Chapter

Gene Therapy as a Modern Method of Treating Naturally Occurring Tendinitis and Desmitis in Horses

Elena Zakirova, Kovac Milomir, Margarita Zhuravleva, Catrin Sian Rutland and Albert Rizvanov

Abstract

Tendon and ligament injuries have always been complex to treat, with recovery often taking many months, if successful at all. This chapter looks at recent work undertaken using regenerative medicine, specifically gene therapy and the advances that have been made in equine therapy. It looks at the process from plasmid construction, in vitro testing through to trialing the equine-specific plasmid construct in horses with superficial digital flexor tendon (tendinitis) and suspensory ligament branch injuries. It also looks at the rationale for utilizing vascular endothelial growth factor (VEGF164) and a basic fibroblast growth factor (FGF2) for these trials and the cellular effects and potential mechanisms of actions.

Keywords: horse, gene therapy, tissue regeneration, superficial digital flexor tendon, tendon injuries, suspensory ligament

1. Introduction

Tendon and ligament injuries are the most common traumas in horses (*Equus caballus*) irrespective of the age and breed [1]. Based on the statistics, injuries in sport horses can achieve 86% of a total morbidity rate, with 37% of them accounting for muscle, tendon and ligament pathologies. As a rule, musculoskeletal injuries require long-term recovery for 9–12 months [2]. Tendon and ligament injuries result in a loss of performance of the horses and frequently cause discomfort or pain. Therefore, the animals are unable to participate in competitions for a long period of time. Complications of these injuries include chronic musculoskeletal diseases. They result in degenerative-dystrophic damage of collagen fibers of tendons as well as adjacent and underlying tissues. Incomplete tissue recovery leads to recurrent injuries in 80% of horses with treated tendon micro- and macroruptures within 3–12 months after the first injury [3].

Methods of regenerative medicine are used for appropriate regeneration of damaged tissue in animals. These include the administration of stem cells [4, 5] and recombinant proteins, as well as gene therapy. These methods are presently the most advanced and promising approaches to manage musculoskeletal disorders [6]. However, regenerative medicine is mainly targeted toward the treatment of human disorders. Animals are mostly considered as models to test drugs and devices intended for human use. Drugs developed for human use can be ineffective for the

treatment of animal diseases due to partial homology of physiological processes. When given to animals, such products can cause long-term immunological disorders, decrease the efficacy of a subsequent treatment or even cause adverse side effects including anaphylactic shock.

In a veterinary practice, an autologous graft rejection can be avoided in 85% of cases [7]. The likelihood of immune responses in animals to the administration of allogenic or autologous species-specific stem cells is also low [8–10]. However, full homology can be of vital importance when applying more advanced therapeutic approaches such as gene therapy.

Gene therapy is a novel, rapidly developing trend in regenerative medicine and veterinary, which can provide continuous stimulation of regeneration. When this approach is used, a recipient's body constantly synthesizes its own substances instead of a multiple drug (pharmaceuticals, recombinant proteins and so on) delivery. Gene therapy has been successful in the treatment of various human disorders [11, 12], and it can be used to treat animals [13]. However, species-specific recombinant genes that would provide biological activity and at the same time have no immunological side effects should be developed for this purpose. A therapeutic potential of gene therapy for the treatment of tendinitis and desmitis in sport horses will be discussed in detail in this review, especially those related to a series of papers recently covering gene therapy in horses [14–17].

2. Use of a species-specific plasmid construct in the treatment of traumas in horses

2.1 Description of the plasmid construct

A group of scientists from Russia and Great Britain developed and tested a drug for gene therapy of soft tissue injuries in horses. This gene construct is plasmid DNA (pDNA), encoding animal-specific genes (**Figure 1**). A plasmid construct pBUDK-ecVEGF164-ecFGF2 based on a pBudCE4.1 vector contained codon-optimized sequences of horse genes, a vascular endothelial growth factor (VEGF164) and a basic fibroblast growth factor (FGF2) under eukaryotic promoters (EF-1α and CMV promoters, respectively) [14].

