A coordinated reciprocal interaction between epithelium and mesenchyme is involved in salivary gland morphogenesis. The submandibular glands (SMGs) of Wnt1-Cre/R26R mice have been shown positive for mesenchyme, whereas the epithelium is β-galactosidase-negative, indicating that most mesenchymal cells are derived from cranial neural crest cells. Platelet-derived growth factor (PDGF) receptor α is one of the markers of neural crest-derived cells. In this study, we analyzed the roles of PDGFs and their receptors in the morphogenesis of mouse SMGs. PDGF-A was shown to be expressed in SMG epithelium, whereas PDGF-B, PDGFRα, and PDGFRβ were expressed in mesenchyme. Exogenous PDGF-AA and -BB in SMG organ cultures demonstrated increased levels of branching and epithelial proliferation, although their receptors were found to be expressed in mesenchyme. In contrast, short interfering RNA for Pdgfa and -β as well as neutralizing antibodies for PDGF-AB and -BB showed decreased branching. PDGF-AA induced the expression of the fibroblast growth factor genes Fgf3 and -7, and PDGF-BB induced the expression of Fgf1, -3, -7, and -10, whereas short interfering RNA for Pdgfa and Pdgfb inhibited the expression of Fgfβ3, -7, and -10, indicating that PDGFs regulate Fgf gene expression in SMG mesenchyme. The PDGF receptor inhibitor AG-17 inhibited PDGF-induced branching, whereas exogenous FGFβ7 and -10 fully recovered. Together, these results indicate that fibroblast growth factors function downstream of PDGF signaling, which regulates Fgf expression in neural crest-derived mesenchymal cells and SMG branching morphogenesis. Thus, PDGF signaling is a possible mechanism involved in the interaction between epithelial and neural crest-derived mesenchyme.

Platelet-derived growth factors (PDGFs),² originally identified as mitogenic factors for smooth muscle cells, fibroblasts, and glia cells, are also known as paracrine growth factors that mediate epithelial-mesenchymal interactions in various tissues (1). This growth factor family is known to have four members at present, PDGF-A, -B, -C, and -D, all of which assemble into homo- or heterodimer forms. PDGFs bind to two tyrosine kinase receptors, PDGFRα and -β, with PDGF-A, -B, and -C binding to PDGFRα as high affinity ligands and PDGF-B and -D binding to PDGFRβ (2).

PDGFRα is expressed in the embryo by several different types of progenitor cells that proliferate and migrate in response to PDGF (3). For example, PDGFRα is expressed in precursor cells that become cranial neural crest (NC) cells and is thought to be required for migration of those cells into the branchial arches. PDGFRα is also expressed by smooth muscle progenitors in developing lungs (4) and widely throughout the embryonic mesenchyme (5). PDGFRα-deficient mice have a variety of defects in crest-derived tissues, including gross craniofacial, skeletal, and cardiac abnormalities (6–10). These mice are deficient in functional cardiac NC cells, although it is unclear whether the deficiency is in the number of cells that reach target tissues, in NC cell differentiation, or functional target sites, such as cardiac and nasal septation defects and cleft face, cleft palate, and tooth defects (5, 10–13). PDGFRα signaling has also been implicated in the migration and survival of cranial NC cells.

In explant experiments, PDGF-A enhanced NC cell motility without affecting proliferation, and stimulated cultured NC cells to secrete matrix metalloproteinase-2 (MMP-2) and its activator membrane-type MMP (14, 15). However, conditional mutant NCC-PDGFRα embryos do not exhibit defects in NC survival, migration, or proliferation, suggesting that PDGFRα is required for post-migratory NC functions (12). Although PDGF-B and PDGFRβ knock-out mice die around the time of

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2 The abbreviations used are: PDGF, platelet-derived growth factor; PDGFR, PDGF receptor; E<sub>n</sub>, embryonic day; SMG, submandibular gland; NC, neural crest; BrdUrd, bromo-2-deoxyuridine; ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; ANOVA, analysis of variance; siRNA, short interfering RNA; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; PBS, phosphate-buffered saline; ECM, extracellular matrix; MMP, matrix metalloproteinase; RT, reverse transcription; MEK, MAPK/ERK kinase; FGF, fibroblast growth factor; FGFR, FGF receptor.

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** The on-line version of this article (available at http://www.jbc.org) contains supplemental Table 1 and Figs. 1–3.

