Signaling networks in palate development

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Palatogenesis, the formation of the palate, is a dynamic process regulated by a complex series of context-dependent morphogenetic signaling events. Many genes involved in palatogenesis have been discovered through the use of genetically manipulated mouse models as well as from human genetic studies, but the roles of these genes and their products in signaling networks regulating palatogenesis are still poorly known. In this review, we give a brief overview on palatogenesis and introduce key signaling cascades leading to formation of the intact palate. Moreover, we review conceptual differences between pathway biology and network biology and discuss how some of the recent technological advances in conjunction with mouse genetic models have contributed to our understanding of signaling networks regulating palate growth and fusion. © 2014 Wiley Periodicals, Inc.

INTRODUCTION

Mid-facial fusion defects, such as cleft lip with/or without cleft palate, are among the most common congenital birth defects in humans.[1] These defects result from a failure of facial/palatal processes to grow and/or fuse appropriately during the first trimester of human development.[2] Etiology of cleft lip with or without cleft (secondary) palate differs from that of cleft (secondary) palate only.[3] In this review we concentrate on developmental mechanisms that result in formation of the secondary palate.

The secondary palate, which separates the oral cavity from the nasal cavity, consists of an anterior hard palate (bony) and posterior soft palate (muscular).[4] It plays a critical role in breathing, feeding, swallowing, and speech. The secondary palate starts to develop from the maxillary process of the first pharyngeal arch as paired processes called palatal shelves. The key cell types in palate development are the neural crest-derived palatal mesenchyme, the ectoderm-derived epithelial lining, the most apical layer composed of periderm cells, and the cranial paraxial mesoderm-derived myogenic cells in the soft palate. The palatal shelves first grow bilaterally down along the sides of the tongue. Then they rapidly elevate (as the tongue descends to the floor of the mouth), form a contact in the midline and fuse (Figure 1). All of this takes place between weeks 7 and 11 in human gestation (embryonal days E11.5–E16 in mice; see Figure 1). Failure in any of these processes, i.e., growth, elevation, or fusion, and even a post-fusion rupture, results in cleft palate.

During the last 20 years, methodological advances in human genetics, transcriptomics, proteomics, epigenetics, and particularly in mouse genetics, i.e., our ability to manipulate the mouse genome, have provided a wealth of information about roles of individual genes in palatogenesis. Altogether more than 300 genes, and all the important morphogenetic signaling mechanisms, have been implicated in palatal fusion either in humans or in experimental animal models.[5] The most recent studies have started to address mechanisms by which these genes and their product form regulatory networks to regulate palatal shelf growth, patterning, and fusion.[6,7]

EPITHELIAL–MESENCHYMAL INTERACTIONS CONTROL PALATAL SHELF GROWTH AND PATTERNING

Palatal shelf growth and patterning are controlled by epithelial–mesenchymal interactions. Because

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Conflict of interest: The authors have declared no conflicts of interest for this article.
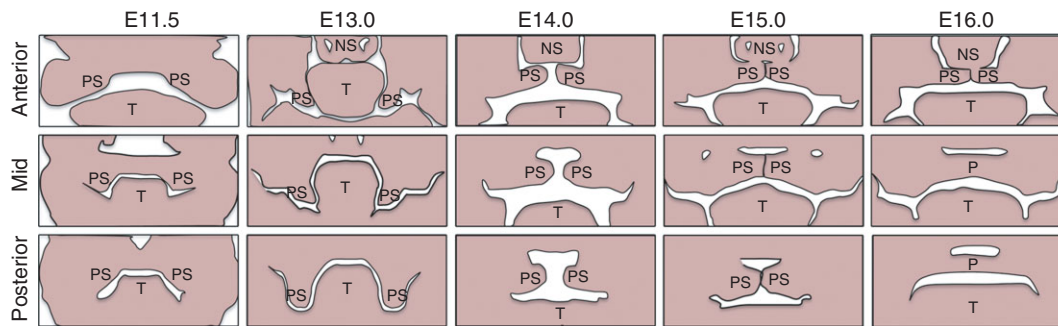
FIGURE 1 | Schematic representation of palatal growth and fusion. Palatal shelves (PS) can be first seen as outgrowths of the maxillary processes of the first pharyngeal arch (E11.5). Then they grow vertically down along the sides of the tongue (T; E13.0), rapidly elevate (E14.0), form a contact with each other and with the nasal septum (NS) in the anterior palate (P) (E15.0), and eventually fuse (E16.0).

outstanding reviews summarizing key pathways involved in palatogenesis have recently been published,[4,5,8] we will provide here only a concise outline of these previous data, and concentrate on the most recent studies elucidating signaling processes that control palatogenesis, and discuss how systems biology can change our view of complex molecular events taking place during palatal fusion.