These genes were selected with good reason as VEGF stimulates synthesis of DNA and proliferation of cells involved in antiapoptotic signaling pathways. It promotes the proliferation and migration of endothelial cells, stimulates angiogenesis and attracts endothelial progenitor cells from bone marrow, stimulates the activity of pericytes and stabilizes newly formed blood vessels. VEGF is also a

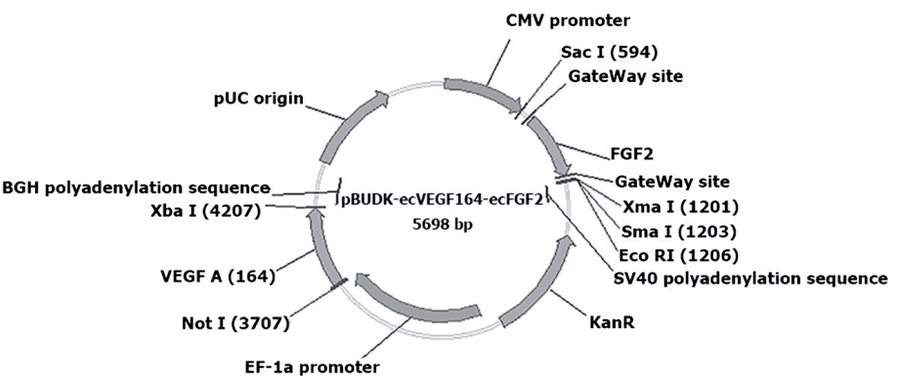

Figure 1.
Map of recombinant plasmid pBUDK-ecVEGF164-ecFGF2 [14].

chemoattractant for smooth muscle cells, monocytes, macrophages and granulocytes. All of these are involved in the process of wound healing. VEGF also increases vessel wall permeability at the site of injury that enhances the formation of granulation tissue [14].

In turn, FGF2 exerts a wide range of mitogenic and angiogenic activities and is a neurotrophic factor. In intact tissues, it is present in a basement membrane of the epithelium and in the subendothelial extracellular matrix of blood vessels. It stimulates cell proliferation, regeneration of nervous, muscle and connective tissues. Also, FGF2 activates de novo formation of blood vessels by triggering the process of angiogenesis [18].

Thus, a mechanism of action of gene therapy comprising VEGF and FGF2 is to stimulate synthesis of proteins in a recipient that enhances the vascularization of damaged tissues. This, in turn, leads to a higher regeneration rate. Both VEGF and FGF2 are well-known growth factors with a wide range of mitogenic and angiogenic activity. They also contribute to regeneration of muscle and connective tissues. What is more important is that in combination these factors demonstrate synergistic effects that surpass those of therapy with just one growth factor [19].

This gene product has been tested for identification and functional activity in mammal cells in the laboratory. Full genetic sequencing and restriction analysis with subsequent agarose gel electrophoresis demonstrated a complete compliance with the claimed structure of pBUDK-ecVEGF164-ecFGF2 (**Figure 2**).

Biosynthesis of recombinant VEGF164 and FGF2 in transfected immortalized HEK293FT cells was confirmed by an immunofluorescence assay with anti-VEGF and anti-FGF2 antibodies (**Figure 3**), which confirmed co-expression of recombinant proteins in transgenic cells [14, 14].

The biological activity of the pBUDK-ecVEGF164-ecFGF2 DNA plasmid was evaluated during *in vitro* experiments in horse stem cells. For this purpose, horse MSCs were isolated under a standard procedure by incubating a subcutaneous adipose tissue homogenate with crab collagenase. The cells obtained were identified as MSCs with flow cytofluorometry-more than 80% of them expressed MSC-specific markers (Thy-1 in 99.8% and CD44 in 83% of the cells) and no CD34 or CD45 was