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PDGF Regulates Branching Morphogenesis

birth, the roles of PDGF-B in adult vascular, kidney, and retina pathogeneses have been elucidated in studies that used conditional knock-out mice, transgenic mice, and mice harboring mutations that alter PDGF activity without causing early lethality (16, 17). Kidneys and retinas are both particularly sensitive to mutations in the PDGFRβ signaling domain, as those affected exhibit the hallmarks of glomerulosclerosis and proliferative retinopathy (17). Together, these results indicate that PDGF-A and -B are critical for regulating organogenesis.

The process of branching morphogenesis is utilized in all animals to form complex-ordered structures and involves dynamic and reciprocal interactions between different tissue types, often through the use of conserved molecular programs (18, 19). In vivo, submandibular gland (SMG) development is initiated as a thickening of oral epithelium on about embryonic day 12 (E12), when an initial epithelium bud on a stalk grows into condensing neural crest-derived mesenchyme. Clefts in the epithelium result in 3–5 epithelial buds by E13.5, then branching morphogenesis occurs with continued proliferation, along with successive rounds of cleft formation, duct elongation, and lumen formation, resulting in a highly branched gland by E14 (20). Branching morphogenesis by mouse SMGs is regulated by multiple growth factors, which has been shown in ex vivo experiments to involve both fibroblast growth factor receptor (21–25) and epidermal growth factor receptor (26, 27) signaling. Transgenic mice lacking FGF genes have major phenotypes, whereas salivary gland aplasia in humans is associated with a mutation in FGF10 (28–30). In addition, mesenchymal FGFs regulate epithelial proliferation and branching morphogenesis of SMGs.

Nevertheless, the activities of PDGFs in salivary gland morphogenesis have not been clearly elucidated. In this study, we studied the roles of PDGF-A and PDGF-B in SMG branching morphogenesis. Both induced FGF expression in mesenchymal cells and also enhanced epithelial proliferation and branching morphogenesis in SMG organ cultures.

EXPERIMENTAL PROCEDURES

Preparation of Tissue Sections and Immunostaining—We used Wnt1-Cre/R26R double transgenic mice, which have been reported previously (34). Whole embryos (E14.5) were stained for β-galactosidase activity using standard procedures and then fixed for 20 min at room temperature in 0.2% glutaraldehyde in phosphate-buffered saline (PBS). Fixed embryos were washed three times in 0.005% Nonidet P-40, 0.01% sodium deoxycholate in PBS. Next they were stained overnight at room temperature using a mixture of 0.4% X-gal, 5 mm potassium ferri cyanide, 5 mm potassium ferrocyanide, and 2 mm MgCl₂ in PBS, then rinsed twice in PBS, and post-fixed in 4% formaldehyde.

The embryos were dehydrated using alcohol and embedded in paraffin. Sections were cut at a thickness of 10 μm and counterstained with Nuclear Fast Red.

E14 ICR mouse heads were dissected and fixed with 4% paraformaldehyde in PBS overnight at 4 °C. The tissues were embedded in OCT compound (Sakura Finetechical Co.) for frozen sectioning and then cut into 8-μm sections with a cryostat (2800 FRIGOCUT; Leica). Immunohistochemistry examinations were performed with the sections, which were incubated in 1% bovine serum albumin/PBS for 1 h before incubation with the primary antibody. We used antibodies directed against PDGF-A (SC-128, Santa Cruz Biotechnology), PDGF-B (AB1550, Abcam), PDGFRα (AB15501, Abcam), PDGFRβ (1469-1, Epitomics), perlecán (MAB1948, CHEMICON), and lamininβ1 (AB3003, Abcam). Primary antibodies were detected by Alexa488- or Alexa594-conjugated secondary antibodies (Molecular Probes). A fluorescent microscope (Biozero-8000; Keyence, Japan) was used for immunofluorescent image analysis.

Semiquantitative and Real Time PCR—Total RNA was isolated using TRIzol reagent (Invitrogen) according to the manufacturer’s protocol. Total RNA (2 μg) was reverse-transcribed into cDNA in 20 μl of 1× first-strand buffer containing 0.5 μg of oligo(dT) as a primer, 500 μM dNTP, and 200 units of SuperScript III (Invitrogen). PCR was performed in 25 μl of 1× first PCR buffer containing 2 μl of reverse transcription products, 1 unit of Ex TaqDNA polymerase (Takara, Japan), 200 μM dNTP, and 0.4 μM of the primer pair. PCR amplification was performed using primers listed in the supplemental Table. The PCR products were separated on 1.5% agarose gels. Real time PCR amplification was performed using primers with SYBR Green PCR master mix with detection by a TaqMan 7700 Sequencer (Applied Biosystems). PCR was performed for 40 cycles, at 95 °C for 1 min, 60 °C for 1 min, and 72 °C for 1 min, as reported previously (35).

