The WNT/MYB pathway suppresses KIT expression to control the timing of salivary proacin differentiation and duct formation

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ABSTRACT

Growth factor signaling is involved in the development of various organs, but how signaling regulates organ morphogenesis and differentiation in a coordinated manner remains to be clarified. Here, we show how WNT signaling controls epithelial morphogenetic changes and differentiation using the salivary gland as a model. Experiments using genetically manipulated mice and organ cultures revealed that WNT signaling at an early stage (E12-E15) of submandibular salivary gland (SMG) development inhibits end bud morphogenesis and differentiation into proacini by suppressing Kit expression through the upregulation of the transcription factor MYB, and concomitantly increasing the expression of distal progenitor markers. In addition, WNT signaling at the early stage of SMG development promoted end bud cell proliferation, leading to duct formation. WNT signaling reduction at a late stage (E16-E18) of SMG development promoted end bud maturation and suppressed duct formation. Thus, WNT signaling controls the timing of SMG organogenesis by keeping end bud cells in an undifferentiated bipotent state.

KEY WORDS: Differentiation, Morphogenesis, Salivary gland, WNT, KIT, MYB, Mouse

INTRODUCTION

Morphogenesis and differentiation are important processes for organ development and homeostasis. Although these two processes are not mutually exclusive, morphogenesis mainly involves cell growth, proliferation and morphological changes to create three-dimensional complex structures, and differentiation allows cells to have specialized functions necessary for the development of distinct organs. Branching morphogenesis is a crucial process exhibited by several tubular organs, including kidneys, lungs, and the mammary and salivary glands (Michael and Davies, 2004; Patel and Hoffman, 2014; Shamir and Ewald, 2014). In addition, in end buds of glandular organs, previously unpolarized epithelial cells become polarized to form microvilli and differentiate into secretory acinar cells. However, it is not clear how a combination of growth factor signaling regulates morphogenesis and differentiation.

The salivary gland has long been used as a model to study branching morphogenesis (Hsu and Yamada, 2010; Tucker, 2007). Fibroblast growth factors (FGFs) are produced in the mesenchymal cells of parenchymal organs and mediate signals by activating FGF receptors expressed in epithelial cells (Steinberg et al., 2005; Tucker, 2007). FGF signaling through the FGF receptor 2 (FGFR2; IIb isoform) is essential for mouse organogenesis, including not only the salivary glands but also the lung and anterior pituitary glands (De Moerlooze et al., 2000). An in vitro study, using submandibular salivary gland (SMG) epithelium, revealed that distinct FGFs exert various roles in SMG branching morphogenesis. In addition, FGFR2 signaling upregulates Kit signaling, and the combination of FGFR2 and Kit signaling expands the number of distal progenitors (Lombaert et al., 2013). However, because most of these studies were performed using in vitro SMG samples within 2 to 3 days of culture, a period when epithelial cells undergo branching morphogenesis but do not differentiate into proacini, how morphogenetic changes and differentiation occur over a long period of culture is not clear.

In the pseudoglandular stage of embryonic day (E) 13, the cord of epithelium of the SMG elongates and grows by repeated end bud branching into the surrounding mesenchyme. In cytodifferentiation processes, aquaporin 5 (AQP5), an early marker of proacinar cells (Larsen et al., 2011; Nelson et al., 2013), is expressed at E15 (the canalicular stage) after the onset of the ducal differentiation markers keratin 7 and 19 (KRT7/19) (Nelson et al., 2013). Parotid secretory protein (PSP; also known as BPIFA2), which is expressed transiently in developing AQP5-positive secretory proacinar cells (Ball et al., 2003), and mucin 10 (MUC10; also known as PROL1), which is expressed in developing proacini and mature mucous acinar cells (Melnick et al., 2001), are first detected at E17 (the terminal bud stage). Thus, to coordinate the timing of cellular proliferation, polarization and differentiation in end buds, fine-tuning mechanisms would need to exist in end buds.

WNT signaling regulates the development of various organs (van Amerongen and Berns, 2006) through the activation of two different signaling pathways, β-catenin-dependent and -independent pathways (Kikuchi et al., 2011). At the early stage of SMG development (E12-E15), WNT/β-catenin-dependent signaling is active in mesenchyme around end buds and around the parasympathetic ganglion, and after E15 WNT/β-catenin signaling downregulates in mesenchyme and is concomitantly upregulated in epithelium of SMG main ducts (Knosp et al., 2015; Patel et al., 2011). Opposing reports exist for the role of the WNT signaling pathway in salivary gland development. SB415286, a glycogen synthase kinase 3 (GSK-3) inhibitor that activates the β-catenin-dependent pathway (Coghlan et al., 2000), suppresses branching morphogenesis of SMG rudiments (Patel et al., 2011), whereas XAV939, a tankyrase inhibitor that inhibits the β-catenin-dependent pathway, also inhibits epithelial branching and growth of SMG rudiments (Haara et al., 2011). Thus, the precise function and mechanism of WNT signaling in SMG development remain to be determined. Here, we investigated the role of WNT signaling in...
SMG development, especially focusing on proacinar differentiation and duct formation.

