Germ Line Gain of Function with SOS1 Mutation in Hereditary Gingival Fibromatosis

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The abbreviations used are: HGF, hereditary gingival fibromatosis; CCNE1, cyclin E1; CCNE2, cyclin E2; EGFR, epidermal growth factor; EGFR, EGFR receptor; ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; PCNA, proliferation cell nuclear antigen; pRb, phospho-Rb; SOS1, Son of Sevenless-1; GEF, guanine nucleotide exchange factor; PC12, pheochromocytoma tumor suppressor protein; pRB, phospho-Rb; SOS1, Son of Sevenless-1; GEF, guanine nucleotide exchange factor; MEK, MAPK/ERK kinase; HA, hemagglutinin; TRITC, tetramethylrhodamine isothiocyanate; TFPD1, -2, transcription factors DP1 and -2; TBP, TATA-box-binding protein; siRNA, small interference RNA.

An SOS1 g.126,142–126,143insC insertion mutation causes a frameshift and early termination of the protein, yielding a chimeric 1,105-amino acid protein that consists of 1,083 SOS1 N-terminal amino acids followed by 22 novel amino acids. This truncation abolishes four C-terminal proline-rich motifs, which are required for Grb2 binding. The form of HGF due to SOS1 mutation is designated HGF1.

SOS1 functions as a guanine nucleotide exchange factor (GEF) that couples receptor tyrosine kinases to the Ras signaling pathway and controls cell proliferation, differentiation, vesicle trafficking, and regulation of the actin cytoskeleton. Under the control of two classes of regulatory proteins, GEF and GTPase-activating proteins, Ras functions as a molecular switch between GDP/GTP cycling. Three Ras-GEFs; SOS, guanine nucleotide-releasing factor, and guanyl nucleotide-relasing protein, have been characterized in controlling Ras activation by catalyzing GDP release and association with GTP (7–11). Two regulatory regions govern the GEF activity of SOS1: a catalytic site that interacts with nucleotide-free Ras and an allosteric site that enhances exchange activity with the binding of nucleotide-Ras (12, 13). Upon activation of receptor tyrosine kinase, Grb2-SOS1 complexes are recruited to the plasma membrane leading to the exchange of GDP for GTP and Ras activation (14). Although the Grb2-SOS1 complex functions exclusively as an Ras activator, SOS1 can also function as a GEF that is specific to the GTPase Rac1. These two distinct catalytic functions of SOS1 are mutually exclusive and reciprocally related (7).

The activation of Ras signal stimulates downstream signaling pathways, including the mitogen-activated protein kinase (MAPK) family, a ubiquitous signal transduction pathway (15, 16). Differences in the duration, intensity, spatial distribution, and temporal qualities of ERK signaling govern distinct biological responses (17–19). For example, transient induction of ERK signaling by EGF leads to proliferation of PC12 cells, whereas sustained signaling induces differentiation (20–25). In response to extracellular stimuli, MAPK signaling exerts control of cell cycle progression. Translocation of activated ERK from the cytoplasm to the nucleus is necessary for activation and stabilization of Elk1, c-Jun, c-Myc, and c-Fos. These transcription factors then regulate the expression of genes, such as cyclin D1 and p21WAF1/CIP1, which are critical for the progression from G1 to S phase (26, 27).

Increased cell proliferation has been reported for HGF1 gingival fibroblasts in both monolayer and three-dimensional matrix cultures (28). Here we report that mutant SOS1 contributes an increased and sustained activation of ERK signaling in...
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HGF1 fibroblasts under serum-starved conditions. Sustained ERK signaling leads to increased expression of cell cycle regulators and transcription factors. RNA interference-mediated SOS1 depletion was used to confirm the association between SOS1 mutation, ERK activation, and gingival fibroblast proliferation. These findings document a gain-of-function SOS1 mutation and provide a molecular mechanism for gingival overgrowth in HGF1.

EXPERIMENTAL PROCEDURES

Isolation of Fibroblasts and Cell Cultures—Human gingival tissues were obtained with informed consent from three normal subjects (control) and three patients (HGF1). All patients were heterozygous carriers of the SOS1 g.126,142–126,143insC mutation. Three sets of gingival fibroblasts from HGF1 patients and age-matched normal controls (ages 20–24 years) were isolated and grown in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum and antibiotics-antimycotic solution (growth medium) at 37°C in a 5% CO2-humidified incubator and maintained up to 10 passages (28). HeLa cells were obtained from ATCC (Manassas, VA).