Western Blotting—SMG organ cultures were incubated in Dulbecco’s modified Eagle’s medium/F-12 supplemented with 1% penicillin and streptomycin and then treated with PDGF-AA or PDGF-BB for 0–60 min at 37 °C. Next, the tissues were washed twice with 1 mm of ice-cold sodium orthovanadate (Sigma), lysed with Nonidet P-40 buffer supplemented with protease inhibitors at 4 °C for 10 min, and centrifuged, after which the supernatants were transferred to a fresh tube. After boiling for 10 min, the proteins were separated by 12% SDS-PAGE and analyzed by Western blotting. Blots were probed using rabbit polyclonal anti-phospho-p44/42 MAPK antibody or rabbit polyclonal anti-p44/42 MAPK antibody (Cell Signaling), with secondary antibody horseradish peroxidase-linked anti-rabbit antibody (Cell Signaling). The phosphorylation of ERK1/2 was detected with ECL Western blotting detection reagents (Amersham Biosciences) and exposure to x-ray film.

Ex Vivo Submandibular Organ Culture—Submandibular and sublingual salivary gland rudiments (referred to as SMGs in this study) were dissected from E13 ICR mice and cultured on Whatman Nucleopore track-etch filters (13 mm, 0.1-μm pore size; VWR, Buffalo Grove, IL) at the air/medium interface. The filters were floated on 200 μl of Dulbecco’s modified Eagle’s medium/F-12 in 50-mm glass-bottom microwell
dishes (MatTek, Ashland, MA). The medium was supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin, 150 μg/ml vitamin C, and 50 μg/ml transferrin. Five SMGs were cultured on each filter at 37 °C in an atmosphere of humidified 5% CO2, 95% air. In case of addition of exogenous PDGFs, either 10 ng/ml of PDGF-AA (Upstate) or 1 ng/ml of PDGF-BB (Systems) was added to the culture medium. The SMGs were photographed after 2, 24, and 48 h, and the end buds were counted at each time point. Each experiment was repeated at least five times. Aliquots of the PCD reactions were analyzed by gel electrophoresis to confirm that a single product of the expected size was amplified. The reactions were run in triplicate and repeated at least three times, with results combined to generate graphs.

BrdUrd Incorporation Assay—After treatment with PDGF-AA or -BB, BrdUrd was added to the plates (10 mM) for 30 min, then the organs were fixed with cold methanol for 10 min, rehydrated in PBS, and incubated for 30 min in 1.5 M HCl. After washing three times in PBS, the organs were incubated with a 1:50 dilution of fluorescein isothiocyanate-conjugated anti-BrdUrd antibody (Roche Applied Science) for 30 min at room temperature. Finally, the organs were washed in PBS three times and the nuclei stained with propidium iodide (Vector Laboratories). BrdUrd-positive cells were examined under a microscope (Biozero-8000; Keyence, Japan) as described previously (36–38).

siRNA Experiments—RNA interference was performed with Stealth Select 3 RNAi (Invitrogen) for mouse PDGF-A and PDGF-B. SMGs were transfected with 200 nM siRNA duplex in regular serum-free culture medium using Oligo-Fectamine reagent (Invitrogen) according to the manufacturer’s protocol (39). An equal concentration of negative control siRNA duplex (Stealth RNAi negative control kit, Invitrogen) or scramble siRNA for PDGF-A and PDGF-B was used for the control SMGs.

Addition of PDGF Antibodies, Recombinant FGFRs, and Kinase Inhibitor—E13 SMGs were cultured for 48 h as described above with neutralizing antibodies to PDGFs or recombinant FGFRs. Neutralizing antibodies to PDGF-AB (06-127, Upstate) and PDGF-BB (AB10845, Abcam) were used for neutralization of PDGF-AA, PDGF-AB, and PDGF-BB activities, as well as a goat IgG control (SC2028-L, Santa Cruz Biotechnology), and added to culture media at concentrations of 0.2–10 μg/ml. Next, 10 μg/ml of rFGFR1b (655-FR, R & D Systems) or 2 μg/ml of rFGFR2b (708-MF, R & D Systems) was added. Furthermore, 375 nm of AG-17 (Calbiochem) and 0.75 μM of SU5402 (Calbiochem) were added to the culture medium at the beginning of each experiment. FGF1 (500 ng/ml), FGF2

