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RNA INTERFERENCE IN DENTISTRY: A CRITICAL REVIEW

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ABSTRACT

RNA interference (RNAi) is emerging as a promising technology in dentistry. This paper focuses on the applications and future prospects of RNAi, which opens new horizons in the diagnosis and treatment of dental diseases. RNA interference shows potential as a new and effective method of gene silencing that can selectively target and shut off the post-transcriptional expression of mRNAs. Thus, RNAi may play a big role in dental therapeutics because of its potential to modulate tissue regeneration, bone resorption, oral cancer development, tooth repositioning, and insurgence of craniofacial deformities. This paper updates the current knowledge and future aspects of RNAi in the field of dentistry.

KEY WORDS: RNA interference dental disorders, tissue regenerations gene silencing.

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INTRODUCTION

Current treatments for dental caries, periodontal diseases, periapical infections, and oral cancer are based on traditional approaches that presents with limitations in terms of efficacy and applicability. For instance, dental caries are currently treated by removing the affected tissue and replace it with biocompatible materials. However, dental restorations are not necessarily permanent and may require renovation and replacement[1]. Periodontal diseases and periapical infections are treated with antibiotics and eventually by surgical approaches aimed at removing the cause of infection and establishing an adequate and more functional anatomy. However, these approaches are rarely regenerative in nature and when so they are not able to re-establish the normal anatomy[2, 3]. Oral cancer is also treated by means of surgical removal and chemotherapy. Rarely, these approaches can be conservative as cancer needs to be aggressively removed. RNA interference (RNAi), as an emergent tool in bio-medicine, may represent a novel tool in dentistry as well, as novel and more effective and efficient approaches are needed to treat caries, periapical infections, periodontal diseases, and oral cancer.

RNAi: historical perspectives

RNAi was first observed in petunias, when Richard Jorgensen and colleagues attempted to intense the flowers' purple color by introducing a pigment-producing gene. In place of intensifying the color, the gene suppressed it. White patches or was completely white colors were observed in the resulting flowers[4]. In continuous, curiosity was further provoked when a similar effect was detected in the fungus *Neurospora crassa*. Introduction of extra copies of carotenoid pigment genes was done to enhance orange pigment in the fungus. This experiment failed when the orange pigment gene was suppressed. The effect was called quelling. At the same time, Guo and Kemphus were investigating the function of the *par-1* gene in the *Caenorhabditis elegans* nematode worm and inhibited the production of par-1 protein using antisense technology, in which a small synthesized strand of RNA that is complementary to a specific sequence in *par-1* mRNA attaches and halts the mRNA from being translated into the protein. Injecting an antisense RNA into the worm gave the predictable result: the embryos all died. But they were confused when the injection of the sense strand (the similar sequence as the *par-1* mRNA) which is a standard negative control for the experiment, also lead in embryonic lethality. Andrew Fire and Craig Mello had detected a similarly confusing result in their experiments, injected both antisense and sense RNAs into the worms to understand if there was an additive effect, but found that double-stranded RNA (dsRNA) was much more effective and silenced the target gene more competently than either strand alone. This result became identified as RNA interference[5]. It is an outwardly ancient defense mechanism against external double-stranded RNA (dsRNA). It is an extremely conserved gene silencing mechanism found in many eukaryotic species such as flies, nematode and mammals[6]. RNA interference can act at translational[7], transcriptional[8] and post transcriptional level[9].The natural role of RNAi and its related processes seem to protect the genome against invasion of mobile genetic elements such as transposons and viruses as well as helps in the organized development of eukaryotic organisms. RNAi also have developed to stop the jumping of genetic elements called transposons within a cell's DNA. Transposons cause destructions by jumping from one position to another on a genome, sometimes originate mutations that can produce cancer or other diseases[10]. RNA interference also have the ability to control transcription in the cell nucleus and acts as an ancient defense mechanism in plants by degrading viral RNA during the growth of viral genomes in infected plants[11, 12]. A similar example of virus-induced gene silencing was found when an RNA nepovirus, tomato black ring virus was injected in *Nicotiana clevelandii*[13].

