain. These three chains bind at their carboxyl termini. Disulphide bonds form the chains through mediation of the enzyme protein-disulphide isomerase (PDI). Before the chains get folded, they undergo several posttranslational modifications: 4-prolyl hydroxylation, 3-hydroxylation of proline residue 986 and the α1(I) triple helical domain, hydroxylation processes of lysine residues by lysyl hydroxylase and finally the glycosylation of galactosyl- and glucosyl-transferases. With the help of the chaperones: PDI, BiP, and HSP47, the molecules get folded into a triple helix. After the folding of the helix, posttranslational modification occurs. These processes happen inside the ER, traffic to the Golgi apparatus afterwards, get packaged into secretory vesicles and travel to the extracellular matrix. Finally, amino- and carboxy-terminal propeptides get removed and a substrate is formed for bone mineralization [5]. A defect in the FKBP65 protein due to missense, nonsense and frameshift in *FKBP10*, often leads to delayed type 1 procollagen secretion and accumulation in the ER [6]. The PPIase activity is responsible for folding of the proline-rich tropoelastin and the folding of the type 1 collagen heterotrimer, but this activity is marginal [3]. Research has showed that loss of function of the FKBP65 protein causes a delay in type 1 procollagen secretion, disruption of the heterotrimer structure and decreased hydroxylation of telopeptide lysyl residues result in abnormal cross-linking of collagen [5, 7]. Loss of function of the FKBP65 protein does not only create abnormal crosslinking, but is accompanied by LH2. LH2, created

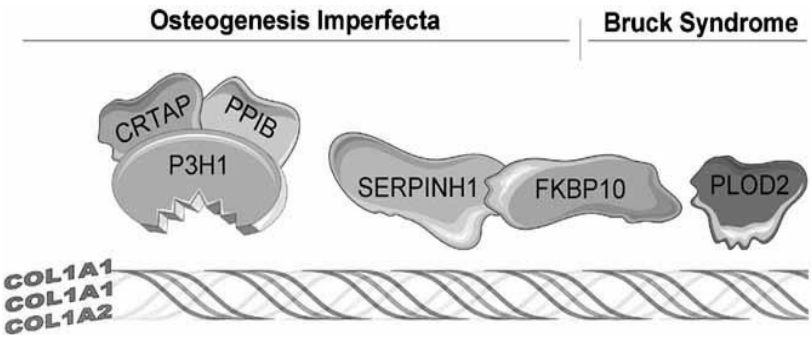

Figure 1: Genes and corresponding proteins involved in osteogenesis imperfecta (OI) and Bruck Syndrome (BRKS). Mutations in the COL1A1/COL1A2 gene are resulting in the recessive or dominant mutations of OI. BRKS1 is caused by a mutation in the *FKBP10* gene (chromosome 17q21) and BRKS2 is caused by a mutation in the *PLOD2* gene (chromosome 3q24) [2].

by the gene *PLOD2*, is a crucial protein for the formation of crosslinks. LH2 can physically interact with the FKBP65 protein, which stimulates dimerization and activation of LH2 [8].

Diagnosis and current treatment

BRKS syndrome is clinically characterized by features that are mostly the same as the other rare diseases OI and arthrogryposis multiplex congenita (AMC). BRKS is currently diagnosed as patients whom have congenital large joint contractures, bone fragility, multiple bone fractures that start at infancy or early childhood. Additional features can be postnatal short stature, pterygia, limb deformities and scoliosis. In contrast to other related bone diseases, patients with BRKS usually have normal sclerae, normal dentinogenesis, no hearing loss and normal intelligence [9]. The orthopedic manifestations of patients with Bruck Syndrome can be confirmed by radiography.

