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REVIEW

Advances of Wnt signalling pathway in dental development and potential clinical application

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ABSTRACT. Wnt signalling pathway is widely studied in many processes of biological development, like embryogenesis, tissue homeostasis and wound repair. It is universally known that Wnt signalling pathway plays an important role in tooth development. Here, we summarized the function of Wnt signalling pathway during tooth initiation, crown morphogenesis, root formation, and discussed the therapeutic potential of Wnt modulators.

KEYWORDS. crown morphogenesis, Dentin regeneration, root formation, Wnt signalling

INTRODUCTION

The Wnt signalling pathway is widely studied in many processes of biological development, including embryogenesis, tissue homeostasis and wound repair. It controls several developmental processes via regulating cell proliferation, differentiation, polarization, and apoptosis. The Wnt signalling pathway is a complex system that consist of 19 ligands (Wnts), 10 Frizzled receptors (Frz) and 2 co-receptors (Lrp5/6), as well as antagonists (WISE, Dkks, Notum, sclerostin, WIFs, sFRPs) and co-activators (Norrin, R-Spondins).[1–3] For the canonical Wnt signalling pathway, soluble Wnt protein ligands bind to Frz receptors to mediate their biological effects, leading to the accumulation of β-catenin and its translocation into nuclei to activate downstream transcriptions.[4] The non-canonical Wnt signalling pathway includes Wnt-PCP and Wnt-Ca^{2+} pathway, which extensively participate in biological

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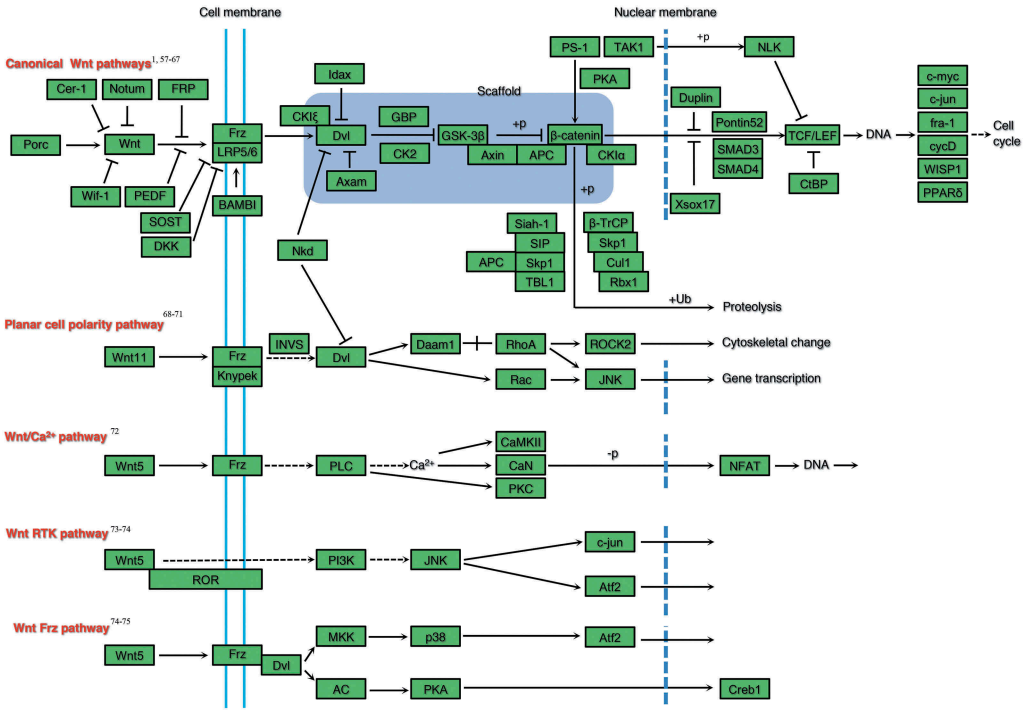
processes; nevertheless, the core mechanisms remain unclear.