RESULTS

Activation of WNT signaling suppresses end bud differentiation of SMG in vivo

To examine the role of WNT signaling in morphogenesis and differentiation of SMGs in vivo, we used Ctnnb1<sup>Ex3<sup>fl/fl</sup></sup> mice, in which exon 3 of the β-catenin gene (Ctnnb1) is flanked by loxp sites (Harada et al., 1999). In these mice, the expression of Cre recombinase stabilizes β-catenin and activates WNT signaling. Ctnnb1<sup>Ex3<sup>fl/fl</sup></sup> mice and Ctnnb1<sup>Ex3<sup>fl/fl</sup></sup>;Rosa26-Cre/ERT2<sup>2α</sup> mice were crossed, and pregnant females administered tamoxifen on E12. Embryos were collected at E17 and then SMG development was analyzed. In this study, distal spherical epithelia consisting of AQP5-negative cells or cells with diffusely distributed AQP5 were defined as ‘end buds’, whereas distal spherical epithelia consisting of cells with AQP5 accumulated in apical membranes and with PSP were defined as ‘proacini’. Whole-body appearances and total epithelial area of SMG embryos were unchanged in Ctnnb1<sup>Ex3<sup>+/+</sup></sup> (control) and Ctnnb1<sup>Ex3<sup>ΔΔ</sup></sup> mice (Fig. 1A,B), but the size of distal spherical epithelia was increased and the ratio of distal acinar-like structures to end buds was reduced (Fig. 1B). In SMGs at E17 from Ctnnb1<sup>Ex3<sup>ΔΔ</sup></sup> mice, multiple acinar-like structures, defined by the existence of a central lumen, and polarized ducts were clearly observed in distal and proximal regions, respectively (Fig. 1B). By contrast, distal epithelia in SMGs from Ctnnb1<sup>Ex3<sup>ΔΔ</sup></sup>Δ mice formed solid, end bud-like structures. In Ctnnb1<sup>Ex3<sup>ΔΔ</sup></sup>Δ SMGs, β-catenin accumulation in distal proacini and the increase in the mRNA of Lef1, a target gene of WNT/β-catenin signaling, were detected by immunostaining and real-time PCR, respectively (Fig. 1C). Meanwhile, β-catenin accumulation in proximal ductal cells was minimal (Fig. 1C), probably because of region-specific differences in the expression of Cre-ERT2.

In distal epithelia of control SMGs, AQP5 and an apical marker, atypical protein kinase C (aPKC), localized to the central region of cuboidal cells where PSP was also expressed, whereas in distal epithelia of Ctnnb1<sup>Ex3<sup>ΔΔ</sup></sup>Δ SMGs, AQP5 and aPKC were diffusely distributed throughout the cell membrane or cytoplasm; the presence of PSP-positive cells was minimal (Fig. 1D,E). Consistent with these results, mRNA levels of Aqp5, Psp and Muc10, and protein levels of AQP5 and PSP, were reduced in Ctnnb1<sup>Ex3<sup>ΔΔ</sup></sup>Δ SMGs compared with those in control Ctnnb1<sup>Ex3<sup>+/+</sup></sup> SMGs at E17 (Fig. 1F,G). By contrast, the expression of Env4 (a downstream target gene of FGFR2 signaling) was unchanged in Ctnnb1<sup>Ex3<sup>ΔΔ</sup></sup>Δ and Ctnnb1<sup>Ex3<sup>ΔΔ</sup></sup>Δ mice (Fig. 1F). In addition, distal progenitor markers, Sox10 and Krt14, but not Kit, were clearly increased in epithelium of SMGs from Ctnnb1<sup>Ex3<sup>ΔΔ</sup></sup>Δ embryos at E17 and apoptosis was not observed in these SMGs (Fig. 1H). However, no differences in morphology and differentiation between SMGs of wild-type and mutant embryos at E15 were observed (Fig. S1A). These results suggest that forced activation of β-catenin signaling markedly suppresses proacinar differentiation as well as the morphological maturation of end buds in SMGs.

WNT signaling keeps end buds in an undifferentiated state

An E13 SMG rudiment was cultured on a membrane filter. The expression of E-cadherin (a general epithelial marker; also known as cadherin 1), Sox10, AQP5 and PSP at days 0, 2, 4 and 6 were immunohistochemically investigated. In the control rudiment, epithelial cells in end buds at day 0 were AQP5 negative but Sox10 positive (Fig. S1B). AQP5-positive cells were observed in 20.1%, 94.6% and 97.4% of end bud cells at days 2, 4 and 6, respectively (Fig. 2A). AQP5 expressed in distal epithelia was distributed throughout the cytosol and cell membrane at day 4, and accumulated to the apical membrane surface at day 6, indicating that end bud cells were polarized and formed proacinar structures from day 4 to 6 (Fig. 2A). In control rudiments, the mRNA levels of Aqp5 increased between days 2 and 4, and those of Psp and Muc10 increased in an exponential manner between days 4 and 6 (Fig. S1C). Consistent with these results, PSP was detected in proacini at day 6 (Fig. 2A).

In rudiments treated with the GSK-3 inhibitor CHIR99021, distal end buds were enlarged. Only 10.6% of end bud cells were AQP5 positive at day 4, with this positive staining remaining low even at day 6. The formation of proacinar structures and the expression of PSP between days 4 and 6 were suppressed (Fig. 2A). Consistently, CHIR99021 decreased protein and mRNA expression of AQP5, PSP and MUC10 (Fig. 2B,C). CHIR99021 also increased mRNA expression of Krt14, Muc10 and Sox10, but decreased Kit mRNA at day 4 (Fig. 2C). KRT14-positive and SOX10-positive cells were indeed increased in the distal end buds of CHIR99021-treated rudiments at day 4 (Fig. 2D; Fig. S1D).