The clinical characterizations of patients with BRKS already manifest in fetuses. Abnormalities like bowed femurs and multiple joint contractures can be detected by prenatal ultrasound testing [10]. To get a diagnosis for a patient with a phenotype that resembles BRKS, molecular analysis can be performed. Genetic testing provides the possibility to get a diagnosis based on the exact mutation, which is needed to discriminate between BRKS1 and BRKS2, whom are phenotypically indistinguishable [11]. Multiple genetic tests are suitable for identifying mutations in these genes, but targeted next-generation sequencing or sanger sequencing seem to be the most efficient and accurate methods that are currently used. With targeted next generation sequencing, a targeted panel test is designed for genes that are related to OI, including *FKBP10*. Sanger sequencing can be used to determine the exact sequence of the gene of interest, by first amplifying the DNA and subsequently adding ddNTPs (fluorescent-labelled oligonucleotides) [12, 13].

Treatment

For patients with BRKS, not many treatment options are available. Because it is rare form of OI, treatment of these two diseases is quite similar. Obvious measures can be taken to alleviate pain and ease everyday life, such as using assistive devices like a wheelchair or canes, physical therapy, and orthopedic care. Before 1998, the treatment of OI was based on orthopedic surgery aiming at correcting deformities and fractures to stabilize bone construction. After 1998, researchers found that clinical treatment with bisphosphonates had significantly positive effects on quality of life and the reduction of fractures in patients with OI, which became part of the treatment of BRKS. Bisphosphonates are a potent antiresorptive agents that inhibit the osteoclast function and is the first in line treatment for patients with osteoporosis. Phosphonate groups (-PO3) can bind to hydroxyapatite crystals in bone; they have a high affinity for bone mineral, and therefore effective in inhibiting osteoclast-function. The use of bisphosphonates leads to increased bone mineral density, decreased bone turnover and reduced risk of fractures. The most wide-studied and used bisphosphonate is cyclic pamidronate [14]. A more recent treatment option that has been described in the literature is zoledronate. This compound is also a bisphosphonate and can be given intravenously. Treatment with zoledronate in patients with OI has been shown to: increase bone mineral density, decrease the number of fractures, reduction of (chronic) pain and increasement of height over and period of 1-3.5 years. This treatment comes with mild side-effects: it is reported that OI patients that used zoledronic acid encountered flu-like reactions. There are no published reports where bisphosphonates are used by patients with BRKS. Although, as mentioned earlier, BRKS patients may be responsive to treatment because of the similarities between the diseases. Because the lack of controls, number of cases and clinical heterogeneity, research regarding treatment options are very limited [15].

Future treatment options

The current treatment options for BRKS, such as orthopedic care and dosing of intravenous bisphosphonates, are falling short of giving a substantial solution for alleviating or resolving symptoms over a longer period of time. Because of the rarity of the disease, specific treatment options are not extensively researched. There are existing techniques that have the potential to downregulate the production of proteins that have mid- to long-term effects on improvement of the symptoms patients with BRKS suffer from. In the case of patients with BRKS1, that have abnormal formation of the FKBP65 protein, several techniques may be suitable for creating a normal functioning protein. Some existing cell and gene-targeted therapies are technically suitable for realising a substitution of a mutated *FKBP10* gene to a normal functioning gene. One of these techniques is stem cell transplantation. A research study that has investigated this method already, is Boost Brittle Bones Before Birth (BOOSTB4). BOOSTB4 is an European trial that focusses on mesenchymal stem cell (MSC) transplantation as a therapy for severe forms of OI. This trail recently suggested implementing this technique and setting up an European network where patients with severe OI are able to receive MSCs in Stockholm, London, Cologne and Utrecht/Leiden [16, 17]. At the gene level, repair of gene-abnormalities such as deletions or insertions in a transient or permanent manner are gaining interest rapidly. The most common and popular method is CRISPR/Cas. This system is a highly precise genomic editing tool that operates with a sequence-specific nuclease to cut out and replace specific sequences in the genome [18, 19]. A similar method of gene-editing is the use of small interfering RNAs (siRNAs). siRNAs are short double-stranded RNAs that specifically target mRNA sequences to subsequently degrade them [20].