Plasmid Construction, Transfection, and Subcellular Fractionation—The full-length human SOS1 expression plasmid (pCGN-HASOS1) was a gift from D. Bar-Sagi (State University of New York, Stony Brook, NY). The vector control plasmid was generated by HindIII digestion to release the HA-SOS1 insert and self-ligated. The QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) was used with oligonucleotide (5’-AGCATCTGCACCAAATTCTTCCcAAGAACACCGTTA-3’, GenBank™ accession number L13857) to generate the mutated pCGN-HASOS1 construct that carried the HGF1 mutation (small case and cytosine insertion). The mutation was confirmed by DNA sequencing. For transient transfection, 15 × 10⁵ cells/well were seeded in 6-well plates a day before experiments. Expression constructs (2 μg) were transfected into primary gingival fibroblasts using JetPEI reagent (ISC Bioexpress, Kaysville, UT) or into HeLa cells by Lipofectamine 2000 (Invitrogen). Transfection was terminated 48–72 h post-transfection, and total cellular lysates were obtained (29). To study the subcellular distribution of SOS1, 100 × 10⁵ HeLa cells were plated in 10-cm dishes 24 h before transfection. Eight micrometers of indicated expression constructs were used, and transfected cells were harvested 48 h post-transfection. Cellular fractions were isolated using the ProteoExtract Subcellular Proteome Extraction kit (EMD Biosciences, La Jolla, CA).

Cell Proliferation and Ras Activation Assays—For proliferation assays, 1 × 10⁵ cells/well of primary gingival fibroblasts were seeded in 48-well plates. After 24 h the growth medium was replaced with Dulbecco’s modified Eagle’s medium containing 20 mM HEPES (starving medium) for overnight. The medium was then replaced with growth medium and maintained for 9 days. Cells from triplicate wells were trypsinized at each time point, and the total cell number was determined by using a Coulter Counter (Beckman Z series). To monitor Ras activation, cells were seeded in 150-mm culture dishes, grown to 85% confluence, and serum-starved for 16 h. After treatment with EGFR (25 ng/ml) for 0.5–6.0 h at 37°C, cellular extracts were collected in lysis buffer (25 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10 mM MgCl2, 1 mM EDTA, 1% Nonidet P-40, 0.5% sodium deoxycholate, 10% glycerol, 2.0 mM phenylmethylsulfonyl fluoride, 20 μM Na3VO4, 10 μM NaF, 1 μg/ml phosphatase A, and 10 μg/ml aprotinin plus protease inhibitor tablet (Complete tablet, Roche Applied Science). Ras activity was measured using a Ras Activation kit (Upstate, Charlottesville, VA). For each reaction, 600 μg of whole cell lysate was incubated with Raf-1 Ras binding domain-agarose (15 μg) for 1 h at 4°C. The complexes were collected by centrifugation and washed five times with buffer (25 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10 mM MgCl2, 1 mM EDTA, 10% glycerol, 20 μM NaF, and 1% Nonidet P-40). Protein complexes were released with SDS sample buffer, separated by 4–12% NuPAGE, and transferred to a polyvinylidene difluoride membrane. Proteins were detected by mouse anti-Ras antibody (0.05 μg/ml) and goat anti-mouse horseradish peroxidase-conjugated secondary antibody (Bio-Rad, 1:5000).

Culture Treatment, Western Blot Analyses, and Indirect Immunofluorescence Staining—To monitor the level of phosphorylated ERK1/2, the cultures were either maintained in growth medium or switched to starving medium for 16 h before addition of EGF (Upstate) with indicated concentrations and incubation times. In some experiments, cultures were treated with AG1478 (EGFR inhibitor, 10 μM, EMD Biosciences) or PD98059 (MEK inhibitor, 10 μM, EMD Biosciences) 30 min prior to the addition of EGF as indicated (Fig. 2, legend). At each time point, cultures were washed with cold phosphate-buffered saline, and whole cellular extracts were prepared by adding lysis buffer directly into monolayer cultures. Isolation of nuclear extracts and Western blotting were conducted as previously described (29). After washing with phosphate-buffered saline with 0.1% Tween 20, the primary antibodies were detected with a polyclonal goat anti-rabbit or anti-mouse IgG coupled with horseradish peroxidase. Primary antibodies included mouse anti-HA (16B12, 1:1500, Covance, Berkeley, CA), mouse anti-β-actin (1:800, Sigma), mouse anti-SOS1(N) (1:250, epitope at N-terminal, BD Biosciences Pharmingen), rabbit anti-SOS1(C) (1:500, epitope at C-terminal), rabbit anti-Egr-1 (1:300), rabbit anti-EGFR (1:300), rabbit anti-phospho-retinoblastoma (pRB, Ser-780), rabbit anti-retinoblastoma (RB), and mouse anti-PCNA (1:500) from Santa Cruz Biotechnology, rabbit anti-extracellular signal-regulated kinase (ERK1/2, 1:2000), and rabbit anti-phospho-ERK1/2 (1:2000) from Upstate. Signals were detected by using ECL Western blotting Detection Reagents (Amersham Biosciences) and exposed to x-ray film (XAR, Kodak). All experiments were conducted at least three times and quantitated using a FluorChem digital imaging system (Alpha Innotech, San Leandro, CA) and National Institutes of Health Image 1.63 software.

