

The FOS Transcription Factor Family Differentially Controls Trophoblast Migration and Invasion*

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Background: Trophoblast cells invade the uterus during pregnancy to promote blood flow to the conceptus.

Results: The transcription factor FOS inhibits trophoblast invasion, whereas FOS-like (FOSL)-1 promotes invasion.

Conclusion: The intracellular balance of FOS family transcription factors modulates trophoblast invasive potential.

Significance: Understanding the regulation of trophoblast invasion is crucial for determining the etiology of several placenta-associated obstetrical complications.

Extravillous trophoblast invasion is a fundamental component of human placentation. Invading trophoblast cells promote blood flow to the conceptus by actively remodeling the uterine vasculature. The extent of trophoblast invasion is tightly regulated; aberrant invasion is linked with several obstetrical complications. However, the transcriptional networks responsible for controlling the extent of trophoblast invasion are not well defined. Previous studies have identified high levels of FOS (FOS, FOSB, FOS-like (FOSL) 1, and FOSL2) proteins in extravillous trophoblast cells. These proteins form part of the activating protein-1 (AP-1) transcription factor complex and are implicated in regulating gene networks controlling cellular invasion in diverse biological systems. Therefore, we hypothesized that FOS family proteins play a role in regulating trophoblast invasion. We assessed expression of FOS family proteins in trophoblast cell lines and human placentae at different gestational ages. FOS, FOSB, and FOSL1 proteins were robustly increased in trophoblast cells subject to wound-based migration assays as well as Matrigel-based invasion assays. FOS knockdown resulted in cessation of proliferation and an induction of migration and invasion concomitant with robust expression of matrix metalloproteinase (MMP) 1, MMP3, and MMP10. Conversely, FOSL1 knockdown abrogated trophoblast migration and invasion and inhibited the production of MMP1, MMP3, and MMP10. In human placenta, FOS was expressed in proximal anchoring villi in conjunction with phospho-ERK. FOSL1 was temporally expressed only in the distal-most extravillous trophoblast cells, which represent a migratory cell population.

Therefore, FOS and FOSL1 exert opposing effects on trophoblast invasion.

Extravillous trophoblast invasion during the first half of pregnancy is an essential component of human placentation. Invading trophoblast cells migrate into the uterus, infiltrate the uterine spiral arterioles, displace the endothelium lining these vessels, and destroy the surrounding smooth muscle and elastic laminae (1). This remodeling results in dilated, flaccid conduits capable of supplying the conceptus with sufficient maternal blood during the latter stages of pregnancy. Trophoblast invasion is physiologically normal and is tightly regulated to ensure that the growing conceptus receives a sufficient supply of maternal blood without excessive maternal hemorrhage. Aberrant trophoblast invasion is linked with several obstetrical complications. For example, deficient trophoblast invasion is a key culprit in the etiology of pre-eclampsia and intrauterine growth restriction (2), whereas trophoblast hyperinvasion into maternal tissues is linked with choriocarcinoma, invasive moles, and placenta creta (3).

The transcriptional networks that facilitate trophoblast taxis are not well defined. Because of their inherently invasive nature, trophoblast cells have often been compared with highly invasive carcinoma cells, and in many cases, both exhibit similar gene expression signatures that regulate cellular invasion (4, 5). For example, trophoblast cells and carcinomas both express a diverse spectrum of matrix metalloproteinases (MMPs),⁴ which facilitate matrix remodeling and promote cellular locomotion in both systems. Therefore, it stands to reason that the transcriptional regulation of many genes associated with cancer metastasis and trophoblast invasion may also be shared. The activating protein-1 (AP-1) complex potentially fits this scenario.

Activation of AP-1 causes rapid modulation of gene expression, including that of several MMP genes, in response to a variety of extracellular stimuli (6, 7). The net outcome is the modulation of factors affecting fundamental cellular processes,

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⁴ The abbreviations used are: MMP, matrix metalloproteinase; FOSL, FOS-like; AP-1, activating protein-1.

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TABLE 1
List of primers used for RT-PCR

Gene	Forward primer	Reverse primer	Size
<i>FOS</i>	TCTCCAGCATGGGCTCGCCT	CAGGCAGGTCGGTGAGCTGC	bp
<i>FOSB</i>	GCAGGAAGACTGCACAGAAA	AGGAGTCCACCGAAGACAGA	501
<i>FOSL1</i>	GCCCACTGTTTCTCTTGAGC	GATGGAGAGTGTGGCAGTGA	306
<i>FOSL2</i>	TATCCCGGGAACCTTGACAC	GACGCTTCTCCTCTCTCA	169
18 S	GCAGCCCAAACCTAACCTCAC	TAGCCATAAGGTCGCTCTC	369
			123

including proliferation, survival, and migration. AP-1 complexes can consist of JUN (JUN, JUNB, and JUND) and FOS (FOS, FOSB, FOSL1, and FOSL2) family members. Although JUN proteins are capable of homodimerization, FOS family members exclusively heterodimerize with JUN proteins (6). Accumulation of JUN and FOS proteins is promoted by phosphorylation-mediated stabilization of existing proteins as well as transcriptional regulation (8, 9). *In vitro* studies have determined that, in many situations, JUN-FOS heterodimers are more stable and have stronger DNA binding activities than JUN-JUN homodimers (10–12). Thus, expression of FOS proteins contributes to the robust activity of AP-1-regulated genes.

In the context of cellular invasion, each FOS family member is implicated in the control of genes affecting cell locomotion, particularly in cancer cells (13–21). However, little is known about the role of FOS family members in the placenta. In the mouse, which exhibits invasive hemochorial placentation akin to humans, FOSL1-deficient mice exhibit placental and extra-embryonic abnormalities leading to early embryonic death (22). Similarly, knockdown of *FOSL1* in rat placenta and rat-derived trophoblast stem cells causes defects in trophoblast invasion *in vivo* and *in vitro* (23). FOS is expressed in mouse placenta, but mice deficient in FOS are viable and fertile despite having reduced placental and fetal weights (15). FOSL2 knock-out mice are also viable but exhibit placental structural and signaling defects that indirectly impact differentiation of embryonic tissues from which they ultimately succumb (24). No defect in placentation has been reported in FOSB knock-out mice. In human placenta, FOS, FOSB, FOSL1, and FOSL2 have been detected spatiotemporally at high levels in extravillous trophoblast cells (25–27). Moreover, AP-1 transcriptional activity is required for the promotion of MMP production by human trophoblast cells (28, 29). However, the biological significance of individual FOS components in human trophoblast cells is not known. To address this, we studied the expression of FOS family members in trophoblast cells *in vitro* and in placental sections *in situ* and utilized knockdown approaches to determine the role of individual FOS family members in trophoblast migration and invasion.

EXPERIMENTAL PROCEDURES

Cells—BeWo, Jar, and JEG3 trophoblast cells initially derived from metastatic lesions of choriocarcinoma (30–32) were obtained from the American Type Culture Collection. The immortalized human first trimester extravillous trophoblast lines HTR-8/SVneo (HTR8) (33), Swan 71 (34), and SGHPL4 (35) were kind gifts from Professors Charles Graham (Queen's University, Kingston, Ontario, Canada), Gil Mor (Yale University, New Haven, CT), and Guy Whitley (St. George's, Univer-

sity of London, London, UK), respectively. The TCL1 term trophoblast cell line was kindly provided by Professor Mark Sullivan (Imperial College London, London, UK) (36). BeWo and SGHPL4 cells were maintained in DMEM/F-12 medium (Sigma-Aldrich), whereas JEG3, Jar, HTR8, Swan 71, and TCL1 were maintained in RPMI 1640 medium (Invitrogen). All media were supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin, and 100 μ M streptomycin (all from Sigma-Aldrich). Cells were passaged by light trypsinization prior to reaching confluence and were maintained at 37 °C in an atmosphere consisting of 5% CO₂ for no more than 20 successive passages. 293FT human embryonic kidney cells were purchased from the American Type Culture Collection and maintained in DMEM supplemented with 10% FBS, 100 units/ml penicillin, and 100 μ M streptomycin.

RT-PCR—Relative levels of RNA were assessed by RT-PCR. Total cellular RNA was extracted using TRI Reagent (Sigma-Aldrich) according to the manufacturer's protocol. 500 ng of total RNA was utilized for reverse transcription using a High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA). cDNAs were diluted 1:10 and subjected to conventional PCR or quantitative RT-PCR analysis using primers detailed in Tables 1 and 2, respectively. Conventional PCR was performed for 30 cycles (denature, 94 °C for 30 s; anneal, 55 °C for 30 s; extension, 72 °C for 30 s), and products were resolved by electrophoresis in ethidium bromide-containing 1% agarose gels. Quantitative RT-PCR was performed using SYBR Green PCR Master Mix (Applied Biosystems). Amplification and fluorescence detection were carried out using an ABI Prism 7500 real time PCR system (Applied Biosystems). Cycling conditions included an initial holding step (95 °C for 10 min) followed by 40 cycles of two-step PCR (95 °C for 15 s and then 60 °C for 1 min) followed by a dissociation step (95 °C for 15 s, 60 °C for 15 s, and then a sequential increase to 95 °C). Relative mRNA expression was calculated by the comparative $\Delta\Delta$ Ct method using 18 S rRNA as a reference RNA.