With science-based evidence about the efficiency of existing techniques that are technically applicable to BRKS, questions regarding treatment options for this rare genetic disease arise: are stem cell transplantation and gene-editing techniques like CRISPR/Cas and siRNA potentially superior in alleviating symptoms to current treatment methods, and suitable for curing BRKS?

Stem cell transplantation

Stem cell transplantation (SCT) is the replacement of stem cells in bone marrow. Stem cells have two functions: the capacity to self-renew and to differentiate into a variety of cells types, which makes the cells multipotent. Replacement of stem cells in patients with abnormal protein production due to genetic errors, can be done through donation of healthy (allogenic) stem cells or through gene-editing adjusted autogenic stem cells. New self-generating stem cells are then able to produce the desirable protein. The most well-known stem cells are hemopoietic stem cells (HSCs). HSCs are used in the treatment of various types of cancer, specifically leukaemia, because HSCs are the precursors of platelets, red- and white blood cells. In case of patients with bone-specific pathologies like BRKS, stem cells that differentiate to bone cells are important: these include pluripotent stem cells (iPSCs), mesenchymal stromal cells (MSCs) and adipocyte-derived stromal progenitor cells (ASCs). The current research, however, seems to be focussing on MSCs. MSCs were initially identified in adult bone marrow, adipose tissue and umbilical cord. The MSC lineage is indirectly responsible for the production of the proteins that are related to bone-related issues: cells like osteoblasts are primarily responsible for the production of collagen and collagen-related proteins. Key features of MSCs are that the cells are not very immunogenic, and are able to migrate to specific target site to engraft. MSCs come in different forms, including fetal MSCs. Research showed that fetal MSCs have advantages over adult MSCs in multiple ways: they have a greater colony-forming capacity, higher proliferative capacity, a superior proliferative potential, produce more osteogenic genes, and induce more calcium production (*in vitro*). Research showed that prenatal intravenous infusion of autologous or allogeneic MSCs appears to be safe, without high amounts of side-effects [16, 21]. Since anomalies in patients with

BRKS already occur in the womb, *in utero* stem cell transplantation (IUSCT) may be a solution. In recent decades, prenatal imaging, molecular diagnostics and prenatal surgery techniques have rapidly improved, making *in utero* treatment more accessible. Ultrasound techniques enable physicians to administer MSCs into the umbilical vein of the fetus, which is a similar procedure as the established fetal blood transfusion method [22]. Preclinical animal studies show that IUSCT of different stem cells, including MSCs, into the intra-amniotic fluid and the spinal cords of rats, rabbits, chickens and sheep, is safe and effective [23]. The clinical trials with in IUSCT in fetuses are predominantly done through the umbilical cord, and have shown to be effective [21]. The BOOSTB4 project already started with pre- and postnatal infusion of adult allogenic MSCs in fetuses and children with OI: the children that received the treatment improved their growth and reduced the number of fractures. The only observed side-effect was an allergic reaction after 12 infusions [21, 24]. The upside of MSC treatment is that HLA-mismatching is acceptable for transplantation due to the low immunogenicity. In other transplantations, like HSC, HLA-matching is required for the succession of the transplantation. Although MSCs have advantages other stem cells do not have, several limitations and hurdles exist. MSCs have low potency in *in vivo* condition compared to *in vitro* (differentiation stability is hard to control), a low homing rate, low cell viability after transplantation and no sufficient expression of all factors [20].

siRNA

siRNA gene therapy offers a precise and personalized treatment of diverse diseases. This method is similar to gene-editing with CRISPR/Cas, but discriminates on the technical front. RNA interference (RNAi) is a natural defence mechanism of organisms to prevent the invasion of exogenous genes. Small interfering RNAs (siRNA) are double-stranded RNAs that divide in single-strand to bind to target mRNAs. To induce the RNAi present, siRNA has to be completely complementary to the target mRNA. The siRNA agents get help of an enzyme called endoribonuclease dicer (helicase) that reduces the length of long dsRNA or hairpin DNA. One strand of the remaining siRNA that is most suited and used subsequently, is cleaved to the RISC complex. The RISC complex binds to the target complementary mRNA, AGO2 cleaves to phosphodiester backbone, and subsequently the target mRNA gets degraded by exonucleases

