Extracellular Signal-Regulated Kinase 2 and CHOP Restrict the Expression of the Growth Arrest-Specific p20K Lipocalin Gene to G₀

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The activation of the growth arrest-specific (gas) p20K gene depends on the interaction of C/EBP β with two elements of a 48-bp promoter region termed the quiescence-responsive unit (QRU). Here we identify extracellular signal-related kinase 2 (ERK2) as a transcriptional repressor of the p20K QRU in cycling chicken embryo fibroblasts (CEF). ERK2 binds to repeated GAAAG sequences overlapping the C/EBP β sites of the QRU. The recruitment of ERK2 and C/EBP β is mutually exclusive and dictates the expression of p20K. C/EBP homologous protein (CHOP) was associated with C/EBP β under conditions promoting endoplasmic reticulum (ER) stress and, to a lesser extent, in cycling CEF but was not detectable when C/EBP β was immunoprecipitated from contact-inhibited cells. During ER stress, overexpression of CHOP inhibited p20K, while its downregulation promoted p20K, indicating that CHOP is also a potent inhibitor of p20K. Transcriptome analyses revealed that hypoxia-responsive genes are strongly induced in contact-inhibited but not serum-starved CEF, and elevated levels of nitroreductase activity, a marker of hypoxia, were detected at confluence. Conditions of hypoxia (2% O₂) induced growth arrest in subconfluent CEF and markedly stimulated p20K expression, suggesting that the control of proliferation and gas gene expression is closely linked to limiting oxygen concentrations associated with high cell densities.

In response to unfavorable conditions of proliferation, cells exit the cell cycle and enter a state of reversible growth arrest known as G₀. The G₀ state of quiescence is characterized by the activation of a group of genes referred to as growth arrest-specific (gas) genes (1). The products of gas genes contribute directly to growth arrest (2, 3), enhance survival under conditions of oxidative stress (4), are components of the extracellular matrix (5–7), are involved in lipid metabolism (8–11), and prepare the cell to reenter the cell cycle (12, 13). Our understanding of the signals and mechanisms regulating the expression of gas genes is incomplete. A subset of gas genes are regulated at the posttranscriptional level, while others depend on transcriptional activation for expression under conditions of contact inhibition or serum starvation (6, 14–18).

We previously characterized the induction of the p20K lipocalin gene by cell density. In chicken embryo fibroblasts (CEF), p20K is expressed predominantly at confluence and, to a lesser extent, in response to serum/medium depletion (17, 19). Transcriptional activation of the p20K gene depends on a 48-bp region of the promoter termed the quiescence-responsive unit (QRU) (17). C/EBP β binds to two elements of the QRU and is sufficient to induce the expression of p20K when overexpressed in cycling cells (20).

C/EBP β is activated in response to several stimuli and plays an important role in biological processes unrelated to growth arrest. For instance, we reported that the activity of C/EBP β is induced in CEF transformed by Rous sarcoma virus (RSV), i.e., under conditions where p20K is not expressed (21). Mitogenic stimulation also controls the activity of C/EBP β and promotes the expression of genes of the G₀/G₁ transition. AP-1, a factor controlling the expression of interleukin-8 (IL-8) and cyclin D1, inhibits the induction of p20K and plays a major role in this process (22). Cells overexpressing c-Jun, JunD, or Fra-2, the main components of AP-1 in cycling CEF, do not express p20K, are unable to enter G₀, and undergo apoptosis at a high cell density. Normal CEF entering G₀ downregulate the activity of AP-1 by a number of mechanisms that include the repression of c-Jun, JunD, and Fra-2 expression.

The expression of a dominant negative mutant of C/EBP β blocks the induction of p20K and enhances the activity and expression of AP-1 proteins in CEF. Therefore, AP-1 and C/EBP β play opposing roles in the expression of gas genes in CEF (22).

Despite these advances, the signals promoting the transcriptional activation of p20K and the factors interacting or antagonizing C/EBP β in this process remain poorly understood. In this report, we identify extracellular signal-regulated kinase 2 (ERK2) as a transcriptional repressor binding directly to the QRU in cycling cells and competing with C/EBP β for recruitment to the p20K promoter. Heterodimers consisting of C/EBP homologous protein (CHOP) and C/EBP β were prominent under conditions of endoplasmic reticulum (ER) stress but were also detected, albeit at lower levels, in actively dividing cells. In contrast, CHOP-C/EBP β dimers were absent in density-arrested CEF. Forced expression of CHOP reduced p20K levels in growth-arrested cells, while downregulation of CHOP reestablished p20K expression under conditions of ER stress. Therefore, CHOP also functioned as a factor limiting p20K expression. We characterized the transcriptomes of cycling CEF and CEF rendered quiescent by serum starvation or contact inhibition. While genes induced by starvation were identified in these analyses, we focused the present studies on the program of contact-inhibited cells. As determined by gene

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TABLE 1 Oligonucleotides used for preparation of shRNA vectors

Oligonucleotide	Sequence ^a
A640	GAGAGGTGCTGCTGAGCGACAGCTGAGTGCACACAACGAGTAGTGAAGCCACAGATGTA
A387	GAGAGGTGCTGCTGAGCGTGGGGGTGCACAGCAGGAAGATAGTGAAGCCACAGATGTA
B640	ATTCACCACCACTAGGCAGCAGCTGAGTGCACACAACGAGTACATCTGTGGCTTCACT
B387	ATTCACCACCACTAGGCACGGGGGTGCACAGCAGGAAGATACATCTGTGGCTTCACT
C	GGCGGGGCTAGCTGGAGAAGATGCCTTCCGGAGAGGTGCTGCTGAGCG
D	GGGTGGACGCGTAAGAGGGGAAGAAAGCTTCTAACCCCGCTATTCACCACCACTAGGCA

^a Target sequences are underlined.

profiling, hypoxia-responsive genes (genes for carbonic anhydrase [CA] IX, CA XII, and enolase 2) were markedly induced by contact inhibition but not by starvation. Hypoxia (2% oxygen) was a potent inducer of growth arrest and p20K expression in CEF. Moreover, high cell density reduced the availability of oxygen as indicated by the activity of nitroreductase, a marker of hypoxia. Therefore, the expression of p20K is restricted to G₀ by ERK2 and CHOP and is induced under conditions promoting reversible growth arrest, such as high cell density and limiting oxygen concentrations.

MATERIALS AND METHODS

Cell culture. Early-passage ($n < 10$) CEF were cultured at 41.5°C in complete medium, consisting of Dulbecco's modified Eagle's medium (DMEM) with 5% heat-inactivated (57°C for 30 min) "cosmic" calf serum (CCS; HyClone, Logan, UT), 5% tryptose phosphate broth, 2 mM L-glutamine, 0.2 µg/ml streptomycin, and 0.2 U/ml penicillin (Life Technologies). CEF were also starved in DMEM without serum (serum-free medium) after being washed twice with serum-free medium. Nutrient depletion was also induced by culturing cells in complete medium over several days without nutrient/medium replenishment. Hypoxia was induced by culturing CEF for 24 h in 1 to 2% O₂. CEF were treated with the MEK inhibitor PD184352 (0.1 to 2 µM; Sigma-Aldrich). ER stress was induced by treating CEF with 1 µg/ml tunicamycin or thapsigargin (Sigma-Aldrich) for 12 to 48 h.

Live-cell detection of nitroreductase activity. Nitroreductase activity was used as an indicator of hypoxia in CEF and was examined by fluorescence microscopy using commercially available reagents (ENZ-51042; Enzo Life Sciences, Farmingdale, NY). Subconfluent CEF under conditions of normoxia or subjected to 2% O₂ for 24 h were analyzed and compared to density-arrested CEF. CEF treated with the hypoxia-mimetic desferrioxamine (DFO) at 0.2 mM were used as a control in these experiments.