Western Blotting—Evaluation of protein expression was examined by Western blotting. Whole cell lysates were prepared using 62.5 mM Tris-HCl (pH 6.8), 10% glycerol, 2% SDS, and 50 mM DTT. Approximately 10 μ g of cellular protein was loaded for 10% acrylamide-containing SDS-PAGE and transferred to PVDF membranes. Membranes were subsequently probed using antibodies specific for FOS (sc-7202, Santa Cruz Biotechnology, Santa Cruz, CA), FOSB (2251, Cell Signaling Technology, Danvers, MA), FOSL1 (sc-28310, Santa Cruz Biotechnology), FOSL2 (5365-1, Epitomics, Burlingame, CA), phospho-Ser²⁶⁵-FOSL1 (5841, Cell Signaling Technology), phospho-ERK (4370, Cell Signaling Technology), phospho-

TABLE 2

List of primers used for quantitative RT-PCR

Gene	Forward primer	Reverse primer	Size
<i>CDKN1A</i>	CTGCCAAGCTCTACTTCC	CCACATGGTCTTCTCTGCT	bp
<i>CDKN1B</i>	CCGGTAACTCTGAGGACAC	TGCAGGTCGCTTCTTATTC	117
<i>CDKN1C</i>	AGCTGCACTCGGGATTT	CTTCTCAGGCGCTGATCTCT	104
<i>CDKN2A</i>	CGGGTCGGGTAGAGGAG	CGTAACTATTCGGTGGCTTG	136
<i>CDKN2C</i>	CCCGATTTGAAAGACCGAAC	CAGCTTGAAACTCCAGCAA	69
<i>CDKN3</i>	CACCTGCTATGGAGACTTGG	GGCTGTCTATGGCTTGTCT	96
<i>FOS</i>	CCAACCTGCTGAAGGAGAAG	AGATCAAGGAAGCCACAGA	96
<i>FOSB</i>	TTGCACCTTACTTCCCCAAC	AGGAGTCCACCGAAGACAGA	117
<i>FOSL1</i>	GCCCACTGTTTCTCTTGAGC	GATGGAGAGTGTGGCAGTGA	179
<i>FOSL2</i>	CCTCCATGTCCAACCCATAC	GACGCTTCTCTCTCTTCA	169
<i>JUN</i>	GCAGCCCAAACCTAACCTCAC	TAGCCATAAGGTCGGCTCTC	173
<i>JUNB</i>	TGGAACAGCCCTTCTACCAC	AGGCTCGGTTTCAGCAGTTT	123
<i>JUND</i>	CGCCTGGAAGAGAAAGTGAA	GTTGACGTGGCTGAGGACTT	102
<i>MMP1</i>	GCCACAACCTGCCAAATGGGCTT	GGAGCTGTAGATGTCTTGGGGT	116
<i>MMP2</i>	TGTGTCTTCCCCTTCACTT	GATCTGAGCGATGCCATCAA	136
<i>MMP3</i>	TCATTTTGGCCATCTCTTCC	GATCTGAGCGATGCCATCAA	399
<i>MMP7</i>	AAAGAGATCCCCCTGCATT	CCAGCTCGTACCTCATTTCC	119
<i>MMP8</i>	AGGCCAAGGTATTGGAGGAG	GTCCAGCGTTTATCTCATC	180
<i>MMP9</i>	CGCCTCTGGAGGTTTCGACGTG	GTAATTTGCGGAGGTGTTGG	69
<i>MMP10</i>	GTGGAGTTCCTGACGTTGGT	CGTAGCCCACTTGGTCCACCTG	198
<i>MMP11</i>	CTCACCGAGAAGGGATGT	AGCCTGGAGAATGTGAGTGG	180
<i>MMP12</i>	TGGTTTTTGGCCGTGGAGCTCAT	CAGTGGGTAGCGAAAGGTGT	175
<i>MMP13</i>	AGGAGATGAAGACCCCAACC	GAATGGCCAATCTCGTGAACAGCA	186
<i>MMP14</i>	CCTCGGCTCGGCCAAAGCA	AAAAACAGCTCCGCATCAA	161
<i>MMP15</i>	CAGGCCACACCTTCTTCTC	AGCGATGGCCGCTGAGAGTGA	126
<i>MMP16</i>	GCAGCACAAGCACATCACTT	CCAGTATTTGGTGCCCTTGT	181
<i>MMP17</i>	AAGAGAACCTGTCGTGGAG	CACATCACGTTTGGCCATTT	165
<i>MMP19</i>	ATGCCAGACCCTTGCAGTAG	CCTTGGAGAAGTCGATCTGG	171
<i>MMP20</i>	ATTCCACCTCCCAAGATG	CATAGTCCCCCTTGAAGCA	81
<i>MMP21</i>	GGGACCCTATCCAAATCCTC	ACAGAGTTCAGGGATGGATG	123
<i>MMP23</i>	AGGTTCTACCCGATCAACC	GTGAGGGCCTGATCCTTGT	153
<i>MMP24</i>	CCTATGACTCACGGGCATCT	GAGCCACACGCCTTCTTC	180
<i>MMP25</i>	GCAGCAACTCTATGGGAAGG	CACATCGGGGTTTCTTCATC	140
<i>MMP26</i>	GTGAGAATGGAGATGCAGA	AGGGGCCTTGAAGAAGAAA	187
<i>MMP27</i>	GCATACGAGAACCCAGAGA	GGCATGGCCTAAGATACCAC	98
<i>MMP28</i>	AGACACCGGACCAAAATGAG	ACCAAATGCCACAAAGAAAG	192
18 S	GCAATTATTTCCCATGAACG	GTTGCTCCCAACTGGAAGG	158
		GGCCTCACTAAACCATCCAA	123

JNK (4668, Cell Signaling Technology), phospho-p38 (4511, Cell Signaling Technology), or α -tubulin (CP06, EMD-Millipore Corp., Billerica, MA). All antibodies were used at a 1:1000 dilution except for α -tubulin, which was used at 1:5000. Following a 1-h incubation with species-appropriate horseradish peroxidase-conjugated antibodies, membranes were immersed in Luminata Classico enhanced chemiluminescence reagent (EMD-Millipore Corp.) followed by detection using GeneMate basic blue autoradiography film (Bioexpress, Kaysville, UT).

Immunocytochemistry—To immunolocalize FOS family members expressed by trophoblast cells in culture, cells were fixed in 24-well plates using 4% paraformaldehyde, permeabilized, and probed with antibodies specific for FOS, FOSB, or FOSL1. The antibodies used were the same as those described above for Western blotting. All antibodies were diluted 1:200. Following a 1-h incubation with species-appropriate fluorescence-conjugated antibodies, cells were incubated with 4',6-diamidino-2-phenylindole (DAPI; Molecular Probes, Carlsbad, CA) to detect nuclei and visualized using a Leica DMI 4000 microscope equipped with a charge-coupled device camera (Leica Microsystems GmbH, Welzlar, Germany).

Wound Assay—*In vitro* scratch wound assays were conducted to assess directional cell migration. They have been described in detail elsewhere (37). Briefly, confluent monolayers of cells were incubated with 5 μ M mitomycin-C (Sigma-Aldrich) for 2 h to inhibit cell proliferation and washed extensively, and then cells were disrupted by horizontally dragging a

pipette tip across the surface of the monolayer. Wells were washed with phosphate-buffered saline (PBS), pH 7.4 to remove detached cells, and fresh medium was added to the wells. Subsequently, an image of the scratch was recorded in phase using a Leica DMI 4000 microscope. To ensure that the image was consistently taken in the same region of the scratch, a small vertical scratch in the monolayer was included and used as a landmark. The time the initial image was taken was considered $t = 0$ h. Images were captured at multiple times over the course of wound closing. Cells were returned to 37 °C, 5% CO₂ between imaging sessions. To measure the area of wound closure in images taken at specified times, cell frontiers bordering the wound were traced using ImageJ software (38). The percentage of wound closure was calculated based on the equation $[(W_i - W_z)/W_i] \times 100\%$ where W_i is the area of wound at $t = 0$ h and W_z is the area of wound following z h of culture.

To assess FOS family member expression in trophoblast cells following scratch wounding by RT-PCR and Western blotting, multiple scratch wounds were introduced into a monolayer of trophoblast cells by dragging a comb with 20 teeth/inch in a circumferential motion. Following washing with PBS, the medium was changed, and cells were returned to 37 °C, 5% CO₂ for 0, 1, 3, or 6 h prior to RNA or protein extraction.

Matrigel-based Invasion Assay—To assess trophoblast invasion through Matrigel, Transwell inserts (6.5-mm diameter, 8- μ m pore; BD Biosciences) were coated with 400 μ g/ml phenol red-free Matrigel (BD Biosciences) diluted in serum-free

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RPMI 1640 medium for 3–4 h. The medium was removed prior to plating cells. 4×10^4 trophoblast cells were then plated on top of the gelled Matrigel in each chamber and placed in 24-well plates containing normal culture medium. Plates were placed at 37 °C, 5% CO₂. After 24 h, excess cells and Matrigel were removed from the top of the chamber using a cotton swab, and cells on the bottom of the membrane were stained using Diff-Quik (Dade Behring, Newark, DE) according to the manufacturer's recommendations. Invaded cells were counted under a Leica MZFLIII stereomicroscope.

Short Hairpin RNA (shRNA) Constructs, Expression Vectors, and Production of Lentiviruses—FOS, FOSB, and FOSL1 shRNA constructs in pLKO.1 vectors were obtained from Sigma-Aldrich. Control shRNA that does not target any known mammalian gene was obtained from Addgene (pLKO.1-shScramble, plasmid 1864, Addgene, Cambridge, MA). Sequences representing the sense target site for each of the shRNAs used in the analyses are as follows: FOS, GCGGAGACAGACCAACTAGAAC; FOSB, GCCGAGTCTCAATATCTGTCTC; FOSL1, CCTCAGTCTCATCGCAAGAGTAC; Control, CCTAAGGTTAAGTCGCCCTCG. pLX304 lentiviral constructs containing the complete cDNA sequence of FOS were obtained from DNASU plasmid repository (HsCD00434995; DNASU, Tempe, AZ). Empty pLX304 plasmids were obtained from Addgene (plasmid 25890) and used as controls. Third generation lentiviral packaging vectors (pMDLg/pRRE (plasmid 12251) and pRSV-Rev (plasmid 12253)) and a vesicular stomatitis virus glycoprotein envelope vector (plasmid 12259) were acquired from Addgene. The generation of lentiviral particles was reported previously (39). Briefly, 293FT cells were transiently transfected using Lipofectamine 2000 (Invitrogen) with the shRNA-containing transducing vector and the third generation plasmids described above. Culture supernatants containing lentiviral particles were harvested every 24 h for a total of 48–72 h. Supernatants were centrifuged to remove cell debris, filter-sterilized, and stored at –80 °C until used.

In Vitro Lentiviral Transduction—Trophoblast cells were exposed to lentiviral particles for 24–48 h in the presence of 6 µg/ml Polybrene (Sigma-Aldrich) in normal growth medium, selected with puromycin dihydrochloride (Invitrogen; 3.5 µg/ml final concentration) for 2–5 days for cells transduced with pLKO.1 plasmids and blasticidin (Invitrogen; 3.5 µg/ml final concentration) for 5–7 days for cells transduced with pLX304 plasmids, and then subjected to experimental analyses.

Proliferation Assay—To quantitatively determine the proliferative capacity of manipulated trophoblast cells, shRNA-incorporated cells were plated in triplicate in 96-well plates and incubated at 37 °C, 5% CO₂ for 2 h while cells adhered to the plate. This was referred to as $t = 0$ h. Other triplicate wells were incubated for 24 or 48 h. After the appropriate incubation, cells were fixed in 4% paraformaldehyde, stained using toluidine blue in 5% ethanol, washed extensively, and allowed to dry. Absorbed dye was then released by addition of a solution containing 2% sodium dodecyl sulfate, and the resulting color was gauged by measuring absorbance at 550 nm using a spectrophotometer. The absorbance in these samples was then plotted against a standard curve generated by performing this assay

with known cell numbers. Trophoblast cells used in the production of the standard curve were initially counted under a microscope using a hemocytometer and then subjected to a 1:1 dilution series.

