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Gene Therapy for Fibrodysplasia Ossificans Progressiva: Feasibility and Obstacles

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Fibrodysplasia ossificans progressiva (FOP) is a rare and devastating genetic disease, in which soft connective tissue is converted into heterotopic bone through an endochondral ossification process. Patients succumb early as they gradually become trapped in a second skeleton of heterotopic bone. Although the underlying genetic defect is long known, the inherent complexity of the disease has hindered the discovery of effective preventions and treatments. New developments in the gene therapy field have motivated its consideration as an attractive therapeutic option for FOP. However, the

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immune system's role in FOP activation and the as-yet unknown primary causative cell, are crucial issues which must be taken into account in the therapy design. While gene therapy offers a potential therapeutic solution, more knowledge about FOP is needed to enable its optimal and safe application.

Keywords: fibrodysplasia ossificans progressiva, gene therapy, heterotopic ossification, ALK2 mutation, RNA

FIBRODYSPLASIA OSSIFICANS PROGRESSIVA (FOP; MIM no. 135100) is a rare genetic disease affecting soft connective tissues. The prevalence is reported to be 1 in 1.3 million –2 million.[1] FOP is characterized by muscles, tendons, and ligaments that turn into bone through an endochondral ossification process.[2] Bone formation typically transpires through so-called flare-ups (Fig. 1), a local inflammatory response which subsequently triggers local chondrogenesis and osteogenesis.[3] In addition to the flare-ups, there is also a level of basal chronic heterotopic ossification (HO) present in FOP patients.[4] During life, FOP follows a progressive pattern first affecting the axial skeleton and later the appendicular skeleton, although it varies greatly between patients. Eventually, this highly complex disease leads to devastating contractures and severe disability and causes premature death in FOP patients due to thoracic insufficiency syndrome, trauma, or sepsis.[5]

The underlying cause of FOP is a heterozygous, usually *de novo*, R206H gain-of-function mutation in the ubiquitously expressed bone morphogenetic protein (BMP) type I receptor activin receptor-like kinase 2 (ALK2) (Fig. 2). This mutation alters the properties of the receptor by converting it to a form that is both mildly constitutively active at the basal state and hyperactive to BMP signaling in the activated state as evidenced by the phosphorylation of the downstream SMAD1/5/8 effector proteins.[6,7] Also, the mutation renders the ALK2 receptor aberrantly responsive to Activin A, which induces phosphorylation of SMAD 1/5/8, leading to bone formation where it normally would not occur.[8]

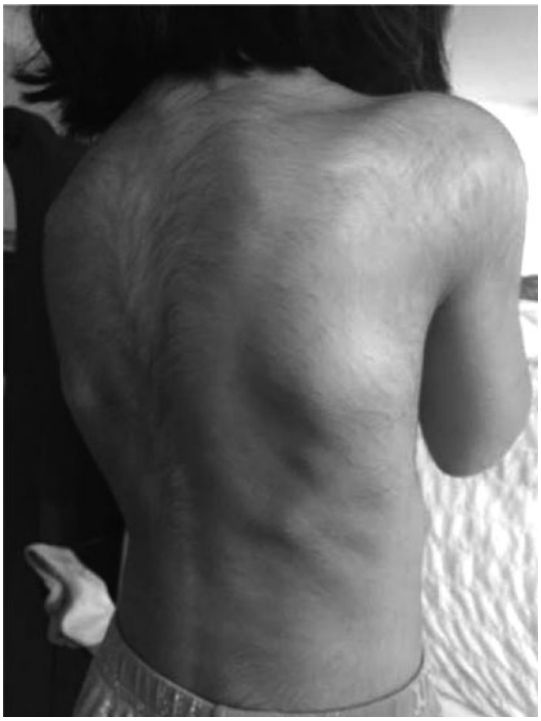

Figure 1. A flare-up with swelling of the back of a young girl diagnosed with FOP. Image is reproduced with the written consent of the patient and her parents. FOP, fibrodysplasia ossificans progressiva.