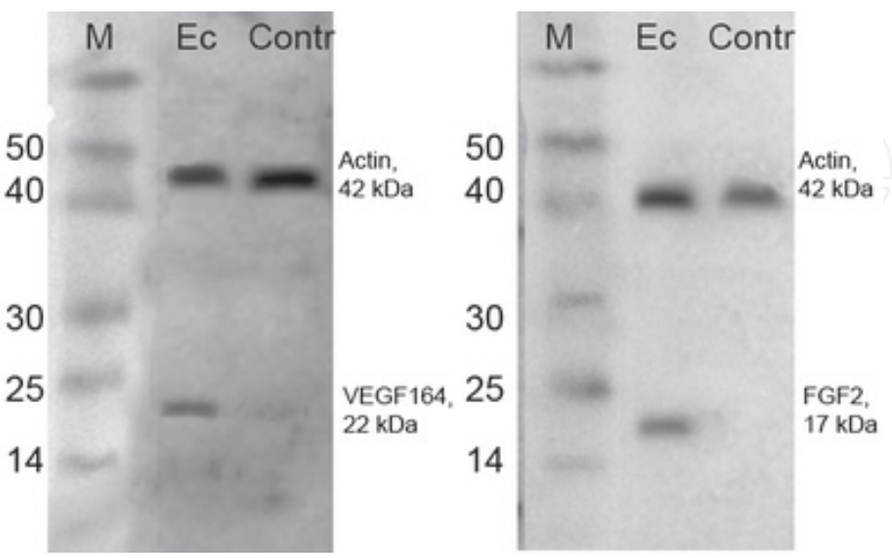

Figure 2.
Analysis of VEGF164 and FGF2 biosynthesis by immunoblotting in HEK293FT cells after transfection. Electrophoresis in 12% SDS-PAGE gel was performed in Laemmli system. Antibodies against human actin, VEGF and FGF2 were used. Bands correspond to human actin (42 kDa), horse VEGF164 (22.3 kDa) and horse FGF2 (17.2 kDa). M-molecular weight protein marker (GE LifeSciences RPN756E); Ec-HEK293FT cells transfected with pBUDK-ecVEGF164-ecFGF2; control nontransfected cells [14].

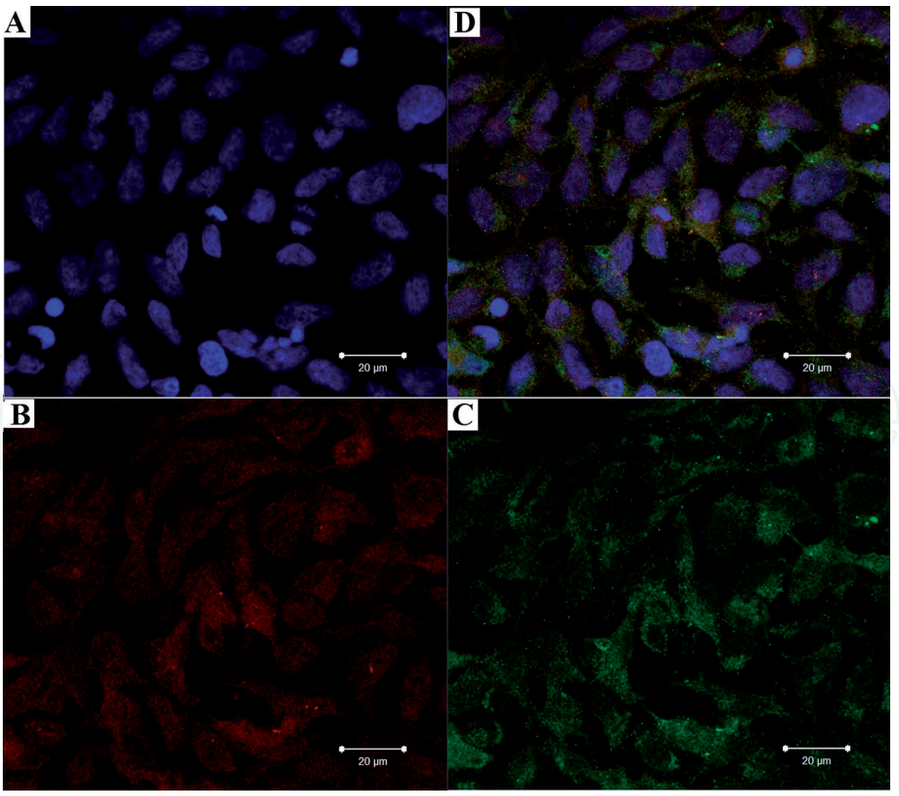

Figure 3.
Immunofluorescence analysis of VEGF164 and FGF2 biosynthesis in HEK293FT cells, 48 h after transfection. (A) Negative control: HEK293FT cells without pDNA transfection, nuclei-stained DAPI (blue). (B)–(D) HEK293FT cells transfected with pBUDK-ecVEGF164-ecFGF2. b) Staining with primary antibody against VEGF and secondary antibody, conjugated with a fluorescent label Alexa Fluor 555 (red). (C) Staining with primary antibody against FGF2 and secondary antibody, conjugated with a fluorescent label Alexa Fluor 488 (green). (D) Overlay image of a, c and d: VEGF (red), FGF2 (green), cell nuclei stained with DAPI (blue) [14].

expressed (data not provided). Thus, according to literature comparisons and based upon the laboratory data, the cells obtained were equine MSCs [20].

