Int. J. Dev. Biol. 65: 497-504 (2021)
https://doi.org/10.1387/ijdb.200307LG

THE INTERNATIONAL JOURNAL OF DEVELOPMENTAL BIOLOGY
www.intjdevbiol.com

Canonical Wnt signaling regulates branching morphogenesis of submandibular gland by modulating levels of lama5

LIMING GOU*, XIAOCHUN REN and PING JI*

Chongqing Key Laboratory of Oral Diseases and Biomedical Sciences, and Department of Oral and Maxillofacial Surgery, The Stomatological Hospital of Chongqing Medical University, Chongqing, China

ABSTRACT Branching morphogenesis is a crucial developmental mechanism for the formation of the typical bush-like structure of the submandibular gland (SMG). However, the detailed mechanism underlying this process remains to be fully understood. Here, we have investigated whether cross-talk may exist between the Wnt/beta-catenin signaling pathway and lama5 during the branching process in SMG development. An embryonic mouse SMG organ culture model was established, and the validity of this model was confirmed. The roles and possible interactions of the Wnt/beta-catenin signaling pathway, FGF signaling, and lama5 in the branching process were investigated by morphogenesis assays and gene expression patterns. Here, we show that the E12 or E13 SMG organ culture model can be used as an ideal approach to study the process of branching morphogenesis. Our branching morphogenesis assay revealed that the epithelial branching process can be promoted when the canonical Wnt pathway is inhibited and significantly suppressed when the wnt pathway is over activated. Further experiments indicated that FGF signaling most likely acts upstream as a negative regulator of the canonical Wnt pathway during the branching process, whose effect could be partially reversed by Wnt3a. Finally, we show that Wnt/beta-catenin signaling regulates branching morphogenesis through Lama5. We conclude that the Wnt/beta-catenin signaling pathway acting downstream of FGF signaling can serve as a negative regulatory mechanism in the process of SMG branching morphogenesis through Lama5.

KEY WORDS: *salivary gland, organ culture, Wnt/β -catenin signal transduction pathway, laminin*

Introduction

The typical bush-like structure of the submandibular gland (SMG) consists of a network of epithelial branches, which was formed by repeated furcation in the distal ends of the epithelial buds, a process termed branching morphogenesis(Borghese, 1950). This process is repeated in a reiterative manner and results in an exceedingly branched epithelial structure within a minimal packed volume in order to substantially expand the epithelial surface area for secretion or absorption(Gjorevski and Nelson, 2010). A similar process is exhibited as a crucial developmental mechanism for the formation of many other vertebrate organs(Lu and Werb, 2008).

The submandibular gland has been applied as a model to study the regulatory mechanisms of branching morphogenesis for decades. Extensive research has shown that the branching process of salivary gland epithelium is regulated by a complex network of parallel and broadly related signaling pathways involved in the regulation of collagen deposition, epithelium-mesenchyme interaction, and cell proliferation, migration and adhesion(Harunaga *et al.*, 2011, Jaskoll *et al.*, 2001, Sakai *et al.*, 2005), which include EGF(Jaskoll *et al.*, 2004b, Kashimata and Gresik, 1997, Morita and Nogawa, 1999), FGF(Hoffman *et al.*, 2002, Jaskoll *et al.*, 2005, Jaskoll *et al.*, 2004b), Shh(Jaskoll *et al.*, 2004a), *etc.*

The Wnt/beta-catenin signaling pathway which is also called the canonical Wnt signaling pathway is an ancient and evolutionarily

Abbreviations used in this paper: SMG, submandibular gland.

***Address correspondence to:** Liming Gou. Department of Oral and Maxillofacial Surgery, Chongqing Key Laboratory of Oral Diseases and Biomedical Sciences The Stomatological Hospital of Chongqing Medical University, Chongqing 401147, China. Tel: +86-23-8886-0110. Fax: 86-23-8860-0222.
E-mail: goulm@hospital.cqmu.edu.cn - https://orcid.org/0000-0002-4551-975X
Ping Ji. Department of Oral and Maxillofacial Surgery, Chongqing Key Laboratory of Oral Diseases and Biomedical Sciences, The Stomatological Hospital of Chongqing Medical University, Chongqing 401147, China. Tel.: +86-23-8886-0110. Fax: 86-23-8860-0222.
E-mail: jiping@hospital.cqmu.edu.cn - https://orcid.org/0000-0002-2608-1035

Submitted: 17 November, 2020; *Accepted:* 17 February, 2021; *Published online:* 17 February, 2021.

