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Transitory FGF Treatment Results in the Long-Lasting Suppression of the Proliferative Response to Repeated FGF Stimulation

Ashleigh Poole,[1] Nicholas Knowland,[1] Emily Cooper,[1] Rebecca Cole,[1] Hongchuan Wang,[1] Lucas Booth,[1] Doreen Kacer,[1] Francesca Tarantini,[2] Robert Friesel,[1] and Igor Prudovsky[1]*

[1]*Center for Molecular Medicine, Maine Medical Center Research Institute, Maine Medical Center, Scarborough, Maine*

[2]*Department of Clinical and Experimental Medicine, University of Florence, Florence, Italy*

ABSTRACT

FGF applied as a single growth factor to quiescent mouse fibroblasts induces a round of DNA replication, however continuous stimulation results in arrest in the G1 phase of the next cell cycle. We hypothesized that FGF stimulation induces the establishment of cell memory, which prevents the proliferative response to repeated or continuous FGF application. When a 2–5 days quiescence period was introduced between primary and repeated FGF treatments, fibroblasts failed to efficiently replicate in response to secondary FGF application. The establishment of "FGF memory" during the first FGF stimulation did not require DNA synthesis, but was dependent on the activity of FGF receptors, MEK, p38 MAPK and NFκB signaling, and protein synthesis. While secondary stimulation resulted in strongly decreased replication rate, we did not observe any attenuation of morphological changes, Erk1/2 phosphorylation and cyclin D1 induction. However, secondary FGF stimulation failed to induce the expression of cyclin A, which is critical for the progression from G1 to S phase. Treatment of cells with a broad range histone deacetylase inhibitor during the primary FGF stimulation rescued the proliferative response to the secondary FGF treatment suggesting that the establishment of "FGF memory" may be based on epigenetic changes. We suggest that "FGF memory" can prevent the hyperplastic response to cell damage and inflammation, which are associated with an enhanced FGF production and secretion. "FGF memory" may present a natural obstacle to the efficient application of recombinant FGFs for the treatment of ulcers, ischemias, and wounds. J. Cell. Biochem. 115: 874–888, 2014.

KEY WORDS: FGF; DNA SYNTHESIS; CELL "MEMORY"; HDAC; NFκB; CELL MIGRATION

Fibroblast growth factors (FGF), which signal through specific FGF receptors (FGFR) 1–4, induce DNA synthesis in quiescent cells, stimulate cell migration, and cause a drastic change of cellular morphology, including cell polarization and reorganization of the actin cytoskeleton [Friesel and Maciag, 1999]. Despite strong immediate effects of FGFs in vitro, attempts to use them for tissue repair have been marginally successful thus far [Barrientos et al., 2008]. The application of recombinant FGF1 and FGF2 released from implanted gels either moderately stimulated wound healing [Kawaguchi et al., 2010] or had no significant effect [Kusuhara et al., 2011]. We found that long-term FGF1 stimulation of mouse fibroblasts in culture resulted in an initial wave of DNA replication and mitoses, which was followed by cell blockage in the G1 phase of the next cell cycle [Andreeva et al., 2004] despite the continuous activation of FGFR1 and Erk1/2.

We hypothesized that as a result of a single FGF stimulation the cell loses the ability of proliferative response to the repeated application of FGF. This phenomenon could repress the hyperplastic response to tissue damage or inflammation, which are associated with the release of ubiquitously expressed FGF1 and FGF2 [Khurana et al., 2004; Ribeiro et al., 2012]. It could also explain why recombinant FGF often only modestly affects wound healing. In the present study, we found that Swiss 3T3 fibroblasts and several other types of cells maintain "memory" about FGF for several days after the initial stimulation, and as a result respond to the repeated FGF stimulation with drastically reduced proliferation. The establishment of "FGF memory" does not

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*Correspondence to: Igor Prudovsky, PhD, DSc, Center for Molecular Medicine, Maine Medical Center Research Institute, Maine Medical Center, 81 Research Dr., Scarborough, ME 04074. E-mail: prudoi@mmc.org
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depend on DNA synthesis during the first round of stimulation and requires the activation of MEK and p38 MAPK as well as NFκB signaling and histone deacetylase activity.

MATERIALS AND METHODS

CELL CULTURES

Swiss 3T3 (ATCC, Manassas, VA) cells were maintained in DMEM (HyClone, Logan, UT) supplemented with 10% bovine calf serum (HyClone) and 1% antibiotic/antimycotic mixture (GIBCO, Grand Island, NY). Quiescence was induced by culturing cells in DMEM containing 0.2% bovine calf serum and 5 units/ml heparin (Sigma, St. Louis, MO). Similar cell culture conditions were used for 10T1/2 mouse mesenchymal stem cells (ATCC). LEII immortalized mouse lung endothelial cells [Friesel and Maciag, 1988], mouse ear-derived mesenchymal stem cells (gift of Robert Koza, MMCRI), and human adipose-derived stem cells (gift of Thomas Tulenko, Rowan University) were maintained in DMEM supplemented with 10% fetal calf serum (HyClone). Quiescence was induced via serum starving in DMEM containing 0.2% fetal calf serum and 5 units/ml heparin. For spontaneous transformation, Swiss 3T3 cells were cultivated in the medium with 10% fetal calf serum (HyClone) and left after achieving 100% confluency for a week without replating. This procedure was repeated 10 times, at this point cultures were overgrown with spontaneously transformed cells unable to reach quiescence neither at high cell density nor in low serum.

CELL STIMULATION WITH GROWTH FACTORS AND TREATMENT WITH INHIBITORS

Stimulation schedules for the standard repeated FGF1 stimulation experiment were as follows:

- *Q:* 168 h of quiescence
- *QF:* 132 h of quiescence followed by 36 h FGF1 stimulation in the absence of other growth factors
- *QFQQ:* 48 h quiescence, 36 h FGF1 stimulation, and 84 h of additional quiescence
- *QFQF:* 48 h quiescence, 36 h FGF1 stimulation, 48 h of intermediate quiescence followed by 36 h of repeated FGF1 stimulation

In a variation of the repeated stimulation experiment, the intervening quiescence period between FGF1 stimulations was extended from 48 to 120 h. In all experiments, we used recombinant human FGF1 prepared as described [Forough et al., 1991] at 10 ng/ml in DMEM with 5 U/ml heparin and 0.2% bovine calf serum. The same four basic stimulation schedule conditions were followed in repeated stimulation experiments for other polypeptide growth factors: FGF2, PDGF-BB (both from R&D Systems, Minneapolis), and IGF1 (gift of Cliff Rosen, MMCRI). In several experiments, Swiss 3T3 cell populations were treated with various inhibitors throughout the 36 h of primary FGF1 stimulation. Among these compounds were histone deacetylase inhibitor trichostatin A (TSA) (Sigma), DNA replication inhibitors thymidine in high concentration and aphidicolin (Sigma), DNA methylation inhibitor azacytidine (Sigma), G9a histone methylase inhibitor BIX01294 (Sigma), JNK inhibitor II (Calbiochem, San Diego, CA), p38 MAPK inhibitor SB202190 (Selleckchem, Boston, MA), MEK inhibitor U0126 (Selleckchem), NFκB inhibitors celastrol (Cayman Chemical, Ann Arbor, MI) and BMS-345541 (Sigma), and FGFR inhibitor, PD1666866 (a generous gift from R.L. Panek, Pfizer). In the experiment with the protein synthesis inhibitor cycloheximide (Sigma), the primary FGF stimulation period was decreased to 18 h to prevent cell death. Upon induction of quiescence as well as withdrawal of growth factors, the cells were washed twice with DMEM media containing 5 units/ml heparin.

DNA SYNTHESIS STUDY

Throughout the final 36 h of each stimulation condition, the cells were exposed to 10 μg/ml bromodeoxyuridine (Sigma). Once stimulation schedules were completed, the cells were fixed for a minimum of 10 min in 100% ethanol, washed with PBS, and DNA was denatured by incubation in 1 N HCl at 55°C for 30 min. The residual acid was then washed with PBS. Non-specific binding of antibodies was prevented by a 30 min pre-exposure to blocking buffer (5% bovine albumin, 0.1% Triton X-100, 0.1% sodium azide in PBS), followed by an 1 h incubation in a 1:200 dilution of monoclonal mouse anti-bromodeoxyuridine antibody (Dako, Carpinteria, CA) in blocking buffer. The cells were then washed with PBS and incubated for 30 min in 1:100 dilution of FITC-conjugated anti-mouse IgG antibodies (Vector, Burlingame, CA). Counting of BrdU-positive nuclei in Swiss 3T3 populations was performed using an Olympus IX70 microscope with a combination of fluorescence and phase contrast. Two coverslips were studied per each experimental point. For each coverslip, 500 nuclei were counted; the number of fluorescent nuclei was recorded to acquire BrdU incorporation percentages. LEII cells, which have low refraction, were imaged using an Olympus camera and associated CellSens Standard software attached to the fluorescent microscope. The cell counter plugin for Image J software (NIH) was used for counting BrdU-positive nuclei in LEII populations. Percentage of BrdU labeled cells with 95% confidence interval were calculated for each condition. Each experiment was repeated at least three times.

CELL MIGRATION ASSAY

Linear scratches in cell monolayers were made using a 1,000 μl pipette tip. Photographs of scratches were taken at 0, 24, and 32 h after monolayer wounding. Per each experimental condition, three independent wells were studied and in each of them fifteen microscopic fields were photographed using the 10× objective. The mean distances covered by the migrating fronts of monolayers and corresponding SEM were calculated.

CONFOCAL FLUORESCENCE MICROSCOPY

The effects of primary and secondary FGF stimulation on the actin skeleton of Swiss 3T3 cells and on the intracellular localization of NFκB were studied using confocal fluorescence microscopy. The cells were fixed with 4% neutral formalin, pre-incubated in blocking permeabilizing buffer (PBS with 5% BSA and 0.1% Triton X100), and then stained with Oregon green-conjugated phalloidin and TOPRO3 (both from Invitrogen, Carlsbad, CA). To study the nuclear translocation of NFκB, fixed and permeabilized cells were first incubated with rabbit anti-NFκB antibodies (Abcam, Cambridge, MA) and then stained with Alexa 488-conjugated anti-rabbit IgG

antibodies (Invitrogen) and TOPRO3. Cell images were taken using Leica SP1 confocal microscope at the MMCRI confocal microscopy facility.

