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Research Models in Dentine Development and Regeneration

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Dentine is a major component of teeth and is responsible for many of their functions, such as mastication and neural sensation/transduction. Over the past decades, numerous studies have focused on dentine development and regeneration using a variety of research models, including in vivo, ex vivo and in vitro models. In vivo animal models play a crucial role in the exploration of biochemical factors that are involved in dentine development, whereas ex vivo and in vitro models contribute mainly to the identification of biophysical factors in dentine regeneration, of which mechanical force is most critical. In the present review, research models involved in studies related to dentine development and regeneration were screened from publications released in recent years and summarised comprehensively, particularly in vivo animal models including prokaryotic microinjection, Cre/LoxP, CRISPR/Cas9, ZFN and TALEN, and scaffold-based in vitro and ex vivo models. The latter were further divided by the interactive forces. Summarising these research models will not only benefit the development of future dentine-related studies but also provide hints regarding the evolution of novel dentine regeneration strategies.

Key words: *dentine development, dentine regeneration, research models, scaffolding system, transgenic mice*
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As the major component of teeth, dentine is responsible for various functions such as enamel support, pulp protection, neural sensation and transduction; however, due to caries lesions, trauma and abrasion, dentine loss has become one of the most common issues in dental clinical work, leading to dentine hypersensitivity, occlusion deficiency and even changes in countenance. To deal with these problems, artificially synthesised materials such as resins or ceramic prostheses are usually employed, but although these materials manage to restore the appearance and some of the functions of dentine, they fail to reconstruct the sensation of the dentine–pulp complex, and their long-term effects are not guaranteed[1,2]. Therefore, functional regeneration of dentine is still required and is of critical importance.

To perform functional dentine regeneration, it is vital to recognise the key factors involved in dentine development. Dentine development is known to be a complicated and delicate biological process. At different stages, regulatory biochemical factors vary regarding their types, expression patterns and rendered effects. For example, Runx2 is upregulated in the early stages of dentine development and downregulated in the later stages, whereas osterix is continuously and highly expressed at all stages of dentine development[3]. Thus, dentine development involves a series of genetic

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modulations and activation/inactivation of signalling pathways that comprise an elaborate regulatory network. A careful illustration of this network will aid not only the prevention and treatment of dentine-related developmental diseases but also the functional reparation or regeneration of dentine.

In addition to biochemical cues, understanding of the fact that biophysical factors also contribute to dentine development and regeneration has increased in recent decades. It is widely known that in regenerative strategies, the biophysical characteristics of a scaffold determine the accommodating niche of stem cells and the release profiles of growth factors[4]. Meanwhile, during dentine development, the biophysical cues also participate in the commitment of the dental mesenchyme. For example, a study proposed that mesenchyme condensation in the dental papilla initiated odontogenesis[5].

Research models are generally divided into in vivo, ex vivo and in vitro. Exploration of the biochemical network usually requires a combination of animal models and subsequent in vitro or ex vivo confirmation, among which animal models play the most vital role. Numerous studies focusing on dentine development have employed this approach and proven the effects of dentine sialophosphoprotein (DSPP), bone morphogenetic proteins (BMPs) and transforming growth factors (TGFs), etc. In the meantime, studies of biophysical cues in dentine regeneration and reparation depend largely on ex vivo and in vitro approaches. In particular, the development of novel biofabrication approaches such as micropatterning[6] and mathematical modelling approaches[7,8] have vastly facilitated these studies.

Thus, all three models supplement each other and work together to improve knowledge regarding dentine development and reparation; however, to the best of the present authors' knowledge, no systematic review of these approaches has been carried out to date. To fill this knowledge gap, approaches employed in the literature relating to dentine development or regeneration were screened and summarised in this review. First, animal models used to identify biochemical factors in studies relating to dentine development are introduced, including their history, basic theories and applications. Second, the exploration of biophysical factors via ex vivo and in vitro approaches is illustrated, focusing on the design of the systems and their application in studies relating to dentine regeneration. This review aims to provide insight regarding the tools for future studies concerning dentine development and reparative endodontics.

Biochemical cues: animal models

Of the three types of experimental models, in vivo animal models are considered the gold standard for speculating human responses because of animals' similarities to humans in terms of biological structures and genes. In particular, they are more reliable in the exploration of the dynamics of biochemical factors including genes and regulatory molecules compared to in vitro or ex vivo methods[9].

Laboratory animals are a group of standardised animals that are used extensively in various experimental studies, both basic science and preclinical, owing to their accuracy, reproducibility and rationality[10]. In order to fulfil predetermined experimental aims, researchers employ laboratory animals and construct specific animal models. Among these, genetically modified animal models, which have marked a significant breakthrough, are relatively advanced models that are powerful and customisable. Researchers use various gene editing approaches to modify the genome of laboratory animals by inserting or deleting target DNA segments, resulting in customised models with specific biological, pathological and cellular characteristics that imitate those of human diseases.

In 1980, Gordon et al[11] created the first transgenic mouse via prokaryotic microinjection of fertilised eggs. Subsequently, the combination of the gene targeting technique based on homologous recombination devised by Thomas and Capecchi[12] and Smithies et al[13], and the embryonic stem cell technique created by Evans and Kaufman[14] led to the first gene knockout mice in 1989[15]. Over the subsequent decades, the booming development of transgenic mice and gene manipulation approaches have provided powerful tools for biological exploration and disease-related studies.

To date, commonly used gene-modified approaches include prokaryotic microinjection, Cre/LoxP, CRISPR/Cas9, ZFN and TALEN. Although each approach has its inherent advantages and disadvantages, they all contribute to the exploration of tooth development and related diseases (Fig 1).