Results were adjusted for loading controls. Immunofluorescent staining was conducted as previously described (30). After transfection, gingival fibroblasts were maintained in either growth or starving medium for 16 h and fixed with paraformaldehyde (3.7%) for 15 min at room temperature. Cells were incubated with anti-HA (1:1000) for 1 h at room temperature. After washing, the cells were incubated with goat anti-mouse conjugated with TRITC IgG antibody for 30 min. After wash-
ing, the cell nuclei were stained with 4′,6-diamidino-2-phenylindole, dihydrochloride (Sigma) for 5 min at room temperature and washed three additional times. Slides were examined with fluorescence microscopy (Olympus, IX71), and images were processed using Adobe Photoshop CS.

**Real-time PCR**—Total RNA from equivalent cell densities of control and HGF1 fibroblast cultures maintained either in starving medium for 16 h or switched to growth medium for 1 day were prepared by TRIzol solution (Invitrogen) and the star substrate (SuperArray Bioscience). Membranes were detected with chemiluminescence using a copalyl diphosphate-DE). Reverse transcription-PCR experiments were conducted as previously described (32) with modification. TaqMan probes and PCR primers were purchased from ABI Biosystems (Foster City, CA). These included SOS1 (Hs00362308_m1), E2F1 (Hs00153451_m1), E2F2 (Hs00 231667_m1), transcription factor DP1 (TFDP1, Hs00955488_m1), transcription factor DP2 (TFDP2, Hs00232366_m1), cyclin E1 (CCNE1, Hs0023356_m1), and cyclin E2 (CCNE2, Hs00180319_m1). The RNase P or TATA-box-binding protein (TBP) was used as endogenous control for normalization. QPCR Human Reference Total RNA (Strategene) was used as a calibrator in all quantitative reverse transcription-PCR experiments. Relative levels of the indicated transcripts in each sample were calculated as 2−ΔΔCT, where ΔΔCT = [target gene’s CT − TBP CT]sample − [target gene’s CT − TBP CT]calibrator. Experiments were performed at least three times with triplicate samples.

**RNA Interference Knock-down and Focus Oligoarray Analysis**—Duplex RNA of SOS1 target sequences flanking the cytosine insertion were synthesized with the Silencer siRNA Construct- tion Kit (Ambion). Transient transfection experiments were conducted as previously described (30). Briefly, 18 × 10^6 cells/well of gingival fibroblasts were plated in 6-well plates 24 h before transfection. Double-stranded siRNA alone or together with SOS1 expression constructs (1 μg/35-mm) was introduced into HeLa cells by Lipo-2000 (Invitrogen). The siRNA for Luciferase (5′-CTTAGCTGAGTACTCTCGA) (31) was used as a control. Total cellular lysates were collected after 48-h transfection and subjected to Western blot analyses. To monitor the effects of knock-down on proliferation, fibroblasts were seeded at 8000/well into 48-well plates. Cultures were transfected with indicated siRNA (50 nM) overnight and replaced with fresh growth medium (day 0). At each time point, the total number of cells was determined as described above. Experiments were performed at least three times with triplicate samples.

**RESULTS**

**Expression of Mutant SOS1 in Gingival Fibroblasts**—The insertion mutation of SOS1 in HGF1 results in an early termination as illustrated in Fig. 1A. To verify the presence of truncated SOS1 in HGF1 fibroblasts, antibody with the epitope against the N-terminal region of SOS1 (SOS1(N)), detected one asterisk indicates significant difference between HGF1 and control (p < 0.05).
corresponds to the full-length SOS1 and the smaller ~130-kDa band approximates the calculated molecular weight of the truncated mutant SOS1. To confirm this, expression constructs of wild-type and mutant SOS1 were transfected into primary gingival fibroblasts. As shown in Fig. 1B (lower panel), the expressed wild type (arrow a, lanes 2 and 5) and mutant (arrow b, lanes 3 and 6) bands were detected at the same positions as the endogenous full-length (lanes 1 and 3) and truncated SOS1 (lanes 4 and 5). Western blotting results revealed the intensity of the mutant (lower) band was 40% less than the full-length (upper) band in HGF1 samples (Fig. 1B). The lower amount of mutant SOS1 could result from the instability of either its transcript or its protein product. Real-time PCR experiments were conducted to monitor the total levels of both wild-type and mutant SOS1 transcript in HGF1 fibroblasts. Overall levels of SOS1 transcript were 40% lower in HGF1 fibroblasts than in control fibroblasts (Fig. 1C). The TBP transcript served as an internal control. The lower levels of mutant SOS1 protein in HGF1 fibroblasts likely reflect a less stable mutant SOS1 transcript. Because all three sets of controls and HGF1 patients showed similar results, only results from one set of control and HGF1 fibroblasts are presented.