Mechanisms of RNA interference

The fundamentals of RNAi process is the slicing of dsRNA into smaller pieces of a defined length by the enzyme called Dicer. The Dicer is an RNase III like nuclease which then slices the dsRNA into two classes of smaller RNAs— small interfering RNAs (siRNAs) and microRNAs (miRNAs) —that are about 21–23 nucleotides in length with 2 nucleotide 3' overhangs with 5' phosphate and 3' hydroxyl terminal. The ATP-dependent enzyme called helicase relaxed this duplex RNAs[14] and one strand (antisense strand) of the siRNA make complex with RNA induced silencing complex (RISC) [15]. The antisense strand amalgamated into siRNA and RISC complex monitors the siRNA and RISC complex to aim mRNA transcript thus target mRNA comprising a perfectly complementary sequence binds on to this siRNA after which the target mRNA is cleaved by Argounate 2 protein component of RISC[16, 17]. Silencing of gene can be completed by injecting the synthetic siRNAs in the cell (see Fig: 1), which are initially recognized by dsRNA binding protein, RNAi Defective family member-4(RDE-4)[18] and then submitted to Dicer.

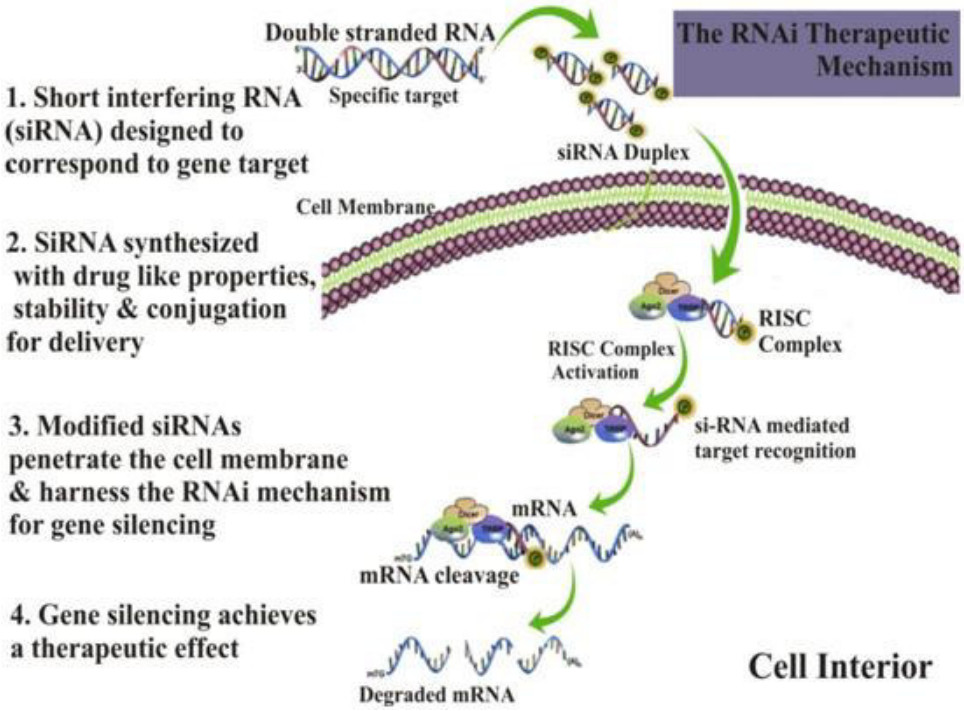

Figure 1
Mechanism of RNA interference

❖ ***Potential applications of RNAi in dentistry.***

• ***RNAi to inhibit periapical inflammation and periapical bone resorption***

One of the reasons for periapical bone destruction is intervened by interleukin (IL)-1alpha which is prospectively modulated by a group of cytokines. Studies showed that most of the Th1-type cytokines are activated in response to bacterial infection and showed an increase in mRNA and/or protein expression in periapical lesions. Kawashima & Stashenko found that inflammatory bone resorption may be up regulated in vivo by Th1-type mediators, and down regulated by Th2-type mediators. Role of IL-1 is in the pathogenesis of bone destructive diseases, including osteoporosis, rheumatoid arthritis and periodontitis, approaches for controlling IL-1 function directly and/or indirectly may offer a means of treating these diverse disorders through RNAi technology [19]. Additional factor is Cathepsin K (Ctsk); a lysosomal cysteine protease intensely expressed by osteoclasts and plays a crucial role in the degradation of protein components of the bone matrix. Result of Cathepsin K mutations cause human syndrome pycnodysostosis, in which bone resorption is impaired [20]. Gao B et al used the AAV RNAi knockdown system to examine the therapeutic potential of Cathepsin K silencing *in vivo* to reduce endodontic disease progression, bone resorption, and inflammation in periapical lesions in a well-established mouse model. They found that AAV-sh-Cathepsin K (AAV-sh-Ctsk) was responsible for impairment of osteoclast function in vivo and moreover reduced 88% of bacterial infection-stimulated bone resorption by[21]. *Atp6i* codes a subunit of the