**FIGURE 1. Expression of PDGFs and PDGFR in mouse SMGs.** Neural crest-derived salivary gland mesenchyme expressed PDGF-B and PDGFRα and -β. A, SMGs from E14.5 Wnt1-Cre/R26R mice were stained with X-gal (blue) and counter-stained with Fast Red (red). B, staining of E14 SMGs for PDGF-A and -B and PDGFRα and -β. Abbreviations used are as follows: mes, mesenchyme; tb, terminal bud; d, duct. C, high magnification of E14 SMGs double-stained with the antibodies for PDGF and PDGFR, and the basement membrane molecules perlecan or laminin β1. Abbreviations used are as follows: epi, epithelium; BM, basement membrane. D, schema used for the separation of E13 SMG epithelium and mesenchyme by Dispase. E, mRNA expressions of Pdgf and Pdgfr in SMG epithelium and mesenchyme were analyzed by RT-PCR. F, expression level of each gene was quantified using bands of the PCR products and compensated using the internal control hypoxanthine phosphoribosyltransferase (HPRT). Expression levels of Pdgfa in epithelium, Pdgfb, and Pdgfra and -β in mesenchyme were set at a value of 1 for comparison. Statistical analysis was performed by ANOVA (n = 4 per group; *, p < 0.01).
PDGF Regulates Branching Morphogenesis

On E13, SMGs are known to have three epithelial buds and begin to undergo branching morphogenesis. During this stage, PDGF-A and -B and PDGFRα and β can be found expressed in dissected SMGs, whereas mRNA for Pdgfa and Pdgfrα is expressed during all developmental stages. In this study, Pdgfb and Pdgfrβ expression was shown to be the highest on E15 (supplemental Fig. 1, A and B). To confirm the expression pattern of PDGFs and their receptors observed in our immunohistochemical analysis, epithelia and mesenchymal tissue samples from E13 SMGs were dissected and analyzed by Western immunoblotting. ERK1/2 phosphorylation in E13 SMGs after stimulation of PDGF-AA and -BB was analyzed by Western blotting. Immunohistochemistry was performed to confirm the expression pattern of PDGFs and their receptors observed in our immunohistochemical analysis. To clarify the border between epithelium and mesenchyme, we performed immunostaining with antibodies for perlecain and laminin β1, which are components of the basement membrane. PDGF-A was mainly expressed in epithelia in the ducts and terminal buds of SMGs, although it was weakly detected in mesenchyme (Fig. 1A). This result indicates that embryonic SMG mesenchyme is derived from cranial NC cells.

Expression of PDGF-A, PDGF-B, PDGFCαR, and PDGFBαR in Developing SMGs—Next, we examined the expressions of PDGF-A and -B, and PDGFRα and β in E14 SMGs using immunohistochemistry. To clarify the border between epithelium and mesenchyme, we performed immunostaining with antibodies for perlecain and laminin β1, which are components of the basement membrane. PDGF-A was mainly expressed in epithelia in the ducts and terminal buds of SMGs, although it was weakly detected in mesenchyme (Fig. 1A). This result indicates that embryonic SMG mesenchyme is derived from cranial NC cells.

Salivary Gland Mesenchymal Cells Are Derived from Cranial Neural Crest—To determine the role of PDGFs in SMG branching morphogenesis, we attempted to determine the fate of PDGFRα-positive NC cells in normal salivary glands using the Wnt1-Cre/R26R mouse strain that results from being crossed with the ROSA26 conditional reporter (R26) transgene. R26R exhibits constitutive β-galactosidase expression in all cells when activated by ubiquitously expressed Cre, and it is ideal for monitoring Cre-mediated expression and performing cell lineage analysis. By utilizing the Wnt1 promoter, Cre expression was restricted to the precursors of NC cells. On E14.5, β-galactosidase-positive cells were restricted to mesenchymal cells in the SMG but not in the epithelium (Fig. 1A). This result indicates that embryonic SMG mesenchyme is derived from cranial NC cells.

RESULTS

Salivary Gland Mesenchymal Cells Are Derived from Cranial Neural Crest—To determine the role of PDGFs in SMG branching morphogenesis, we attempted to determine the fate of PDGFRα-positive NC cells in normal salivary glands using the Wnt1-Cre/R26R mouse strain, which is a Wnt1-Cre transgenic mouse strain that results from...
MEK-ERK1/2 pathway; therefore, we analyzed the phosphorylation of ERK1/2 in SMG organ cultures. Phosphorylation of ERK1/2 was observed at 5 min after stimulation with PDGF-AA and then disappeared after 30 min, whereas that of ERK1/2 was observed at 5 and 60 min after stimulation with PDGF-BB (Fig. 2A). Next, we separated epithelium and mesenchyme tissues from the SMG organ cultures, and stimulated them with either PDGF-AA or PDGF-BB. Phosphorylation of ERK1/2 was observed in SMG mesenchymal cells but not in the epithelium (Fig. 2B). These results suggest that functional PDGFRα and -β are expressed in SMG mesenchyme and transmit PDGF signaling into mesenchymal cells.