Proliferation assays. Cells were collected by treatment with trypsin. Two-hundred-microliter aliquots of cell suspension in 10 ml of diluent were counted in a Coulter counter (with the lower limit set to 10 µm). The averages for triplicate samples were used to determine proliferation. Error bars in figures represent standard deviations of the means.

Construction of retroviral vectors for CHOP downregulation by shRNA. The 21-nucleotide (nt) target sequences of the CHOP gene were chosen using the Genscript design tool. The 5' base of the sense strand was altered in all cases so that it mismatched the guide strand base to mimic the structure found in the endogenous microRNA 30 (miRNA30). The results described in this report were obtained with a short hairpin RNA (shRNA) construct targeting nucleotides 640 to 660 of the CHOP mRNA (designated "640"; the target sequence is underlined in Table 1) but were replicated with a separate shRNA construct targeting nucleotides 387 to 407 (designated "387"; underlined in Table 1) (data not shown). Hairpins for the first miRNA cloning site were generated by PCR, using 10 ng each of the gene-specific oligonucleotides A (A640 or A387) and B (B640 or B387) together with 100 ng of two generic flanking oligonucleotides, C and D, in a 50-µl reaction mixture with ProofStart polymerase (Qiagen)

(Table 1). The PCR conditions were 5 min at 95°C followed by 25 cycles of 30 s at 94°C, 30 s at 55°C, and 45 s at 72°C, using a GeneAmp 2700 PCR system (Applied Biosystems). PCR products were purified, digested with NheI and MluI, and subcloned into pRFPRNAiC(U6-). The miRNA expression cassettes for CHOP were then subcloned from pRFPRNAiC(U6-)-CHOP into a modified RCASARNAi vector as a NotI-ClaI fragment (ARK-Genomics) (23, 24).

Northern blotting and RT-qPCR analyses. Northern blotting was performed as described before (22). For reverse transcription-quantitative PCR (RT-qPCR) analyses, RNA samples were treated with DNase I, reverse transcribed, and analyzed using commercially available reagents (ProtoScript cDNA synthesis kit [New England BioLabs] and Perfecta SYBR green FastMix low-ROX reaction mix [Quanta Biosciences]), a real-time PCR instrument from Stratagene (MX3000P), and the following primers: for carbonic anhydrase IX (CAIX), forward primer TAGGTTGGGCAAGGGAGAACCC (nt 5 to 27) and reverse primer CGGCAATGTTGAACCCGGGCA (nt 110 to 90); for carbonic anhydrase XII (CAXII), forward primer GGCAAGGATCCTCCACCACACCT (nt 748 to 770) and reverse primer TCGCCGGAGATGCCTTCCG (nt 876 to 857); for enolase II (ENOII), forward primer CCCCCTGCAGGTCTGAACGC (nt 1276 to 1295) and reverse primer AGTTGTGTCCAGCAAAGCGTGCT (nt 1368 to 1346); and for p20K lipocalin (p20K), forward primer GCCCAGCAGGAGGAATGCA (nt 519 to 538) and reverse primer AGCAGCTCCGAGCTTTGGCA (nt 618 to 599).

Gene profiling. Total cellular RNA was isolated and analyzed with an Affymetrix chicken GeneChip representing 32,773 transcripts and 28,418 genes. cRNA synthesis, labeling, and microarray hybridization were conducted at the McMaster Centre for Functional Genomics (CFG) at McMaster University (Hamilton, Canada). Probe set data normalization and expression summaries were generated using the Affymetrix PLIER algorithm. The statistical significance of differences in expression was determined by two-way analysis of variance (ANOVA), using contact inhibition and serum starvation as factors ($\alpha = 0.05$). Twofold or greater changes in gene expression were determined by the unpaired *t* test, which was performed on all pairwise comparisons between experimental conditions, with correction for multiple testing (Bonferroni-corrected $\alpha = 0.05$). Probe sets whose mean signal under a given condition did not exceed the minimum signal threshold above background under at least one such condition in the pairwise tests were discarded.

Western blotting and immunofluorescence microscopy. Results described for Western blot and immunofluorescence analyses were obtained in three or more independent experiments, using cells isolated from different chicken embryos and according to procedures described before (22). Protein lysates were generated from confluent or subconfluent cultures by seeding CEF at a density of 1×10^5 cells per 100-mm dish and preparing samples by cell lysis in SDS sample buffer at different times after seeding. The following primary antibodies and dilutions were used in these analyses: chicken p20K, 1:2,000 (Western blotting) and 1:300 (immunofluorescence assay) (19); chicken CHOP, 1:1,500 (25); chicken C/EBPβ, 1:1,500 (Western blotting) and 1:100 (immunoprecipitation) (21); ERK2, 1:2,000 (Western blotting) and 1:100 (immunofluorescence assay) (clone 1B3B9; Millipore); HIF1α, 1:500 (ab2185; Abcam); and β-tubulin, 1:50,000 (T7816; Sigma-Aldrich, St. Louis, MO).

FIG 1 (A) Expression of p20K during proliferation, at confluence, and in response to nutrient depletion. Subconfluent CEF were cultured for several days, and protein lysates were prepared at different days after seeding. CEF reached confluence on day 3, and cell numbers began to decrease on day 9 after seeding. The levels of p20K and CHOP, a marker of starvation, were examined by Western blotting. (B) Transcripts for p20K and CHOP were examined in cycling CEF, subconfluent CEF starved in serum-free medium for 48 h, confluent CEF maintained in complete medium (C.I.), and confluent CEF transferred to serum-free medium for 48 h (C.I. & Starved). RNA loading was examined by probing the blot for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA. (C) Unsupervised hierarchical clustering of genes demonstrating a minimum of a 2-fold change in expression between at least two conditions. (D) Validation of mRNA expression for p20K, carbonic anhydrase 9 (CA9), carbonic anhydrase 12 (CA12), and enolase 2 (ENO2) by RT-qPCR. Transcript levels are indicated in relative units for cycling and contact-inhibited (C.I.) CEF.

Following several washes, the blots were incubated with a 1:25,000 dilution of a secondary anti-rabbit, anti-mouse, or anti-goat IgG antibody conjugated with horseradish peroxidase (HRP) at room temperature in Tris-buffered saline (TBS) with 5% milk. Chemiluminescence signals were revealed by incubation with the HRP substrate Luminata Forte according to the protocol provided by the supplier (Millipore).

For immunofluorescence microscopy, cells were first seeded onto glass slides in 60-mm plates and subjected to various conditions of normoxia or hypoxia. Cells were fixed by utilizing 3.7% formaldehyde in phosphate-buffered saline (PBS) for 10 min at room temperature. Cells were permeabilized with 0.1% Triton X-100 in PBS on ice for 5 min, incubated in 5% fetal bovine serum in PBS for 1 h, and finally incubated

TABLE 2 Pairwise comparisons of numbers of differentially expressed probes for cycling CEF, serum-starved CEF, contact-inhibited (CI) CEF, and starved and CI CEF

Direction of regulation	No. of differentially expressed probes for indicated pair					
	(CI and starved)/CI	Starved/CI	(CI and starved)/cycling	CI/cycling	Starved/cycling	(CI and starved)/starved
Upregulated	466	510	413	638	277	23
Downregulated	199	218	414	935	206	29
Total	665	728	827	1,573	483	52

FIG 2 (A) Western blotting of p20K in CEF cultured under normoxic conditions (21% O₂) and various conditions of hypoxia (1 to 3% O₂). ERK was utilized as a loading control. (B to E) CEF treated with a fluorescent indicator of hypoxia (Cyto-ID) were analyzed under different culture conditions. Subconfluent CEF treated with the hypoxia-mimetic desferrioxamine (DFO; 0.2 mM) and with the diluent 0.1% DMSO were used as positive and negative controls, respectively. For conditions of hypoxia, subconfluent CEF were cultured in 2% oxygen for 24 h; all other samples were examined at normoxia. (F to I) Nuclei were stained with Hoechst stain. (J and K) CHOP and p20K mRNA levels were quantified by RT-qPCR and normalized to GAPDH expression. Relative expression is depicted for subconfluent/cycling (cyc) or contact-inhibited (CI) CEF cultured for 24 h at hypoxia (Hyp; 2% O₂) or normoxia (Norm; 21% O₂). Error bars represent the standard deviations for three independent experiments. ***, $P < 0.001$ by one-way ANOVA with the Bonferroni *post hoc* test versus the control (cycling normoxia); **, the value was significantly different ($P < 0.001$) in a separate *t* test analysis.

with the primary antibody overnight at 4°C. Anti-rabbit IgG–fluorescein isothiocyanate (FITC) or anti-mouse IgG–CF594 secondary antibody at a dilution of 1:100 was used in these experiments. Nuclei were stained with DAPI (4',6-diamidino-2-phenylindole; Thermo Fisher) at a concentration of 300 nM for 5 min in the dark.