osteoclast proton pump, is crucial for the extracellular acidification that is necessary for osteoclast-mediated bone resorption [22]. Atp6i knockout mice have intense osteoporosis and absence of tooth eruption; hence, this gene is a potential target for obstructing osteoclast function [23]. Ma J et al explored the therapeutic potential of synchronized knockdown of *TIRC7*and *Atp6i* in the inhibition of bone loss of periapical induced by polymicrobes in the mouse model through the usage of locally delivered AAV-*Atp6i* RNAi, which caused silencing of both *Atp6i* and its isoform, *TIRC7.* This was the first study to examine the application of AAV-mediated knockdown of *Atp6i* as a probable target for gene therapy to treat endodontic disease. Their results validated that endodontic disease development, bone resorption, and inflammation in periapical tissues can inhibit by AAV-mediated Atp6i/TIRC7 knockdown, indicating for their potential application in gene therapy which may expressively improve the health of patients suffering from endodontic disease[1].

• ***RNAi for treatment of periodontal diseases***

Hongbing Jiang et al explored the possibility that adeno-associated virus (AAV)-mediated RNAi knockdown of Atp6i/TIRC7 could be used to target bone resorption and gingival inflammation in periodontal disease. To test this hypothesis they used a mouse model of periodontitis based on the infection of the oral pathogen *Porphyromonas gingivalis* W50 (*P. gingivalis)*. They found that Atp6i depletion impaired extracellular acidification and osteoclast-mediated bone resorption and suggested that AAV-shRNA-Atp6i/TIRC7 therapy may evident very helpful in the treatment of patients who suffer from *P. gingivalis*-mediated periodontal disease [24].

RNAi to intercept craniofacial deformities

RNAi has been use to examine the role of Bmp-4 during midfacial morphogenesis. Shumana and Gong were the first to investigate the use of RNAi to study the developing wild-type murine midface in vivo. This study was projected to show the efficient delivery of RNAi plasmid to the developing embryonic midface via maternal tail-vein injection and to equate the midface development of control embryos with Bmp-4RNAi exposed embryos. They assumed that delivery of RNAi in the developing murine embryonic midface could be attained via maternal tail-vein injection at 7.5 days post conception, and results in decreased Bmp-4 expression leads to modifications in morphology of the developing facial processes and prevent fusion of the midface. Levels of Bmp-4 mRNA and protein in the embryonic midface were examined with RT-PCR and immunohistochemistry, separately and it was found that injecting RNA plasmids via maternal tail-vein to pregnant mothers consistently delivered plasmids to the developing stage of embryonic midface with an average 46% (±12.2) knockdown in Bmp-4 RNA levels [25].

