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Research Article

Differential effects of v-Jun and c-Jun proteins on v-*myb*-transformed monoblasts

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Abstract. The v-*myb* oncogene of avian myeloblastosis virus transforms myelomonocytic cells in vitro. The line of v-*myb*-transformed chicken monoblasts BM2 can be induced to terminal differentiation using phorbol esters. The fact that Jun proteins are up-regulated in the phorbol ester-treated BM2 cells prompted us to investigate the role of the Jun proteins in regulation of myeloid differentiation. We ectopically expressed v-*jun* and c-*jun* in BM2 cells and evaluated their effects on differentiation and proliferation. c-Jun up-regulated the transactivation activity of v-Myb and induced a proliferation block and differentiation of BM2 cells. In contrast, v-Jun down-regulated v-Myb transactivation causing no dramatic effects on BM2 cells. This confirms that there is no strong correlation between transcriptional activation and strength of oncogenic transformation by v-Myb. Both c-Jun and v-Jun proteins affected sensitivity of BM2 cells to retinoic acid and phorbol ester. Sensitivity of BM2 cells to retinoic acid was enhanced by both Jun proteins, while sensitivity to phorbol 12-myristate 13-acetate was reduced by v-Jun. These data suggest thate Jun plays a major role in macrophage differentiation.

Key words. v-Myb; Jun; differentiation; proliferation; retinoic acid; phorbol ester.

Introduction

A variety of proto-oncogenes such as c-*jun*, c-*fos*, and c-*myb*, c-*myc* function to stimulate cell growth, and in mutated or deregulated form contribute to cellular transformation [1–3]. The Jun and Fos proteins are components of the transcription factor AP-1 [4]. AP-1 regulates transcription of several genes through its ability to bind specifically to the DNA sequence TGAG(C)TCA also known as the (TPA) response element (TRE). Thus, Jun and Fos proteins seem to mediate biological effects of TPA [5–7]. In fact, TPA and a variety of growth factors stimulate AP-1 activity in multiple cell systems [8–13]. Up-regulation of c-*jun* expression has been reported in multiple leukemic cell lines such as HL-60, THP-1, and U-937 during TPA-induced monocytic differentiation [14, 15]. Dexamethasone, an agent that blocks TPA-induced monocytic differentiation of U-937 cells, was shown to inhibit the increase in c-*jun* expression [16]. Similarly, blockage of c-Jun function by a dominant negative mutant interferes with cytosine arabinosite (Ara-C)-mediated differentiation in U-937 cells [17], implying that c-Jun is required for monocytic differentiation. Indeed, induction of c-*jun* gene expression during myeloid

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cell differentiation has been reported [15, 18–20]. Furthermore, ectopic c-*jun* expression in human U-937 and murine WEHI-3B D^+ myelomonocytic leukemia cells resulted in partial macrophage differentiation [21, 22]. On the other hand, c-Jun can act as an oncoprotein when expressed in a deregulated way [4]. For example, over-expression of c-Jun induces transformation of chicken embryo fibroblasts [23]. In addition to regulation of cellular proliferation and differentiation, c-Jun was also shown to be involved in control of apoptosis [24–26]. These data suggest that c-Jun/AP-1 is an important factor in determining the fate of the cell.

Viral *jun* (v-*jun*) is the transforming gene of the avian retrovirus ASV-17 [27]. v-Jun differs from c-Jun by three mutations that enhance the oncogenic potential of the protein: a 27-amino-acid deletion of the delta region, and two amino acid substitutions affecting regulatory sites in the C-terminal region of the Jun protein [23, 28, 29]. Over-expression of v-Jun triggers transformation in both animal models and primary embryo fibroblasts of mammalian or avian origin [23, 30–32]. Transformation requires the presence of the carboxy-terminal bZip domain as well as the amino-terminal transcriptional regulatory domain(s) of Jun [33]. v-Jun was found to act antagonistically to c-Jun on TRE-controlled promoters causing suppression of transactivation by c-Jun [34], so c-Jun-controlled cellular processes can be significantly affected by v-Jun.

