MDPI

Review

Gene Therapy in Pediatric Orthopedics

Emmanuel Olaonipekun [1,*] **, Anthony Lisyansky** [2] **, Robin Olaonipekun** [1] **, Bouchra Ghania Merabia** [3] **, Karim Gaber** [4] **and Waleed Kishta** [4]

1 School of Medicine, Royal College of Surgeons, D02 YN77 Dublin, Ireland; robolaonipekun@gmail.com
2 Faculty of Science, Western University, London, ON N6A 3K7, Canada; anthony.lis135@gmail.com
3 Faculty of Science, McGill University, Montréal, QC H3A 1G1, Canada; bouchra.merabia@mail.mcgill.ca
4 Division of Orthopedic Surgery, Department of Surgery, McMaster University,
   Hamilton, ON L8S 4L8, Canada; karim.gabber@gmail.com (K.G.); kishtaw@mcmaster.ca (W.K.)
* Correspondence: emanola419@gmail.com

Abstract: Gene therapy is gaining traction as an effective treatment for several deleterious disorders by delivering genetic material using viral or non-viral vectors to correct mutated genes. Research in the field focuses primarily on the treatment of cancers; however, it shows great promise for treating diseases related to pediatric orthopedics. This review aims to describe gene therapy's application, efficacy and safety in pediatric orthopedics. This paper will examine common pediatric orthopedic disorders including Duchenne muscular dystrophy, osteogenesis imperfecta, spinal muscular atrophy and osteosarcoma. Overall, gene therapy for spinal muscular atrophy and Duchenne muscular dystrophy has made great advances with approved gene therapy drugs already in use, while therapy for osteogenesis imperfecta and osteosarcoma treatments is still widely preclinical but still promising. As a whole, gene therapy is rapidly advancing in the field of pediatric orthopedics; however, further research is crucial in continuing and spreading these advancements and for the treatment of other debilitating pediatric-related orthopedic disorders.

Keywords: gene therapy; pediatric orthopedics; osteogenesis imperfecta; Duchenne muscular dystrophy; osteosarcoma; spinal muscular atrophy

1. Introduction

Gene therapy allows for the modulation and correction of specific problem genes which are mutated in severe pathologies. The term "gene therapy" is loosely defined by many sources. The FDA defines it as "products whose effects are transferred through transcription/translation of genetic material via administration as nucleic acids, viruses, or genetically engineered microorganisms" [1]. Typically, gene therapy is categorized into two broad types: somatic and germ-line therapy. In somatic therapy, genetic material is inserted in target cells, but the resulting changes are not passed along to the next generation; in contrast, germ-line gene therapy involves the transfer of therapeutic or modified genes in target cells to subsequent generations [1]. The concept of gene therapy was first conceptualized and trialed in 1928 with Frederick Griffith's "Griffith experiment" [1]. Griffith's trial focused on the transformative qualities of bacteria, demonstrating that bacteria could be injected with pathogenic genetic properties, which enhanced their virulence [2]. These trials laid the groundwork which was further explored by Joshua Lederberg and his team in 1947. Ledergberg, who won a Nobel prize in 1958 for his work in bacterial genetics, discovered the transformative and conjugative properties of bacteria [1]. Watson and Crick's discovery of DNA structure in 1953 and Marshall Nirenberg's discovery of the "triple code of DNA" in 1961 were subsequent and important breakthroughs in genetic therapy. Following these discoveries, the area of genetic therapy has seen numerous advancements. In the mid-1990s, scientists began to see increased success and potential with the use of viral vectors for genetic therapy [2]. This led to trials in animals and humans which yielded variable

responses [2]. Adenoviral vectors, retroviral vectors and naked plasmids are currently the most popular vectors used in genetic therapy as they correlate with the most ideal results [1]. Today, cancer is also the most researched field with regard to gene therapy; however, other fields like orthopedics also have extensive ongoing trials [1]. Pediatric orthopedics is a subset of orthopedics that focuses on pathologies involving children aged 0–18 years old. In pediatric orthopedics, gene therapy is an area of great promise. Drug trials are currently ongoing for the treatment of severe conditions like Duchenne muscular dystrophy (*DMD*), osteogenesis imperfecta (OI), spinal muscular atrophy (SMA) and osteosarcoma, with a few of these drugs reaching the point of commercial use (Table 1). This paper seeks to review some of the existing gene therapy interventions present in pediatric orthopedics and outline prospects for the field as a whole.

Table 1. Pediatric orthopedic disease name, MIM identification, ongoing gene therapy trials and corresponding trial identification numbers.

Disease Name	MIM Number	Treatment Type	Name of Treatment	Phase of Clinical Trial	Clinical Trial Number
Duchenne Muscular Dystrophy	#310200	*Microdystrophin* gene replacement	SRP-9001	Phase I	NCT04626674
			PF-0693992	Phase III	NCT04281485
			SGT-001	Phase I/II	NCT03368742
		GALGT2 gene replacement	rAAVrh74.MCK.GALGT2	Phase I/IIa	NCT03333590
		Anti sense oligonucleotides	Eteplisern	FDA approved	N/A
			Golodirsen	FDA approved	N/A
			Casimersen	FDA approved	N/A
			Viltolarsen	Phase II/III and IV	NCT04060199
			scAAV9.U7.ACCA	Phase I/IIa	NCT04240314
		CRISPR/Cas9 Therapy	CRISPR/Cas9	Pre clinical	N/A
Osteogenesis Imperfecta	#166200	Gene silencing and iPSC use	COL1A1 silencing	Pre clinical	N/A
		CRISPR/Cas9 and iPSC use	COL1A1/2 editing	Pre clinical	N/A
		Yamanka factor and iPSC use	N/A	Pre clinical	N/A
Spinal Muscular Atrophy	#253300	*SMN1* gene replacement	Onasemnogene Abeparvovec	FDA approved	N/A
Osteosarcoma	#259500	Tumor supressor gene targeting	TP53 knockout	Pre-clinical	N/A
		proto-oncogene targeting	MDM2 targeting	Pre-clinical	N/A
		Mi-RNA delivery	MiR-29b delivery	Pre-clinical	N/A
		Mi-RNA delivery	Mi-R-520a-3p delivery	Pre-clinical	N/A