- ***RNAi applications in tissue regeneration***

The traditional concept of restoration of diseased dental/pulp tissues by inert materials is being challenged by latest advancements in pulp biology and regenerative strategies leading to the generation of new vital tissue. However, differentiation of true odontoblast is still speculative, and the approach is largely limited to immature teeth with an open tip. A more efficient approach may be provided by the adoption of the tissue engineering concepts associated to RNA interference strategies [26]. RNAi may favor tissue regeneration through the silencing of genes that are responsible for negative control of cell proliferation and cell differentiation or genes that induce inflammation or apoptosis[27]. New mechanism was developed by researchers in 2006 to silence gene expression in dental mesenchymal cells and assess gene role in tooth growth using a lentivirus mediated RNAi. knock down of Msx1 or Dlx2 expression in the dental mesenchyme truly recapitulates the tooth phenotype of their targeted mutant mice. They also verified that silencing of Barx1 expression in the dental mesenchyme results in stop of tooth development at the bud stage, validating a important role for Bar × 1 in tooth formation[28].Hef *et al*., in 2009 examined the effects of Notch‐Delta1 RNAi on the proliferation and differentiation of human dental pulp stem cells (DPSC's) *in vitro*. They found inhibition of self‐renewal capacity of DPSCs due to poor notch signals and induction of DPSCs differentiation under odontoblast differentiation inducing conditions. These findings proposed that DPSC's/Delta 1‐RNAi might be relevant to stem cell therapies and tooth tissue regeneration. Recent study showed that in a murine calvaria model, tumor necrosis factor‐α‐targeted siRNA caused the suppression of osteolysis induced by metal particles, thus introducing novel way to the application of RNAi technology in dental implant therapy and orthopedics [29]. In terms of bone regeneration, Gazzerro *et al.,* have showed the increase in the bone morphogenetic protein‐2 stimulatory effect on Smad 1/5/8 phosphorylation and on alkaline phosphatase activity by down regulating gremlin through RNAi in MC3T3 osteoblastic and ST‐2 stromal cells, results in enhance Runx‐2 expression and osteocalcin, thus increases Wnt signaling, probably increase bone formation *in vivo* [30]. Wang *et al*., in 2010 stated the inhibition of both bone resorption and osteoclast formation caused by short term suppression of RANK expression without off‐targeting effects as a result of delivery of siRNA targeting receptor activator of nuclear factor‐kappaB (RANK) to both primary bone marrow cell cultures and RAW 264.7 [31]. Taken together, these studies prove that RNAi, if adequately used, may inhibit bone resorption and substitute tissue regeneration. The use of RNA-interference based therapeutics for tissue regeneration is still in its budding stage. However, RNAi promises to be an efficient therapeutic tool and may be successful in periodontal and dental tissue engineering.

❖ ***RNAi therapy in the treatment of oral cancer***

RNAi approaches for cancer therapy is to knock out the expression of a target gene to stop tumor growth and kill the cancer cells selectively without damaging normal ones. Followings are some of the recent works on the efficacy of RNAi in the treatment of oral cancer.

- ***Treatment of Oral Cancer by silencing p53R2 gene***

Effect of RNAi-mediated p53R2 reduction on growth and 5-FU sensitivity of oral cancer cells and normal fibroblasts.

The fluoropyrimidine drug 5-fluorouracil (5-FU) is extensively used in the treatment of gastrointestinal, breast, head and neck cancers [32]. In fact, the combination of 5-FU with other anticancer agents such as cisplatin and methotrexate as a neoadjuvant chemotherapy has enhanced the response rate for advanced oral cancer [33]. Though, patients who have a low response to 5-FU-based chemotherapy, suffers a delay in initiating the most effective treatment resulting to the late overall diagnosis. Thus, there is an urgent need of new treatment strategies to improve the efficacy of anticancer agents and drugs. The tumor suppressor gene p53 is the most commonly mutated gene in human cancers [34]. Recently, the p53-inducible p53R2 gene has been studied and revealed to play a crucial role in DNA repair and synthesis after DNA damage [35]. Moreover, the expression and activity of p53R2 has been reported to be associated with the anticancer agent resistance of human cancer cells [32]. It was also reported that the presence of p53R2 expression was a predictive factor for the sensitivity to preoperative radio chemotherapy in oral squamous cell carcinoma [36]. The p53R2 gene encodes the ribonucleotide reductase (RR) which is induced by some stress signals activating p53, for instance DNA-damaging agents. The p53R2 gene product increases the deoxynucleotide triphosphate (dNTP) molecules in the nucleus, which accelerates DNA repair and synthesis [37]. In a recent study, a human breast cancer cell line MCF-7, three human oral cancer cell lines (SAS, HSC-4 and Ca9-22), and a normal human fibroblast cell line

NHDF were used to test the effects of silencing the expression of p53R2 by means of RNAi. It was found that the cancer cell lines with higher p53R2 expression were more resistant to 5-FU. RNAi-mediated p53R2 reduction enhanced chemo sensitivity and inhibited growth selectivity in cancer cell lines but not in normal fibroblasts. These outcomes were suggested that basal transcription of p53R2 could be associated with the sensitivity to anticancer agents [38].