Sustained Activation of MAPK in HGF1 Gingival Fibroblasts—Because SOS1 plays a critical role in the Ras/MAPK/ERK signaling pathway (22), the effect of the SOS1 mutation on Ras signaling was evaluated. Although Ras activity was low in serum-starved control fibroblasts, it increased rapidly after EGF treatment and subsequently deceased, approaching basal levels by 30 min (Fig. 2A). In contrast, Ras activity was 5-fold higher in HGF1. Upon EGF stimulation, Ras activity in HGF1 fibroblasts showed higher levels than control in all respective time intervals suggesting mutant SOS1 remained active even under serum-starved conditions, leading
to higher Ras activity in response to growth factor stimuli. We next studied how the SOS1 mutation altered signal transduction through the MAPK/ERK pathway. As shown in Fig. 2B, panel a (lanes 2–5), transient activation of ERK1/2 with increasing concentrations of EGF resulted in a dose-related increase in both control and HGF1 fibroblasts. The magnitude of ERK1/2 activation was at least 30% greater in HGF1 than in control fibroblasts at each respective dosage of EGF treatment (Fig. 2B, panel b). In untreated cells, whereas the ERK1 signal was weak but detectable only in HGF1 samples, the ERK2 signal alone showed ~3-fold greater intensity in HGF1 than in control fibroblasts (Fig. 2B, panel a, lane 1). These data suggest Ras remains active and leads to a sustained activation of ERK1/2 signal in serum-starved HGF1 fibroblasts. Activation of ERK1/2 by EGF was evaluated in the presence of the selective pharmacological inhibitors AG1478 and PD98059 (32, 33). The presence of PD98059 or AG1478 resulted in a similar degree of reduction on phospho-ERK signal in both cell types (Fig. 2C, panel a, lanes 1–4). The overall phospho-ERK1/2 level remained higher in HGF1 than in control fibroblasts (Fig. 2C, panel b). The duration of ERK activation was studied through the continued induction of EGF. In the presence of EGF, the phospho-ERK signal increased and peaked at the 1-h time point in both cell types (Fig. 2D). The signal gradually returned to basal levels after 6 h (Fig. 2D, panel a). Although the pattern of phospho-ERK response to EGF induction was similar in both cell types, the level was 50% higher in HGF1 than in control fibroblasts (Fig. 2D, panel b).

The effect of PD98059 on the duration of EGF-induced ERK signaling is shown in Fig. 2E. Activation of ERK signaling was reduced in the presence of MEK inhibitor in both cell types (Fig. 2E, panel a). However, the pattern of reduction was different in HGF1 compared with control cells (Fig. 2E, panel b). After 20-min incubation with inhibitor, 30% of phospho-ERK remained in control fibroblasts, whereas >80% of phospho-ERK remained in HGF1 fibroblasts. The phospho-ERK signal was sustained up to 2 h in HGF1 but not in control fibroblasts. At 4 h, phospho-ERK decreased to basal levels in controls, however, significant levels of active ERK signal remained in HGF1 fibroblasts. These results demonstrate that, in the absence of growth factors, Ras together with its downstream ERK1/2 signaling remained active and sustained in HGF1 fibroblasts. Transient induction by EGF elicited a stronger response, both in magnitude and duration, indicating the functional consequence of the SOS1 mutation in HGF1 fibroblasts.

**FIGURE 2. Ras activity and ERK signaling in HGF1 fibroblasts.** Ras and ERK activation assays. A, serum-starved control and HGF1 fibroblasts were either untreated (0 min) or treated for 5, 15, and 30 min with EGF (50 ng/ml). Ras activation was evaluated by the binding of Ras to Raf-Ras binding domain and blotted with Ras antibody. Total Ras protein in whole cell lysate (WCL) is shown in the lower panel. The activation is expressed as -fold change over untreated control. B–E, ERK activation assays. B and C, panel a, serum-starved fibroblasts were either treated with EGF (B, panel a, lanes 1–5: 0, 5, 10, 25, and 50 ng/ml) for 10 min or preincubated with Me2SO (C, panel a, lane 2). PD98059 (lane 3), or AG1478 (lane 4) for 30 min before addition of EGF (50 ng/ml) for 10 min (lanes 2–4). Cellular extracts were probed with antibodies of phospho-ERK1/2 and total ERK1/2 as a control. B and C, panel b, quantification of data from a. The data of control (solid bar) and HGF1 fibroblasts (striped bar) were normalized with respective ERK1/2 and expressed as the -fold changes over control (lane 1). D, a, cellular extracts of serum-starved fibroblasts treated with EGF (50 ng/ml) for indicated time intervals were probed with anti-phospho-ERK1/2 and β-actin antibodies. b, quantification of data from a. The data of control (dashed line) and HGF1 fibroblasts (solid line) were normalized with each respective β-actin and expressed as the -fold changes over control fibroblasts. E, panel a, serum-starved fibroblasts were treated with EGF (50 ng/ml) for 10 min, and medium was replaced with fresh medium containing PD 98059 (10 μM) (lanes 3–6 and lanes 9–12) and harvested at the indicated time points. Cellular extracts were probed with antibodies of phospho-ERK1/2 and total ERK1/2 as a control. Panel b, quantification of data from a. The data of control (dashed line) and HGF1 fibroblasts (solid line) were normalized with each respective EPIK1/2 and expressed as the -fold changes over control (lane 1).
wild-type-transfected fibroblasts were similar to fibroblasts transfected with three times less mutant construct (lanes 2 and 4). These results provide evidence that the presence of mutant SOS1 leads to higher levels of phospho-ERK signal in gingival fibroblasts.