IMMUNOBLOT ANALYSIS

Lysates were prepared from Swiss 3T3 monolayers that had undergone the four basic FGF1 stimulation conditions described above, after 18 h of the final FGF stimulation The cells were washed and scraped in ice-chilled PBS and centrifuged for collection at 2,500 rpm for 10 min. NPB buffer (20 mM Tris, 250 mM sucrose, KCl 60 μM, 20 mM EDTA, 1.5 M NaCl, 1% TritonX-100, 0.1% deoxycholic acid, and a 1:10 dilution of protease inhibitor cocktail from Sigma) was used to lyse the cells. Relative protein concentrations were determined with Coomassie Plus Protein Assay Kit (Pierce, Rockford, IL) using a DU 640 spectrophotometer (Beckman, Fullerton, CA) at an excitation wavelength of 595 nm. The lysate was then mixed with an equal volume of SDS–PAGE sample buffer and incubated at 95°C for 10 min. Equivalent sample amounts were resolved in the l2% PAGE and transferred to membranes Hybond-P (GE Healthcare, Little Chalfont, UK). The membranes were blocked in 5% fat-free dry milk diluted in TBS-Tween buffer at 42°C for 2 h and then blotted with the appropriate primary mouse or rabbit antibody overnight at 4°C. Membranes blotted with mouse anti-GAPDH antibodies (Sigma) served as loading controls. Antibodies against the following proteins were used: rabbit antibodies against phosphorylated Erk1/2 (Sigma) and cyclin D1 (Santa Cruz Biotechnologies, Santa Cruz, CA), mouse monoclonal antibodies against p21 and p27 (both from BD Biosciences, San Jose, CA) and cyclin A (Millipore, Temecula, CA). The bound primary antibodies were visualized using horseradish peroxidase-conjugated goat antibodies against rabbit or mouse IgG (BioRad, Hercules, CA) and the ECL detection system (Amersham, Piscataway, NJ).

HETEROKARYON ANALYSIS OF CELL PROLIFERATION BLOCKAGE

Fusion of FGF-primed and "naïve" Swiss 3T3 cells was used to analyze FGF memory maintenance. FGF priming was achieved by incubating confluent cultures of Swiss 3T3 cells for 48 h in quiescence medium, treating them with FGF1 during the following 36 h and then returning to quiescence for 48 h. Partner naïve Swiss 3T3 cells were first incubated for 72 h in full culture medium with 0.6 μg/ml BrdU, which resulted in the labeling of 100% nuclei. After that, cells were transferred to quiescence medium for 48 h. Quiescent FGF-primed and naïve cells were dislodged by trypsin, resuspended in full culture medium, centrifuged, resuspended in cold PBS, counted, mixed 1:1 and then fused using the HVJ Envelope Cell Fusion Kit GenomOne-CF™ (Cosmo Bio Co., Ltd., Tokyo, Japan) according to the manufacturer protocol. Fused cell were suspended in full cell culture medium and plated on fibronectin-coated glass coverslips. One hour later, they were transferred to quiescence medium and incubated for 48 h. After that, the cells were transferred to fresh quiescence medium with 10 ng/ml FGF1 and 5 μg/ml EdU, incubated for 36 h, fixed with 4% neutral formaldehyde, treated for 20 min at room temperature by 4 M HCl, washed in PBS and then stained for BrdU as described in the DNA Synthesis Study section except that fluorescence staining was achieved using Alexa 546-conjugated anti-mouse IgG antibodies (Invitrogen). Then, EdU incorporation was detected using the Click-iT EdU Assay kit (Invitrogen). Fluorescence microscopy was used to identify the following types of fused cells: (1) Naïve × naïve homodikayons (both nuclei red); (2) primed × primed homodikaryons (both nuclei devoid of red fluorescence); and (3) primed × naïve heterodikaryons (one of two nuclei is red). Percentages of EdU labeled (green fluorescence in the nuclei) fused cells and corresponding 95% confidence intervals were calculated for 1, 2, and 3.

STAINING FOR SENESCENCE-ASSOCIATED β-GLACTOSIDASE (SA-β-GAL) ACTIVITY

Cells were formalin fixed at the end of each standard treatment scheme, after 36 of the final FGF1 stimulation, and stained for SA-β-gal activity according to [Dimri et al., 1995].

RT-PCR

RNA was prepared from Swiss 3T3 cells, which had undergone four standard treatment schedules, after 18 h of the final FGF1 stimulation, using the RNeasy kit (Qiagen, Hilden, Germany). Expression of HDACs was assessed by RT-PCR using the SuperScript kit (Invitrogen). The following pairs of primers were utilized:

. hdac1 sense: 5′-GGA GAT CTA CCG TCC TCA CAA-3′
. hdac1 antisense: 5′-GCC ATC GCC ATG GTG AAT ATC A-3′
. hdac2. sense: 5′-GGA GGA CTA CAT CAT GCC AAG AA-3′
. hdac2. antisense: 5′-GCT AGG CTG GTA CAT CTC CAT CA-3′
. hdac3. sense: 5′-GGC CAT TAG TGA GGA ACT TCC-3′
. hdac3. antisense: 5′-TCC ACA TCA CTT TCC TTG TCG-3′
. hdac4. sense: 5′-GCT CTC CCA GCT CTC CAG CA-3′
. hdac4. antisense: 5′-GTT GTG AGC TGC TGC ACC GT-3′
. hdac5. sense: 5′-GCC ACG GAC TCC TCT GCA TAG C-3′
. hdac5. antisense: 5′-GGA TGA GCA GCT GCT GCT CC-3′
. hdac6. sense: 5′-TCA GGT CTA CTG TGG TCG TT-3′
. hdac6. antisense: 5′-TCT TCA CAT CTA GGA GAG CC-3′
. hdac7. sense: 5′-GTT CAC CAT GGC AAC GGC AC-3′
. hdac7. antisense: 5′-ACT GCC TGG GAA GAA GTT GCC-3′
. hdac8. sense: 5′-ACC GAA TCC AGC AAA TCC TCA-3′
. hdac8. antisense: 5′-ATA AAA TTC TTC CCC CCA ACT TGC-3′
. hdac9. sense: 5′-ACT GGT TCC ACA GCA GCG CAT AC-3′
. hdac9. antisense: 5′-GTT CCT TCA GCA GTA GGT GCT GC-3′
. hdac10. sense: 5′-CTG TGC TAA CAG GAG CTG TGC ACA-3′
. hdac10. antisense: 5′-CAT GCT CAT AGC GGT GCC AAG AGA-3′

β-actin was used as a loading control.

RESULTS

PROLIFERATIVE RESPONSE OF SWISS 3T3 CELLS TO REPEATED FGF STIMULATION

Studies conducted by Andreeva et al. [2004] demonstrated a strong reduction in DNA replication upon continuous stimulation of Swiss 3T3 cells with FGF1. The initial question asked in the present study was whether cells acquire memory of FGF1 stimulation and respond with significantly diminished DNA synthesis to the repeated FGF1 application after a period of quiescence. In a standard experiment, four stimulation schedules were followed (Fig. 1A). One population of Swiss 3T3 cells was quiescent for the entire 168 h period of the experiment. Another population underwent 132 h of quiescence followed by FGF1 stimulation for the final 36 h of the schedule. In the