2. Gene Therapy in Duchenne Muscular Dystrophy (#310200)

Duchenne muscular dystrophy (*DMD*) is a severe X-linked recessive muscle-wasting disease that results from mutations in the *DMD* gene on chromosome 21 [3]. The *DMD* gene forms dystrophin protein, an integral muscle structure and integrity component. Without sufficient dystrophin, muscles are exposed to increased damage leading to progressive

muscle wasting and loss of muscle function. *DMD* is a pediatric-laden disease with a diagnosis usually occurring in children aged 3–5 [3]. The disease complications of *DMD* typically progress during childhood, leading to mortality in young adults in their early 20s [3]. Due to the nature of the disease, *DMD* care can often be complex, and treatment usually involves a multi-disciplinary team (MDT) treating cardiorespiratory, endocrine, orthopedic and rehabilitative complications. Due to its poor survivorship, many interventions are being researched to improve outcomes for patients with this disease; gene therapy is an emerging field in this regard.

2.1. Gene Replacement Therapy

The goal of GRT is to transfer corrective material into cells to alleviate disease symptoms. Corrective material in these cases can be delivered via viral/non-viral delivery systems. In *DMD*, GRT is not aimed at correcting specific mutations but rather restoring muscle function via the injection of truncated or muscle-protective enzymes [4]. To this avail, many gene replacement therapies/treatments aimed at perfecting truncated/muscle-protective enzymes to be used in *DMD* treatment are currently ongoing.

2.1.1. Microdystrophin Targeting

Most of the trialed GRTs in *DMD* focus on the *micro-dystrophin* gene. The *micro-dystrophin* gene is a truncated version of the *dystrophin* gene. Because of its altered form, the gene produces dystrophin proteins which are a third of their expected size. These proteins have a decreased capacity to strengthen and protect muscle fibers, leading to their injury. *Micro-dystrophin* targeting trials range from phase 1 to phase 3 and have expected finish dates from 2023 to 2028. Currently, three drugs are being developed for GRT targeting *micro-dystrophin* in *DMD*: SRP-9001 by Sarepta Therapeutics, PF-06939926 by Pfizer and SGT-001 by Solid Biosciences [4]. SRP-9001 is transmitted using vector rAAVrh74 and uses promoter MHCK7 to enhance cardiac dystrophin expression [4]. SRP-9001 first showed promise in Study 102, a two-part phase 2 placebo-controlled trial. The first part of the trial involved SRP-9001 meeting the biological outcome of micro-dystrophin protein production, the second part had *DMD* patients present with statistically significant scores on the North Star Ambulatory Assessment (NSAA) when treated with SRP-9001 compared to the external control group [4]. These results led to SRP-9001 initiating the EMBARK study whose primary outcomes were to see if basepoint NSAA could be changed after 52 weeks of use. In addition to the EMBARK study, SRP-9001 also finished the 1-year phase ½ trial (NCT03375164) with promising results [5]. The trial involved SRP-9001 administration via a peripheral limb vein with daily administration of prednisolone started 1 day before SRP-9001 administration and tapered off for 30 days after [5]. The trials showed SRP-9001 to be well tolerated with minimally adverse effects while eliciting improvements in baseline NSAA scores and system creatine kinase levels [5]. SRP-9001 is also currently undergoing a phase 1 open-label trial called ENDEAVOR which is expected to finish in 2024 [4]. PF-0693992, unlike SRP-9001, uses AAV9 instead of rAAVrh74 as a vector. Its safety following IV administration is currently being tested in a phase 1b open-label clinical trial. Pre-trial data for the study presented PF-06939926 to have an acceptable safety profile with treatment-related side effects thrombocytopenia, dehydration and acute kidney injury all resolving within 15 days of presentation [4]. In the same phase 1b open-label trial, PF-0693992 also resulted in an NSAA score of +1 in the experimental group compared to the −4 seen in the control cohort group. This, similar to what was seen in SRP-9001, prompted an ongoing phase 3 study for PF-0693992 which is examining basepoint NSAA scores after 52 weeks of use [4]. SGT-001 contains a neuronal nitric oxide domain which aids it in preventing ischemia-related muscle injury. It is currently being evaluated in the phase ½ trial IGNITE *DMD*. IGNITE *DMD* is a trial aimed at testing SGT-001's effect on SV95C, an assessment of post-administration peak ambulatory performance in *DMD* patients five years old or older [6]. The study involves patients receiving one IV shot of SGT-001 and being subsequently followed for about 5 years [7] (NCT03368742). So far, the study has

been promising, with IGNITE *DMD* patients demonstrating average improvements in SV95C scores of 8.8–9.5% compared to baseline, 23.9–24.6% compared to natural history and 26.0–26.7% compared to the control patients [6]. These patients have also expressed stable and increased micro-dystrophin as well as continued localization of B-sarcoglycan and nNOS compared to their baseline results [4].

2.1.2. GALGT2 Targeting

Although *micro-dystrophin* targeting is the mainstay of gene replacement therapy in *DMD*, success has also been seen in trials targeting the *GALGT2* gene. One such study which finished in 2021 has since progressed to a phase ½ trial due to its promising results. The *GALGT2* gene encodes for the GALGT2 enzyme which glycosylates α-dystroglycan in skeletal muscles, increasing dystroglycan-binding proteins like dystrophin. Targeting of this gene has proven useful and could be a future alternative to *micro-dystrophin* gene targeting for *DMD* treatment. In 2018, an AAVrh74-mediated *GALGT2* GRT trial under the control of a Muscle Creatinine Kinase (MCK) promoter finished with positive results [4]. In the trial, no organ damage was seen at 1 and 3 months after drug administration, and widespread positive transduction was seen with mice injected with the drug. These results were positive and show promise in the area of *GALGT2* targeting.