EMSAs. Electrophoretic mobility shift assays (EMSAs) were performed as described before (22). To generate probes, single-stranded oligonucleotides were labeled with a biotin 3'-end DNA labeling kit (Pierce) per the manufacturer's protocol. The sequences of the QRU probes used for EMSA are provided in the figures. Binding reactions were carried out using 100 fmol of a biotinylated double-stranded DNA (dsDNA) probe and 500 ng of purified protein in 20 μ l of binding buffer (10 mM Tris, pH 7.5, 100 mM NaCl, 1 mM EDTA, 25% glycerol, 1 mM dithiothreitol [DTT], and Halt inhibitor cocktail [Pierce]). Recombinant ERK2 was produced in *Saccharomyces cerevisiae* as described by Hu and coinvestigators (26). The yeast strain BY7471 was transformed with a high-copy-number expression vector encoding glutathione *S*-transferase

FIG 3 (A) CEF proliferation under conditions of hypoxia (2% O₂) or normoxia (21% O₂) over a period of 7 days. Error bars represent the standard deviations for four independent counts. (B) Western blotting of HIF-1 α and p20K expression in CEF cultured in 5% "cosmic" calf serum (CCS; HyClone) or 10% fetal bovine serum (FBS) and incubated at hypoxia (2% O₂) or normoxia (21%) over a period of 24 h. ERK was used as a loading control. (C) CEF proliferation was examined over a period of 40 h for cells in 10% FBS versus 5% CCS under conditions of hypoxia (2% O₂). The error bars represent the standard deviations for four independent samples.

(GST)–His₆ fusion proteins. Separate vectors encoding human ERK2 (pEGH-A-ERK2) and human MEK1 (pEGH-A-MEK1) were cotransformed to generate activated ERK2. ERK2 dephosphorylation was performed by adding calf intestine phosphatase (14 U/ml of extract; New England BioLabs) to yeast extracts (7 μ g/ μ l) and incubating them for 30 min at different temperatures, as indicated in the text.

Chromatin immunoprecipitation (ChIP) assays. Cells were fixed in 1% formaldehyde for 10 min, followed by the addition of 1.25 M glycine. Cells were collected and resuspended in 1 ml of SDS lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris, pH 8.1, 1 $\times$ Halt protease phosphatase inhibitor cocktail [Pierce]). Aliquots of 400 μ l were then sonicated for a total of 6 min in 20-s pulses. Samples were precleared using salmon sperm DNA-blocked protein A beads (Millipore) and diluted to a 1-ml final volume by using dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA,

FIG 4 (A) Sequences of the putative hypoxia-responsive elements (HRE) of the p20K gene promoter region. (B) Schematic representations of reporter constructs of the p20K promoter analyzed in transient-expression assays (C) under conditions of normoxia (21% O₂) or hypoxia (2% O₂). CAT, chloramphenicol acetyltransferase. (D) Western blotting of p20K and a dominant negative form of C/EBPβ (LIP) in CEF cultured at normoxia or hypoxia (2% O₂). Data are shown for uninfected CEF and CEF infected with the control virus RCAS(B) or a virus expressing the dominant negative form of C/EBPβ [RCAS(B) Δ184-C/EBPβ]. ERK was used as a loading control.

16.7 mM Tris-HCl, pH 8.1, 167 mM NaCl, and Halt inhibitor cocktail [Pierce]). A sample was saved, and the remaining chromatin solution was immunoprecipitated using antibodies (2 μg) described above. Antibody complexes were pulled down using blocked protein A beads for 1 h (note that 2 μg of rabbit anti-mouse IgG [Jackson ImmunoResearch] was added to all samples to increase the affinity of mouse antibodies for protein A) and then subjected to a series of washes, once with low-salt immune complex wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.1, 150 mM NaCl), once with high-salt immune complex wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.1, 500 mM NaCl), once with LiCl immune complex wash buffer (0.25 M LiCl, 1% NP-40, 1% deoxycholic acid, 1 mM EDTA, 10 mM Tris-HCl, pH 8.1), and twice with TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). Following washes, bound complexes were eluted using 200 μl of elution buffer (0.1 M NaHCO₃, 0.005% SDS). Samples were then de-cross-linked overnight at 65°C and treated with RNase A and proteinase K. The DNA was ethanol precipitated and resuspended in TE buffer. PCR amplification of the QRU and a control region located within intron 1 of the p20K gene was performed using the following primers: QRU forward, 5'-CATCCCCTCTTCATTCTCCA-3'; QRU reverse, 5'-CACTGCTATTGTTGGCATGG-3'; p20K intron forward, 5'-GGTGTGCTGAGTATTTGAGGTG-3'; and p20K intron reverse, 5'-AAATTACTCTGGGGGCTGA-3'.

Transient-expression assays. Chloramphenicol acetyltransferase assays were performed as described before (22). ¹⁴C-labeled chloramphenicol species were imaged using a Typhoon Trio scanner (GE Healthcare), quantified using ImageQuant software, and analyzed by the *t* test. For luciferase assays, pGluc-derived reporter constructs and an RSV-βgal control plasmid were cotransfected into normal CEF by utilizing the DEAE-dextran method for 6 h and then were shocked using a 10% dimethyl sulfoxide (DMSO)-PBS shock for 2 min (27). Cells were split the following day and placed under conditions of normoxia or hypoxia (2% oxygen) for a period of 24 h. CEF were then lysed in 100 μl of 250 mM Tris (pH 6.8) and 1% NP-40. Ten microliters of lysate was assayed for *Gaussia* luciferase by using a *Gaussia* luciferase assay kit in accordance with the manufacturer's recommendations (New England BioLabs). Samples were subsequently normalized by β-galactosidase (β-Gal) activity.

RESULTS

Control of p20K expression by hypoxia and cell confluence.

Confluent chicken embryo fibroblasts (CEF) express high levels of the growth arrest-specific p20K lipocalin gene (19) (Fig. 1A). However, this expression is transient and inhibited when cells are kept at confluence for several days without medium/nutrient replenishment. The downregulation of p20K is associated with induction of the CHOP transcriptional regulator, a marker of starvation and ER stress, and with a progressive decline in cell numbers (25, 28) (Fig. 1A and data not shown). Interestingly, cycling CEF expressed low levels of CHOP, while contact-inhibited CEF were characterized by nearly undetectable expression of this protein. Similar patterns of p20K and CHOP expression were observed at the mRNA level, with high levels of p20K transcripts observed in contact-inhibited CEF and high levels of CHOP mRNA detected in serum-starved but not contact-inhibited CEF (Fig. 1B). Basal and low levels of CHOP transcripts were also found in cycling CEF. These results suggest that CHOP and p20K are components of different biological responses regulated by different pathways.