Growth and Patterning of the Secondary Palate

Many studies have shown that gene expression patterns and gene functions are remarkably different in the developing anterior and posterior palate, which give rise to the hard- and soft palate, respectively (Figure 2). The anterior–posterior (AP) boundary in the developing palate is on the level of the most posterior ruga.

Anterior Palate Development

It has been demonstrated that Sonic Hedgehog (Shh), an important morphogen, plays a crucial organizing role in development and growth of the anterior secondary palate.[9,10] *Shh* is expressed in periodic stripes on the oral side of the developing palate; these stripes correspond to future palatal rugae (transverse ridges on the hard palate).[11,12] Abrogation of *Shh* in the palatal epithelium (by K14-Cre) or its receptor *Smoothened* (*Smo*) in the palatal mesenchyme (using *Osr2-Cre*) has been shown to result in cleft palate and in reduced expression of a number of genes relevant for palatal shelf growth, e.g., *Fgf10*, *Bmp2*, *Msx1*, and *Osr2*.[10] Interestingly, mesenchymal *Fgf10* expression is also needed for appropriate epithelial Shh signaling via epithelial Fgfr2b activation,[9] and thus Fgf10-Shh signaling axis provides an important regulatory feedback loop between palatal epithelium and mesenchyme to control palatal anterior growth

and patterning.[9] Similarly, it has been shown that *Bmp4* in the palatal mesenchyme is required both for *Shox2*, which is needed for anterior palatal growth (mesenchymal cell proliferation),[13,14] and for *Shh* expression in the palatal epithelium.[15] This provides another signaling loop between the epithelium and mesenchyme to control anterior growth of the secondary palate.[15] Zhou et al. recently demonstrated that deletion of the paired-box gene-9 (*Pax9*) transcription factor in the palatal mesenchyme resulted in cleft palate that was caused by defective palatal shelf growth and elevation.[16] In *Pax9* mutants, *Shh* expression in the palatal epithelium, and *Bmp4*, *Fgf10*, *Msx1*, and *Osr2* expression in the palatal mesenchyme were dramatically reduced. The authors further showed that a novel knock-in allele, expressing *Osr2* in the *Pax9* locus (*Pax9^{Osr2KI}*) was able to rescue the posterior, but not the anterior fusion defects in *Pax9* mutants. Consistent with the lack of anterior phenotypic restoration, the expression levels of many key genes, e.g., *Bmp4*, *Msx1*, and *Shh* were not rescued. The authors concluded that Pax9 acts upstream or parallel to Osr2 and controls several different pathways including Shh, Fgf10, and Bmp4 during development of the anterior palate.