Prokaryotic microinjection

Prokaryotic microinjection is the earliest transgenic technology that was developed to construct transgenic animals[11], and it has been used widely and successfully in numerous explorations. The general steps include acquisition of exogenous target genes, acquisition of fertilised eggs, prokaryotic microinjection, embryo transfer and genotype identification[16]. Specifically,

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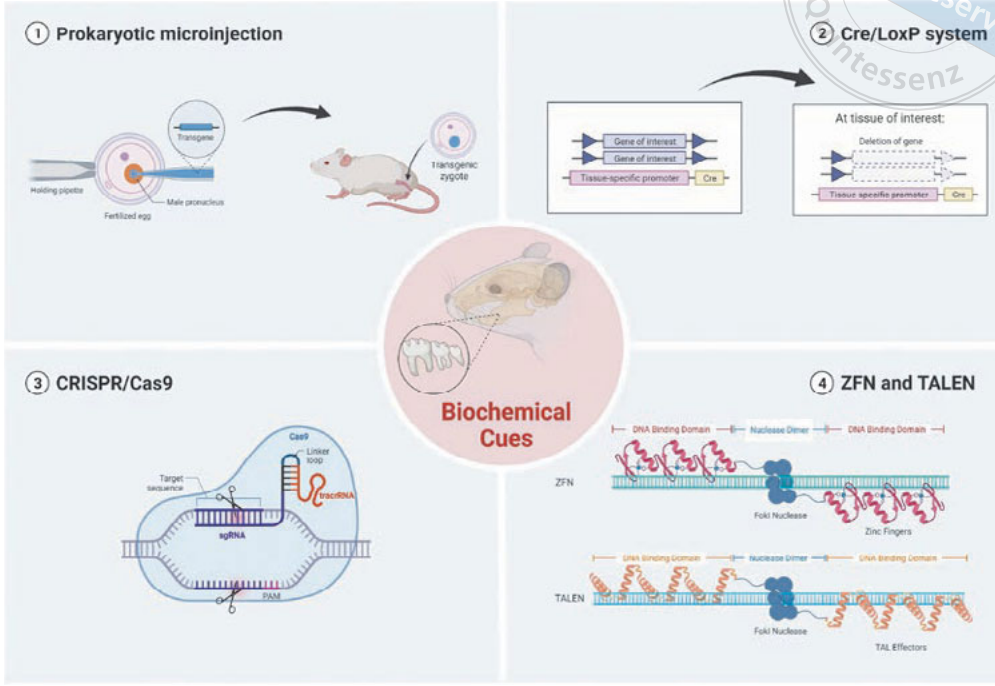

Fig 1 In vivo approaches commonly used in the identification of biochemical cues in dentin development-related and dentin regeneration-related studies: prokaryotic microinjection, Cre/LoxP, CRISPR/Cas and ZFN and TALEN.

gene expression vectors are first constructed using DNA recombination technology to obtain exogenous target genes that exist stably and can be inherited by offspring cells. Then, using prokaryotic microinjection techniques, the plasmid vector containing the target gene fragment is linearised and injected into the male pronucleus of fertilised mouse eggs to randomly integrate them into the host genome. Subsequently, the integrated fertilised eggs are implanted into the oviduct of a pseudopregnant female mouse of the same strain through embryo transfer techniques[17]. Finally, the target transgenic mice are generated by crossbreeding after transgenic founder mice are screened out.

To illustrate the importance and contribution of the prokaryotic microinjection technique in studies related to dentine development, the present authors summarised its applications in identifying the most critical components of dentine, the DSPP/ dentine sialoprotein (DSP)/dentine phosphophoryn (DPP) system.

DSPP is a member of the non-collagenous small integrin-binding ligand N-linked glycoprotein family (SIBLING), which play critical roles in bone and dentine mineralisation[18]. Veis and Perry[19] discovered DPP protein in 1967, and Butler et al[20] discovered DSP protein in 1981. The two proteins were studied irrelevantly until 1997 when MacDougall et al[21] found the DNA sequence encoding DPP was right downstream of that encoding DSP via cDNA cloning and mRNA sequencing, thus illustrating that DSP and DPP originated from a mutual gene *Dspp*. Subsequently, Steiglitz et al[22], Sun et al[23] and von Marschal et al[24] found that DSPP was a non-functional precursor protein that was cleaved into DSP and DPP at the G447 | D448 site in odontoblasts, which were then released into the extracellular matrix; however, the respective functions of DSP and DPP remained ambiguous. With the help of prokaryotic microinjection techniques, researchers used a stepwise approach to explore the function of DSP and DPP in dentine development.

Zhang et al[25] constructed a cDNA encoding DPP and haemagglutinin (HA-DPP cDNA) as the target gene, in which HA was present as a tag to detect DPP expression. The pBC-KS vector containing the HA-DPP with a 3.6 kb Col1a1 promoter was constructed, and the linearised plasmid vector was injected into the pronucleus of fertilised C57BL/6J mouse eggs, and *Col1a1-HA-Dpp* transgenic mice were generated after embryo transfer. Subsequently, this strain of mice was crossed with *Dspp*$^{-/-}$ mice (*Dsp-;Dpp-* mice) to obtain *Dspp*$^{+/-}$*; Dpp*^{Tg} mice, which were then crossed with *Dspp*$^{-/-}$ mice (*Dsp-;Dpp-* mice) to obtain *Dspp*$^{-/-}$*; Dpp*^{Tg} mice (*Dsp-;Dpp+* mice) that expressed only the *Col1a1-HA-Dpp* gene but not endogenous *Dspp*. Compared to *Dsp-;Dpp-* mice that manifested abnormal widening of predentine, irregular dentinal tubules and decreased mineralisation, the *Dsp-;Dpp+* mice possessed dentine with relatively

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increased thickness, volume and mineralised density. Moreover, scanning electron microscopy (SEM) examination and calcein-alizarin red double labelling assays in the *Dsp-;Dpp+* mice showed that the unmineralised and hypo-mineralised areas of dentine decreased, and the dentinal tubules were more regularly aligned and dispersed. Thus, this all implied that the expression of DPP exerted reparative effects on dentine dysplasia caused by *Dspp* deletion.

Based on the aforementioned and relevant studies, researchers inferred that after DPP was synthesised in mature odontoblasts and secreted towards the mineralisation front, it bound not only to collagen fibres but also to large amounts of calcium ions owing to their high content of aspartic acid and serine[21], and as a result, the crystal nucleus of mineralising hydroxyapatite was formed[26]. Thus, DPP played a role in the initial mineralisation of the dentine matrix and the maturation of mineralised dentine by adjusting the size and morphology of the crystal.