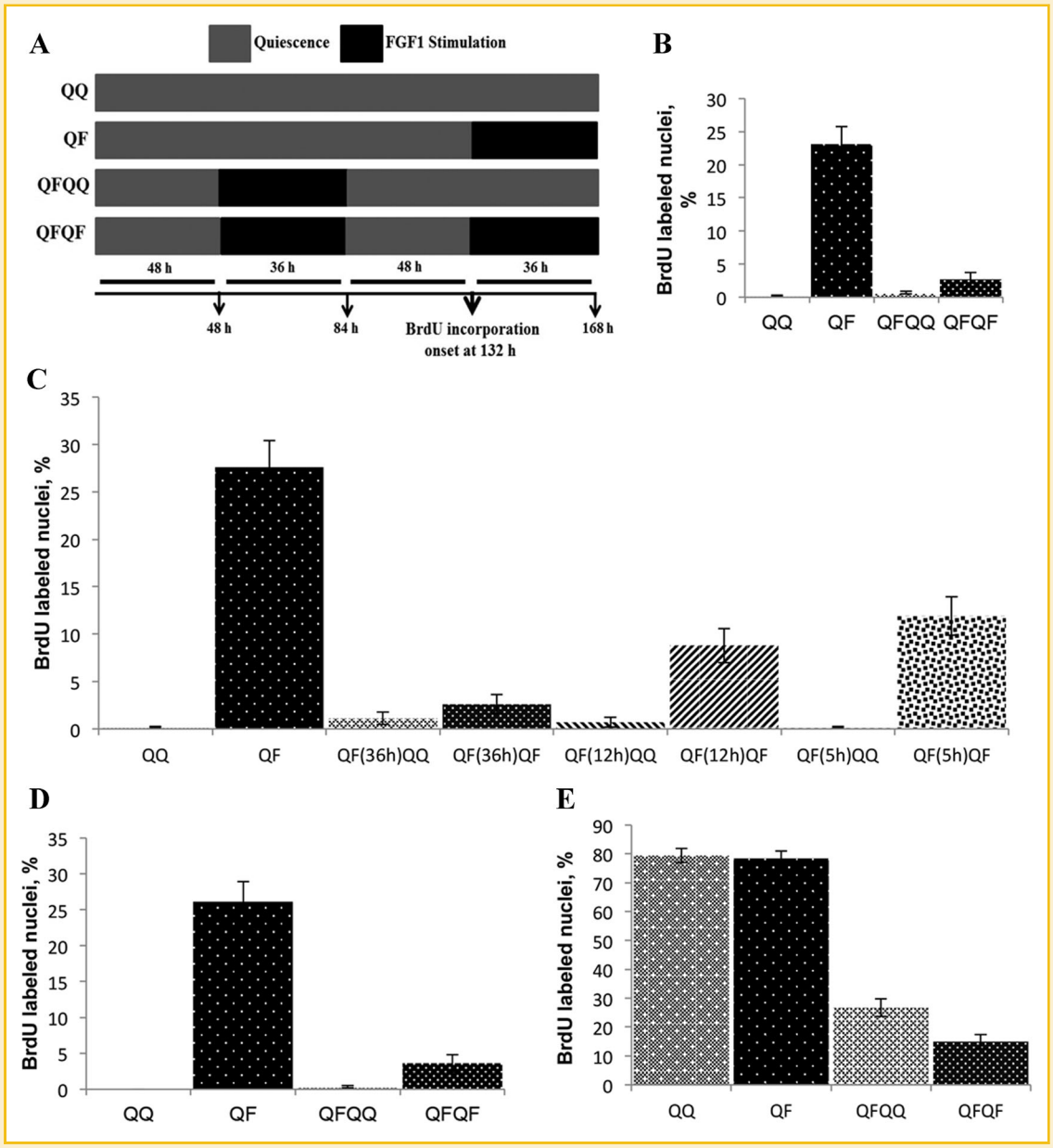

Fig. 1. A: Scheme of the repeated FGF1 stimulation experiment (explanation in the text). B: DNA replication is drastically reduced upon repeated stimulation with FGF1. BrdU incorporation levels were quantified in four different Swiss 3T3 populations. The stimulation schedules were as in (A). BrdU labeling was performed during the final 36 h of each stimulation schedule. Percentage of BrdU labeled cells with 95% confidence interval for each condition are shown. Here and in further graphs the experiments were repeated at least three times and the results of representative experiments are presented. (C) Acute establishment of cell "memory" of initial FGF1 stimulation. Swiss 3T3 cells were used for a repeated stimulation experiment, which in addition to four standard conditions included the four following schedules: QF(12h)QQ—cells were quiescent for 48 h, stimulated with FGF1 for 12 h, and then put back into quiescence for the remaining 108 h; QF(12h)QF—cells were quiescent for 48 h, stimulated with FGF1 for 12 h, then intermediate quiescence of 72 and 36 h of repeated FGF1 stimulation; QF(5h)QQ—cells were quiescent for 48 h, stimulated with FGF1 for 5 h, and then put back into quiescence for the remaining 115 h; QF(5h)QF—cells were quiescent for 48 h, stimulated with FGF1 for 5 h, intermediate quiescence of 79 h and then restimulated with FGF1 for 36 h. BrdU labeling occurred throughout the final 36 h of each stimulation schedule. (D) Cell "memory" of primary FGF1 stimulation is retained throughout an extended intermediate quiescence period. A repeated FGF1 stimulation experiment was performed with Swiss 3T3 fibroblasts, with an intermediate quiescence period extended from 2 to 5 days. (E) Spontaneous transformation of Swiss 3T3 cells does not prevent FGF "memory" formation. A standard repeated FGF1 stimulation experiment was performed with spontaneously transformed Swiss 3T3 cells.

third schedule, the cells were made quiescent for 48 h and then stimulated for 36 h with FGF1 followed by quiescence for the remainder of schedule. The fourth schedule of repeated FGF1 stimulation was as follows: 48 h of quiescence, 36 h primary FGF1 stimulation, 48 h intermediate quiescence, and then 36 h of secondary FGF1 stimulation. BrdU was present in media for the final 36 h of each stimulation schedule. Analysis of BrdU incorporation demonstrated a ten-fold reduction in DNA synthesis after secondary FGF1

stimulation, as compared to primary stimulation (Fig. 1B). Next, we determined how quickly the "memory" of the initial FGF1 stimulation was established. The primary stimulation period was reduced from 36 to 12 h or 5 h and, as before, BrdU incorporation throughout secondary stimulation was determined. Both 12 and 5 h primary stimulations resulted in a significant decrease in DNA replication upon repeated stimulation with FGF1 (Fig. 1C). It is important to note that this decrease becomes stronger with extended primary stimulation time. However, even 5 h of primary stimulation was sufficient to provoke an almost threefold reduction in DNA synthesis upon secondary FGF1 stimulation; thereby indicating that onset of the cellular "FGF memory" is acute.

In the following series of experiments, the longevity of the "FGF memory" was investigated by increasing the intermediate quiescence period between stimulations from 48 to 120 h. Extension of the intermediate quiescence period failed to produce a rescue in DNA replication upon repeated FGF1 stimulation (Fig. 1D), indicating that the "FGF memory" is stable for at least 120 h.

The maintenance of 3T3 cells at high density for over 10 passages results in the overgrowth of spontaneously transformed cells, which have lost the ability to achieve quiescence at low serum concentration. We produced spontaneously transformed Swiss 3T3 cells and assessed their response to primary and secondary FGF1 stimulation. While initial FGF1 treatment failed to increase the ratio of DNA synthesizing cells, which was already high, secondary stimulation resulted in a drastic inhibition of DNA replication, to a level well below the initial "quiescence" (Fig. 1E).

SWISS 3T3 CELLS ARE NOT UNIQUE IN THE "MEMORIZATION" OF FGF

The establishment of cell "memory" of FGF stimulation has been firmly proven for spontaneously immortalized Swiss 3T3 mouse embryo fibroblasts. To assess the extent of this phenomenon, we performed the FGF restimulation experiments with other non-transformed cell cultures: LE II mouse lung endothelial cells (Fig. 2A), 10T1/2 mouse mesenchymal stem cells (Fig. 2B), mouse ear-derived mesenchymal stem cells (Fig. 2C) and human adipose-derived stem cells (Fig. 2D). All of these cell types demonstrated a strong reduction of DNA synthesis in response to repeated FGF1 stimulation, as compared to primary stimulation DNA synthesis levels.

CELL "MEMORY" AND OTHER GROWTH FACTORS

Because various FGFs, including FGF1 and FGF2, signal through common receptors, we expected that the phenomenon of cell "memory" is not unique for FGF1. Indeed, we found that the restimulation experiments with FGF2 gave the results identical to those with FGF1. The proliferative response to the secondary FGF2 stimulation after an intermediate 48 h quiescence period was more than 10-fold lower than to the primary stimulation (Fig. 3A). However, unlike FGFs, the experiments with the PDGF-BB restimulation did not demonstrate the formation of cell "memory" of PDGF stimulation. Indeed, we did not find a significant difference between the levels of DNA synthesis induced by the primary and secondary PDGF-BB stimulations (Fig. 3B). On the contrary, PDGF-BB treatment

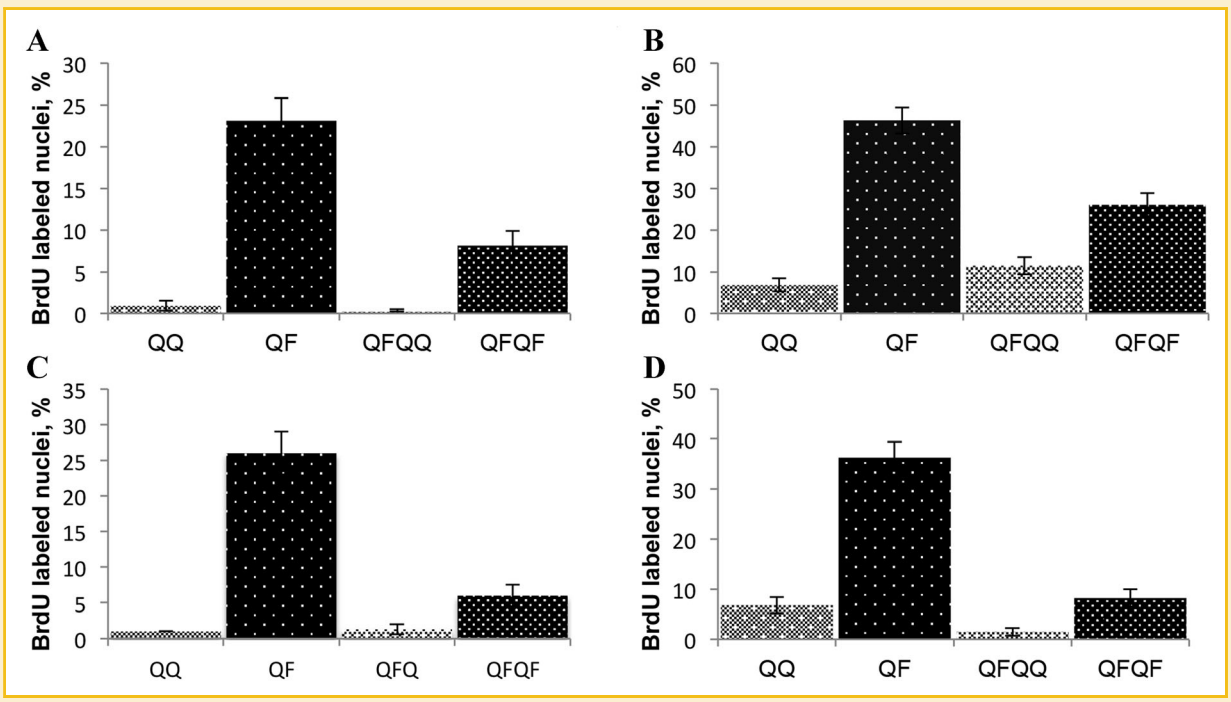

Fig. 2. "Memorization" of primary FGF1 stimulation in various cells. Standard repeated FGF1 stimulation experiments were performed with Lee II immortalized mouse lung endothelial cells (A), 10T1/2 mouse mesenchymal stem cells (B), ear-derived mouse mesenchymal stem cells (C), and adipose-derived human stem cells (D). Percentage of BrdU labeled cells with 95% confidence interval for each condition are shown. The experiments were repeated at least three times and the results of representative experiments are presented.