2.2. Antisense Oligonucleotides

As the term "gene therapy" is a bit loosely defined, the use of antisense oligonucleotides (ASOs) in *DMD* is occasionally debated. The general definition for this therapy is the treatment of disease via the transfer of genetic material into cells [8]. As stated by the YU lab, although they act on genetic diseases, ASOs are not considered gene therapy because they act on RNA, not DNA [9]. Other studies, however, acknowledge ASOs as a form of genetic therapy, and thus, their inclusion is ambiguous. In *DMD*, ASOs are used as a method of skipping exons via binding to a region of mRNA; this is especially beneficial in *DMD* as the majority of *DMD* mutations are located between exons 43 and 53, allowing for widespread application of treatments [4]. There are a few FDA-approved ASO treatments available; these include Eteplisern, Casimersen and Golodirsen, all developed by Sarepta Therapeutics, and Vitolarsen, developed by Nippon Shinyaku in collaboration with the National Centre of Neurology and Psychiatry [4]. Eteplisern was the first FDA-approved drug for *DMD*. The drug is a Phosphorodiamidate morpholino oligomer (PMO) which binds to the complementary exon 51 on preMRNA dystrophin, causing exon skipping during the dystrophin mRNA splicing process [4]. Despite the specificity of its function, it applies to a wide range of patients as 13–14% of *DMD* patients have mutations that benefit from skipping exon 51 [4]. In July 2011, a 12-participant study showed that the drug did not elicit any serious disadvantages while restoring dystrophin levels; the success of this study was followed up upon FDA request, leading to a phase 3 trial called PROMOVI, which similarly demonstrated Eteplisern's ability to restore dystrophin levels despite a lack of statistically significant restoration [4]. Following these results, Eteplisern was approved for public use; however, ongoing confirmatory studies to establish its efficacy are still being run by Sarepta Therapeutics [4]. Golodirsen is another PMO developed for the treatment of *DMD*. It facilitates the skipping of exon 53 in the dystrophin gene, which assists in the treatment of around 7.7% of *DMD* patients. It first garnered attention in a phase ½ trial which showcased its use resulting in increased dystrophin production in the skeletal muscles of patients [4]. These results led to its accelerated approval by the FDA. Despite its approval, Golodirsen carries the risk of renal toxicity, as seen in some animal studies involving the drug. Because of this, the FDA mandated renal function monitoring when this drug is used [4]. Casimersen is a PMO that facilitates the skipping of exon 45 in the *DMD* gene, which benefits around 8.1% of all *DMD* patients [4]. It was approved by the FDA following its promising results of dystrophin increase in a double-blind placebo-controlled study (NCT02500381). Similar to Golodirsen, however, this drug was shown to elicit renal toxicity in non-clinical studies, leading to the FDA mandate of renal monitoring when the

drug is taken [4]. Sarepta Therapeutics is currently conducting two confirmatory studies for the drug at the moment. Viltolarsen is a PMO that allows the skipping of exon 53, similar to Golodirsen. Like Golodirsen and Casimersen, Viltolarsen requires renal function monitoring while being taken. In trial NCT02740972, mandated for the drug's accelerated approval process, Viltolarsen yielded statistically significant results for the primary endpoint "time to stand" as well as secondary motor-related functional outcomes [4]. NS Pharma is currently conducting phase 2, 3 and 4 clinical trials for the drug. Apart from ASOs of PMO origin, exon-skipping trials involving the 2OMePS (Drisapersen) chemistry and locked chirality stereo pure ASO structures also exist. Clinical trials involving the latter of the two have not been published yet; however, research into the efficacy of 2OMePS oligonucleotides is extensive [10]. Despite chemical similarities to the drug Nusinersen (2OMOE) used in the treatment of spinal muscular atrophy (SMA), Drisapersen appears to lack a sufficient therapeutic index to drive adequate levels of dystrophin to compensate for its dose-related toxicities [10]. Trials for Drisapersen were almost successful in gaining the drug FDA approval; however, these tries were denied due to concerns about the safety around extensive injection site reactions that continued after cessation of the drug. Due to its similar chemistry with Nusinersen, the method of drug administration used for Drisapersen (subcutaneous) vs. intrathecal (Nusinersen) could be potentially related to the negative effects seen upon the use of the drug [10]. Despite their extensive usefulness in the field of *DMD* treatment, PMO ASOs are limited in their immunogenicity and sensitivity to degradation [4]. An approach to combat these shortcomings is the infusion of ASOs into a U7 snRNP molecule before subject administration. U7 is a uridine-rich ribonucleoprotein that is small in size, is concentrated in the nucleus and does not produce an immune response [4]. ASO infusion with U7 snRNP molecules is currently only useful in the treatment of dystrophin exon 2 duplications. In these cases, the ASOs are delivered using scAAV9 vectors leading to wild-type mRNA and asymptomatic patients [4]. Currently, this combination is being tested in trial NCT04240314 for safety and efficacy.

2.3. CRISPR/Cas9 Therapies

Since its discovery about a decade ago, clustered regularly interspaced short palindromic repeat, CRISPR-associated (CRISPR/Cas9) has been a groundbreaking tool in the field of precision medicine [11]. The CRISPR/Cas9 system is currently used to precisely edit mutagenic genes at specific genomes. As *DMD* is mainly caused by point mutations (30% of patients) and exon deletions (70%), the increasing precision and functionality of CRISPR/Cas9 treatments present the tool as a potential remedy to some presentations of the disease [11]. Currently, clinical trials related to CRISPR/Cas9 and *DMD* do not exist; however, the current sentiment is that clinical implementation of these trials is close to feasibility, with around 35 non-clinical CRISPR-related studies existing as of 22 October 2021 [11]. Currently, experimental trials for CRISPR/Cas9 are focused on two main areas, mediating single- and double-strand DNA breaks. Double-strand breaks involve inducing specific breaks in the DNA leading to the restoration of dystrophin expression in the cells of *DMD* patients [11]. The main issue with double-stranded DNA breaks is their high mutagenic potential and tendency to lose genetic information. Additionally, if there is imprecision with double-stranded DNA techniques, genetic mutations and INDELs can occur [11]. On the contrary, single-stranded DNA techniques are used to avoid genetic damage that can occur from the use of double-stranded break techniques [11].

In sum, many genetic interventions exist/are being trialed for the treatment of *DMD*. The two main areas focused on in these therapies are antisense oligonucleotides (ASOs) and gene replacement therapies (GRTs) [4]; other therapies like CRISPR editing also exist but have not heralded the success of the previous methods.