Genetic modification of horse MSCs with pDNA pBUDK-ecVEGF164-ecFGF2 showed that transfected cells possess a higher ability to form a capillary-like networks on the Matrigel™ matrix as compared to intact cells ($p < 0.005$) (**Figure 4**).

2.2 Use of the plasmid construct in vivo

Due to a high incidence of tendon and ligament injuries in horses, a high rate of recurrent traumas and a prolonged period of recovery that normally lasts for several months and up to 15 months with severe injuries, these injuries are a medical and surgical challenge. Even when modern technologies are applied, in many cases, damaged tendons and ligaments demonstrate biochemical and ultrastructural abnormalities after 12 months and preinjury biomechanical properties are not completely restored [21].

A total of 12 horses were given gene therapy [15, 16] through in vivo trials of the treatment. Out of them, eight horses had naturally occurring injuries of the superficial digital flexor tendon (SDFT; tendinitis) and four horses had suspensory ligament branch (SLB) desmitis. All the horses had spontaneous SDFT and SLB injuries and were included into the study from 2015 to 2017 undergoing treatment in the veterinary clinic "New Century" at the Moscow State Academy of Veterinary and Biotechnologies, Moscow.

Gene therapy of the four horses with injured SLBs showed that before treatment all horses had pain in the injured leg. By day 40 after treatment, no animals had any sings of inflammation at the site of injury, nor was there a change in the skin surface temperature within the area of injury, swelling or tenderness when palpated. By day 20 after treatment, lameness significantly reduced as compared to the baseline. By 12 weeks and during subsequent follow-up examinations, no horses were lame.

Ultrasound parameters in damaged SLB began to improve 20 days after the onset of treatment, this positive tendency remaining thereafter. Parameters such as changes of the zone of damage, echogenicity and fiber alignment made this especially evident. When the treated horses started doing a program of physical exercise, the ligament architecture constantly improved, as indicated by their longitudinal alignment and length (**Figure 5**).

Based on the examination results, only one horse had no significant ultrasound improvements in the first 90 days after pDNA injection. On days 20 and 40, this horse had new hypoechoic lesions that indicate a nonstable healing process. By 120–180 days after treatment, this horse had a noticeable ultrasound improvement in the site of injury.

Color Doppler ultrasonography (CDU) demonstrated evidently increased blood supply by day 20 after pDNA injection. This tendency remained up to day 40 and

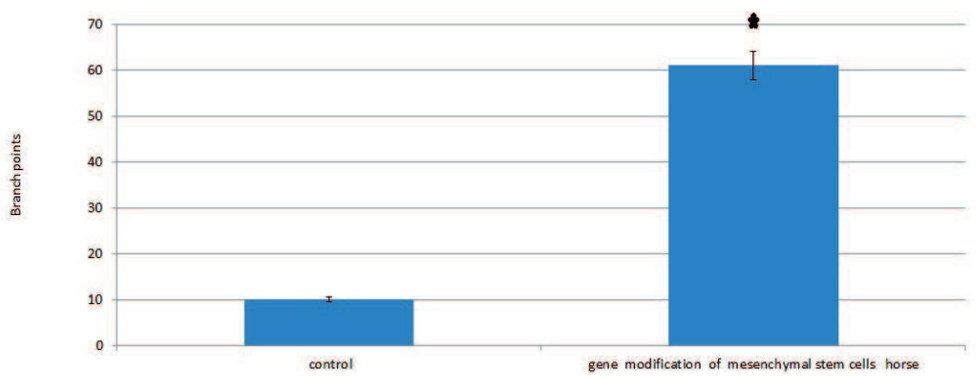

Figure 4.
In vitro *angiogenesis assay using Matrigel to characterize the proangiogenic effect mediated by genetic modification of mesenchymal stem cells.*