- ***RNAi for inhibition of metastasis and progression of oral squamous cell carcinoma***

u-PAR (urokinase-type plasminogen activator receptor) is overexpressed in many human malignant tumors as well as in oral squamous cell carcinoma (OSCC) and plays a significant role in an array of cancer key cellular events as an adaptable signaling orchestrator. In a study, a retroviral vector expressing u-PAR-specific siRNA was introduced into OSCC xenografts of nude mice to monitor its inhibitory effects on OSCC [39]. The siRNA targeting u-PAR extremely suppressed tumor growth decreased the expression of proliferation-related gene (Ki-67) and augmented cell apoptosis in OSCC. Additionally, the mRNA and protein expression of VEGF-C, VEGF-D, MMP-2, MMP-9 and VEGFR-3, which have been shown to be involved with oral cancer invasion and metastasis, was concurrently down regulated as determined by quantitative real-time RT-PCR and Western blot.

- ***RNAi targeting Bmi-1 in oral squamous cell carcinoma***

Bmi-1 is a polycomb group protein that was recognized as c-myc cooperating oncogene in murine lymphomagenesis. Bmi-1 protein and RNA expression levels are strikingly enhanced in the oral squamous cell carcinomas (OSCC) cells in contrast to normal human oral keratinocytes (NHOK). Enhanced Bmi-1 expression was also identifying in situ in the archived oral mucosal tissues with precancerous and cancerous histopathology, together with that of mild epithelial dysplasia. Thus; Bmi-1 expression arises at a very early stage in oral carcinogenesis and its endogenous knock down in actively proliferating SCC4 cells and NHOK by RNA interference strictly holds up the division of the cells of OSCC. Thus, suppressing the expression of Bmi-1 is effective in reducing cancer cells activity [40].

- ***RNAi targetingLAT1 in human oral cancer cells***

Amino acid transporters are crucial for growth and proliferation in all living cells. L amino acid transporters systems are the most important nutrient transport system among the amino acid transporters. It is responsible for the Na+-independent transport of neutral amino acids, including numerous essential amino acids. Over expression of L-type amino acid transporter 1 (LAT1) is responsible for cell growth in malignant tumors. For the sequence-specific inhibition of gene expression in a wide variety of eukaryotes, siRNA can be used as a promising technology. The study suggested that the inhibition of LAT1 (amino acid transporter) expression led to the inhibition of KB cell growth by inducing an intracellular reduction of neutral amino acids such as L-leucine, essential for cell growth; and furthermore provides an insight into the growth inhibition of oral cancer cells *via* LAT1 siRNA, the LAT1 inhibitor. Thus, LAT1 could be a novel target for the suppression of tumor cell growth, including oral cancer cells. RNAi has been found very effective for silencing LAT1 expression on human oral squamous cell carcinoma [41].

❖ ***Limitations of RNAi***

An advance in RNAi technology provides benefits to both basic and applied research. Yet, limitations exist in terms of maintenance of high levels of efficiency [42]. The efficacy of siRNA-mediated suppression of gene expression depends on a number of factors, which are mentioned below:

- Designing of an effective siRNA sequence is the initial constraint of RNAi technology.
- To achieve optimal silencing of the target gene accurate structure of the siRNA, and/or protein are needed.
- There is lack of efficient methods for delivering the siRNA to the target cells in vivo.
- Nonspecific and off-target effects of siRNAs present an inherent limitation of RNAi. Indeed, initial studies reported that introduction of a siRNA molecule into a cell can have multiple effects other than those caused by gene-specific silencing [43, 44]. Two common types of nonspecific effects have been detected.

➢ First, off-target effect is observed when specific genes except targeted gene show altered expression in response to a siRNA.
➢ Second, siRNAs can activate alternative dsRNA-responsive cellular pathways causing up-regulation of a large number of genes usually associated with pathways of innate immune system, together with interferon-stimulated genes (ISGs).

Basic guidelines of good experimental practice can minimize the nonspecific and off-target effects of siRNAs

1) Exploiting available utilize information and algorithms the most effective and specific siRNA possible should be designed so that it can be used at very low concentrations.

2) Using different control siRNAs against measurable irrelevant genes, and finally rescuing the phenotype caused by an siRNA by ectopic expression of a version of the gene that cannot be silenced by the siRNA [45].

3) Developing several siRNAs against the same target since it is unlikely that they all would have same sequence-dependent off-target effects.

Nanotechnology solves the delivery-associated problems of the RNAi-based therapies.