3. Gene Therapy in Osteogenesis Imperfecta (#166200)

Osteogenesis imperfecta (OI) is a connective tissue disorder involving dysregulated type 1 collagen synthesis [12]. The disease has five predominant clinical manifestations,

with the mildest being type I and the most lethal being type II. Types III and IV are severe forms of the disease but typically yield patient survival past the neonatal period. Type V, like type I, is often mild and characterized by moderate phenotypical manifestations with calcification of interosseous membranes [12]. Type 1 OI is the most common presenting form of the disease. Complications of the disease typically result from insufficient type 1 collagen or excess mutated collagen formed from *COL1A1* gene mutations [12]. These complications typically result in brittle bones, i.e., decreased bone density and increased susceptibility to bone fracture. The main treatment for OI is bisphosphonates [13]; however, Denosumab, synthetic parathyroid hormone and growth hormone (GH) are also used in pediatric therapy [12]. The MDT in OI management typically includes neurosurgeons, general physicians, orthopedic surgeons and physiotherapists [14]. Emerging fields regarding OI treatment include gene and cell therapy aimed at type 1 collagen mutations seen in the disease. Due to its novelty, Schindler and colleagues discussed the need for further research on gene therapy's side effects in OI before it could become a viable treatment option for the disease. Patients recruited for ongoing phase 1 and phase 2 genetic therapy trials in OI are typically adults and older children, with less focus on pediatric cases due to this population's increased vulnerability. Research in the field would benefit from testing directed at younger children, however, as they are the population most afflicted by this disease [15]. Overall, the current consensus is that genetic therapy for OI is in its experimental stages with a few promising treatment options gaining traction [12].

3.1. Gene Silencing + iPSC Use in OI

In their 2015 paper, Besio and colleagues asserted that most advances concerning genetic therapy in OI were focused on mutant gene suppression aimed at converting qualitative type I collagen defects into quantitative ones associated with milder phenotypic outcomes. They asserted that ideally, reversing the mutation process would be the most effective form of genetic therapy for OI; however, due to complexity, allele silencing was much more viable as a treatment option [16]. Genetic (allele) suppression is typically aimed toward alleles associated with collagen mutations; the goal of this process is to induce haploinsufficiency resulting in less severe phenotypical manifestations of the disease in the form of "silent" quantitative manifestations [16]. The main interventions used in allele suppression for OI involve antisense technologies such as antisense oligo-deoxyribonucleotides (ODNs), ribozymes, short interfering RNA (siRNA) and short hairpin RNA (shRNA) [16]. When Besio's paper was published, most of the existing work available involved ex vivo and in vivo trials, and to a lesser extent studies using murine models. A study included in their paper illustrated the positive effects of ex vivo allele silencing therapy and the positive results it can yield upon re-transplantation. In this study, 75 copies of induced pluripotent stem cells (iPSCs) were isolated from six OI patients ranging from age 4 to 15, these iPSCs were then successfully reprogrammed to silence dominant negative mutations (non-wild type) leading to haploinsufficiency. The results of this study were promising as the engineered iPSCs produced correctly formed bone and collagen in vivo [17].

Botor and colleagues also saw similar results in their 2021 study where they noted allele suppression could lead to quantitative non-manifesting presentations rather than qualitative severe manifestations of the disease [12]. Botor's team also examined the efficacy of other methods focused on silencing mutant collagen transcripts, i.e., ODNs, siRNAs and hammerhead ribozymes [12]. One such study by the team examined siRNA silencing and its effects on mutant *COL1A1* expression. In the study, a 50% reduction in genetic mutations and a 40% reduction in mutant proteins were observed following siRNA intervention. Despite its positive results, gene suppression in OI is still limited by a few factors, one being the diverse locations of mutations leading to OI and another being technical details regarding the application of silencing molecules or their carrier agents [12]. Gene silencing therapy is still promising, however, because minimal levels of functional collagen are associated with exponential decreases in the manifestations of OI, and thus, if gene therapy

can somewhat remedy patient mosaics to leave them with functional collagen, the positive benefits would be exponential [18].

Apart from genetic silencing, the area of allele modification using iPSCs also showed promise as a treatment option for OI. As denoted by Besio's team, iPSCs carry less strenuous HLA matching requirements compared to MSCs and embryonic stem cells [16]. Botor and colleagues also denoted iPSCs as a fascinating area for OI treatment due to their ability to differentiate into a wide range of cells from all three germ layers, i.e., the ectoderm, mesoderm and endoderm. Because of their high degree of differentiability, iPSCs are usually beneficial in a wide array of genetic diseases. In OI, iPSCs have mainly been trialed in cases for type 1 manifestations of the disease as mutations with the *COL1A1* gene are typically the most common. In these trials, iPSCs are typically harvested from human fibroblasts, but they can also be harvested from murine embryonic cells [12]. The major hurdle in this area of therapy is the need for HLA matching before treatment. While iPSCs require less matching than MSCs and embryonic cells, HLA matching is still required to minimize alloimmune response post-transplantation [12].