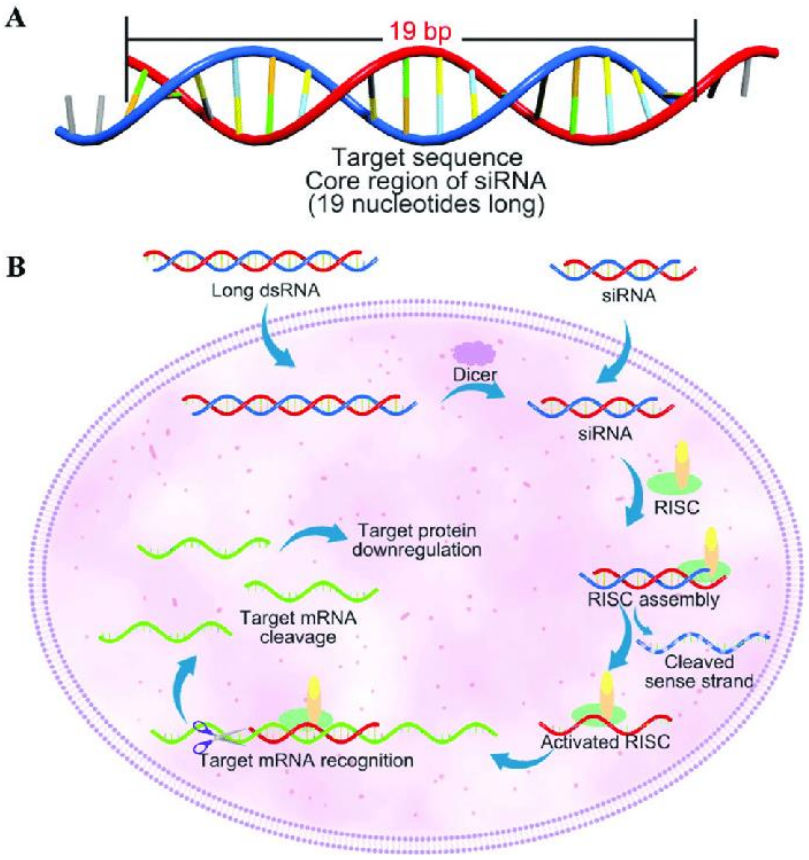

Figure 2: Mechanism of siRNA gene silencing. A dicer molecule cuts long double-stranded RNA (dsRNA), which cleaves to the RISC complex. The RISC complex is able to cleave the sense strand, which activates the RISC-siRNA complex. The activated RISC-complex can recognize and cleave the target mRNA which causes inactivation of the target sequence. Source: Shen J, Zhang W, Qi R, Mao ZW, Shen H. Engineering functional inorganic-organic hybrid system advances in siRNA therapeutics. *Chemical Society Reviews*. 2018 Mar 21;47(6): 1969-1995. Figure 3, Structure and mechanism of siRNA: p.1987.

(figure 2). The advantage of siRNA therapy is that the separate components are highly modifiable: phosphate, ribose, and base groups can all be edited, so that designed siRNAs are applicable to almost every gene. Because of this modification ability, siRNA can be designed for very specific sequences, and thus for very rare genetic disorders. siRNA therapy has been successful and commercialized for two rare-genetic diseases already: ONPATTRO® is designed for hereditary transthyretin amyloidosis (ATTRv), and GIVLAARI™ for acute hepatic porphyria (AHP) [25].