ISSN: Online 1696-3547, Print 0214-6282

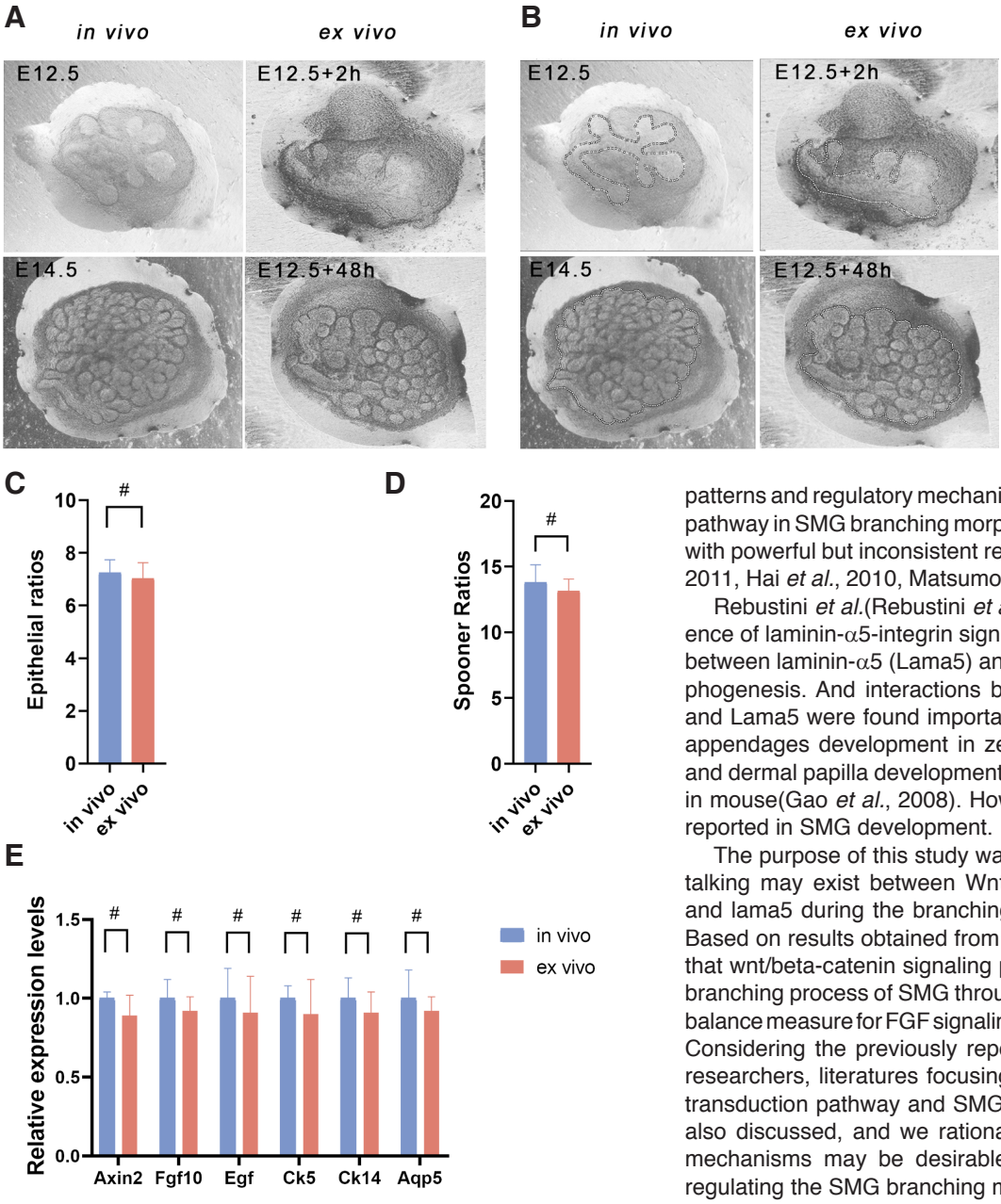

Fig. 1. Establishment of *ex vivo* submandibular gland culture model and assessment of validity. (A) In vivo *glands and cultured glands captured by inverted microscope; original magnification, ×40.* **(B)** *The epithelial areas of glands were marked and calculated by ImageJ.* **(C)** *Comparison of the relative epithelial areas between the* in vivo *glands and cultured glands.* **(D)** *Comparison of the relative Spooner Ratios between the* in vivo *glands and cultured glands.* **(E)** *Comparison of the relative expression levels of the key regulating genes between the* in vivo *glands and cultured glands. #, p>0.05.*

conserved pathway that regulates numerous processes through several distinct pathways in animal development (Nusse, 2005). It is now well established from a variety of studies, that this pathway is activated by the binding of Wnt ligands to a co-receptor complex consisting of frizzled and LRP5/6 proteins. Activating of the receptor complex inhibits the GSK-3 beta kinase, and finally leads to nucleus accumulation of the transcriptional coactivator beta-catenin which subsequently binds to members of the LEF/TCF transcription factor family. Eventually, this series of events will activate down-stream targets of Wnt signaling. And through this pathway, Wnt signaling regulates several key developmental processes(Bridgewater *et al.*, 2008, Chu *et al.*, 2004). Since many components of the Wnt signaling pathway are also found in SMG as reported by Hoffman *et al.(Hoffman et al., 2002)*, the role of Wnt signaling in SMG branching morphogenesis has provoked the interest of many researchers. Studies focusing on the expression patterns and regulatory mechanisms of wnt/beta-catenin signaling pathway in SMG branching morphogenesis have been carried out, with powerful but inconsistent results being reported(Häärä *et al.*, 2011, Hai *et al.*, 2010, Matsumoto *et al.*, 2016, Patel *et al.*, 2011).

Rebustini *et al.*(Rebustini *et al.*, 2007) demonstrated the influence of laminin-α5-integrin signaling pathway and the interaction between laminin-α5 (Lama5) and FGFR on SMG branching morphogenesis. And interactions between canonical Wnt signaling and Lama5 were found important for epithelial patterning during appendages development in zebrafish(Nagendran *et al.*, 2015) and dermal papilla development during early hair morphogenesis in mouse(Gao *et al.*, 2008). However, no similar study has been reported in SMG development.

The purpose of this study was to investigate whether a cross-talking may exist between Wnt/beta-catenin signaling pathway and lama5 during the branching process in SMG development. Based on results obtained from morphogenesis assay, we found that wnt/beta-catenin signaling pathway negatively regulates the branching process of SMG through Lama5, which may serve as a balance measure for FGF signaling in regulating branching process. Considering the previously reported conflicting results by other researchers, literatures focusing on the Wnt/beta-catenin signal transduction pathway and SMG branching morphogenesis were also discussed, and we rationally imply that more complicated mechanisms may be desirable for Wnt/beta-catenin signaling regulating the SMG branching morphogenesis.

Results

Establishment and assessment of SMG organ culture model

The mouse SMG can be excised from the embryo at E12 or E13 and it is because that this excised SMG grows and branches so beautifully in ex vivo culture, recapitulating many aspects of *in vivo* development, that it has been used frequently as an approach to mimic the *in vivo* process for the study of the general process of branching morphogenesis(Larsen *et al.*, 2006, Molnick and Jaskoll, 2000). In this study we compared not only the end bud numbers as reported by most previous studies but the expression levels of several key regulating genes and the epithelial areas between the *ex vivo* model and *in vivo* gland.

To compare epithelial areas or end bud numbers, for *ex vivo* model the E12.5 SMG was obtained and cultured *ex vivo* for 48

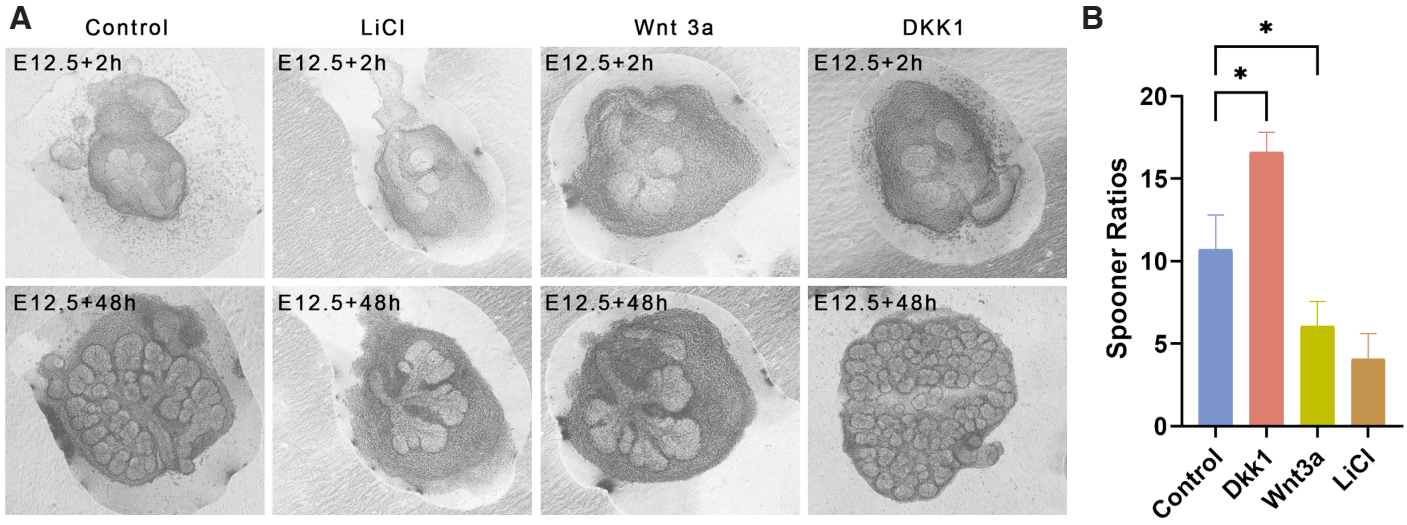

Fig. 2. Branching morphogenesis assay showed that up regulating Wnt/β -catenin signaling inhibits embryonic submandibular gland branching morphogenesis. (A) *LiCl and Wnt3a suppressed branching morphogenesis by activating the canonical wnt signaling pathway, and Dkk1 promoted the branching process through inhibiting the wnt signaling.* **(B)** *Comparison of the relative Spooner Ratio between the experiment groups and control group. *, P>0.05.*