There are Myb proteins, similar to the Jun family of proteins, that act as sequence-specific transcription factors regulating differentiation, cell growth, and death [35]. One member of this family is the c-Myb protein that is essential for hematopoiesis [36]. c-Myb is expressed at high levels in immature hematopoietic cells of all lineages and its expression decreases as cells differentiate [37–39]. Constitutive c-Myb expression can block differentiation of various erythroid and myeloid cell lineages, suggesting that c-Myb may control hematopoietic differentiation [40–43]. Indeed, deregulated c-Myb expression was found in most leukemias or lymphomas with immature phenotype [38] and in many pancreatic carcinomas [44]. A central part of the c-*myb* proto-oncogene, v-*myb*, has been transduced by the avian myeloblastosis virus (AMV), causing monoblastic leukemia in chickens [45] and transformation of myelomonocytic cells *in vitro*. Cells of an established line of chicken monoblasts (BM2) transformed by the v-*myb* of AMV are committed to differentiate into macrophages [46]. Indeed, either phorbol esters or liganded retinoic acid receptors can promote their terminal differentiation [47–50].

To explore the role of Jun proteins in differentiation and proliferation of v-*myb*-transformed cells, we transfected c-*jun* and v-*jun* genes under the control of a human metallothionein IIA promoter into v-*myb*-transformed BM2 chicken monoblasts and isolated several clones expressing c-Jun and v-Jun. We compared the effects of c-Jun and v-Jun on the phenotype of BM2 cells and correlated them with the activity of endogenous v-Myb oncoprotein.

Materials and methods

Plasmid construction

v-*jun* cDNA was cut from the plasmid RCASVJ-1 [23] (a gift from T. Bos) using *Cla*I. Recessed termini were filled using the Klenow fragment of DNA polymerase I and ligated to the *Xba*I linkers. The *Xba*I-resistant fragment harboring v-*jun* cDNA was cloned into the *Xba*I site of the vector pMT-IRES-CD4 [51] forming the pMT-vjun-CD4 plasmid. c-*jun* cDNA was cut out of the plasmid RCASCJ-1 [23] (a gift from T. Bos) using *Cla*I; recessed termini were filled using the Klenow fragment of DNA polymerase I and cloned into the Klenow-filled *Xba*I site of the vector pMT-IRES-CD4 [51] forming the pMT-cjun-CD4 plasmid. Restriction enzymes, *Xba*I linkers, the Klenow fragment of DNA polymerase I, and T4 DNA ligase were purchased from New England Biolabs.

Cell transfection and cultivation

Conditions of cultivation of BM2 cells are described elsewhere [42]. For stable transfection, Fugene 6 reagent (Boehringer Mannheim/Roche Biochemicals) was used. Fugene 6 reagent (3 µl) was added to a sterile tube containing 97 µl of serum-free OPTIMEM I medium (GIBCO BRL/Life Technologies) and incubated for 5 min at room temperature.

Two micrograms of pMT-vjun-CD4 (or pMT-cjun-CD4) plasmid DNA mixed with 0.5 µg pSV2Neo were then added to the tube, dropwise, and incubated for 15 min at room temperature; this solution was used for transfection of 2×10^6 of exponentially growing BM2 cells. The next day, G418 (Sigma-Aldrich) was added together with 3 ml of regular medium containing chicken and fetal calf sera to obtain a final concentration of 500 µg/ml. Stable transfectants appeared within 2 weeks and underwent negative and positive selection with anti-CD4-coated paramagnetic beads (Dynal) as described previously [51]. Cloning was performed by limiting dilution.

Immunoblotting

Ten percent sodium dodecyl sulphate-polyacrylamide electrophoresis (SDS-PAGE) and immunoblotting were performed as described elsewhere [42]. The blots were probed using a polyclonal antiserum directed against the highly conserved DNA-binding domain of c-Jun and v-Jun (Santa Cruz, sc-44), 4E3 and 10A3 of the anti-Myb monoclonal antibody mixture [52] (a gift from J. Sleeman) or anti-Mim-1 polyclonal antibody [53] (a gift from S. Ness). Blots were developed with either goat anti-rabbit secondary antibody or goat anti-mouse sec-

ondary antibody, both conjugated to alkaline phosphatase as recommended by the manufacturer (Promega).