When SMG rudiments were treated with IWP2, which inhibits WNT secretion (Chen et al., 2009), distal end buds at day 2 were enlarged and end bud cells positive for AQP5 increased to 51.5% (Fig. 2A). By day 4, distal epithelia formed central lumens, and expressed AQP5 at the apical membrane surface as well as cytosolic PSP, suggesting that distal spherical epithelial structures were already proacini by the inhibition of WNT signaling (Fig. 2A). IWP2 accelerated protein and mRNA expression of proacinar cell markers (Fig. 2B,C; Fig. S1C) and decreased distal progenitor markers, except for Sox10 and Kit mRNA (Fig. 2C,D). CHIR99021 inhibited the PSP expression observed in IWP2-treated SMG rudiments, suggesting that IWP2-induced phenotypes of premature differentiation are the result of inhibition of WNT/β-catenin signaling (Fig. S1E). CHIR99021 and IWP2 promoted and suppressed, respectively, the incorporation of 5-ethyl-2'-deoxyuridine (EdU), a marker of proliferating cells in KRT14-positive distal progenitors (Fig. 2D). These CHIR99021- and IWP2-induced phenotypes suggest that WNT signaling expands distal progenitors, prevents early maturation of end buds, and keeps them in an undifferentiated state.

WNT signal activity decreases during SMG development

Axin2 (a target gene of the WNT/β-catenin pathway) mRNA levels in SMG rudiments were downregulated towards day 6 (Fig. 3A). To examine whether WNT signaling is active or not in the end bud epithelium, rudiments were mechanically separated into end buds, primary ducts, and mesenchyme in the distal region, and the correct separation of the regions was confirmed by the assessment of Sox10 (end bud marker), Krt19 (duct marker) and vimentin (mesenchyme marker) mRNAs (Fig. 3B). Although Axin2 mRNA levels were lower in end buds than in mesenchyme and primary ducts, WNT/β-catenin signaling was still active and downregulated towards day 6 in end buds and IWP2 further decreased Axin2 mRNA expression not only in primary ducts but also in end buds (Fig. 3C,D).

WNT ligand mRNAs varied in the SMG mesenchyme and epithelium at E13 according to an existing microarray data set of the developing SMG (http://sgmap.niddc.nih.gov/sgmap/sgexp.html). Among these, Wnt2b mRNA was highly expressed in the SMG mesenchyme at day 0, but levels decreased towards day 4 (Fig. 3C). Wnt7b mRNA was highly expressed in primary ducts, with greatly increased expression levels between days 4 and 6 (Fig. 3C).
Fig. 1. Activation of WNT signaling suppresses end bud differentiation of SMG in vivo. (A) Treatment regime and representative appearances of Ctnnb1\(^{flox3/+}\) (left) and Ctnnb1\(^{flox3/3}\) (right) embryos at E17 are shown. (B) Tissue sections of SMGs at E17 of Ctnnb1\(^{flox3/+}\) and Ctnnb1\(^{flox3/3}\) mice were stained with Hematoxylin and Eosin. Black solid or dashed boxes indicate area of enlarged images of distal and proximal regions, respectively, in cross-sections through the middle of SMGs. Arrowheads indicate acinar-like structures. The areas of total epithelia and distal spherical epithelia, and the ratio of acinar-like structures to end bud from SMGs of control Ctnnb1\(^{flox3/+}\) (n=3) and Ctnnb1\(^{flox3/3}\) (n=3) mice were counted (graphs below). (C) SMGs at E17 from Ctnnb1\(^{flox3/+}\) (n=3) and Ctnnb1\(^{flox3/3}\) (n=3) mice were stained with anti-β-catenin antibody. White solid or dashed boxes indicate area of enlarged images of distal and proximal regions, respectively. mRNA levels of Lef1 were measured by real-time PCR and expressed as fold change compared with Ctnnb1\(^{flox3/+}\) SMGs. (D) SMGs at E17 were stained with the indicated antibodies. White boxes show enlarged images. Dashed lines indicate the outline of proacini. (E) SMGs at E17 were stained with anti-aPKC antibody and DRAQ5. (F) SMGs at E17 of Ctnnb1\(^{flox3/+}\) (n=3) and Ctnnb1\(^{flox3/3}\) (n=3) mice were collected, and real-time PCR analyses for the indicated mRNAs were performed. (G) Lysates of SMG rudiments from Ctnnb1\(^{flox3/+}\) (n=2) and Ctnnb1\(^{flox3/3}\) (n=2) mice at E17 were probed with the indicated antibodies. (H) SMGs at E17 of Ctnnb1\(^{flox3/+}\) and Ctnnb1\(^{flox3/3}\) mice were stained with the indicated antibodies and DRAQ5. Results are expressed as mean±s.e.m. **P<0.01. Scale bars: 1 cm (A); 100 μm (B-D); 25 μm (E); 20 μm (H).
Fig. 2. WNT signaling keeps end buds in an undifferentiated state. (A) E13 SMG rudiments were treated with CHIR99021 or IWP2 for 2, 4 or 6 days. Rudiments were stained with the indicated antibodies. Regions of distal epithelia are magnified and shown in the small panels to the right. The percentage of cells positively stained for AQP5 compared with total E-cadherin-stained cells in the end bud are shown on the panel (n≥280 cells from six end buds). Confocal z-stack images of SMGs at day 6 in vitro are shown at the far right. (B) SMG rudiments were treated with CHIR99021 or IWP2 for 2, 4 or 6 days, and lysates of rudiments were probed with the indicated antibodies. (C) SMG rudiments were treated with CHIR99021 or IWP2 for 4 days. Real-time PCR analyses for the indicated mRNAs were performed (n=6 rudiments). (D) SMG rudiments were treated with CHIR99021 or IWP2 for 4 days, and then incubated with EdU for 30 min. SMGs were stained with anti-KRT14 antibody and DRAQ5. The percentage of KRT14-positive cells out of DRAQ5-stained cells (n≥900 cells from 15 end buds) or EdU-positive cells out of KRT14-positive cells (n≥300 cells from 15 end buds) in end buds are shown. Results are expressed as mean±s.e.m. *P<0.05, **P<0.01. Scale bars: 50 μm (A, large panels); 20 μm (A, small panels); 100 μm (A, far right panels); 50 μm (D).
Secreted frizzled-related protein 1 (sFRP1), a negative regulator of WNT signaling and a target gene of FGF signaling, is expressed in end buds and promotes branching morphogenesis (Patel et al., 2011). Sfrp1 mRNA was indeed expressed in the end bud epithelium at day 0 and temporarily increased at day 2, but decreased towards day 6 (Fig. 3C). Thus, WNT/β-catenin signal activity in the end bud epithelium is active, although it is low possibly because of the balance of the expression levels of WNT ligands and their antagonists.

