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Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR): A critical overview on the most promising applications of molecular scissors in oral medicine

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Abstract: The scientific community is continuously working to translate the novel biomedical techniques into effective medical treatments. CRISPR-Cas9 system (Clustered Regularly Interspaced Short Palindromic Repeats-9), commonly known as the "molecular scissor", represents a recently developed biotechnology able to improve the quality and the efficacy of traditional treatments, related to several human diseases, such as chronic diseases, neurodegenerative pathologies and, interestingly, oral diseases. Of course, dental medicine has notably increased the use of biotechnologies to ensure modern and conservative approaches: in this landscape, the use of CRISPR-Cas9 system may speed and personalize the traditional therapies, ensuring a good predictability of clinical results. The aim of this critical overview is to provide evidence on CRISPR efficacy, taking into specific account its applications in oral medicine.

Introduction

The scientific community has increasingly focused attention towards the last innovations in medicine. Over the years, scientific knowledge has been ever more created in specific sectors, such as the human genetics, also thank to the breakthrough boosted by the modern technologies working on genome editing. In fact, through artificial nucleases such as zinc-finger (ZNF) and activators of the nucleases of the effector of the transcription (TALENs), researchers can modify defective genes without any pharmacological support (Zhang *et al.*, 2019; Huang *et al.*, 2020; Yu *et al.*, 2021a, 2021b).

The last scientific research has focused on CRISPR-Cas9 system (Clustered Regularly Interspaced Short Palindromic Repeats-9), most commonly known as "molecular scissors" (Bao *et al.*, 2019). CRISPR is a versatile approach that allows physicians to edit nearly several loci in the human genome; this ability is currently investigated to develop innovative therapies against a large number of diseases. This system confers resistance to the phages containing sequences similar to the ones showed in CRISPR system; CRISPR *loci* typically consist of short fragments of DNA of viral origin, identically repeated (CRISPR-RNA), trans-activating the crRNA (trans-crRNA) and a series of genes coding for CAS endonucleases (Gong *et al.*, 2021; Liu *et al.*, 2021). These endonucleases using a leading strand of RNA (crRNA) and cutting DNA in specific *loci*, allow the insertion of the sequence where it is necessary (Jiang and Doudna, 2017).

Currently, there in a notable increase of research focused to the development of this system in the treatment human diseases, such as HIV/AIDS (Xiao *et al.*, 2019), Malaria, Epstein Barr Viruses (Rodriguez-Rodriguez *et al.*, 2019), chronic granulomatous diseases (de Ravin *et al.*, 2017), heart diseases (Gifford *et al.*, 2019), Alzheimer's disease (Kurochkin *et al.*, 2018), and recently also a possible application in the diagnosis of SARS-CoV-2 related syndromes (Esbin *et al.*, 2020) and specific dental diseases (Divaris, 2019; Gong *et al.*, 2020).

On the other hand, it has been observed a correlation between patient with diabetes, inflammatory bowel disease, obesity, and oral diseases (Le Bars *et al.*, 2017).

The aim of this critical overview is to provide evidence on CRISPR efficacy, taking into specific account its applications in oral medicine.

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CRISPR/Cas9 in oral diseases

Oral tissues are generally contaminated by bacteria able to promote, or worsen, several oral pathologies (Curtis *et al.*, 2020). Dental decays, periodontitis and other gingival infections are often co-caused by bacterial flora hosted in dental plaque: many oral bacteria have been demonstrated to be linked to several systemic infections (Agbo-Godeau, 2019).