Matrigel-based Tube Forming Assay—The ability of trophoblast cells to form tubes when placed on Matrigel has been described previously (40–42) and is thought to relate in part to their capacity for pseudovasculogenesis. To determine the effect of FOS family members on the ability of trophoblast cells to form tubelike structures on Matrigel, trophoblast cells (1.5×10^5 cells/ml) expressing control shRNA or shRNA for FOS, FOSB, or FOSL1 were placed onto gelled Matrigel (~7.5 mg/ml). Images were taken following incubation for 24 and 48 h by light microscopy (Leica DMI 4000). The total numbers of tubes within 400× microscopic fields were recorded. At least four non-overlapping fields were used for analysis.

Chromatin Immunoprecipitation—Chromatin immunoprecipitation (ChIP) analysis was performed as described previously (23). Briefly, cells were plated on Matrigel-coated 150-mm plates for 6 h. Cells were then fixed with 1% formaldehyde, and purified nuclear lysates were sonicated using a Bioruptor (Diagenode, Denville, NJ) to prepare DNA fragments at a size of ~200–400 bp. Lysates (~75 µg of chromatin) were immunoprecipitated with 10 µg of rabbit FOSL1 antibodies (sc-605, Santa Cruz Biotechnology) as used by our laboratory previously (23) followed by incubation with Protein G-conjugated Sepharose beads (GE Healthcare). Chromatin incubated with 10 µg of rabbit IgG (550875, BD Biosciences) was used as a nonspecific control. Immunoprecipitated chromatin fragments were eluted from Sepharose beads. DNA-protein interactions were reverse cross-linked and purified using a QiaQuick PCR purification kit (Qiagen). Purified DNA fragments were assessed by quantitative PCR using MMP1, MMP3, or MMP10 promoter-specific primers and SYBR Green PCR Master Mix (Applied Biosystems). Primers were designed using Primer3 software to span the region surrounding a characterized AP-1 response element located 71, 68, and 67 bp upstream of the MMP1 (TGAGTCA), MMP3 (TGAGTCA), and MMP10 (TGAATCA) transcription start sites, respectively (43). Primer sequences were as follows: MMP1 (forward, ATCTGCTAGGAGTCACCATTTTC; reverse, TCCTTGCCCTTCAGAAAGC; 110-bp product spanning –143 to –33); MMP3 (forward, TGTTTGGAATGGTCTGCT; reverse, AAAGAGTGACAGTGTGTTTGG; 85-bp product spanning –110 to –25); MMP10 (forward, TTAAATGTTTGTGAACATTCAGACTT; reverse, TACCCCCTAGTGGCCTTTCT; 102-bp product spanning –109 to –07). Relative occupancy/enrichment was normalized to input samples by use of the $\Delta\Delta C_t$ method.

Immunohistochemistry—Paraffin-embedded blocks of first trimester (gestational age, 6 or 12 weeks) and term (39-week) human placenta were obtained from the Research Centre for Women's and Children's Health Biobank (Mount Sinai Hospital, Toronto, Ontario, Canada). All collections were performed with appropriate consent and were approved by the University of Toronto and the University of Kansas Medical Center research human ethics boards. Placenta that were removed due to elective termination of pregnancies (first trimester tis-

sues) or elective caesarian deliveries (term) were deemed to be from healthy pregnancies. Blocks were sectioned at 5- μ m thickness. Subsequently, sections were deparaffinized and rehydrated in a graded series of ethanol washes, subjected to antigen retrieval at 95 °C in Decloaking agent (RV1000M, Biocare Medical, Concord, CA), and probed with antibodies specific for FOS (1:200), FOSB (1:50), FOSL1 (1:100), or phospho-ERK (1:200). The antibodies used for immunohistochemistry were the same as those described above for Western blotting and immunocytochemistry. To identify proliferating cells, some sections were probed for proliferating cell nuclear antigen (diluted 1:200; sc-56, Santa Cruz Biotechnology). Sections were immersed in species-appropriate fluorescence-conjugated secondary antibodies followed by incubation with FITC-conjugated pan-cytokeratin antibody (1:400; Sigma-Aldrich) to identify trophoblast cells and DAPI to localize nuclei. Sections were then mounted with Fluor-G medium and examined using a Leica DMI 4000 microscope.

Statistical Analysis—Statistical comparisons between two means were performed with Student's *t* test. Comparisons of multiple groups were evaluated with analysis of variance. The source of variation from significant F-ratios was determined with Dunnett's multiple comparison test. Results were deemed statistically significant when *p* was <0.05. All experiments were conducted at least in triplicate and were replicated at least two to three times. Graphing and statistical analyses were performed using GraphPad Prism 6.0.

RESULTS

FOS Family Gene Expression in "Quiescent" Trophoblast Cell Lines—We initially screened seven human trophoblast cell lines for their respective expression of FOS family members, namely three lines derived from choriocarcinoma (BeWo, JEG3, and Jar), three lines from immortalized first trimester extravillous trophoblast cells (HTR8, Swan 71, and SGHPL4), and one line derived by immortalization of term cytotrophoblast cells (TCL1). Each cell line was cultured according to published standard culture conditions. These conditions will be referred to as quiescent for the duration of the report. Under quiescent conditions, *FOS* and *FOSB* exhibited low to undetectable mRNA expression. *FOSL2* was present at low but detectable levels in all trophoblast lines studied except for the term trophoblast cell line TCL1. *FOSL1* expression was not detected in JEG3, Jar, or TCL1 cells; was detected at low levels in BeWo cells; and was constitutively robust in the lines derived from first trimester extravillous trophoblast cells (Fig. 1). For positive controls, BeWo trophoblast cells stimulated with 250 μ M 8-bromo-cyclic adenosine monophosphate were used.

FOS Family Expression in Trophoblast Cell Lines following Stimulation of Migration—Because of the unique FOS family transcription factor profile in first trimester extravillous trophoblast cell lines, we pursued additional experiments using two of these lines, Swan 71 and HTR8 trophoblast cells. To assess FOS family expression in these cells during migration, confluent monolayers were mechanically disrupted at regular intervals analogous to incorporation of multiple *in vitro* scratch wounds. In preliminary analyses, we determined that both Swan 71 and HTR8 trophoblast cells begin to move into the

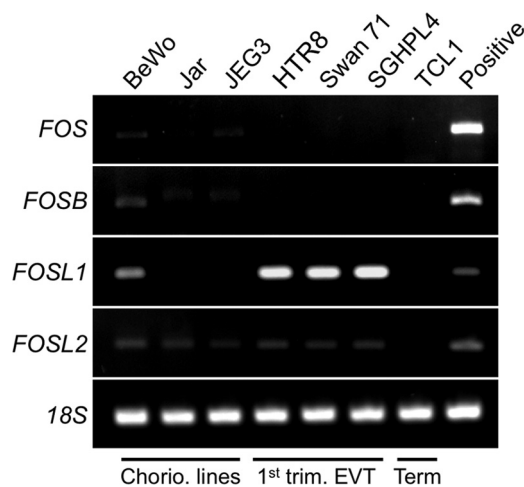


FIGURE 1. Transcript expression of FOS family members in seven trophoblast cell lines. RNA was extracted from three cell lines derived from choriocarcinoma (*Chorio. lines*; BeWo, Jar, and JEG3), three cell lines derived from first trimester extravillous trophoblast (*1st trim. EVT*; HTR8, Swan 71, and SGHPL4), and a cell line derived from term trophoblast cells (*Term*; TCL1). All cell lines were grown according to standard culture conditions and were plated for at least 24 h. BeWo trophoblast cells exposed to 250 μ M 8-bromo-cyclic adenosine monophosphate for 24 h were used as a positive control. 18 S rRNA was used as a loading control.

wound site ~3–4 h following introduction of a scratch wound, and both completely close the wound within 24 h. Immediately following wounding, Swan 71 trophoblast cells exhibited a transient but robust increase in *FOS* and *FOSB* transcript levels of ~25- and ~35-fold, respectively, 1 h following wounding compared with unscratched cells (Fig. 2A; *p* < 0.05). *FOS* and *FOSB* transcript concentrations were also elevated 3 h following wounding but returned to basal levels by 6 h. Comparatively, *FOSL1* and *FOSL2* mRNA levels remained unchanged. Similar results were obtained using HTR8 trophoblast cells (Fig. 2A). Induction of *FOS* and *FOSB* proteins paralleled that of mRNA (Fig. 2B). Interestingly, *FOSL1*, but not *FOSL2*, protein substantially increased following introduction of the scratch wound. Accumulation of *FOSL1* protein was slower than that of *FOS* or *FOSB* but was more stable, remaining high even after 6 h. Upon further analysis, it was determined that *FOSL1* phosphorylation at Ser²⁶⁵ (phospho-Ser²⁶⁵-*FOSL1*) also increased following scratch wounding (Fig. 2B). Phosphorylation of *FOSL1* at Ser²⁵² and Ser²⁶⁵ is known to stabilize *FOSL1* by protection from proteasome-mediated degradation (9, 44). Therefore, *FOSL1* accumulation in human trophoblast cells may also be influenced by post-translational modification.

To determine the localization of *FOS*, *FOSB*, and *FOSL1* in monolayers subjected to scratch wounding, we performed immunocytochemical analysis for each factor 1 or 3 h following scratch wounding (Fig. 2C). Unwounded cells were used as a control. Cells immediately adjacent to the wound site exhibited remarkably high levels of *FOS* and *FOSB*, whereas cells distant from the wound site had low or absent expression of these factors. *FOSL1* was increased in both cells adjacent and distant to the wound site, consistent with our earlier observations of constitutively high expression of *FOSL1* in quiescent cells (Fig. 2C).

