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TUMORIGENESIS AND NEOPLASTIC PROGRESSION

CREB-Induced Inflammation Is Important for Malignant Mesothelioma Growth

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Malignant mesothelioma (MM) is an aggressive tumor with no treatment regimen. Previously we have demonstrated that cyclic AMP response element binding protein (CREB) is constitutively activated in MM tumor cells and tissues and plays an important role in MM pathogenesis. To understand the role of CREB in MM tumor growth, we generated CREB-inhibited MM cell lines and performed *in vitro* and *in vivo* experiments. *In vitro* experiments demonstrated that CREB inhibition results in significant attenuation of proliferation and drug resistance of MM cells. CREB-silenced MM cells were then injected into severe combined immunodeficiency mice, and tumor growth in s.c. and i.p. models of MM was followed. We observed significant inhibition in MM tumor growth in both s.c. and i.p. models and the presence of a chemotherapeutic drug, doxorubicin, further inhibited MM tumor growth in the i.p. model. Peritoneal lavage fluids from CREB-inhibited tumor-bearing mice showed a significantly reduced total cell number, differential cell counts, and pro-inflammatory cytokines and chemokines (IL-6, IL-8, regulated on activation normal T cell expressed and secreted, monocyte chemotactic protein-1, and vascular endothelial growth factor). *In vitro* studies showed that asbestos-induced inflammasome/inflammation activation in mesothelial cells was CREB dependent, further supporting the role of CREB in inflammation-induced MM pathogenesis. In conclusion, our data demonstrate the involvement of CREB in the regulation of MM pathogenesis by regulation of inflammation. *(Am J Pathol 2014, 184: 2816–2827; http://dx.doi.org/10.1016/j.ajpath.2014.06.008)*

Malignant mesothelioma (MM) is a very aggressive cancer originating from the mesothelial lining of the peritoneal, pleural, or pericardial cavity.[1] The incidence of MM continues to increase worldwide because of the long latency period of MM development.[2] MM is difficult to diagnose at an early stage and is resistant to conventional and multimodal treatments. A combination of cisplatin and pemetrexed is the current first-line chemotherapy regimen for MM patients.[3] Doxorubicin (Dox) was the first successful chemotherapeutic drug tested in MM and is currently administered in combination with other treatment strategies.[4,5] Recent focus for MM treatment includes immunotherapy, growth factor receptors, signaling molecular pathways, angiogenic pathways, and epigenetic modulator targeting (reviewed by Mossman et al[6]). Moreover, gene therapy is seen as a potential therapeutic possibility for MM (reviewed by Tagawa et al[7]). As the population of MM patients is growing worldwide, there is a strong need for the development of new and effective therapies.

Various signaling molecules have been involved in the pathogenesis of MM, and targeting them by small-molecule inhibitors or gene therapy is an ongoing strategy in the development of chemotherapeutics. An important step in this direction was our identification of extracellular signal–regulated kinases, which play important roles in MM

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pathogenesis, and their inhibition by small-molecule inhibitors in combination with chemotherapeutic drugs could have significant effects on MM tumor growth.[8–10] Cyclic AMP response element binding protein (CREB) is a transcription factor that mediates signals from calcium, cytokines, and cellular stressors by regulation of gene expression.[11] Although CREB-dependent gene expression plays significant roles in the regulation of various aspects of the central nervous system, little knowledge exists about the role of CREB in cancers. Recent limited reports have demonstrated a significant emerging role of CREB in some cancers. For example, patients with acute lymphoid leukemia or acute myeloid leukemia show CREB overexpression in their bone marrow samples, and CREB overexpression is associated with a poor outcome in AML patients.[12] Another CREB family member, CREB2, was significantly elevated in breast carcinoma compared to corresponding normal breast tissue and may potentially be involved in the development of cancer.[13] Furthermore, CREB overexpression and activation has been linked to negative prognosis in nonsmokers with non–small cell lung cancer[14] and melanoma metastasis.[15]

We recently reported that asbestos activates CREB in mesothelial cells, and MM cells and tumor tissues show constitutively activated CREB.[16] Here, using xenograft mouse models and genetically CREB-silenced MM cell lines [small hairpin (sh) CREB], we demonstrate that CREB promotes MM tumor growth in mouse models. Additionally, we demonstrate that Dox in the presence of CREB silencing is more effective in MM tumor reduction compared with Dox alone. Moreover, inflammatory profiles assessed in peritoneal lavage fluid (PLF) of i.p. tumor-bearing mice showed significant inhibition in total and differential cell counts, as well as pro-inflammatory cytokines, chemokines, and growth factor levels in shCREB groups. *In vitro* data validated *in vivo* findings that showed that asbestos-induced inflammasome activation in human mesothelial cells, which could be a source of many pro-inflammatory cytokines, is CREB dependent. Conclusively our data show that CREB controls MM tumor development and growth by multiple mechanisms, predominantly by regulating inflammation.

Materials and Methods

Cell Culture and Treatment with Asbestos and/or Inhibitors

Human peritoneal mesothelial LP9/TERT-1 (LP9) cells[17] were a gift from James Rheinwald (Brigham and Women's Hospital, Harvard University, Boston, MA). Human MM cell lines H2373, H2595, H2461, and HP-1 were contributed by Harvey Pass (New York University, New York, NY).[18] HMESO cells, originally designated H-MESO-1, were isolated by Reale et al.[19] All cells were cultured as reported previously.[9]

Cell lines were validated by short tandem repeats DNA fingerprinting using the Cell ID System (Promega Corp., Madison, WI). The short tandem repeats profiles are of human origin and did not match known DNA fingerprints in the Cell Line Integrated Molecular Authentication database (*http://bioinformatics.istge.it/clima*) but will serve as a reference for future work.

The physical and chemical characterization of the National Institute of Environmental Health Sciences reference sample of crocidolite asbestos has been reported previously.[20] Fibers were added to cells in medium to achieve the desired final concentration of 5 $\mu g/cm^2$ area dish (represented as 75 in terms of surface area at some places) as described previously,[21] a concentration causing apoptosis and compensatory proliferation of surrounding pleural mesothelial cells.[22] Protein kinase A (PKA) inhibitor H89 was purchased from Calbiochem (La Jolla, CA) and was added at an effective concentration reported previously.[16] Control cultures received medium without inhibitor but with vehicle ($\leq$0.1% dimethyl sulfoxide) instead and were treated identically. Dox was purchased from Sigma-Aldrich Corp. (St. Louis, MO), and cisplatin was purchased from Alfa Aesar (Ward Hill, MA). All experiments were performed in duplicate or more.

Creation of shCREB MM Lines

Confluent HMESO or H2373 cells were transfected with either CREB1 or scrambled control Sure Silencing Plasmids (4 shConstructs per gene per cell line were used) from SABiosciences (Frederick, MD), using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) as previously described.[8] After this procedure, shCREB1 clones exhibiting inhibition of >70% to 80% CREB1 expression were obtained. Inhibition of CREB1 in transfected MM cell lines was determined by quantitative real-time PCR as well as by Western blot analysis.