CRISPR loci are frequently located on sites residing in human oral microbiota (Toyomane *et al.*, 2021); such loci may be a trigger to modulate the overall behaviour of oral microbiota (Akram *et al.*, 2020). The comparison between healthy patients and those affected by periodontitis has revealed that CRISPR loci found in healthy patients are able to identify a bacterial community characterized by a resistance to the bacteriophages better than the one present in patients affected by periodontitis (Gong *et al.*, 2019; Steier *et al.*, 2019). Cas9 was detected in gingival epithelial cells, gingival fibroblasts, and inflammatory infiltration cells (Steier *et al.*, 2019). The modulation of local inflammation seems to be concretely guided by the association of superoxide dismutase 2 and BIRC3; moreover, some recent trials have suggested an increase of Sod2, Birc3, Casp3 e Casp9 in the gingiva of subjects with periodontitis (Yoon *et al.*, 2018; Deschner *et al.*, 2021). The use of CD40 as a biological target has demonstrated how this system inhibits the development of local inflammation after injection of antibodies of anti-CD40 in the inflammatory bowel diseases (Anka Idrissi *et al.*, 2021; Wang *et al.*, 2020a). Similarly, recent studies on periodontitis have highlighted a pivotal pathogenetic role of the oxidative stress reactive oxygen species (ROS); ROS are produced by bacteria (Oveisi *et al.*, 2019; Sulijaya *et al.*, 2019; Zeng *et al.*, 2019) and a key factor for oxidative stress is the regulation of Nerf2, which has been reported to have a protective role in many oral pathologies. Furthermore, Keap1 allows the translocation of Nerf2 in the cell core, where it activates the transcription of genes with antioxidant function. Therefore, Nrf2 activity is critical for the balance of redox homeostasis of the cell (Shaw and Chattopadhyay, 2020). Moreover, Nrf2 activation seems to reduce the activation of the Nlp3 inflammasome, by suppressing the production of ROS (Liu *et al.*, 2017; Saha *et al.*, 2020).

CRISPR also allows the deletion of NLRP3 inflammasome by stopping its activation during the transition process from epithelium to mesenchymal in the fibrosis (Alyaseer *et al.*, 2020). As regards craniofacial abnormalities, instead, in zebrafish, CRISPR technique has been used to inhibit WNT-associated gene lrp5 (Hao *et al.*, 2019).

In the studies on craniofacial development, CRISPR-based genome modification system accelerated the formation of animal models thanks to the rapid manipulation of specific genes (Wu *et al.*, 2019). Knockout mice generated by CRISPR/Cas9 have showed the importance of Golgb1 and MSX 1 in the development of teeth, palate, and normal cilia function in zebrafish (Zheng *et al.*, 2021; Bergen *et al.*, 2017). In fact, mutations of the transcriptional factor MSX1 cause craniofacial malformations and agenesia of teeth (Goto *et al.*, 2016).

Genome-editing with CRISPR-Cas9 represents the most important starting point for the resolution of the previously untreatable diseases (Zhan *et al.*, 2019). An example is the identification of various therapeutic targets, such as p75NTR and its receptor, in the treatment of the oesophageal squamous cell carcinoma and tooth morphogenesis (Shen *et al.*, 2019; Zhao *et al.*, 2019). The receptor p75NTR carries out different functions impacting cell survival, apoptosis, differentiation (Meier *et al.*, 2019) it is expressed in the neural crest cell population (Wislet *et al.*, 2018), mouse alveolar bone cells (Wang *et al.*, 2020b) and human oesophageal keratinocyte stem cells (Daltoe *et al.*, 2020). Another intriguing use of this system is within the protocol that allows to study the human epithelial cells exosomes released from the oral mucosa infected by EV71 (Enterovirus 71) (Wang *et al.*, 2020c). Thus, the well-recognized genome editing ability of the CRISPR-Cas system has triggered significant advances in CRISPR diagnostics and potential treatments related to oral medicine.

CRISPR/Cas9 and modified oral-derived stem cells

There are different aspects of stem cells that can be strongly influenced by CRISPR (Table 1). The changes promoted by CRISPR-Cas9 on mesenchymal stem cells can be useful for the correction of several pathological defects. Stem cells have been isolated from different human tissues, including the dental tissues (Berebichez-Fridman and Montero-Olvera, 2018; Granz and Gorji, 2020; Yoshida *et al.*, 2020): DPSC (dental pulp stem cells), PDLSC (periodontal ligament stem cells), SHED (mesenchymal stem cells from human exfoliated deciduous teeth), GMSC (mesenchymal gingival stem cells), DFSC (dental follicle stem cells), and SCAP (apical papilla stem cells) have active role in the healing of bone tissues, such as the