hours, the epithelial ratios or spooner ratios were determined by dividing the epithelial area or bud number at 48h by that at 2h; For *in vivo* gland the epithelial ratios or spooner ratios were determined by dividing the epithelial area or bud number of the E14.5 gland by that of the E12.5 gland (Fig. 1A). The bud numbers were counted manually, and the epithelial areas was manually marked and calculated by ImageJ (Fig. 1B). Mean ratios were determined, data were arcsin transformed to insure normality and homoscedasticity, and compared by paired *t* test for all embryos studied(Jaskoll *et al*., 2004b, Spooner *et al*., 1989).No significant differences in epithelial ratios and spooner ratios were found between the *ex vivo* model and *in vivo* gland (Fig. 1C and 1D). Meanwhile, no statistically significant differences were found between the E14.5 glands and E12.5+48h explants in the expression levels of regulation gene *Fgf10* and *Egf*, and marker gene *Ck5*, *Ck14* and *Aqp 5*. The expression levels of *Axin2* mRNA indicated that there are no differences in the activity of canonical Wnt signaling between the two groups. Taking together, these results demonstrated that there are no differences in both the phenotypes and gene expression levels between *ex vivo* models and *in vivo* glands, and that the cultured SMG would be applied as an ideal model for the research of branching morphogenesis.

Branching morphogenesis assay reveals a negative regulating role of Wnt/beta-catenin signaling pathway in epithelial branching and that Wnt/beta-catenin signaling pathway is negatively regulated by FGF signaling

A regulation role of canonical Wnt signaling in the development of SMG epithelium has been reported in previous studies but with confusing results(Häärä *et al*., 2011, Hai *et al*., 2010, Matsumoto *et al*., 2016, Patel *et al*., 2011). We reexplored this question using the branching morphogenesis assay established as above.

We first tried to constitutively activate beta-catenin signaling by inhibiting GSK3-beta. LiCl was added in the culture media and four concentrations (5mM, 10mM, 20mM and 25mM, respectively) of LiCl were tested. While concentrations below 10mM had no apparent effect on the branching process and those above 25mM exhibited toxic effects, 20mM of LiCl kept the rudiments growing and had a negative effect on the branching process which significantly inhibited epithelial branching. This effect was fully recapitulated by addition of 100ng/ml Wnt3a instead of LiCl (Fig. 2A second and third columns). To further examine the hypothesis, we then used recombinant Dkk1 protein to down-regulate Wnt/beta-catenin signaling. Dkk1 specifically inhibits Wnt/beta-catenin signaling, by forming a complex with the Wnt co-receptor Lrp5/6(Bafico *et al*., 2001, Semënov *et al*., 2001) and Kremen transmembrane proteins(Mao *et al*., 2002) outside the cells, which promotes the internalization of LRP, making it becoming unavailable for Wnt reception. Three concentrations of Dkk-1 (150ng/ml, 200ng/ml and 250ng/ml, respectively) were tested. Enhanced branching morphogenesis was monitored at concentrations > 200ng/ml (Fig. 2A fourth column). These results indicated that the branching morphogenesis of the embryonic SMG was negatively regulated by the canonical Wnt signaling pathway.

The FGF families are found to positively regulate the branching morphogenesis of mouse salivary epithelium (Jaskoll *et al*., 2005, Jaskoll *et al*., 2004b, Morita and Nogawa, 1999). While it is reported that Wnt/beta-catenin signaling acts upstream of FGF signaling to regulate proximal-distal patterning in the lung (Shu *et al*., 2005), we conducted experiments to further our understanding of the interactions between FGF and Wnt/beta-catenin signaling during the regulation of the ex vivo branching process of SMG.

As reported previously, we found FGF10 induced a significant increase in the number of branching epithelial end bud in E12.5 + 2 SMGs (Fig. 3A second column). This effect could be reinforced by DKK1 (Fig. 3A third column) and reduced by Wnt3a (Fig. 3A fourth columns). These effects are statistically significant (Fig. 3B). These results suggested an interaction between FGF and Wnt/beta-catenin signaling during the branching process of SMG, which could be demonstrated by the qPCR results. As shown in Fig. 3C, FGF signaling significantly decreased the mRNA level of *Axin2* after 48h cultivation, while treatment with SU5402 resulted in an almost 1700% increase in *Axin2* mRNA level. These data suggested that the Wnt/beta-catenin signaling pathway is negatively regulated by