Proliferation Assay

Transfected HMESO or H2373 cells (shCon and shCREB1) were plated at the same density and were allowed to grow for 72 hours in 12 well plates. Cells were trypsinized and counted at 0, 24, 48, and 72 hours with a hemocytometer.

MTS Assay

Human MM cells (shCon and shCREB) were treated with different concentrations of Dox for 24 hours, and cell viability was measured in cells using the colorimetric MTS assay CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega Corp., Fitchburg, WI) as per the manufacturer's recommendations.[9]

Transformation Assay

Anchorage-independent growth of transfected HMESO MM cells was assessed by a colony-formation assay in soft agar using the CytoSelect Cell Transformation assay (Cell Biolabs, Inc., San Diego, CA) as previously reported.[10]

Migration Assay

Migration of MM cells was assessed using 6-well Transwell polycarbonate filters (Corning Costar Corp., Corning, NY) with an 8-μm pore size as described previously.[10]

S.C. Xenograft Mouse Model

HMESO cells stably transfected with either shCREB1 or shCon were injected into 4 s.c. sites (5×10^6 cells per injection site) on the dorsa of 6-week-old Fox Chase severe combined immunodeficiency (SCID) mice (Charles River Laboratories Inc., Wilmington, MA) ($n = 5$ or 6 per group). At the end of the experiment, all mice were weighed, euthanized with i.p. injections of sodium pentobarbital, and necropsied to determine possible gross metastases.[10] Tumor volumes were calculated as described previously.[23] All experiments using mice were approved by the Institutional Animal Care and Use Committee at the University of Vermont College of Medicine (Burlington, VT).

Assessment of Human MM Tumor Development in an i.p. SCID Mouse Model

CREB-inhibited (shCREB) or control (shCon) HMESO cells (5×10^6 cells in 50 μL 0.9% NaCl, pH 7.4) were injected into the lower left quadrant of the peritoneal cavity of 6-week-old male Fox Chase SCID mice ($n = 5$ or 6 mice per group). Two weeks after cell injection, two groups (shCon-Dox and shCREB1-Dox) started receiving 0.5 mg/kg Dox i.p., t.i.w. for 2 weeks. The other two groups (shCon-saline and shCREB-saline) received equal volumes of saline for the same period of time. Four weeks after MM cell injections, mice were euthanized as described above. PLF was collected, and animals were closely examined for the presence of tumors. Weights and volumes of tumors were determined as reported previously.[24] Briefly, tumor volumes were measured using calipers and calculated using the following formula:

$$(\pi \times \text{long axis} \times \text{short axis} \times \text{short axis})/6 \qquad (1)$$

and were not based on tumor weights. As i.p. MM tumors developed attached to mesentery, individual numbers could not be counted.

Generation of CREB-Overexpressing MM Lines and Injection into Mice

CREB-overexpressing HMESO MM lines were generated by the transfection of plasmid-containing CREB1 human cDNA clone (transfection ready; OriGene Technologies, Inc., Rockville, MD) or empty vector (PCMV6NEO; OriGene Technologies, Inc.) into cells and then after the selection process and limited dilution as described above. These cells were injected i.p. into mice as discussed above, and tumor growth was followed for 4 weeks.

Determination of Inflammatory Cell Profiles in PLF in the I.P. SCID Mouse Model

After the euthanization of the mice, PLF was collected as described previously.[24] Cells were stained with white blood cell stain and counted. For total cell counts, we stained PLF samples with white blood cell stain, suggesting that total cell counts included all nucleated cells, including mesothelial/mesothelioma cells. During differential cell counting, we saw clumps of mesothelioma cells that were not included in the blood cell counts. After proper processing, PLF was used for cytokine, chemokine, and growth factor analysis and for differential cell count assessment.[24] PLF was selected over serum for inflammatory marker assessment, as our previous experiments (unpublished data) showed that inflammatory changes are detectable in PLF, but are not significantly reflected in serum, at week 4 of experimentation.

Cytokine, Chemokine, and Growth Factor Assessment in PLF

To quantify cytokine and chemokine levels in PLF from mice, a multiplex suspension protein array was performed using a Human Cytokine 27-plex panel (Bio-Rad Laboratories, Inc., Hercules, CA) as described previously.[25] Concentrations of each cytokine and chemokine were determined using Bio-Plex Manager version 3.0 software. Data were expressed as pg cytokine/mL medium.

TUNEL Staining in Tumors

Paraffin sections of shCon and shCREB tumors collected from the *in vivo* model were dehydrated and processed for antigen retrieval (Dako, Carpinteria, CA). Slides were then transferase dUTP nick end labeling (TUNEL) stained using Roche's (Indianapolis, IN) In Situ Cell Death Detection Kit, POD, using the recommended protocol for processing difficult tissue. Images of TUNEL-stained tumor tissue were taken using a 40× objective with an Olympus BX50 upright light microscope (Olympus America Inc., Lake Success, NY) and an attached QImaging Retiga 2000R Digital CCD Camera (Advanced Imaging Concepts, Inc., Princeton, NJ).

Western Blot Analysis

Western blot analysis was performed as described previously,[26] using antibodies specific to total and phosphorylated CREB [rabbit polyclonal anti-phosphoCREB, 1:500; rabbit polyclonal anti-CREB, 1:1000 (Cell Signaling Technology, Inc., Beverly, MA); and total β-actin 1:2000 (Abcam plc, Cambridge, UK)]. QuantityOne was used to quantify band density, and phosphorylated protein levels were normalized to respective total protein levels (ie, phosphoCREB/CREB). Blots are representative of at least two different experiments.

Detection of Caspase-1 p20 and HMGB1 in Supernatants (Medium) of Mesothelial and MM Cells

Medium was collected (500 μL) and concentrated using Amcion ultracentrifugal filters with a 10K membrane (EMD Millipore, Billerica, MA) as described previously.[21] Western blots were performed as previously described[26] on both cell lysates and concentrated medium. A rabbit polyclonal high-mobility group box 1 protein (HMGB1) antibody (Abcam plc) was used at a dilution of 1:5000. Rabbit polyclonal antibodies for caspase-1 p20 (Cell Signaling Technology, Inc.) were used at 1:500 dilutions. Western blot analysis performed in medium had no normalization control (eg, β-actin), and quantitation represents the band density of each sample.

Enzyme-Linked Immunosorbent Assay for IL-1β and IL-18

The Quantikine Human IL-1β/IL-1f2 Immunoassay (R&D Systems, Inc., Minneapolis, MN; measures predominantly mature IL-1β) was used on concentrated cell medium, prepared as described previously,[21] and the assay was performed according to the manufacturer's instructions. IL-18 release was measured using the Human IL-18 enzyme-linked immunosorbent assay kit (MBL International Corp., Woburn, MA; measures predominantly active IL-18, 0.7% pro IL-18).[21] Values were expressed as pg/mL of IL-1β or IL-18 from the original supernatant (nonconcentrated).