TABLE 1

Factors/genes influenced by CRISPR and their main role in biomedical processes

Factors/ genes	Role	CRISPR/Cas9 applications	References
Sox2	Cell Pluripotency	Remodelling specific gene locus	Liu *et al.* (2018)
Oct4	Cell Pluripotency	Remodelling specific gene locus	Liu *et al.* (2018)
Sod2	Periodontal Inflammation/periodontitis	Modulate specific molecular functions	Yoon *et al.* (2018)
Birc3	"	"	Yoon *et al.* (2018)
Wnt + lrp5	Signal transduction pathways	Delete wnt in studies of cranial cells of the neural crest	Ji *et al.* (2019)
Golgb1	Development teeth and palate	Possible treatment for resolution oral diseases	Bergen *et al.* (2017)
Msx1	"	"	Zheng *et al.* (2021)
P75NTR	Survival, apoptosis, and cell differentiation	Squamous oral carcinoma	Shen *et al.* (2019)

post-extractive sockets; moreover, PDLSC and GMSC have demonstrated osteogenic and regenerative potential *in vitro* (Yoshida *et al.*, 2020). On the other hand, GMSC have been investigated and successfully used to promote regeneration in subjects with severe periodontal defects (Table 2) (Zhou *et al.*, 2020; Liu *et al.*, 2019; Shi *et al.*, 2017). In recent studies, it has been observed that stem cells modified by CRISPRS may support a better and faster tissue regeneration (Sürün *et al.*, 2020; Ben Jehuda *et al.*, 2018): the biological pathways potentially involved in this biological behaviour is related to the increase of the expression of Oct4, Sox2, and Klf7 (Liu *et al.*, 2018; Corbineau *et al.*, 2017; Yang *et al.*, 2020). To date, researchers are working on the potential application of genome editing to the treatment of several systemic disorders (Frangoul *et al.*, 2021). As an example, the sickle cell anaemia has already taken advantage from other techniques, such as Zinc-finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs) two chimeric nucleases linked to a non-specific DNA cleavage domain (Demirci *et al.*, 2019). In the patients with Fanconi anaemia, somatic cells have been genetically reprogrammed towards oral-derived induced-Pluripotent Stem Cells (iPSCs) with CRISPR/Cas9 system to treat patients with gene-therapy; also, the Diamond-Blackfan anaemia has shown interesting results from studies based on the inactivation of the p53 gene into modifies pre-erythrocytes obtained from such iPSCs (Kapralova *et al.*, 2020). Recently, CRISPR-Cas9 system has been used to achieve efficient treatments based on gene silencing and insertion of gene sequences in oral-derived MSCs (Komor *et al.*, 2017). Interestingly, significant results have been achieved with the use of CRISPR-Cas9 system in the therapy of diabetes mellitus associated with Wolfram syndrome, a rare and untreated disease associated with a defect of WFS1 gene (Maxwell *et al.*, 2020).

Other strategies are related the production of oral-derived stem cells carrying a correction of faulty gene expressed on LDL receptors that cause homozygous familial hypercholesterolemia (Okada *et al.*, 2019). Finally, it has been obtained a significant slowdown of the aging process, and a doubling in life expectancy, in mice-models suffering by progeria, treated with iPSCs modified with CRISPS-Cas9 technique (Santiago-Fernández *et al.*, 2019).

Discussion

In the last decades, a consistent scientific literature has widely debated about a novel way to approach translational medicine. Recently, several biological pathways have been investigated because of their impact on important clinical aspects. Interestingly, scientific researchers have discovered a number of biochemical and genetic mechanisms belonging to ancestral pathways able to deeply modulate the biology of complex organisms, such as the pathway that makes the bacteria able to deactivate their ability to damage the host organism (Gong *et al.*, 2019; Chen *et al.*, 2019). In this landscape, it was recently identified the biological strategy able to turn-off CRISPR system: it was called "anti-CRISPR" mechanism. In detail, the phages are able to produce the anti-CRISPR proteins named ACR: those proteins are able to stop the molecular scissor Cas, avoiding that Cas will be able to cut and modify the human genome (Marino *et al.*, 2020). The function of anti-CRISPR mechanisms was investigated and fully demonstrated, basically, administering AcrII4 to human cells, obtaining that the anti-CRISPR mechanism was enabled and working (Kim *et al.*, 2018). Currently, the biological strategies introduced by the recent discovery of the anti-CRISPR system has been turned into studies aimed to understand the so called "off-target systemic effects" (Shin *et al.*, 2017). In a study on this matter, it was described an anti-CRISPR pathway that blocks the genomic editing in every organ of the body, unless in the liver; in fact, in the hepatocytes it has been found a tissue-specific micro-RNA (microRNA-122), still not completely understood (Lee *et al.*, 2019).

