Regulation of Expression of Sprouty Isoforms by EGF, FGF7 or FGF10 in Fetal Mouse Submandibular Glands

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Abstract: Branching morphogenesis of the fetal mouse submandibular gland (SMG) is regulated by signaling through the ErbB and FGF families of tyrosine kinase receptors, whose members activate the ERK-1/2 pathway. The four Sprouty (Spry) proteins are inhibitory modulators of ERK-1/2. There is little information on their expression during pre- and postnatal development of the SMG. Qualitative RT-PCR detected mRNAs for Spry1, 2, and 4 from embryonic day 13 (E13) through postnatal day 7 (P7), but only trace amounts of Spry1 and 2 in adult SMGs. More sensitive quantitative RT-PCR revealed that transcripts for all four Spry isoforms are expressed, and each shows individual patterns of variation across fetal and early postnatal stages, and that there are very low levels of Spry1 and 2, but no Spry3 and 4, in adult glands. EGF, FGF7 and FGF10 upregulate expression of mRNA for Spry1, but only FGF7 upregulates Spry2 mRNA. EGF strongly induces an activating phosphorylation of all four Spry isoforms, but both FGFs do so only minimally. Quantitative RT-PCR of samples collected by laser capture microdissection showed that transcripts for Spry1 are confined to the epithelium of E13 SMG rudiments. The isoform-specific temporal variation in the patterns of expression of Spry1, 2, 3 and 4 suggests a potentially important role for these negative modulators of growth-factor driven ras/ERK-1/2 signaling at stages when the SMG is most actively undergoing branching morphogenesis.

Key words: submandibular gland, Sprouty, EGF, FGF7, FGF10

Introduction
Branching morphogenesis (BrM) is one of the basic processes for the formation of many organs, such as the lungs, the kidneys, and all exocrine glands including the three major salivary glands. The submandibular gland (SMG) of the fetal mouse is a widely used model system to study BrM, since it is capable of undergoing branching in vitro1,2. The mouse SMG initiates development on day 12 of gestation (E12; the day of discovery
of vaginal plug was taken as E0) as a downgrowth of the oral epithelium into the underlying mesenchyme, forming an elongated stalk ending in an expanded endbud. A cleft develops on the distal tip of the endbud, and the epithelium bifurcates as two epithelial stalks elongate from this site and again end in expanded endbuds. This process of clefting and elongation of the epithelium is done repeatedly until the final appropriate extent of branching is reached. Eventually the elongated portions form lumens and become ducts, and the endpieces differentiate into acini. This process is dependent on epitheliomesenchymal interactions mediated by integrins and extracellular matrix, and growth factors and their receptors.

Several growth factors are known to be required for full development of the fetal SMG, including EGF, FGF7, and PDGF. The members of all three of these families of ligands activate membrane spanning receptors that are tyrosine kinases (RTKs), that trigger intracellular signaling cascades. The most commonly used pathways are those leading to activation of ERK-1/2, PLCγ1, and PI3K/Akt. All three of these signaling casettes have been shown to be important for BrM of the fetal mouse SMG.

Sprouty (Spry) was first discovered as an antagonist of fibroblast growth factor (FGF) signaling in branching of Drosophila airway, and of epidermal growth factor (EGF) signaling in eye and wing development via inhibition of the Ras/Raf/MEK/ERK pathway. There are four member of the Spry family called Spry1, 2, 3, and 4, and they have been shown to be general inhibitors of RTK signaling mediated by the Ras/Raf/MEK/ERK pathway. It is known that the phosphorylation of tyrosine residues of Spry molecules is required for their activation. However, depending on the phosphorylation site, the functions of the individual Spry proteins vary.

Expression of Spry genes 1, 2, and 4 has been detected in the perinatal mouse SMG epithelium by in situ hybridization. However, nothing further is known about these proteins in the development of the SMG. To further analyze possible roles for these negative modulators of the ras/ERK-1/2 pathway activated by many of the growth factor systems regulating development of this gland, we have examined expression of the four Spry isoforms across the entire time span of the gland's fetal development, and in early postnatal life and in adults.