Epithelial WNT signaling modulates FGF-dependent end bud differentiation

To understand the physiological relevance of WNT activity in the end bud epithelium, mesenchyme-free SMG epithelium at E13 was cultured in medium containing FGFs. Consistent with previous observations (Steinberg et al., 2005), individual FGFs demonstrated individual actions on SMG development when SMG epithelium was cultured in the presence of FGFs for 1 or 2 days (Fig. S1F). In particular, distal epithelium formed acinar-like structures when treated with FGF7, FGF10, or a combination of FGF1 and FGF10 (FGF1-FGF10) or FGF1 and FGF7 (FGF1-FGF7) during days 3 to 4, suggesting that FGF10 or FGF7 induces end bud differentiation into proacini (Fig. S1F,G). FGF1-FGF10 or FGF1-FGF7 induced phenotypes with elongated ducts, multiple branching, and distal acinar-like structures at day 4 (Fig. S1G).

In the presence of FGF1, FGF7 promoted proacinar differentiation in a dose-dependent manner (Fig. S1H). Because FGF1-FGF7 promoted acinar-like structure formation more efficiently than FGF1-FGF10, SMG epithelium was treated with FGF1-FGF7 in the following experiments.

SMG epithelium cultured with FGF1-FGF7 for 4 days exhibited increased numbers of proacini expressing AQP5 apically, whereas a small number of AQP5-positive cells were sparsely distributed in distal end buds cultured with FGF1 alone (Fig. 4A). FGF1-FGF7
significantly increased not only the mRNA levels of proacinar cell markers, but also those of distal progenitor cell markers, compared with FGF1 alone (Fig. 4B). After expressing WNT3A or treating with CHIR99021, AQP5 was expressed throughout the plasma membrane or in the cytosol of the distal tips of end buds, where PSP was not detected (Fig. 4C,D). These manipulations reduced Psp and Muc10 mRNA levels, and increased Sox10 and Myc mRNA levels (Fig. 4C,D). However, WNT5A, a ligand that activates the β-catenin-independent pathway, did not affect the formation of distal acinar-like structures and the mRNA levels of these markers (Fig. 4C). Taken together, without mesenchymal tissues, WNT signaling through the β-catenin pathway may expand distal progenitors as well as FGF7 signaling, but may prevent functional proacinar differentiation in the end bud epithelium, unlike FGF7 signaling.

WNT signaling suppresses end bud differentiation by inhibiting the PI3K/AKT pathway

During SMG development, ERK (also known as MAPK) and AKT are activated downstream of FGF2 and KIT, respectively (Lombaert et al., 2013). E13 SMG rudiments were incubated with CHIR99021 or IWP2 for 2 days. CHIR99021 and IWP2 decreased and increased, respectively, phosphorylated AKT (pAKT; T308) and pS6K (T389; also known as RPS6KB1; a downstream target of AKT), but did not affect pERK1/2 (MAPK3/1) (T202/Y204) (Fig. 5A). Treatment of SMG rudiments with CHIR99021 for 24 h decreased AKT activity, but 4- and 12-h treatment did not affect it (Fig. S2A), suggesting that WNT signaling inhibits AKT indirectly. When SMG rudiments were treated with various signal inhibitors for the last 48 h of a 7-day culture, CHIR99021, FIH1 (an FGFR inhibitor), ISCK03 (a KIT inhibitor) and LY294002 [a phosphatidylinositol-3-kinase (PI3K) inhibitor] clearly suppressed PSP and AQP5 expression, and these inhibitors also inhibited AKT (Fig. 5B). IWP2-induced PSP expression and pAKT(S473) staining were inhibited by ISCK03 and LY294002 (Fig. 5C; Fig. S2B). Under these conditions, CHIR99021, IWP2 and ISCK03 did not induce apoptosis in SMG rudiments (Fig. S2C), confirming that there is no toxicity at the concentrations used. WNT signaling is unlikely to inhibit the FGF2/SMG pathway because CHIR99021 did not inhibit the expression of FGFR signaling target genes, including En4, En5, Spry2 and Ccnd1 (Fig. S2D). Taken together, these data indicate that WNT signaling might regulate the KIT/AKT pathway indirectly.