CRISPR/Cas9

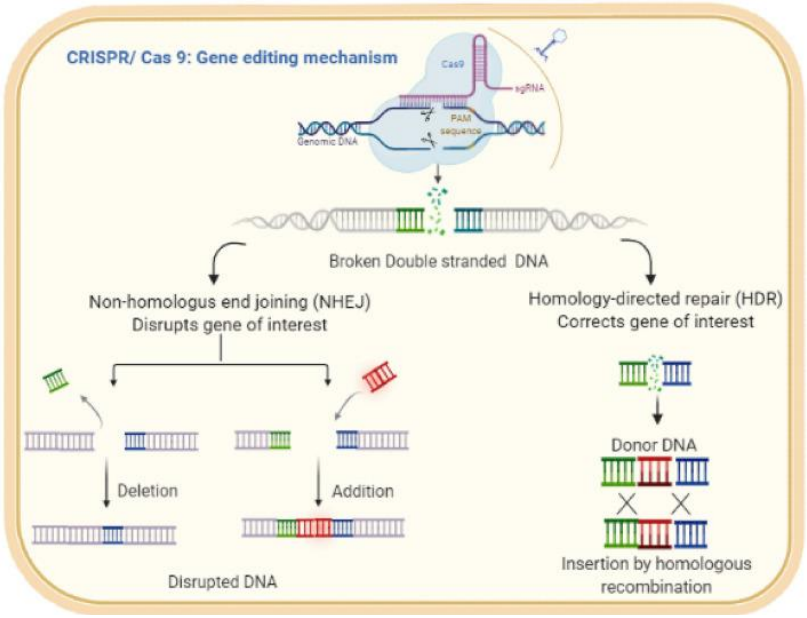

Figure 3: Mechanisms of CRISPR/Cas9 system. sgRNA cleaved to a specific gene-targeted sequence enables double-strand breaks on the target DNA. Strand breaks DNA can be repaired either by non-homologous end joining (NHEJ) or by homology-directed repair [26].

The CRISPR/Cas system is one of the present-day mostly used gene-editing tools in molecular biology. The addition of CRISPR/Cas complex into genomic DNA enables the system to cut out specific DNA sites, and replace them with DNA of interest (figure 3). Clustered regularly interspaced short palindromic repeats (CRISPR) are an integrated part of bacteria and have defensive purposes. These clustered inverted repeats function as a defensive mechanism for bacteria, and are also functional in a CRIPSR/Cas system. Engineered RNA- or DNA binding proteins can target specific sequences in the genome; CRISPR RNA (crRNA) or single-guide RNA (sgRNA) can be designed that are complementary to the genomic sequence of interest. The Cas protein, of which Cas9 is the most widely studied, is an effector protein that can bind to sgRNA. Cas9 is able to cut specific sequences, causing the formation of blunt ends because of double-stranded DNA breaks. During non-homologous end joining or recombination, a donor template DNA can repair the site where Cas9 caused these blunt ends (figure 3) [26]. CRISPR/Cas9 plays a big role in the therapeutic industry, having cured many diseases, including genetic-related diseases. For example, with the use of CRISPR/Cas9, dystrophin point mutation correction prevented muscular dystrophy development in mice, adjusted cystic fibrosis transmembrane conductor receptor (CFTR) in human stem cells, and inactivated the *FREP1* gene in *anopheles gambiae* suppressing malaria parasite infection.

Deliverance of gene-editing components into the nucleus

A crucial component to realising a successful DNA change by gene-editing systems, is the transfection of these systems into the nucleus of the cell. Different methods can be used, that are divided into two categories: non-viral and viral transfection. Non-viral transfection predominantly include polymer- and lipid-based systems. The advantages of non-viral transfection are the safety, low production costs, high modifiability and the ability to achieve site-specific delivery by integrating targeting ligands, improvement of serum stability and extension of circulation time by attachment of polyethylene glycol polymer chains (PEG). Despite the advantages, the carry-over of *in vitro* to *in vivo* experiments are lacking. *In vitro* experiments are usually effective, but fail *in vivo* because of toxicity problems, poor pharmacokinetic profiles, nonspecific uptake or immune responses. In contrast, viral vectors are