Materials and Methods

1. Materials

Recombinant EGF, FGF7, and FGF10 were purchased from R & D Systems Inc. (Minneapolis, MN, USA). Anti-Spry1, 2, 3 and 4 antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA), and Protein G magnetic beads were from New England Biolabs (Ipswich, MA, USA).

2. Organ culture

Pregnant mice (ICR strain) were purchased from Japan SLC Inc. (Hamamatsu, Japan). The day of discovery of the vaginal plug was taken as embryonic day 0 (E0). The SMGs were removed from prenatal (E13 to E18) and postnatal (7 days and adult) mice.

SMG rudiments were cultured as described previously. Briefly, approximately 20 SMGs were taken at E13 (one pregnant mouse), and placed on a membrane filter (Nuclepore membrane, 0.1 μm pore size; Whatman International, Brantford UK) floating on 400 μl of DMEM/F-12 medium (GIBCO Invitrogen Cell Culture, Carlsbad, CA, USA) containing 100 units/ml penicillin (GIBCO Invitrogen Cell Culture), 100 μg/ml streptomycin (GIBCO Invitrogen Cell Culture), 150 μg/ml vitamin C (Sigma, St. Louis, MO, USA), and 50 μg/ml transferrin (Sigma). The SMGs were precultured for 3 hrs on the filters in organ-culture dishes (Falcon Becton and Dickinson Labware, Franklin Lake, NJ, USA); then EGF (50 ng/ml), FGF7 (200 ng/ml) or FGF10 (500 ng/ml) was added to the medium, and the rudiments were cultured in 5% CO2/95% air and 80% humidity at 37°C. The experiments were repeated five times (5 pregnant mice were used).

All procedures of animal handling were conducted in accordance with the Guideline for Experimental Animals of Asahi University (Protocol No. 07-011).
3. Total RNA preparation and qualitative RT-PCR

Total RNA was extracted from SMGs using TRI Reagent (Sigma) following the manufacturer's instructions. The following numbers of SMGs were used, with 20–34 SMG rudiments obtained per liter: E13, 6 pregnant mice; E14, 2 pregnant mice; E15–P7, one pregnant mouse at each age; Ad, 3 mice. Total RNA extracts were then dissolved in nuclease-free H2O and digested by DNase I to remove possible DNA contamination. The total RNA (3 μg) was reverse transcribed to cDNA by a Super Script First Strand Synthesis System for RT-PCR (GIBCO Invitrogen Cell Culture) as described previously5. PCR was carried out in triplicate with the following primers: mouse Spry1 (Acc#: NM_011896), forward 5'-GAAAGGACTCATGAAATCATACC-3' and reverse 5'-GTTATCCGAGTAAGAACCTC-3'; mouse Spry2 (Acc#: NM_011897), forward 5'-AAGCCGAGCATAGCATTATTAATCA-3' and reverse 5'-GCAAACCACAAATCCCCTA-3'; mouse Spry3 (Acc#: NM_001030293), forward 5'-CGCTCTACTCACGCTAGCA-3' and reverse 5'-CCTTCAGAGCACCACTTTG-3'; mouse Spry4 (Acc#: NM_011898), forward 5'-GGCCTGTGGAAAGTGTAAGT-3' and reverse 5'-CCGGTTTCTTCCCAATCTAGG'; and mouse β-actin (Acc#: NM_007393), forward 5'-GTGACGTTGACATCCGTAAAGA-3' and reverse 5'-CTCAGGAGGACAATGATCTT-3'. The PCR reactions were carried out for 35 cycles at an initial denaturing temperature of 95°C for 5 min and a final extension at 72°C for 10 min. Each cycle had a denaturing temperature of 94°C for 1 min, an annealing temperature specific for each primer pair for 1 min, and an extension temperature of 72°C for 2 min. The annealing temperatures were 50°C for Spry1 and 2, 58°C for Spry3 and β-actin, and 54°C for Spry4.