Ribonucleic acid interference (RNAi) is a potential molecular tool that has a power to revolutionize the treatment of dental disorders. One main obstacle of applying this technology for clinical application is the absence of site-specific carriers that can efficiently

deliver siRNA to target cells [46]. Advances in molecular biology and nanotechnology are rapidly empowering the development of nanoparticles (NPs) with specific functional properties that address the limitations of traditional disease therapeutic and diagnostic agents [47]. Simultaneously, nanomaterials are being used as drug carriers because of their careful construction (tailored drug release characteristics, low immunogenicity, etc.) yielding improved treatment efficiency and decline of undesirable side effects [48]. Thus, nanotechnology can be used as a promising approach to solve the delivery-associated problems of the RNAi-based therapies. For example, properly engineered magnetic nanovectors can be developed to overcome these obstacles. The fundamental principles of magnetofection (magnetic drug targeting) to nucleic acid delivery by magnetic nanovectors have been recently modified to RNAi. It includes the preparation of a siRNA attached to magnetic carrier, addition of prepared carriers to the cell culture medium or injecting it in the blood stream systemically or applying it to the target tissue, and then applying a magnetic field to straight the vector towards the target cells [49].SPIONs are metallic nanoparticles contain a magnetic iron-oxide core either encapsulated within a metallic shell or polymer or dispersed within a polymer matrix such as PVA, silica, or dextran. The shell is then available for attaching streptavidin, carboxyl groups, antibodies, etc. for further experiment. The particles are generally coated with PEI, binding the nucleic acids on the surface of the SPION via charge interactions for in vitro magnetofection [50]. Number of studies show that the positively charged nanoparticles are most effective for siRNA delivery. This can also make possible by exploiting the magnetic properties of nanovectors. The physicochemical properties of nanovectors, particularly the hydrodynamic size and zeta potential, are known to greatly influence their behavior both in vitro and in vivo, and internalization by cells [51]. Zhang et al reported the assessment and development of a magnetic nanovector construct specific for cancer-cell for effective siRNA delivery and non-invasive monitoring through magnetic resonance imaging (MRI). They reported that the hydrodynamic size and zeta potential of the NP-siRNA nanovectors (NP:siRNA ratio of 10:1) have a hydrodynamic size of 111.9_ 52.4 nm, an average core size of 7.5 nm, and a cationic zeta potential of 19.6_ 9.7 mV. Number of chlorotoxin (CTX) molecules and siRNA per nanoparticle were calculated to be 5 and 3.8, respectively, as determined by gel retardation assays. These nanovector constructs have PEG coated in both the initial shell and the outer layer exposed to solution environment, that contribute to the improved stability of the nanovector construct, even over the pH and NaCl ranges tested. They further demonstrated the targeted siRNA delivery, followed by increased siRNA internalization by target tumor cells and then intracellular trafficking towards improved gene knockdown by NP-siRNA-CTX nanovector through quantitative RT-PCR analysis [46]. Recent research developed a new approach for targeted delivery and expression of siRNA in vivo using DNA-based siRNA expression nanocassettes and receptor-targeted nanoparticles [50]. This new nanoparticle comprises of an amphiphilic polymer-coated QD conjugated to 10 to 20 DNA nanocassettes that enclose a U6 promoter and shRNA gene for *in vivo* siRNA gene expression followed by delivery to target cells. The nanoparticle was conjugated to the amino terminal fragment of uPA-urokinase plasminogen activator, which targets uPAR, its cellular receptor. This receptor is highly expressed in tumor, stromal cells and angiogenic endothelial in many types of human cancer [53].

CONCLUSION

Oral diseases like dental caries, oral cancer and periodontal diseases already have treatments which are painful; having post infection chances but still there is a burning necessity for the improvement of the existing treatments or other flourishing alternative. In this review we showed that RNAi may be effective for treatment of dental diseases. Some genetic disorders like dentinogenesis imperfect, amelogenesis imperfect, osteolysis suppression and tooth development (hypodontia, agenesis) encountered in medical practice represent only the tip of the iceberg. The mechanism of RNAi can inhibit the progression of these genetic disorders to the next generation. Results from basic research and clinical studies suggested that this field of research may soon contribute to more effective therapies in various branches of dentistry. Hence the recent discoveries oozing from RNAi-based methods might prove to be a boon to the society. In near future, RNAi therapy is going to become a method of choice and a novel way of genetic ablation that holds massive hope for improving the ability to disentangle the complex regulatory pathways that regulate the cellular behavior in dentistry.

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