It is universally known that Wnt signalling pathway plays an important role in tooth development. Our recent research using RNA sequencing analysis of rat tooth germs demonstrated that the Wnt/β-catenin signalling is upstream to most of other signalling pathways at the initiation stage of tooth development.[5] Researches about Wnt signalling have made new progress in other tissue, which may have suggestive effects on tooth-related development. Therefore, we summarized the function of Wnt signalling pathway during tooth initiation, crown morphogenesis, root formation, and discussed the therapeutic potential of Wnt modulators.

OVERVIEW OF THE WNT SIGNALLING PATHWAY

The Wnt sigalling pathway can be classified into two distinct categories: the canonical or β-catenin dependent pathway and non-canonical pathway (Fig. 1), some of which have been verified in tooth development, while others remain to be verified. As a class of secretory glycoproteins, at least 19 Wnt proteins are identified to activate Wnt signalling pathway in mammals. Wnt1, Wnt3, Wnt3a, Wnt7a, Wnt7b, and Wnt8a activate the canonical pathway, while Wnt4, Wnt5a and Wnt11 activate the non-canonical pathway.[6] The canonical Wnt pathway controls the cell behavior by regulating transcription of the DNA binding proteins of T cell factor/lymphoid enhancer factor (TCF/LEF) family. In the absence of Wnt proteins, the complex consisting of GSK3, APC and Axin2 binds to β-catenin and phosphorylates it for degradation. Binding the appropriate Wnt proteins to Frz-Lrp5/6 co-receptor complex, it recruits and activates the cytoplasmic signalling protein Dvl which can inhibit the β-catenin destruction complex and maintain the expression of β-catenin. Stabilized cytosolic β-catenin translocates to the nucleus

FIGURE 1. Canonical and non-canonical Wnt signalling pathways known or needed to be proven to participate in rat tooth germ development.[1,56,57,58,59,60,61,62,63,64,65,66,67,68,69,70,71,72,73,74,75]



and interacts with TCF/LEF transcription factors to activate the downstream target genes involved in organ development.[7,8]

Recently, advances in the understanding of the Wnt signalling pathway have been made. For example, our previous study suggested a novel inhibitory mechanism of non-canonical Wnt signalling pathway by the direct interaction between Lrp5/6 and Frz, through which the LRP5/6–Frz complex come into being with the function of maintains both canonical and non-canonical pathways in an inactive status at the basal level.[9] Twa1 was identified as a new member of canonical Wnt signalling pathway, enhancing the Wnt pathway by accumulating nuclear β-catenin.[10] The tumor specific protein C9orf140 compromised Wnt3a induced β-catenin accumulation to negative regulate the Wnt signalling pathway.[11] All the novel advances founded in other tissues or species should be highlighted in tooth development.

ROLE OF WNT IN TOOTH DEVELOPMENT

The development of the tooth germ is the result of a temporal and spatial interaction between odontogenic epithelium and extraembryonic mesenchyme. The canonical Wnt signalling pathway involved in several stages of tooth formation.[12] On day 11.5 of murine embryonic development, expression of members of Wnt signalling pathway, such as Lef1, Wnt10a, Wnt10b, can be detected in both upper and lower odontogenic regions.[13] On day 12.5, nucleus β-catenin is expressed in both the epithelium and underlying mesenchyme. Inhibition of canonical Wnt signalling in this stage may arrest tooth development. For example, overexpression of Dkk1[12], a Wnt signalling inhibitor; conditional deletion of β-catenin driven by Prx-1-Cre[14]; or loss of Lef1[15], a downstream transcription factor of canonical Wnt pathway, lead to the arrest of tooth development at the bud stage. Furthermore, loss of function of Fgf4, a downstream molecule of Lef1/β-catenin, inhibits the proliferation of odontogenic epithelial cells.[16] By contrast, enhanced expression of β-catenin by the K14 promoter in the dental epithelium induces ectopic tooth buds and leads to the formation of supernumerary.[17] On day 13.5, the development of tooth germ enters the cap stage, which there is a dense signalling molecule region called the primary enamel knot. Wnt10, together with Shh, Bmp2, Fgf20, is restricted to a small cluster of placodal cells to form the early signalling center.[18] There is evidence indicating that the enamel knot regulates the further formation of teeth. In the formation of this signalling center, Wnt/β-catenin may be the most upstream signal of Fgf4 and Fgf.20[16,19] Different from the earlier stage, Wnt/β-catenin negatively regulate the odontogenic epithelial cell proliferation and tooth germ development by reducing the expression of Sema3A.[20] On day 14.5, the development of tooth germ enters the bell stage, and a secondary enamel knot can be found in molars at this stage. The patterning of the secondary enamel knots determines the locations and heights of the tooth cusps. Ameloblasts and odontoblasts are generated during the bell stage. Wnt10a is expressed in both primary and secondary enamel knots; meanwhile, from E14.5, the expression of Wnt10a transmits from the epithelium to the mesenchyme and high expression is observed in the preodontoblasts that differentiate into DSPP-expressing odontoblasts.[21] Scientists have also found that Wnt10a inhibits the proliferation of dental papilla cells and promotes their differentiation in the *in vitro* studies.[21,22] Constitutive expression of β-catenin in ameloblasts of mice incisors results in the delayed mineralization and decreased amelogenesis-related proteins MMP20 and KLK.4[23] Transgenic mice have been constructed to identify the function of various molecules during tooth development in this stage. Overexpression of Wnt/β-catenin induces premature differentiation of odontoblasts and causes the teeth to produce large amounts of insufficiently mineralized dentin and low expression of DSPP in OC-Cre; $Catnb^{(+/lox(ex3))}$ mutant mice.[24,25] Conversely, decreased Wnt signalling activity in the early odontoblasts of