4. Quantitative RT-PCR

For each PCR tube, 1.0 μl cDNA from the above reverse transcription reaction mixture (Super-script) was mixed with 0.5 pmole primers (forward and reverse) and 12.5 μl IQ SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA, USA). Primer pairs and temperatures were as follows: mouse Spry1, forward 5'-GAGGTTCTTACTC GGATAAC-3' and reverse 5'-GGTCCAGTCTGTAACAGC-3'; mouse Spry2, forward 5'-AAAGCGACATTATTAATAC-3' and reverse 5'-GCAAACCACAAATCCCCTA-3'; mouse Spry3, forward 5'-AGCCGAGCTGATTATTTGAGCT-3' and reverse 5'-AGAGCCACATCAGACCTTG-3'; mouse Spry4, forward 5'-CTCCTAGTACCATGGCTCC-3' and reverse 5'-GTGCTTTGTCTAGTCTGAC-3'; and mouse β-actin, forward 5'-GTGACGTGACATCCGTAAGA-3' and reverse 5'-CTCAGGAGGACAAATGATCTT-3'. The PCR reactions were carried out for 40 cycles at an initial denaturing temperature of 95°C for 5 min and a final extension at 72°C for 10 min. Each cycle had a denaturing temperature of 94°C for 10 sec, an annealing temperature specific for each primer pair for 30 sec, and an extension temperature of 72°C for 30 sec. The annealing temperatures were 56°C for Spry1, 2, 3 and 4, 52°C for β-actin. The reactions were carried out in a DNA Engine Opticon System (Bio-Rad) and results were analyzed with Opticon Monitor Analysis Software (Bio-Rad). The amplification amount of each Spry reaction was compared to the amount of β-actin amplification as an internal standard, and the final results were expressed as the ratio of these two amounts.

5. Metabolic labeling of Spry proteins with 32P

E13 SMG rudiments were collected from four pregnant mice (128 SMGs) and preloaded with radioisotope by culture under the above conditions for 2.5 h in medium containing 0.5 mCi/ml 32P-orthophosphoric acid, before addition of EGF, FGF7 or FGF10 at the concentrations used above, and culture was continued for 30 min. Incubation was terminated by washing the tissues on the filters with ice-cold PBS containing 1 mM Na3VO4, and the SMG rudiments were collected and homogenized in 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 2.5 mM Na2HPO4, 1 mM β-glycerophosphate, protease inhibitor cocktail (Sigma) and phosphatase inhibitor cocktail 2 (Sigma), and supernatants were processed for immunoprecipitation, as
described previously. Equal amounts of radioactivity (1.77 × 10^4 cpm) were used for immunoprecipitation, and the precipitates were resolved by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and the gels were dried and exposed to X-ray film.

6. Laser capture microdissection

SMG rudiments were collected from E13 mice (96 SMGs) and embedded into OCT compound, and frozen sections (8 μm) were cut in a cryostat. The SMG sections were stained with crystal violet (LCM Staining Kit, Ambion). From each section, samples were collected from three epithelial sites and one mesenchymal site, each containing about 500 cells, using Laser Capture Microdissection (PALM-MBIII, P.A.L.M. Microlaser Technologies AG, Germany). The three epithelial samples were from (1) ducts, (2) endbud epithelium in contact with mesenchyme, and (3) inner epithelial cells of the endbud (see Fig. 6A). Total RNAs were prepared from the captured cells using a RNeasy Micro Kit (QIAGEN Sciences, Germantown, MD, USA), and then cDNA synthesis and real-time RT-PCR were performed as described above.

Results

1. RT-PCR analyses of Spry isoforms

Qualitative RT-PCR of total RNA from intact submandibular glands from embryonic day 13 to 18 (E13 to E18), and from newborns (NB), postnatal day 7 (P7), and adults detected mRNAs for Spry1, 2, and 4, but not for Spry3 (Fig. 1).

Quantitative real-time RT-PCR confirmed the presence of transcripts for Spry1, 2, and 4 and also detected the mRNAs for Spry3 (Fig. 2). The

Fig. 1 RT-PCR for Spry1, 2, and 4 in developing mouse SMG. Total RNAs from SMGs of E13-E18, newborn (NB), postnatal day 7 (P7) and adult (Ad) were transcribed to cDNA, and the target sequences were amplified with each specific primer for target sequences. The PCR products were subjected to agarose gel electrophoresis and visualized using ethidium bromide. The experiments were repeated three times.