Next, we examined the functional importance of PDGFs using organ culture assays. Under the normal condition, SMGs from E13 mice showed an increase in number of terminal buds (Fig. 2C), with the number about 15-fold greater after 48 h as compared with 2 h. However, the addition of PDGF-AA caused an increase in branching morphogenesis in E13 SMGs, which was shown by an increase in the number of terminal buds by ~20% after 24 and 48 h as compared with the control (Fig. 2, D and E). Similar results were observed after stimulation with PDGF-BB (Fig. 2, D and E).

siRNA and Anti-PDGF Antibodies Inhibit Branching Morphogenesis—Decreases in PDGF-AA and -BB gene expression following siRNA treatment were determined by RT-PCR after 20 h. RT-PCR analysis with PDGF-A and -B primers indicated a reduction of ~80% in the messenger level of PDGF-A and about a 90% reduction in that of PDGF-B, as compared with the control SMG cultures (Fig. 3). The addition of PDGF-A and -B siRNA dramatically inhibited branching morphogenesis at 24 and 48 h (Fig. 4A), with the numbers of terminal buds reduced by about 55 and 65%, respectively, as compared after 2 h (Fig. 4, A and B). Negative control and scramble siRNA did not have any effect on the branching morphogenesis of the SMGs (data not shown).

The antibody for PDGF-AB binds to PDGF-AA and -AB, whereas that for PDGF-BB binds to PDGF-BB. Addition of the anti-PDGF-AB antibody to the SMG cultures dramatically inhibited branching morphogenesis at 24 and 48 h (Fig. 4C). Similar results were observed with the addition of the anti-PDGF-BB antibody, although the effects were less pronounced as compared with anti-PDGF-AB (Fig. 4D).
PDGF Regulates Branching Morphogenesis

4, C and D). Rabbit control IgG did not have an effect on the branching morphogenesis of SMGs (data not shown). Together, these results indicate that endogenous PDGF-A and PDGF-B are necessary for the development of SMGs.

PDGF-AA and -BB Enhance and siRNA for PDGF-A and -B Inhibit SMG Epithelial Proliferation—To clarify the mechanisms of branching morphogenesis stimulated by PDGF-AA and -BB, SMG proliferation was determined by measuring BrdUrd incorporation (supplemental Fig. 2A). BrdUrd-positive cells in the terminal bud and duct regions of SMGs were detected using a fluorescence microscope. In contrast, BrdUrd-positive cells were not detected in the mesenchyme with any of the tested conditions (Fig. 5A and supplemental Fig. 2A). These results suggest that PDGF-AA and -BB enhance the proliferation of SMG epithelium and branching morphogenesis, whereas they do not have an effect on mesenchymal proliferation.

To analyze the effects of PDGF-A and -B siRNA during branching morphogenesis, we performed BrdUrd incorporation assays with SMG organ cultures. In the control culture, ~35% of the epithelial cells were BrdUrd-positive, whereas ~3% of mesenchymal cells were positive. In siRNA-treated cells, there was a decrease in the number of BrdUrd-positive cells by about 20% as compared with the control (Fig. 5C). Furthermore, siRNA from PDGF-A and -B inhibited the proliferation of epithelia, especially in the terminal buds, but not in the duct cells (Fig. 5, C and D, and supplemental Fig. 2B). Negative control and scramble siRNA did not have any effect on the proliferation of either epithelium or mesenchymal cells (data not shown). These findings indicate that the mRNA expression levels of PDGF-A and -B are important during branching morphogenesis of SMGs, especially for the proliferation of epithelial cells.

PDGF-AA and -BB Induce Expression of FGFs in SMGs, and siRNA Inhibits FGF Expression Induced by PDGF—PDGF-A is mainly expressed in SMG epithelium, although its receptor is expressed in mesenchyme. In the present experiments, the proliferation of epithelial cells, but not of mesenchyme, was accelerated following stimulation with PDGFs. Based on these results, we speculated that some factors from SMG mesenchyme regulate epithelial cell proliferation stimulated by PDGFs. It was reported previously that FGFs are expressed in mesenchyme and induce branching morphogenesis in SMG cultures (21). Therefore, we investigated the expression of FGFs in SMG mesenchyme and organ cultures after stimulation with PDGF-AA and -BB using real-time PCR assays. PDGF-AA induced the expression of Fgf1, -3, -7, and -10, but not of Fgf2 (Fig. 6A), similar to PDGF-BB (Fig. 6B). Of those FGFs, Fgf7 and -10 were found to be expressed in SMG mesenchymal tissue and enhanced branching morphogenesis in previous studies (21, 25). In the present experiments, PDGF-A and -B siRNA completely abrogated the mRNA expression of FGF-A and -B (Fig. 6, C and D) and inhibited the expression of Fgf7 and -10 (Fig. 6, C and D). Negative control and scramble siRNA did not have any effect on the expression of Fgfs in SMGs (data not shown). These results indicate that the expression of Fgf7 and -10 in SMG mesenchymal cells might be regulated by PDGF-A and -B.