Because JUN family members are heterodimeric binding partners for FOS family members, we also assessed *JUN*, *JUNB*,

The Control of Trophoblast Invasion by FOS Family Members

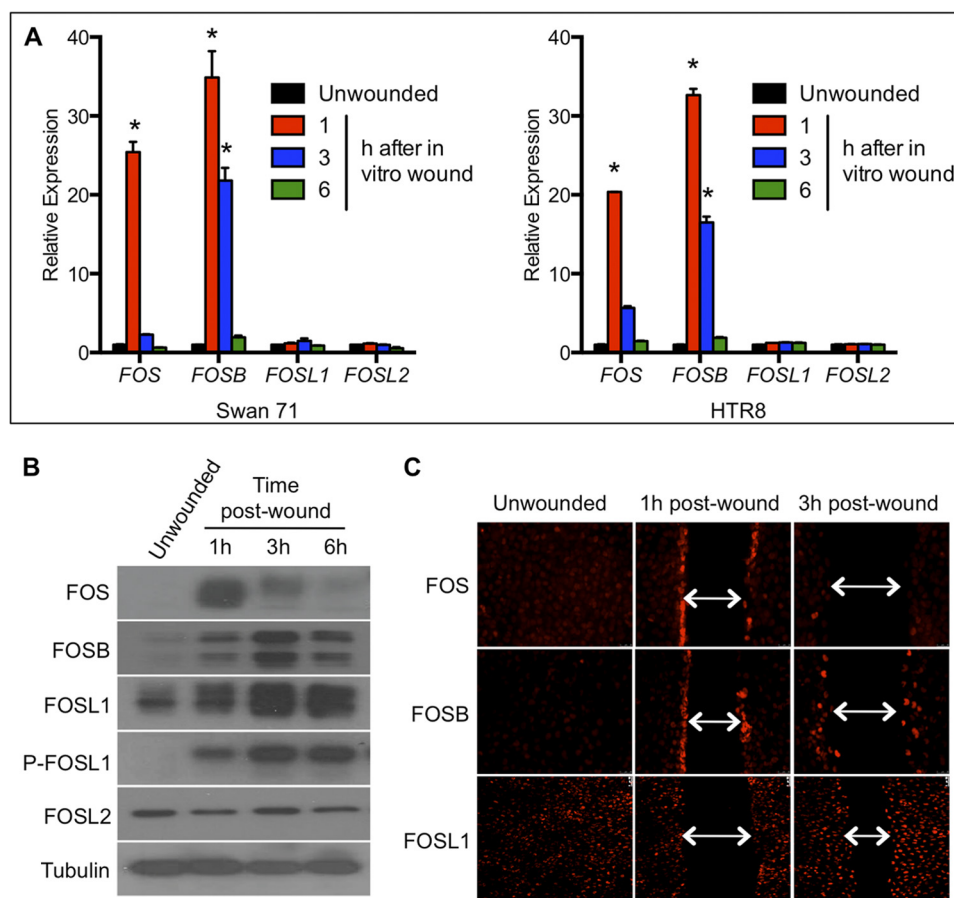


FIGURE 2. Effect of scratch wounding on expression of FOS family members in trophoblast cells. *A*, relative mRNA expression of *FOS*, *FOSB*, *FOSL1*, and *FOSL2* during standard plating conditions (unwounded) and 1, 3, and 6 h following scratch wounding in Swan 71 trophoblast cells (*left*) and HTR8 trophoblast cells (*right*). 18 S rRNA was used as a loading control for transcript. *B*, protein expression of *FOS*, *FOSB*, *FOSL1*, phospho-Ser²⁶⁵-*FOSL1* (*P-FOSL1*), and *FOSL2* during standard plating conditions (unwounded) and 1, 3, and 6 h following scratch wounding. α -Tubulin was used as a loading control for protein. *C*, immunocytochemistry for *FOS*, *FOSB*, and *FOSL1* in unwounded versus wounded monolayers. The wound is shown using a double-sided arrow. Values significantly different ($p < 0.05$) from controls are denoted with an asterisk (*). Error bars represent S.E.

and *JUND* expression in Swan 71 and HTR8 trophoblast cells following scratch wounding. *JUN*, *JUNB*, and *JUND* were all modestly but significantly increased by ~2-fold 1 and 3 h following scratch wounding in Swan 71 trophoblast cells and returned to basal levels 6 h following wounding. In HTR8 cells, only *JUNB* was induced by 2-fold, but it returned to basal levels by 6 h following wounding (data not shown).

ERK-Mitogen-activated Protein Kinase (MAPK) Regulates FOS Family Transcription Factor Accumulation—MAPKs are commonly linked to the regulation of FOS family members (45, 46). Therefore, to ascertain the upstream cascades that induce *FOS*, *FOSB*, and *FOSL1* in trophoblast cells, we assessed phosphorylation levels of p38, JNK, and ERK at 10, 30, and 60 min following monolayer wounding. Phospho-p38, phospho-JNK, and phospho-ERK were undetectable in quiescent cells. After wounding, all three MAPKs were phosphorylated within 10 min and were maximally phosphorylated after 30 and 60 min (Fig. 3A). We then determined mRNA induction of *FOS* and *FOSB* in the presence of the MAPK pharmacological inhibitors U0126 (10 μ M; inhibits MEK-ERK signaling as shown in Fig. 3A), SB203580 (20 μ M; preferentially inhibits p38 downstream pathways but does not affect phosphorylation of p38) (47); and SP600125 (20 μ M; preferentially inhibits JNK phosphorylation

as shown in Fig. 3A) 1 h following wounding. Treatment with U0126 profoundly inhibited wound-induced stimulation of *FOS* and *FOSB* mRNA by >75% (Fig. 3, B and C; $p < 0.05$). Treatment with SP600125 modestly but significantly decreased *FOS* and *FOSB* mRNA induction by 30 and 40%, respectively (Fig. 3, B and C; $p < 0.05$), whereas treatment with SB600125 decreased only *FOSB* mRNA by 30% (Fig. 3C; $p < 0.05$). These findings implicate the MEK-ERK cascade as a primary determinant of *FOS* and *FOSB* mRNA induction following scratch wounding. To determine the effect of MEK-ERK on *FOS*, *FOSB*, and *FOSL1* protein accumulation following scratch wounding, we performed Western blotting on cell lysates collected from vehicle (DMSO)- or U0126-treated Swan 71 cells 1 or 3 h following wounding. Unwounded cells were used as controls. As shown in Fig. 3D, inhibition of MEK-ERK profoundly inhibited *FOS*, *FOSB*, *FOSL1*, and phospho-Ser²⁶⁵-*FOSL1* protein accumulation.

Expression of FOS Transcription Factor Family Members in Trophoblast Cells following Plating on Matrigel—Matrigel-based invasion assays are routinely used to simulate an environment to test the invasive potential of trophoblast cells. However, the intrinsic regulation of trophoblast migration and invasion by dynamic changes in transcription factor expression

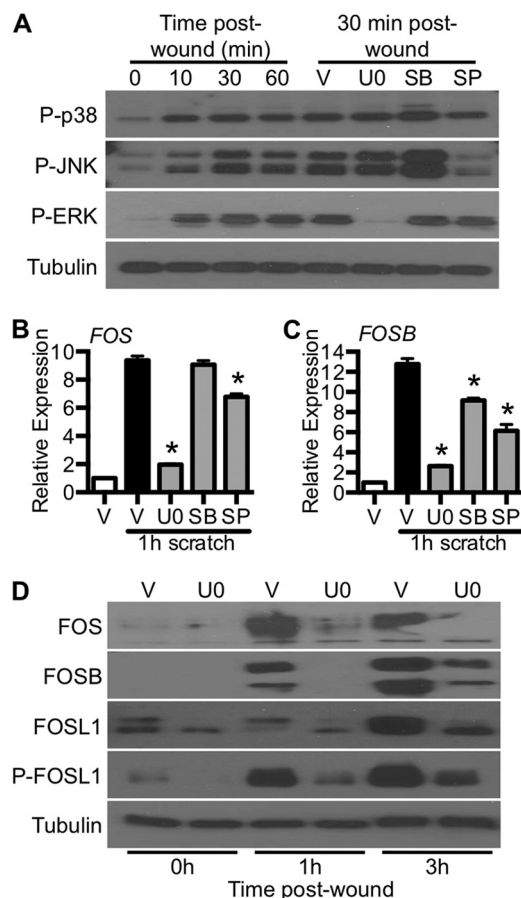


FIGURE 3. ERK-MAPK regulates wounding-induced FOS, FOSB, and FOSL1. *A*, the left four lanes of Western blot show levels of phospho-ERK MAPK, phospho-p38 MAPK, and phospho-JNK MAPK in unwounded cells and 10, 30, and 60 min following wounding, and the right four lanes show efficacy of MAPK inhibitors U0126 (U0) (10 μ M), SB203580 (SB) (20 μ M), and SP600125 (SP) (20 μ M) on phosphorylated MAPK levels 30 min postwound. α -Tubulin was used as a loading control. Vehicle (V) only (DMSO, 1:1000) was used as a control. *B* (*FOS*) and *C* (*FOSB*) mRNA following treatment with U0126 (10 μ M), SB203580 (20 μ M), and SP600125 (20 μ M) is shown. Vehicle (V) only (DMSO, 1:1000) was used as a control. *D*, *FOS*, *FOSB*, *FOSL1*, and phospho-Ser²⁶⁵-*FOSL1* (*P-FOSL1*) expression following treatment with vehicle (V; DMSO, 1:1000) or U0126 (10 μ M) in unwounded cells or 1 or 3 h postwounding. Values significantly different ($p < 0.05$) from controls are denoted with an asterisk (*). Error bars represent S.E.

or activity following plating on Matrigel is poorly understood. Therefore, we assessed expression of FOS family members after plating trophoblast cells on Matrigel. Within 1 h after plating cells on Matrigel, we observed a robust increase in *FOS* and *FOSB* mRNA by ~60- and ~240-fold, respectively, compared with quiescent cells (Fig. 4A; $p < 0.05$ for each). *FOS* and *FOSB* transcripts rapidly declined thereafter and returned to basal levels by 6 h after plating. *FOSL1* mRNA increased by ~12-fold (Fig. 4A; $p < 0.05$), whereas *FOSL2* mRNA did not change significantly after plating on Matrigel. Unlike transcript levels for *FOS* and *FOSB*, *FOSL1* transcript levels remained increased at 6 h following plating (7-fold). Protein levels of *FOS* and *FOSB* were robustly increased by 1 h following Matrigel plating and were still detectable 6 h after plating (Fig. 4B). *FOSB* was still detectable 24 h after plating, although *FOS* expression was not detectable. Protein levels of *FOSL1* were also increased following plating on Matrigel relative to quiescent trophoblast cells. The increase in *FOSL1* protein levels can be partially attributed

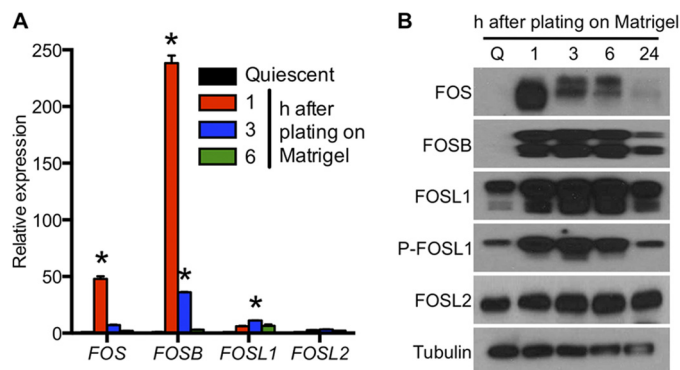


FIGURE 4. FOS, FOSB, and FOSL1 are induced when placed on Matrigel. *A*, relative mRNA levels of *FOS*, *FOSB*, *FOSL1*, and *FOSL2* expressed by Swan 71 cells in quiescent (standard cell culture) conditions versus plating on Matrigel for 1, 3, or 6 h. *B*, Western blot showing protein levels of *FOS*, *FOSB*, *FOSL1*, phospho-Ser²⁶⁵-*FOSL1* (*P-FOSL1*), and *FOSL2* in quiescent (standard cell culture; Q) conditions or following 1, 3, 6, or 24 h of plating on Matrigel. α -Tubulin was used as a loading control for protein. Values significantly different ($p < 0.05$) from controls are denoted with an asterisk (*). Error bars represent S.E.

to the increase in *FOSL1* mRNA, although we also observed an increase in phospho-Ser²⁶⁵-*FOSL1*, suggesting that increased protein stability may also play a role (Fig. 4B). *FOSL2* protein levels showed modest fluctuations in parallel with our observations for *FOSL2* mRNA. Therefore, because *FOSL2* protein expression did not change appreciably following scratch wounding or following Matrigel plating, *FOSL2* was not assessed in subsequent analyses.