Matrix metalloproteinase assay

BM2, BM2vJUN and BM2cJUN cells (1×10^6) were seeded and treated with zinc chloride (1.5×10^{-4} M) for 24 h. The next day, the cells were transferred to zinc-containing serum-free medium at a concentration of 1×10^6 cells/ml and cultivated for further 24 h. The following day, cells were centrifuged, the supernatant divided into 200-µl aliquots and deep-frozen at –70 °C. An aliquot of the supernatant was diluted in sample buffer 3:1 (62.5 mM Tris-Cl pH 6.8, 25% glycerol, 4% SDS, 0.01% bromphenol blue). A non-reduction SDS-PAGE (7.5% acrylamide + 1% gelatine) was then performed at 4 °C. After electrophoresis, the gel was washed with 50 mM Tris-Cl (pH 7.4) containing 2% Triton X-100 three times for 10 min, then washed with 50 mM Tris-Cl (pH 7.4) three times for 5 min and finally incubated in washing solution (0.05 M Tris, 0.2 M NaCl, 5 mM $CaCl_2$, 1% Triton X-100, 0.02% NaN_3 pH 7.4) for 12 h at 37 °C. The gel was stained and fixed with 0.1% amidoblack in a mixture of acetic acid:methanol:H_2O (1:3:6) for 1 h at room temperature and destained in the same solution without amidoblack [54]. Density of individual lines in the gel was measured on a Scanning Densitometer GS 300 (Hoefer Scientific Instruments) and evaluated using GS 365w Densitometer software.

Proliferation, viability and cell cycle analyses

For growth curves, 1×10^6 of BM2, BM2vJUN, and BM2cJUN cells were treated with zinc chloride (1.5×10^{-4} M) for 5 days. Viable cells determined by eosin dye exclusion were counted daily using a hemocytometer. For cell cycle analyses, the cells were cultivated in the presence of inducers, then washed twice in 2 vol of phosphate-buffered saline (PBS), resuspended in 0.5 ml PBS and fixed in 4 ml of 70% ethanol and stored at 4 °C for 24 h. Fixed cells were then centrifuged, washed with PBS and stained in 0.5 ml of Vindelov solution [55] for 30 min at 37 °C. The cells were light-protected until the DNA content was measured by a FACS Calibur system (Becton Dickinson). At least 10,000 cells were analyzed and the percentage of cells in each phase of the cell cycle was determined using ModFit 2.0 software (Verity Software House). Cell debris was excluded by raising the forward scatter threshold appropriately. Single cells were identified and gated by pulse-code processing of the area and the width of the fluorescence signal.

The frequency of dead BM2, BM2vJUN, and BM2cJUN cells treated and untreated with zinc chloride for 1–6 days was determined by flow cytometric measurement of the frequency of propidium iodide-positive cells using the FACS system as described above.

Measurement of oxidative burst

The ability of cells to produce oxygen radicals during an opsonized zymosan-activated oxidative burst was measured by luminol-enhanced chemiluminiscence as described previously [56]. BM2, BM2vJUN, and BM2cJUN cells (1×10^6) treated and untreated with zinc chloride (1.5×10^{-4} M) for 5 days were pelleted by centrifugation, washed with PBS and resuspended in 1 ml of RPMI without phenol red. Chemiluminiscence of phagocytes was measured using an Immunotech LM-01T microplate luminometer (Immunotech). The reaction mixture consisted of 200 µl of cell suspension, 25 µl of luminol stock solution (final concentration of 1 mM), and 25 µl of zymosan particles opsonized in chicken serum (final concentration 2 g/l). The assays were run in duplicate. The chemiluminiscence emission expressed as relative light units was recorded continuously for 60 min at 37 °C. The integral value of the chemiluminiscence reaction, which represents the total reactive oxygen species production by cells, was evaluated.