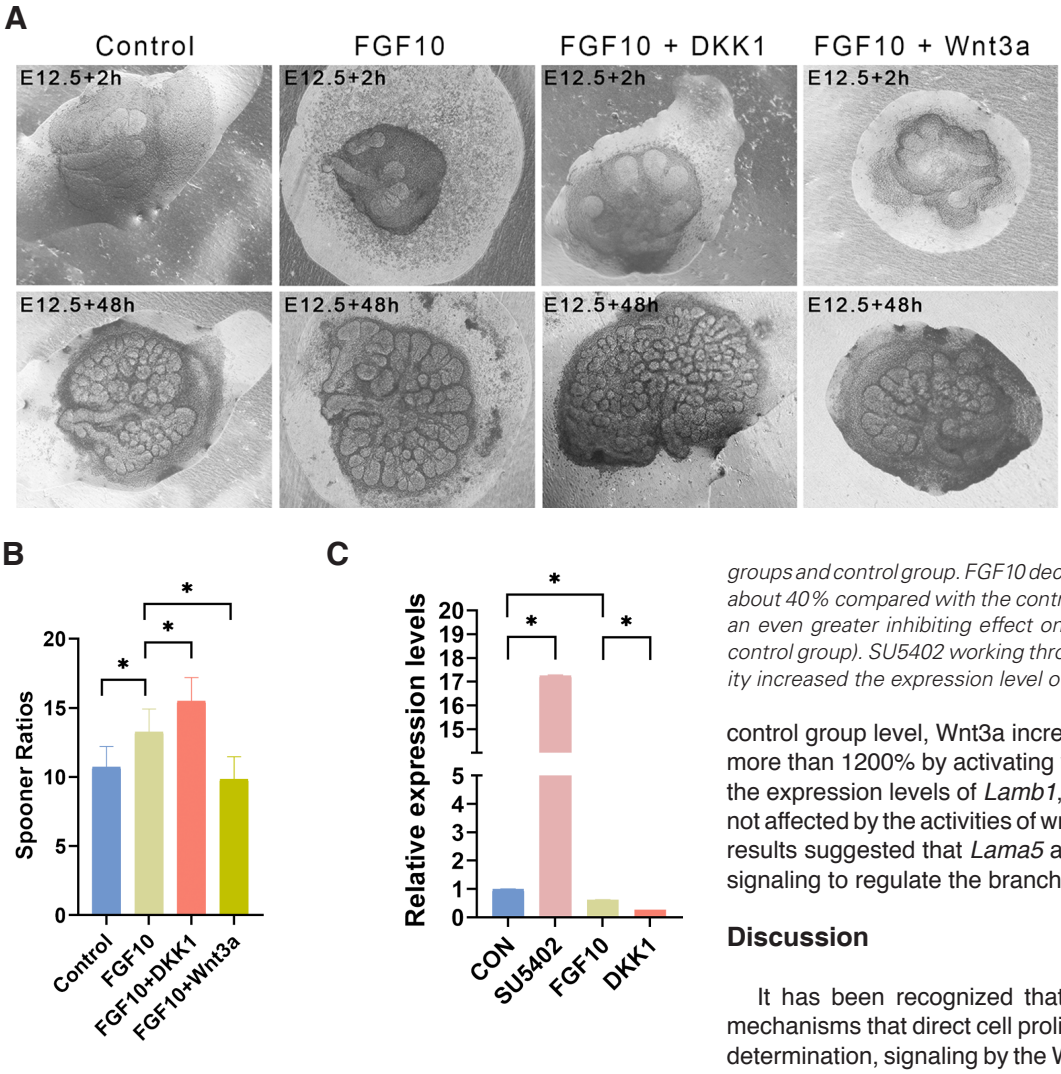

Fig. 3. Canonical wnt signaling was negatively regulated by FGF signaling. (A) *FGF10 promoted the branching morphogenesis, which could be enforced by Dkk1 and reduced by Wnt3a.* **(B)** *Comparison of the relative Spooner Ratio between the experiment groups and control group. Compared with FGF10 group, addition of Wnt3a decreasing about 26% of the end bud number impaired the function of the FGF signaling pathway; on the contrary, addition of DKK1 enforced the function of FGF signaling pathway, which significantly increased the end bud number by about 17%.* **(C)** *Comparison of the expression level of* Axin2 *between experiment groups and control group. FGF10 decreased the expression level of* Axin2 *to about 40% compared with the control group (CON), while DKK1 exhibited an even greater inhibiting effect on the expression of* Axin2 *(to 27% of control group). SU5402 working through inhibiting the FGF signaling activity increased the expression level of Axin2 by about 17 folds. *, P<0.05.*

FGF signaling in branching process.

However, we found in further study that the addition of 200ng/ml DKK1 induced an even greater decrease in the mRNA level of *Axin2* than FGF10 did (Fig. 3C), which may imply that the Wnt/beta-catenin signaling was only partially suppressed by FGF signaling.

Wnt/beta-catenin signaling regulates the branching morphogenesis through LAMA5

The branching morphogenesis assay was further applied to investigated whether a cross-talking between canonical Wnt signaling and LAMA5 exist during the branching process in SMG development as reported in epithelial patterning during appendages development in zebrafish(Nagendran *et al.*, 2015) and dermal papilla development during early hair morphogenesis in mouse(Gao *et al.*, 2008). As shown by branching morphogenesis assay, monoclonal antibody to LAMA5 (Antibody 4G6, at 20ug/ml or 40ug/ml) significantly blocked the inhibiting effect of canonical Wnt signaling in a dose dependent manner (Fig. 4 A,B), which implied that *Lama5* maybe a downstream target for wnt signaling during SMG epithelial branching process. As LAMA5 is a subunit of laminin-511, -521 and -523, we investigated whether the expression of all these subunits were affected by canonical wnt signaling. We found that while Dkk1 treatment only decreased the expression of *Lama5* to 89% of the control group level, Wnt3a increased the expression of *Lama5* to more than 1200% by activating wnt signaling (Fig. 4C). However, the expression levels of *Lamb1*, *Lamb2*, *Lamc1* and *Lamc3* were not affected by the activities of wnt signaling. Taken together, these results suggested that *Lama5* acts downstream of canonical wnt signaling to regulate the branching morphogenesis of SMG.

Discussion

It has been recognized that, being one of the fundamental mechanisms that direct cell proliferation, cell polarity, and cell fate determination, signaling by the Wnt family of secreted lipoproteins has essential roles in multiple tissue morphogenesis during, but not limited to, mammalian animal development(Chu *et al.*, 2004, Dean *et al.*, 2005, Logan and Nusse, 2004, Maretto *et al.*, 2003). We also note that though several studies have discovered the functions of wnt signaling pathway in embryonic SMG development, conflicting results exist in these studies and no research has focused on whether a cross-talking between canonical Wnt signaling and Lama5 during SMG development as in other tissues. Our aim here was to investigate whether a cross-talking may exist between Wnt/beta-catenin signaling pathway and Lama5 during the branching process in SMG development via *ex vivo* SMG organ culture system. First, to assess the validity of the culture model, we compared not only the bud numbers as in previous studies but also the expression of key regulating genes and epithelium areas of the explants with that of the *in vivo* gland, and no significant differences were found. After that, branching morphogenesis assay was carried out, which revealed that the epithelium could not undergo normal branching process when canonical Wnt pathway was blocked by inhibitors or stimulated by activator. Then we found that wnt signaling is negatively regulated by FGF signaling and act upstream of lama5 based on the findings of rescue and neutralization experiments and confirmed by qPCR.

Branching morphogenesis is a key mechanism during develop-

ment to form a bush-like structure required by many organs for normal functions, such as secretion or absorption of substances (Gjorevski and Nelson, 2010), of which salivary gland is a classical research model. The development of mouse submandibular salivary gland (SMG) undergoes branching morphogenesis to turn a single epithelial bud into a functionally efficient, complex, but well-ordered tissue architecture which is comprised of an array of epithelial branches with substantially expanded epithelial surface area for secretion or absorption (Borghese, 1950): During the initial development stage, the mouse SMG first appears as a thickening of the epithelium projecting inwards from the mouth epithelium around the 11.5th day of embryonic development (E11.5), known as the pre-bud stage. The thickening protrudes into the underlying mesenchyme and as the epithelium invaginates it forms a bud linked to the oral surface by a duct from E12.5. This duct will go on to form the main duct of the salivary gland and deep 3D clefts will form in the surface of the primary bud to subdivide it into separate buds, which is followed by proliferative bud outgrowth. Alternating cleft formation and bud outgrowth will continue throughout E13-E15 (Hsu and Yamada, 2010) in a reiterative manner to give rise to a highly branched epithelial structure that maximizes epithelial surface area within a minimal packed volume (Gjorevski and Nelson, 2010). A similar process is conserved as a general developmental mechanism essential for the formation of many other vertebrate organs(Lu and Werb, 2008).