To identify processes associated with contact inhibition, we compared the transcriptomes of cycling CEF, serum-starved and contact-inhibited CEF, serum-starved but subconfluent CEF, and contact-inhibited CEF in complete serum-containing medium (Fig. 1C). All conditions of growth arrest led to extensive changes in gene expression, but differentially expressed genes were more prominent when contact-inhibited CEF were compared to cycling cells (Table 2). Several genes associated with the response to hypoxia, including the carbonic anhydrase IX, carbonic anhydrase XII, and enolase 2 genes, were markedly upregulated in contact-inhibited but not serum-starved CEF. The induction of this class of genes by confluence was confirmed by RT-qPCR (Fig. 1D).

Genes for HIF1α and several factors with proangiogenic activity, including FABP4, FABP5, prokineticin 2, angiopoietin-like 5, SFRP2 (secreted frizzled-related protein 2), and

C1QTNF3 (CTRP3), were also upregulated at confluence, suggesting that oxygen deprivation is a feature of the response to high cell density (see Data Set S1 in the supplemental material) (29–34). As expected, genes involved in DNA replication and progression through the cell cycle (cyclin E2, cyclin M2, PLK3, DNA primase 2, ORC4 and ORC1 [origin recognition complex subunits 4 and 1, respectively], and thymidine kinase 1 genes) were downregulated, while the cyclin-dependent kinase inhibitors p18 (CDKN2C) and p27^{Kip1} (CDKN1B) were induced under conditions of growth arrest.

The induction of hypoxia-responsive genes suggests that oxygen availability regulates the expression of growth arrest-specific genes, such as p20K. In agreement with this model, p20K expression was induced in subconfluent CEF incubated at low oxygen concentrations (1 to 2% O₂) for 24 h (Fig. 2A). Increased levels of cellular nitroreductase activity, an indicator of hypoxia, were observed in confluent CEF, indicating that these cells were under limiting oxygen concentrations (Fig. 2B to E). These conditions of hypoxia were not associated with ER stress, as the basal level of CHOP transcripts was reduced by a 24-h incubation in 2% oxygen (Fig. 2J and K).

Incubation at 2% O₂ inhibited CEF proliferation, raising the possibility that the induction of p20K was not a direct effect of hypoxia but instead the result of growth arrest (Fig. 3A). This was further examined by looking at the kinetics of p20K and HIF1 α expression in response to incubation at 2% O₂. HIF1 α accumulated considerably and rapidly in response to hypoxia, i.e., within 2 h of incubation at reduced oxygen concentrations (Fig. 3B). In contrast, p20K expression was not detected in the first 12 h of incubation. CEF continued to accumulate for 18 h but entered quiescence within 25 h at 2% O₂ (Fig. 3C). This entry into quiescence was marked by the induction of p20K. While hypoxia had similar inhibitory effects on CEF proliferation in medium containing calf or fetal bovine serum, p20K expression was maximally induced when growth factors were more limiting (in calf serum), suggesting that its expression was still affected by the presence of mitogens in the medium.

The results described above suggest that p20K accumulation at 2% O₂ is not a direct effect of hypoxia but the result of growth arrest induced by limiting oxygen concentrations. If this model is correct, the induction of p20K by hypoxia should reflect the QRU- and C/EBP β -dependent activation of the p20K promoter, as observed in contact-inhibited CEF (20). This was examined in transient-expression assays with p20K promoter constructs. Interestingly, two potential hypoxia-responsive elements (HREs), consisting of the canonical HIF binding site and its ancillary sequence, are located upstream of the QRU, at positions –415 and –458 of the p20K gene transcription start site (Fig. 4A) (35). However, constructs lacking the putative HREs were fully inducible under conditions of 2% O₂ (Fig. 4B and C). In contrast, deletion of the 48-bp QRU and C/EBP binding sites abolished the activation of the p20K gene promoter by hypoxia, establishing the requirement for the QRU for induction under these conditions. Consistent with the importance of the QRU, the hypoxia-dependent accumulation of p20K was inhibited by the expression of LIP, the dominant negative form of C/EBP β (Fig. 4D). These results suggest that the QRU- and C/EBP β -dependent induction of p20K reflects the promotion of growth arrest by hypoxia and is not a direct response to low oxygen concentrations.

ER stress antagonizes the density-dependent expression of p20K. The role of CHOP in the control of gene expression by

FIG 5 (A) Western blot showing the effects of downregulation of CHOP by shRNA on p20K expression. The expression of p20K and CHOP was analyzed for both cycling and confluent CEF infected with a group A virus expressing a control shRNA for GFP [RCAS(A)-GFP RNAi] or an shRNA for CHOP [RCAS(A)-CHOP RNAi]. Equal numbers of cells (10^5 cells) were seeded on day 0, and cell lysates were prepared every day for the following 4 days. CEF infected with the control virus [RCAS(A)-GFP RNAi] reached confluence on day 4, while cells infected with the CHOP shRNA virus reached confluence between day 3 and day 4. ERK was used as a loading control. (B) The effect of CHOP overexpression on p20K levels was examined by Western blotting for cells at normoxia or hypoxia (2% O₂ for 24 h). CHOP overexpression was obtained with the RCAS(B)-CHOP retrovirus. ERK was used as a loading control. (C) Relative levels of CHOP and p20K were determined by densitometry.

C/EBP β is context dependent, with CHOP acting as an inhibitor of some genes but cooperating with C/EBP β in the activation of stress-responsive promoters with variants of the C/EBP element (36). The induction of p20K at confluence coincides with a reduc-

FIG 6 (A) Western blotting of p20K and CHOP in cycling or confluent CEF treated with 0.1% DMSO (DM), 1 μ g/ml tunicamycin (TU), or 1 μ g/ml thapsigargin (TH) for 12, 24, or 48 h. CEF reached confluence at 24 h in this experiment. (B) Western blotting of p20K and CHOP in confluent cells infected with a group A virus expressing a control shRNA for GFP [RCAS(A)-GFP RNAi] or an shRNA for CHOP [RCAS(A)-CHOP RNAi] and treated with the diluent 0.1% DMSO (DM), 1 μ g/ml tunicamycin (TU), or 1 μ g/ml thapsigargin (TH). (C) Relative intensities of the p20K and CHOP signals for the samples analyzed in panel B. (D) The association of CHOP and C/EBP β was examined by coimmunoprecipitation and Western blotting. Cell lysates were prepared from cycling CEF (CYC) or contact-inhibited CEF treated with 1 μ g/ml tunicamycin (TU), 1 μ g/ml thapsigargin (TH), or the diluent (0.1% DMSO [DM]). C/EBP β was also immunoprecipitated from a lysate of confluent CEF maintained in the same medium without replenishment and starved for 6 days (STV). Immunoprecipitated C/EBP β was analyzed by Western blotting; the coimmunoprecipitation of CHOP was examined by probing the blot with a CHOP polyclonal antibody. (E) Western blotting of CHOP in cell lysates analyzed under the conditions described for panel D but not subjected to immunoprecipitation.

tion in the basal expression of CHOP (Fig. 1A and 5A). The downregulation of CHOP by shRNA enhanced p20K expression at confluence but led to only a modest upregulation of p20K in cycling CEF (Fig. 5A, lanes 5 and 6). CHOP downregulation also stimulated cell proliferation and enhanced saturation density, with RCAS(A)-CHOP RNAi-infected CEF reaching confluence before control cells (between days 3 and 4), raising the possibility that the effect of CHOP downregulation on p20K was indirect (data not shown). To alleviate this problem, CHOP was overexpressed in subconfluent CEF and p20K expression was examined under conditions of hypoxia. This resulted in a marked inhibition of p20K expression (Fig. 5B and C).