When SMG epithelium was cultured with FGF1-FGF7, phosphatidylinositol-3,4,5-trisphosphate [PIP3; PtdIns(3,4,5)P3], an upstream activator of AKT, was preferentially detected in proacinar rather than ductal regions (Fig. S2E). The addition of CHIR99021 in the last 24 h of a 4-day culture reduced PIP3 levels of proacinar regions. Treatment of SMG epithelium with cycloheximide (CHX), which inhibits protein synthesis, reversed CHIR99021-dependent Psp mRNA downregulation and the inhibition of AKT and its downstream molecule, mTOR (Fig. S2F), suggesting that WNT signaling suppresses the PI3K/AKT pathway through protein synthesis. Notably, in these experiments we added inhibitors to SMG rudiment and epithelium cultures for the last 24-48 h of a 4-day culture with the consequent inhibition of AKT and secretory protein expression. Treatment of SMG epithelium with CHIR99021 resulted in a phenotype that is characterized by the loss of apical localization of AQPS and PSP and is similar to that induced by ISCK03 and LY294002 treatment (Fig. 5D). However, FIH1 caused loss of end bud structure and AQPS expression completely (Fig. 5D). Thus, WNT signaling suppresses proacinar formation and differentiation of SMGs, through the inhibition of the KIT/PI3K/AKT pathway.

WNT signaling suppresses KIT expression through MYB

As well as PIP3, KIT was predominantly detected in the proacinar region rather than in the ductal region of SMG epithelium cultured with FGF1-FGF7 for 4 days (Fig. S2G). Proacini formation was initially observed at the sub-distal boundary region between end buds and ducts in SMG epithelium at day 3 (Fig. S2H). KIT was highly expressed in sub-distal developing proacini rather than in distal undifferentiated end buds (Fig. S2H). As well as SMGs from Cmmbl(Ex3)ΔΔ embryos (see Fig. 1H), CHIR99021 inhibited KIT expression in both SMG rudiments and epithelium (Fig. 5E,F; Fig. S2I). IWP2 treatment increased KIT protein expression at day 2 and 4 in SMG rudiments (Fig. S2J).

KIT-positive distal progenitor cells are expanded by FGF2R2 and KIT pathways during SMG morphogenesis (Lombaert et al., 2013). Consistent with previous observations, ISCK03 suppressed KIT protein expression (Fig. 5F). CHIR99021 did not affect phosphorylation of KIT (Y719) when the total amount of KIT was normalized (Fig. S2K), suggesting that the downregulation of KIT expression induced by WNT signaling does not result from the inhibition of KIT or FGFR kinase activity. In SMGs from Cmmbl(Ex3)ΔΔ embryos at E17, protein expression of KIT and pAKT (T308), but not pERK1/2 (T202/Y204), were indeed decreased (Fig. 5G; Fig. S2L). Taken together, these data indicate that WNT signaling could inhibit the PI3K/AKT pathway through the suppression of KIT expression. In addition, stem cell factor (SCF; also known as KIT ligand, KITL) increased Psp expression in SMG epithelium and rudiments and CHIR99021 inhibited it, implying that the SCF/KIT pathway promotes end bud differentiation into proacini (Fig. 5H). When SMG rudiments were treated with ISCK03 for the last 16 h of a 4-day culture, ISCK03 decreased mRNA expression of Aqp5 and Psp without affecting Sox10 and Ev4 (Fig. S2M), suggesting that the inhibition of proacinar differentiation by WNT-dependent downregulation of KIT is unlikely to result from a decrease of distal end bud progenitors.

The Kit gene promoter contains consensus binding sites for multiple transcription factors, such as AP-2, MYB and SP1 (Ashman, 1999). We examined the expression of these transcription factors in SMG epithelium treated with FGF1-FGF7. CHIR99021 did not affect the expression of mRNAs of Tjap2α (AP-2 alpha), Tjap2b (AP-2 beta), Tjap2e (AP-2 gamma) or Sp1 but did increase Myb mRNA expression (Fig. S3A). Treatment of SMG epithelium with CHX did not abolish Myb mRNA induction by CHIR99021 (Fig. S3B), suggesting that Myb is a direct target gene of WNT signaling. CHIR99021 increased and IWP2 decreased Myb expression at both mRNA and protein levels in SMG rudiments (Fig. 6A; Fig. S3C). Myb mRNA was preferentially expressed in the SMG epithelium rather than the mesenchyme (Fig. S3D). Consistent with this, MYB protein was detected at low levels in distal epithelia but not in proximal duct and mesenchyme of SMGs from Cmmbl(Ex3)ΔΔ/ΔΔ embryos, and MYB increased in distal epithelia of SMGs from Cmmbl(Ex3)ΔΔ embryos (Fig. 6B).