Selective Knock-down of Wild-type and Mutant SOS1 by siRNA—RNA interference experiments were conducted to see if eliminating the endogenous mutant SOS1 reduced ERK signaling and affected fibroblast proliferation. To achieve the specific knock-down on mutant transcript, the siRNA target sequences were designed to flank the mutation site. Three sets of siRNAs were designed to include the extra cytosine base together with either upstream or downstream sequences around the mutation site (Fig. 5A, mS, mR, and mL). To evaluate the specificity and effectiveness of each siRNA, the duplex RNAs were co-transfected with indicated SOS1 expression constructs into HeLa cells. Each siRNA showed different degrees of knock-down effect (Fig. 5B). Almost all expressed full-length SOS1 was depleted in wS siRNA-treated samples (lane 3). Trace levels of SOS1 may represent endogenous SOS1, because it was equivalent to that in the vector control (lanes 1 and 3). Whereas wS showed ~100% depletion, mL exhibited ~60% knock-down effect on wild-type SOS1 (lane 3 and 4). As for the other duplex RNAs, whereas mL reduced about half (lane 6), mR did not have any effect on knock-down of wild-type SOS1 (lane 5). In the presence of expressed truncated SOS1 transcript, the wS and mL siRNAs showed slight or no knock-down effect when compared with controls (lanes 7, 8, and 11). In contrast, both mS and mR siRNAs inhibited all of the expressed truncated SOS1. The wS siRNA was therefore chosen to target the wild-type SOS1 transcript, and mR siRNA was chosen to target mutant transcript due to its nominal effect on wild-type SOS1. To confirm the above observations, different dosages of mR siRNA were co-transfected with either wild-type or mutant SOS1 expression construct into HeLa cells. Fig. 5C shows the level of wild-type SOS1 was gradually reduced as the dosage of mR increased, however, it was still detectable even when 100 nM of mR was applied (lane 6). In contrast, mutant SOS1 transcript was depleted completely when as little as 25 nM of mR was used (lane 9). These results demonstrate that mR duplex RNA could specifically and effectively knock-down the expression of wild-type SOS1.

The knock-down effects of wS and mR siRNA on endogenous SOS1 and ERK signaling are shown in Fig. 5D. Wild-type and mutant SOS1 were depleted 60 and 30%, respectively, by wS siRNA (lane 2). Although the mR siRNA did not affect the level of endogenous wild type, it showed a dose-response reduction on the endogenous mutant SOS1 with almost complete depletion at 50 nM (lane 6). The functional effects of SOS1 knock-down were demonstrated through the level of phospho-ERK signaling (Fig. 5, D and E). When transfected HGF1 fibroblasts were maintained under serum-starved conditions, acti-
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little effect on wild-type SOS1 depletion, the phospho-ERK signal decreased as the concentration of mR increased (lanes 3–6) indicating that, under serum-starved culture conditions, mutant SOS1 contributed to sustained phospho-ERK signaling in HGF1 fibroblasts. These results demonstrate that mR siRNA can specifically target endogenous mutant transcript, with little if any effect on endogenous wild-type transcript of SOS1.

**SOS1 Depletion Results in Decreased Fibroblast Proliferation**—To study the consequences of SOS1 depletion on gingival fibroblast proliferation, fibroblasts were transfected with the indicated siRNAs, and cell proliferation was monitored. Proliferation of control fibroblasts treated with wS (Fig. 6A, striped bar) decreased 20% on day 2 compared with untreated controls (solid bar). On day 4, cell numbers were further decreased. Although cell numbers increased at both days 6 and 8, the number of wS-treated fibroblasts was still ~30% lower than the untreated control fibroblasts. As expected, mR siRNA did not show any effect on the growth of control fibroblasts (unfilled bar). A similar trend appeared when wS and mR duplex RNAs were used together (dotted bar), although the reduction in cell proliferation was greater compared with the cultures treated with wS alone (striped bar). Although depletion of wild-type SOS1 reduced the proliferation of control fibroblasts, after day 4, cell proliferation started to recover, indicating the knock-down effects last ~4 days. When the same experiment was conducted for HGF1 fibroblasts in the presence of either wS or mR siRNAs, cell proliferation also decreased (Fig. 6B). Depletion of wild-type SOS1 (striped bar) resulted in 35% decrease in cell proliferation compared with control HGF1 fibroblasts (solid bar) from day 2 to day 6; however, no significant difference was observed by day 8. Knock-down of mutant SOS1 (unfilled bar) resulted in a >50% reduction in cell proliferation compared with HGF1 controls; however, the cell growth remained low and did not recover on day 8 as for the wild-type SOS1 (wS) knock-down. When HGF1 fibroblasts were treated with both wS and mR siRNAs, cell proliferation decreased ~70 and ~80%...
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**TABLE 1**