Branching Morphogenesis Induced by PDGFs Is Dependent on FGF Signaling—AG17 inhibited PDGF receptor tyrosine kinase and decreased branching morphogenesis in E13 SMG cultures (Fig. 7A). AG17 is known to have an effect on the phosphorylation of the PDGF receptor expressed in mesenchyme and inhibits downstream signaling. In our experiments, ERK1/2 phosphorylation in SMG organ cultures stimulated by PDGF-BB was inhibited by the presence of AG17 and the MEK inhibitor PD98059 (Fig. 7B). Furthermore, the expressions of Fgf7 and -10 were completely inhibited in the presence of AG17 or PD98059 (Fig. 7C). However, when Fgf7 and Fgf10 were added exogenously, SMG branching morphogenesis that was inhibited by AG17 was fully recovered indicating that FGFs function downstream of PDGF signaling (Fig. 7, A and D). Furthermore, Fgf1, -2, and -3 did not affect the SMG branching morphogenesis that was inhibited by AG17, whereas addition of both Fgf7 and -10 was more effective than addition of either Fgf7 or -10.

The known ligands for Fgfr2b are Fgf1, -3, -7, and -10. Both Fgf7 and -10 bind with a high affinity to Fgfr2b (40), whereas Fgf10 binds to Fgfr1b (41). To clarify the effects of PDGF on FGFs induced during SMG organogenesis, we added
PDGF Regulates Branching Morphogenesis

In this study, we analyzed the roles of PDGFs during SMG development. Both epithelial PDGF-A and mesenchymal PDGF-B induced FGF expression in SMG mesenchyme and also enhanced branching morphogenesis.

Vertebrate NC cells include part of a pluripotent cell population that is derived from the lateral ridges of the neural plate during the early stages of embryogenesis (42, 43). During craniofacial development, NC cells migrate ventrolaterally as they populate the branchial arches. As these ectodermally derived cells migrate, they contribute extensively to the formation of mesenchymal structures in the head and neck. The proto-oncogene Wnt1 encodes a short range signal and is only expressed during development of the central nervous system (44, 45).

In a previous experiment that used transgenic mouse lines expressing β-galactosidase under control of the Wnt1 promoter, the population of NC cells that initially emigrated away from the neural tube demonstrated staining for β-galactosidase (46). However, during this Wnt1-LacZ transgenic process, β-galactosidase-positive cells cannot be observed in later embryos, which accurately reflects the cessation of Wnt1 gene expression in the NC progeny, thus preventing its activity following the differentiation of NC cells. Other studies have also shown that crossing of the Wnt1-Cre transgene with the ROSA26 conditional reporter transgene resolves this problem and that NC cells in Wnt1-Cre/ROSA26 mice are β-galactosidase-positive (34, 47). In the present mouse model, nearly all cells in the mesenchyme become β-galactosidase-positive in the developing tooth germ, palate (34), and salivary glands (Fig. 1A). Pdgfrα is also a marker of cranial NC cells (4, 6); however, the molecular basis of this receptor or its ligands in salivary glands has not been clearly elucidated. Furthermore, Pdgfrα mutant mice have a

recombinant FGFR1b (rFGFR1b) and FGFR2b (rFGFR2b) to block endogenous FGFs. Branching morphogenesis by SMGs was dramatically inhibited in the presence of rFGFR2b (Fig. 8, A and B). Furthermore, rFGFR1b also inhibited SMG branching (Fig. 8B). In addition, inhibition of terminal bud growth by rFGFR2b was not rescued by exogenous PDGF-AA or PDGF-BB, although it was partially rescued by excess amounts of FGF7 and -10 (data not shown). SU5402 inhibited FGFR1 signaling and decreased branching morphogenesis by E13 SMGs (Fig. 8C), and FGFR1 was found to be expressed in the epithelial cells of SMGs. After 24 h of SU5402 treatment, the epithelial buds remained small, and did not enlarge or become cleaved (Fig. 8C). Also, PDGF-BB administration did not rescue the inhibi-

branching morphogenesis of SMGs treated with SU5402 (Fig. 8, C and D). These findings demonstrate that FGFR signaling is required for epithelial buds to enlarge and undergo further cleaving, and indicate that PDGF may function as an upstream molecule of FGF during the induction of SMG branching morphogenesis (supplemental Fig. 3).
PDGF Regulates Branching Morphogenesis