MYB is known to function as either a transcriptional repressor or activator in regulating Kit expression in hematopoietic cells (Vandenbark et al., 1996). The putative MYB-binding sites were found in a ~4-kb region upstream of the transcription start site of the Kit gene using ECR browser (Fig. S3E). A chromatin
Fig. 4. Epithelial WNT signaling modulates FGF-dependent proacinar differentiation. (A,B) Epithelia from E13 SMGs were cultured with FGF1 only or FGF1 and FGF7 (FGF1-FGF7) for 4 days in 3D Matrigel culture. SMG epithelia were stained with the indicated antibodies (A). Real-time PCR analyses for the indicated mRNAs were performed (n=4 epithelia) (B). (C) E13 SMG epithelia infected with lentiviruses expressing mock, WNT3A or WNT5A were cultured with FGF1-FGF7 for 5 days and stained with the indicated antibodies. Insets show high magnification images of the boxed areas. Real-time PCR analyses for Psp and Muc10 mRNAs were performed (n=4 epithelia). (D) E13 SMG epithelia were cultured with FGF1-FGF7 with or without CHIR99021 for 4 days and stained with the indicated antibodies. Real-time PCR analyses for the indicated mRNAs were performed (n=4 epithelia). Results are expressed as mean±s.e.m. *P<0.05, **P<0.01. Scale bars: 200 μm (A, top panels); 50 μm (A, bottom panels); 500 μm (C, left three panels); 50 μm (C, right four panels); 500 μm (D, left two panels); 100 μm (D, right four panels).
Fig. 5. WNT signaling suppresses proacinar differentiation by inhibiting the KIT/AKT pathway. (A) SMG rudiments were either left untreated or treated with CHIR99021 or IWP2 for 2 days. Lysates of rudiments were probed with the indicated antibodies. (B) SMG rudiments were cultured for 5 days, and then treated with CHIR99021, FIN1, ISCK03 or LY294002 for a further 2 days. Lysates of the rudiments were probed with the indicated antibodies. (C) SMG rudiments were cultured with IWP2 for 4 days and then treated with or without ISCK03 for a further 24 h. The rudiments were stained with the indicated antibodies. Insets show high magnification images of the boxed areas. The fluorescence intensity of phospho-AKT (S473) in end bud regions was quantified (n≥20 end buds). Lysates of rudiments were probed with the indicated antibodies. (D) SMG epithelium were cultured with FGF1-FGF7 for 2 days in 3D Matrigel, and then treated with the indicated reagents for a further 2 days. Epithelia were stained with the indicated antibodies. (E) SMG rudiments were treated with CHIR99021 for 5 days and stained with the indicated antibodies. (F) SMG rudiments were cultured for 3 days, and then treated with CHIR99021 or ISCK03 for a further 2 days. Lysates of rudiments were probed with the indicated antibodies. (G) Lysates of SMGs from Ctnnb1(Ex3)+/+(n=2) and Ctnnb1(Ex3)Δ/Δ (n=2) embryos at E17 were probed with the indicated antibodies. (H) SMG epithelium were cultured with or without SCF for 3 days and stained with the indicated antibodies. Real-time PCR analyses for the indicated mRNAs were performed (n=4 epithelia). (I) SMG rudiments were treated with CHIR99021 or IWP2 in the presence or absence of SCF for 5 days and lysates of rudiments were probed with the indicated antibodies. Results are expressed as means±s.e.m. *P<0.05, **P<0.01. Scale bars: 50 μm (C); 250 μm (D, bright-field images); 50 μm (D, fluorescence images); 50 μm (E); 250 μm (H, bright-field images); 100 μm (H, fluorescence images).
immunoprecipitation assay revealed that among five possible binding sites MYB does bind to site 1 of the mouse Kit promoter (Fig. 6C; Fig. S3E). Knockdown of Myb by two different shRNAs promoted the formation of AQP5- and PSP-positive proacini and the expression of Psp in SMG epithelia treated with FGF1-FGF7 (Fig. 6D; Fig. S3F,G). MYB knockdown did indeed increase distal epithelial KIT protein and mRNA expression and AKT activation, suggesting that MYB can act as a negative regulator of Kit expression in SMGs (Fig. 6E). Therefore, WNT-induced Myb expression might induce Kit downregulation.

Fig. 6. WNT signaling suppresses KIT expression through MYB. (A) SMG rudiments were treated with CHIR99021 or IWP2 for 6 days, and lysates of rudiments were immunoprecipitated with anti-MYB antibody. Total cell lysate and immunoprecipitates were probed with the indicated antibodies. (B) SMGs of Ctnnb1(Ex3)+/+ and Ctnnb1(Ex3)Δ/Δ embryos at E17 were stained with anti-MYB antibody (brown) and Hematoxylin (blue). Black solid and dashed boxes indicate area of enlarged images of distal and proximal regions of SMGs, respectively. The percentage of end bud cells with cytoplasmic MYB out of total cells in end buds are shown (n≥550 cells from 20 end buds). (C) Chromatin from E16 SMGs was immunoprecipitated with anti-MYB antibody and the precipitates were analyzed by PCR for MYB binding site 1 of the Kit gene promoter. (D) SMG epithelia infected with lentiviruses expressing control or MYB shRNA #1 were cultured for 4 days with FGF1-FGF7, and then stained with the indicated antibodies. Black and white boxes indicate areas of enlarged images. (E) SMG epithelia infected with lentiviruses expressing control or MYB shRNA #1 were cultured for 4 days, and then stained with the indicated antibodies. White boxes indicate areas of enlarged images. The fluorescence intensity of phospho-AKT (S473) in end bud regions was quantified (n=10 end buds). Real-time PCR analyses for Kit mRNA expression were performed (n=4 epithelia). Results are expressed as mean±s.e.m. **P<0.01. Scale bars: 100 μm (B); 200 μm (D); 100 μm (E, top panels); 50 μm (E, bottom panels).
WNT signaling promotes the contribution of end bud cells to duct formation

CHIR99021 increased and IWP2 decreased the width of branched ducts of SMG rudiments (Fig. 7A). WNT3A and CHIR99021 increased the length and width of ducts of SMG epithelium treated with FGF1-FGF7 (Fig. S4A,B). Consistent with these observations, distal enlarged structures found at a depth of 10 μm in SMGs from Ctnnb1 Ex3/Δ mice contained KRT7-positive ductal cells, but distal spherical structures in Ctnnb1 Ex3/+/+ mice did not, suggesting that ductal tissue had expanded to the surface region of SMGs in Ctnnb1 Ex3/+/+ mice (Fig. 7B). FGF1-FGF7 treatment induced proacinar formation and promoted the incorporation of EdU in distal SMG epithelium compared with treatment with FGF1 alone; EdU incorporation was minimal in the ductal region at day 4 (Fig. 7C). Treatment with CHIR99021 and FGF1 also increased the number of EdU-positive cells in the distal epithelia but not in ducts, and induced enlarged ductal structures (Fig. 7C). Treatment with CHIR99021 and FGF1-FGF7 also promoted duct formation. Under these conditions, the number of EdU-positive cells was increased in the distal epithelium but not in ducts (Fig. 7C). Thus, end bud cell proliferation induced by FGF7 might contribute to proacinar formation and that induced by WNT signaling leads to duct formation.