Effect of Individual FOS Transcription Factor Family Members on Trophoblast Proliferation, Migration, and Invasion—To assess the role of *FOS*, *FOSB*, and *FOSL1* in various aspects of trophoblast biology, we transduced Swan 71 trophoblast cells with pLKO.1 plasmids encoding shRNAs specific for individual FOS family members. Cells were briefly selected with puromycin and immediately used for experimental purposes to limit perturbations in clonal bias due to putative proliferation defects. The efficacy of shRNA-mediated knockdown for each FOS family member is presented in Fig. 5A. For these experiments, Swan 71 cells expressing control shRNAs or shRNAs targeting *FOS*, *FOSB*, or *FOSL1* were plated for 1 h on Matrigel. There were no differences in cell viability following individual knockdown of *FOS*, *FOSB*, or *FOSL1*.

We initially assessed proliferation of trophoblast cells following individual knockdown of *FOS*, *FOSB*, or *FOSL1*. Compared with trophoblast cells expressing control shRNA, inhibition of *FOSB* had no discernable effects on trophoblast proliferation. In contrast, *FOS* and *FOSL1* knockdown resulted in a significant reduction of cell proliferation by 80 and 60%, respectively, compared with control cells (Fig. 5B; $p < 0.05$ for both). To determine whether *FOS* and *FOSL1* knockdown affected expression of genes associated with cell cycle progression, we analyzed transcript expression of cyclin-dependent kinase inhibitors (*CDKN1A*, *CDKN1B*, *CDKN1C*, *CDKN2A*, *CDKN2C*, and *CDKN3*) in trophoblast cells expressing shRNAs for *FOS*, *FOSB*, or *FOSL1*. Compared with control cells, knockdown of *FOSB* had no discernible effect on transcript expression of any of these genes, which is consistent with the fact that the shRNA for *FOSB* did not affect trophoblast proliferation. In contrast, following knockdown of either *FOS* or *FOSL1*, expres-

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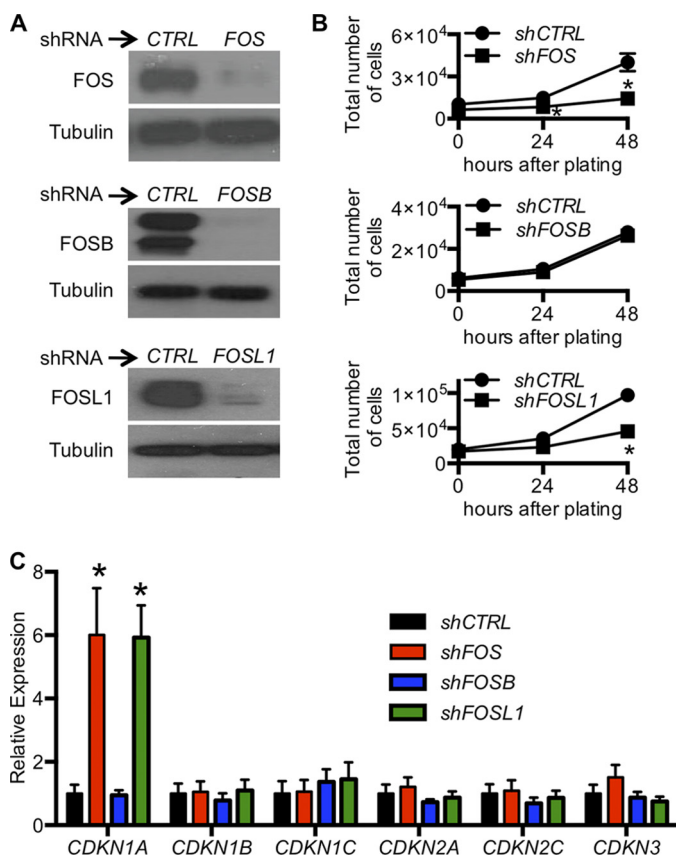


FIGURE 5. Effect of FOS, FOSB, and FOSL1 shRNAs on trophoblast cell proliferation. Swan 71 trophoblast cells were infected with lentiviruses carrying pLKO.1 plasmids encoding shRNAs targeting FOS, FOSB, or FOSL1. *A*, protein expression of FOS (top), FOSB (middle), and FOSL1 (bottom) showing efficacy of knockdown for each individual FOS member compared with control shRNA (CTRL). In all conditions, Swan 71 trophoblast cells were plated on Matrigel for 1 h. *B*, proliferation of trophoblast cells expressing shRNAs for FOS (top), FOSB (middle), or FOSL1 (bottom) compared with cells expressing control shRNA (shCTRL). *C*, transcript expression of the cyclin-dependent kinase inhibitors CDKN1A, CDKN1B, CDKN1C, CDKN2A, CDKN2C, and CDKN3 in trophoblast cells expressing shRNAs for FOS, FOSB, or FOSL1 compared with cells expressing control shRNA (shCTRL). Values significantly different ($p < 0.05$) from controls are denoted with an asterisk (*). Error bars represent S.E.

sion of *CDKN1A* was increased ~6-fold compared with control cells (Fig. 5C; $p < 0.05$ for both). Levels of the other transcripts were unaffected.

Our next analysis involved determining the effect of FOS, FOSB, or FOSL1 on the ability of trophoblast cells to migrate and invade using three independent experiments: scratch wound assay, Matrigel-based invasion assay, and Matrigel-based tube forming assay. Images depicting a wound at the time of scratching (0 h) and 10 h postwounding in cells expressing control shRNA or FOS, FOSB, or FOSL1 shRNAs are shown in Fig. 6A. Following scratch wounding, cells expressing control shRNA migrated into the wound and completely closed it by ~20 h. Similar results were obtained using cells expressing FOSB shRNA. Surprisingly, cells expressing FOS shRNA rapidly moved into the wound and completely saturated the wound site by 10–12 h (Fig. 6B). In contrast, cells expressing FOSL1 shRNA were sluggish in their ability to close the wound site: the gap was still readily apparent even 72 h following scratching. Collectively, these data indicate that cells expressing FOS

shRNA had increased migratory potential, whereas cells expressing FOSL1 shRNA had decreased migratory potential.

Similar roles for FOS family members were observed in the invasion assays. For these experiments, cells were plated on Matrigel-coated Transwell inserts, and the ability of cells to traverse the Matrigel and attach to the underside of the Transwell membrane was scored (representative images are shown in Fig. 6C). Compared with cells expressing control shRNA, cells with FOS knockdown exhibited 4.5-fold increased numbers of cells traversing Matrigel (Fig. 6D; $p < 0.05$). Cells expressing FOSB shRNA exhibited a modest but significant increase in the number of invaded cells (1.5-fold, $p < 0.05$). In contrast, following FOSL1 knockdown, the invasive capacity of trophoblast cells was almost completely inhibited (Fig. 6D; $p < 0.05$).

Finally, we assessed trophoblast morphology on Matrigel using a tube forming assay. Following plating on Matrigel, trophoblast cells clustered tightly together and formed projections that extended into the Matrigel and often connected with adjacent cell clusters analogous to modified tube formations. Images of trophoblast-generated tube formations 24 or 48 h after plating are shown in Fig. 7. Cells expressing control shRNAs as well as cells expressing shRNAs targeting FOS or FOSB immediately (within 6 h) formed tubes on Matrigel. Numbers of tubelike formations were similar among the three groups (Fig. 7). However, cells expressing FOSL1 shRNA were unable to efficiently form tubes but rather formed tight clusters that inefficiently connected to adjacent clusters (Fig. 7; $p < 0.05$).

At 48 h, a striking morphological discrepancy was observed in cells expressing FOS shRNA compared with all other groups. FOS knockdown cells formed more projectiles from cell clusters. Cells in the clusters appeared detached and readily moved into the Matrigel (Fig. 7, white arrows). These morphological observations are consistent with our previous observations that FOS knockdown causes increased migratory and invasive potential. Cells expressing control or FOSB shRNAs formed small projections into the Matrigel (albeit much smaller compared with cells expressing FOS shRNA). In contrast, cells expressing FOSL1 shRNA continued to appear tightly packed with few or no projections extending into the Matrigel, and similar to our observations at 24 h following plating, relatively few tubelike formations were present (Fig. 7).