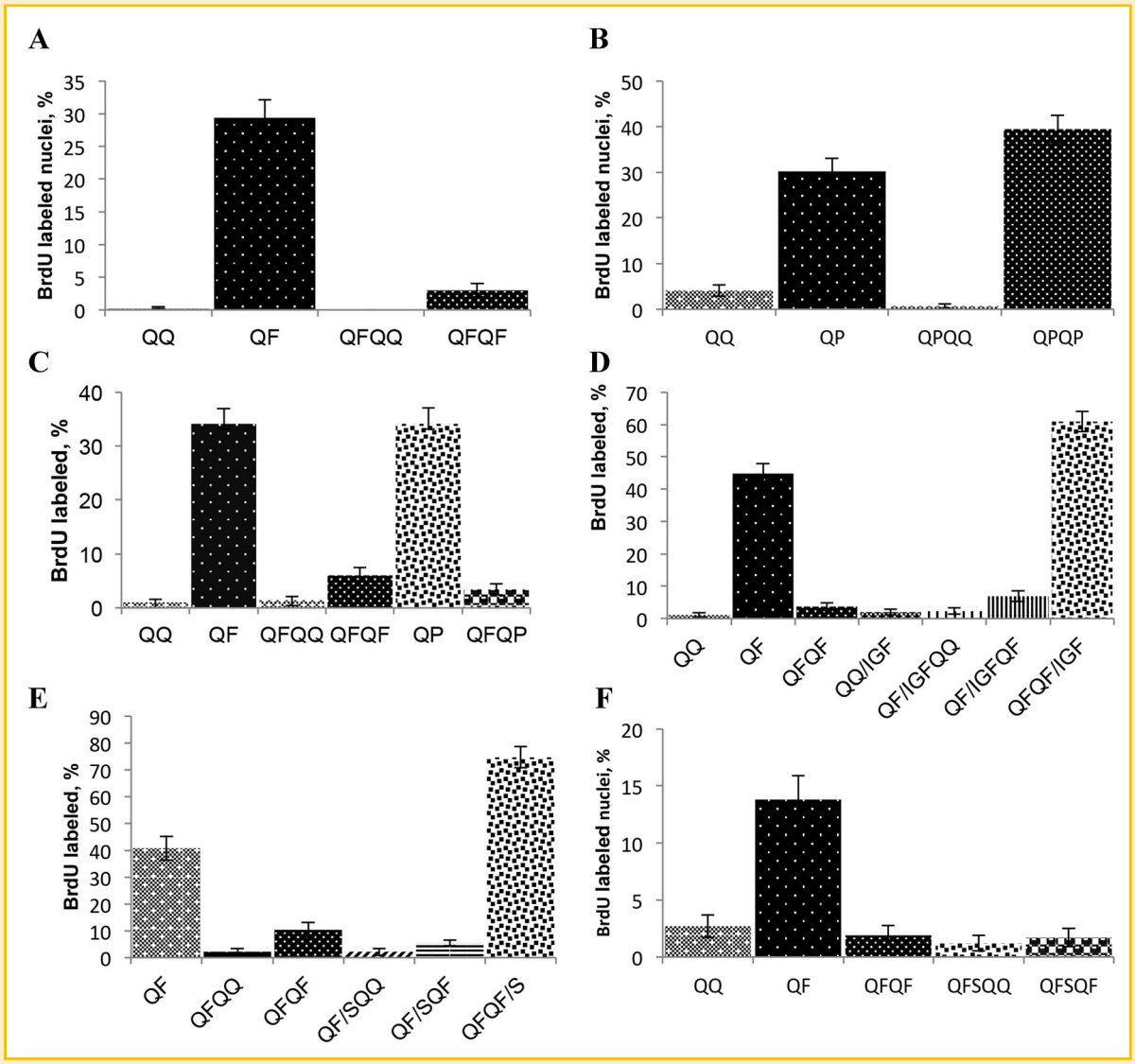

Fig. 3. Cells establish "memory" of FGF2 (A) but not of PDGF-BB (B) stimulation. FGF2 (10 ng/ml) and PDGF-BB (10 ng/ml) repeated stimulation experiments were carried out on Swiss 3T3 cells with schedules analogous to those depicted for FGF1 in Figure 1. Percentage of BrdU labeled cells with 95% confidence interval for each condition are shown. Here and in further graphs the experiments were repeated at least three times and the results of representative experiments are presented. C: Cells previously stimulated with FGF1 respond to PDGF-BB with a drastically reduced DNA synthesis. A standard repeated FGF1 stimulation experiment was performed with two additional schedules: QP—cells were quiescent for 132 h followed by 36 h with PDGF-BB; QFQP—cells were quiescent for 48 h, stimulated with FGF1 for 36 h, followed by 48 h intermediate quiescence, and then PDGF-BB stimulation was applied for 36 h. IGF1 (D) and serum (E) rescue DNA synthesis when applied in combination with FGF1 during the secondary but not during the primary stimulation. D: A standard repeated stimulation experiment was performed including the following additional schedules: QF/IGFQQ—cells were quiescent for 48 h, then stimulated with FGF1 for 36 h in the presence of 10 ng/ml IGF1 followed by an additional 84 h of quiescence; QF/IGFQF—cells were quiescent for 48 h, then stimulated with FGF1 for 36 h in the presence of IGF1, followed by 48 h of intermediate quiescence and then FGF1 stimulation was applied for 36 h; QQ/IGF—cells were quiescent for 132 h followed by an additional 36 h in the presence of IGF1 alone; QFQF/IGF—cells were quiescent for 48 h, then stimulated with FGF1 for 36 h, followed by 48 h of intermediate quiescence and then stimulation with FGF1 was applied for 36 h in the presence of IGF1. E: A standard repeated stimulation experiment was performed containing the following additional schedules: QF/SQQ—cells were quiescent for 48 h, then stimulated with FGF1 for 36 h in the presence of 10% serum, followed by 84 h of additional quiescence; QF/SQF—cells were quiescent for 48 h, then stimulated with FGF1 for 36 h in the presence of 10% serum, followed by 48 h of intermediate quiescence and then FGF1 stimulation was applied for 36 h; QFQF/S—cells were quiescent for 48 h, then stimulated with FGF1 for 36 h, followed by 48 h of intermediate quiescence, and then FGF1 stimulation was repeated for 36 h in the presence of 10% serum. F: Serum stimulation between the primary and secondary FGF1 treatments does not abolish FGF "memory." The following experimental schedules were used in this experiment: QQ—204 h of quiescence; QF—168 h of quiescence followed by 36 h of FGF1 stimulation; QFQQ—48 h of quiescence, 36 h of FGF1 stimulation and 120 h of quiescence; QFSQQ—48 h of quiescence, 36 h of FGF1 stimulation, 36 h with 10% serum, 84 h of quiescence; QFSQF—48 h of quiescence, 36 h of FGF1 stimulation, 36 h with 10% serum, 48 h of quiescence, 36 h of FGF1 stimulation.

of cells, which had been stimulated with FGF1 for 36 h and then underwent a 48 h period of quiescence, resulted in a dramatic decrease in DNA synthesis in comparison with FGF-untreated PDGF-BB stimulated cells (Fig. 3C). These data indicate that the loss of proliferative response to secondary stimulation after FGF treatment is not due to the loss of FGFRs but to some stable changes that reduce growth factor-induced entry to S-phase.

The observed phenomenon of FGF "memory" could be based on induced cell senescence. We assessed the activity of senescence-associated β-galactosidase (SA-β-gal) in FGF1-treated Swiss 3T3 cells. While control quiescent cells (132 h quiescence) did not express SA-β-gal, many cells at 36 h after primary or secondary FGF1 stimulation or at 84 h of intermediate quiescence were SA-β-gal-positive (Supplementary Fig. S2). However, the key characteristic of senescent cells is their inability to grow in response to proliferation inducers. To assess the proliferative potential of FGF primed cells, we applied potent proliferative stimuli. It is well established that the competence growth factors, including FGFs, cooperate with progression factors (insulin, IGFs) to support long-term cell proliferation [Villaudy et al., 1991]. We found that the combination of FGF1 with IGF1 during the restimulation of cells that had been previously treated with FGF1, resulted in a massive induction of DNA replication (Fig. 3D). In contrast, IGF1 applied alone failed to stimulate DNA replication in quiescent Swiss 3T3 cells, regardless of prior FGF stimulation (Fig. 3D). Thus, IGF1 enables cells burdened by the "memory" of FGF to respond to this mitogen. In contrast, application of IGF1 with FGF1 during the primary stimulation did not prevent the formation of FGF "memory," and the response to the restimulation with single FGF1 was still drastically reduced (Fig. 3D). Similar to IGF1, application of 10% bovine calf serum during the initial FGF1 stimulation did not rescue DNA synthesis during the restimulation of Swiss 3T3 cells with FGF1 alone (Fig. 3E), although the addition of 10% serum during the repeated FGF1 stimulation induced a large increase in DNA synthesis (Fig. 3E). Thus, although FGF1-treated cells exhibited SA-β-gal activity, unlike truly senescent cells they were able to resume proliferation following appropriate stimulations. The application of 10% serum for 36 h between the primary FGF1 stimulation and 48 h intermediate quiescence failed to rescue the proliferative response to the subsequent FGF treatment (Fig. 3F). This observation demonstrates that FGF memory can be retained in actively growing serum-stimulated cells.

MORPHOLOGICAL AND SIGNALING RESPONSES IN THE CELLS RESTIMULATED WITH FGF

One may suggest that the inability of cells to respond with a large increase of DNA synthesis to the restimulation with FGF is due to attenuated FGF signaling. Our earlier data that Swiss 3T3 cells continuously stimulated with FGF, although unable to enter the second replication cycle, have high levels of Erk1/2 activation [Andreeva et al., 2004], argue against this suggestion. However, we have specifically assessed the morphology and signaling events in cells restimulated with FGF1 after intermediate quiescence. Figure 4A shows that 84 h after the removal of primary FGF1, cells regained a typical non-polarized cobblestone-like quiescent morphology. Restimulation with FGF1 resulted in a spindle-shaped morphology similar to cells undergoing primary FGF1 stimulation. The confocal fluorescence study demonstrated that after the secondary FGF1 stimulation, Swiss 3T3 cells formed large sheets of submembrane F actin, and this response was more pronounced than after primary FGF1 stimulation (Fig. 4B). Immunoblotting showed that unlike quiescent cells and similar to cells undergoing primary stimulation, the restimulated cells had a high content of phosphorylated Erk1/2 (Fig. 4C). In addition, secondary FGF1 stimulation like the primary treatment induced the production of cyclin D1 (Fig. 4C), which is similar to cells undergoing a long-term continuous FGF1 stimulation [Andreeva et al., 2004]. In contrast, unlike the primary FGF1 treatment, repeated FGF1 stimulation failed to induce the expression of cyclin A, a critical regulator of cell entry to the S-phase (Fig. 4D). Expression of p27, a potent inhibitor of cyclin–CDK complexes was strongly decreased after both primary and secondary FGF1 stimulation (Fig. 4D). On the contrary, while the expression of p21, another cyclin–CDK inhibitor, was very low in quiescent cells, it was dramatically increased after both primary and repeated FGF1 treatment (Fig. 4D). A strong decrease of p21 level in the cells returned to quiescence after the initial FGF1 stimulation does not support the hypothesis that this protein might be responsible for the maintenance of FGF memory. Thus, cells primed with FGF exhibit most of the responses to FGF stimulation characteristic for naïve cells, except the expression of cyclin A. Interestingly, in transformed Swiss 3T3 cells, which have lost the ability to quiesce and exhibit a sharp decrease of DNA synthesis during the intermediated quiescence and repeated FGF1 stimulation (Fig. 1E), the expression of cyclin A was decreased already after the primary FGF1 stimulation (Fig. 4E).