3.2. CRISPR + iPSC Use in OI

Concerning genetic therapy in OI, the use of CRISPR-Cas9 in conjunction with iPSCs is an area showing a lot of promise. CRISPR-Cas9 is an established genome editing tool used in various experimental and human trials [12]. It can potentially enhance the field of bioengineering for gene therapy due to its facilitation of straightforward editing of genetic sequences in human cells on their A→G and C→T base sequences [15]. In a 2019 study by Peng and colleagues, CRISPR/Cas9 editing was hypothesized to contain the potential to repair pathological lesions and completely cure many vascular diseases. Botor and colleagues mirrored these ideas by echoing CRISPR/Cas9's efficacy in vascular disorders and viewing CRISPR/Cas as superior as a gene editing tool for iPSC modification in OI treatment [12]. These speculations were proven successful by Cao and colleagues in their study 3 years later. In their study, Cao's team isolated mutation c.187T > A, suspected to be associated with mutations in *COL1A1/2* genes, from a 5-year-old patient [19]. They then cultured iPSCs from that patient's fibroblast cells and used CRISPR/Cas9 to edit the iPSCs' genetic sequences to rid them of their mutations. These iPSCs were later differentiated into osteoblasts cultured and examined. The study was significant as it resulted in increased normal collagen growth in cultures with decreased c.187T > A levels, illustrating the c.187T > A mutation's relationship with OI. CRISPR/Cas9's efficacy was also demonstrated as the clones corrected using this tool yielded increased normal type 1 collagen expression [19]. Vector use is also important to examine in conjunction with CRISPR/Cas9 therapy in OI treatment. Viral vectors are typically used to deliver desired genes into cells, whereas non-viral vectors are used to target the cell surface [15]. In OI, Adeno-associated viral vectors (AAVs) are preferred due to their high titer, mild immunogenicity and ability to infect most cells [20]. When used with AAVs, CRISPR can be highly transformative in OI treatment [15]. Because naturally induced Adenoviruses have no conventional pharmacological treatments, disseminated infections are an area of concern with the use of AAV vectors. When used with CRISPR/Cas9, however, AAVs successfully assist in facilitating CRISPR/Cas9 delivery for base repair. In terms of non-viral delivery, a few methods have been trialed. Nanoparticles currently remain the most popular [14] as they have low immunogenicity, low toxicity and the ability to transfer large amounts of DNA; these benefits make nanoparticles a viable non-viral option for CRISPR/Cas9-associated gene therapy delivery.

3.3. Yamanaka Factor + iPSC Use in OI

The generation of iPSCs is also an important aspect of OI treatment. iPSCs are typically generated to be used as a medium for introducing genetically modified cells into subjects. Yamanaka (Oct3/4, Sox2, Klf4 and c-Myc (OSKM)) and Thomson factors (Sox2, Lin28a, Nanog (OSLN), etc.) are useful in this regard as they allow for genetic transfection and reversion to a pluripotent state [12]. In the work by Kujawa and colleagues, Yamanaka

factors were programmed into iPSCs to repair *COL1A1* mutations via homologous recombination with star polymers (STARs) used as the carriers of the repaired genetic material [21]. This study was significant as it resulted in an 84% success rate in DNA mutation repair and 87% viability after STAR treatment, displaying the efficacy of Yamanaka factors when used to generate iPSCs. The skin fibroblasts isolated for this procedure were taken from a 3-day-old newborn.

4. Gene Therapy in Spinal Muscular Atrophy (#253300)

Spinal muscular atrophy (SMA) is an autosomal recessive neuromuscular disease caused by the progressive degeneration of motor neurons in the spinal cord [22]. This disease occurs in approximately 1 in 11,000 births and results in hypotonia and weakness [22]. SMA manifests when a homozygous deletion or mutation of the *survival motor neuron 1 (SMN1)* gene is present [22]. This gene is found on chromosome 5q and encodes the SMN protein, which is involved with the function of motor neurons [23]. There are four types of SMA. Type I is the most severe and common form, with an onset of approximately six months of age and death within two years. Infants with SMA type I have severe muscular atrophy and minimal motor skills [22]. Type II SMA has less severe symptoms with onset between 6 and 18 months of age, and type III and type IV SMA are mild forms of the disease occurring later in life [22]. Orthopedic management of SMA aims to treat the musculoskeletal complications of the disease. Spinal management for SMA manifestations including scoliosis and thoracic kyphosis commonly seen in type 1 SMA is scarcely utilized due to this patient population's poor survival and marginal respiratory abilities [22,24,25]. In light of current advancements in gene therapy, however, the possibility of spinal management of SMA type 1 patients is rapidly increasing [24]. Onasemnogene Abeparvovec is an AAV-based gene therapy that uses an AAV9 capsid to deliver a functional copy of the *SMN1* gene intravenously [23]. The initial trial investigating the efficacy of Onasemnogene Abeparvovec in children with diagnosed SMA type I was the open-label, phase I START trial [23]. Of the 15 patients enrolled in the trial, 12 received a therapeutic dose (2.0^14 vg/kg) of Onasemnogene Abeparvovec, and 3 received a low dose (6.7×10^13). Patients additionally received oral prednisolone to combat elevated serum aminotransferase levels. The average age of the patients given the low and therapeutic dose was 6.3 and 3.4 months, respectively [26]. Onasemnogene Abeparvovec increased the survival of patients, with all patients reaching at least 20 months of age at the time of data cutoff [23]. The patients' motor function increased significantly (score over 40 on the CHOP INTEND scale), and the children achieved new motor milestones. In the high dose cohort, 11 of the 12 patients achieved the ability to sit unassisted for at least 5 s, and 9 were able to sit for greater than 30 **s**. Eleven patients achieved head control, nine were able to roll over and two could stand and walk independently [26]. These significant motor achievements have never been achieved in historical cohorts [26]. An ongoing long-term follow-up study on the phase 1 START trial reported that all the high-dose cohort patients enrolled in the follow-up study (10 out of 12 in the original study) were alive, did not require permanent ventilation, and maintained their motor milestones 5.2 years after treatment [23]. STRIVE-US and STRIVE-EU are open-label, multicenter, phase III trials that followed the START trial and showed similar findings. Patients in the STRIVE trials were diagnosed with SMA type I and had a one-time intravenous infusion of Onasemnogene Abeparvovec (1.1×10^14 vg/kg) [23]. At the time of dose administration, the average age of patients was 3.7 months in STR1VE-US and 4.1 months in STR1VE-EU [23]. Patients in both trials showed improvement in motor function, with 95% of STR1VE-US patients and 73% of STR1VE-EU patients reaching CHOP INTEND scores of over 40. Head control was achieved in 85% of patients in STR1VE-US and 78% of patients in STRIVE-EU. Independent sitting for more than 30 s was achieved by 64% of patients in STR1VE-US and 46% in STR1VE-EU, and a small percentage of patients could stand and walk [23]. Onasemnogene Abeparvovec was tested on infants under six weeks old with presymptomatic SMA in the SPR1NT trial; the patients received Onasemnogene Abeparvovec 1.1×10^14 vg/kg [23]. The patients with presymptomatic SMA treated with