In the meantime, the functions of DSP remain relatively ambiguous. Gibson et al[27] injected the pBC KS+ plasmid vector containing DSP cDNA into the pronucleus of fertilized C57BL/6J mouse eggs and obtained *Dspp*$^{-/-}$*;Dsp*^{Tg} mice (*Dsp+;Dpp-* mice) expressing only the *Dsp* gene but not endogenous *Dspp*. Radiographic, microcomputed tomography (microCT), SEM and histological analysis showed that compared to *Dsp-;Dpp-* mice, the *Dsp+;Dpp-* mice possessed larger pulp chambers, thinner dentine and thicker predentine; meanwhile, the dentinal tubules were more disorganised, irregularly dispersed and in a significantly smaller amount. Besides, biglycan, which was supposed to be found solely in predentine in normal mice, was shown to be expressed in the whole predentine-dentine layer[28], implying that dentine mineralisation barely occurred in *Dsp+;Dpp-* mice. Thus, this work indicated that DSP functioned as a suppressor of the early mineralisation of predentine.

However, in another study that also used prokaryotic microinjection to study the role of DSP, Suzuki et al[29] drew an opposite conclusion. They enzymatically linearised the pIRES-DsRed vector containing the DSP expression fragment to obtain the corresponding target DNA solution and injected it into the fertilized eggs of FVB/NJ mice to obtain *Dsp*^{Tg} mice, and then crossed them as described above to obtain *Dspp*$^{-/-}$*;Dsp*^{Tg} mice (*Dsp+; Dpp-* mice). SEM and microCT showed that compared to *Dsp-;Dpp-* mice, the *Dsp+;Dpp-* mice displayed thinner predentine and thicker dentine and were thus more resistant to abrasion and unlikely to have pulp exposure. Therefore, the detailed roles of DSP need to be explored further.

Although prokaryotic microinjection techniques can be used to generate transgenic mice effectively, they also possess disadvantages including the need for expensive and sophisticated equipment, high sensitivity to personnel and technology, and uncontrollable integration sites or copy numbers of exogenous genes, which often result in mutations such as the deletion or misrecombination of DNA fragments and cause serious pathological defects[30]. In recent years, precise gene editing technologies such as Cre/LoxP, CRISPR/Cas9, ZFN and TALEN have flourished and have addressed these challenges to some extent.

Cre/LoxP

The Cre/LoxP system is a powerful method for manipulating biological genetic information, and transgenic animal models based on this technique have become one of the most commonly used tools for tooth development studies[31,32]. The Cre/LoxP system is composed of two LoxP sites, which are 34bp DNA segments comprising two 13bp palindromic sequences and an 8bp core sequence, and a Cre-recombinase that identifies loxP sites and mediates the deletion of DNA segments between the adjacent homotropic LoxP sites[33]. Two mice are required to breed a Cre/LoxP mouse, one with the expression of Cre-recombinase and one with two LoxP sites inserted upstream and downstream of the target gene. When the two types of mice mate, the DNA segments between the LoxP sites are sheared and deleted in cells or tissues expressing Cre-recombinase. Since Cre-recombinase is expressed under the control of promoters, by selecting promoters that are uniquely expressed in specific cells or at specific developmental stages, the Cre-recombinase is intentionally activated and deletion of target genes is achieved[31].

Growth factors play critical roles in the development of individuals, and the TGF-β superfamily[34], which includes TGF-β, activin, BMPs and growth differentiation factors (GDFs), has received increasing attention in recent years. Among these, BMPs are reported to be most closely related to dentine development[35,36]. The present authors described the application of the Cre-LoxP system in studies on dentine development by introducing the BMP-Smad signalling pathway.

When odontoblasts start to differentiate, BMP2 is expressed in the dental mesenchyme. Malik et al[37] conditionally knocked out *Bmp2* in neural crest cells based on the Cre-LoxP system using Wnt1 as the promoter to investigate the role of BMP2 in early odontogenesis. In Wnt1-cre;Bmp2$^{fl/fl}$ mice, thinner dentine, disorganised dentinal tubules and ectopic mineralisation in the pulp

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cavity were observed via microCT and SEM. Meanwhile, DSPP expression was significantly downregulated as shown by immunofluorescence staining and polymerase chain reaction (PCR), suggesting that a BMP2 deficiency caused abnormalities during early tooth development and resulted in dentine dysplasia.

BMP receptors are classified as type I and type II according to their cytoplasmic regions. Type I receptors include BMPR1A, ACVR1 and BMPR1B, and type II receptors include BMPR-II, ACVR-II and ACVR-IIB[38]. Among them, the roles of BMPR1A and ACVR1 in tooth development have been investigated to the best of the present authors' knowledge, whereas the effects of other receptors such as BMPR1B and BMPR-II have only been explored in osteogenesis. For example, Zhang et al[39] used the Cre-LoxP system to conditionally knock out *Acvr1* in mesenchymal cells using *Osterix* as the promoter, and generated Osterix-Cre;Acvr1$^{fl/-}$ mice. Haematoxylin and eosin (HE) staining and microCT displayed thinner crown dentine and thicker predentine in the molars and osteodentine in the incisors, in which a transition from *Dsp* to *Bsp* expression was detected. Moreover, Wnt antagonists Dkk1 and Sost were downregulated, and β-catenin was upregulated in the cKO incisors but remained unchanged in molars. Therefore, it was suggested that ACVR1 played different roles, promoting the differentiation of dental mesenchyme to odontoblasts in molars but inducing a cell fate shift of odontoblasts to osteoblasts in incisors. Feng et al[40] and Liu et al[41] used Gli1, Nestin and OC as promoters to knock out Bmpr1a, and found that all mice lines exhibited dentine defects including thinner dentine and changed morphologies of odontoblasts, although the degree of defect varied.

Smad4 is the only Co-Smad protein that has been discovered to date, which is one of the downstream signalling factors of the TGF-*β*/BMP signalling pathway[42]. Since previous studies had identified *Col1* and *Osteocalcin (OC)* as early marker genes and *Dspp* as a late marker gene in odontogenesis[43,44], Kim et al[45] used *Col1*, *OC* and *Dspp* as promoters and knocked out *Smad4* at different stages to obtain *Smad4*-cKO mouse lines. In all three strains, the mice were found to exhibit dwarf odontoblasts, thinning coronal dentine and decreased expression of *Col1a1*, *OC* and *Dsp* by HE, immunohistochemistry (IHC) and microCT. Among them, the phenotypes were more significant in the Smad4^{OC} and Smad4^{Col} mice and relatively mild in Smad4^{Dspp} mice. These results suggest that Smad4 is required during dentine development in a stage- and site-dependent manner.