operating differently and have other (dis)advantages. Viral vectors include lentiviruses, adenoviruses and adeno-associated viruses (AAVs), and are much more efficient in transferring RNA-encoding vectors into the nucleus of mammalian cells than non-viral vectors. Viruses can take up RNA sequences into their vector. Viral vectors have a high transduction efficiency, which makes this method more stable and reliable, in terms of transfection, compared to non-viral methods. Next to this, permanent transfection of RNA in the host genome is possible. Although promising features of viruses can have a big impact on the succession of gene-editing, major drawbacks are accompanied by the use of viral vectors. While viral gene delivery is highly efficient, high immunogenicity and insertional mutagenesis can be highly troublesome. High production costs and low packaging capacity are also limiting factors when using viruses. Some cell lines are harder to transfect than others. When future research focusses on treatment with mesenchymal stem cell transplantation combined with gene-editing techniques, the crucial component is the optimization of transfection [27, 28].

Gene-editing of MSCs

It has been demonstrated that stem cell transplantation and gene-editing techniques like CRISPR/Cas and siRNA have been successful in tackling gene-related diseases. The CRISPR/Cas system has proven to play a significant role in curing many genetic diseases in animal-models (brittle bones/*oim* mice) and humans, just like siRNA therapy [20, 25, 27]. Theoretically, treatment of BRKS can be done by autogenetic transplantation of MSCs combined with gene-editing systems: replacement/repair of the *FKBP10* gene (knock-out of mutated gene and knock-in of correct functioning gene afterwards) should provide the generation of a correct functioning FKBP65 protein. However, since allogenic transplantation appears to be safe, this is not necessary. Nonetheless, gene-editing systems like CRISPR/Cas9 editing can assist in and improve the therapeutical efficiency of MSC transplantation. Genetically engineered MSCs have the ability to improve direction to target site (homing), transplantation survival, guided differentiation into the osteogenic lineage and the migration to specific locations like exosomes by stimulating the expression of ligands [29]. This also accounts for siRNA therapy. siRNA therapy has been used in combination with MSCs to increase therapeutic effects in several diseases. Just like CRISPR/Cas9, siRNA therapy has shown to alter migration or homing properties of stem cells. Altering MSC gene expression by siRNA therapy is a relatively new field of research, but some studies have showed success already. For example, one study genetically engineered MSCs that contained exosomes with high expression of CXC chemokine receptor type 4 (CXCR4) to target cancer cells *in vivo,* and were successful in doing so. Even in human embryonic cells, researchers successfully knocked-down targeted genes with phenotypical changes shortly afterwards [30]. Although siRNA is a powerful tool to alter gene function, translation to therapeutics has struggled to overcome the hurdle of efficient and reliable delivery to the target [31]. One of the main hurdles of injecting siRNA and CRISPR/Cas9 successfully, is transfection. The primary concern in the manufacturing of genetically modified MSCs is the use of viral vectors. As mentioned, viral vectors are immunogenic, independent of the type of cell transfected, which is problematic. Thus, perfecting non-viral delivery has gained popularity. Recent developments show improvement of nanocarrier technology, like lipid nanoparticle (LNP) transfection (which is used in GIVLAARI™ and ONPATTRO®), during *ex vivo* culturing of MSCs [32]. The same success has been demonstrated in the use of deblock copolymers of siRNA into MSCs [33].

Discussion

The question that remains to be answered is whether stem cell transplantation and gene-editing techniques like CRISPR/Cas and siRNA are superior in alleviating symptoms to current treatment methods, and if they are potentially suitable for curing BRKS. Gene and cell manipulation methods including SCT, CRISPR/Cas and siRNA have been broadly used in the treatment of various diseases