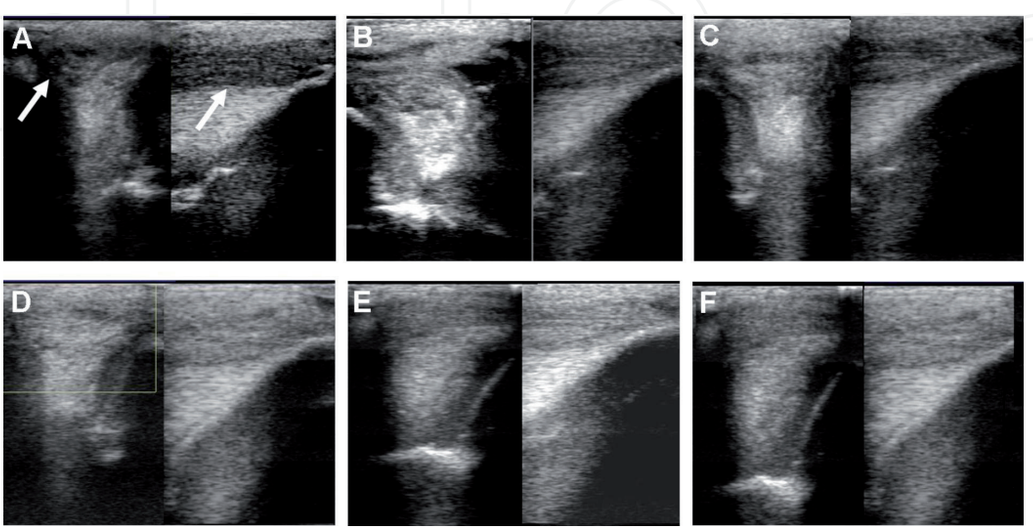

Figure 5.
Ultrasound images prior to plasmid DNA encoding VEGF164 and FGF2 genes on day 0 (A), 20 (B), 40 (C), 90 (D), 180 (E) and 300 (F) after administration in horse with SLB desmopathy. Arrows indicate lesion.

was high until day 90. By day 180 after plasmid injection, CDU parameters reduced to baseline values in most horses (with the baseline set at values within an intact limb of the same animal). There was no significant correlation between the soft tissue damage severity prior to treatment and post-treatment CDU parameters.

Ultrasound parameters of SDFT lesions in most horses began to improve 20 days after treatment [15]. This positive tendency remained during the follow-up period. With the onset of training, healing of the damaged tissue increased in the tendon treated. This manifested as a longitudinal alignment of fibers and an increase in their length [16].

After treatment, the echogenicity of the damaged SDFT constantly and significantly decreased from day 0 to day 60 in all horses except one. In 3 months after the beginning of treatment, the echostructure was more uniform in most horses, with collagen fibers arranged in parallel to the longitudinal axis.

A linear fiber pattern in horses with SDFT injuries also improved during the study, but this was happening more slowly when compared to the echogenicity. Within nine months, there were scarcely any signs of tendon damage in most of the horses with the SDFT injury. They had correct alignment and a well-arranged longitudinal pattern of fibers.

Doppler ultrasonography demonstrated a significant improvement in blood supply of the affected areas by day 20 [16]. This tendency continued for 90–120 days, with a peak that was reached on day 40. After postinjection day 180, the vascularization decreased to baseline levels (as in healthy limbs of the same animal). There was no significant correlation between the injury severity before treatment and CDU parameters afterwards. There were no significant differences in CDU images between horses with SDFT and SLB injuries after treatment; CDU changes were strictly individual. CDU changes can be due to hypervascularity being natural in the process of healing. Normally, tendons and ligaments are hypovascular [22]. A short-term increase in blood flow results in response to damage-associated tissue hypoxia. We propose that the gene therapy enhanced this effect markedly.

To identify possible side effects, all horses were constantly examined by a veterinarian in the clinic from the time of plasmid administration until 12 months later. Horses did not have any side effects to the pDNA administration, and horse age, gender and the duration of lameness had no effect on the outcome of gene therapy. The main differences in clinical outcomes were determined by the extent and site of the animal's soft tissue damage sustained before treatment. The study results showed that only one horse with a serious injury of the SLB and body did not respond to treatment, and it was lame for the first 3 months after the onset of therapy. Only one horse that recovered after gene therapy (initially with SDFT tendonitis) suffered a repeated injury at the same site 6 months after treatment [15, 16]. In the 12-month follow-up after treatment, owners of the other horses rated gene therapy results as good or excellent in terms of sporting success.