Oro-Nasal Patterning of the Anterior Secondary Palate

As outlined above, *Shh* is specifically expressed in the oral palatal epithelium, while Shh receptors (*Ptch* and *Smo*) and downstream effectors (*Msx1*, *Osr1*, and *Osr2*) display a graded expression in the adjacent mesenchyme along the oro-nasal axis.[17,18] In contrast, the distal-less homeobox-5 (*Dlx5*) and *Fgf7* genes are specifically expressed in the nasal mesenchyme of the palatal shelf, and the authors suggested that the Dlx5-regulated Fgf7 signaling is critically important in negatively regulating the mesenchymal Shh signaling and palatal oro-nasal patterning[17] (Figure 2).
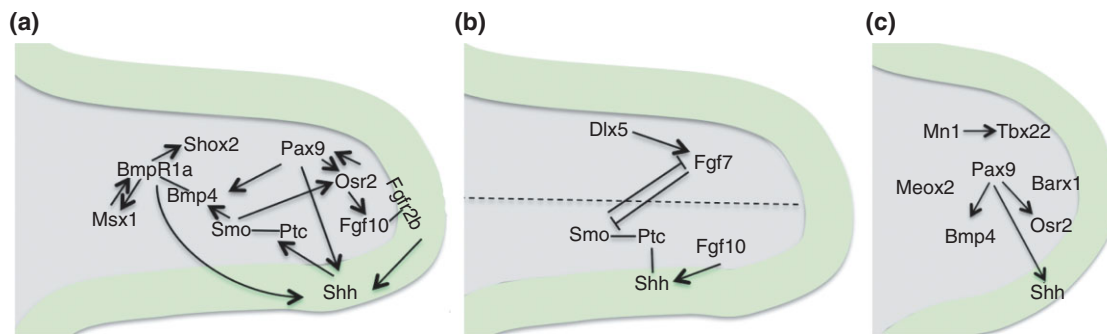
FIGURE 2 | Signaling circuits governing palatal shelf growth and patterning. (a) Epithelial–mesenchymal interactions via Pax9-regulated Shh-Bmp and Shh-Fgf feedback loops control growth and patterning of the anterior palate. (b) Oro-nasal patterning of the anterior secondary palate is regulated via Fgf7-mediated Shh repression. (c) Pax9-regulated expression of *Bmp4* and *Osr2* in the posterior palatal mesenchyme and *Shh* in the posterior palatal epithelium is required for appropriate posterior growth of the secondary palate. In addition, Meox2, Barx1, and Mn1-Tbx22 signaling module regulate posterior palatal growth. Gray, palatal mesenchyme; Green, palatal epithelium.

Posterior Palate Development

The posterior muscular palate (soft palate) functions during swallowing and speech. In addition to neural crest-derived mesenchymal cells, the posterior palate mesenchyme is populated by myogenic cells derived from the cranial paraxial mesoderm.[19] The importance of muscle function to palatogenesis was recently demonstrated by Rot-Nikcevic et al., who showed that mouse embryos lacking striated muscles display cleft palate.[20] Compared to the molecular control of the anterior palate development, much less is known about signaling processes governing the growth and patterning of the posterior secondary palate. Transcription factors *Tbx22* and *Meox2* are specifically expressed in the posterior palate, while *Barx1* and *Mn1* are expressed along the entire AP axis, although the expression of these genes is stronger in the posterior than in the anterior mesenchyme,[21–23] and concordant with the posterior expression domain, mice deficient in *Tbx22* suffer from a submucous cleft palate.[24] Zhou et al. recently showed that unlike previously thought, *Bmp4* is expressed both in the anterior and posterior palatal mesenchyme, and its expression is dependent on Pax9 but not on *Msx1* in the posterior palate.[16] Moreover, their results implied that both *Osr2* expression in the posterior palatal mesenchyme and *Shh* expression in the posterior palatal epithelium (in the developing sensory papilla) is regulated by Pax9.[16]

SIGNALING PATHWAYS CONTROLLING PALATAL EPITHELIAL DIFFERENTIATION AND MIDLINE SEAM DISAPPEARANCE

Along with palatal shelf growth, there is growth of maxillary and mandibular processes. This allows the tongue to slide down and forward, which is required for palatal shelf elevation and reorientation.[25] Pioneering studies of Walker and Fraser and their recent detailed refinement and complementation by Yu and Ornitz showed that mechanisms of the anterior and posterior palatal shelf elevation are different.[26,27] While the anterior palatal shelves are elevated by a 'flipping-up' mechanism, the posterior palatal shelves undergo tissue-remodeling movement. Molecular mechanisms controlling these events are still poorly known.[4]

After elevation, the palatal shelves meet in the midline and become adherent.[28] This event is tightly controlled, because inappropriate adherence prevents palatal shelf elevation resulting in cleft palate.[29,30] Periderm cells, joined to each other by tight junctions, have an important role in controlling palatal shelf adherence and epithelial differentiation.[31,32] This thin (one cell) layer of flattened cells is thought to function as a protective layer or insulator preventing aberrant adhesions. However, the loss of peridermal cells is required at sites of fusion, e.g., the tips of apposing palatal shelves for appropriate epithelial differentiation and adherence.[32] Recent studies have shown that mouse embryos lacking *Fgf10*, *Jagged2*, *Irf6*, *Ikka (chuk)*, or *Tbx1* show aberrant oral adhesions between the tongue and palatal shelves,[29,30,33,34] and it has been suggested that Jagged2-Notch and p63-Irf6 signaling regulate maintenance of periderm cells during palatogenesis[29,30,35] (Figure 3(a)).