OC-Cre; Wls(CO/CO) mutant mice cause a reduction of dentin deposition, resulting in a thinner dentin wall and wider pulp champer.[26] PKP1 is the effector of Wnt pathway that controls the differentiation process of ameloblasts by regulating cell adhesion complex.[27] $Wls^{Shh\text{-}Cre}$ conditional knockout mice revealed that the Wnt ligand in dental epithelium is crucial for tooth differentiation in the late development stage.[28] Mice overexpressed MMP20 have higher levels of β-catenin and fibroblasts invading the position of enamel, such that the original enamel becomes soft, thin and irregular.[29]

Animal studies on tooth development are no longer limited to rodents, scientists have done some investigations on large animals like miniature pigs.[30–32] In situ hybridization, immunohistochemistry and microarray analysis of miniature pig tooth germs at cap stage, early bell stage and late bell stage revealed that Wnt signalling pathway play an essential role in the tooth development.[30,32] Part of the expression pattern of Wnt signalling molecules in miniature pig tooth germs were similar to that of human and mice, such as Wnt3a, Wnt5a, β-catenin, Axin2, and Lef1 expressed in the cap and early bell stage.[32] However, the expression of Wnt3a and Wnt5a in ameloblasts and Tcf4 in odontoblasts/pre-odontoblasts was never reported neither in human or mice.[32] Due to the similarity between human and pigs in anatomy, physiology, and immune responses,[33] much more researches should be done on pigs in the future.

ROLE OF WNT IN THE ROOT FORMATION

It has been shown that Wnt/β-catenin is not only involved in the tooth crown formation but also involved in the process of root formation accompanied with spatial and spatially specific expression. The expression and distribution of Wnt associated molecules was detected. Wnt3a can be detected in the HERS of mice mandibular first molar at postnatal 2 weeks but disappeared at 5 weeks.[34] Bae et al. found that Wnt10a is specifically expressed in the root odontoblasts and β-catenin is expressed in odontoblasts, HERS cells, and periodontal ligament cells during the transition/root stage of mice tooth development.[35] Paradoxically, the β-catenin degradation complex component Axin2 can be observed around the root sheath and dental papilla at PN 10 in mouse molars, and the expression remains at a high level in the root as this region continued to development and differentiate while the expression reduced in the odontoblasts.[36] The expression of Axin2 indicated that Wnt signalling may be suppressed in the root development, which was contrary to those of Bae's research[35] mentioned above, possibly because other pathways were also involved in the root formation process and the mechanism of Wnt signalling in root development was unclear.