Fig. 2 Quantitative analyses of Spry mRNAs in developing mouse SMG by real-time RT-PCR. The amplification amount of each of the Spry cDNAs (A: Spry1, B: Spry2, C: Spry3, and D: Spry4) which were synthesized from E13-E18, newborn (NB), postnatal day 7 (P7) and adult (Ad) mouse SMGs using reverse transcriptase were analyzed. Amount of each Spry gene was expressed as the ratio of the amounts of Spry and β-actin cDNAs. The experiments were repeated three times, with four SMGs per age group.
pattern of expression is specific for the mRNA of each isoform across the course of the gland’s development. Spry1 mRNA is expressed at an unchanging level from E13 through P7, but is dramatically reduced in adult glands (Fig. 2A). Levels of Spry2 transcripts are high at E13 and E14, then decline from E15 to birth, rise again at P7, and are lowest in adult glands (Fig. 2B). Spry3 transcripts are highest at E13, but remain low from E14 to birth; there is a slight increase at P7, but they are essentially absent in adults (Fig. 2C). Spry4 mRNA levels are highest at E13 and gradually decline thereafter, and are apparently absent in the adult (Fig. 2D). The ranges of CT (CT = 25–29) values for Spry1 and Spry2 are lower than those of Spry3 and Spry4.

2. Morphological changes by three major growth factors

Spry proteins are specific inhibitors of the Raf-ERK-1/2 intracellular signaling cascade, which is activated by many receptor tyrosine kinases, including both the ErbB and FGFR families of receptors. As previously reported by us and others,5,18,19 EGF increases branching morphogenesis in E13 SMGs cultured in vitro (Fig. 3A, B), but the two FGF have only a slight or minimal effect on this process in this gland (Fig. 3C, D).

3. Inductions of transcription and phosphorylation of Spry mRNA by growth factors

We found, furthermore, that all three of these ligands of these two RTK families significantly upregulate expression of Spry1 and Spry2 mRNAs, with FGF7 having the strongest effect (Fig. 4A, B). Spry proteins must be phosphorylated in order for them to inhibit Raf-ERK-1/2 signaling20. However, levels of Spry proteins are very low, and are very difficult to detect by immunoblotting studies. To determine if these proteins are phosphorylated in response to EGF or FGF7 or FGF10, we exposed E13 SMGs to these growth factors.
Factors in the presence of $^{32}$P-orthophosphate. The supernatants of these glands were then subjected to immunoprecipitation with antibodies specific for each of the four Spry isoforms, and the immunoprecipitates were resolved by electrophoresis and transferred to membranes for autoradiography. The radioactive tag greatly improved the sensitivity of these immunoprecipitation techniques, and all four Spry isoforms were then detectable in the fetal SMGs (Fig. 5). EGF had the greatest effect and strongly phosphorylated all four Spry proteins. FGF7 mildly stimulated Spry2, 3 and 4, and FGF10 had a slightly greater effect on Spry1 and 3.

4. Localization of Spry transcript in E13 SMG rudiments

Since levels of the Spry proteins are so low, we were unable to demonstrate their tissue localization by immunocytochemical techniques (data not shown). Thus, we probed the cellular localization by laser capture microdissection and RT-PCR. Samples were chosen from four sites (Fig. 6A): the epithelium of ducts, the outer epithelium of the endbud in contact with mesenchyme, the inner endbud epithelium, and the mesenchyme. All three epithelial compartments showed the same amount of Spry1 transcripts, but the mesenchyme had only negligible message (Fig. 6B).

Discussion

The present findings demonstrate that the four members of the Spry family are expressed in the fetal SMG, that mRNAs for Spry1, 2 and 4 are
present at higher levels than the mRNA for Spry3, and that EGF or FGF7 or 10 not only upregulate expression of these genes, but also can stimulate phosphorylation and activation of these proteins.