Using the same techniques, researchers also explored the roles of numerous other factors including DMP1[46], DLX3[47] and TGF-β[48], and found that knockout or knockdown of these factors usually led to similar phenotypes, such as disrupted odontoblast polarity and thinner dentine, implying the participation of various signalling pathways in dentine development.

Compared with the knockout mice constructed via traditional prokaryotic microinjection, the conditional knockout mice constructed via the Cre-loxP system can largely avoid embryonic or neonatal death of mice and effectively decrease the chances of blind or random integration of gene fragments. Moreover, genes can be removed in specific tissues or at particular stages, which is crucial for studying the spatial and temporal expression pattern of target genes.

CRISPR/Cas9

In 2012, Jinek et al[49] integrated the crRNA (CRISPR RNA) and tra-crRNA (trans-activating crRNA) from the natural CRISPR/Cas system into a single strand (single guide RNA, sgRNA) and developed the CRISPR/Cas9 technique. With this advanced approach, accurate DNA editing was achieved successively in both animals and cells[50]. From then on, the CRISPR/Cas9 technique received a blowout spurt and has become one of the most popular tools in gene editing.

The CRISPR/Cas9 system is composed of two components: the gRNA that recognises a specific DNA sequence and the Cas9 protein that cuts the DNA sequence[51]. The gRNA and Cas9 bind and form a ribonucleoprotein (RNP) complex. The customised gRNA recognises the protospacer adjacent motif (PAM) and binds to the target DNA sequence, which enables Cas9 to cut the double-stranded DNA efficiently. Afterwards, DNA recombination via non-homologous end joining (NHEJ) or homology-dependent repair (HDR) is achieved, leading to targeted gene editing. Generally, cells will repair the broken DNA via NHEJ; however, mismatches such as base insertions or losses occur frequently. As a result, the downstream sequence of the cut site loses its function and gene deletion is obtained. Moreover, by introducing an extra gene repair template that contains the target gene sequence to be inserted and corresponding homologous arms at either side, the DNA double-strand can also be repaired via HDR and the target sequence can be inserted.

Because of its non-absolute specificity, gRNA may recognise other similar sequences in the genome, leading to off-target editing. Still, compared to the traditional Cre/LoxP system that requires gene targeting and is full of complexities and uncertainties, CRISPR/Cas9 possesses higher targeting accuracy and other

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advantages including simple construction, easy operation, short trial cycle, low expense, high adaptability, efficient capability and simultaneous targeting of multiple genes. It is a revolutionary technique that has been employed successfully in various biomedical studies[52]. For example, this novel technique has made it possible to explore the role of *Dspp* in dentine development further in a more precise and competent way.

Based on clinical and radiographic appearance, hereditary dentine disorders can be divided into two types: dentinogenesis imperfect (DGI), which is further divided into DGI-I, DGI-II and DGI-III, and dentine dysplasia (DD), which is further divided into DD-I and DD-II. Among them, DGI-II is the most common. It is known that *Dspp* is the gene responsible for these diseases except for DGI-I. Further analysis of the mutations in *Dspp* that lead to hereditary dentine disorders suggests that they can be generally divided into two types: missense or nonsense mutations at the 5' end that encodes DSP segment, and frameshift mutations at the 3' end that encodes the DPP segment[53].

The missense mutation of *Dspp* at site 17 where leucine replaces proline (c.50C>T,p.P17L, g.50C>T) was found to lead to both DGI-II[54] and DGI-III[55] phenotypes. To identify the underlying mechanism, Liang et al[56] designed an sgRNA that was located on the antisense strand directly opposite to codon 19 of the mouse *Dspp* gene, which was equivalent to codon 17 of the human *Dspp* gene, and a typical PAM sequence (AGG) was immediately adjacent to its 3' end, as well as a single-stranded oligodeoxynucleotide as a repair template that contained the centrally located desired pathogenic mutation (CCG>CTG) as well as a silent mutation (GCC>GCG) flanked by two arms of homologous genomic sequence, respectively. Radiographic, microCT and SEM examinations showed that young Dspp^{P19L} mice displayed phenotypes similar to those of DGI-III, including larger pulp chambers, thinner dentine at the chamber roof and thicker dentine at the chamber floor. In contrast, aged Dspp^{P19L} mice exhibited phenotypes similar to those of DGI-II, including smaller pulp chambers and heavier abrasion. Moreover, the odontoblast processes in Dspp^{P19L} mice retracted and peritubular dentine was deposited continuously with aging. Thus, mutations of the *Dspp* gene at different stages present with different phenotypes.

In addition, it was found that compared to normal mice, DSP/DSPP signals increased significantly in odontoblasts but were weakly expressed in dentine extracellular matrix in Dspp^{P19L} mice. Besides, DSPP normally colocalises with GM130, a Golgi apparatus matrix protein, whereas the P19L-DSPP colocalised mainly with calreticulin, an ER matrix protein, indicating that P19L-DSPP failed to be efficiently transported from ER to Golgi, leading to its accumulation in ER and damage in DSPP secretion. Moreover, considering that DSPP contains large amounts of aspartic acid and glutamate residues, its accumulation may result in ER stress and pathological unfolded protein responses[57].

Two years later, Liang et al[53] again employed the CRISPR/Cas9 technique to knock out the guanine right after the fourth amino acid in the DPP coding sequence and generated DSPP^{-1fs} mice with a -1 frameshift. Compared to Dspp^{P19L} mice in which the morphology of odontoblasts remained normal while dentine became thinner with a rough inner surface, SEM, nanohardness testing and histological staining revealed that the DSPP^{-1fs} mice displayed more severe dentine abnormalities. Phenotypes observed in the DSPP-1fs mice included the loss of the columnar morphology of odontoblasts and the dentinal tubules, which were similar to the appearance of reparative dentine formed after odontoblast death.

The development of CRISPR/Cas9 enables precise editing of *Dspp*. By illustrating the changes resulting from mutations of single base pairs, researchers are gaining a much deeper understanding of dentine dysplasia caused by *Dspp* mutations, which offers enormous potential and hope of clarifying the underlying mechanism and preventing the occurrence of these diseases.