Profiling MMPs in Trophoblast Cells following Stimulation of Migration: Potential Targets of FOS Family Transcription Factors—We performed a comprehensive examination of known human MMP transcripts 6 h after plating on Matrigel compared with the expression of these enzymes under quiescent conditions. We chose to use 6 h after Matrigel for our experiments because FOS, FOSB, and FOSL1 are all detectable at or before this time and because cells would have sufficient time to adhere, spread, and induce appropriate genes that promote invasion. MMP1, MMP2, MMP3, MMP9, MMP10, MMP11, MMP14, MMP15, MMP16, MMP17, MMP19, MMP24, and MMP25 were detected at significant levels by quantitative PCR (Ct <30 cycles), whereas MMP7, MMP8, MMP12, MMP13, MMP20, MMP23A, MMP27, and MMP28 did not meet the threshold detection levels (Ct >30 cycles). As

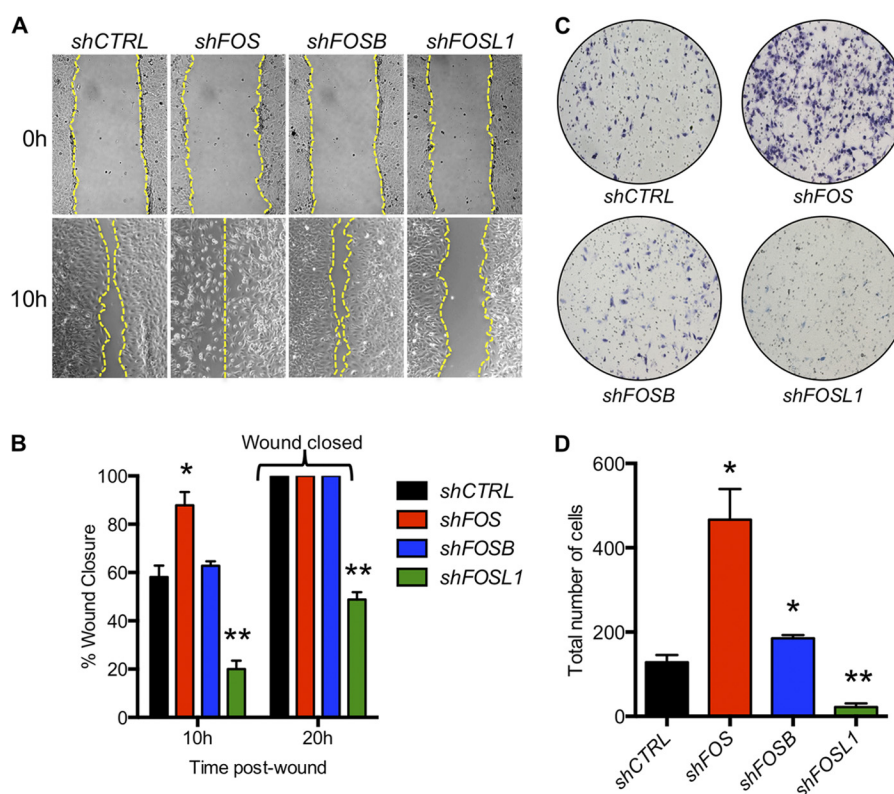


FIGURE 6. Effect of FOS, FOSB, and FOSL1 shRNAs on trophoblast cell migration and invasion. *A*, Swan 71 trophoblast cells expressing control shRNA (*shCTRL*) or shRNAs for *FOS*, *FOSB*, or *FOSL1* were plated to form confluent monolayers and treated with 5 μM mitomycin-C to inhibit proliferation, and monolayers were disrupted using a pipette tip. Images depicting wounds at $t = 0$ h are shown in the *top panels*, whereas images depicting the cell frontiers at $t = 10$ h are shown in the *bottom panels*. In each image, the edges of the wound are denoted with a *dashed yellow line*. Quantification of percentage of wound closure at $t = 10$ h and $t = 20$ h is shown in *B*. 100% wound closure was observed at $t = 20$ h in trophoblast cells expressing control, *FOS*, and *FOSB* shRNAs. *C* and *D*, Swan 71 trophoblast cells expressing control shRNA (*shCTRL*) or shRNAs for *FOS*, *FOSB*, or *FOSL1* were plated on Matrigel-coated Transwell inserts and incubated for 24 h. Representative images of invaded cells attached to the bottom of the membrane is shown in *C*. The mean number of cells that traversed the membrane is shown in *D*. Values significantly increased ($p < 0.05$, $n = 3$) from controls are denoted with an *asterisk* (*), whereas values significantly decreased ($p < 0.05$) from controls are denoted with a *double asterisk* (**). Error bars represent S.E.

shown in Fig. 8A, plating on Matrigel induced the expression of four MMPs in a statistically significant manner: MMP1 (6-fold), MMP3 (7.5-fold), MMP10 (5-fold), and MMP16 (2.5-fold; $p < 0.05$). MMP1, MMP3, and MMP10 were also induced following scratch wounding (MMP1, 7-fold; MMP3, 3-fold; MMP10, 3.5-fold; $p < 0.05$; Fig. 8B). MMP9 was also induced following scratch wounding by 3-fold ($p < 0.05$). Because MMP1, MMP3, and MMP10 were robustly elevated following both Matrigel plating and scratch wounding, we next determined whether disruption of individual FOS family members would affect the expression levels of these MMPs. Knockdown of *FOSB* in trophoblast cells caused a modest but significant elevation of MMP1 by 3-fold after plating on Matrigel (Fig. 9A; $p < 0.05$). MMP3 and MMP10 were not significantly affected following *FOSB* knockdown. In contrast, knockdown of *FOS* expression produced a robust increase in the expression levels of MMP1 (35-fold), MMP3 (5-fold), and MMP10 (5-fold; Fig. 9A; $p < 0.05$). Conversely, knockdown of *FOSL1* dramatically reduced the expression of MMP1 (75%; Fig. 9A; $p < 0.05$), MMP3 (90%; Fig. 9A; $p < 0.05$), and MMP10 (90%; Fig. 9A; $p < 0.05$). *FOSB*, *FOS*, or *FOSL1* knockdowns had no discernable effect on MMP16 or on any MMPs that were unchanged by Matrigel plating.

Because *MMP1*, *MMP3*, and *MMP10* were dramatically reduced in trophoblast cells expressing *FOSL1* shRNA, we per-

formed chromatin immunoprecipitation to determine whether *FOSL1* is capable of binding to previously characterized AP-1 heptameric sites (TGAGTCA or TGAATCA) located ~ 70 base pairs upstream of the transcription start site of each gene. We found that, relative to nonspecific control antibody, *FOSL1* binding was enriched within the proximal promoter regions of *MMP1* (Fig. 9B; 2.5-fold; $p < 0.05$), *MMP3* (Fig. 9B; 7-fold; $p < 0.05$), and *MMP10* (Fig. 9B; 4-fold; $p < 0.05$).

FOS Suppresses FOSL1 Expression—Because of the seemingly opposite results observed with trophoblast migration, invasion, and MMP expression in cells expressing *FOS* versus *FOSL1* shRNA, we checked the expression of each FOS family member in Swan 71 cells expressing control, *FOS*-, *FOSB*-, or *FOSL1*-specific shRNAs following 1 h of plating on Matrigel. As expected, cells expressing shRNAs for *FOS*, *FOSB*, or *FOSL1* dramatically reduced the expression of their intended target (Fig. 10A). *FOS* knockdown also modestly reduced *FOSB* expression and vice versa, indicating that these proteins may positively regulate each other. Strikingly, cells expressing *FOS* shRNA had dramatically enhanced levels of *FOSL1* and phospho-Ser²⁶⁵-*FOSL1* protein (Fig. 10A). Interestingly, we did not observe a change in *FOSL1* mRNA expression in *FOS* knockdown cells, suggesting increased post-translational stabilization of *FOSL1* when *FOS* is inhibited (Fig. 10B). To determine whether *FOS* is capable of directly reducing *FOSL1* phosphor-

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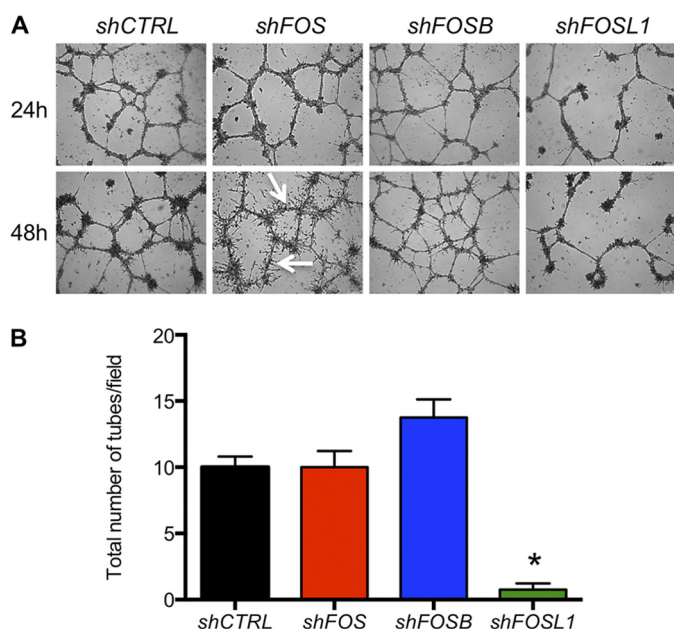


FIGURE 7. Effect of FOS, FOSB, and FOSL1 shRNAs on tube formation by trophoblast cells. Swan 71 trophoblast cells expressing control shRNA (*shCTRL*) or shRNAs for *FOS*, *FOSB*, or *FOSL1* were placed in Matrigel-coated plates, and representative images taken at 24 or 48 h are shown in *A*. The white arrows in bottom *shFOS* panel identify cells migrating away from the tube formations. *B*, the total number of tubes formed at 24 h was scored. Values significantly different ($p < 0.05$) from controls are denoted with an asterisk (*). Error bars represent S.E.

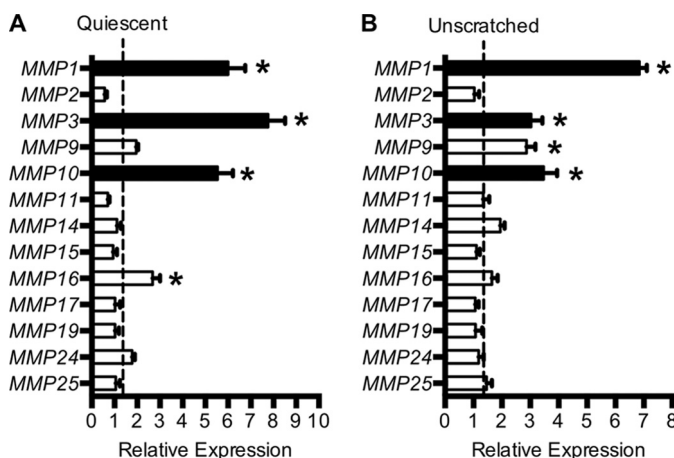


FIGURE 8. Expression of MMPs following plating on Matrigel or scratch wounding. *A*, relative mRNA expression of MMPs in Swan 71 trophoblast cells during quiescent (standard cell culture, indicated by the dashed line) conditions or following plating on Matrigel for 6 h. *B*, Swan 71 trophoblast cells were grown to confluence and then subjected to multiple scratch wounds. RNA was extracted at 6 h following wounding. Monolayers that were not subjected to wounding were used as controls (unscratched, indicated by the dashed line). Only those MMPs that were detected at above threshold levels are included. Values significantly increased ($p < 0.05$) from controls are denoted with an asterisk (*). MMP1, MMP3, and MMP10 are shown in black bars because they were significantly increased in both *A* and *B*. Error bars represent S.E.

ylation and accumulation, we ectopically expressed FOS in trophoblast cells and noted a marked down-regulation of FOSL1 and phospho-Ser²⁶⁵-FOSL1 despite a modest but significant increase in *FOSL1* mRNA levels (Fig. 10, *C* and *D*; $p < 0.05$).