The present study is the first to measure the amounts of mRNAs for Spry1, 2, 3, and 4 that are endogenously expressed at all developmental stages in the mouse SMG. The results of real-time RT-PCR showed that mRNAs for Spry1, 2, 3, and 4 were expressed in the SMG rudiments from E13 to E18 and newborn, postnatal 7 days and adult mice. All Spry mRNAs were decreased at adult ages. Because mRNAs of Spry1, 2, 3, and 4 are highly expressed in fetal and early postnatal stages, they most likely are involved mainly in modulating developmental processes of fetal mouse SMGs.

Spry transcripts are known to localize to epithelium in developing mouse SMG, lung, kidney, and tooth by in situ hybridization\textsuperscript{16}. The transcripts were most strongly expressed in the distal leading tips of the epithelial endbuds of the SMG and lung, but were not seen in the mesenchyme. We collected total RNA from epithelial and mesenchymal tissues from E13 SMG sections using Laser Capture Microdissection, and we showed that Spry1 is highly expressed in epithelium by real-time RT-PCR, but not in the mesenchyme, consistent with Zhang et al.\textsuperscript{16}

Mailleux et al.\textsuperscript{21} reported that both Spry2 mRNA expression and the size of the epithelial endbuds are increased in E12 lung rudiments cultured in the presence of FGF10. Spry2 most likely regulates organogenesis of the lung by suppressing ERK signals stimulated by FGF10. Mai et al.\textsuperscript{22} reported agenesis of the SMG and lacrimal glands in mice with a missense mutation in the Fgfr2 gene. Patel et al.\textsuperscript{23} found increased levels of the mRNAs for Spry1 and 2, but not Spry4, in mesenchyme-free fetal SMG epithelium in Matrigel cultured in the presence of a synthetic sulfated oligosaccharide, which presumably forms a complex with FGFs to activate the FGFR1b.

However, there has been no information about the actual amounts of the mRNAs for Spry isoforms induced by direct stimulation by growth factors in the developing mouse SMG. We showed that mRNAs for Spry1 and Spry2 were significantly increased by the administration of EGF, FGF7 or FGF10 in E13 SMG rudiments. These data suggest complex regulatory interactions between these growth factors and these downregulators of ERK-1/2 signaling during organogenesis of fetal mouse SMG.

Other investigators using cultured cell systems found that several growth factors were able to stimulate the phosphorylation of tyrosine residues of Spry proteins, and these phosphorylations are known to be required to activate the inhibitory functions of Spry proteins. In this study, we showed that all three growth factors (EGF, FGF7 and FGF10) stimulated phosphorylation of Spry1, 2, 3, and 4. EGF was the strongest stimulator for the phosphorylation of Sprys, whereas the two FGFs were weaker stimulators, and had no effect on Spry2. These results imply that the modulating effects of Sprys on ERK signaling would vary depending on which growth factor is active in triggering this intracellular signaling cascade, and may be relevant in explaining differences in morphogenesis events in fetal mouse SMGs induced by different growth factors, such as EGF or the FGFs.

There results show that the levels of expression and/or phosphorylation of Sprys are regulated by the growth factors, and that the Spry isoforms may function as modulators of development of the mouse SMG through modulating the ras/ERK-1/2 signaling network. Given that Spry proteins act as inhibitory modulators of the Raf-ERK-1/2 signaling pathway that is involved in many networks triggered by many RTKs responsive to a wide variety of growth factors, it is at present not possible to define the precise roles that Spry proteins play in the fetal mouse SMG, but their presence unquestionably has functional importance since the levels of individual Spry isoforms differ from each other across development. The observation that the growth factors used in this study resulted both in increased phosphorylation and activation of ERK1/2, as well as increased expression of the Spry isoforms, suggests that the latter may be acting as modulators in negative feedback loops in this signaling cascade. There are no known inhib-
itors of the Spry family members, so an approach using siRNAs would likely be most effective in pursuing these investigations\textsuperscript{17}. Indeed, by using knockout or transgenic mice for Spry1 it has been demonstrated that this isoform acts to oppose and balance the stimulatory effects of GDNF on branching morphogenesis in the developing kidney\textsuperscript{24}. It is reasonable to suggest that the Spry family of proteins may play similar roles in the developing SMG.

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References