Morphogenesis of this bush-like highly branched epithelial structure in SMG is regulated by the functional integration of parallel and broadly related signaling pathways regulating cell proliferation, migration and adhesion(Jaskoll *et al.*, 2001, Sakai *et al.*, 2005), which include EGF(Jaskoll *et al.*, 2004b, Kashimata and Gresik, 1997, Morita and Nogawa, 1999), FGF(Hoffman *et al.*, 2002, Jaskoll *et al.*, 2005, Jaskoll *et al.*, 2004b), Shh(Jaskoll *et al.*, 2004a), *etc.*

Since many components of the Wnt signaling pathway are found present in developing SMG as reported by Hoffman *et al.(Hoffman et al., 2002)*, the activities of Wnt signaling during SMG development was investigated by several researchers. Using BATGAL Wnt reporter transgenic mice, Hai *et al.*(Hai *et al.*, 2010) found that Wnt/beta-catenin signaling was involved in the postnatal development and regeneration process of SMG through regulating the activity of salivary gland stem/progenitor cells, and Wnt signaling was localized only in a few cells at the basal layer of intercalated ducts in the newborn SMGs. However, they also reported that no Wnt activation was observed in the parenchyma of salivary glands during the entire embryonic development, which is inconsistent with the findings reported by Häärä *et al.*, (Häärä *et al.*, 2011), who found that Wnt activity was active though entirely confined to the mesenchyme surrounding the branching epithelium at the early branching stage, then Wnt signaling could be detected in the ductal epithelium in addition to mesenchyme when the main salivary duct starts to differentiate, and from E16

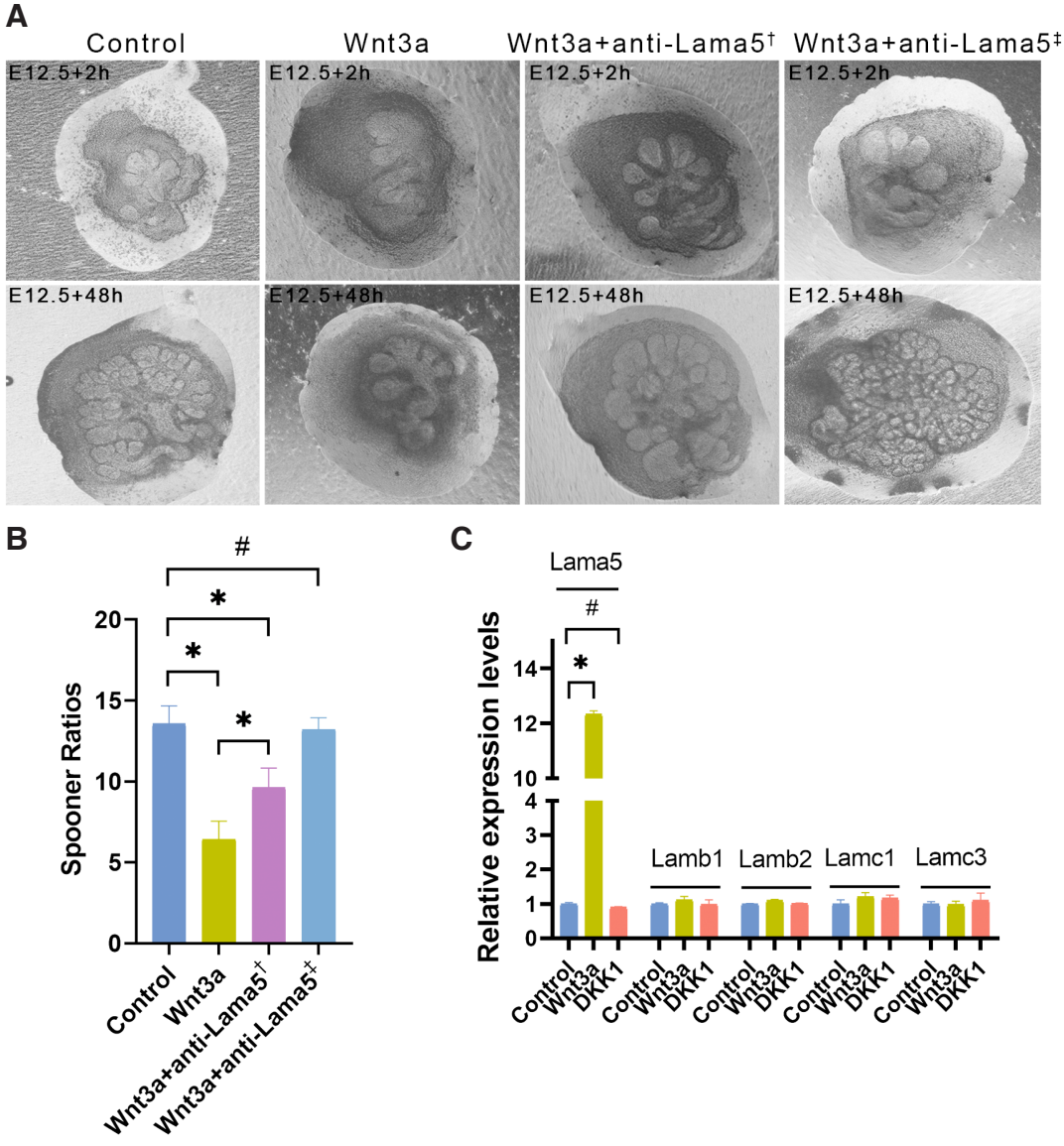

Fig. 4. Lama5 acted downstream of canonical wnt signaling to regulate the branching morphogenesis of cultured submandibular gland. (A) *Lama5 neutralizing antibody significantly reversed the inhibiting effect of canonical wnt signaling via a dose dependent manner.* **(B)** *Comparison of the relative Spooner Ratio between the experiment groups and control group.* **(C)** *Activating canonical wnt signaling could significantly increase the expression level of* Lama5, *but not that of* Lamb1, Lamb2, Lamc1, *and* Lamc3. *#, p>0.05. *, P<0.05. †, 20ug/ml. ‡, 40ug/ml.*

onwards, the Wnt activity was exclusively epithelial and localized to the developing ducts. The latter results commonly accepted now are further supported by Patel *et al.,* (Patel *et al*., 2011) who found that wnt/beta-catenin signaling is active first in the mesenchyme and later in the ductal epithelium at the time of lumen formation. However, one thing should be noted is that a different Wnt reporter line, Axin2LacZ rather than BATGAL mice, was used by Häärä *et al.,* and Patel *et al.,* According to a systematic comparison of the expression patterns of three classical and commonly used canonical wnt reporter lines, TOPGAL, BATGAL, and Axin2LacZ mice, contrasting expression patterns of Wnt signaling reporters was found(Al Alam *et al*., 2011). The same situation exists not only in developing lungs but in developing mammary glands(Jarde and Dale, 2012), and may have been widely ignored by many researchers as reviewed by Barolo(Barolo, 2006). So it would be rational to speculate that maybe an inappropriate reporter line was applied by Hai *et al.*. Now that it is realized that the canonical Wnt pathway activity is required mainly in the mesenchyme when branching process dominates and is strictly limited to the duct epithelium and is much less active when the ducts begin to develop and the branching process to diminish, a regulatory role of wnt signaling can be expected. In the same study performed by Häärä *et al.,* mentioned above, it is reported that when the mesenchymal Wnt activity was inhibited by Wnt signaling inhibitors XAV939 or CKI-7 in E13 wild-type salivary gland, the epithelial branching would be significantly reduced simultaneously. They furtherly confirmed these results by mesenchymal conditional deletion of beta-catenin *in vivo* in the Dermo-Cre+; beta-catenin$^{-/l}$ mice(Häärä *et al*., 2011). However, opposite results are observed by our study and by Patel *et al.,* (Patel *et al*., 2011): The epithelial branching process would be promoted when the canonical Wnt pathway was inhibited, and be significantly suppressed when wnt pathway is over activated. This suppressing effect of canonical wnt signaling during SMG branching process are consistent with those found in the lung and lacrimal gland(Dean *et al*., 2005). We first supposed that this suppressing effect of canonical Wnt pathway was a balance mechanism to the well-known FGF and EGF pathways in regulating the SMG branching process. The FGF families are found positively regulating the branching morphogenesis of SMG epithelium (Jaskoll *et al*., 2005, Jaskoll *et al*., 2004b). Further results obtained by qRT-PCR experiment in our study indicated that FGF signaling acts upstream of the Wnt/beta-catenin signaling pathway, and most likely acts as a negative regulator of the canonical Wnt pathway during the branching process. Moreover, we found that this effect of FGF signaling could be partially, but not all, reversed by Wnt3a. Considering that the interaction between FGF and canonical Wnt signaling is context dependent(Patel *et al*., 2011, Shu *et al*., 2005), further researches are needed to elucidate the details of this cross-talking.