Nonspecific esterase assay

BM2, BM2vJUN, and BM2cJUN cells (1×10^6) were cultivated in the presence or absence of zinc chloride (1.5×10^{-4} M) for 3 days. Cells of each sample (3×10^5) were washed twice with PBS and incubated with 0.5 µg/ml 5(6)-carboxyfluorescein diacetate (Fluka) in Hank's balanced salt solution (HBSS, without phenol red) (Sigma-Aldrich) at room temperature for 10 min [57]. The cells were then washed once with PBS, resuspended in HBSS without phenol red, kept on ice and immediately measured by FACS (ex. 488 nm, em. 530 nm). At least 15,000 cells were analyzed and the median wavelength of fluorescence was measured by CellQuest 3.1 software (Becton Dickinson). Cell debris was excluded by raising the forward scatter threshold appropriately. The viable-cell population gated using forward versus side scatter parameters was analyzed.

Cell adhesion

BM2, BM2vJUN, and BM2cJUN cells (1×10^6) were cultivated in the presence or absence of zinc chloride (1.5×10^{-4} M). At day 1 and 6 of cell cultivation, medium containing cells growing in suspension was carefully removed and placed in a tube. Adherent cells were washed twice with PBS to ensure that only strongly attached cells remained in the dish. Next, adherent cells were removed by 1 mM EDTA in PBS. Both adherent and nonadherent cells were counted in a hemocytometer.

Tests of sensitivity to TPA and retinoic acid

BM2, BM2vJUN, and BM2cJUN cells (1×10^6) were treated with TPA (Sigma-Aldrich; 250 ng/ml) for 2 days or with all-*trans* retinoic acid (RA; Sigma-Aldrich) (1×10^{-6} M) and zinc chloride (Sigma-Aldrich) (1.5×10^{-4} M) for 1–6

days. To make a 1 mg/ml stock solution, TPA was diluted in dimethyl sulfoxide (DMSO). RA was diluted in ethanol to provide an appropriate stock solution (1×10^{-3} M).

Phagocytosis assay

BM2, BM2vJUN, and BM2cJUN cells (1×10^6) were cultivated in the presence of zinc chloride (1.5×10^{-4} M) and RA for 5 days. Thirty microliters of suspension containing 3×10^7 Dynabeads M-270 Epoxy (Dynal) was then added to the medium for 18 h. Harvested cells were washed with 5 ml PBS, suspended in 1 ml of PBS and transferred to the tube containing 2 ml of Histopaque (Gibco BRL/Life Technologies). Following centrifugation (400 g/30 min), the upper layer was removed and a white opalescent layer of cells was transferred to a new tube containing 10 ml of PBS. Following the next centrifugation (350 g/5 min), the cell pellet was suspended in 5 µl of PBS. The cell suspension (3 µl) was dropped on pre-stained slides (Testsimplets; Roche). The number of cells that engulfed none, one, and two Dynabeads was counted by light microscopy.

Transcription activation assay

For transient transfection of BM2CD4, BM2vJUN, and BM2cJUN cells, 5 µg of the EW5luc plasmid containing multiple Myb-binding sites [58] was used, together with 5 µg of cmv-βgal plasmid. Both plasmids and 5×10^6 exponentially growing BM2, BM2vJUN, or BM2cJUN cells were transferred into 400 µl of regular medium containing sera and 1.25% DMSO in a sterile electroporation cuvette and electroporation was performed as described [48]. Electroporated cells were transferred into a 10-cm dish containing 10 ml of growth medium with 1.25% DMSO. The next day, the cells were washed with regular media to remove the DMSO, and zinc chloride (1.5×10^{-4} M) was added. Twenty-four hours later, the cells were washed with PBS, harvested and processed for luciferase- and β-galactosidase assays as described [48]. Relative light units were normalized for transfection efficiency using β-galactosidase activity as an internal control.