Once the apposing palatal shelves have adhered, the midline epithelial seam (MES) must be removed to obtain a mesenchymal confluence.[28] This will mostly happen via programmed cell death (apoptosis), although a possibility that one or both of the two other suggested mechanisms, i.e., epithelial–mesenchymal transformation and migration, would also play some
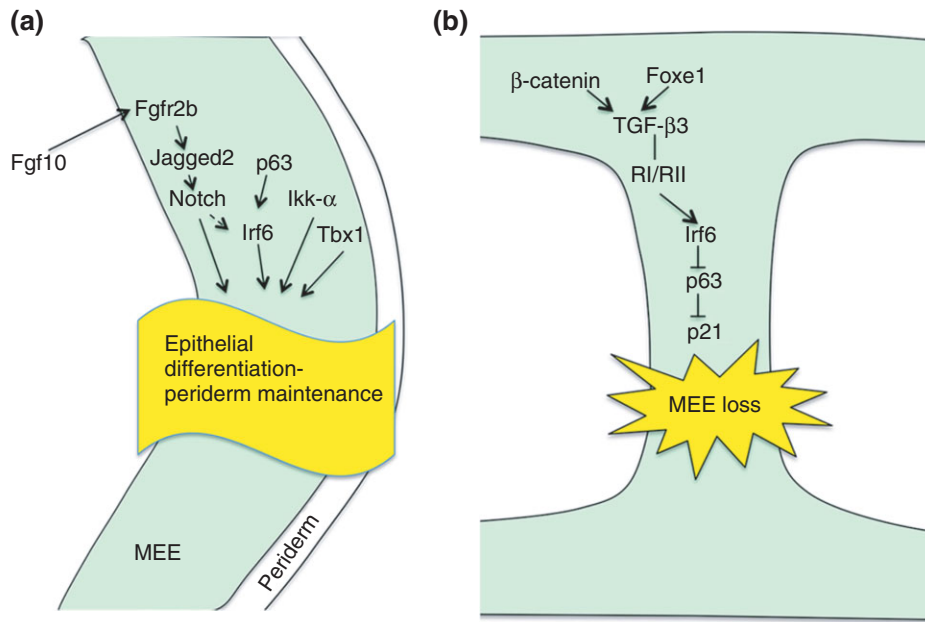
FIGURE 3 | (a) Molecular control of palatal epithelial (green) differentiation. Fgf-Notch signaling, P63-Irf6 signaling, Tbx1, and Ikk-α regulate differentiation or the prefusion palatal epithelium (green). (b) Medial edge epithelial loss is mediated via a signaling cascade involving TGF-β3, Irf6, p63, and p21 in the palatal midline epithelial seam (green).

role in MES removal cannot be totally excluded.[31,36,37] Nevertheless, it is clear that TGF-β signaling plays a critical role in MES removal. *Tgfb3* is strongly and specifically expressed in the MEE,[38,39] and mice deficient in *Tgfb3* show 100% penetrant cleft palate.[40,41] Moreover, palatal epithelial cells defective in genes encoding TGF-β type I or type II receptors fail to undergo apoptosis resulting in a failure of the MES to disappear.[37,42] How *Tgfb3* expression is regulated in the MEE is poorly known. It was recently shown that epithelial-specific *Ctnnb1* (the gene encoding β-catenin) mutants displayed cleft palate and loss of *Tgfb3* expression suggesting that canonical Wnt signaling via β-catenin is required for appropriate MEE-specific *Tgfb3* expression.[43] Venza et al. showed that mutations in the *FOXE1* gene result in *Bamforth-Lazarus* syndrome characterized with craniofacial defects including cleft palate,[44] and that *Tgfb3* is a direct target of Foxe1.[44]