Comparison of sustained versus transient ERK signaling on the expression of cell cycle regulators

| Symbol | GenBank\textsuperscript{TM} | Description (gene name) | EGF-treated/untreated\textsuperscript{a} | HGF1/normal control\textsuperscript{b} |
|--------|-----------------------------|-------------------------|------------------------------------------|-------------------------------------|
| CCNC   | NM 005190                   | Cyclin C                | 2.1                                      | 7.2\textsuperscript{b}             |
| CCND1  | NM_053056                   | Cyclin D1               | 1.3                                      | 4.1                                 |
| CCND2  | NM_001759                   | Cyclin D2               | 1.8                                      | 4.6                                 |
| CCNE1  | NM_001238                   | Cyclin E1               | 1.5                                      | 3.5                                 |
| CCNE2  | NM_004702                   | Cyclin E2               | 2.4                                      | 5.2                                 |
| E2F1   | NM_005225                   | E2F transcription factor 1 | 1.8                                      | 2.3                                 |
| E2F2   | NM_004091                   | E2F transcription factor 2 | 2.0                                      | 2.9                                 |
| PCNA   | NM_182649                   | Proliferating cell nuclear antigen | 2.6                                      | 3.6                                 |
| RB1    | NM_000321                   | Retinoblastoma 1 (including osteosarcoma) (Rb) | 5.1                                      | 3.8                                 |
| RBBP8  | NM_002894                   | Retinoblastoma binding protein 8 (CTIP) | 4.1                                      | 15.6                                |
| TFPD1  | NM_007111                   | Transcription factor DP-1 (DP1) | 4.8                                      | 6.4                                 |
| TFPD2  | NM_006286                   | Transcription factor DP-2 (DP2) | 7.2                                      | 11.3                                |

\textsuperscript{a} The -fold change of "normal plus EGF versus untreated normal fibroblasts" and "HGF1 versus normal fibroblasts."

\textsuperscript{b} A difference of >2-fold between two groups is shown in boldface.

on days 2 and 4, respectively, compared with the HGF1 control cells (dotted versus solid bars). These data suggest 1) targeted knock-down of the mutant SOS1 transcript reduced proliferation of HGF1 fibroblasts and 2) knock-down of mutant SOS1 had a greater effect on reduction of fibroblast proliferation than the knockdown of wild-type SOS1. These findings suggest mutant SOS1 is responsible for the increase of fibroblast proliferation seen in HGF1.

Up-regulation of Cell Cycle Regulatory Proteins in HGF1 Gingival Fibroblasts—Sustained ERK signaling in fibroblasts is associated with cell cycle control (35, 36). To test if prolonged ERK signaling affects cell cycle progression in primary gingival fibroblasts, we used a focused microarray containing 112 genes involved in cell cycle regulation. Under serum-starved conditions, HGF1- and EGF-treated control fibroblasts represent sustained and transient ERK signaling, respectively. To monitor the effects of either transient or sustained ERK signaling on the expression level of cell cycle regulators, two experimental groups were studied: 1) EGF-treated versus untreated control fibroblasts and 2) HGF1 versus control fibroblasts. As shown in Table 1, most transcripts showing a >2-fold increase in HGF1 over control fibroblasts were involved in the cell cycle progression and transition for G\textsubscript{1} phase and from G\textsubscript{1} into S phase. In most cases, the relative increase in differential expression was greater in the HGF1 versus control group than in the EGF-treated versus untreated control group, particularly, for cyclins C, C\textsubscript{1}, D\textsubscript{1}, D\textsubscript{2}, E\textsubscript{1}, and E\textsubscript{2} and RBBP8 (CTIP) (Table 1). Although the transcription factors E2F1 and E2F2 were increased ~2-fold, their dimerization partners DP1 and DP2 were up-regulated ~4- and 7-fold, respectively. These results reveal that, even under serum-starved conditions, key proteins involved in cell cycle regulation continued to be expressed, suggesting that these genes may be downstream targets of the sustained ERK signaling in HGF1 fibroblasts.

To validate the microarray findings real-time PCR and Western blotting were performed. Cyclin E-Cdk2 and Rb-E2F-DP complexes are known to promote cell cycle transition from G\textsubscript{1} into S phase in mammalian cells (37, 38). When gingival fibroblasts cultures were serum-starved overnight, more phosphorylated Rb isoforms were detected in HGF1 fibroblasts than in controls (Fig. 7A, lanes 1 and 3). After switching to growth medium, a major phospho-Rb, most likely p130, was detected in both cell types, and the overall phospho-Rb was higher in HGF1 fibroblasts (lanes 2 and 4) indicating sustained phosphorylation. Expression of cyclin E1 (CCNE1) was 3-fold greater in HGF1 (Fig. 7B, unfilled bar) than in control fibroblasts (solid bar) under serum-starved conditions, consistent with microarray results. Similar increases were found in both control (striped bar) and HGF1 (dotted bar) fibroblasts grown in growth medium. Interestingly, cyclin E2 (CCNE2) expression was 2.5-fold higher in HGF1 fibroblasts (unfilled bar) than in control fibroblasts (solid bar) under serum-starved conditions. After switching to growth medium, levels of cyclin E2 increased significantly: 15- and 46-fold higher in control (striped bar) and HGF1 (dotted bar) fibroblasts, respectively. In addition, cyclin E2 levels were 8-fold higher in HGF1 (dotted bar) than in control fibroblasts (striped bar) under growth medium suggesting cyclin E2 plays a role in the overgrowth of HGF1.