Results

Derivation of v-Myb-transformed BM2 monoblasts inducibly expressing c-*jun* and v-*jun*

BM2 cells were transfected with the chicken and viral *jun* genes under the control of a human metallothionein IIA promoter (pMT-cjun-CD4 and pMT-vjun-CD4, respectively) by lipofection. BM2 cells harboring stably integrated c-*jun* and v-*jun* were isolated by selection for G418 resistance. Pools of G418-resistant cells were then subjected to negative selection with anti-CD4-coated magnetic beads in order to eliminate cells which constitutively co-expressed the exogenous *jun* and CD4 genes [51]. Following the induction of the CD4-negative cells with zinc, inducible cells were then positively selected with anti-CD4 magnetic beads and cloned. A total of four G418-resistant BM2cJUN clones and six BM2vJUN clones were isolated. All of these CD4-positive clones inducibly expressed Jun proteins as shown by immunoblotting (fig. 1). BM2 cells transfected with the vector pMT-IRES-CD4, BM2CD4, were prepared previously [59]. Low levels of Jun protein were detectable in BM2vJUN and BM2cJUN clones cultivated in zinc-free medium because of the basal level of transcription from the metallothionein promoter. The amount of Jun proteins found in BM2vJUN and BM2cJUN cells was stable for at least 6 days of cultivation in medium containing zinc inducer (not shown).

Matrix metalloproteinases are up-regulated in BM2cJUN and BM2vJUN cells

Matrix metalloproteinases (MMPs) may potentially regulate many important cellular processes including, for example, cell migration, proliferation, and apoptosis [60]. c-Jun was shown to participate in regulation of MMP in immortalized cells [61]. To test the capability of Jun proteins to regulate MMPs in BM2cJUN and BM2vJUN cells, the activity of MMPs secreted from these cells was measured by zymography. We found that both v-Jun and c-Jun protein induced MMP activity and caused an approximately two fold increase in BM2vJUN and a seven fold increase in BM2cJUN cells (fig. 2). Since AP-1-binding sites are located in the promoter regions of multiple MMPs [62], this result proves that exogenous Jun proteins can participate in formation of functional AP-1 in BM2vJUN and BM2cJUN cells.

Morphology of BM2vJUN and BM2cJUN cells

To test the effects of over-expressed Jun proteins in BM2cJUN and BM2vJUN cells, we first examined the morphology of these cells by phase-contrast microscopy. We found that many zinc-treated BM2cJUN cells enlarged and became adherent within 24 h (fig. 3). These features were retained for at least 4 days. Following further treatment, the number of adherent BM2cJUN cells decreased. They either returned to their original blastic morphology or died. In contrast, the response of BM2vJUN cells to zinc treatment was weaker. The cells also developed adherence but at a lower frequency. In addition, adhesive cells were smaller in size and tended to return to the original morphology within 48 h of zinc treatment. Control BM2 and empty vector-transfected BM2CD4 cells did not respond to zinc treatment at all. No clone-specific variations in behavior of BM2vJUN, BM2cJUN, and BM2CD4 cells were observed. This suggests that it is the c-Jun protein that induces morphological changes in BM2 cells. v-Jun protein is capable of inducing similar changes is c-Jun but does so less efficiently.