FUSION OF FGF PRIMED AND NAÏVE CELLS INHIBITS THE PROLIFERATIVE RESPONSE TO FGF STIMULATION

The maintenance of "FGF memory" may be passive, that is, dependent exclusively on stable silencing of gene(s) needed for cell entry to the S phase after FGF restimulation. Alternatively, "FGF memory" may be supported by an active mechanism, which requires the continuous persistence of diffusible intracellular proteins preventing the proliferative response to the secondary FGF stimulation. The active character of "FGF1 memory" maintenance would not exclude a stable epigenetic reprogramming resulting in the permanent production of such proteins. Heterokaryon analysis is an established method for studying the general regulation of cell proliferative status [Zelenin and Prudovsky, 1989], and we used it to assess whether FGF memory is maintained through a passive or active mechanism. To distinguish the nuclei of partner cells in heterokaryons, naïve cells were prelabeled with a low dose of BrdU and then transferred to quiescence. The partner cells were primed with FGF1 for 36 h and then transferred to quiescence medium for 48 h. Naïve and primed cells were fused with Sendai virus envelopes, plated, transferred for 48 h to the quiescence medium, and then stimulated for 36 h with FGF1 in the presence of EdU. BrdU labeling was used to distinguish the nuclei of primed (BrdU negative) and naïve (BrdU positive) cells. The entry of heterokaryons and control homokaryons to DNA synthesis was detected by EdU incorporation in their nuclei (Fig. 4F). As expected, DNA synthesis in primed homodikaryons was five times lower than in naïve homodikaryons (Fig. 4G). DNA synthesis in heterodikayons containing one naïve and one primed nucleus was as low as in primed homodikaryons. These results demonstrate that

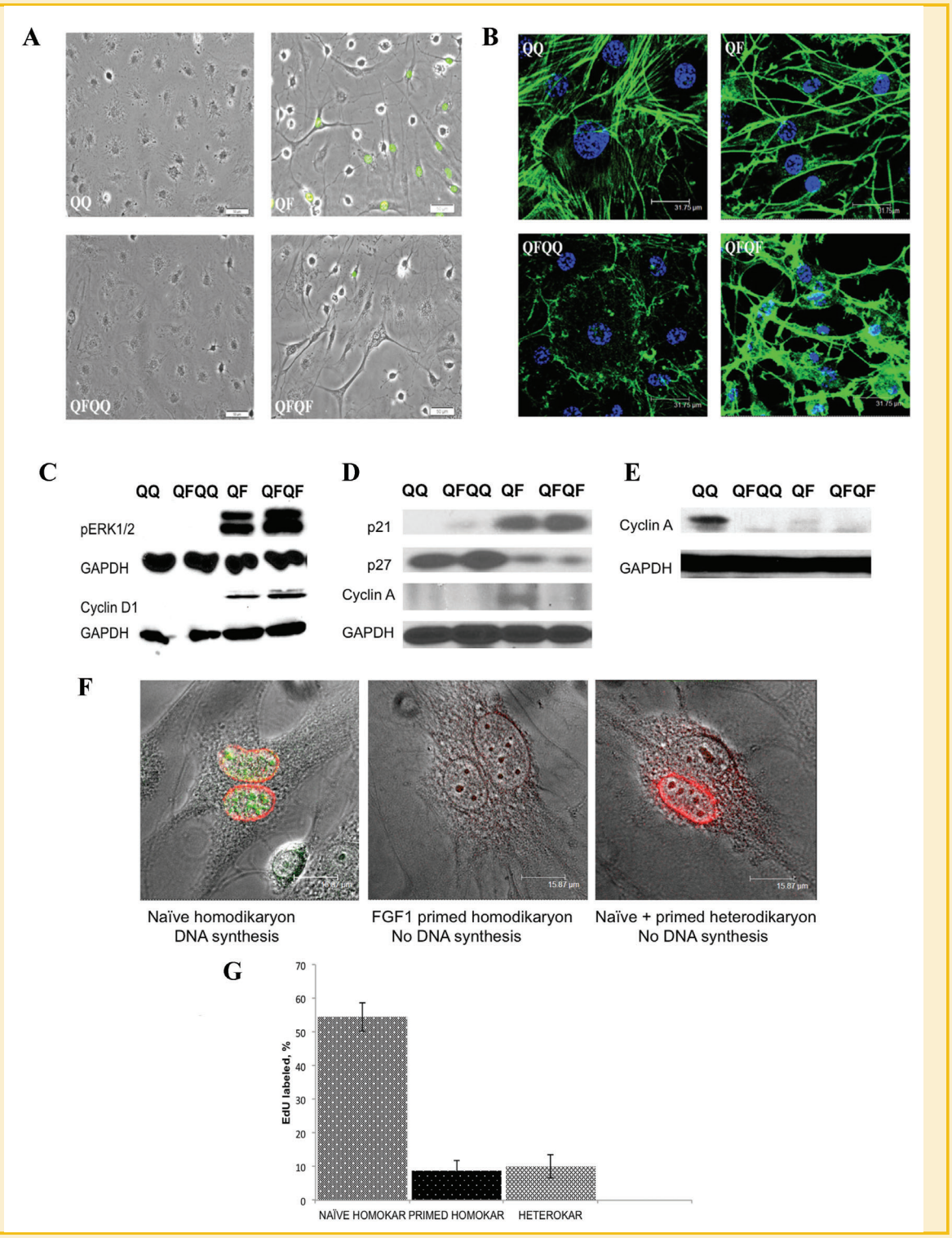

Fig. 4. A: Morphological changes in response to repeated FGF1 stimulation are strongly pronounced. A standard repeated FGF1 stimulation experiment was performed on Swiss 3T3 cells. Combined phase contrast and BrdU immunofluorescence images are presented. Bar: 50 μm. (B) Repeated FGF1 stimulation induces an exaggerated restructuring of actin cytoskeleton. A repeated FGF1 stimulation experiment was performed on Swiss 3T3 cells. Following formalin fixation, cells were co-stained with FITC-phalloidin for F-actin (green) and TOPRO3 for DNA (blue). Confocal fluorescence images are presented. Bar: 32 μm. Phosphorylation of Erk1/2 (C) and expression of Cyclin D1 (C), p21 (D), p27(D), and cyclin A(D) in response to repeated FGF1 stimulation. Repeated FGF1 stimulation experiments were performed on Swiss 3T3 cells. To assess Erk1/2 phosphorylation, the duration of final FGF1 stimulation was 45 min. Cyclin D1, p21, p27, and Cyclin A expression was determined after 18 h of final FGF1 stimulation. Total cell lysates were resolved by SDS–PAGE and immunoblotted for phospho-Erk1/2, Cyclin D1, p21, p27, cyclin A, or GAPDH. E: Expression of cyclin A in transformed Swiss 3T3 cells in response to repeated FGF1 stimulation. F: Heterokaryon analysis of FGF "memory" maintenance. Quiescent naïve Swiss 3T3 cells prelabeled with BrdU were fused with unlabeled quiescent FGF1 primed cells and stimulated with FGF1 in presence of EdU as described in "Materials and Methods" section. Confocal immunofluorescence microscopy in combination with phase contrast was used to identify heterokaryons and study DNA synthesis. BrdU—red. EdU—green. Bar: 16 μm. G: In heterokaryons, FGF primed cells suppress the FGF-induced DNA synthesis in partner naïve nuclei. Percentages of Edu labeled homo- and heterodikaryons with 95% confidence intervals are shown. The experiment was repeated twice with similar results.

FGF1 memory is maintained in an active manner, apparently due to the production of diffusible intracellular factors which can prevent the entry of nuclei to the S phase.

CELLS EXHIBIT ENHANCED MIGRATION AFTER SECONDARY FGF STIMULATION

Since, unlike DNA synthesis, the morphological response to the secondary FGF stimulation was unchanged, we next compared the migratory behavior of Swiss 3T3 cells after the primary and repeated FGF treatment. Uniform wounds were produced in the monolayers of Swiss 3T3 cells immediately before the final stimulation with FGF1 and the average distance traveled by the monolayer fronts after 36 h was determined. Interestingly, the migration of cells after secondary FGF stimulation was significantly faster than cells in cultures after primary FGF treatment (Fig. 5). Thus, while FGF pretreatment strongly reduces the proliferative response to secondary FGF stimulation, the migratory potential of these cells is not only preserved but significantly enhanced.