the gene therapy showed age-appropriate achievement of motor skills, and none required ventilatory or feeding support [23]. In clinical trials, Onasemnogene Abeparvovec was tolerated well in patients with SMA type I. An analysis of all four clinical trials showed pyrexia, upper respiratory tract infection and increased liver function as the most common adverse effects of the treatment [23]. Elevated alanine aminotransferase and aspartate aminotransferase levels were the most common serious adverse effects [23]. Clinical trials demonstrate that Onasemnogene Abeparvovec is an effective treatment for SMA type I, with increased efficacy in treating patients under six weeks with presymptomatic SMA. Onasemnogene Abeparvovec is approved as a treatment in Canada and the USA for patients under two years old with 5q SMA and a biallelic mutation in the *SMN1* gene [23]. In Japan, gene therapy treatment is also approved for patients under two years old, including presymptomatic patients [23]. Onasemnogene Abeparvovec is a recommended treatment option for 5q SMA type I in patients under six months old by the UK National Institute for Health and Care Excellence [23]. Dosing of Onasemnogene Abeparvovec is recommended as a single intravenous infusion of $1.1 \times 10\char`^14$ vg/kg over 60 min with oral prednisone 1 mg/kg/per day for 30 days to combat elevated aminotransferase levels [23]. To this day, Onasemnogene Abeparvovec is the only approved gene therapy treatment for SMA [23]. Its long-term safety is still unknown, but it is a promising treatment, as seen in various clinical trials and real-world settings [23].

5. Gene Therapy in Osteosarcoma (#259500)

Osteosarcoma (OS) is a bone cancer originating in mesenchymal tissue [27]. It commonly occurs in patients between 10 and 25 years old and is primarily found in rapid-growth long bones of the arms, legs, knees and shoulders. The current significant therapies for OS include surgery, chemotherapy and radiotherapy in some cases [28]. Gene therapy, however, aims to treat OS at its fundamental cause: genetic mutation [27].

5.1. Tumor Suppressor Genes in Osteosarcoma

Tumor suppressors like p53 and Rb are well known to contribute to OS formation when mutated and thus have been studied as possible gene therapy targets for treatment [29]. *TP53* stands out as a promising gene therapy target. Over 90% of OSs have *TP53* with sequence mutations in the gene or structural variations in the protein. Knocking out mutant *TP53* in human OS cell lines using CRISPR-Cas9 showed a reduction in OS cell proliferation, induced apoptosis, suppressed cell motility and increased sensitivity to chemotherapy [30]. Additionally, the knockout of *TP53* with CRISPR-CAS9 enhanced the effects of the chemotherapy drug doxorubicin in multidrug-resistant OS cell lines, signifying that the knockout of mutant *TP53* can increase the chemosensitivity of multidrug-resistant OS [30].

5.2. Proto-Oncogenes in Osteosarcoma

Another gene therapy approach for treating OS is to target tumor-associated proto-oncogenes and silence their expression to inhibit tumor growth [31]. Proto-oncogenes that can become abnormally activated in OS include *MDM2*, *SAS* and *c-myc* [31]. Hu et al. used a CRISPR-dCAS9-KRAB system with CKM and TERT promoters to target the *MDM2* proto-oncogene and inhibit its expression in human OS cells [31]. CRISPR-dCAS9 can bind to a specific location guided by sgRNA and act as a scaffold to recruit effector molecules. These researchers fused the dCAS9 protein with the KRAB fusion protein, a transcriptional repressor domain that can silence gene expression. The CKM and TERT promoters drive the expression of dCas9-KRAB and the sgRNA in malignant OS cells, explicitly targeting the *MDM2* proto-oncogene [31]. The study found that the CRISPR-dCAS9-KRAB system could specifically target and silence the *MDM2* gene. The system inhibited proliferation, promoted apoptosis and inhibited motility in OS cells without affecting normal cells. Testing this method in vivo on nude mice using lentiviral vectors

resulted in smaller tumors, signifying that CRISPR-dCAS9 could inhibit the growth of OS tumors in vivo [31].

5.3. MiRNA in Osteosarcoma

A novel gene therapy treatment being studied for OS is the in vivo delivery of microR-NAs (miRNAs). MiRNAs are gene expression regulators and can be potentially developed to suppress tumor formation and promote bone remodeling [32]. MiR-29b is shown to have tumor-suppressive properties, plays a role in bone remodeling and bone regeneration, and has been shown to inhibit angiogenesis [32]. Freeman et al. developed a pBAE nanoparticle delivery vector containing miR-29b, and when transfected into a tumor spheroid model, it showed an anti-tumor and anti-angiogenic effect [32]. MiR-29b transfection also showed a significant increase in mineralization, verifying the bone remodeling properties of miR-29b [32]. Following these results, the researchers delivered the miR-29b nanoparticles using an HA-based delivery system to tumors in an orthotopic murine model of OS [32]. The treated mice showed slower tumor growth, and when the miR-29b was delivered alongside systemic chemotherapy, it showed a 45% reduction in tumor volume compared to chemotherapy alone [32]. Combinational therapy increased the survival of the mice by 50% from 24 days (only chemotherapy) to 32 days (combination therapy) [32]. Looking at the bone remodeling potential of miR-29b delivery, the researchers compared it to *BMP-2* delivery, which is known to induce bone remodeling [32]. The combined miR-29b and doxorubicin treatment showed significant bone volume increases and better bone distribution maintenance than *BMP-2* and doxorubicin [32]. Overall, this study highlights the therapeutic potential for the localized delivery of miR-29b and pBAE nanoparticles to slow OS growth tumor growth and increase the efficacy of chemotherapy treatment while concurrently correcting the tumor-induced dysregulation of bone homeostasis [32]. Mi-R-520a-3p is another miRNA that shows efficacy in inducing apoptosis of OS cancer by the downregulation of recombinant interleukin 6 receptors but requires an effective vector to protect the miRNA from degradation and to target the tumor cells [33]. Li and colleagues developed a non-viral vector using γ-Fe2O3 due to its low toxicity, high stability and magnetic resonance imaging ability [33]. They combined this vector with folic acid (FA), which can target the folate receptors on cells usually found in higher levels on tumor cells, making it an effective tumor-targeting molecule [33]. The finished nanoprobe was named miRNA-Fe_2O_3@PDA-FA and could be used for various applications such as MRI, photothermal therapy and gene therapy [33]. The delivery of miRNA-520a-3p using the miRNA-Fe_2O_3@PDA-FA system suppressed OS tumor growth in nude mice. Additionally, the combination therapy of miRNA-Fe_2O_3@PDA-FA + photothermal therapy showed even more prominent tumor inhibition, signifying that this combination therapy can be a very effective treatment for OS [33]. Gene therapy treatment for OS is in its early stages and is still far from clinical trials [27]. Results in preclinical studies are promising with many different treatment methods, but each technique has some limitations. CRISPR-Cas9 targeting TP53 requires more evaluation in preclinical trials as the exact mechanisms by which the inhibition of mutant TP53 inhibits proliferation, colony formation and migration are unknown [30]. The CRISPR-dCAS9-KRAB system must be further optimized to improve its specificity and efficacy, as there is a potential for off-target effects [31]. Research must be conducted to improve the delivery of the system to OS cells in vivo and to examine toxic side effects that may occur [31]. Dosing regimens must be further explored for localized delivery of miR-29b and the mechanism by which it induces apoptosis in OS cells [32]. Overall, gene therapy for OS has shown promising results in preclinical studies, so further research and, eventually, clinical studies will provide more insight into making it a safe and effective treatment.