Though conflicting results are found considering the expression manner and regulatory role of canonical Wnt signaling during SMG epithelial branching, several possible mechanisms of canonical Wnt signaling regulating SMG branching process are reported. Eda/Edar/NF-κB pathway is essential for embryonic SMG development(Jaskoll *et al*., 2003), and Häärä *et al.*(Häärä *et al*., 2011) found that canonical Wnt signaling can promote the branching process through *Eda*. However, Matsumoto *et al.*(Matsumoto *et al*., 2016) reported that canonical Wnt signaling restrained the branching process by suppressing *Kit* expression through up-regulation of *Myb* transcription factor. We think that a mesenchyme-epithelium cross-talking can be presumed for the mesenchymal canonical wnt signaling to regulate epithelial branching process. As interactions between canonical Wnt signaling and laminin α5 were found essential for epithelial patterning during appendages development in zebrafish(Nagendran *et al*., 2015) and dermal papilla development during early hair morphogenesis in mouse(Gao *et al*., 2008), we demonstrated in the present study that Lama5 might be the mediator of canonical Wnt signaling in regulating the branching process of SMG epithelium.

In conclusion, though several conflicting opinions have been reported as discussed above and more complicated mechanisms would be expected for the Wnt/beta-catenin signal transduction pathway to regulate submandibular gland branching morphogenesis, our present work demonstrated that Wnt/beta-catenin signaling pathway acting downstream of FGF signaling may serve as a negative regulatory mechanism in the process of SMG branching morphogenesis through Lama5.

Materials and Methods

Mouse strain and tissue collection

Adult mice of the ICR strain was used and raised at 25°C in a constant photoperiod (14L:10D). The guidelines for the Care and Use of Animals in Research were followed, and this research was approved by the Research Ethics Committee of West China Hospital of Stomatology of SiChuan University and Stomatological Hospital of Chongqing Medical University. Female mice were mated with fertile males of the same strain. The morning of finding a vaginal plug was designated as day 0 (E0). Pregnant females were anesthetized at 1130-1230h on day E12.5 of gestation with ether and euthanized by cervical dislocation. Embryos were dissected in cold phosphate buffered saline (PBS) and staged according to Theiler(Theiler, 1989). Mandible sections were isolated, and submandibular glands with attached sublingual glands (hereafter referred to as SMG) were removed from these sections under a dissecting microscope.

Culture system

Rudiments of E12.5 SMG were cultured on membranes at the air/medium interface at 37°C in a humidified 5% CO_2/95% air atmosphere for up to 48 hours. Six glands were placed on a Nuclepore Track-Etch Membrane (pore size 0.1 μm; diameter 13mm; Whatman) floating on 200μl DMEM/F12 (1:1) supplemented with vitamin C (150μg/ml), transfferin (50μg/ml), penicillin (100U/ml) and stretomycin (100μg/ml). The medium was replaced every 24 hours. In supplementation studies, Wnt3a (150ng/ml; #1324-WN/CF; R&D Systems) or LiCl and Dickkopf-1 (Dkk-1) (#5897-DK-010/CF; R&D Systems) were used as the exogenous activator and specific inhibitor for Wnt/beta-catenin signaling pathway, respectively. Each was resuspended in PBS. For each experiment, at least six paired E12.5 SMGs were used for each treatment. Control glands were cultured with an equal volume of the vehicle.

As toxic effects were observed with LiCl, toxicity was recognized as an apparent darkening of the epithelial cells when imaged by bright field mi-

TABLE 1

LIST OF PRIMERS USED FOR qPCR ANALYSIS

Primer name	Forward	Reverse
Axin2 Mus	5′- CTCCCCACCTTGAATGAAGA -3′	3′-ACTGGGTCGCTTCTCTTGAA-5′
Fgf10 Mus	5′- TGCGGAGCTACAATCACCTC -3′	3′-GCATGTCACAGGACCTCTATTG-5′
Egf Mus	5′-TTGACAAGTGGCAGGAGGTC-3′	3′-AACGTGACCAACAAGTAGCG-5′
Ck5 Mus	5′-TGGACCAGTCAACATCTCTGTC -3′	3′-GTCAGTTCCTCCGTACTCGA-5′
Ck14 Mus	5′-GAACCACGAGGAGGTGGC-3′	3′-GTCAGGGTCGAGTCGTACTT-5′
Aqp5 Mus	5′-GGCCATCTTGTGGGGATCTAC-3′	3′-TCTTCTGGTAGCTCGGCTGC-5′
Beta-actin Mus	5′- CACTGTCGAGTCGCGTCC -3′	5′-GTGGTCAAGCGGTACCTACT-3′

croscopy and was defined operationally as a state at which the SMG would not resume proliferation after 24h if the inhibitor or activator contained media were replaced with fresh media(Jaskoll *et al*., 2004b). Cultured rudiments were photographed at 2, 24, and 48 hours. The spooner ratios (end bud number/initial bud number) and epithelial ratios (epithelial areas at end time/ epithelial areas at start time) were determined using ImageJ software for each group. Because a notable difference in SMG epithelial branch number is seen among littermates, we compared the number of terminal buds and epithelial areas in the left and right glands (treated and control) from each embryo for all embryos studied.

Rescue and neutralization experiments

Paired E12.5 SMG primordia were cultured in 25μM SU5402 (#572630-500UGCN; MerckMillipore), a FGFR1 tyrosine kinase inhibitor that does not inhibit EGF,PDGF or the insulin receptor, for at least 48 h, or in 200ng/ml recombinant mouse FGF10 (#6224-FG-025/CF; R&D Systems) for an initial period of 3h and then each pair was cultured in FGF10, or FGF10 + 150ng/ml Wnt3a, or FGF10 + 200ng/ml DKK1 for at least 48h. These Wnt3a and DKK1 concentrations were shown in previous supplementation studies to induce a significant effect on branching morphogenesis in E12.5 + 3 SMGs compared to controls.