PROTEIN SYNTHESIS, BUT NOT DNA REPLICATION IS REQUIRED FOR FGF MEMORY FORMATION

Primary stimulation with FGF1 resulted in DNA synthesis. To determine the contribution of S phase progression to FGF "memory" formation, repeated stimulation experiments were performed that included an additional schedule in which DNA synthesis was blocked throughout the primary stimulation. It was achieved using the chemical inhibitor aphidicolin or an excess of thymidine, which halted cell entry to the S-phase. Both aphidicolin (Fig. 6A) and high thymidine (Fig. 6B) application during primary stimulation failed to rescue DNA synthesis in response to repeated FGF1 stimulation. Conversely, the application of cyloheximide, an inhibitor of protein synthesis, during the primary FGF stimulation, almost completely

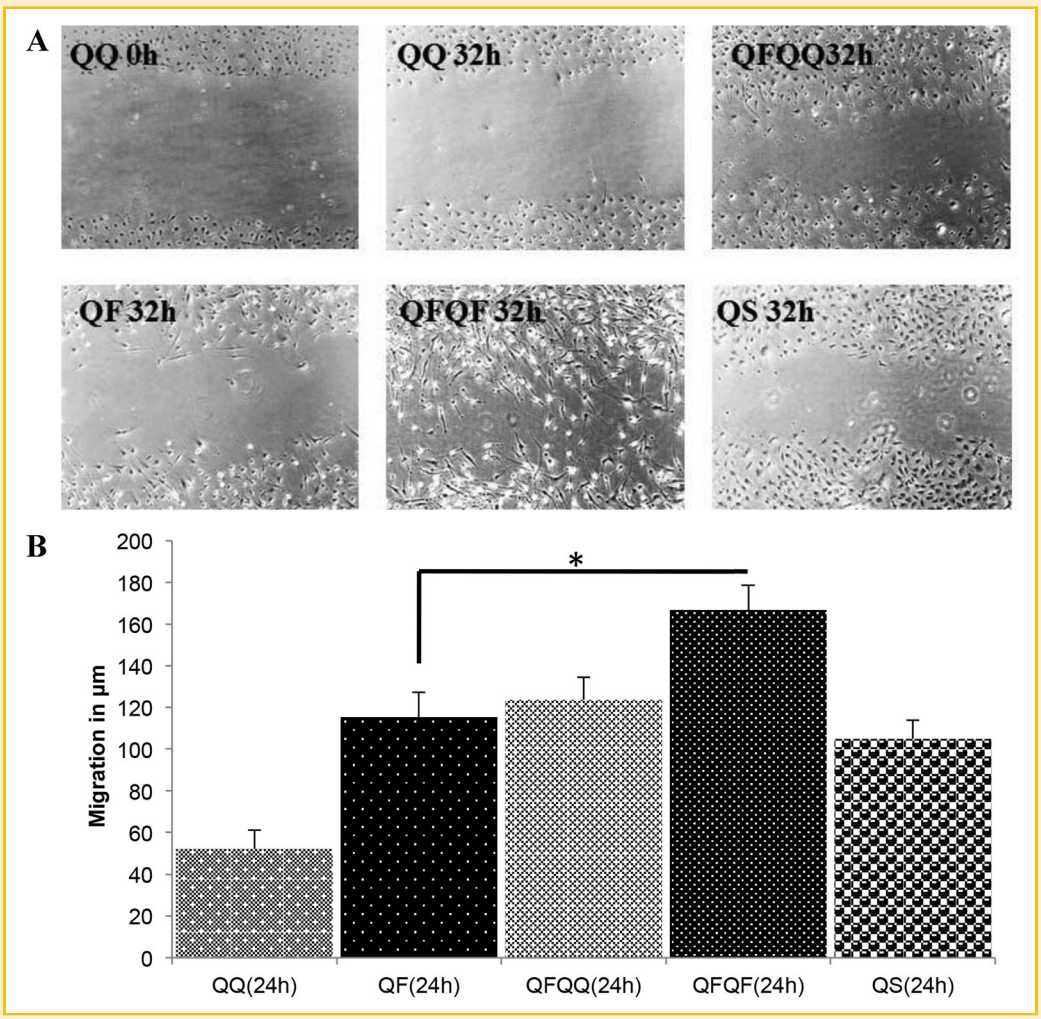

Fig. 5. Repeated FGF stimulation results in enhanced cell migration. A standard repeated FGF1 stimulation experiment was performed. At the end of intermediate quiescence period, linear scratches in cell monolayers were made as described in Materials and Methods section and then the usual stimulation schedules were followed. One additional schedule was included: QS—cells monolayers were quiescent for 132 h, then wounded and stimulated with 10% serum. Photograph of scratches were taken at 0, 24, and 32 h after monolayer wounding (see representative 0 and 32 h photos in A). Mean distances (with corresponding SEM) covered by migrating monolayer fronts by 24 h are presented on B. $^{*}P < 0.05$. The experiment was repeated three times with similar results.

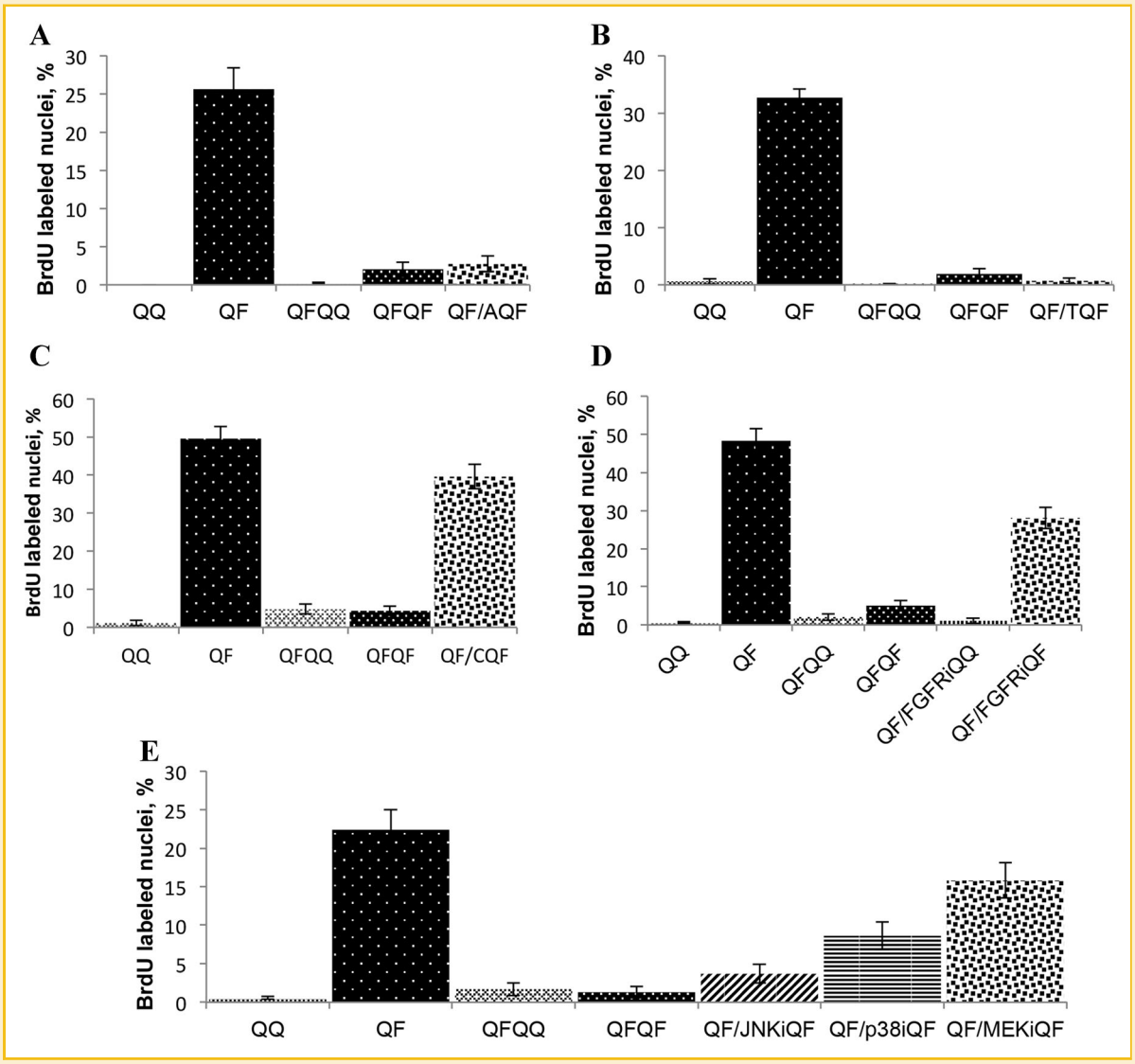

Fig. 6. A: Inhibition of DNA synthesis by aphidicolin throughout primary stimulation does not rescue the DNA synthesis following repeated FGF1 stimulation. A standard repeated FGF1 stimulation experiment was performed with one additional schedule: QF/AQF—cells were quiescent for 48 h, then stimulated with FGF1 for 36 h in the presence of 10 μM aphidicolin, followed by 48 h of intermediate quiescence and then stimulation was repeated for 36 h. Percentage of BrdU labeled cells with 95% confidence interval for each condition are shown. Here and in further graphs the experiments were repeated at least three times and the results of representative experiments are presented. B: Thymidine block throughout primary FGF1 stimulation fails to rescue DNA synthesis in response to repeated stimulation. A standard repeated stimulation experiment was performed with one additional schedule: QF/TQF—cells were quiescent for 48 h, then stimulated with FGF1 for 36 h in the presence of a 2 mM thymidine, followed by 48 h of intermediate quiescence and then repeated FGF1 stimulation for 36 h. C: Protein synthesis inhibition throughout the primary FGF1 stimulation rescues DNA synthesis in response to repeated stimulation. A standard repeated FGF1 stimulation experiment was modified by decreasing the time of primary FGF1 treatment to 18 h and introducing an additional schedule: QF/CQF—48 h quiescence, 18 h of FGF1 stimulation in presence of 10 μg/ml cycloheximide, 48 h quiescence, 36 h FGF1 stimulation. D: FGF receptor inhibition throughout primary FGF stimulation rescues DNA synthesis levels in response to repeated FGF stimulation. A standard repeated FGF stimulation experiment was performed with two additional schedules: QF/FGFRiQQ—cells were quiescent for 48 h, then stimulated with FGF1 in the presence of 1 μM FGF receptor inhibitor PD166866 for 36 h, followed by an 84 h of quiescence; QF/FGFRiQF—cells were quiescent for 48 h, then stimulated with FGF1 in the presence of 1 μM PD166866 for 36 h, followed by a 48 h intermediate quiescence and secondary stimulation with FGF1 for 36 h. E: Inhibition of p38 MAPK and MEK throughout primary FGF1 stimulation rescues DNA synthesis in response to secondary stimulation. A standard repeated stimulation experiment was performed with the additional schedules: QF/JNKiQF—cells were quiescent for 48 h, then stimulated with FGF1 in the presence of 20 μM JNK inhibitor II, followed by 48 h of intermediate quiescence and finally 36 h of FGF1 stimulation; QF/p38iQF—cells were quiescent for 48 h, then stimulated with FGF1 in the presence of 10 μM of the p38 MAPK inhibitor SB202190, followed by 48 h of intermediate quiescence and finally 36 h of FGF1 stimulation; QF/MEKiQF—cells were quiescent for 48 h, then stimulated with FGF1 in the presence of 10 μM of the MEK inhibitor U0126, followed by 48 h of intermediate quiescence and finally 36 h of FGF1 stimulation.

rescued the proliferative response to secondary stimulation (Fig. 6C). Thus, the establishment of cell "memory" of FGF does not require DNA replication but is dependent on protein synthesis.