Conditions resulting in strong activation of CHOP, such as ER stress induced by tunicamycin or thapsigargin, abolished p20K expression. This was true for cells entering growth arrest at confluence or kept in a contact inhibition state for more than 24 h (Fig. 6A). Partial expression of p20K was restored when CHOP levels were reduced by an shRNA in tunicamycin- but not thapsigargin-treated cells, where higher levels of CHOP expression were observed (lanes 5 and 10 in Fig. 6B and C, respectively). Coimmunoprecipitation experiments confirmed that CHOP was associ-

ated with C/EBP β during proliferation, starvation, and ER stress but could not be detected under contact inhibition conditions, when CHOP levels were lowest (Fig. 6D, lane 8, and E). Reduced levels of the two *trans*-activating forms of C/EBP β (designated LAP⁺ and LAP) were also observed in cells treated with tunicamycin and thapsigargin (Fig. 6D, lanes 9 and 10). These results suggest that CHOP functions as a potent inhibitor of p20K by interacting with C/EBP β in various cellular contexts.

ERK2 binds to the QRU and inhibits expression of p20K during proliferation. The downregulation of CHOP by shRNAs was not sufficient to obtain full induction of p20K in cycling CEF, raising the possibility that an additional factor(s) interferes with the transactivation of the p20K QRU under these conditions (Fig. 5A). The central region of the QRU, located between but overlapping the two C/EBP β binding sites, is similar to the ERK2 binding element described by Hu and coinvestigators (26). ChIP assays confirmed that ERK2 was recruited to the p20K promoter under conditions of proliferation but was not detectable when CEF were growth arrested at 2% O₂ or by contact inhibition (Fig. 7B and data not shown). C/EBP β showed the opposite pattern and was

FIG 7 Interaction of ERK2 and C/EBPβ with the p20K QRU. (A) Sequence of the QRU, with the positions of the two C/EBPβ binding sites (A site and B site) and the two ERK2 binding sites (EBS) indicated. (B) ERK2 and C/EBPβ antibodies were used in ChIP assays to probe for the recruitment of ERK2 and C/EBPβ to the QRU for CEF under conditions of normoxia or hypoxia (24 h in 2% O₂). Mouse IgG and the corresponding rabbit preimmune serum (P.I) were used as negative controls for the ERK2 and C/EBPβ antibodies, respectively. Primers were used to PCR amplify the QRU, while a region located in intron 1 of the p20K gene was used as a negative control. In, input. (C) Recombinant ERK2 binds to the QRU in EMSA. An asterisk indicates the position of the nucleoprotein complex. The competitor was a 250-fold excess of unlabeled double-stranded oligonucleotide added to the binding reaction mixture. GST was utilized as a negative control. The consensus ERK2 binding sites are underlined, while nucleotides altered in the EBS mutant oligonucleotides are highlighted in red. A control yeast lysate subjected to mock purification was also analyzed in parallel (GST). The specificity of the ERK2 nucleoprotein complex was determined by adding ERK2-specific antibodies or a control IgG to the binding reaction mixture. WT, wild type; MT, mutant. (D) The binding of ERK2 to the QRU was examined after dephosphorylation by CIP incubated at different temperatures or by expressing ERK2 without coexpression of MEK (lane 6). The dephosphorylation of ERK2 was confirmed by the Western blot shown at the bottom.

detected only in response to hypoxia and in the absence of ERK2 binding.

The association of ERK2 with the p20K gene promoter appears to be direct, as recombinant ERK2 formed a complex with the central domain of the QRU in electrophoretic mobility shift assays (Fig. 7C). An excess of the central domain oligonucleotide that included substitutions of key nucleotides within the candidate

ERK2 binding sites did not compete for formation of this complex (Fig. 7C, lane 5). Binding was also impaired when recombinant ERK2 was preincubated with an ERK2-specific antibody (Fig. 7C, lane 7).

The interaction of ERK2 with the QRU was phosphorylation independent, as dephosphorylation of activated ERK2 produced in yeast by treatment with calf intestine phosphatase (CIP) did not

FIG 8 Competition of ERK2 and C/EBPβ for QRU occupancy. (A) The recruitment of ERK2 and C/EBPβ to the QRU was examined in ChIP assays using chromatin isolated from CEF infected with the control virus RCAS(A) or a C/EBPβ-encoding RCAS(A) virus. A murine IgG and the corresponding rabbit preimmune (P.I) serum were used as negative controls for ERK2 and C/EBPβ immunoprecipitation, respectively. Primers were used to PCR amplify the QRU region or a region located in intron 1 of the p20K gene as a negative control. (B to G) Immunolocalization of ERK2 (B and C) and C/EBPβ (D and E) in CEF infected with a control virus [RCAS(A)] (B, D, and F) or a virus expressing C/EBPβ [RCAS(A)-C/EBPβ] (C, E, and G). (F and G) DAPI was used for nuclear staining. (H) Western blotting of p20K and phospho-ERK (P-p42/44 MAPK) in CEF treated with 0.1% DMSO or 0.1 μM or 2 μM MEK inhibitor for 24, 48, or 72 h. ERK was used as a loading control. (I) Activities of reporter constructs of the p20K gene promoter, including (–177) or lacking (–169) the QRU, in response to ERK2 overexpression in transient-expression assays of cycling and contact-inhibited CEF. In this experiment, an expression vector for ERK2 or the parental control vector was cotransfected with the reporter constructs. (J) Promoter activities in constructs containing the wild-type QRU (WT QRU) and constructs containing mutations in the putative ERK2 binding site of the QRU (EBS mutant) were analyzed by transient-expression assays under conditions of normoxia or hypoxia (24 h in 2% O₂). The parental reporter vector, lacking a QRU region, was used as a negative control. *, $P < 0.01$. RLU, relative light units.

interfere with the interaction of ERK2 with the QRU (Fig. 7D). Likewise, ERK2 was still capable of binding the QRU when it was produced in yeast in the absence of ectopically expressed MEK (Fig. 7D, lane 6). Finally, bacterially expressed ERK2 was also capable of binding the QRU, as determined by EMSA (data not shown).

The results described above suggest that ERK2 competes with C/EBPβ for QRU occupancy under conditions of proliferation. To test this model, C/EBPβ was overexpressed from a retroviral vector generating conditions leading to the ectopic expression of p20K in CEF (20). ChIP assays confirmed that overexpressed C/EBPβ displaced ERK2 on the p20K gene promoter (Fig. 8A). ERK2 remained largely nuclear in these cells, suggesting that the absence of ERK2 on the QRU was not the result of intracellular relocalization caused by C/EBPβ overexpression (Fig. 8C). We also generated nuclear extracts from C/EBPβ-overexpressing cells and analyzed the effects of increasing amounts of purified ERK2 on the interaction of C/EBPβ with the QRU by EMSA. As shown in Fig. 9, C/EBPβ was effectively displaced and replaced by ERK2

when increasing amounts of this kinase were added to the binding reaction mixture and analyzed by EMSA.

We attempted to downregulate the expression of ERK2 by using RCASBP-shRNA retroviral vectors, but CEF quickly entered senescence under these conditions. The shRNA-mediated downregulation of ERK2 also led to a marked reduction in C/EBPβ expression and a lack of p20K inducibility (Fig. 10). In contrast, chemical inhibition of MEK impaired CEF proliferation but did not cause senescence (our unpublished results). The expression of p20K was stimulated by MEK inhibition but required more than 24 h of treatment (Fig. 8H). As determined by ChIP and immunofluorescence assays, this delayed induction of p20K reflected the slow and progressive nuclear exclusion of ERK2 (Fig. 11). Interestingly, MEK inhibition abolished the basal expression of CHOP in actively dividing CEF (Fig. 8H). As determined by transient-expression assays, forced expression of ERK2 reduced the activity of the QRU in cycling and contact-inhibited CEF (Fig. 8I), while substitutions of key nucleotides affecting the ERK2 binding sites enhanced the activity of the QRU in cycling cells (Fig. 8J), EBS