Expression of FOS, FOSB, and FOSL1 in Human Placenta—To gain perspective of the spatiotemporal localization of FOS, FOSB, and FOSL1 in human placenta, we performed immuno-

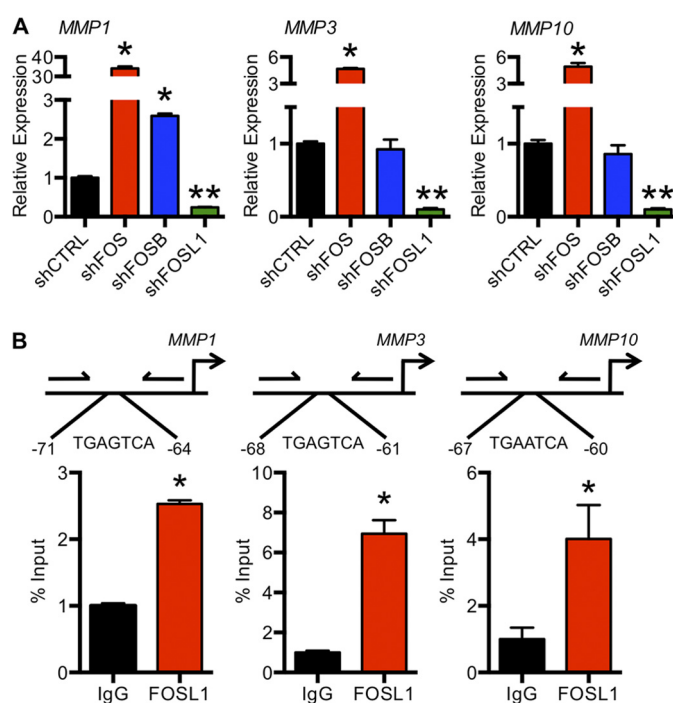


FIGURE 9. FOS family members differentially affect expression of MMPs following plating on Matrigel. *A*, relative mRNA expression of *MMP1*, *MMP3*, and *MMP10* in Swan 71 trophoblast cells expressing control shRNA (*shCTRL*) or shRNAs for *FOS*, *FOSB*, or *FOSL1* after plating on Matrigel for 6 h. *B*, relative occupancy of FOSL1 on the promoter regions of *MMP1*, *MMP3*, and *MMP10*. A diagram depicting the primer sites in relation to the characterized AP-1 sites is indicated. Values significantly increased ($p < 0.05$) from controls are denoted with an asterisk (*), whereas values significantly decreased ($p < 0.05$) from controls are denoted with a double asterisk (**). Error bars represent S.E.

histochemistry using antibodies specific for each of these transcription factors on paraffin-embedded sections of human placenta collected at 6 (Fig. 11, *A, D, G, J*, and *M*), 12 (Fig. 11, *B, E, H, K*, and *N*), and 39 (Fig. 11, *C, F, I, L*, and *O*) weeks of gestation. Fig. 11, *A, B*, and *C* depict images that detail landmark structural components at 6 (Fig. 11*A*), 12 (Fig. 11*B*), and 39 (Fig. 11*C*) weeks of gestation. At 6 weeks of gestation, trophoblast cells located within the core of the cell columns emanating from villi were intensely immunopositive for FOS (Fig. 11*D*). This staining closely co-localized with phospho-ERK (Fig. 11*J*) and the proliferation marker proliferating cell nuclear antigen (not shown). Villous cytotrophoblast cells were also positive for FOS, phospho-ERK, and proliferating cell nuclear antigen but at lower levels than column extravillous trophoblast cells. FOSB was diffusely present within the core of the cell columns (Fig. 11*G*). Syncytiotrophoblast, representing the outer strata of the trophoblast bilayer on the periphery of the villi, was intensely positive for FOSB at all gestational ages. FOSL1 was lowly expressed at 6 weeks of gestation. Expression was limited to cells on the periphery of the cell columns.

At 12 weeks of gestation, strong expression of FOS and FOSB was present within proximal extravillous trophoblast cells in the core of the cell columns (Fig. 11, *E* and *H*, respectively). Similar to our observation at 6 weeks of gestation, FOS expression co-localized with phospho-ERK expression (Fig. 11*N*). The number of FOS- and FOSB-immunopositive cells seemed to subside near the periphery of the extravillous trophoblast outgrowth where cells seemed detached from the cell column and

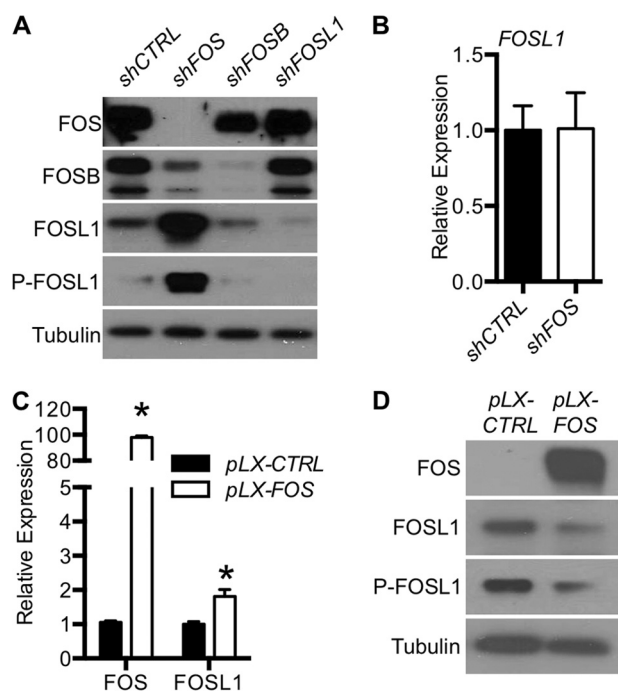


FIGURE 10. FOS knockdown affects FOSL1 accumulation. A, Swan 71 trophoblast cells expressing control shRNA (*shCTRL*) or shRNAs targeting *FOS*, *FOSB*, or *FOSL1* were plated on Matrigel for 1 h. Proteins were subsequently extracted and analyzed by Western blotting for FOS, FOSB, FOSL1, or phospho-Ser²⁶⁵-FOSL1 (*P-FOSL1*). α -Tubulin was used as a loading control. B, *FOSL1* transcript levels in control shRNA (*shCTRL*) or shRNA targeting *FOS*. C, relative mRNA expression comparing *FOS* and *FOSL1* in trophoblast cells ectopically expressing FOS. D, Western blot showing FOS, FOSL1, or P-FOSL1 in trophoblast cells ectopically expressing FOS. α -Tubulin served as a loading control. Values significantly different ($p < 0.05$) from control are denoted with an asterisk (*). Error bars represent S.E.

were gaining migratory capacity. At this site, FOSL1 was highly expressed (Fig. 11K).

In term placentae (39 weeks), FOS, FOSB, and phospho-ERK were detected in invaded interstitial extravillous trophoblast cells situated within the placental bed (Fig. 11, F, I, and O, respectively). Similar to our earlier observations, FOSB was also detected in syncytiotrophoblast. FOSL1 expression was notably absent in term human placenta (Fig. 11L).

DISCUSSION

In this report, we have demonstrated that FOS family members distinctively contribute to the control of trophoblast invasion. The results insinuate that extravillous trophoblast behavior is highly dependent on the expression of specific FOS family members and that different AP-1 permutations impact extravillous trophoblast phenotypes in discrete ways.

In our initial screen of seven trophoblast cell lines, we identified high levels of FOSL1 in all three first trimester extravillous trophoblast lines. Each of these lines was derived by immortalization of isolated first trimester trophoblast cells (48). They all express cytokeratin, consistent with their classification as epithelial cells, and are highly invasive *in vitro* but also display features consistent with cells that have transitioned from an epithelial to a mesenchymal phenotype (49). Our *in situ* analysis identified FOSL1-positive extravillous trophoblast cells at the distal-most edges of the anchoring villi in first trimester placentae, but whether they share mesenchymal fea-

tures and are thus related to the first trimester immortalized extravillous cell lines requires further investigation. At this juncture, it is difficult to ascertain whether FOSL1 expression is a consequence or a direct cause of the mesenchymal characteristics associated with human first trimester extravillous cell lines. In support of the latter, FOSL1, but not FOS, was shown to be a key mediator in epithelial-mesenchymal transition in human breast and colon cancer lines (50, 51). Moreover, ectopic expression of FOSL1 causes adenocarcinoma cells to adopt morphological changes reminiscent of mesenchymal cells together with modulation of gene expression signatures and enhanced cell motility and invasion (19). Thus, high levels of FOSL1 expressed in first trimester extravillous trophoblast cell lines may be at least partly responsible for their mesenchymal morphology, high levels of *in vitro* invasiveness, and unique gene expression profiles. In line with this, we found that *FOSL1* knockdown substantially affected morphology, motility, and invasiveness of these cells.

Based on comparing relative accrual of transcript and protein, we determined that increased FOS and FOSB expression was likely mediated primarily through transcript induction or stabilization, whereas FOSL1 accumulation was mediated likely by post-translational modifications (e.g. phosphorylation) and protein stabilization. Accumulation of all FOS family transcription factors was dependent on ERK activity, which is consistent with other reports (46). In human placenta, however, only FOS expression correlated with phospho-ERK in extravillous trophoblast cells. FOS and phospho-ERK also co-localized with the proliferation marker proliferating cell nuclear antigen, strongly signifying that an ERK-FOS pathway may be important in stimulating proliferation of extravillous trophoblast cells. Accordingly, *FOS* knockdown substantially abrogated the capacity of trophoblast cells to proliferate. FOS expression is vital for the proliferative capacity of many cell types in part by regulating the transcriptional activation of cyclin genes and repression of cyclin-dependent kinase inhibitors (52–55). However, in other cells, FOS seems to be dispensable (56, 57) or even down-regulates proliferation (58, 59). The effect of FOS on proliferation may depend to some degree on the intracellular abundance of other FOS and JUN proteins (56, 60). Interestingly, we found that *FOSL1* knockdown also significantly inhibited proliferation albeit to a lesser extent than *FOS* knockdown. The similar proliferation phenotypes observed with *FOS* and *FOSL1* knockdown may be explained by shared transcriptional targets. Indeed, genetic complementation studies in mice have shown an overlapping function between FOS and FOSL1 in bone formation (61). In this study, we determined that knockdown of either *FOS* or *FOSL1* resulted in a robust increase of the cyclin-dependent kinase inhibitor *CDKN1A*. *CDKN1A* encodes p21 (CIP1/WAF1), a potent inhibitor of cell cycle progression, whose expression is negatively associated with trophoblast proliferation (62). Additional studies are needed to determine whether increased expression of *CDKN1A* is directly responsible for the suppressed proliferation following knockdown of either *FOS* or *FOSL1*. In extravillous trophoblast cells, FOS and FOSL1 may each have a role in the regulation of genes governing progression through the cell cycle and consequently are both relevant for the promotion of proliferation.