It is still unclear what other factors contribute to the unpredictable and episodic activity of the disease, although an important role is attributed to the immune system.[9–12] The R206H (c.617G>A) mutation can be found in more than 95% of the classic form of FOP patients. Currently, at least 13 other mutations have been found in the glycine-serine rich or kinase domain of ALK2 that cause FOP, which appear to lead to different phenotypes than the ''classic'' FOP. Nonetheless, they are all heterozygous missense mutations, which enhance receptor signaling (Fig. 1).[13]

Presently, it is not known when the disease may become active, although (minor) trauma is one of the most predictive triggering factors. Several drugs are currently being investigated in clinical trials and represent different molecular strategies.[14] These include blocking antibodies that stop Activin A from triggering the mutant ALK2 receptor (REGN2477),[15,16] ALK2 kinase inhibitors (AZD0530; IPN60130),[17,18] mTOR inhibitors which modulate the inflammatory response to tissue injury and aim to affect the early hypoxic stages involved in chondrogenesis (rapamycin)[19] and retinoic acid receptor gamma agonists, which block the chondrogenic signaling required for endochondral bone formation (palovarotene).[20,21] All of these experimental approaches have been shown to be effective in FOP mouse models.[15,18,22,23]

Given the nature of this mutation and the importance of the ALK2 receptor in homeostasis and development of the skeletal system, pharmaceutical interference with the receptor can be expected to cause numerous potential side effects. Demonstrating the effect of intervention with these drugs in clinical studies has already appeared to be more difficult than initially expected in terms of acceptable risks, expected benefits, and lack of comprehensive understanding of the natural history of the disease. Clinical trials are currently being conducted to further evaluate the safety and efficacy of the aforementioned drugs. However, at the time of this article, no efficacy and safety data have

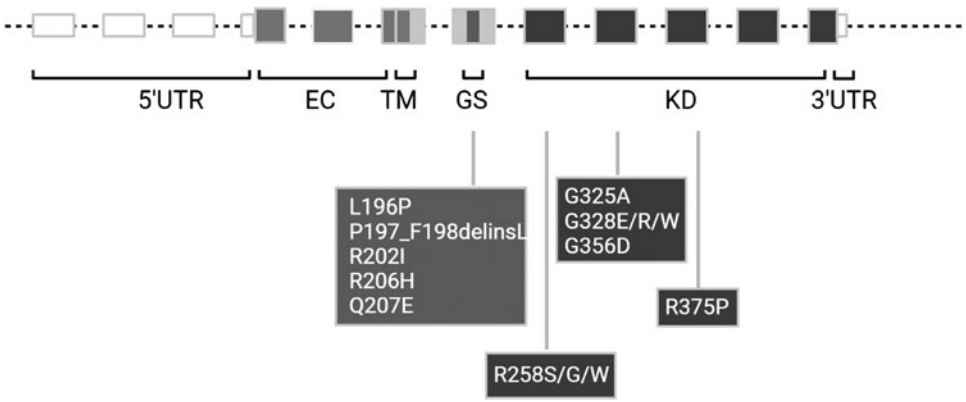

Figure 2. Overview of FOP mutations in the exons of the different domains of the ACVR1 gene. Figure 2 was created with biorender.com. EC, extracellular; GS, glycine-serine rich; KD, kinase domain; TM, transmembrane; UTR, untranslated region.

yet been published and, with the exception of approval of palovarotene in Canada, no drugs have been approved by regulatory authorities elsewhere.

The complexity of finding safe and effective treatments specific to the known genetic cause is why gene therapy is being explored as a new treatment option in FOP. For many monogenic diseases, the gene therapy horizon is being intensively explored as it offers attractive possibilities which seem tangible in the near future; this has motivated the investigation and investment in the gene therapy approach. Considering the therapeutic benefits of commercialized gene therapy on several monogenic diseases such as lipoprotein lipase deficiency,[24] inherited retinal dystrophy,[25] and spinal muscular atrophy,[26] it is plausible that current gene therapy options could be beneficial for the treatment of FOP caused by a monogenic gain-of-function mutation in the ALK2 receptor. In this perspective, we summarize the different gene therapy options and their expected suitability in FOP (Table 1).