6. Conclusions

Overall, the existing literature for gene therapy in pediatric orthopedics shows promise. Various drug trials have progressed to the point of FDA approval, including Eteplisern

and Golodirsen for *DMD* and Onasemnogene Abeparvovec for SMA. Other areas of gene therapy are also being explored, such as using iPSCs and CRISPR in many pediatric diseases. Due to its potential, many trials are also beginning for gene therapy in pediatric-related orthopedic diseases such as limb–girdle muscle dystrophy and myotonic dystrophy type 1. The field of pediatric orthopedics will continue to grow if the successes of current trials continue to bring increasing awareness to the field. Future studies in the field should be longitudinal with humans as the main subjects to explore the long-term side effects of existing interventions. If genetic therapy continues its trajectory, it could become a viable treatment option for many pediatric and orthopedic diseases worldwide.

References

1. Wirth, T.; Parker, N.; Ylä-Herttuala, S. History of gene therapy. *Gene* **2013**, *525*, 162–169. [CrossRef]
2. Malech, H.L.; Garabedian, E.K.; Hsieh, M.M. Evolution of Gene Therapy, Historical Perspective. *Hematol. Oncol. Clin. N. Am.* **2022**, *36*, 627–645. [CrossRef] [PubMed]
3. Patterson, G.; Conner, H.; Groneman, M.; Blavo, C.; Parmar, M.S. Duchenne muscular dystrophy: Current treatment and emerging exon skipping and gene therapy approach. *Eur. J. Pharmacol.* **2023**, *947*, 175675. [CrossRef] [PubMed]
4. Chamakioti, M.; Karantzelis, N.; Taraviras, S. Advanced Gene-Targeting Therapies for Motor Neuron Diseases and Muscular Dystrophies. *Int. J. Mol. Sci.* **2022**, *23*, 23094824. [CrossRef]
5. Mendell, J.R.; Sahenk, Z.; Lehman, K.; Nease, C.; Lowes, L.P.; Miller, N.F.; Iammarino, M.A.; Alfano, L.N.; Nicholl, A.; Al-Zaidy, S.; et al. Assessment of Systemic Delivery of rAAVrh74.MHCK7.micro-dystrophin in Children With Duchenne Muscular Dystrophy: A Nonrandomized Controlled Trial. *JAMA Neurol.* **2020**, *77*, 1122–1131. [CrossRef]
6. Solid Biosciences. Solid Biosciences Presents New SGT-001 IGNITE DMD Study Results at World Muscle Society 2022 Congress Demonstrating Improvements in Ambulatory Function. Available online: https://www.solidbio.com/about/media/press-releases/solid-biosciences-presents-new-sgt-001-ignite-dmd-study-results-at-world-muscle-society-2022-congress-demonstrating-improvements-in-ambulatory-function (accessed on 9 November 2023).
7. clinicaltrials.gov. Microdystrophin Gene Transfer Study in Adolescents and Children With DMD—Full Text View—ClinicalTrials.gov. Available online: https://classic.clinicaltrials.gov/ct2/show/NCT03368742 (accessed on 9 November 2023).
8. Scheller, E.L.; Krebsbach, P.H. Gene therapy: Design and prospects for craniofacial regeneration. *J. Dent. Res.* **2009**, *88*, 585–596. [CrossRef]
9. The Yu Lab. Antisense Therapy. Available online: https://www.theyulab.org/antisense-therapy#:~:text=While%20they%20act%20on%20genetic (accessed on 9 November 2023).
10. Takeda, S.; Clemens, P.R.; Hoffman, E.P. Exon-Skipping in Duchenne Muscular Dystrophy. *J. Neuromuscul. Dis.* **2021**, *8* (Suppl. 2), S343–S358. [CrossRef] [PubMed]
11. Happi Mbakam, C.; Lamothe, G.; Tremblay, G.; Tremblay, J.P. CRISPR-Cas9 Gene Therapy for Duchenne Muscular Dystrophy. *Neurotherapeutics* **2022**, *19*, 931–941. [CrossRef]
12. Botor, M.; Fus-Kujawa, A.; Uroczynska, M.; Stepien, K.L.; Galicka, A.; Gawron, K.; Sieron, A.L. Osteogenesis Imperfecta: Current and Prospective Therapies. *Biomolecules* **2021**, *11*, 11101493. [CrossRef] [PubMed]
13. Marom, R.; Rabenhorst, B.M.; Morello, R. Osteogenesis imperfecta: An update on clinical features and therapies. *Eur. J. Endocrinol.* **2020**, *183*, R95–R106. [CrossRef]
14. Marr, C.; Seasman, A.; Bishop, N. Managing the patient with osteogenesis imperfecta: A multidisciplinary approach. *J. Multidiscip. Healthc.* **2017**, *10*, 145–155. [CrossRef]

15. Schindeler, A.; Lee, L.R.; O'Donohue, A.K.; Ginn, S.L.; Munns, C.F. Curative Cell and Gene Therapy for Osteogenesis Imperfecta. *J. Bone Miner. Res.* **2022**, *37*, 826–836. [CrossRef]