Expressions of E2F1, E2F2, TFPD1, and TFPD2 were also evaluated, because activation of the E2F family is the key step for cell cycle progression from G\textsubscript{1} into S phase. Under serum-starved conditions, E2F1 transcript levels were similar for control and HGF1 fibroblasts (Fig. 7C, solid and unfilled bars). After switching to growth medium, E2F1 expression increased in control (10-fold) and HGF1 (40-fold) fibroblasts, respectively. Interestingly, the relative levels of E2F2 were 80-fold greater than E2F1 in both types of fibroblasts under serum-starved conditions. TFPD1 expression levels were 30% higher in HGF1 than in control fibroblasts in both serum-starved and growth conditions (Fig. 7D). No differences were found for TFPD2 expression between the two cell types regardless of culture conditions; however, after switching from starving to growth condition, it decreased 75% in both cell types. Taken together, these data suggest that 1) cyclin E2, E2F1, and TFPD1 play a major role in cell cycle regulation in gingival fibroblasts and 2) the cell cycle is more active in HGF1 fibroblasts than in control fibroblasts and may underlie the higher proliferation rates in HGF1 fibroblasts.

One of the immediate-early gene products induced by ERK signaling is the early growth response gene (Egr-1) (39). Under serum-starved conditions, trace amounts of Egr-1 were detected in control fibroblasts (Fig. 8A, lane 1), whereas signif-
FIGURE 7. Expression of cell cycle regulators in control and HGF1 fibroblasts. Fibroblast cultures were maintained either in starving medium (S) overnight or switched to growth medium for 1 day (S + G). A, nuclear extracts from control and HGF1 fibroblasts were resolved in NuPAGE and probed with phospho-Rb (pRb) antibody, and total Rb was used as control. B–D, total RNA from control and HGF1 fibroblasts were isolated, and the expression levels of CCNE1 and CCNE2 (B), E2F1 and E2F2 (C), and TFDP1 and TFDP2 (D) were monitored by reverse transcription-PCR in control (solid and striped bars) and HGF1 (unfilled and dotted bars) fibroblast cultures maintained in serum-starved (S, solid and unfilled bars) or switched to growth medium (S + G, striped and dotted bars). The data are from three separate experiments with triplicate samples and are presented as averages (± S.D.) of the relative level over control fibroblasts in starving medium (control/S).

FIGURE 8. Expression of Egr-1 and PCNA in gingival fibroblasts. A, control and HGF1 fibroblasts were maintained in starving medium overnight (S) or in starving medium overnight and switched to growth medium for 1 day (S + G). Cellular extracts were blotted with antibodies of Egr-1 and β-actin. B, HGF1 fibroblasts were transfected with siRNA (50 nm) of luciferase (lane 1), wS, mR, or wS and mR together for 48 h, and cellular extracts were probed with antibodies of Egr-1, and PCNA, with total ERK1/2 as a control.

DISCUSSION

In this study, we have elucidated the mechanism of gingival overgrowth in HGF1 with SOS1 mutation. Our studies demonstrate that the overall level of SOS1 transcript is lower in HGF1 fibroblasts suggesting the mutant transcript is significantly less than the wild-type transcript. Although the relative level of wild-type SOS1 transcript appears to be higher than the mutant transcript in HGF1 fibroblasts (Fig. 1B), targeting mutant transcript by mR siRNA produced more profound effects on reduction of cell proliferation than wS (Fig. 6B), suggesting that the increased proliferation of HGF1 fibroblasts was chiefly dependent upon the function of mutant SOS1.

Both the N- and the C-terminal domains are involved in down-modulation of SOS activity (40). SOS1 lacking the C-terminal domains and targeted to the plasma membrane exhibits increased GEF activity and triggers the Ras signaling cascade without external stimuli (41–45). In HGF1 fibroblasts, without growth factors stimuli, we observed the truncated SOS1 distributed in the membrane/organelle faction. Although such subcellular fractionation analyses do not rule out the possibility that mutant SOS1 could associate with endomembranes, immunostaining of endogenous SOS1 and HA-tagged SOS1 showed that mutant protein could distribute to the plasma membrane in serum-starved fibroblasts. The mechanism for the translocation remains unknown. It is unlikely that the novel 22 C-terminal residues resulting from the frameshift mutation play a role in membrane targeting, because it failed to direct green fluorescent protein to the plasma membrane (data not shown). Recently, SOS1 substitution mutations found in some Noonan syndrome patients (46, 47) are critical in the maintenance of SOS1 autoinhibition and lead to elevation of Ras activity. In HGF1, the truncation mutation abolishes the C-terminal pro-

![Image](https://example.com/image1.png)

![Image](https://example.com/image2.png)
line-rich domains and could also release the autoinhibition state of SOS1.