Caspase-1 Activity Assay

Caspase-1 activity was measured using the Caspase-1 Colorimetric Assay Kit (R&D Systems, Inc.) as previously reported.[21] Protein concentrations were determined by the Bio-Rad protein assay[27] using the remaining lysate.

Quantitative Real-Time PCR

Total RNA was prepared using an RNeasy Plus Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's protocol as described previously.[26] Total RNA (1 μg) was reverse-transcribed with random primers using the Avian Myeloblastosis Virus Reverse Transcriptase kit (Promega Corp., Madison, WI) according to the recommendations of the manufacturer. Gene expression was quantified by TaqMan quantitative real-time PCR using the Prism 7700 Sequence Detector (PerkinElmer/Applied Biosystems Inc., Foster City, CA) as described previously.[10] Duplicate/triplicate assays were performed with RNA samples isolated from at least two independent experiments.

Affymetrix Gene Profiling

Microarrays were performed on samples from three independent experiments as described previously.[28] Each of the samples was analyzed on a separate array, that is, $n = 3$ arrays per MM cell line (three independent biological replicates) (shCon and shCREB). A Human Genome U133A 2.0 Array (Affymetrix, Inc., Santa Clara, CA) was scanned twice (HP GeneArray Scanner; Hewlett-Packard, Palo Alto, CA), the images overlaid, and the average intensities of each probe cell compiled. Microarray data were analyzed using GeneSifter software (VizX Labs, Seattle, WA). This program used a *t*-test for pairwise comparison and a Benjamini-Hochberg test for false discovery rate (5%) to adjust for multiple comparisons. A twofold cutoff limit was used for significance (Gene Expression Omnibus, *http://www.ncbi.nlm.nih.gov/geo*, accession no. GSE51447).

Statistical Analysis

In all *in vitro* assays, at least two independent samples were examined at each time point per group in duplicate or triplicate experiments. Data were evaluated by either analysis of variance using the Student-Neuman-Keuls procedure for adjustment of multiple pairwise comparisons between treatment groups, the nonparametric Kruskal-Wallis and *U*–tests, or a two-tailed *t*-test. P values ≤ 0.05 were considered statistically significant. The difference in tumor growth rates in the s.c. MM model was assessed using a hierarchical regression model to take into account the correlation between repeated measurements on the same tumor and multiple tumors in the same animal. In this analysis, the regression coefficient describing tumor growth is modeled as a function of treatment group as well as random variation due to differences between animals and tumors on the same animal.

Results

CREB Silencing in MM Cell Lines Causes Reduced Tumorigenic Properties

We generated two stably inhibited CREB MM lines (HMESO and H2373) and assessed them for various *in vitro* tumorigenic properties (Figure 1). Both MM lines showed significant inhibition in CREB levels as measured at the RNA and protein levels (Figure 1A). CREB silencing in both MM cell lines demonstrated inhibition in proliferation (Figure 1B) and increased Dox sensitivity (Figure 1C). In addition, colony formation and migration were inhibited by CREB attenuation in HMESO MM cells (Figure 1, D and E).

CREB Inhibition Causes Attenuated Tumor Growth in Xenograft Mouse Models

After *in vitro* testing, stably inhibited CREB MM cells (HMESO) were injected into mice either s.c. or i.p. as described earlier. Figure 2A shows the remarkably different tumor sizes from the shCon and shCREB groups in the s.c. model. Tumor in this model grew for 45 days, and the median tumor volumes were 0.62172 and 0.00052 in the shCon and shCREB groups, respectively. Significantly

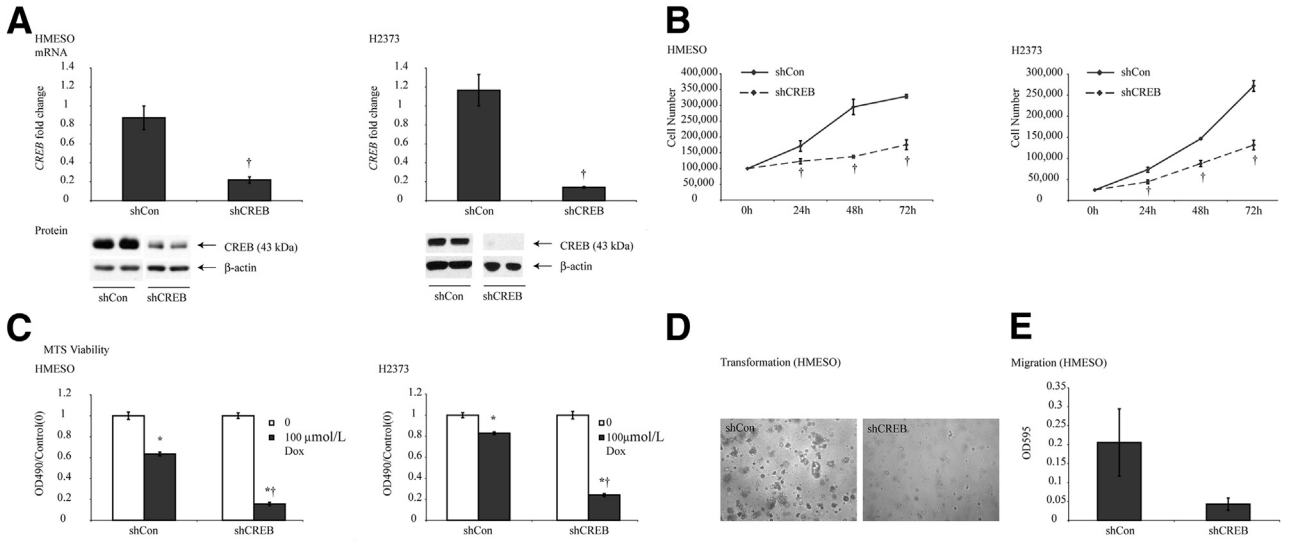

Figure 1 Creation and characterization of shCREB malignant mesothelioma (MM) lines. **A:** shCREB HMESO and H2373 MM lines show decreased levels of CREB after shCREB transfection. **B**—**E:** CREB inhibition represses proliferation of MM lines (**B**), makes MM lines more sensitive to the chemotherapeutic drug Dox (**C**), causes attenuation of colony formation by MM cells (**D**), and reduces MM cell migration (**E**). Data are expressed as means ± SEM (**A**—**C**, **E**). $n = 2$ to 6. $^{*}P < 0.05$ versus untreated (0) of same sh group; $^{\dagger}P < 0.05$ versus shCon of same treatment group. OD, optical density, in nm.

greater inhibition in MM tumor growth rate was observed in the shCREB group compared with the shCon group in the same model, as assessed by a biostatistician as described in Statistical Analysis section (Figure 2B). Tumor volumes in the shCREB group were very small (0, 0.00028, 0.001, 0.00094, 0.00087, 0.00098, 0.00174, and 0.00560) but not zero as appears from the scale. The tumor volumes in the two groups were also significantly different at various time points. TUNEL staining to assess cell death in tumors showed increased staining in CREB-inhibited tumors (Figure 2C); representative sections from same animals stained for hematoxylin and eosin and phosphoCREB are also included in Figure 2C. The differences in cell viability may have been the result of pCREB, hematoxylin and eosin, and TUNEL staining performed on different sections representing different areas of the same tumor.