As outlined above, epithelium-specific deletion of genes encoding TGF-β type I and II receptors resulted in defective palatogenesis.[37,42] Therefore, it was rather surprising that epithelium-specific deletion of *Smad4*, which is a critical intracellular TGF-β signal transducer, did not result in detectable defects in palatal fusion.[45] Xu et al. further demonstrated that simultaneous deletion of *Smad4* and inhibition of p38Mapk resulted in persistent MES in explant cultures suggesting that both Smad-dependent and Smad-independent TGF-β signaling act redundantly

for successful palatal epithelial fusion.[45] Iwata et al. recently showed that *Irf6* is a direct target of Smad4 in the palatal epithelium, and that overexpression of *Irf6* in the palatal epithelium rescued the palatal defect seen in epithelial-specific *Tgfbr2* mutants.[46] Moreover, they showed that Irf6 is needed to suppress *dNp63*, which is a prerequisite for *p21* (*Cip1*) expression, cell cycle arrest and subsequent MEE loss[46] (Figure 3(b)).

NETWORK BIOLOGY AND PALATOGENESIS

Pathway Biology Versus Network Biology

As outlined above, genetically manipulated mouse models have helped to identify genes, pathways and signaling modules controlling both palatal shelf growth, patterning, and fusion. While providing valuable conceptual models, these molecule-centric 'pathway biology' approaches are often highly simplified, ignoring context-dependent interactions and are thus potentially misleading. Completion of genome sequencing projects of several different species, and subsequent technological advances in expression profiling (microarray and RNA-Seq), proteomics (mass spectrometry, protein arrays), analyses of protein–protein interactions (yeast two-hybrid screen, mass spectrometry), and protein–DNA interactions (Chip-Seq) have provided basic tools for the understanding of complex regulatory networks. From these
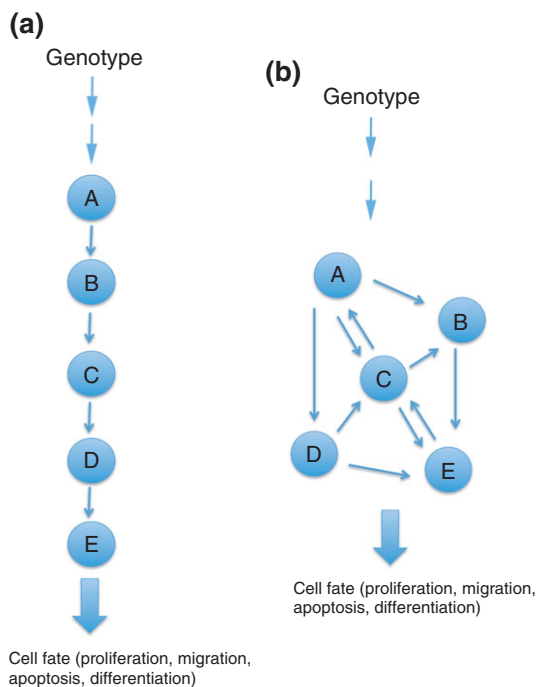
(a)

Genotype

(b)

Genotype

Cell fate (proliferation, migration, apoptosis, differentiation)

Cell fate (proliferation, migration, apoptosis, differentiation)

FIGURE 4 | Schematic comparison of a pathway biology model (a) to a network biology model (b). Arrows depict interactions between signaling molecules. In a pathway biology model (a), genomic information regulating a cell fate is mediated by a linear pathway, where every downstream function is directly affected by an upstream signaling molecule. In a network biology model (b), genomic information controlling a certain cell fate is mediated by a network of interacting molecules.

advances, new paradigms have emerged that are collectively called systems biology or network biology (Figure 4).

On the basis of chosen high-throughput methods, networks can be further divided into transcription regulation-, protein-, signaling-, metabolic-, and regulatory networks or different combinations of intergrated networks (as reviewed by Emmert-Streib and Glazko[47]). A common feature of these interactome networks is that they are highly complex and context dependent placing multidimensional and multidirectional networks between genotypes and phenotypes.[48] Network biology is rapidly contributing to our understanding of complex biological processes. However, due to the extreme complexity and the volume of the generated data, their interpretations would not have been possible without parallel advances in computational biology. Together these developments have also made it possible to apply machine learning and other virtual biology approaches to develop new hypotheses and to prioritize and to test existing hypotheses in situations where experimental biology is not feasible, for instance, due to the high cost or ethical reasons.