FIG 9 Recombinant ERK2 competes with C/EBP β for interaction with the QRU. Increasing amounts of recombinant ERK2 (0.125 to 2.5 μ g) were used to compete with C/EBP β for formation of a QRU nucleoprotein complex in electrophoretic mobility shift assays. The position of the ERK2 complex is shown after incubation of recombinant ERK2 with the QRU probe in binding buffer (lane 2). Nuclear lysates (containing a total of 5 μ g of proteins) prepared from C/EBP β -overexpressing cells generated a strong nucleoprotein complex with the QRU that was disrupted by the addition of increasing amounts of recombinant ERK2. GST (2.5 μ g; lane 4) was added as a negative control for ERK2 binding to the nuclear lysate of RCAS(A)-C/EBP β -infected CEF. No nucleoprotein complex was detected when the QRU probe was incubated with GST in the absence of the RCAS(A)-C/EBP β -infected CEF nuclear lysate (data not shown).

mutant). Since the ERK2 and C/EBP β binding sites overlap, the lower activity of the EBS mutant under conditions of hypoxia suggests that substitutions of key nucleotides affecting ERK2 interaction also reduce, albeit modestly, the interaction of the QRU with C/EBP β (our unpublished results). These results confirmed the inhibitory role of the central domain of the QRU. Significantly, the EBS mutant QRU construct was no longer inducible by growth arrest under low-oxygen conditions (Fig. 8J). These results indicate that the expression of p20K is under inhibition by the MEK pathway, with ERK2 functioning as a transcriptional repressor binding to the QRU in actively dividing cells.

DISCUSSION

Control of the p20K gas gene by C/EBP β and the MEK pathway. The promotogenic and prosurvival functions of C/EBP β have been described in several contexts, but C/EBP β also cooperates with ATF-4 to induce the expression of death-associated protein kinase 1 and the onset of apoptosis in gamma interferon (IFN- γ)-treated cells (37, 38). In CEF, C/EBP β is required for the control of genes activated by the v-Src tyrosine kinase but is also critical for the expression of the growth arrest-specific p20K lipocalin gene under contact inhibition conditions (20, 21). To exert this pleiotropic function, C/EBP β interacts with unrelated transcription factors

controlled by multiple signaling pathways and governing different programs of gene expression. In RSV-transformed CEF, C/EBP β cooperates with AP-1 and NF- κ B to mediate the constitutive induction of IL-8 (21, 24, 27, 39). In contrast, the upregulation of c-Jun, JunD, or Fra-2 and the increase in AP-1 activity antagonize the action of C/EBP β in the control of growth arrest-specific genes (22). To date, the exact mechanism underlying the opposing roles of AP-1 and C/EBP β in CEF is unclear. Likewise, the factors determining the specificity of gene activation by C/EBP β are not completely understood. In this report, we establish ERK2 as a factor binding to the quiescence-responsive unit (QRU) and inhibiting the expression of the p20K gas gene in actively dividing cells. Using ChIP assays, we demonstrated that ERK2 and C/EBP β interact with the QRU in a mutually exclusive manner, with ERK2 binding detected under conditions of proliferation and p20K repression (Fig. 7 and 8). We reported previously that forced expression of C/EBP β by use of the RCASBP virus is sufficient to promote strong p20K expression in cycling CEF (20). These conditions abrogated the association of ERK2 with the p20K QRU, suggesting that ERK2 and C/EBP β compete for QRU occupancy in the control of p20K expression (Fig. 8A and 9).

Recombinant ERK2 interacted with the repeated GAAAG element of the QRU in EMSAs, suggesting that ERK2 controls p20K expression directly by binding to sequences overlapping the C/EBP β binding sites (Fig. 7C). This mode of action of ERK2 was first described by Hu and coinvestigators for promoters of IFN- γ -inducible genes in human cells (26). While our studies used different cellular models, it is intriguing that ERK2 functions as a transcriptional repressor of genes activated by C/EBP β in both systems. The identification of additional target genes will indicate if the transcriptional repressor activity of ERK2 is limited to C/EBP β -controlled genes or is also important for promoters regulated by other transcription factors.

To confirm these findings, we attempted to downregulate the expression of ERK2 with shRNAs but failed to obtain cells that could be propagated in culture. CEF infected with ERK2 shRNA viruses quickly entered senescence, lost C/EBP β expression, and did not express p20K (Fig. 10). A similar phenotype of premature senescence has been described for mouse embryo fibroblasts lacking both *erk1* and *erk2* gene function (40). Since a gene encoding ERK1 has not been found in the chicken genome, it is possible that the *erk1/erk2* gene function is solely dependent on ERK2 in this species (41). Surprisingly, CEF treated with the MEK inhibitor PD184352 were viable, did not enter senescence, and expressed high levels of p20K, albeit after a long delay. Since phospho-ERK levels and cell proliferation were markedly reduced in PD184352-treated CEF, ERK2 may antagonize senescence, at least in part, in a phosphorylation-independent manner. The slow kinetics of CHOP downregulation (Fig. 8H) and ERK2 nuclear exclusion likely account for the delay of p20K induction in response to PD184352, a model consistent with the results of ChIP assays performed with the ERK2 antibody in normal CEF (Fig. 11).

Conditions generating ER stress, such as prolonged starvation or treatment with tunicamycin or thapsigargin, led to a marked induction of the C/EBP homologous protein (CHOP) factor and to repression of p20K (Fig. 1 and 5). CHOP is a bifunctional protein that forms heterodimers with C/EBP β to induce the expression of stress-responsive genes, such as carbonic anhydrase (CA) VI (36). In the CA VI promoter, the CHOP-C/EBP β dimer binds to a variant of the C/EBP regulatory element promoting gene ac-

FIG 10 (A) Western blotting of p20K, ERK2, and C/EBP β in CEF transfected with a group A virus expressing either a control shRNA for GFP [RCAS(A)-GFP RNAi] or an shRNA for ERK2 [RCAS(A)-ERK2 RNAi]. Cell samples were collected the day after transfection at confluence (confluent) and at every successive passage under cycling conditions, for a total of 5 passages (passage 1 to passage 5). (B) Proliferation of CEF infected with a group A virus expressing either a control shRNA [RCAS(A)-GFP RNAi] or an shRNA for ERK2 [RCAS(A)-ERK2 RNAi] for 6 days. Data represent the averages $\pm$ standard deviations for four independent counts. (C) Phase-contrast and bright-field micrographs of CEF displaying senescence-associated (SA) β -galactosidase activity for cells infected with a group A virus expressing either a control shRNA [RCAS(A)-GFP RNAi] or an shRNA for ERK2 [RCAS(A)-ERK2 RNAi], taken 6 days after transfection of retroviral plasmid DNA.

tivation. However, the dimerization with CHOP can also interfere with the function of C/EBP β on promoters lacking this element and normally activated by other C/EBP β dimers, thus causing gene repression. Forced expression of CHOP inhibited p20K in response to hypoxia, in agreement with a role for CHOP as an inhibitor of p20K (Fig. 5B and C). The mechanism promoting CHOP expression in cycling cells is poorly characterized but may reflect a direct interaction of AP-1 with a TPA response element of the CHOP promoter, as described for mammals by Holbrook and coinvestigators (42). In agreement with this model, CHOP ex-

pression was abolished by the expression of a dominant negative mutant of c-Jun in cycling CEF (our unpublished results).