The absence of Wnt/β-catenin signalling has a negative effect on the differentiation of root dentin. Using the odontoblast-specific Ctnnb1 deletion, Zhang et al. found that ablation of β-catenin in odontoblasts led to aberrant HERS formation,[37] as well as the formation of dentin and periodontal tissues is greatly hampered in the molar roots and the lingual portion of incisor in loss function of β-catenin.[38] Tissue-specific inactivation of β-catenin in developing odontoblasts produces molars lacking roots and aberrantly thin incisors. Furthermore, root odontoblast differentiation is disrupted, followed by the loss of some HERS inner layer cells; the result suggests that there is a cell-autonomous requirement for Wnt/β-catenin signalling in the dental mesenchyme for root formation.[39] Mechanism research has shown that the absence of Wnt signalling affected the secretion of dentin matrix and odontoblasts polarization.[26,40] Vogel et al. found that the crown morphology of Notum-deficient mice was normal, but their surfaces were covered with severe dysplasia dentin and the roots were short.[41] As a secretory enzyme, Notum regulates the Wnt signalling pathway by removing part of the palmitic Wnt protein. This result indicated that the inhibition of the Wnt/β-catenin signal leads to the obstruction of root dentin and cementum development. On the contrary, over activation of β-catenin during

root development led to a shorter molar roots, thinner root dentin than the WT mice, as well as the downregulation of biglycan and DSPP and upregulation of DMP1 and DKK.1[25] These results indicated that the overactivated Wnt/β-catenin signalling inhibited the root dentin formation which is different to crown dentin. It can be seen that the regulation of Wnt/β-catenin on odontoblasts differentiation and matrix secretion in different stages of tooth development needs further study.

WNT AND THE ABNORMALITY OF TOOTH DEVELOPMENT

In mice, deletion of β-catenin in the odontoblasts leads to the incisor becoming much more smaller than the wild-type ones.[39] Inactivation the expression of Gpr177 (Wnt ligand secretion mediator) in mouse odontogenic epithelium abrogates the dental epithelial tooth-forming capability and leads to the tooth development arrest at early cap stage.[42] WIF1is a secretory Wnt inhibitor that is restrictly expressed in the enamel knot. Inhibiting the expression of WIF1 causes increased apoptosis and arrest of tooth development.[43] Lef1/Tcfs regulates the development of tooth by combining with an upstream signal which is the enhancer of Shh, and deletion of the enhancer results in supernumerary teeth.[44] Bcl9 and Bcl9l, co-transcription factors of the Wnt signalling pathway, highly expressed in the cytoplasm of ameloblasts, and deletion of these co-transcription factors leads to enamel malformation.[45] Conditional knockout of β-catenin in the mouse incisor epithelium makes it short and blunt, with soft and irregular enamel.[46] The *in vitro* cell experiment shows that the absence of β-catenin leads to defective differentiation, up-regulating E-cadherin and migration inhibition in ameloblasts.[46] By contrast, the overactivation of Wnt signalling results in supernumerary teeth.[47] The abnormal phenotypes of tooth development dysplasia connected with Wnt signalling pathway are summarized in Table 1.

TABLE 1. Abnormal phenotypes of tooth development dysplasia connected with Wnt signalling pathway

Gene	OMIM	Disease
Apc	175100	Gardner syndrome, Adenomatous polyposis coli
Axin2	608615	Oligodontia-colorectal cancer syndrome
Dvl1	118210	Charcot-Marie-Tooth disease type 2A
Kremen1	609898	Ectodermal dysplasia including oligodontia
LRP6	616724	Tooth agenesis
Wnt7a	276820	Al-Awadi-Raas-Rothschild syndrome
Wnt10b	617073	Tooth agenesis
Wnt10a	257980	Odontoonychodermal dysplasia
	150400	Tooth agenesis

EFFECT OF THE WNT SIGNALLING PATHWAY ON DENTIN REGENERATION

The essential role of the Wnt signalling pathway in tooth development has attracted scientists' attention and has led to focus on its function in dentin regeneration. Hunter *et al.* found that the ideal healing of dental pulp after injury was closely related to the high expression of Wnt signalling molecules.[48] Yoshioka *et al.* observed an accumulation of β-catenin in the pulp beneath the cavity of 9-weeks-old rat molars, suggesting that the preparation of cavity may activate the Wnt/β-catenin pathway to participate in the dentin regeneration process.[49] The Wnt signalling activator LiCl and GSK3β inhibitor Tideglusib promoted the formation of tertiary dentin *in vivo*.[50,51] These natural tooth repairing processes are potential new approaches to clinical tooth restoration. However, the presence of Wnt/β-catenin signalling does not always mean that it promotes odontoblast differentiation and dentin regeneration. For example, the overexpression of Wnt10a significantly increased the proliferation of DPSCs, but decreased the expression of odontoblast differentiation-related genes, such as DSPP, DMP1, ALP, and COL1A1, suggesting that overexpression of Wnt10a negatively regulates the differentiation of DPSCs into odontoblasts.[52]