For neutralization experiment, the monoclonal antibody to laminin α5(Song *et al*., 2013, Sorokin *et al*., 1997) (Antibody 4G6, at 20ug/ml or 40ug/ml) or IgG were added into the culture mediums prior to adding Wnt3a.

For each experiment, at least six paired E12.5 SMGs were used for each treatment. Finally, the explants were collected, and the numbers of terminal buds were analyzed as described above.

Analysis of RNA by qPCR

Quantitative reverse transcription- PCR was carried out by real-time PCR with the SYBR Green reporter. Total RNA was isolated from freshly dissected or cultured SMGs using Rneasy Mini Kit (Qiagen) and was subsequently reverse transcribed to cDNA with the SuperScript First-Strand Synthesis System (Invitrogen). The quantitative real-time RT-PCR was performed using the SYBR premix EX Tap II kit (Takara) according to the manufacturer's instruction. The reaction mix was subjected to quantitative real-time PCR to detect levels of the corresponding target genes. The primer sequences used were shown in Table 1. The relative expression levels were quantified and analyzed using Bio-Rad iCycler iQ software. The comparative threshold cycle (Ct) method was used to calculate amplification fold. Beta-actin gene was used as a reference control gene to normalize the expression value of each gene. Triple replicates were performed for each group of SMGs, and average expression value was computed for subsequent analysis. The relative expression level of the genes was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

Statistical analysis

All quantitative assays were performed in triplicate and/or repeated three times. Analysis results were expressed as mean ~ SD. Statistical significances were determined by student's *t* test or the paired student's *t* test. A value of $p< 0.05$ was considered statistically significant.

Acknowledgments

This study was supported equally by the National Nature Science Foundation of China (No. 31871464 to Ping Ji) and Chongqing Postdoctoral Science Foundation (No. cstc2019jcyj-bshX0018 to Liming Gou).

Conflict of interest

The authors declare no competing conflicts of interests.

References

AL ALAM, D., GREEN, M., TABATABAI IRANI, R., PARSA, S., DANOPOULOS, S., SALA, F.G., BRANCH, J., ELAGHA, E., TIOZZO, C. and VOSWINCKEL, R. (2011). Contrasting expression of canonical Wnt signaling reporters TOPGAL, BATGAL and Axin2 (LacZ) during murine lung development and repair. *PLoS One* 6: e23139.

BAFICO, A., LIU, G., YANIV, A., GAZIT, A. and AARONSON, S.A. (2001). Novel mechanism of Wnt signalling inhibition mediated by Dickkopf-1 interaction with LRP6/Arrow. *Nat Cell Biol* 3: 683-686.

BAROLO, S. (2006). Transgenic Wnt/TCF pathway reporters: all you need is Lef? *Oncogene* 25: 7505-7511.

BORGHESE, E. (1950). The development *in vitro* of the submandibular and sublingual glands of Mus musculus. *J Anat* 84: 287.

BRIDGEWATER, D., COX, B., CAIN, J., LAU, A., ATHAIDE, V., GILL, P.S., KUURE, S., SAINIO, K. and ROSENBLUM, N.D. (2008). Canonical WNT/β -catenin signaling is required for ureteric branching. *Dev Biol* 317: 83-94.

CHU, E.Y., HENS, J., ANDL, T., KAIRO, A., YAMAGUCHI, T.P., BRISKEN, C., GLICK, A., WYSOLMERSKI, J.J. and MILLAR, S.E. (2004). Canonical WNT signaling promotes mammary placode development and is essential for initiation of mammary gland morphogenesis. *Development* 131: 4819-4829.

DEAN, C.H., MILLER, L.-A.D., SMITH, A.N., DUFORT, D., LANG, R.A. and NISWANDER, L.A. (2005). Canonical Wnt signaling negatively regulates branching morphogenesis of the lung and lacrimal gland. *Dev Biol* 286: 270-286.

GAO, J., DEROUEN, M.C., CHEN, C.H., NGUYEN, M., NGUYEN, N.T., IDO, H., HARADA, K., SEKIGUCHI, K., MORGAN, B.A., MINER, J.H. *et al.,* (2008). Laminin-511 is an epithelial message promoting dermal papilla development and function during early hair morphogenesis. *Genes Dev* 22: 2111-24.

GJOREVSKI, N. and NELSON, C.M. (2010). Branch formation during organ development. *WIRES Syst Biol Med* 2: 734-741.

H R, O., FUJIMORI, S., SCHMIDT-ULLRICH, R., HARTMANN, C., THESLEFF, I. and MIKKOLA, M.L. (2011). Ectodysplasin and Wnt pathways are required for salivary gland branching morphogenesis. *Development* 138: 2681-2691.

HAI, B., YANG, Z., MILLAR, S.E., CHOI, Y.S., TAKETO, M.M., NAGY, A. and LIU, F. (2010). Wnt/β -catenin signaling regulates postnatal development and regeneration of the salivary gland. *Stem Cells Dev* 19: 1793-1801.

HARUNAGA, J., HSU, J. and YAMADA, K. (2011). Dynamics of salivary gland morphogenesis. *J Dent Res* 90: 1070-1077.

HOFFMAN, M.P., KIDDER, B.L., STEINBERG, Z.L., LAKHANI, S., HO, S., KLEINMAN, H.K. and LARSEN, M. (2002). Gene expression profiles of mouse submandibular gland development: FGFR1 regulates branching morphogenesis *in vitro* through BMP-and FGF-dependent mechanisms. *Development* 129: 5767-5778.

HSU, J.C.-F. and YAMADA, K.M. (2010). Salivary gland branching morphogenesis—recent progress and future opportunities. *Int J Oral Sci* 2: 117.

JARDE, T. and DALE, T. (2012). Wnt signalling in murine postnatal mammary gland development. *Acta Physiol* 204: 118-127.

JASKOLL, T., ABICHAKER, G., WITCHER, D., SALA, F.G., BELLUSCI, S., HAJIHOSSEINI, M.K. and MELNICK, M. (2005). FGF10/FGFR2b signaling plays essential roles during *in vivo* embryonic submandibular salivary gland morphogenesis. *BMC Dev Biol* 5: 11.

JASKOLL, T., LEO, T., WITCHER, D., ORMESTAD, M., ASTORGA, J., BRINGAS, P., CARLSSON, P. and MELNICK, M. (2004a). Sonic hedgehog signaling plays an essential role during embryonic salivary gland epithelial branching morphogenesis. *Dev Dynam* 229: 722-732.

JASKOLL, T., WITCHER, D., TORENO, L., BRINGAS, P., MOON, A.M. and MELNICK, M. (2004b). FGF8 dose-dependent regulation of embryonic submandibular salivary gland morphogenesis. *Dev Biol* 268: 457-469.