How does WNT signaling coordinate a balance of size and morphology between ducts and proacini? To address these issues, the photoconvertible fluorescent protein, Kikume Green-Red (KikGR), was expressed in SMG epithelium cultured with FGF1-FGF7. Distal end bud cells were labeled with red fluorescence from with green fluorescence after KikGR photoconversion using violet light at 405 nm (Tsutsui et al., 2005). In the control SMG epithelium, the distal half of end bud cells were labeled with red fluorescence after photoconversion at day 1 (Fig. 8A). Red-labeled cells derived from end buds were observed in developing sub-distal proacini and the ductal region at day 3 (48 h after photoconversion; Fig. 8B; Fig. S4C), suggesting that undifferentiated end buds are bipotent and contribute to both proacini differentiation and duct formation. CHIR99021 dramatically increased the number of red-labeled cells in the ductal region (Fig. 8B). When distal epithelia forming proacinar structures were labeled with red fluorescence (red-KikGR) after photoconversion at day 3, red-labeled cells were not observed in the ductal region at day 5, suggesting that distal differentiated proacinar cells might be unipotent and no longer contributed to duct formation (Fig. S4D,E). Meanwhile, distal epithelia labeled with red-KikGR after photoconversion at day 3 in the presence of CHIR99021 were still observed in the ductal region at day 5 (Fig. S4E).

End bud cells seemed to contribute to secondary- and tertiary-branched duct formation. The contribution of primary duct cells to SMG morphogenesis was examined using Dil, which is a highly lipophilic fluorescent dye (Sparks et al., 2000). Under the conditions that allowed labeled end bud cells to expand in the ductal region in the presence of FGF1-FGF7 (Fig. S4F), labeled primary duct cells expanded in the same region but were never observed in secondary- and tertiary-branched ductal regions or end buds with or without CHIR99021 (Fig. S4G). These results suggest that end bud cells, unlike primary duct cells, are involved in branched duct formation at the early stage of SMG morphogenesis.

DISCUSSION

In this study, we demonstrated how WNT signaling coordinates regulation of morphology and differentiation during SMG development. Our results suggested that WNT signaling controls the timing of proacinar differentiation and duct formation, in cooperation with FGF and KIT signaling.

WNT-dependent maintenance of undifferentiated cells in end buds

The mesenchyme surrounding end buds produces some WNT proteins, including WNT2B. WNT signaling activity in end bud epithelium is kept at low levels, probably due to sFRP1 induced by FGF signaling, and furthermore decreases towards the late stage probably due to reduction in WNT2B. However, WNT signaling activity is important for suppressing premature end bud differentiation into proacini, and for promoting undifferentiated cell proliferation at an early developmental stage until the E15 canalicular stage in vivo, which corresponds to SMG rudiments at day 4. WNT signaling maintains proximal ductal progenitor cells (Knosp et al., 2015). In addition, of the distal progenitor markers Sox10, Myc, Krt14 and Kit, all of which are increased by FGF signaling, WNT signaling increased the former three markers but decreased KIT.

KIT is a hematopoietic stem cell marker, as well as a progenitor marker of epithelial organs, such as mammary tissues and salivary glands (Kajstura et al., 2011; Kent et al., 2008; Lim et al., 2009; Lombaert et al., 2008). Since KIT and PIP3 were expressed in proacini at higher levels than in end buds, and KIT expression was upregulated during proacinar structure formation, the regulation of KIT expression appears to be crucial for SMG development. Our results show that WNT signaling inhibits the PI3K/AKT pathway by decreasing KIT protein without inhibiting FGF2 signaling. Therefore, WNT signaling suppresses the expression of distal proacinar markers by keeping end bud cells as undifferentiated progenitor cells. SCF, but not FGF10, increases Sox10 mRNA, whereas FGF10, but not SCF, increases Myc mRNA (Lombaert et al., 2013). Given that WNT signaling inhibits KIT signaling, but not FGF signaling, WNT and KIT signaling could upregulate the transcription of the Sox10 gene independently.

WNT-dependent upregulation of MYB could be involved in the mechanism behind WNT-dependent decreases in KIT protein. The product of the Myb (also known as c-myb) gene is highly conserved through evolution and was originally identified as a proto-oncogene associated with avian leukemia (Mucenski et al., 1991; Oh and Reddy, 1999). Although MYB is primarily expressed in immature hematopoietic cells, it is also detected in other cells. Myb can function as both a transcriptional activator and repressor (Graf, 1992; Nakagoshi et al., 1989) and is known as one of regulators of Kit gene expression (Vandenbark et al., 1996). The Kit promoter contains several potential binding sites for MYB, which have positive or negative roles in regulation of Kit transcription (Vandenbark et al., 1996). We found that MYB is upregulated by the WNT/β-catenin pathway in the distal SMG epithelium and that it binds to the promoter region of the Kit gene. WNT signaling suppresses Kit expression by MYB protein expression and the SCF/KIT pathway constitutes a positive-feedback loop by upregulating KIT expression. In this manner, WNT signaling could efficiently suppress KIT protein expression, resulting in the inhibition of proacinar morphogenetic changes and differentiation.