Zinc finger nucleases (ZFN) and transcription activator-like effector nucleases (TALEN)

Unlike the CRISPR/Cas9 system that relies on sgRNA to recognise target sequences, the zinc finger nucleases (ZFN) technique and transcription activator-like effector nucleases (TALEN) technique recognise proteins.

ZFN is composed of two parts: the zinc finger proteins (ZFs) that recognise and bind to specific gene sequences, and the Fok I endonucleases that cut the genes. ZFs have three independent repeats that each recognise three bases; thus, a ZF DNA binding motif can recognise a 9bp gene sequence, and two ZFs recognise an 18bp gene sequence[58]. After either ZF recognises the specific sequence on one of the two DNA strands, Fok I endonucleases cut the target gene by forming a dimer[59], leading to the breakage of DNA strands at particular sites, followed by DNA reparation via NHEJ or HDR[60]. In this way, the genome is manipulated.

Chiba et al[61] cut a base pair at specific sites via the ZFN technique, leading to a frameshift mutation of AmeloD, a transcription factor of the bHLH family,

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and bringing the stop codon forward, which generated conditional *AmeloD* knockout mice. MicroCT examination in combination with HE staining showed that its deletion caused decreases in dentine volume and thickness, and dysplasia in the development of both the crown and root[61].

The merits of ZFN include the lack of exogenous DNA introduction and wide application in various models, as NHEJ and HDR exist in basically all species[60]; however, since the assembly of the ZFN sequence relies on current databases, its application is limited. Furthermore, the design of ZFN is intricate. Off-target effects also occur because of the low specificity and affinity of ZF motif when binding with DNA. When there are extensive off-target occurrences that DNA breakage overweighs repair, cells or animals may die, and these problems need to be solved in further studies[62].

Similar to ZFN, TALEN is formed by Fok I and a protein that recognises and binds to specific DNA sequences, the transcription activator-like (TAL) effector. Similarly, Fok I cuts the DNA strands, which get repaired by NHEJ and HDR[63]. The difference lies in the DNA-binding motifs. The recognition motif of TAL contains 34 amino acids with conservative sequences except for the twelfth and thirteenth ones, which are called repeat variable diresidue (RVD)[64,65]. Each RVD corresponds to four different types of base pairs[66,67], as a result, the specific TAL recognises and binds to target DNA bases. Thus, by connecting different TAL motifs based on target sequences, and the sequence that encodes FokIoendonuclease, a customised TALEN is constructed.

SIX1 (Sine Oculis Homeobox 1) is a transcription factor and belongs to the homologous box gene family. It plays a critical role in the development of sensory organs and craniofacial tissue. Takahashi et al[68] employed the TALEN technique and generated *Six1*-cKO mice. It was observed that odontoblast differentiation in mandibular incisors was damaged and DSPP expression in dentine matrix was decreased. Besides, the pre-odontoblast marker DKK1 which is normally expressed only in the labial side was observed at both labial and lingual sides in *Six1*-cKO mice. These phenotypes indicated that Six1/DKK1 participated in odontogenesis and dentine formation.

Although the TALEN technique also has off-target effects, it is easier to design compared to ZFN. Theoretically, it is possible to design and construct a specific TALEN nuclease for a random DNA sequence. Besides, the cytotoxicity and expense are lower. However, since a TALEN recognition motif is required for each base pair on the target sequence, significant work needs to be performed for TALEN construction, and the delivery into cells is also challenging[63,69,70].

From the abovementioned examples, it can be seen that animal models play fundamental roles in studies relating to dentine development; however, dentine development is such an intricate process that it is regulated by thousands of genes and proteins among which extremely complicated interactions exist within the niche. This intricacy has made it somewhat challenging to identify and explore the role of one single component using only animal models. Thus, ex vivo or in vitro examinations are generally required following in vivo studies to confirm the conclusions. Compared to animal models, ex vivo or in vitro approaches have outstanding merits like relative simplicity, convenience of use, quick response to stimuli and the capacity to explore specific genes or regulatory molecules. Since such work has been thoroughly summarised previously, an in-depth discussion was not included in the present study.

Biophysical cues: ex vivo/in vitro approaches

It is not only various biochemical factors participate in dentine development and regeneration; so too do biophysical factors. In particular, the importance of mechanical stimuli from inherent or exogenous microenvironments has been increasingly proven in recent decades. Due to the limitations of current in vivo techniques, using them to explore the effects of biophysical factors remains an overwhelming challenge. Thus, researchers have turned to ex vivo and in vitro approaches. Traditionally, scaffolds or substrates of specific features are designed to explore the role of a concerned biophysical factor, and numerous scaffolding systems have been developed in recent decades. Excellent reviews summarising these systems based on scaffold types or features have been published previously, to which the present group also contributed[71,72]. Therefore, in the present study, ex vivo and in vitro approaches will not be redundantly reviewed according to scaffold types.

In recent years, researchers have increasingly come to the agreement that biophysical factors indeed function by exerting mechanical forces on cells or tissues cultured within the niches. Cells respond to the forces via transmembrane mechanosensation receptors and intracellular mechanotransduction signalling pathways. Thus, in this section, approaches commonly used to identify biophysical factors in studies related to dentine development or regeneration were systematically interpreted according to the interactive forces between the scaffolds and cells (Fig 2).

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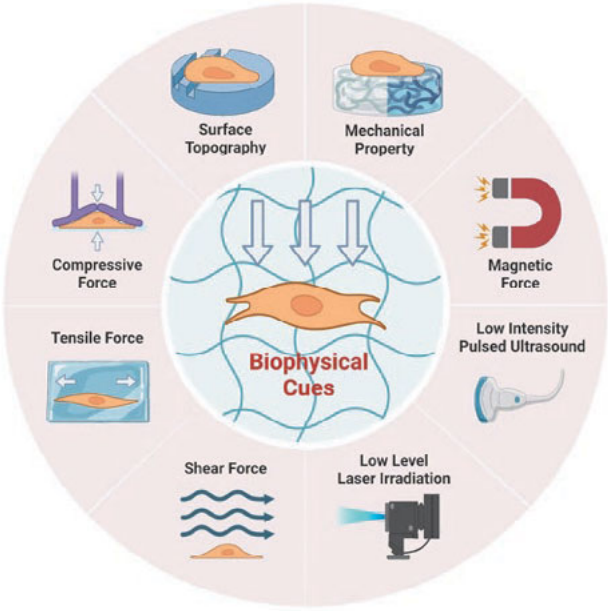

Fig 2 Ex vivo or in vitro approaches commonly used in the identification of biophysical cues in studies relating to dentine development and regeneration. Approaches are illustrated according to the interactive forces.