FGFR SIGNALING, MAPK ACTIVITY AND NFκB SIGNALING ARE REQUIRED FOR THE ESTABLISHMENT OF FGF "MEMORY"

The necessity of protein synthesis for formation of "memory" of FGF stimulation led us to the question of which components of the FGF1 signaling pathway are required for this phenomenon. In the first series of experiments, we assessed the requirement of FGFR activity. A repeated stimulation experiment was performed that included all four standard schedules, as well as two additional schedules in which FGF receptor signaling was chemically inhibited throughout the primary FGF1 stimulation period. In one of the additional schedules, the cells remained in quiescence following primary FGF1 stimulation and FGFR inhibition; in the other, they underwent repeated FGF1 stimulation following intermediate quiescence. Inhibition of FGFR throughout primary stimulation with the specific inhibitor PD166866 produced a strong rescue of DNA replication in response to repeated FGF1 stimulation (Fig. 6D). This result implies that FGFR signaling directly leads to memory formation; therefore downstream components of FGF signaling pathway were considered further.

FGFR signaling activates MAPK p38, ERK1/2, JNK [Maher, 1999; Makino et al., 2010]. We investigated the contribution of these kinases to the establishment of cell "memory" of FGF stimulation. Repeated stimulation experiments containing all standard schedules and three additional schedules were performed. They included primary stimulation in the presence of a chemical inhibitor of one of the investigated kinases followed by intermediate quiescence and repeated FGF1 stimulation in the absence of kinase inhibitors. We found that the three kinases unequally contributed to cellular memory of primary FGF1 stimulation (Fig. 6E). JNK inhibition throughout the primary stimulation failed to produce a significant rescue in S phase progression upon repeated FGF1 stimulation. Inhibition of p38 MAPK throughout the primary stimulation produced a partial rescue of DNA synthesis in response to secondary stimulation. An even stronger rescue was associated with the inhibition of MEK, an upstream kinase of ERK1/2 signaling cascade (Fig. 6E). Therefore, p38 MAPK and to a greater extent ERK1/2 appear to be involved in the formation of "memory" of FGF1 stimulation.

NFκB signaling, which plays important roles in the regulation of cell proliferation, is known to be activated by the Ras-Erk signaling pathway [Han et al., 2006]. We used NFkB signaling inhibitors to elucidate whether it is required for the formation of cellular memory of FGF stimulation. Two inhibitors of NFκB were used: celastrol (Fig. 7A) and BMS-345541 (Fig. 7B). For each inhibitor, repeated stimulation experiments were performed, including an additional schedule for NFκB inhibition during primary FGF stimulation. Both inhibitors produced a strong rescue in DNA replication upon secondary FGF1 stimulation. Activation of the NFκB signaling results in the translocation of NFκB to cell nuclei [Hatada et al., 2000]. Confocal immunofluorescence study demonstrated that while more than 99% of quiescent cells had NFκB-negative nuclei, primary FGF1 stimulation resulted in the presence of NFκB in most cell nuclei (Fig. 7C). Interestingly the predominant majority of cell nuclei remained NFκB positive 84 h after the removal of FGF1 and the return to quiescence medium. Strong nuclear NFκB positivity was maintained after the secondary FGF stimulation.

HDAC ACTIVITY IS INVOLVED IN THE ESTABLISHMENT OF "FGF1 MEMORY"

The existence of "cell memory" of primary FGF stimulation indicates that FGF treatment may induce epigenetic changes that prevent the efficient induction of DNA synthesis in response to secondary stimulation. These modifications can involve changes in DNA methylation, histone methylation, and histone acetylation. Swiss 3T3 treatment with the DNA methyltransferase inhibitor azacytidine throughout the primary FGF stimulation did not prevent the formation of "FGF memory" (Fig. 8A). Similarly, BIX-01294, the inhibitor of histone methyltransferase G9a, which blocks the dimethylation of histone 3 (H3) lysine 9, failed to block the establishment of FGF "memory" (Fig. 8B). The involvement of epigenetic modifiers histone deacetylases (HDAC) in the regulation of vertebrate development by FGF was reported previously [Xu et al., 2000]. Therefore, we next assessed the effects of cell treatment with trichostatin A (TSA), a potent HDAC inhibitor, on "FGF memory." As in experiments with azacytidine and BIX-01296, Swiss 3T3 cells were treated with TSA during primary FGF stimulation (Fig. 8C). TSA pretreatment resulted in a strong rescue of DNA synthesis in response to secondary stimulation. This result indicates that HDAC activity is involved in the formation of cell "memory" of FGF stimulation. In an attempt to identify the HDAC(s) involved in the formation of FGF memory, we used RT-PCR to analyze the effect of primary and secondary FGF1 stimulation on the expression of HDAC1-10. The expression of HDAC 1, 2, 3, 7, and 8 was not perturbed by FGF stimulation, and the expression of other HDACs was not detectable (Supplementary Fig. S1). RNAi analysis will be needed to identify specific HDAC(s) required for the formation of FGF memory.

DISCUSSION

We found that temporary FGF stimulation resulted in the establishment of cell "memory," which prevents the efficient induction of DNA synthesis in response to secondary FGF treatment. The "memory" of FGF stimulation was retained by immortalized mouse embryo Swiss 3T3 fibroblasts for at least 5 days. The ability to "remember" FGF stimulation was also exhibited by mesenchymal stem cells, lung endothelial cells, and adipose-derived stem cells. Thus, FGF "memory" is not restricted to Swiss 3T3 cells. Unlike FGF1 and FGF2, PDGF stimulation did not induce the formation of cell "memory." Interestingly, although serum and IGF1 applied simultaneously with FGF1 during secondary stimulation rescued DNA synthesis, their application in the course of primary FGF stimulation did not prevent the establishment of FGF "memory." The drastic decrease of proliferation in transformed Swiss 3T3 cells restimulated with FGF1 and the preservation of "FGF memory" after an intermediate period of growth in high serum additionally demonstrate the resilience of the discovered phenomenon to both cell transformation and strong proliferative stimulation. While secondary FGF stimulation failed to efficiently induce DNA synthesis, it resulted

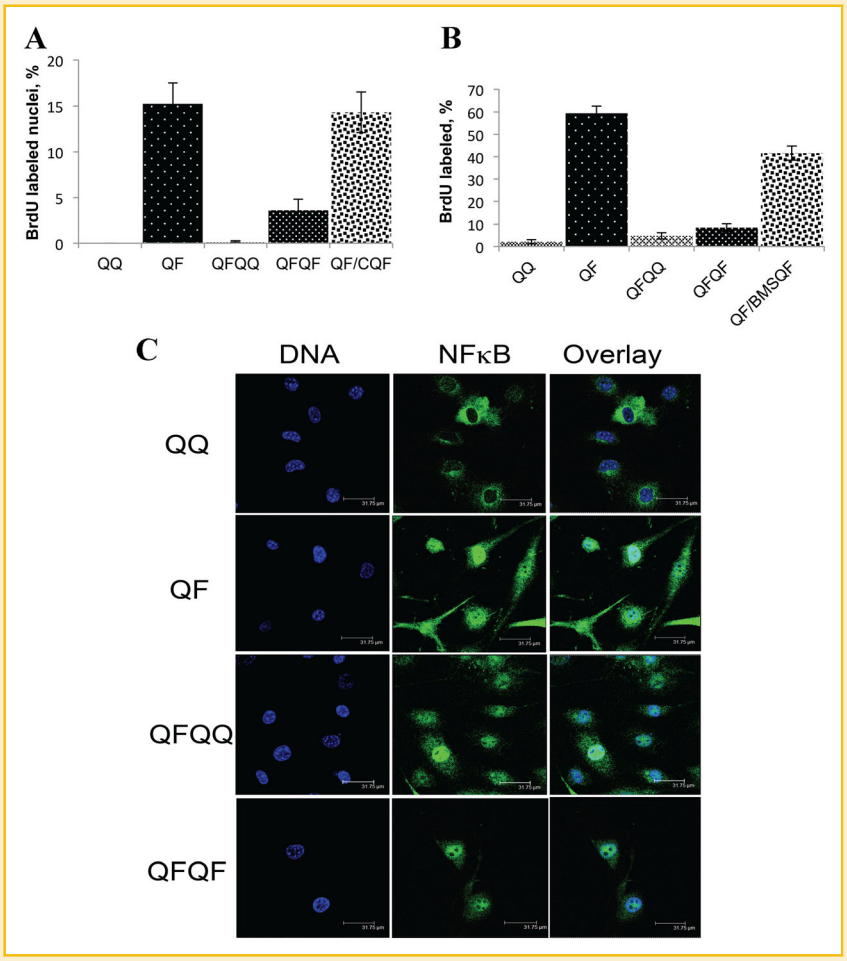

Fig. 7. A: Inhibition of NFκB signaling with celastrol throughout primary stimulation with FGF1 rescues DNA synthesis in response to secondary stimulation. A standard repeated stimulation experiment was performed with one additional schedule: QF/CQF—cells were quiescent for 48 h, then stimulated with FGF1 for 36 h in the presence of 500 nM celastrol, made quiescent for another 48 h and then restimulated with FGF1 for 36 h. Percentage of BrdU labeled cells with 95% confidence interval for each condition are shown. Here and in (B) and (C), the experiments were repeated at least three times and the results of representative experiments are presented. B: Inhibition of NFκB signaling with BMS-345541 throughout primary FGF1 stimulation rescues DNA synthesis after secondary stimulation. A standard repeated stimulation experiment was performed with one additional schedule: QF/BMSQF—cells were quiescent for 48 h, then stimulated with FGF1 for 36 h in the presence of 5 μM BMS-345541, made quiescent for another 48 h and then restimulated with FGF1 for 36 h. C: FGF stimulation results in the persistent nuclear localization of NFκB. A standard repeated stimulation experiment was performed with Swiss 3T3 cells plated on coverslips. Fixed cells were permeabilized and fluorescently stained for NFκB (green) and DNA (blue). Bar: 32 μm.

in high level of Erk1/2 phosphorylation, and, at later time point—cyclin D1 expression. These results, together with a strongly decreased proliferative response of cell previously stimulated with FGF to treatment with PDGF, a growth factor signaling through a different receptor, indicate that FGF "memory" cannot be explained by stable internalization or inactivation of FGFR. At the same time, unlike the primary, secondary FGF stimulation failed to induce the expression of cyclin A. This deficiency apparently prevents the efficient transition from the G1 to S phase. Interestingly, "FGF memory" only limits the proliferative response to the secondary stimulation, whereas morphological changes and migration are enhanced.