I.p. injection of CREB-inhibited MM cells also resulted in significantly reduced tumor weights and volumes compared with those in control tumors (Figure 2, D and E). Treatment with Dox resulted in further reductions in tumor weights and volumes (Figure 2, D and E), suggesting that combined CREB inhibition and chemotherapy is better than either of them alone. The volumes of PLF were not significantly different between the two groups.

To show that overexpression of CREB has an effect on tumor growth opposite that of CREB silencing, we generated CREB-overexpressing MM cell lines and injected them i.p. into SCID mice. As expected, CREB overexpression had no inhibitory effect on MM tumor weights and volumes (Figure 2, F and G).

The use of two different *in vivo* models (i.p. and s.c.; Figure 2) to show that CREB is involved in MM tumorigenesis reinforced our findings.

Reduced Inflammation by CREB Silencing in the I.P. MM Tumor Model

PLF from i.p. human MM tumor—bearing SCID mice were assessed for inflammatory responses. Total cell counts in PLF from control and Dox-treated mice were not significantly different [total cell counts reflect only immune cells, macrophages, neutrophils, and eosinophils (lymphocyte counts were not included as SCID mice do not have lymphocytes); mesothelial/mesothelioma cells were not included in the total cell counts]. However, CREB inhibition alone and in combination with Dox showed a decrease in total cell number (Figure 3A). Differential cell counts also showed the same pattern (Figure 3B). We studied a panel of human cytokines in PLF by BioPlex analysis. Use of the human panel ensured that the cytokines measured were derived from MM tumors and not from the host (mice). CREB silencing attenuated many proinflammatory cytokines, chemokines, and growth factors [e.g., IL-6, IL-8, IP-10, monocyte chemotactic protein 1, macrophage inflammatory protein 1β, chemokine (C-X-C motif) ligand 5/regulated on activation normal T cell expressed and secreted, and vascular endothelial growth factor] compared with controls (Figure 3C). In contrast, Dox treatment significantly increased the levels of IL-6, IL-8, IP-10, macrophage inflammatory protein 1β, and regulated on activation normal T cell expressed and secreted (Figure 3C). The combined effect of Dox and CREB inhibition in many cases was significantly better than that with either of them alone (Figure 3C). Very low levels of IL-1β were detected in the PLF, which is consistent with our previous findings,[24] and there were no significant differences across groups. In addition, IL-18 (another inflammasome-related cytokine) could not be measured as it was not a part of the BioPlex panel.

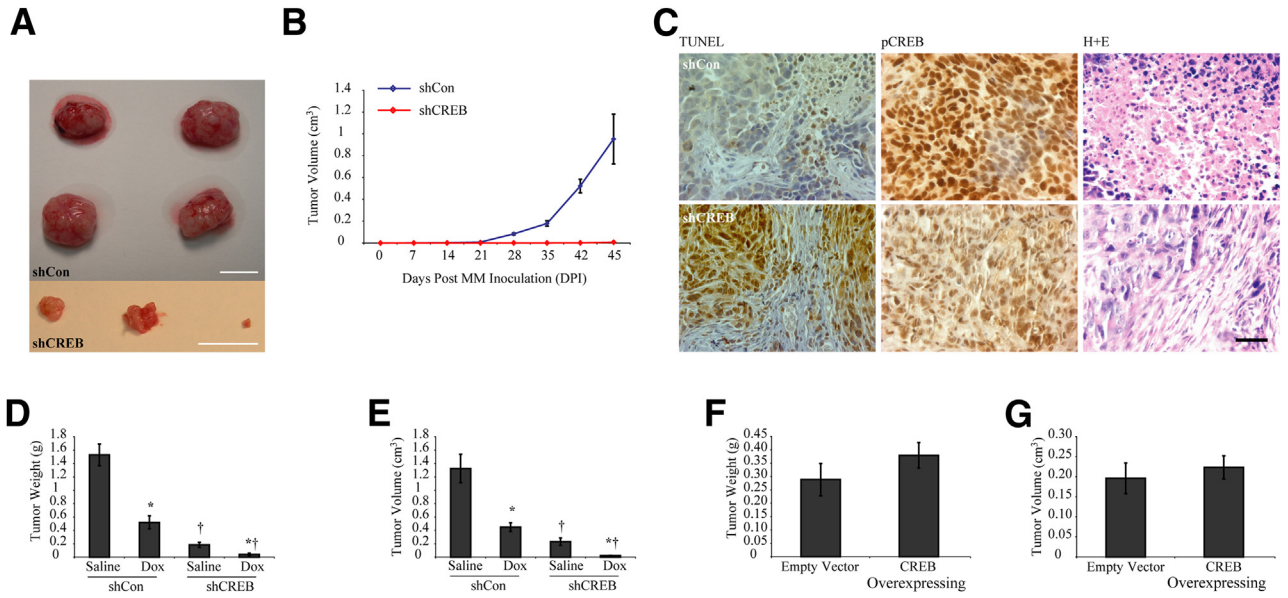

Figure 2 CREB inhibition attenuates *in vivo* malignant mesothelioma (MM) tumor growth in xenograft mouse models. **A:** Excised control or shCREB tumors. **B:** Tumor growth rate in a s.c. model. **C:** TUNEL staining of tumors from the s.c. model, phosphoCREB, and hematoxylin and eosin (H+E) stained sections from same animals. **D** and **E:** Combined effects of CREB inhibition and 0.5 mg/kg chemotherapeutic drug Dox on peritoneal MM tumor weights (**D**) and volumes (**E**). **F** and **G:** CREB overexpression has no inhibitory effect on MM tumor growth in i.p. model. Data are expressed as means ± SEM (**B**, **D**–**G**). $n = 5$ to 6. $^{*}P < 0.05$ versus untreated (0) of same sh group; $^{\dagger}P < 0.05$ versus shCon of same treatment group. Scale bars = 1 cm (**A**) and 50 μm (**C**).