In general, gene addition aims to introduce genes encoding missing proteins or encoding corrective proteins in the event that defective proteins are produced by a genetic mutation. For FOP, where the pathological mutations cause a gain-of-function, gene therapy could conceivably apply four strategies, including gene replacement, gene silencing, combination of gene replacement and silencing, and gene editing (Table 1). First, introduction of healthy proteins via gene replacement can be used to compete against proteins with gain-of-function autosomal dominant mutations such as the classic ALK2 mutation in FOP. Second, gene silencing aims to suppress the expression of abnormal proteins at the messenger RNA level by using ribonucleic acid interference (RNAi). This strategy can be useful for FOP mutant allele-specific silencing of the ALK2 receptor.[27,28]

Third, a combinatory approach of gene replacement and silencing removes abnormal proteins and expresses healthy proteins simultaneously. This strategy can be used to replace the *ACVR1* mutation in FOP with normal ALK2. Finally, gene editing aims to correct DNA mutations in the genome by using the clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) system.[29,30] This strategy can be used to correct the ALK2 mutation in FOP at the genomic levels. However, a caveat to consider in using these therapeutic strategies in FOP is the lack of definitive identification of the HO-triggering cell types in the body.

In light of this, the critical question is whether it is possible to specifically correct the ALK2 mutation in the cells involved in the various phases of the disease, which might be a solution, but at the moment still not feasible in patients. In addition, targeting the locally affected tissue during a FOP flare-up may also pose difficulties since HO consists of normal bone tissue (at an ectopic site) which

Table 1. *Gene therapy options in fibrodysplasia ossificans progressiva for in vivo treatment*

	Approach	*Target*	*Effect*
Gene replacement	Expression of wild-type ALK2	mRNA	Normal ALK2 competes against mutant ALK2 receptor
Gene silencing	Mutant ALK2-specific RNAi	mRNA	Suppression of mutant ALK2 receptor expression
Gene replacement and silencing	Combination of the two above	mRNA	Combined effect of the two above
Gene editing	CRISPR/CAS-mediated correction of ALK2 mutation	DNA	Only normal ALK2 receptor is produced

ALK2, activin receptor-like kinase 2; CAS, CRISPR-associated protein; CRISPR, clustered regularly interspaced short palindromic repeats; mRNA, messenger RNA; RNAi, ribonucleic acid interference.

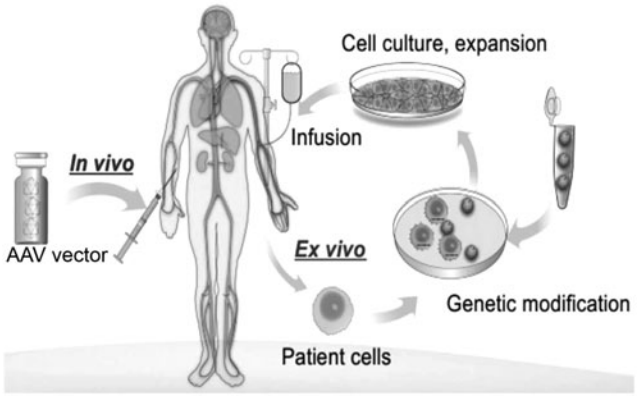

Figure 3. Two ways to express therapeutic genes in target cells and/or tissues. (1) *Ex vivo* gene therapy: genetic modification is executed on isolated patient cells using a viral vector, and after cell expansion in the culture, treated cells are introduced to patients via infusion. (2) *In vivo* gene therapy: AAV vector carrying a therapeutic gene is directly introduced to patient via systemic or local administration. AAV, adeno-associated virus.

may be difficult to selectively target. These issues may be circumvented by improving tissue-specific tropism of the vectors that deliver therapeutic genes.