Stationary force

Surface topography

The surface topography of a scaffold indicates the features it presents to the accommodating cells, and it has been proven to play critical roles in cell fate determination. Of these, two inseparable features, the superficial morphology and the residing area/volume of a scaffold, have mainly been explored with regard to the regulation of dental stem cell behaviours.

Concerning the residing area/volume, Bachhuka et al[73] constructed gradients of different-sized gold nanoparticles to simultaneously examine the influence of nanoparticle density and size on dental pulp stem cells (DPSCs) on a single substrate and found the density of nanotopography features was positively correlated to DPSC adherence and proliferation. In addition, Du et al[74] constructed a bilayer poly(lactide-co-glycolide) (PLGA) scaffold with small pores on the closed side (1 to 5 μm) and large pores on the open side (45 μm) via the diffusion-induced phase separation technique and found that DPSCs showed improved F-actin stress fibre alignment, increased spreading area, elongated appearance, predominant nuclear YAP localisation and spontaneous osteogenic differentiation on the closed side. Graziano et al[75] generated PLGA membranes and treated them with N-metil-pyrrolidone to create hierarchic concave pits (primary pits: 80 to 120 μm in diameter and 40 to 100 μm in depth, and secondary pits: 10 to 20 μm in diameter and 3 to 10 μm in depth) where cell-matrix interactions increased. They found that DPSCs seeded on the concave-rich PLGA substrates exhibited nuclear polarity, released greater amounts of BMP-2 and vascular endothelial growth factor (VEGF), expressed higher alkaline phosphatase (ALP) activity, and resulted in better osteogenesis compared to smooth substrates.

As to superficial morphology, DPSCs are generally believed to exert better differentiation potential on rough or extrusive surfaces. For example, *Dspp* was upregulated on the printed (rough) polylactic acid (PLA) scaffold compared to moulded (smooth) substrates[76]. Portone et al[77] generated graphene oxide-coated polycaprolactone (PCL) nanofibres (mean diameter 500 nm) with different orientations, and found that randomly orientated nanofibres were able to revert neuronal precommitment and trigger osteoblastic differentiation, whereas uniaxially aligned fibres were fibroblast-directed. Similarly, Ha et al[78] generated gelatine methacryloyl hydrogels with microgrooves and ridges of 60 μm and 120 μm via photolithography. They found that the micropatterned hydrogels guided the self-alignment of SCAPs and promoted a higher degree of odontogenic differentiation than non-patterned hydrogels[78]. In addition, Alksne et al[79] fabricated 3D printed scaffolds with wavy and porous topographies. The wavy ones were formed by closely assembled PLA threads (188 μm in diameter) with grooves, whereas the porous ones were formed by PLA threads (500 μm in diameter) with 300-μm-wide pores[79]. DPSC morphology was modulated and spontaneous osteogenic differentiation was observed on both surfaces without biochemical induction[79].

In tooth development, odontogenesis is comprised of an odontoblast polarisation (morphology transformation) process and an odontoblast differentiation (function maturation) process. Nowadays, most work is focused on the differentiation process, whereas the polarisation process, which determines the formation of the tubular architecture of dentine, remains largely unclear due to the lack of appropriate research models.

To address this challenge, a series of studies were conducted recently in an attempt to solve this mystery[80-82]. First, Ma et al[80] created bioinspired 3D nanofibrous matrices with highly organised tubular architecture via a laser-drilling technique. With the maskless micropatterning technology, this 3D tubular hierarchical scaffold successfully induced the regeneration of tubular dentine that possessed a well-organised microstructure as its natural counterpart[80]. In contrast,

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the non-tubular control group only induced osteodentine formation, indicating the decisive role of the 3D tubular structure[80]. Chang et al[81] further improved this system by establishing a nanofibrous tubular 3D micropatterning system that confined one single cell on each microisland for single DPSC polarisation. They found that DPSC polarisation was a dynamic and sequential process and that the nanofibrous tubular architecture was another critical factor in initiating DPSC polarisation[81]. Furthermore, cytoskeletal rearrangements were found to modulate DPSC polarisation and cell tension was involved. Chang et al[82] continued to employ this scaffold as a platform and proved that nanofibrous architecture, tubule size and microisland area all contributed to the initiation of DPSC polarisation by regulating the formation of DPSC processes and the translocation of Golgi apparatus, while microisland geometry exerted negligible effects and gravity accelerated the polarisation process.

Niu et al[83] obtained similar conclusions with a distinct approach. They developed a microfluidic chip with microchannels that mimicked dentinal tubules and interconnected sites that were used to culture odontoblasts. The 2-μm channels were found to successfully induce the growth of odontoblast processes, while the 4-μm or larger channels only promoted the migration of odontoblasts into the microchannels, confirming the role of tubule size in odontoblast polarisation.

Mechanical properties

The stiffness of the substrate also plays an important role in regulating the behaviour of DPSCs. Qu et al[84] showed that DPSCs on nanofibrous gelatine scaffold of high stiffness had a more organised cytoskeleton, larger spreading area, higher expression of odontogenic differentiation marker genes and more mineral deposition than those of low stiffness. By further integrating matrices of the two stiffness into a single scaffold for pulp-dentine regeneration, they observed that biomineralisation only occurred in the high-stiffness peripheral area and formed a ring-like structure that surrounded the non-mineralised central area; thus, a complete construct similar to natural pulp-dentine complex was regenerated successfully[84].