FIGURE 7. The PDGFR inhibitor AG-17 inhibited SMG branching morphogenesis, which was rescued by exogenously added FGF7 and -10. A, E13 SMGs were cultured with AG17 with or without 500 ng/ml FGF7 or 1 μg/ml FGF10 for 24 and 48 h. B, ERK1/2 phosphorylation at 5 min after stimulation with PDGF-BB with or without AG-17, and the MEK inhibitor PD98059 in E13.5 SMGs was analyzed. C, FGF7 and -10 expressions in SMG organ cultures with or without AG-17 and PD98059 (PD) were analyzed by real time PCR. D, quantification in A; the numbers of terminal buds at 24 and 48 h were compared with those at 2 h. The effects of FGF1, -3, -7, and -10 in branching morphogenesis of SMGs at 48 h in the presence of AG-17 were also analyzed. Statistical analysis was performed by ANOVA (n = 4 per group; *, p < 0.01).

PDGFs might regulate the proliferation of SMG epithelium and branching morphogenesis.

In another study, microarray-based gene expression profiles of SMGs showed that Fgfr1b and -2b were expressed in E13 SMG epithelium (21), whereas other Fgfrs, i.e. Fgfr1c, -2c, -3, and -4, were expressed in mesenchyme. Fgfr2b-null and soluble FGF2 transgenic mice do not develop salivary glands (48, 49). In addition, SU5402, an FGF1 signaling inhibitor, decreases epithelial proliferation and branching morphogenesis when added to SMG organ cultures. These results suggest that FGF1b and -2b signalings are important for epithelial proliferation and SMG branching morphogenesis. Fgf2, -3, -7, and -10 are each expressed in mesenchyme, whereas Fgf1, -8, and -13 are expressed in both epithelium and mesenchyme (21). In this study, PDGF-AA and -BB induced the expression of Fgf1, -3, -7 and -10 (Fig. 6). Also, PDGF-A and -B siRNA caused a slight decrease in Fgf1, -3, -7, and -10 in cultured SMGs, indicating that PDGFs regulate the expression of FGFs in SMG mesenchymal cells.

FGF7 and -10 and BMP7 each have positive regulatory effects on the number and size of SMG epithelial buds, whereas FGF2 and BMP4 have negative regulatory roles, as they potentially decrease epithelial cell proliferation and may define the sites of cleft formation (21). In our study, an inhibitory FGF, FGF2, was decreased following stimulation by PDGF-AA and -BB and increased by PDGF-A and -B siRNA, which agrees with the notion of enhancement of branching morphogenesis by PDGFs. FGF7- and FGF10-mediated FGF2b signaling regulates SMG epithelial morphogenesis ex vivo, suggesting that localized proliferation and increased expressions of FGF1b/FGF1 and MMP2, as well as increases in both FGF1 and MMP2 activities are required for FGF-mediated epithelium morphogenesis (25). FGF10 is involved in Harderian and lacrimal gland development, where it induces epithelial proliferation but not branching morphogenesis (50, 51). In SMG epithelium, FGF10 promotes duct elongation by localized proliferation at the tip of the duct through ERK1/2-dependent pathways (25). However, the regulatory mechanisms of FGF7 and -10 expressions in SMG mesenchyme are not clearly understood. In our experi-
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Furthermore, PDGF-BB enhances the expression of laminin in vascular smooth muscle cells (53), and PDGFs were found to enhance the abundance of collagen IV and fibronectin mRNA in glomerular mesangial cells (54). These results suggest that PDGFs regulate the expression of ECM molecules. Transient and localized expressions of fibronectin fibrils provide a mechanism for site-specific accumulation of ECM at the site of SMG epithelial clefts. In a previous study, a decrease in fibronectin expression by siRNA was shown to reduce cleft formation, whereas exogenous fibronectin was not affected (55).