JASKOLL, T., ZHOU, Y.M., CHAI, Y., MAKARENKOVA, H.P., COLLINSON, J.M., WEST, J.D., HAJIHOSSEINI, M.K., LEE, J. and MELNICK, M. (2001). Embryonic submandibular gland morphogenesis: Stage-specific protein localization of FGFs, BMPs, Pax6 and Pax9 in normal mice and abnormal SMG phenotypes in FgfR2-iiic+/Δ, BMP7–/–and Pax6–/–mice. *Cells Tissues Organs* 170: 83-98.

JASKOLL, T., ZHOU, Y.M., TRUMP, G. and MELNICK, M. (2003). Ectodysplasin receptor-mediated signaling is essential for embryonic submandibular salivary gland development. *Anat Rec Part A* 271: 322-331.

KASHIMATA, M. and GRESIK, E.W. (1997). Epidermal growth factor system is a physiological regulator of development of the mouse fetal submandibular gland and regulates expression of the α6-integrin subunit. *Dev Dynam* 208: 149-161.

LARSEN, M., WEI, C. and YAMADA, K.M. (2006). Cell and fibronectin dynamics during branching morphogenesis. *J Cell Sci* 119: 3376-3384.

LIVAK, K.J. and SCHMITTGEN, T.D. (2001). Analysis of relative gene expression data

using real-time quantitative PCR and the 2– ΔΔCT method. *Methods* 25: 402-408.

LOGAN, C.Y. and NUSSE, R. (2004). The Wnt signaling pathway in development and disease. *Annu. Rev. Cell Dev. Biol.* 20: 781-810.

LU, P. and WERB, Z. (2008). Patterning mechanisms of branched organs. *Science* 322: 1506-1509.

MAO, B., WU, W., DAVIDSON, G., MARHOLD, J., LI, M., MECHLER, B.M., DELIUS, H., HOPPE, D., STANNEK, P. and WALTER, C. (2002). Kremen proteins are Dickkopf receptors that regulate Wnt/β -catenin signalling. *Nature* 417: 664-667.

MARETTO, S., CORDENONSI, M., DUPONT, S., BRAGHETTA, P., BROCCOLI, V., HASSAN, A.B., VOLPIN, D., BRESSAN, G.M. and PICCOLO, S. (2003). Mapping Wnt/β -catenin signaling during mouse development and in colorectal tumors. *Proc Natl Acad Sci USA* 100: 3299-3304.

MATSUMOTO, S., KURIMOTO, T., TAKETO, M.M., FUJII, S. and KIKUCHI, A. (2016). The WNT/MYB pathway suppresses KIT expression to control the timing of salivary proacinar differentiation and duct formation. *Development* 143: 2311-2324.

MOLNICK, M. and JASKOLL, T. (2000). Mouse submandibular gland morphogenesis: a paradigm for embryonic signal processing. *Crit Rev Oral Biol M* 11: 199-215.

MORITA, K. and NOGAWA, H. (1999). EGF-dependent lobule formation and FGF7-dependent stalk elongation in branching morphogenesis of mouse salivary epithelium in vitro. *Dev Dynam* 215: 148-154.

NAGENDRAN, M., ARORA, P., GORI, P., MULAY, A., RAY, S., JACOB, T. and SONAWANE, M. (2015). Canonical Wnt signalling regulates epithelial patterning by modulating levels of laminins in zebrafish appendages. *Development* 142: 320-30.

NUSSE, R. (2005). Wnt signaling in disease and in development. *Cell Res* 15: 28-32.

PATEL, N., SHARPE, P.T. and MILETICH, I. (2011). Coordination of epithelial branching and salivary gland lumen formation by Wnt and FGF signals. *Dev Biol* 358: 156-167.

REBUSTINI, I.T., PATEL, V.N., STEWART, J.S., LAYVEY, A., GEORGES-LABOUESSE, E., MINER, J.H. and HOFFMAN, M.P. (2007). Laminin alpha5 is necessary for submandibular gland epithelial morphogenesis and influences FGFR expression through beta1 integrin signaling. *Dev Biol* 308: 15-29.

SAKAI, T., LARSEN, M. and YAMADA, K.M. (2005). Morphogenesis and branching of salivary glands: Characterization of new matrix and signaling regulators. *Oral Biosci Med* 213: 105-113.

SEM NOV, M.V., TAMAI, K., BROTT, B.K., K HL, M., SOKOL, S. and HE, X. (2001). Head inducer Dickkopf-1 is a ligand for Wnt coreceptor LRP6. *Curr Biol* 11: 951-961.

SHU, W., GUTTENTAG, S., WANG, Z., ANDL, T., BALLARD, P., LU, M.M., PICCOLO, S., BIRCHMEIER, W., WHITSETT, J.A. and MILLAR, S.E. (2005). Wnt/β -catenin signaling acts upstream of N-*myc*, BMP4, and FGF signaling to regulate proximal–distal patterning in the lung. *Dev Biol* 283: 226-239.

SONG, J., LOKMIC, Z., L MMERMANN, T., ROLF, J., WU, C., ZHANG, X., HALLMANN, R., HANNOCKS, M.-J., HORN, N., RUEGG, M.A. *et al.,* (2013). Extracellular matrix of secondary lymphoid organs impacts on B-cell fate and survival. *Proc Natl Acad Sci USA* 110: E2915-E2924.

SOROKIN, L.M., PAUSCH, F., FRIESER, M., KR GER, S., OHAGE, E. and DEUTZMANN, R. (1997). Developmental regulation of the laminin alpha5 chain suggests a role in epithelial and endothelial cell maturation. *Dev Biol* 189: 285-300.

SPOONER, B.S., BASSETT, K.E. and SPOONER JR, B.S. (1989). Embryonic salivary gland epithelial branching activity is experimentally independent of epithelial expansion activity. *Dev Biol* 133: 569-575.

THEILER, K. (1989). The House Mouse: Atlas of embryonic development, 2nd edn. New York: Springer - Verlag.

Further Related Reading, published previously in the *Int. J. Dev. Biol.*

Branching morphogenesis - historical first evidences
Domenico Ribatti and Diego Guidolin
Int. J. Dev. Biol. (2020) 64: 397-407
https://doi.org/10.1387/ijdb.200020dr

Prep1 (pKnox1) transcription factor contributes to pubertal mammary gland branching morphogenesis
Lara Sicouri, Federica Pisati, Salvatore Pece, Francesco Blasi and Elena Longobardi
Int. J. Dev. Biol. (2018) 62: 827-836
https://doi.org/10.1387/ijdb.180278fb

Epithelial-mesenchymal interactions: a fundamental Developmental Biology mechanism
Domenico Ribatti and Marcello Santoiemma
Int. J. Dev. Biol. (2014) 58: 303-306
https://doi.org/10.1387/ijdb.140143dr

The contribution of Roberto Montesano to the study of interactions between epithelial sheets and the surrounding extracellular matrix
Domenico Ribatti
Int. J. Dev. Biol. (2010) 54: 1-6
https://doi.org/10.1387/ijdb.082727dr

NK-2 class homeobox genes and pharyngeal/oral patterning: Nkx2-3 is required for salivary gland and tooth morphogenesis
Christine Biben, Cheng-Chun Wang and Richard P Harvey
Int. J. Dev. Biol. (2002) 46: 415-422
http://www.intjdevbiol.com/web/paper/12141427