CREB Inhibition Attenuates Asbestos-Induced Inflammasome Activation in Human Mesothelial Cells

Inflammation plays determining roles in asbestos-induced lung diseases, including MMs. Recent reports show that NLR family, pyrin domain containing 3 (NLRP3) inflammasome activation plays a contributory role toward asbestos-induced inflammation in macrophages[29] as well as in mesothelial cells.[21] To assess the role of CREB in asbestos-induced activation of NLRP3, and therefore in asbestos-induced inflammation, in mesothelial cells, we used a PKA inhibitor, H89, to block CREB activation by asbestos. This inhibitor was chosen based on our previous findings that asbestos-induced CREB activation in mesothelial cells is PKA mediated.[16] The inhibition of CREB activation by H89 caused significant inhibition in asbestos-induced NLRP3 priming and activation (Figure 4A). As NLRP3 activation is measured by caspase-1 activation, here we measured caspase-1 activation by two techniques, enzyme activity assay and caspase-1 p20 release in the medium (Figure 4A), and using both techniques we showed that CREB inhibition attenuated asbestos-induced NLRP3 activation. Asbestos-induced secretion of the pro-inflammatory marker HMGB1 in medium was also significantly attenuated by CREB inhibition (Figure 4A). Furthermore, the secretion of IL-1β and IL-18, two key pro-inflammatory cytokines released as a result of inflammasome activation, was also significantly attenuated by CREB inhibition (Figure 4A). To further confirm the role of CREB in inflammasome priming and activation, we used the CREB-silenced human MM cell line (shCREB) described above. In CREB-silenced MM cells (HMESO), significantly decreased IL-1β steady-state mRNA levels were observed (Figure 4B). PYCARD, another subunit required to form a complex with NLRP3 for inflammasome activation, was also significantly inhibited in CREB-silenced MM cells (Figure 4B). No significant effect of CREB silencing on NLRP3 steady-state mRNA levels was observed (data not shown). In the assessment of caspase-1 activation, measured by p20 release into the medium, untreated CREB-silenced MM cells had slightly (HMESO) (Figure 4B) increased levels of caspase-1 compared with shCon. Treatment with the chemotherapeutic drug Dox (5 μmol/L) or cisplatin (100 μmol/L) did not significantly alter caspase-1 activation (Figure 4B). HMGB1, another pro-inflammatory molecule that may be secreted in response to inflammasome activation, also showed no significant response in MM cells (Figure 4B) after CREB inhibition. However, HMGB1 levels were significantly lower in the drug-treated shCREB group compared with those in the respective control groups (Figure 4B). IL-1β secretion was not significantly affected by CREB silencing or drug exposure in HMESO cells (data not shown). The data presented here suggest that CREB can regulate asbestos-induced inflammation in mesothelial cells by modulating inflammasomes, which may thereby interfere with the development of MM. In MM (HMESO) cells, CREB silencing caused decreased levels of IL-1β and PYCARD mRNA as well as increased levels of caspase-1 release, resulting in reduced inflammation and increased cell death, which may inhibit the progression of MM.

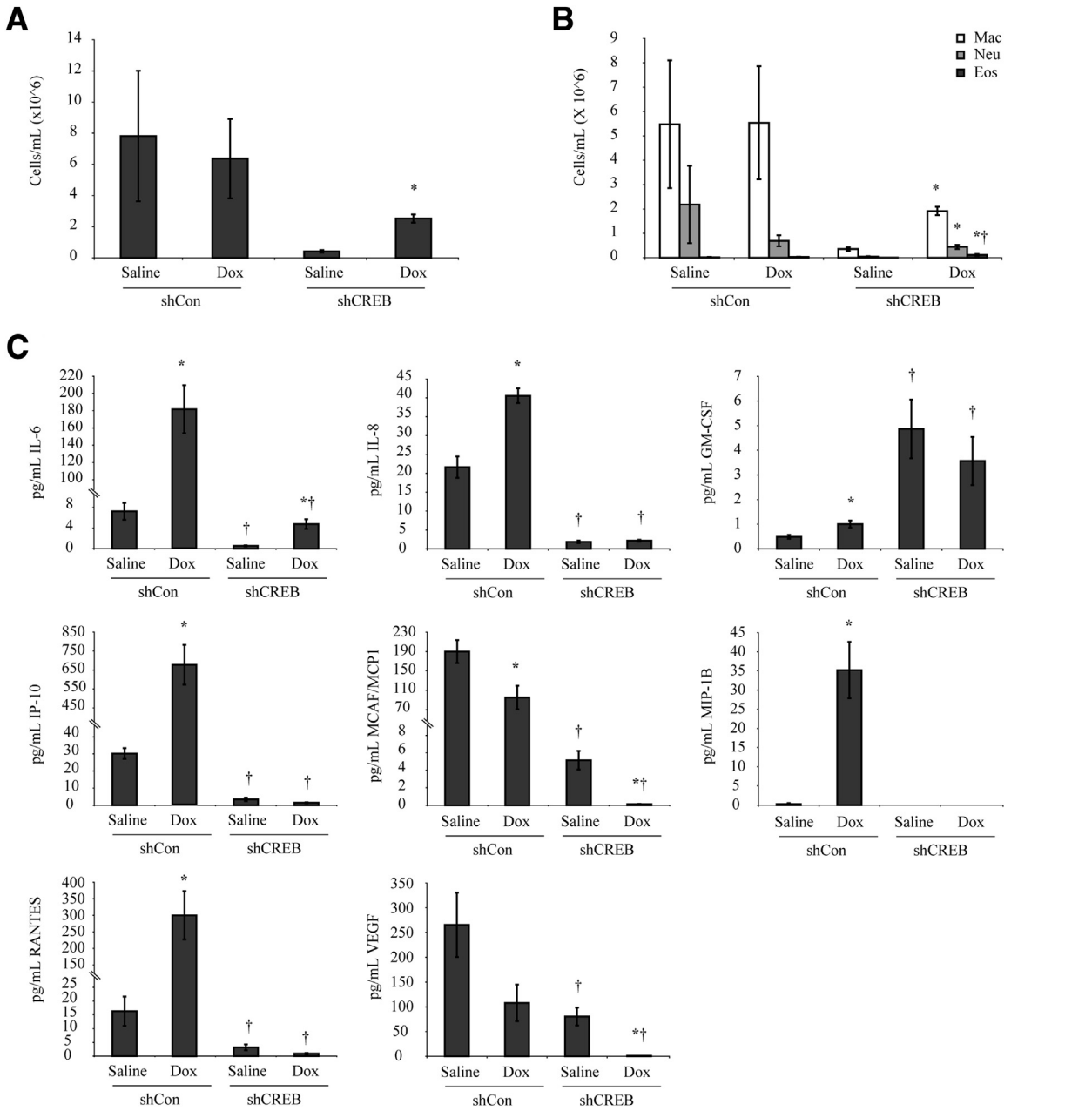

Figure 3 Inhibition of CREB attenuates inflammation in human tumor—bearing SCID mice. **A:** Total cell counts in peritoneal lavage fluid (PLF) of tumor-bearing mice. **B:** Differential cell counts in PLF of tumor-bearing mice. **C:** Human proinflammatory cytokine, chemokine, and growth factor levels, as measured by BioPlex, in PLF of tumor-bearing mice. Data are expressed as means ± SEM. $n = 5$ to 6. *$P < 0.05$ versus untreated (0) of same sh group; †$P < 0.05$ versus shCon of same treatment group.