High cell density promotes hypoxia in CEF. Gene profiling and RT-qPCR analyses revealed that several genes associated with the response to hypoxia, including carbonic anhydrase IX, carbonic anhydrase XII, and enolase 2, are induced in density-arrested CEF (Fig. 1). Nitroreductase activity, an indicator of hypoxia, was also elevated at confluence, indicating that a high cell density results in a significant depletion of oxygen levels. Since protein synthesis and cell proliferation are dependent on oxygen

FIG 11 (A) Analysis of ERK2 and C/EBP β interaction with the p20K QRU during MEK inhibitor (MEKi) treatment (PD184352), as determined by ChIP assay. ERK2 and C/EBP β antibodies were used to immunoprecipitate ERK2 or C/EBP β protein-DNA complexes in CEF treated with either 2 μ M MEK inhibitor or the DMSO diluent (as a control). Samples were taken every 24 h for a total of 72 h. Mouse IgG and the corresponding rabbit preimmune (PI) serum were used as negative controls. Primers were used to PCR amplify the QRU region, while a region located within intron 1 of the p20K gene was PCR amplified as a negative control. (B) Immunolocalization of ERK2 and p20K in CEF treated with the MEK inhibitor (2 μ M) or the DMSO diluent. Photos were obtained every 24 h for a total of 72 h. DAPI was used to stain nuclei.

availability, hypoxia may promote growth arrest in confluent CEF monolayers. A similar relationship between high cell density, hypoxia, and the control of protein synthesis has been observed in mammalian cells (43). Interestingly, p20K was first identified in quiescent chicken heart mesenchymal cells, in which its expression was also strongly affected by cell density (19). While the function of p20K remains to be characterized, these results suggest a role for this protein in the response to hypoxia. In agreement with this model, the results of recent experiments indicate that p20K enhances cell survival under conditions with limiting oxygen concentrations (our unpublished results). Thus, the induction of p20K may be part of an adaptive response to the lack of oxygen, changing conditions of proliferation, and lipid metabolism. This is under investigation.

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REFERENCES

- Schneider C, King RM, Philipson L. 1988. Genes specifically expressed at growth arrest of mammalian cells. *Cell* 54:787–793. [http://dx.doi.org/10.1016/S0092-8674\(88\)91065-3](http://dx.doi.org/10.1016/S0092-8674(88)91065-3).
- Goldstein S, Moerman EJ, Jones RA, Baxter RC. 1991. Insulin-like growth factor binding protein 3 accumulates to high levels in culture medium of senescent and quiescent human fibroblasts. *Proc Natl Acad Sci U S A* 88:9680–9684. <http://dx.doi.org/10.1073/pnas.88.21.9680>.
- Del Sal G, Ruaro ME, Philipson L, Schneider C. 1992. The growth arrest-specific gene, *gas1*, is involved in growth suppression. *Cell* 70:595–607. [http://dx.doi.org/10.1016/0092-8674\(92\)90429-G](http://dx.doi.org/10.1016/0092-8674(92)90429-G).
- Kops GJ, Dansen TB, Polderman PE, Saarloos I, Wirtz KW, Coffey PJ, Huang TT, Bos JL, Medema RH, Burgering BM. 2002. Forkhead transcription factor FOXO3a protects quiescent cells from oxidative stress. *Nature* 419:316–321. <http://dx.doi.org/10.1038/nature01036>.
- Coppock DL, Kopman C, Scandalis S, Gilleran S. 1993. Preferential gene expression in quiescent human lung fibroblasts. *Cell Growth Differ* 4:483–493.
- Mauviel A, Santra M, Chen YQ, Uitto J, Iozzo RV. 1995. Transcriptional regulation of decorin gene expression. Induction by quiescence and repression by tumor necrosis factor- α . *J Biol Chem* 270:11692–11700.
- Casado FJ, Pouponnot C, Jeanny JC, Lecoq O, Calothy G, Pierani A. 1996. QRI, a retina-specific gene, encodes an extracellular matrix protein exclusively expressed during neural retina differentiation. *Mech Dev* 54:237–250. [http://dx.doi.org/10.1016/0925-4773\(95\)00482-3](http://dx.doi.org/10.1016/0925-4773(95)00482-3).
- Bedard PA, Yannoni Y, Simmons DL, Erikson RL. 1989. Rapid repression of quiescence-specific gene expression by epidermal growth factor, insulin, and pp60v-src. *Mol Cell Biol* 9:1371–1375. <http://dx.doi.org/10.1128/MCB.9.3.1371>.
- Bohmer FD, Mieth M, Reichmann G, Taube C, Grosse R, Hollenberg MD. 1988. A polypeptide growth inhibitor isolated from lactating bovine mammary gland (MDGI) is a lipid-carrying protein. *J Cell Biochem* 38:199–204. <http://dx.doi.org/10.1002/jcb.240380307>.
- Provost PR, Marcel YL, Milne RW, Weech PK, Rassart E. 1991. Apolipoprotein D transcription occurs specifically in nonproliferating quiescent and senescent fibroblast cultures. *FEBS Lett* 290:139–141. [http://dx.doi.org/10.1016/0014-5793\(91\)81244-3](http://dx.doi.org/10.1016/0014-5793(91)81244-3).
- Iyer VR, Eisen MB, Ross DT, Schuler G, Moore T, Lee JC, Trent JM, Staudt LM, Hudson J, Jr, Boguski MS, Lashkari D, Shalon D, Botstein D, Brown PO. 1999. The transcriptional program in the response of human fibroblasts to serum. *Science* 283:83–87. <http://dx.doi.org/10.1126/science.283.5398.83>.
- Lih CJ, Cohen SN, Wang C, Lin-Chao S. 1996. The platelet-derived growth factor alpha-receptor is encoded by a growth-arrest-specific (gas) gene. *Proc Natl Acad Sci U S A* 93:4617–4622. <http://dx.doi.org/10.1073/pnas.93.10.4617>.
- Sang L, Collier HA, Roberts JM. 2008. Control of the reversibility of cellular quiescence by the transcriptional repressor HES1. *Science* 321:1095–1100. <http://dx.doi.org/10.1126/science.1155998>.
- Fornace AJ, Jr, Nebert DW, Hollander MC, Luethy JD, Papathanasiou M, Fargnoli J, Holbrook NJ. 1989. Mammalian genes coordinately regulated by growth arrest signals and DNA-damaging agents. *Mol Cell Biol* 9:4196–4203. <http://dx.doi.org/10.1128/MCB.9.10.4196>.
- Krauss RS, Housey GM, Hsiao WL, Johnson MK, Rotenberg SA, Borner CM, Weinstein IB. 1990. The role of protein kinase C in signal transduction and cellular transformation. *Prog Clin Biol Res* 340D:175–182.
- Krauss RS, Weinstein IB. 1991. A novel, plasmid-based system for study-