It is even more evident that the anti-CRISPR strategy may represent a safeguard mechanism, highly used by simple organisms like the bacteria, which may have pathogenetic role, still not fully under-stood, in complex metabolisms also involving the human tissues. The anti-CRISPR mechanisms may have an overall function to promote inflammation in several acute conditions; in fact, they are often recognized as "non-self" by the human immune system, and this specific condition would result in a severe inflammatory response involving several organs (Liu *et al.*, 2020).

In conclusion, CRISPR and anti-CRISPR are two specular mechanisms that offer the potential control of human genome. Unfortunately, both can result in collateral severe consequences that make them not safe to manage. Researchers are closely working toward the identification of biological molecules able to turn-off Cas9 in a safe procedure, in order not to activate the immune response (Hille *et al.*, 2018). One the major challenges is to clarify the mechanisms to by-pass the immune system, thus making CRISPR and anti-CRISPR able to work in human organism;

TABLE 2

Oral-derived Stem cells influenced by CRISPR and their main role in biomedical processes

Stem cells	Site	CRISPR/Cas9 applications	References
DFSC	Dental follicle	Dental tissues repair	Zhou *et al.* (2020)
GMSC	Gingiva	Gingival means	Shi *et al.* (2017)
SCAP	Apical papilla	Restore of injured tissues of multiple organs	Zhou *et al.* (2020)
SHED	Human exfoliated deciduous teeth	(Re)Generate dentin	Yoshida *et al.* (2020)
DPSC	Dental pulp	(Re)Generate a dentin/pulp-like complex	Yoshida *et al.* (2020)
PDLSC	Periodontal ligament	Odontogenic differentiation of local stem cells	Zhou *et al.* (2020)

currently, the investigations are based on the molecules involved in the activation of these mechanisms, and on their physiological effects (Fu *et al*., 2021). Many enzymes have been certainly associated to CRISPR: one interesting example is the Cpf1 enzyme that is similar to Cas9; it has a number of deactivated forms that can be useful for the study of Cas13 transcriptional effects (Safari *et al*., 2019) and RNA adenosine deaminase transfer. To fully understand these mechanisms, we need to fully understand the role of genome editing not only related to the potential advantages, but more importantly to the hypothetical damages; in fact, several enzymes can work on DNA, also converting the AT couple to GC in the target position inside the genome without breaking the double-stranded of DNA (Gaudelli *et al*., 2017). Nevertheless, also this system may have some issues; in fact, genome editing efficiency may be influenced by several factors, also able to create mutations elsewhere in the genome, known as 'off-target' modifications.

We strive to safely manage these biological scissors, and the complete understanding of their use will certainly change the way of performing the future medicine.

Conclusions

In this overview, we have tried to clarify the latest breakthrough on CRISPR, as it represents a valuable tool in the field of genetics and epigenetics. The impact of CRISPR and anti-CRISPR systems can improve the understanding of several oral pathology and may impact the way to make diagnosis and therapy of all the main diseases. Unfortunately, such systems allow to directly manipulate the human genome, for example eliminating a gene responsible for a specific pathology, even if we do not fully know the collateral impact of these modifications; nevertheless, there are many unanswered questions on the ethical implications regarding embryonic stem cell lines/germ lines. Undoubtedly, CRISPR-Cas system may be involved in more further strategies, rather than the only immunological tasks. Promising insights could be carried out from studies aimed at a deeper understanding of this tool on several impacting oral diseases, such as the periodontitis, targeting the research on why *P. gingivalis* plays a pivotal role in that class of diseases. Also, pharmacological target may be studied for several inflammatory pathologies affecting the oral mucosa; in fact, cutting-edge CRISPR/Cas9-based technology may transform the field of oral pathology research by efficiently introducing genetic alterations, so to investigate the main genes function in experimental models of different oral pathologies. The hope is to develop more consistent knowledge in the translational applications of genome editing applied to oral pathology and, more in general, to medical sciences.

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