Different Kinases and Phosphatases Regulate CREB Activation in MM Cells

We have previously shown that MM cells show constitutively activated CREB[16]; however, upstream pathways leading to CREB activation in MM cells remain unknown. To investigate upstream pathways that may regulate the CREB pathway in MM cells, we selected various inhibitors that have been demonstrated to regulate CREB phosphorylation in different cell types. We used five different MM cell lines[16] and inhibitors of epidermal growth factor receptor (AG1478), extracellular signal—regulated kinase 1/2 (U1026), PKA (H89), phosphatidylinositide 3-kinase (LY), and c-Met (c-Met kinase inhibitor II). No single inhibitor was effective in all MM cell lines (Figure 5). We assessed the levels of phosphatase and tensin homolog in various MM cell lines with constitutive CREB activation[16] because phosphatases can also regulate the activation of CREB, and a recent report suggested CREB as a target of phosphatase and tensin homolog phosphatase.[30] The HMESO cell line was the only MM cell line that showed decreased phosphatase and tensin homolog levels compared

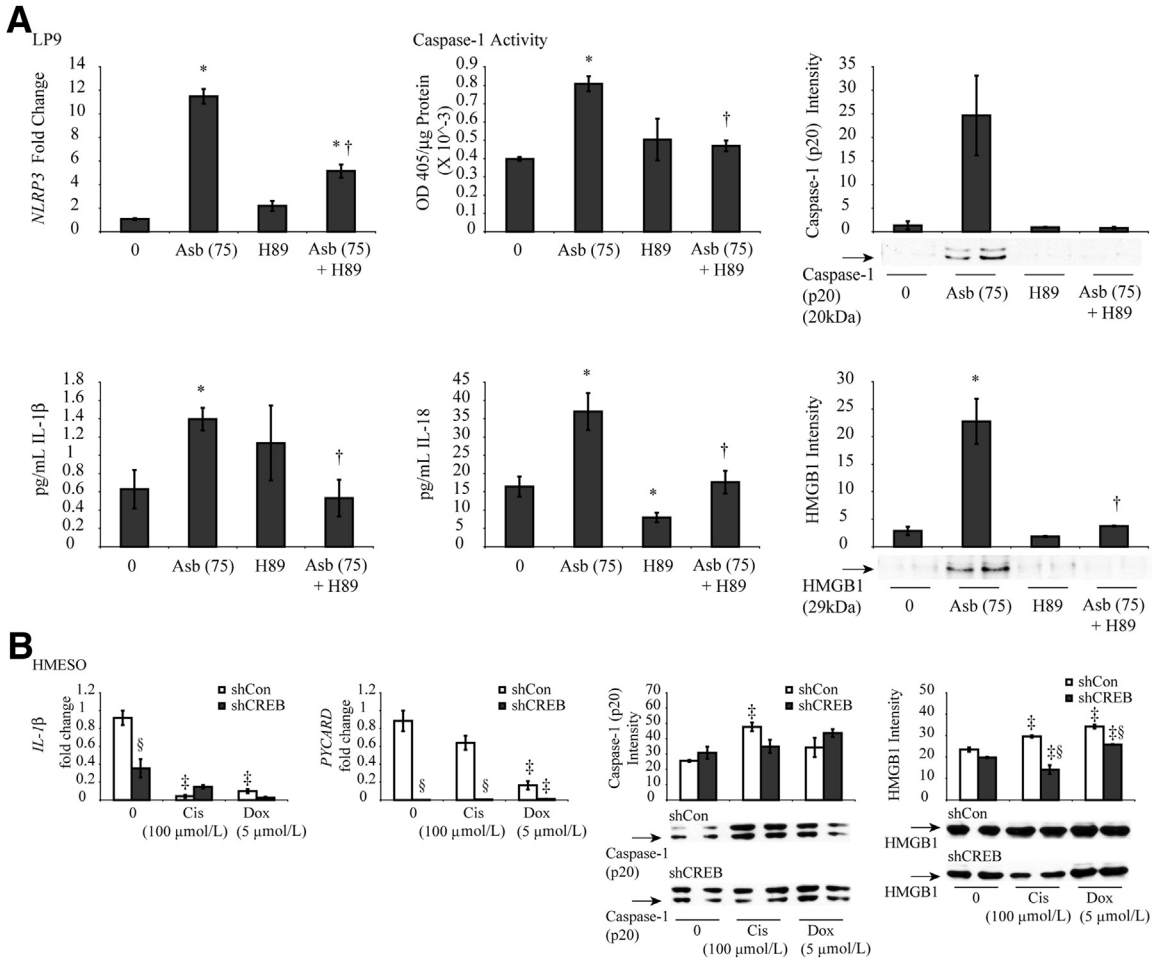

Figure 4 CREB regulates asbestos-induced inflammasomes in human mesothelial cells. **A:** Asbestos-induced NLRP3 priming is inhibited by attenuation of CREB activation by 10 μmol/L H89. Asbestos-induced caspase-1 activation is attenuated by CREB inhibition. Asbestos-induced IL-1β, IL-18, and HMGB1 levels in medium are also inhibited by CREB inhibition. **B:** CREB inhibits HMESO cells (shCREB) showing decreased IL-1β and apoptosis-associated speck-like protein containing a CARD (PYCARD) mRNA levels and secreted caspase-1 p20 and HMGB1 levels compared with control HMESO cells (shCon). Data are expressed as means ± SEM. $n = 2$ to 3. $^{*}P < 0.05$ versus untreated (0) group; $^{\dagger}P < 0.05$ versus Asb (75) group; $^{\ddagger}P < 0.05$ versus untreated (0) of same sh group; $^{\S}P < 0.05$ versus shCon of same treatment group.

with those in mesothelial cells (LP9) (Figure 5F). From this experiment, we conclude that it is difficult to inhibit the activation of CREB in MM cells by a single small-molecule inhibitor. A superior approach is to inhibit CREB levels by genetic manipulation (shRNA) or to inhibit the interaction of active CREB to its partner CREB-binding protein (KG501).