The Control of Trophoblast Invasion by FOS Family Members

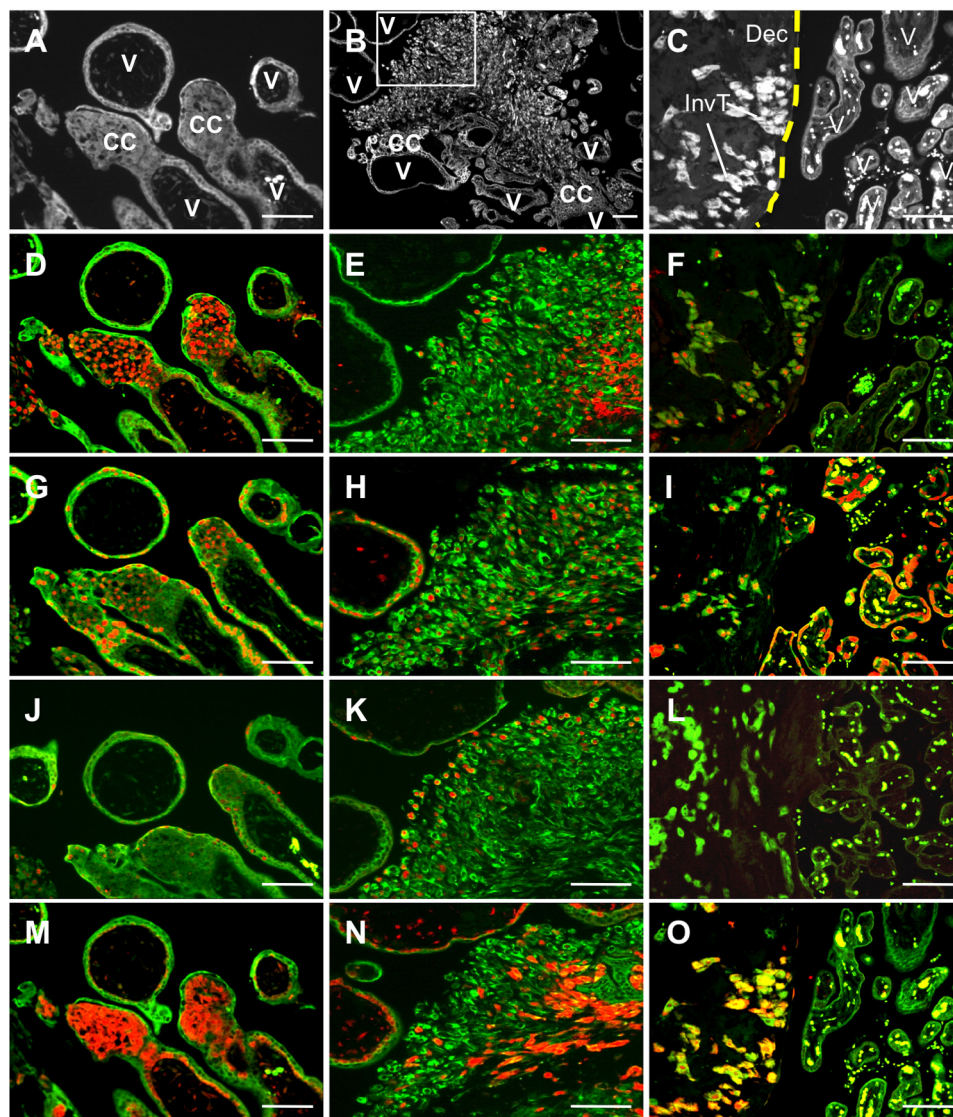


FIGURE 11. Immunolocalization of FOS, FOSB, FOSL1, and phospho-ERK in human placenta at 6, 12, and 39 weeks of gestation. Images on the *left* (A, D, G, J, and M) are serial sections processed from placentae at 6 weeks of gestation. Images in the *middle* (B, E, H, K, and N) are serial sections processed from placentae at 12 weeks of gestation. Images on the *right* (C, F, I, L, and O) are serial sections processed from placentae at 39 weeks of gestation. Images detailing major structural landmarks are shown for 6 weeks (A), 12 weeks (B), and 39 weeks (C). CC, trophoblast cell column; V, villous; Dec, decidua; InvT, invaded trophoblast. The *yellow dashed line* in C demarcates the boundary between the decidua and placenta. FOS immunohistochemistry (*red*) is shown in D, E, and F. FOSB immunohistochemistry (*red*) is shown in G, H, and I. FOSL1 immunohistochemistry (*red*) is shown in J, K, and L. Phospho-ERK immunohistochemistry (*red*) is shown in M, N, and O. Cytokeratin is immunostained *green* to depict epithelial (trophoblast) cells. Scale bars, 100 μ m.

One of the most intriguing findings from this study was that FOS actively suppressed the invasive phenotype of trophoblast cells. At first glance, the transient accumulation of FOS following scratch wounding or Matrigel plating seems counterintuitive. However, the capacity to control invasion is a defining feature distinguishing the regulated invasiveness of trophoblast cells from cellular invasiveness during pathological situations like carcinoma progression. The mechanism by which FOS suppresses the invasive phenotype of trophoblast cells is at this point speculative and requires additional studies. However, we noted a substantial accumulation of FOSL1 in trophoblast cells following knockdown of FOS. Moreover, ectopic expression of FOS caused decreased FOSL1 expression. Therefore, it is possible that the repressive actions of FOS on trophoblast invasion may stem from restraining FOSL1 accumulation and will be the subject of future investigations. If this hypothesis is true, this

places greater emphasis on FOSL1 as a master regulator of gene expression networks that promote trophoblast invasion. Fittingly, in first trimester human placentae, FOS was highly detected in most extravillous trophoblast cells within the proximal cell columns, consistent with its essential role in trophoblast proliferation. Distally, FOS expression waned whereas FOSL1 expression increased specifically in cells that were detaching from the column and gaining migratory capacity.

Promotion of trophoblast invasion during the first half of pregnancy is essential to establishing a sufficient maternal-fetal blood supply, but equally as important for maternal health is appropriate cessation of trophoblast invasion during the latter half of pregnancy. In term placentae, FOSL1 expression was not detectable in any trophoblast cells, including those that had embedded within the decidua. Conversely, FOS and FOSB were readily detected in these cells. It is possible that cessation of

invasion in the latter half of pregnancy is accomplished in part by re-expression of FOS (and FOSB), which actively represses FOSL1 accumulation. Interestingly, high expression of FOSL1 is observed in gestational disorders associated with hyperinvasive trophoblasts, including molar pregnancy and choriocarcinoma (26). In this case, insufficient FOS expression may pathologically fail to disable FOSL1 accumulation in trophoblast cells, causing sustained expression of gene networks that promote invasion.

Putative transcriptional targets of FOS family members include a multitude of genes affecting cell proliferation, survival, and migration. In this report, we studied FOS family-mediated control of MMP expression as an illustration of disparate regulation of these genes by individual FOS family members. We focused on MMPs because they are highly linked with cell invasion and tissue remodeling in a variety of biological systems, including extravillous trophoblast cells (63–65), and several of these proteases contain well characterized AP-1 binding sites within their proximal promoter regions. In this study, we found that MMP1 (collagenase-1), MMP3 (stromelysin-1), and MMP10 (stromelysin-2) were rapidly induced after trophoblast cells were subjected to a wound-based migration assay or plated on Matrigel. These MMPs are situated in series on chromosome 11, and each has a conserved AP-1 site within their respective proximal promoter regions: 5'-TGAGTCA-3' 71 and 68 bp upstream of *MMP1* and *MMP3*, respectively, and 5'-TGAATCA-3' 67 bp upstream of *MMP10* (66). The structural similarities and chromosomal proximity suggests that these genes arose through duplication. Occupation of this site by AP-1 is essential for transcription of these MMPs (7). In this study, we noted enrichment of FOSL1 at this AP-1 site. We also observed that *FOSL1* knockdown in trophoblast cells substantially reduced the expression of *MMP1*, *MMP3*, and *MMP10*, whereas *FOS* knockdown had the opposite effect. Therefore, FOSL1 binding to this AP-1 site is likely necessary for robust transcription of these genes. However, a high degree of cooperativity with other cis-acting elements, including other putative AP-1 sites located more distally along the promoters of these genes (66), as well as interactions with other transcription factors (67) is also required for cell- and tissue-specific control of MMP expression. Although the regulation of MMPs was used in this study as a paradigm of how individual FOS family members can differentially modulate their expression in trophoblast cells, we predict that other genes regulated by AP-1 transcriptional activity that encode proteins promoting invasion may also display differential regulation by individual FOS family members.

In conclusion, it is clear that individual FOS transcription factor family members have diverse roles in various facets of trophoblast biology. Although FOSB was readily induced along with FOS and FOSL1, we did not observe a substantial phenotypic change in trophoblast behavior following *FOSB* knockdown. It is possible that other FOS family members are capable of sufficient compensation in its absence. Moreover, although FOSB expression was diffusely present within the extravillous trophoblast population, high levels were also detected in syncytiotrophoblast. Therefore, more information is required to determine exactly what role FOSB plays in placentation. How-

ever, we provide compelling evidence that FOS and FOSL1 engage in a physiological “tug of war” to regulate the extent of trophoblast invasion. *In vivo*, their expression in extravillous trophoblast cells is mutually exclusive and is identified at the right place and at the right time to exert their respective actions on trophoblast proliferation and motility. Our data build on a previous report by our laboratory that identified FOSL1 as a master transcriptional regulator of trophoblast invasion in the rat (23). Therefore, FOSL1 may have a conserved role in the regulation of trophoblast invasion in species exhibiting hemochorial placentation. Much more information is still required to fully understand how FOS and FOSL1 fit into a hierarchical cascade of events controlling trophoblast invasiveness, including whether dysregulation of these factors is implicated in aberrant trophoblast invasion linked with the etiology of placenta-associated obstetrical complications.

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