Chuang et al[85] found that adjustment in the modulus of polyisoprene substrates by changing coating thickness or incorporating inorganic particles influenced DPSC differentiation. Upregulation of osteocalcin (OCN) and COL1a1 and increased biomineralisation without the induction of dexamethasone were observed on hard substrates, including both spun-cast thin polyisoprene substrates and filled thick polyisoprene substrates[85]. Minerals were aggregated into large clusters on thin substrates and uniformly distributed on filled thick substrates, and intracellular modulus of DPSCs also changed to accommodate to changes in the modulus of interactive substrates[85]. Similarly, a study showed that DPSCs were more likely to undergo osteoblastic/odontogenic differentiation and proliferation when seeded on stiff substrates, which involved the Wnt signalling pathway[86].

Compressive force

During embryo development, mesenchyme condensation triggers the organogenesis of many tissues including cartilage, bone, muscles, kidneys and lungs. It was believed that mesenchyme condensation also initiated odontogenesis. Researchers proposed that odontoblast polarisation and differentiation were the result of rapid proliferation and condensation of dental papilla mesenchymal cells, which generated mechanical compaction among the cells; however, this hypothesis could hardly be verified via in vivo approaches. The rapid development of ex vivo tissue culture methods, bioengineering techniques and mathematic models have recently made it possible to explore the role of compression on dentine development.

Mammoto et al[5] created a mechanical compression device to explore the effect of compaction on the mesenchyme. The polydimethylsiloxane (PDMS) device had a soft base and piston and was permeable to air and water, and thus created a suitable microenvironment for ex vivo culture. Mesenchymal tissues from the first pharyngeal arch freshly isolated from embryonic day 10 were placed within the chamber of the device and compacted with a force of 1 kPa by a 30-g metal cube on the PDMS piston[5]. Compared to the uncompacted group, the mRNA expression of *Pax9*, *Msx1* and *BMP4* of the compacted tissues was significantly increased, indicating that mechanical compression enhanced mesenchyme differentiation[5].

Miyashita et al[87] cultured hDPSCs on a customised silicone membrane with microchannels that were 10 μm in depth, 5 μm in diameter and 10 μm in pitch, resembling the morphology of dentinal tubules, and employed a compression bioreactor to exert cyclic loading force on the membrane. They found that the odontogenic markers *Bmp7* and *Wnt10* were upregulated and the phosphorylation levels of ERK1/2 and p38 were increased, implying that the MAPK signalling pathway was involved in promoting odontogenesis by mechanical compaction[87]. They also found that DPSCs formed

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odontoblast-like cellular processes within the microchannels, displaying a polarised morphology similar to in vivo conditions[87]. Together, these results proved that mechanical compaction was capable of directing the odontogenic differentiation and polarisation of DPSCs.

Mammoto et al[88] confirmed the effect of mechanical compaction on odontogenesis based on micropatterning method. To recapitulate the mesenchyme condensation process of the dental papilla in the initial stage of dentine development, they generated micropatterns via a photolithography technique and cultured undifferentiated dental mesenchymal cells (E10) within the fibronectin-coated micropatterns in different densities[88]. They found that *Pax9* was highly expressed on the high-density micropatterns compared to the low-density group, and that when the cells originally cultured with a high density were replated in a low density, *Pax9* was downregulated, indicating that the maintenance of mesenchyme condensation was a prerequisite for odontoblast differentiation[88]. In addition, they proved that the microfilament cytoskeleton played a critical role in regulating dental mesenchyme condensation and noted that cell condensation led to changes in intracellular microfilament arrangement and activation of p38/MAPK pathway, which subsequently activated the SP1 transcriptional factor and promoted the synthesis of type VI collagen[88]. As a result, the condensed status of cells was maintained and odontogenesis was stabilised[88].

Hashmi et al[89] reinforced this conclusion by employing a bioengineering scaffold. They generated a 3D thermosensitive GRGDS-modified PNIPAAm hydrogel that shrank in volume and displayed smaller interconnected micropores when heated from 34°C to 37°C, and cultured BMSCs within the hydrogel[89]. Afterwards, the cell-scaffold complex was implanted under the mice subrenal capsules[89]. ALP activity, calcium nodule formation and DSP expression were improved in the experimental group, and the mineralisation level of the newly formed tissues was significantly higher as examined by elemental map and SEM scanning[89]. This indicated that compressive force generated from the scaffold transition induced odontogenic differentiation and mineralisation of BMSCs[89].

Tensile force

Clinical studies have found that tensile forces generated by orthodontic treatment induce ectopic dentine mineralisation in the pulp chamber[90]. To understand the underlying mechanisms, researchers have adopted devices like Flexcell to explore the relationship between odontogenesis and tensile stress.

Liao et al[91] employed the Flexcell system to exert tensile force on human pulp-derived odontoblast-like cells (hOBs) and found that while the odontogenic markers including *Dspp*, *Opn*, *Ocn* and *Runx2* were upregulated, the expression level of intracellular sclerostin was decreased. To further identify the influence of tension-relevant sclerostin on odontogenesis, they overexpressed sclerostin in hOB cells via plasmid transfection and found the odontogenic markers were downregulated with the STAT3 pathway involved, indicating that sclerostin impacted odontogenic differentiation[91]. When inhibitors that specifically target the ERK1/2 and proteasome pathways were utilised, tensile stress downregulated sclerostin via ERK1/2 and proteasome pathways, respectively[91]. Other researchers also generated *Sost*$^{-/-}$ mice using the Cre/LoxP technique[92], which displayed lower pulp volume and accelerated reparative dentine formation; meanwhile, the DPCs derived from *Sost*$^{-/-}$ mice had increased mineralisation. These results further confirmed the effect of tensile force and sclerostin on odontogenic differentiation.

With a similar Flexcell approach, Lee et al[93] concluded that tension promoted the odontogenic differentiation of hDPCs via the HO-1 pathway. Compared to the control group, hDPCs that underwent tensile stress within the Flexcell system exhibited increased expression of *Ho-1*, *Dspp*, *Dmp-1* and *Opn*, and this increase disappeared when a HO-1 siRNA was used[93]. Moreover, tensile force induced the nuclear translocation of Nrf2, which was hindered when hDPCs were processed with inhibitors targeting PI3K and NF-κB, implying that mechanical tension induced odontogenic differentiation via Nrf2 and HO-1[93].