CREB Silencing Alters Various Tumorigenesis-Related Genes in MM Cells

As CREB is a transcription factor and plays important roles in various gene regulations, we screened MM cell lines for the genes regulated by CREB. Microarray analyses were performed on MM cell lines (HMESO and H2373) stably inhibited for CREB and respective controls. Selected tumorigenesis-related genes significantly altered by CREB inhibition in HMESO MM cells are presented in Table 1. Proliferation- and growth-related genes p21(cyclin-dependent kinase inhibitor 1A)-activated kinase 7 (*PAK7*), Met proto-oncogene [c-MET/hepatocyte growth factor receptor (HGFR)], v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene (oncogenic KIT), and Polo-like kinase 2 (*PLK*) were significantly inhibited by CREB silencing. Several genes directly or indirectly involved in the process of angiogenesis [tyrosine kinase with immunoglobulin-like and endothelial growth factor–like domains1 (TIE), melanoma cell-adhesion molecule (*MCAM*/CD146), pro-inflammatory chemokine (c-c) ligand 5 (*CCL5*/regulated on activation normal T cell expressed and secreted)] that are overexpressed in many cancers and play important roles in angiogenesis, proliferation, and invasion of tumor cells were all significantly attenuated by CREB inhibition. Furthermore, CREB silencing also down-regulated some adhesion and migration-related genes, such as *CD24* and matrix metalloprotease 2 (*MMP2*), that control the metastasis of tumors. We also validated three different genes (Table 1) by quantitative real-time PCR from RNA obtained from a different experiment. Taken together, our

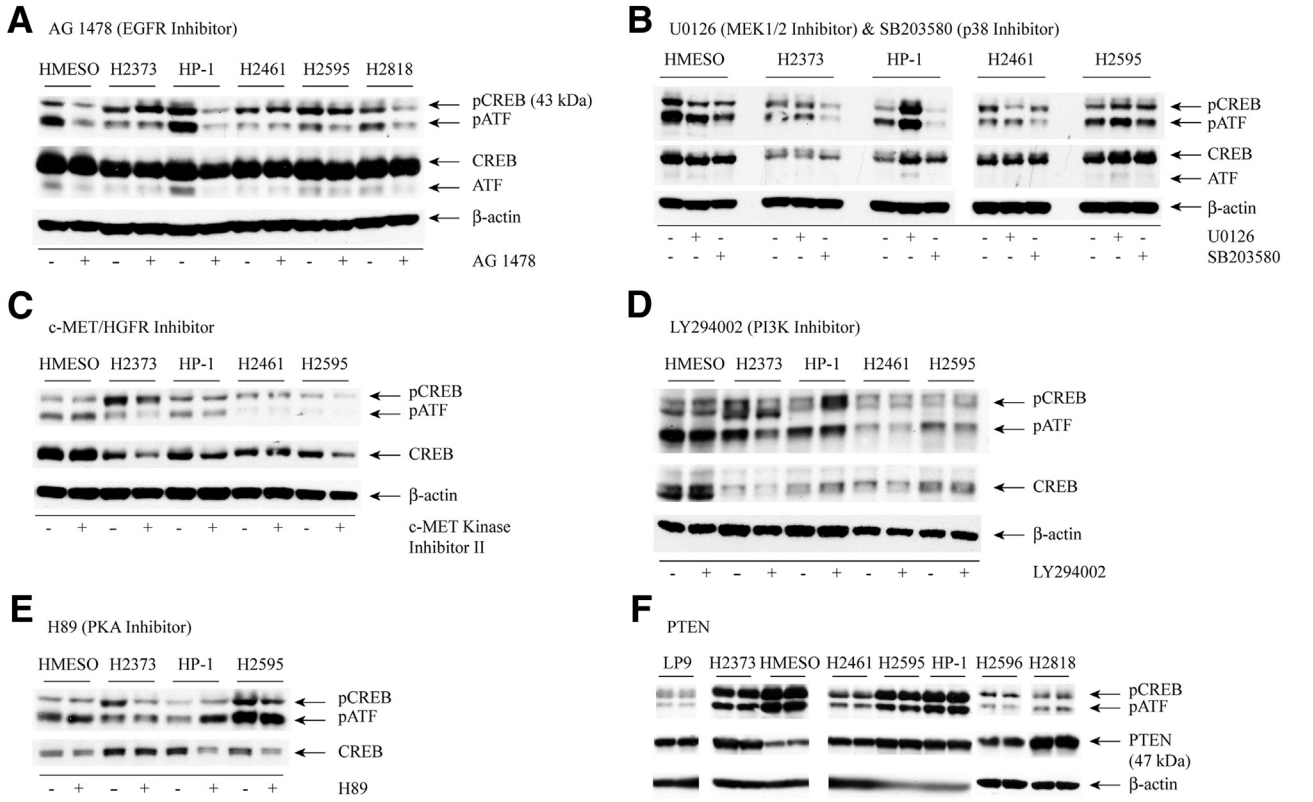

Figure 5 CREB regulation in malignant mesothelioma (MM) cells is multifaceted. **A**–**E:** Different human MM cell lines exposed to various small-molecule inhibitors show varied responses. **F:** Phosphatase and tensin homolog inhibition is observed in one in five MM lines tested.

findings suggest that CREB may regulate MM tumorigenesis by regulating many relevant genes.

Discussion

MM is a deadly cancer without any effective treatment and it is therefore important to investigate any potential lead observed in MM pathogenesis or therapeutic strategies. Our previous studies have demonstrated that CREB is constitutively activated in MM cells and in tumor tissues and that its inhibition by siRNA can result in increased apoptosis and decreased migration.[16] These findings encouraged us to look further into the role of CREB in MM tumor growth using *in vivo* models. Because of the highly heterogeneous nature of MMs, we could not identify a common kinase or phosphatase pathway for CREB activation (Figure 5) in the different MM cell lines. As a solution to this problem, we generated shCREB in two MM cell lines (HMESO and H2373) to generate CREB1-silenced MM cell lines and then used these lines to study the effect of CREB on MM tumorigenesis.

In vitro characterization of stable lines showed a significant effect of CREB inhibition on the proliferation, drug resistance, and migration of human MM cells. Many significant gene changes observed by microarray analysis (Table 1) performed on these MM cell lines may be correlated with observed *in vitro* anti-tumorigenic effects. For example, decreases in the expression of *PAK7*, a proliferation and survival signal–related gene; *c-MET*, a receptor for hepatocyte growth factor that stimulates chemotaxis and growth of MM cells[30]; and oncogenic *v-kit,* a proliferation-related gene often mutated in gastrointestinal stromal tumors,[31] by CREB inhibition may in part be involved in the decreased proliferation and transformation observed in shCREB MM cell lines. In addition, significant attenuation in migration and motility related genes such as *MMP2* and *CD24*[32] by CREB inhibition may play a role in reduced migration in shCREB MM cells.

The injection of stable MM cells inhibited for CREB into SCID mice significantly attenuated human MM tumor growth in two *in vivo* (i.p. and s.c.) xenograft models (Figure 2). The use of two different *in vivo* models strengthens our findings. Reduced tumor growth in CREB-inhibited lines could be attributed in part to increased cell death in shCREB s.c. tumors, as shown by TUNEL staining (Figure 2). Inhibition of proliferation- and growth-related genes as a consequence of CREB silencing, as mentioned above, may also be involved in reduced tumor growth in both models.