There are essentially two routes to express therapeutic genes in target cells and/or tissues (Fig. 3: adapted from G.G.). Genetically modified cell therapy is an *ex vivo* treatment approach that extracts target cells from the affected tissue of the patients, followed by genetic manipulation via vector-assisted transduction and reintroduction into the tissue. By contrast, *in vivo* gene therapy aims for the direct delivery of therapeutic genes to target tissues using either a viral vector (*i.e.,* recombinant adeno-associated virus [rAAV] or a nonviral vector [such as liposomes or nanoparticles]).

All FOP cells in the body with the potential to differentiate into bone need to be repaired as any untreated cell is a potential source of flare-up and HO. Therefore, *ex vivo* cell therapy, followed by reintroducing genetically manipulated cells back into the body is unlikely to substantially benefit FOP, because the presence of reintroduced cells will not affect the cells that contain the mutation. Consequently, *in vivo* gene therapy is considered the most likely treatment option in FOP as presently conceived.

Since immunological triggers are known to pose a high risk for HO induction in FOP, viral vector options need to be very carefully considered. Each viral vector type has its advantages and disadvantages in terms of transduction efficiency, duration of gene expression, transgenic capacity and potential side effects (Table 2).[31]

Among them, rAAV has a long track record for safety and efficacy in relevant preclinical and clinical studies in non-FOP contexts and has been evaluated in over 130 clinical trials and 2,000 patients worldwide.[31,32] AAV, a small (26 nm) nonenveloped parvovirus with a single-stranded genome of ~4.7 kb in length,[33] has high transduction efficiency, persistent transgene expression, relatively low postinfection immunogenicity, and importantly no association with any human diseases, which make it an attractive viral vector for use in gene therapy.[34] In addition, a systemic disease such as FOP requires a systemic delivery via the vasculature and takes advantage of AAV's transvascularity and tissue-specific tropism.[31]

However, a high-dose administration of the AAV vector can potentially trigger an immunomodulatory effect in FOP complicating reliable delivery of the gene of interest to the target cell(s) and may potentially compromise the subsequent safety of this method as well as any potential therapeutic effect.[35] In addition, flare-ups are unpredictable and have different phases of development with involvement of other target cells and their microenvironment. During the HO developmental process, cellular hypoxia occurs and a periodic diminished blood supply is suspected, which adds an additional level of complexity in deciphering the anatomical locations and the target cells that the vector must be designed to reach.[36]

Cotreatment with an immunosuppressor or an FOP inhibitor or the development of a new AAV vector that does not trigger FOP-associated flare-ups may be able to address these issues. Alternatively, liposomes and nanoparticles are nonimmunogenic gene therapy vectors, but they can be rapidly degraded, cleared in the circulation, have short biological half-lives, and generally exhibit nonspecific uptake by cells.[34]

The CRISPR/Cas9 system has been developed as a genome-editing tool that can correct DNA mutations un-

Table 2. *Comparison of different viral vectors in transduction efficacy, duration of expression, transgenic capacity, and potential side effects*[31]

	AAV	*Retrovirus*	*Lentivirus*	*Adenovirus*
Broad host range (infects many cell types)	Yes (tissue-specific tropism)	Yes (dividing cells only)	Yes	Yes
Infects both dividing and nondividing cells	Yes	No (dividing cells only)	Yes	Yes
Genome integration (genotoxicity)	No	Yes	Yes (integrase-deficient versions available)	No
Very high level of protein expression	No	No	No	Yes
Insert size capacity	2.5 kb	2.5–5.0 kb	2.5–5.0 kb	3.0–8.0 kb
Typical titer	10^{12}–10^{13} GCs/mL	10^{6} IFU/mL	10^{7}–10^{8} IFU/mL	10^{9} IFU/mL

AAV, adeno-associated virus; GCs, genome copies; IFU, infectious units.

derlying human diseases. In principle, many heterozygous mutations can be individually corrected by homology-directed repair (HDR) using an exogenous DNA template.[37] Recently, the AAV-compatible Cas9 nuclease (SaCas9), derived from *Staphylococcus aureus*, has been engineered for *in vivo* gene editing as SaCas9 and fits within the genome packaging limits of AAV.[38] However, since the SaCas9 nuclease shows a low HDR-mediated gene-editing efficiency and being a bacterial protein, its expression triggers immune responses in animal cells. Consequently, an alternative gene therapy technique likely needs to be considered, the so-called RNA genetic techniques.