Inhibitor analysis demonstrated that the establishment of FGF "memory" depended upon FGFR signaling during primary stimulation. Interestingly, it required the activation of not only MEK-Erk1/2 pathway but also p38 MAPK. However, the most drastic rescue of DNA synthesis in response to secondary FGF stimulation was achieved by inhibition of NFκB signaling during primary FGF treatment. Rozenblatt-Rosen et al. [2002] reported that NFkB signaling activation accompanies the inhibition of chondrocyte proliferation induced by FGF. It is noteworthy that both Erk1/2 [Kang et al., 2008] and p38 MAPK [Rajaiya et al., 2008] can activate NFκB signaling and that activated NFκB can form transcription repressing complexes with HDAC [Liu et al., 2010]. In accordance with that, TSA, a potent inhibitor of HDAC, also efficiently rescued the DNA synthesis in response to repeated FGF stimulation.

These results are corroborated by confocal immunofluorescence studies, which demonstrated that primary FGF1 stimulation resulted in translocation of NFκB into the majority of Swiss 3T3 cell nuclei, which remained NFκB positive for up to 84 h after the removal of

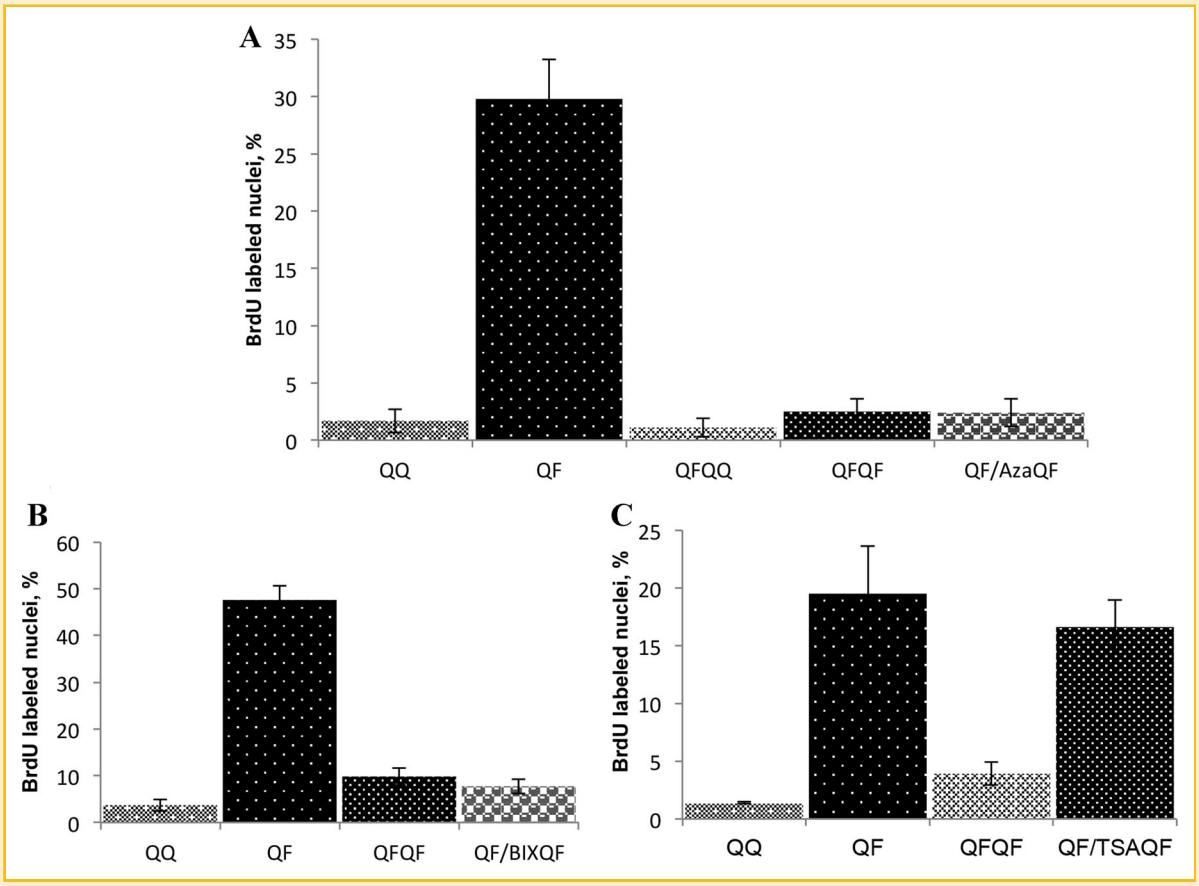

Fig. 8. A: Inhibition of DNA methylation throughout primary FGF1 stimulation does not interfere with FGF "memory." A standard repeated stimulation experiment was performed with one additional schedule: QF/AzaQF—48 h of quiescence, 36 h of FGF1 stimulation in the presence of 5 μg/ml Azacytidine, 48 h of intermediate quiescence, and finally 36 h of repeated FGF1 stimulation. Percentage of BrdU labeled cells with 95% confidence interval for each condition are shown. Here and in further graphs the experiments were repeated at least three times and the results of representative experiments are presented. B: Inhibition of histone methyltransferase G9a throughout primary FGF1 stimulation does not interfere with FGF "memory" formation. A standard repeated stimulation experiment was performed with one additional schedule: QF/BIXQF—48 h of quiescence, 36 h of FGF1 stimulation in the presence of 5 μM BIX-01294, 48 h of intermediate quiescence, and finally 36 h of repeated FGF1 stimulation. C: Inhibition of HDAC activity throughout primary stimulation with FGF1 rescues DNA synthesis in response to secondary stimulation. A standard repeated stimulation experiment was performed with one additional schedule: QF/TSAQF—48 h of quiescence, 36 h of FGF1 stimulation in the presence of 333 nM TSA, 48 h of intermediate quiescence, and finally 36 h of repeated FGF1 stimulation.

FGF1 and return to quiescent conditions. On the other hand, the heterokaryon analysis demonstrated an active character to the maintenance of FGF "memory," which is manifested by the persistence of unidentified diffusible intracellular factors preventing the efficient proliferative response to the secondary FGF stimulation.

We suggest that FGFR-dependent signaling mediated by Erk1/2 and p38 MAPK results in the NFκB- and HDAC-dependent deacetylation of some regulatory genetic elements, which is maintained for extended periods and prevents the efficient entry of cells into the S-phase upon repeated FGF or PDGF stimulation. However, this limitation can be superseded if IGF is applied in the course of the secondary FGF treatment. The genes involved in the establishment and maintenance of FGF memory remain to be elucidated.

The existence of FGF "memory" in vivo is yet to be determined. Some available data indirectly suggests this possibility. The application of recombinant FGF1 and FGF2 for repair of different organ and tissue damages has been studied and developed during the last 20 years [Khurana and Simons, 2003; Barrientos et al., 2008]. However, the results of these studies are still inconclusive in spite of improvement in the methods of FGF delivery including the use of polymer implants slowly releasing FGFs [Barrientos et al., 2008]. Indeed, unlike PDGF-BB, FGFs have not yet been approved by FDA for clinical use [Barrientos et al., 2008]. Moreover, a recent phase III clinical study of FGF1 treatment of limb ischemia in diabetic patients failed to confirm its healing effect. One may suggest that the inefficiency or low efficiency of recombinant FGFs reported in a number of studies could be due to the insufficient proliferation caused by the rapid establishment of FGF "memory."

Under normal conditions, the availability of endogenous FGFs in the organism is under strict control at the level of transcription [Ford-Perriss et al., 2001]. In addition, unlike most of other members of FGF family, ubiquitously expressed FGF1 and FGF2 are devoid of signal peptides and thus are exported through tightly regulated nonclassical secretion mechanisms [Prudovsky et al., 2008; Nickel and Rabouille, 2009]. Tissue damage can result in increased FGF

production and release from local cells and also from invading macrophages [Zhang et al., 1993; Ganat et al., 2002; Rossini et al., 2005]. We suggest that "FGF memory" is required for precise regulation of cell proliferation in the organism, which prevents excessive cell proliferation and hyperplasia. It is noteworthy that FGFs are involved in the formation, growth, and metastasis of many tumors [Korc and Friesel, 2009]. In addition to the loss of cell cycle control, the enhanced production of IGFs characteristic for various tumors [Gallagher and LeRoith, 2011] may overcome the FGF memory and result in continuous proliferation of tumor cells stimulated by locally produced FGF and IGF. Especially interesting is the observed "uncoupling" of the proliferation (which is inhibited) and migration (which is enhanced) in the cells undergoing a secondary FGF stimulation. One can suggest that while the cells are expending less energy on DNA synthesis they can devote more of it to migration. This may also have significance to the in vivo situation. Indeed FGF release shortly after wounding may stimulate DNA synthesis, while continuous FGF export could enhance cell migration needed to fill the wound.

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