16. Besio, R.; Forlino, A. New frontiers for dominant osteogenesis imperfecta treatment: Gene/cellular therapy approaches. *Adv. Regen. Biol.* **2015**, *2*, 27964. [CrossRef]

17. Deyle, D.R.; Khan, I.F.; Ren, G.; Wang, P.-R.; Kho, J.; Schwarze, U.; Russell, D.W. Normal collagen and bone production by gene-targeted human osteogenesis imperfecta iPSCs. *Mol. Ther. J. Am. Soc. Gene Ther.* **2012**, *20*, 204–213. [CrossRef]

18. Cabral, W.A.; Marini, J.C. High proportion of mutant osteoblasts is compatible with normal skeletal function in mosaic carriers of osteogenesis imperfecta. *Am. J. Hum. Genet.* **2004**, *74*, 752–760. [CrossRef]

19. Cao, Y.; Li, L.; Ren, X.; Mao, B.; Yang, Y.; Mi, H.; Guan, Y.; Li, S.; Zhou, S.; Guan, X.; et al. CRISPR/Cas9 correction of a dominant cis-double-variant in *COL1A1* isolated from a patient with osteogenesis imperfecta increases the osteogenic capacity of induced pluripotent stem cells. *J. Bone Miner. Res.* **2023**, *38*, 719–732. [CrossRef] [PubMed]

20. www.cellbiolabs.com. Adeno-Associated Virus (AAV) Provides Advantages for Gene Delivery | Cell Biolabs. Available online: https://www.cellbiolabs.com/news/adeno-associated-virus-aav-provides-advantages-gene-delivery#:~:text=Home- (accessed on 9 November 2023).

21. Fus-Kujawa, A.; Mendrek, B.; Bajdak-Rusinek, K.; Diak, N.; Strzelec, K.; Gutmajster, E.; Janelt, K.; Kowalczuk, A.; Trybus, A.; Rozwadowska, P.; et al. Gene-repaired iPS cells as novel approach for patient with osteogenesis imperfecta. *Front. Bioeng. Biotechnol.* **2023**, *11*, 1205122. [CrossRef]

22. Arnold, W.D.; Kassar, D.; Kissel, J.T. Spinal muscular atrophy: Diagnosis and management in a new therapeutic era. *Muscle Nerve* **2015**, *51*, 157–167. [CrossRef] [PubMed]

23. Blair, H.A. Onasemnogene Abeparvovec: A Review in Spinal Muscular Atrophy. *CNS Drugs* **2022**, *36*, 995–1005. [CrossRef]

24. Mercuri, E.; Finkel, R.S.; Muntoni, F.; Wirth, B.; Montes, J.; Main, M.; Mazzone, E.S.; Vitale, M.; Snyder, B.; Quijano-Roy, S.; et al. SMA Care Group Diagnosis and management of spinal muscular atrophy: Part 1: Recommendations for diagnosis, rehabilitation, orthopedic and nutritional care. *Neuromuscul. Disord. NMD* **2018**, *28*, 103–115. [CrossRef]

25. Schwentker, E.P.; Gibson, D.A. The orthopaedic aspects of spinal muscular atrophy. *J. Bone Jt. Surg. Am. Vol.* **1976**, *58*, 32–38. [CrossRef]

26. Mendell, J.R.; Al-Zaidy, S.; Shell, R.; Arnold, W.D.; Rodino-Klapac, L.R.; Prior, T.W.; Lowes, L.; Alfano, L.; Berry, K.; Church, K.; et al. Single-Dose Gene-Replacement Therapy for Spinal Muscular Atrophy. *N. Engl. J. Med.* **2017**, *377*, 1713–1722. [CrossRef]

27. Zhao, X.; Wu, Q.; Gong, X.; Liu, J.; Ma, Y. Osteosarcoma: A review of current and future therapeutic approaches. *Biomed. Eng. Online* **2021**, *20*, 24. [CrossRef] [PubMed]

28. Tan, M.L.; Choong, P.F.; Dass, C.R. Osteosarcoma: Conventional treatment vs. gene therapy. *Cancer Biol. Ther.* **2009**, *8*, 106–117. [CrossRef] [PubMed]

29. Broadhead, M.L.; Clark, J.C.; Choong, P.F.; Dass, C.R. Making gene therapy for osteosarcoma a reality. *Expert Rev. Anticancer Ther.* **2010**, *10*, 477–480. [CrossRef]

30. Tang, F.; Min, L.; Seebacher, N.A.; Li, X.; Zhou, Y.; Hornicek, F.J.; Wei, Y.; Tu, C.; Duan, Z. Targeting mutant TP53 as a potential therapeutic strategy for the treatment of osteosarcoma. *J. Orthop. Res. Off. Publ. Orthop. Res. Soc.* **2019**, *37*, 789–798. [CrossRef] [PubMed]

31. Hu, Y.; Zhang, H.; Guo, Z.; Zhou, J.; Zhang, W.; Gong, M.; Wu, J. CKM and TERT dual promoters drive CRISPR-dCas9 to specifically inhibit the malignant behavior of osteosarcoma cells. *Cell. Mol. Biol. Lett.* **2023**, *28*, 52. [CrossRef] [PubMed]

32. Freeman, F.E.; Dosta, P.; Shanley, L.C.; Ramirez Tamez, N.; Riojas Javelly, C.J.; Mahon, O.R.; Kelly, D.J.; Artzi, N. Localized Nanoparticle-Mediated Delivery of miR-29b Normalizes the Dysregulation of Bone Homeostasis Caused by Osteosarcoma whilst Simultaneously Inhibiting Tumor Growth. *Adv. Mater.* **2023**, *35*, e2207877. [CrossRef] [PubMed]

33. Li, X.; Wang, S.; Gao, Q.; Li, N.; Dong, S.; Gao, Y.; Wang, Z.; Zhang, B.; He, X. MiRNA-520a-3p combined with folic acid conjugated Fe_2O_3@PDA multifunctional nanoagents for MR imagine and antitumor gene-photothermal therapy. *Nanotechnology* **2023**, *34*, acd5d9. [CrossRef] [PubMed]