Despite enormous promise, network biology paradigms have not been intensely applied to complex developmental processes, e.g., palatogenesis. This is largely due to the fact that developmental events are highly context dependent, both spatially and temporally. Therefore, it has been challenging to isolate sufficient quantities of highly purified transient cell types, e.g., palatal medial edge epithelial cells, without introducing changes to their endogenous gene expression profiles or interfering with pre-existing protein–protein or protein–DNA interactions.

Initial attempts to understand gene regulatory networks during palatogenesis have focused on epigenetic and expression screens on samples harvested from palatal tissues containing both the epithelium and mesenchyme. Pelikan et al. used conventional microarray approaches on samples harvested on prefusion palatal shelves of control and neural crest-specific *Tgfbr2* mutants (*Tgfbr2/Wnt1-Cre*) during palatogenesis.[6] *Tgfbr2/Wnt1-Cre* mutants display cleft palate resulting from reduced growth of prefusion palatal shelves.[49] Because most of the mesenchyme in palatal shelves is derived from the cranial neural crest,[50] these studies allowed identification of genes that are differentially expressed between control mice and mice lacking the gene encoding the TGF-β type II receptor in the palatal mesenchyme. The authors performed gene ontology, transcription factor binding prediction, and miRNA enrichment analyses and analyzed predicted functional relationships of differentially expressed genes using pathway analyses. They grouped the differentially expressed genes based on different functions, e.g., cell cycle regulation, micro-tubule function, and cholesterol synthesis,[6] and have used these data to further dissect the role of TGF-β type II receptor-mediated signaling in the palatal mesenchyme.[51,52]

Ozturk et al. combined database analysis and RNA-Seq to identify cleft palate genes in a *Tgfb3* knockout model.[53] The original data analysis revealed a large number of genes that were differentially expressed between control and mutants throughout palatal fusion. By analyzing differentially expressed genes within the set of 322 cleft palate-associated genes, the authors could identify eight unique genes that all followed the Smad-dependent pathway.

Seelan et al. analyzed methylated promoters and microRNA expression profiles using array-based platforms, and analyzed their data using computational gene interaction predictions.[7] The authors discussed the benefits of understanding epigenetic gene regulation during palatal fusion, and pointed out that some teratogenic compounds, e.g., an antiepileptic drug, valproic acid, alter chromatin conformational state

and its use during early pregnancy results in birth defects including cleft palate in humans.

As mentioned above, major limitations in applying modern high-throughput methods to embryological processes are the lack of a temporal control in analysis of dynamic developmental events and the difficulty in obtaining pure populations of cells without introducing a myriad of artifactual variables. The current methods, e.g., fluorescence activated cell sorting (FACS), laser capture or manual microdissection and immunopanning all have obvious limitations: FACS and immunopanning remove cells from their natural environment, which will likely introduce changes to endogenous gene expression programs, while dissection methods are prone to operator errors and when combined with laser capture, often compromise the quality and quantity of the harvested specimen. Novel methods based on in vivo labeling of cell type-specific transcripts, e.g., INTACT, Ribo-tag, TRAP have attempted to address some of these problems.[54–56] A recently published TU-tagging method is particularly interesting, because in addition to allowing cell-type specific in vivo labeling, it can be used to identify newly synthesized mRNAs and non-coding RNAs, including microRNAs, in intact mouse embryos or in postnatal mice.[57] Moreover, this can be accomplished in specific cell types and at specific times.[57]

CONCLUSIONS

Advances made in mouse genetics during the last 25 years have largely contributed to our understanding of molecular mechanisms controlling palatal shelf growth, patterning, and fusion. They have helped to identify genes required for palatogenesis, and have provided information about pathways in which these genes are functioning during palate development and fusion. These studies have also revealed some significant limitations in classical molecule- or pathway-centric approaches. At the same time, several methodological advances in high throughput- and computational techniques have contributed to an emergence of new paradigms of network biology. While these novel integrated approaches are changing the way we think about biological processes, their application to highly dynamic context-dependent processes taking place during embryonal development, e.g., palatogenesis, has been challenging. Notwithstanding, initial studies have addressed changes in transcriptomes in particular mouse cleft palate models and contributed to understanding of the role of epigenetics during palate development. As new tools and techniques become available, our understanding of molecular mechanisms regulating palate development will likely advance rapidly, which will also help us to better understand pathogenetic mechanisms of cleft palate syndrome, one of the most common birth defects in humans.

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