Mutant SOS1 in HGF1 fibroblasts is capable of sustained activation of Ras/MAPK signaling in the absence of growth factors stimuli. Activation of ERK signaling was not blocked by AG1478 suggesting mutant SOS1 was already present in the plasma membrane, contributing to sustained activation of Ras signaling. The presence of PD98059 also reduced but did not completely block ERK signaling. These findings suggest that mutant SOS1 may activate ERK signaling through other pathways or that HGF1 cells reduced their sensitivity to the kinase inhibitor through an unknown mechanism.

Mutations in key components of the MAPK pathway that result in sustained ERK activation correlate with tumor formation (48, 49). Furthermore, high transformation activity in NIH3T3 fibroblasts and skin tumor formation in transgenic mice were shown by artificially constructed C-terminal truncated forms of SOS1 (43, 44, 50). It is believed that the strong promoter activity used in these constructs produces high levels of mutant protein expression, which leads to strong ERK signaling and transformation activity. Therefore, a threshold level of expression might be necessary to transform cells. We speculate that the benign form of overgrowth in HGF1 can be explained as following: the low level of SOS1 mutant with partial release from its autoinhibition state could result in sustained activation with attenuated ERK signal, which is sufficient to lead to higher proliferation but not strong enough to cause transformation.

In primary fibroblasts, overexpression of the key players in the MAPK pathway such as Ras and MEK results in sustained Ras/ERK signaling and cell growth arrest (51, 52). When we compared sustained (HGF1) versus transient (control treated with EGF) activated ERK signaling in gingival fibroblasts, the higher expression of cyclin C, D, and E family members in HGF1 fibroblasts (Table 1) suggests these cyclin proteins are the downstream targets of the sustained ERK signaling. Interestingly, the expression of cyclin E2 is low or undetectable in non-transformed cells and elevates significantly in tumor-derived cells (53). We found that levels of cyclin E2 increase markedly in both cell types after medium switch indicating cyclin E2 plays a major role in transition from G1 to S phase in gingival fibroblasts (Fig. 7B).

E2F and TFDP also play a pivotal role in the control of cell cycle progression (54, 55). Different E2F-DP complexes bind to different pRB family members, pRb, p107, and p130 (53). Upon hyperphosphorylation of Rb, the E2F-DP complex is released and activates their downstream target genes (56). We observed higher levels of phospho-Rb in HGF1 fibroblasts under both serum-starved and growth conditions. When serum-starved fibroblasts were switched to growth conditions, E2F1 and TFDP1 levels, not E2F2 and TFDP2, were significantly increased (Fig. 7, C and D). It is likely that E2F2 plays a major role in cell cycle control in response to the sustained ERK signal. Both transient and sustained activation of ERK induce expression of immediate-early genes such as fos, jun, and myc; however, only sustained ERK signaling leads to phosphorylation and stabilization of immediate-early gene-encoded proteins (17, 18). We observed that the increase of Egr-1 expression in HGF1, and the depletion of either wild-type or mutant SOS1, reduced the level of both Egr-1 and PCNA, one of the target genes of the E2F-DP complex (Fig. 8).

The findings in this study reveal a Grb2-independent function of mutant SOS1 and suggest a mechanism for HGF1 fibroblast proliferation (see supplemental Fig. S1). Unlike SOS1 mutations in Noonan syndrome, where no gingival overgrowth was reported (46, 47), the truncated SOS1 in HGF1 results clinically in gingival overgrowth. It is unclear why different SOS1 mutations result in different clinical phenotypes. The SOS1 insertion mutation in HGF1 results in a truncated, chimeric protein, whereas the Noonan syndrome mutations are substitution mutations that alter a single amino acid. In addition, why the overgrowth phenotype of HGF1 is restricted to gingival tissues remains unclear. At least four alternative isoforms of SOS1 have been reported in different tissues and cell lines (49). We have observed two SOS1 isoforms (I and II) expressed in gingival fibroblasts, with the majority consisting of isoform II, which contains an extra 15 C-terminal residues that are not present in isoform I (57), (data not shown). It is unlikely these alternative isoforms contribute to the tissue-specific phenotype observed in HGF1, because the early termination of SOS1 occurs upstream of the 15-amino acid difference and would therefore affect both isoforms. It is possible that specific E2F-DP-Rb complexes together with their target genes may be present in gingival fibroblasts and contribute to the localized gingival phenotype. In conclusion, the SOS1 mutation in HGF1 appears to be a gain of function that drives an increase in gingival fibroblast proliferation. Human HGF1 offers a unique model and opportunity to study the SOS1/Ras/MAPK signaling pathway and its biological outcome in a disease state, and the application of RNA interference on allele-specific depletion of SOS1 also provides a potential tool for controlling the gingival overgrowth.

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