3. Discussion on the use of gene therapy in horses

One should emphasize that the disappearance of lameness with treated tendinitis or desmitis in a horse does not mean absolute tissue regeneration. In these studies, rapid and mostly complete regeneration of both the tendon and ligament occurred within 2–3 months of treatment, which included a single injection of pBUDK-ecVEGF164-ecFGF2. This was confirmed by increased echogenicity and homogeneity at the site of injury, as well as an increased percentage of parallel collagen fibers.

Thus, the study data are encouraging and demonstrated a positive effect of using pDNA encoding horse-specific proteins at early stages of healing of traumatic tendinitis and desmitis, when injected into the site of injury. In part, this can be explained by coincidence with conditions and stages of normal tendon healing. However, the horses included into the study had moderate or severe tendon injuries. It is well known that such injuries are associated with a poor prognosis in response to standard treatments.

A drawback of these clinical studies is that they did not identify an exact mechanism of action of direct gene therapy with pBUDK-ecVEGF164-ecFGF2 on the regeneration of damaged horse tendons and ligaments. Since the horses had fully recovered, the investigators considered possible histological interventions to take tissue samples as inappropriate. If histological samples could be taken looking at the cell types, checking for inflammatory reactions and cells associated with inflammation and immune responses would be advantageous for confirming the lack of immune response at a cellular level. In addition, investigating the healing mechanism via histology by looking at collagen type and wound repair would further the knowledge in this area. Adding RNA and protein expression studies would also help understand the mechanisms involved in this therapy. The pDNA administration used also avoids possible side effects associated with vector-mediated insertional mutagenesis when integration into the patient's genome is the long-term aim. This is not necessary in these disorders as long-term correction/replacement is not required. As previous reports of treatment results of such tendinitis and desmitis in horses are lacking, results of this gene therapy cannot be compared with those of other treatment methods.

Therefore, gene therapy, as one of the most advanced technologies in medicine, is a promising treatment for hereditary diseases and in addition offers new possibilities for a clinical management of numerous orthopedic disorders, including tendon and ligament injuries [23–25]. The use of direct gene therapy with species-specific growth factors is quite promising for the treatment of orthopedic disorders not only in horses but also in other animal species and in people [17]. The successful use of direct gene therapy with a similar plasmid construct based on dog-specific VEGF164 genes and bone morphogenetic protein (BMP2) to treat an anterior cruciate ligament injury in large dogs has been previously reported [26]. Moreover, there is a case report on using gene therapy to treat patients with critical lower limb ischemia [27]. Finally, plasmid DNA pl-VEGF165 (approved as Neovasculgen), encoding human VEGF165, has demonstrated its safety and efficacy in the treatment of atherosclerotic peripheral arterial disease in patients with chronic lower limb ischemia without side effects [28]. The high efficacy and safety of direct gene therapy have been demonstrated in all of these cases. There are also numerous benefits of using pDNA rather than recombinant viruses. Plasmids are relatively easy to construct, can be produced in large quantities and provide a safe method of delivery with low levels of immunogenicity associated with delivery. They can often be kept at room temperature for long periods of time, which is especially useful in clinical settings. Although they have lower levels of gene transfer, the studies carried out in the horse show that delivery is appropriate and efficient in these circumstances as it was delivered directly to the injured area.

VEGF and FGF2 gene therapy's direct effects on the regeneration of tendon and ligament injuries in horses should be further evaluated in a larger number of experimental animals, for a longer follow-up period and in a randomized controlled clinical study. Complete and more detailed results could also be obtained by histological examination and immunohistochemistry of samples and biopsy materials given the right conditions. Factors such as gene expression levels in tissues, collagen analysis, identification and quantification, the functional and intracellular distribution of

proteins and further studies of pathological biochemistry will help identify the main mechanisms of action.

4. Conclusions

The introduction of gene therapy in veterinary clinics becomes ever more possible; however, there are issues that require solutions. The future of veterinary gene therapy seems promising thanks to the studies described, and many other therapies are likely to be approved for use in both human and animal medicine [17].

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Conflicts of interest

The authors declare no conflicts of interest.

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