- ing gene rearrangements in mammalian cells. *Mol Cell Biol* 11:3915–3924. <http://dx.doi.org/10.1128/MCB.11.8.3915>.
17. Mao PL, Beauchemin M, Bedard PA. 1993. Quiescence-dependent activation of the p20K promoter in growth-arrested chicken embryo fibroblasts. *J Biol Chem* 268:8131–8139.
 18. Smith CM, Steitz JA. 1998. Classification of gas5 as a multi-small-nucleolar-RNA (snoRNA) host gene and a member of the 5'-terminal oligopyrimidine gene family reveals common features of snoRNA host genes. *Mol Cell Biol* 18:6897–6909. <http://dx.doi.org/10.1128/MCB.18.12.6897>.
 19. Bedard PA, Balk SD, Gunther HS, Morisi A, Erikson RL. 1987. Repression of quiescence-specific polypeptides in chicken heart mesenchymal cells transformed by Rous sarcoma virus. *Mol Cell Biol* 7:1450–1458. <http://dx.doi.org/10.1128/MCB.7.4.1450>.
 20. Kim S, Mao PL, Gagliardi M, Bedard PA. 1999. C/EBPbeta (NF-M) is essential for activation of the p20K lipocalin gene in growth-arrested chicken embryo fibroblasts. *Mol Cell Biol* 19:5718–5731. <http://dx.doi.org/10.1128/MCB.19.8.5718>.
 21. Gagliardi M, Maynard S, Bojovic B, Bedard PA. 2001. The constitutive activation of the CEF-4/9E3 chemokine gene depends on C/EBPbeta in v-src transformed chicken embryo fibroblasts. *Oncogene* 20:2301–2313. <http://dx.doi.org/10.1038/sj.onc.1204354>.
 22. Gagliardi M, Maynard S, Miyake T, Rodrigues N, Tjew SL, Cabannes E, Bedard PA. 2003. Opposing roles of C/EBPbeta and AP-1 in the control of fibroblast proliferation and growth arrest-specific gene expression. *J Biol Chem* 278:43846–43854. <http://dx.doi.org/10.1074/jbc.M304085200>.
 23. Das RM, Van Hateren NJ, Howell GR, Farrell ER, Bangs FK, Porteous VC, Manning EM, McGrew MJ, Ohyama K, Sacco MA, Halley PA, Sang HM, Storey KG, Placzek M, Tickle C, Nair VK, Wilson SA. 2006. A robust system for RNA interference in the chicken using a modified microRNA operon. *Dev Biol* 294:554–563. <http://dx.doi.org/10.1016/j.ydbio.2006.02.020>.
 24. Wang L, Rodrigues NA, Wu Y, Maslikowski BM, Singh N, Lacroix S, Bedard PA. 2011. Pleiotropic action of AP-1 in v-Src-transformed cells. *J Virol* 85:6725–6735. <http://dx.doi.org/10.1128/JVI.01013-10>.
 25. Maynard S, Ghosh R, Wu Y, Yan S, Miyake T, Gagliardi M, Rethoret K, Bedard PA. 2015. GABARAP is a determinant of apoptosis in growth-arrested chicken embryo fibroblasts. *J Cell Physiol* 230:1475–1488. <http://dx.doi.org/10.1002/jcp.24889>.
 26. Hu S, Xie Z, Onishi A, Yu X, Jiang L, Lin J, Rho HS, Woodard C, Wang H, Jeong JS, Long S, He X, Wade H, Blackshaw S, Qian J, Zhu H. 2009. Profiling the human protein-DNA interactome reveals ERK2 as a transcriptional repressor of interferon signaling. *Cell* 139:610–622. <http://dx.doi.org/10.1016/j.cell.2009.08.037>.
 27. Dehbi M, Mbiguino A, Beauchemin M, Chatelain G, Bedard PA. 1992. Transcriptional activation of the CEF-4/9E3 cytokine gene by pp60v-src. *Mol Cell Biol* 12:1490–1499. <http://dx.doi.org/10.1128/MCB.12.4.1490>.
 28. Ron D, Habener JF. 1992. CHOP, a novel developmentally regulated nuclear protein that dimerizes with transcription factors C/EBP and LAP and functions as a dominant-negative inhibitor of gene transcription. *Genes Dev* 6:439–453. <http://dx.doi.org/10.1101/gad.6.3.439>.
 29. Monnier J, Samson M. 2010. Prokineticins in angiogenesis and cancer. *Cancer Lett* 296:144–149. <http://dx.doi.org/10.1016/j.canlet.2010.06.011>.
 30. Khoury M, Drake A, Chen Q, Dong D, Leskov I, Fragoso MF, Li Y, Iliopoulou BP, Hwang W, Lodish HF, Chen J. 2011. Mesenchymal stem cells secreting angiopoietin-like-5 support efficient expansion of human hematopoietic stem cells without compromising their repopulating potential. *Stem Cells Dev* 20:1371–1381. <http://dx.doi.org/10.1089/scd.2010.0456>.
 31. Yu CW, Liang X, Lipsky S, Karaaslan C, Kozakewich H, Hotamisligil GS, Bischoff J, Cataltepe S. 2016. Dual role of fatty acid-binding protein 5 on endothelial cell fate: a potential link between lipid metabolism and angiogenic responses. *Angiogenesis* 19:95–106. <http://dx.doi.org/10.1007/s10456-015-9491-4>.
 32. Elmasri H, Ghelfi E, Yu CW, Traphagen S, Cernadas M, Cao H, Shi GP, Plutzky J, Sahin M, Hotamisligil G, Cataltepe S. 2012. Endothelial cell-fatty acid binding protein 4 promotes angiogenesis: role of stem cell factor/c-kit pathway. *Angiogenesis* 15:457–468. <http://dx.doi.org/10.1007/s10456-012-9274-0>.
 33. Courtwright A, Siamakpour-Reihani S, Arbiser JL, Banet N, Hilliard E, Fried L, Livasy C, Ketelsen D, Nepal DB, Perou CM, Patterson C, Klauber-Demore N. 2009. Secreted frizzled-related protein 2 stimulates angiogenesis via a calcineurin/NFAT signaling pathway. *Cancer Res* 69:4621–4628. <http://dx.doi.org/10.1158/0008-5472.CAN-08-3402>.
 34. Yi W, Sun Y, Yuan Y, Lau WB, Zheng Q, Wang X, Wang Y, Shang X, Gao E, Koch WJ, Ma XL. 2012. C1q/tumor necrosis factor-related protein-3, a newly identified adipokine, is a novel antiapoptotic, proangiogenic, and cardioprotective molecule in the ischemic mouse heart. *Circulation* 125:3159–3169. <http://dx.doi.org/10.1161/CIRCULATIONAHA.112.099937>.
 35. Kimura H, Weisz A, Ogura T, Hitomi Y, Kurashima Y, Hashimoto K, D'Acquisto F, Makuuchi M, Esumi H. 2001. Identification of hypoxia-inducible factor 1 ancillary sequence and its function in vascular endothelial growth factor gene induction by hypoxia and nitric oxide. *J Biol Chem* 276:2292–2298. <http://dx.doi.org/10.1074/jbc.M008398200>.
 36. Ubeda M, Wang XZ, Zinszner H, Wu I, Habener JF, Ron D. 1996. Stress-induced binding of the transcriptional factor CHOP to a novel DNA control element. *Mol Cell Biol* 16:1479–1489. <http://dx.doi.org/10.1128/MCB.16.4.1479>.
 37. Sebastian T, Johnson PF. 2006. Stop and go: anti-proliferative and mitogenic functions of the transcription factor C/EBPbeta. *Cell Cycle* 5:953–957. <http://dx.doi.org/10.4161/cc.5.9.2733>.
 38. Gade P, Roy SK, Li H, Nallar SC, Kalvakolanu DV. 2008. Critical role for transcription factor C/EBP-beta in regulating the expression of death-associated protein kinase 1. *Mol Cell Biol* 28:2528–2548. <http://dx.doi.org/10.1128/MCB.00784-07>.
 39. Cabannes E, Vives MF, Bedard PA. 1997. Transcriptional and post-transcriptional regulation of kappaB-controlled genes by pp60v-src. *Oncogene* 15:29–43. <http://dx.doi.org/10.1038/sj.onc.1201162>.
 40. Voisin L, Saba-El-Leil MK, Julien C, Fremin C, Meloche S. 2010. Genetic demonstration of a redundant role of extracellular signal-regulated kinase 1 (ERK1) and ERK2 mitogen-activated protein kinases in promoting fibroblast proliferation. *Mol Cell Biol* 30:2918–2932. <http://dx.doi.org/10.1128/MCB.00131-10>.
 41. Lefloch R, Pouyssegur J, Lenormand P. 2008. Single and combined silencing of ERK1 and ERK2 reveals their positive contribution to growth signaling depending on their expression levels. *Mol Cell Biol* 28:511–527. <http://dx.doi.org/10.1128/MCB.00800-07>.
 42. Guyton KZ, Xu Q, Holbrook NJ. 1996. Induction of the mammalian stress response gene GADD153 by oxidative stress: role of AP-1 element. *Biochem J* 314:547–554. <http://dx.doi.org/10.1042/bj3140547>.
 43. Jin HO, An S, Lee HC, Woo SH, Seo SK, Choe TB, Yoo DH, Lee SB, Um HD, Lee SJ, Park MJ, Kim JI, Hong SI, Rhee CH, Park IC. 2007. Hypoxic condition- and high cell density-induced expression of Redd1 is regulated by activation of hypoxia-inducible factor-1alpha and Sp1 through the phosphatidylinositol 3-kinase/Akt signaling pathway. *Cell Signal* 19:1393–1403. <http://dx.doi.org/10.1016/j.cellsig.2006.12.014>.