Control of proacinar differentiation and duct formation by WNT signaling

Cell behaviors such as motility have been shown to differ in outer columnar bud epithelial cells, inner polymorphic bud cells, and duct cells of SMG rudiments (Hsu et al., 2013). Because this previous
study was carried out for only 8 h after preparing SMG rudiments at E13, we examined the involvement of SMG epithelial cells in branching and proacinar structure formation at a later stage. In experiments using the SMG epithelium labeled with KikGR and DiI, undifferentiated cells derived from end buds at days 1-3 were observed in ducts, but differentiated cells at days 3-5 were not, suggesting that undifferentiated epithelial cells might be bipotent and contribute to duct formation. It is currently unknown whether...

Fig. 7. WNT signaling promotes distal epithelial proliferation and duct formation. (A) SMG rudiments at E13 were cultured with or without CHIR99021 or IWP2 for 4 days, and then stained with anti-KRT7 antibody. The widths of ducts connected to end buds were measured and expressed as fold changes compared with control SMGs (n=15 ducts). Blue boxes indicate areas of enlarged images. (B) SMG rudiments at E17 were stained with anti-KRT8 and anti-KRT7 antibodies, and confocal images at a 0 or 10 μm depth from the topmost surface are shown. Dotted lines indicate the outline of distal enlarged structures. (C) SMG epithelia were cultured with FGF1 or FGF1-FGF7, in the presence or absence of CHIR99021, for 4 days, and then incubated with EdU for 30 min. SMGs were stained with DRAQ5. The percentage of EdU incorporated into cells compared with DRAQ5-stained cells in end buds or proximal ducts is shown (n≥500 cells from ten end buds). Results are expressed as mean±s.e.m. *P<0.01. Scale bars: 200 μm (A); 100 μm (B); 500 μm (C, top eight panels); 50 μm (C, bottom eight panels).
positive effects of WNT signaling on duct formation are related to a reduction in KIT protein levels.

At a late SMG developmental stage (E16∼), WNT signaling in the end bud epithelium is reduced. Therefore, by suppressing WNT signaling activity, distal undifferentiated cells may start to differentiate into matured epithelial cells, showing apical and basal polarity and expressing secretory proteins. Such matured epithelial cells may be unipotent and form proacini at sub-distal regions by FGF and KIT signaling, indicating that differentiated cells could no longer be involved in duct formation. However, differentiated unipotent epithelial cells in the proacini might still proliferate to contribute to the expansion of the proacinar region. Thus, WNT signaling activity in end bud epithelial cells may regulate their differentiation state and potential by modifying other signaling pathways, including the SCF/KIT pathway, contributing to end bud-proacinar differentiation and end bud-ductal transition (Fig. 8C). However, the interaction between epithelium and mesenchyme is important for organ development (Minoo and King, 1994; Santosh and Jones, 2014) so it is still necessary to consider effects from mesenchyme.
Our present findings explain some of the discrepancies in previous reports (Haara et al., 2011; Patel et al., 2011) on phenotypes induced by WNT signaling activation or inhibition in an early stage of SMG development. Forced activation of WNT signaling suppressed clefting and end bud branching (Coghlan et al., 2000). Since inhibition of the PI3K/AKT or SCF/KIT pathways also suppressed cleft formation and end bud branching (Larsen et al., 2003; Lombaert et al., 2013), excessive activation of WNT signaling may have inhibited branching morphogenesis through the suppression of KIT levels. In addition, it has also been reported that WNT signaling inhibition prevents branching morphogenesis (Haara et al., 2011). Cell movement within acini structures of postnatal day 1 SMG, in which WNT signaling activity might be low, was severely reduced (Larsen et al., 2006). Thus, WNT signaling inhibition might suppress epithelial cell movement, cleft formation, and end bud branching because of the premature differentiation of distal proacinar structures (see Fig. 2A). Therefore, it seems that similar SMG morphological phenotypes were observed, irrespective of WNT signaling activation and inhibition. Thus, stage-dependent WNT signaling activity controls the differentiation state and potential of end bud cells by cooperating with FGF and KIT signaling pathways and thereby regulates the timing of salivary proacinar differentiation and duct formation.

**MATERIALS AND METHODS**

**Mice**

Protocols used for all animal experiments in this study were approved by the Animal Research Committee of Osaka University, Japan (No. 21-048-1). All animal experiments were carried out according to guidelines for the care and use of experimental animals of Osaka University. Ctnnb1(Ex3)Δ/Δ mice were crossed with Ctnnb1(Ex3)Δ/+;Rosa26-Cre/ERT2Tg mice, and conditional Ctnnb1(Ex3)Δ/Δ; Rosa26-Cre/ERT2Tg mice, and control Ctnnb1(Ex3)Δ/Δ; Rosa26-Cre/ERT2Tg mice were obtained. The day the vaginal plug was observed was considered as E0. See supplementary Materials and Methods for further details of tamoxifen administration.

**SMG rudiment culture**

Embryonic SMGs (SMG rudiments) isolated from ICR mice at E13 were cultured at an air-liquid interface on ThinCert™ tissue culture inserts with F12 medium. After isolated epithelia were placed in 20 μl of growth factor-absorbed with salmon sperm DNA/protein A-agarose beads. See supplementary Materials and Methods for further details of DiI labeling.

Immunohistochemical analysis

Immunohistochemical analysis was carried out as previously described (Matsumoto et al., 2014). See supplementary Materials and Methods for further details of procedures.

Immunofluorescence staining

Immunofluorescence staining was carried out as previously described (Matsumoto et al., 2014). See supplementary Materials and Methods for further details of procedures.
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Supplementary information
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