The assessment of inflammation in the PLF of i.p. tumor-bearing mice revealed a significant effect of CREB inhibition on total and differential cell counts as well as on pro-inflammatory human cytokines, chemokines, and growth factors (Figure 3). The decreased infiltration of

Table 1 Effect of CREB Inhibition (shCREB) on Tumorigenesis-Related Genes in HMESO Cells Compared with Controls (shCon) in Microarrays

Gene name/symbol	Fold decrease	Function	Validation by quantitative real-time PCR
p21(CDKN1A)-activated kinase 7/*PAK7*	23.16	Proliferation/cell survival	Validated
Tyrosine kinase with immunoglobulin-like and EGF-like domains, Tie-2/*TEK*	11.92	Angiogenesis	
Melanoma cell adhesion molecule, CD146, MUC18/*MCAM*	9.65	Cell adhesion/angiogenesis	
CD24 molecule/*CD24*	6.49	Cell adhesion/cell motility	
RANTES/*CCL5*	3.2	Inflammation, angiogenesis	
Met proto-oncogene, cMET, HGFR/*MET*	3.02	Proliferation/growth	
Polo-like kinase 2/*PLK1*	2.81	Oncogenesis	
v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene, c-Kit/*KIT*	2.75	Oncogenesis	
Matrix metalloprotease 2/*MMP2*	2.65	Migration/invasion	Validated
Interleukin-6/*IL6*	2.42	Inflammation	Validated

CDKN1A, cyclin-dependent kinase inhibitor 1A; EGF, endothelial growth factor; HGFR, hepatocyte growth factor receptor; RANTES, regulated on activation normal T cell expressed and secreted.

macrophages, neutrophils, and eosinophils in the PLF of shCREB tumor-bearing mice could be due to the reduced levels of monocyte chemotactic protein-1 and macrophage inflammatory protein 1β observed in the same group.

Another chemokine, regulated on activation normal T cell expressed and secreted/*CCL5*, attracts eosinophils and is known to be secreted by many cell types, including mesothelial cells,[33] and promotes angiogenesis. The pro-angiogenic cytokines CCL5, chemokine (C-X-C motif) ligand 8 (IL-8), and vascular endothelial growth factor, which play important roles in MM tumor growth,[34,35] were significantly attenuated by CREB silencing. In support of our findings, the regulation of chemokine (C-X-C motif) ligand 8 by CREB has been reported previously[36] in non−small cell lung cancer. Vascular endothelial growth factor regulation by CREB either directly or indirectly has also been documented in cancer and noncancer cells.[36,37] Moreover, CREB has previously been shown to regulate inflammatory responses either by regulating direct gene transcription of pro-inflammatory genes (*IL2, IL6, IL10*, and TNF-α) or by inhibiting NF-κB activation by blocking the binding of CREB-binding protein to the NF-κB complexes.[38] Another pro-inflammatory cytokine, IL-6, which is regulated by CREB[38] and is heavily involved in the carcinogenesis process of many cancers including MM,[39] was significantly down-regulated in the PLF of shCREB mice. Our findings here demonstrate that CREB-induced inflammation may play a significant role in MM tumor growth, and CREB inhibition may be the crucial first step in MM therapy.

Dox was the first successful chemotherapeutic drug tested in MM and is currently administered in combination with other strategies.[4,5] We selected Dox for our studies to use in combination with CREB inhibition. Here, the observed findings of Dox-induced increases in levels of various cytokines and chemokines could be due to the fact that Dox can activate CREB[16] as well extracellular signal−regulated kinases[34,36] in MM cells. In addition to Dox, another chemotherapeutic drug, tamoxifen, has also been reported to activate CREB.[40] Our data here suggest that chemotherapeutic drugs may work better in combination with CREB inhibitor than alone. Our *in vitro* (Figure 1) as well as *in vivo* (Figure 2) data also show that CREB inhibition can sensitize MM cells and tumors to Dox-induced cell death (or tumor reduction). In support of our findings, recent studies have reported that increased CREB expression and/or phosphorylation can result in anthracyclin/Dox drug resistance in hepatocellular carcinoma and myeloid leukemia,[41−43] again emphasizing that CREB inhibitor in combination with chemotherapeutic drugs may work better than drugs alone.

To validate the role of CREB in asbestos-induced inflammation, we performed *in vitro* studies using human mesothelial cells (origin cell for MM). Our work has previously demonstrated that asbestos can prime and activate NLRP3 inflammasome in mesothelial cells involving an autocrine loop.[21] Inflammasome activation is one of the several ways that asbestos can activate the process of inflammation in mesothelial cells.

To understand the role of CREB in asbestos-induced inflammasome priming and activation, we used PKA inhibitor (H89), a kinase that has been shown to phosphorylate CREB in mesothelial cells in response to asbestos exposure.[16] Our results demonstrate that asbestos-induced NLRP3 priming and activation in part are regulated by CREB (Figure 4A).

Along with the pro-inflammatory cytokines IL-1β and IL-18, a pro-inflammatory marker, HMGB1, was released as a result of NLRP3 activation by asbestos and was also inhibited by CREB attenuation. HMGB1, depending on its location, can perform double functions. It can act as an oncogenic molecule or as a tumor suppressor.[44] In support of our findings, the involvement of CREB-binding protein in lipopolysaccharide (LPS)-induced relocation and release of HMGB1 in murine macrophages has recently been reported.[45] CREB inhibition significantly attenuated HMGB1 release but had no effect on intracellular levels of HMGB1 (data not shown).

The role of CREB in IL-1β-induced C-X-C chemokine gene overexpression in non−small cell lung cancer and in IL-18 transcriptional regulation in the development of T helper type 1 has been previously documented.[36,46] As

previously reported,[16] human MM cells show constitutive activation of CREB, and in the present study, inhibition of CREB with shRNA caused significant inhibition in steady-state mRNA levels of IL-1β. Our data substantiates the role of CREB in IL-1β–induced C-X-C chemokine gene overexpression in non–small cell lung cancer, as reported earlier.[38] Low levels of secreted IL-1β were detected in medium of shCon cells, and shCREB had no further effect on IL-1β levels (data not shown). Similarly, in the PLF from *in vivo* study, IL-1β levels were very low, and no significant differences across different groups were observed (data not shown). This finding is supported by our published and unpublished studies in which HMESO cells had low NLRP3 steady-state mRNA levels (required to process IL-1β) (unpublished data) and in which HMESO tumors secreted very low levels of IL-1β in a xenograft model.[24] The *in vitro* studies support the role of CREB in asbestos-induced inflammation by inflammasome activation in mesothelial cells and a possible role in the initiation of MM. In addition, significantly reduced IL-1β steady-state mRNA levels by CREB silencing in HMESO MM cells may in part be involved in the observed reduced inflammation and tumor growth. The *in vitro* pro-inflammatory effect of CREB is also evident from reduced asbestos- or chemotherapeutic drug–induced HMGB1 levels in CREB-inhibited cells.

Conclusion

Our findings suggest that CREB regulates MM tumor growth by multiple-gene regulation. More importantly, CREB-regulated inflammation may play a determining role in the process of MM development and progression. Our study also suggests that chemotherapeutic drugs alone can enhance the process of drug resistance or inflammation due to CREB activation. Based on these findings, it may be advisable to attenuate CREB function along with chemotherapeutic drugs for MM therapy. As a future direction of our research, we plan to conduct preclinical studies using CREB-interaction inhibitor (KG-501) in combination with cisplatin or pemetrexed to slow MM tumor growth.

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