already. Although gene and cell manipulation have advantages, problems and downsides are part of the current research and applications. Regarding stem cell therapy, transplantation of MSCs appears to be a safe intervention. A systematic review and meta-analysis published in 2012 reported that acute infusional toxicity is not observed in the studies where unmatched allogenic MSCs were dosed, which supports the idea of MSCs being 'immune-privileged'. Due to the low expression of MHC class ll proteins, rejection in allogenic transplantation is mostly prevented; MHC class l may be a different story. MHC class l could have immunogenic characteristics. If immunorejection in allogenic MSC transplantation forms a potential problem, knockdown of the light chain of MHC l class molecule β2-microglobulin (B2M) may be a solution [20]. Finally, using dimethylsulfoxide as cyropreservative (cooling down cell-components for preservation) may be toxic, but this phenomena was only seen once. Other side-effects are relativity mild, like allergic reactions of fever due to intravenous injections [34]. The superior form of MSCs seems to be fetal MSCs, as they provide many advantages compared to adult MSCs for transplantation, including osteogenic therapeutical potential [16]. Since anomalies already occur in the womb, *in utero* or prenatal stem cell transplantation is probably best to prevent the formation of new fractures and bone-abnormalities. Rapid technical improvement of prenatal imaging makes it possible to inject stem cells with MSCs, guided by ultrasound, and has proven to be effective in doing so [22, 23]. Although postnatal treatment is superior in some respects, like taking away the potential of hurting the mother and haveing a more stable infusion of MSCs, there are arguments that are worth mentioning choosing IUSCT rather than postnatal injection of MSCs. These advantages include: 1) a relatively smaller dose is required because the size of a fetus and 2) MSCs can manoeuvre in arterial circulation rather than being trapped in the lungs postnatally [35]. Combining these two treatments may be even better: in practice, in two patients who received pre- and postnatal allogenic fetal MSCs transplantation, it showed to be effective regarding the reduction of fractures, and the treatment appears to be safe [24]. Although the results of pre- and postnatal MSC transplantation show promising results, the therapy remains highly experimental, in particular for patients with OI; patients with BRKS did not even get such a treatment yet. Next to (fetal) MSCs transplantation, CRISPR/Cas9 engineering is expected to deliver accurate and precise results in a therapeutical way to tackle gene-related problems. Most research and success is obtained with *in vitro* studies. But lately, with *in vivo* AAV-based CRISPR/Cas9 gene-editing, quite some achievements have been made in regard to the treatment of other forms of OI. Non-homologous end joining (NHEJ) (figure 3) has shown to be highly accurate and efficient in gene-editing and a lot of clinical data has been gathered on the topic [27]. One major problem is the threat of potential off-target effects. siRNA has similar potential, but the absence of ability to knock-in genes may be problematic, since patients with BRKS have two mutant copies. Nonetheless, siRNA has proven its therapeutical value, and can surely assist in perfecting the potential of MSCs transplantation. An overarching problem of these gene-editing techniques is the transfection of particles into the nucleus. Since using viral factors are potentially immunogenic, optimizing non-viral transduction is probably better to pursue. On the other hand, solving the problem of realising consistent, highly accurate non-viral transfection is hard [28, 30].

Conclusion and outlook

In conclusion, future treatment options like stem cell transplantation, *in utero* and postnatally with progenitor cells such as (fetal) mesenchymal cells, gene-editing with CRISPR/Cas9 and siRNA are powerful tools that have proven to be efficient in treating various gene-related diseases, and have great potential regarding therapeutical application for different forms of OI, including BRKS. The main challenges are: optimizing non-viral transfection of MSCs, transfecting CRISPR/Cas9 and siRNA editing into bone-related cells, resolving the problem of off-target effects and reducing the use of

immunogenic and toxic components used in SCT and gene-editing. The main limitation is the lack of evidence with *in vivo* studies and clinical trials regarding BRKS and other forms of OI.

For future research, I advocate researchers to start with mapping all the problems that come with using these techniques, with the aim of implementing it into the clinic. First and foremost, the potential of pre- and post-natal allogenic (fetal) MSCs transplantation has to be investigated further, as it seems that this holds therapeutical potential, such as demonstrated by the BOOSTB4 project. Gene-editing systems can definitely contribute to the treatment of BRKS, especially CRISPR/Cas9, since this technique is able to knock-out and knock-in genes.

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