Cai et al[94] drew a contrasting conclusion regarding the role of tension on odontogenesis. Applying a uniaxial circulating tensile force of 2000 με and 1 Hz on isolated DPSCs, they detected decreased expression of DSPP and BSP via q-PCR and immunocytochemistry staining, suggesting tension played an inhibitory role on odontogenesis[94]. The reason for such discrepancy remains unclear, and further in-depth studies are required.

Apart from Flexcell, other appliances were also used. For example, Chen et al[95] generated aligned polyglycolic acid (PGA) fibrous scaffolds that were applied with static tension via a dental arch expansion appliance and observed that when DPSCs were seeded into the scaffolds, tendon-related markers were significantly upregulated and tendon-like constructs were formed in a mouse model, implying that tensile force also encouraged DPSCs to differentiate towards tenocytes.

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Shear force

Within dentinal tubules, odontoblast processes are constantly immersed in dentinal fluids. When exogenous stimuli like sudden temperature changes or mechanical operations are exerted on dentine, dentinal fluid flow occurs instantaneously[96]. Thus, odontoblasts undergo shear stress frequently.

Sun et al[97] constructed a model that stimulated odontoblasts with fluid flow shear stress within a customized parallel plate flow chamber. With the shear force, the intracellular calcium content increased rapidly within odontoblasts, whereas this phenomenon disappeared when GsMTx4, a specific blocker of the mechanosensitive Piezo1 ion channel, was added to the culture system[97]. When cells were cultured in an extracellular microenvironment where calcium ions were deprived, the shear force failed to trigger increase in calcium content; this indicated that shear stress promoted calcium influx via Piezo1 and confirmed the mechanosensation function of odontoblasts[97]. Moreover, it was shown that odontoblasts released adenosine triphosphate (ATP) under fluid flow, which activated not only trigeminal neurons but also adjacent odontoblasts via P2Y and phospholipases C-coupled ATP/ADP receptors, modulating both mechanotransduction and odontogenesis of odontoblasts[98]. Thus, a signalling network that enabled rapid communication was generated at the dentine-pulp complex.

Honda et al[99] further confirmed the effect of shear stress in tooth development with heterogenous cell clusters. They isolated, minced and enzymatically dissociated developing tooth tissues including dental epithelium, dental papilla and dental follicle into pieces, then cultured the heterogenous cells on PGA fibrous mesh pre-coated with type I collagen[99]. The cell-PGA complex was placed on a Bio-shaker where the two-way flow of the culture medium produced shear stress, then the complex was implanted under the submentum of immune-deficient rats[99]. In the experimental group, the protein levels of BSP and Vimentin and the mRNA expression of *Runx2*, *Col1a*, *Opn*, *Ocn* and *Dspp* were enhanced significantly[99]. Organised columnar cells and mineralised structures resembling dentinal tubules, enamel and bone were detected, and tooth development was much faster than in the control group[99]. All these results indicated that shear stress promoted tooth development.

Other forces

With advancements in physics, recent years have witnessed the creation and development of many novel machines and technologies. Some of these technologies, for example ultrasound and lasers, have been adopted in the dental field and shown great potential in the treatment and therapy of dental diseases. They have also influenced dental tissues or dental stem cells via forces, but since these forces cannot be simply described as compression, tension or shear forces, they are only summarised briefly in the present study.

Low-intensity pulsed ultrasound (LIPUS)

As a mild physiotherapy, low-intensity pulsed ultrasound (LIPUS) has been reported to accelerate wound healing and tissue regeneration by generating continuous and superposed shockwaves. In the dental field, LIPUS has been widely explored in dentine reparation. Zuo et al[100] treated mice molar defects with LIPUS and found that the disrupted odontoblast layer was repaired and tertiary dentine with orderly aligned, spindle odontoblast-like cells in the periphery was formed, indicating that LIPUS possessed the capacity to repair early dentine defects.

Low-level laser irradiation (LLLI)

Compared to other types of lasers that generate enormous amounts of heat and traumatise tissues, low-level laser irradiation (LLLI) barely causes any photothermal phenomena owing to its low capacity. Currently, LLLI is widely used in dental clinics to treat wounds, temporomandibular disorder, pain and swelling after tooth extraction, dentine hypersensitivity and mucosal diseases. With regard to odontogenesis, Theocharidou et al[101] found that LLLI promoted the expression of biomarkers including DSP, BMP-2 and Runx2 in DPSCs, and increased cell proliferation on porous Mg^{2+}-based bioceramic scaffolds.

Magnetic forces

The precise assembly of cytoskeletons is a prerequisite for cell polarisation and tissue regeneration. In recent years, researchers have managed to modulate cell polarisation by controlling the organisation of cytoskeletal elements intracellularly. For example, Zhang et al[102] constructed a MS-ABPAda⊂HACD complex that promoted the directional polymerisation of microfilaments. This complex was comprised of β- CD-modified hyaluronic acid polymers and magnetic nanospheres that were modified with actin-binding peptides (ABPs) and adamantane (Ada). The ABPs could efficiently target actin, whereas β- CD and Ada promoted self-assembly

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of the nanofibres via non-covalent forces. It was shown by a confocal microscope and transmission electron microscope that this complex determined the organisation of microfilaments by promoting actin self-assembly in parallel to the magnetic field, and thus induced the directional extension of DPSC processes and cell polarisation.

Conclusion

Commonly used animal models including prokaryotic microinjection, Cre/LoxP, CRISPR/Cas9 and ZFN and TALEN were described in detail in the present study. However, in vivo models are complex systems considering that signalling factors interact with and compensate each other intimately, and it is impossible to isolate one signalling factor and explore its role. Meanwhile, the effects of biophysical factors cannot be studied using these models. Ex vivo or in vitro models present advantages in overcoming limitations of in vivo models; however, ex vivo or in vitro models also have unavoidable deficiencies. A major one is that cells or tissues used in vitro or ex vivo are often artificially modulated or transformed, leading to unpredictable changes in biological functions. To summarise, although each model has its own merits and disadvantages, they all contribute enormously to studies relating to dentine development and regeneration.

Conflicts of interest

The authors declare no conflicts of interest related to this study.

Author contribution

Drs Ya Lu SUN, Xi Heng LI, Shuang Shuang WANG and Hu En LI searched the literature and drafted the manuscript; Dr Bei Chang organised the structure and revised the manuscript; Dr Hong Chen SUN proposed the idea and revised the manuscript.

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