CONCLUSIONS AND PERSPECTIVES

The development of tooth and roots involves several signalling pathways and growth factors. Our review mainly focuses on the Wnt signalling pathways during tooth development.

(1) As is known, the Wnt signalling pathway can be classified into the canonical pathway and the non-canonical pathway. In the course of tooth development, scientists have focused more on the canonical Wnt signalling pathway. Recently, researches on the non-canonical Wnt signalling is increased. For example, both the ameloblasts and odontoblasts are polarized during tooth development, while non-canonical Wnt signalling pathway plays an important role in the cell polarized process.[9,34] Thus, the function of the non-canonical Wnt signalling pathway may take part in the cell polarization during tooth development requires further study.

(2) More importantly, there are often contradictory results when it comes to the role of Wnt signalling in tooth formation. For example, human mutations in APC and Axin2, the two components of the same β-catenin degradation complex, provoke opposite syndromes, hyperdontia, and hypodontia, respectively.[47] Likewise, in non-mammalians, activation of Wnt signalling has been shown either yes[53] or no[54] to stimulate replacement tooth formation.

(3) Studies of tooth development in large animals, like miniature pigs, revealed that Wnt signalling do play an important role in the development process, and the expression patterns were different from the mice to some extent. The similarities and differences between pigs, mice, and human make the miniature pig tooth germ an excellent model in the tooth morphogenesis and regeneration research.

(4) In terms of the research methods, in situ hybridization and immunohistochemistry are traditional methods in scientific research. Tooth germ in vitro culture and renal capsule transplantation enable the role of Wnt signalling molecules in tooth development at the organ level to be investigated. RNA sequencing is a highly sensitive and accurate tool for detecting the whole transcriptome expression, enabling researchers to detect the expression changes in disease status, treatment response and any other required conditions. It is also applied in the researches of tooth germs development of both mice[55] and rats[5] in our previous studies. With the further study of Wnt signalling pathway, we hope to provide more theoretical evidence for tooth regeneration. Since the tooth morphogenesis is the result of sequential and reciprocal interactions between the dental epithelium and the underlying mesenchyme, the RNA-seq of separated dental epithelium and mesenchyme would be more powerful. This would be our next target in tooth development research.

(5) The Wnt signalling pathway is not only involved in the tooth development but also in the regeneration of reparative dentin. For example, GSK3 antagonists promote the natural processes of reparative dentin formation to completely restore dentin in the mouse model.[51] This novel approach has made Wnt signalling-related drugs ideally translatable in clinical therapy for dentistry. At present, there are no Wnt signalling pathway-related clinical trials for dental diseases, and we believe that it will appear in the near future.

In conclusion, both canonical and non-canonical Wnt signalling pathways are important in the regulation of tooth development. Drugs related to the components of the Wnt signalling pathway may be effective for tooth regeneration. Whether target Wnt signalling is

ready for clinical treatment of tooth development dysplasia and regeneration of reparative dentin or not? With a deeper understanding of the role of Wnt signalling in tooth development, we hope that Wnt signalling targeting drugs will be successfully applied in dental therapy within this decade.

HIGHLIGHTS

The role of Wnt signalling pathway in crown morphogenesis

The role of Wnt signalling pathway in root formation

The role of Wnt signalling pathway in dentin regeneration

AUTHOR CONTRIBUTIONS

X. L.: collection and assembly of data, data analysis; X. L. and S. L.: manuscript writing; J. Y.: samples collection of rat tooth germs; S. L. and S. Z.: conception and design, manuscript editing, and final approval of manuscript.

DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

No potential conflicts of interest were disclosed.

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