Laminin is widely expressed in the basement membrane and is also important for organogenesis. Lama5-null embryos die late during the process of embryogenesis with multiple defects, including exencephaly, syndactyly, absent kidneys and eyes, and lung, tooth, and salivary gland morphogenesis defects (56–58). Lama5-null mutants show decreased branching and expression of Fgfr1b and Fgfr2b in SMGs. Also, siRNA for FGFR decreases the expression of lama5, and exogenous FGF10 rescues branching morphogenesis of SMG, suggesting that FGFR signaling provides positive feedback for lama5 expression. It has also been shown that Adam17-null mice have reduced SMG terminal bud branching, and Mmp14-null embryos show decreases in SMG branching morphogenesis, indicating that ECM and ECM remodeling regulated by MMPs may be important for SMG branching morphogenesis (59, 60). Expression profiling of MMPs in salivary epithelium and mesenchyme revealed that most MMPs are expressed in the mesenchyme, except for MMP2 and the two membrane-bound MMPs, MMP14 and MMP15 (25). Epithelial localization of MMP2 around the periphery of the expanding bud and elongating duct, as well as inhibition of morphogenesis by an MMP inhibitor in the epithelium in a previous study, suggests that MMP2 activation is important for matrix remodeling and epithelial morphogenesis, whereas ECM remodeling, ECM production, and degradation by MMPs may be important for SMG branching morphogenesis.

Important roles for PDGF-A during connective tissue morphogenesis are highlighted by the murine Patch mutation (Ph), which includes a deletion of the α-subunit of the PDGF receptor. Individuals with this patch mutation show severe craniofacial abnormalities (7), and tissues from Ph/Ph embryos exhibit

FIGURE 8. Recombinant FGFR and FGFR1 inhibitor SU5402 inhibited SMG branching morphogenesis. A, E13 SMGs were cultured with recombinant (r) FGFR1b (rFGFR1) and recombinant FGFR2b for 24 and 48 h. B, quantification in A; the numbers of terminal buds at 24 and 48 h were compared with those at 2 h. C, E13 SMGs were cultured with the FGFR1 inhibitor SU5402 with or without PDGF-BB for 24 and 48 h. D, quantification in C; the numbers of terminal buds at 24 and 48 h were compared with those at 2 h. Statistical analysis was performed by ANOVA (n = 4 per group; *, p < 0.01).

ments, exogenously added PDGF-AA and -BB increased SMG branching morphogenesis and FGF expression (Fig. 2, D and E, and Fig. 6, A and B). Furthermore, the PDGFR signaling inhibitor AG-17 inhibited the phosphorylation of ERK1/2 induced by PDGF-BB, along with the expressions of Fgf7 and -10 in salivary mesenchyme. AG-17 also inhibited SMG branching morphogenesis, indicating that PDGFR signaling in salivary mesenchyme is important for FGF7 and -10 expressions, as well as SMG branching morphogenesis. In addition, exogenously added FGF7 recovered the number of terminal buds in the presence of the AG-17, whereas PDGF-BB did not rescue the branching of SMGs in the presence of the FGFR1 inhibitor SU5402 nor with rFGFR1b and -2b, indicating that PDGF signaling occurs upstream of that of FGFRs (supplemental Fig. 3).

Remodeling of the extracellular matrix (ECM) and cell surface by matrix metalloproteinase (MMP) and the ADAM metalloproteidase family is a critical process in branching morphogenesis (52). MMPs have multiple functions, including cleavage of ECM proteins such as collagen, fibronectin, and laminin.
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significant decreases in both MMP-2 and membrane-type MMP as compared with tissues from normal embryos (14). In tooth morphogenesis, loss of the Pdgfra gene does not have an effect on the proliferation and differentiation of odontoblasts in the cranial neural crest-dentodigastic mesenchyme, although it does disturb the formation of the extracellular matrix and organization of odontoblast cells in the cusp-forming area, resulting in a dental cusp growth defect (13). Furthermore, the dramatic reduction in MMP-2 in the NC-derived dental mesenchyme of Pdgfra null mutants suggests a critical role for this extracellular proteinase in normal tooth development. Together, these findings suggest that the expression of epithelial MMP-2 may be regulated by mesenchymal FGFRs via PDGF signaling. Direct evidence of the relationship between PDGFs and MMPs during salivary gland morphogenesis is currently under investigation in our laboratory.

In conclusion, we specified specific roles of PDGFs during normal development, specifically during SMG branching morphogenesis. PDGF-AA and -BB enhanced SMG branching and induced Fgf1, -3, -7, and -10 in neural crest-derived mesenchymal tissues in SMGs. Furthermore, FGF expression and branching morphogenesis induced by PDGFs were inhibited by siRNA for PDGF-A and -B, as well as the PDGFR inhibitor AG-17. Our results indicate that epithelial branching induced by FGF may be regulated by PDGFs in a novel manner via epithelial-mesenchymal interaction.

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