RNA was conventionally thought to be a transient messenger (mRNA) for the passive translation of genetic information encoded by DNA into protein sequences. However, mRNA comprises only a small fraction of the RNA types and their functions in the cell. Other types of RNAs also exist which can turn genes on and off, support chemical reactions, cut and build other RNAs, and constitute the protein-building machines of cells by transporting and linking amino acids. Taking this into account, RNA therapies can provide efficient silencing of target mRNA expression by inhibitory RNA (RNAi) (*i.e.,* siRNA, shRNA, miRNA)-mediated degradation. Similar to DNA gene therapy, RNAi approaches also require a vector for delivery into cells, especially since RNA is unstable and is easily degraded in the bloodstream.

For this reason, RNA therapy can be relatively short-lasting, while high levels of expression can induce cytotoxicity and inflammation by perturbing the RNAi machinery or leading to significant off-target silencing. To circumvent these issues, AAV-compatible miRNA scaffolds (artificial miRNA) have been developed to increase the duration of RNAi expression, limit RNAi-related toxicity, and enable efficient gene knockdown, while reducing off-target silencing by 10-fold compared to conventional RNAi.[31] RNA therapy might theoretically be preferable in treating flare-ups, although the problem of not knowing which cell types to target remains.

In summary, after years of setbacks,[39] the field of gene therapy has now achieved some success with effective applications of DNA-modulating therapy in clinical trials in previously difficult to treat hereditary diseases such as certain forms of immunodeficiency, neurological disorders, musculoskeletal disorders, blindness, hemoglobinopathies, coagulation disorders, and cancer.[31,40] RNA-related gene therapy exists in the form of two mRNA-based therapies for hereditary transthyretin-mediated (ATTR) amyloidosis—a potentially fatal disease characterized by abnormal protein accumulation in nerves and organs, including the heart,[41] and Nusinersen,[42] which targets a fatal inherited condition called spinal muscular atrophy. Regrettably, the application of Nusinersen is hampered by high costs. Eteplirsen, a treatment for Duchenne muscular dystrophy,[43] has been approved by the Food and Drug Administration (FDA).

One of the biggest barriers[44] in the above mentioned RNA therapies has been the delivery of RNA to the correct cells. The above mentioned genetic diseases, present relatively accessible affected tissues and cells, which can be distinctly targeted compared to FOP. Fundamental problems in gene therapy for FOP are the identity of the proper targets and the safety and durability of the gene targeting system. An analogy can be made with metastatic cells in cancer. Any untreated cell is still a potential source of a flare-up and HO. Since it has recently been shown that the mutant ALK2 can lead to aberrant gain of BMP signaling in different cell lines and tissue progenitor cells, with different regeneration capacities,[45,46] successful and comprehensive gene therapy for FOP needs may require the targeting of broader range of cell types.

Therefore, for a complex disease such as FOP, deeper insight into the underlying causative cell type(s) and the factors involved in the different phases of HO is a paramount prerequisite for efficient and safe gene therapy design. Gene therapy has the clear advantage of achieving the direct correction of the genetic cause in monogenic diseases such as FOP, which is lacking in current strategies. This justifies its pursuit as a novel therapeutic modality. While gene therapy could be a promising tool in the distant future, there are still significant obstacles to overcome until a safe therapy can be offered to the patients.

Considering the complexity of FOP, it can be envisioned that its efficient treatment will involve a combination strategy of gene and pharmacological therapy. As we learn more about the nature of chronic and traumatic FOP, it will be possible to evaluate the benefit of vector-mediated therapeutic gene and pharmacological treatment in each for optimal therapeutic outcome. Finally, the discovery of the underlying factors and the natural course of the disease, in combination with the developing variety of drug studies and new ongoing options, are all very much needed to advance FOP treatment and should receive due attention in the next decade.

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