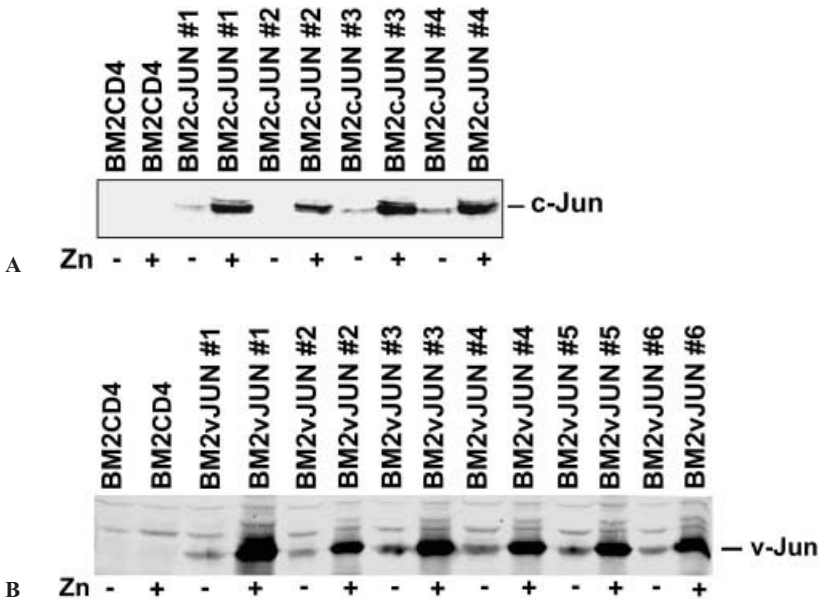

Figure 1. Expression of c-Jun (*A*) and v-Jun (*B*) in v-Myb-transformed monoblasts. A total of 2×10^6 empty vector-transfected BM2 cells (BM2CD4) and pMT-cjun-CD4- or pMT-vjun-CD4-transfected BM2 cells (independent clones of BM2cJUN, and BM2vJUN, respectively) were either treated with zinc chloride (+ Zn) or left untreated (–Zn) for 24 h. Proteins extracted from harvested cells were resolved in 10% SDS-PAGE and analyzed by immunoblotting with anti-Jun polyclonal antibody.

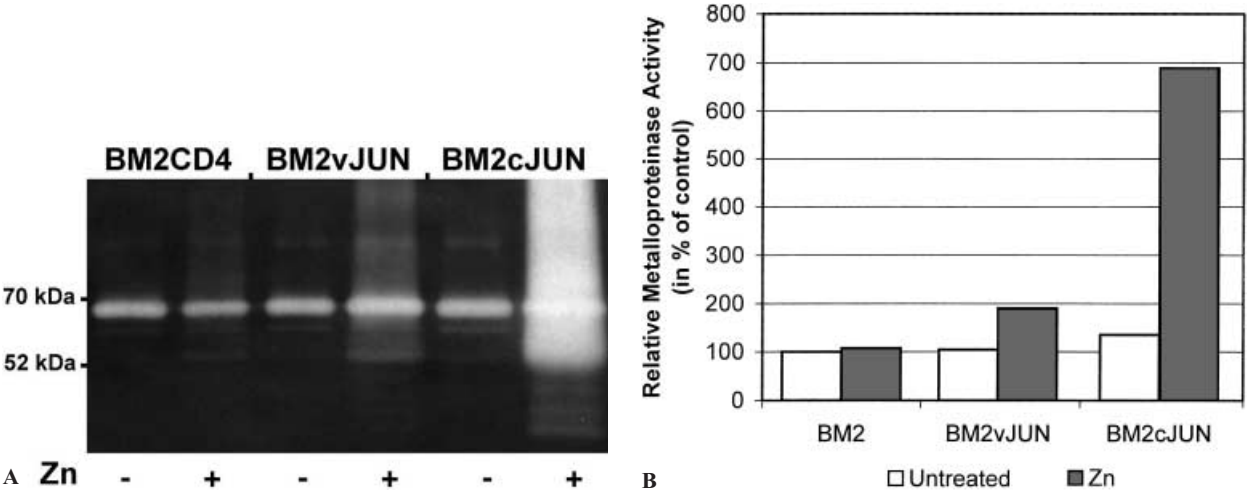

Figure 2. MMP activity is up-regulated in BM2vJUN and BM2cJUN cells. A total of 1×10^6 cells were cultivated in the presence or absence of zinc chloride for 24 h. Cell extracts were resolved by SDS-PAGE under non-reducing conditions and stained with amidoblack as described in Materials and methods. The bands representing matrix metalloproteinases in the gel (*A*) were analyzed by densitometry (*B*).

Physiology of BM2cJUN and BM2vJUN cells

To investigate the effects of Jun proteins on the physiology of BM2 cells, we performed several tests targeting mainly their proliferative capability and ability to differentiate. First, the same number of BM2, BM2cJUN, and BM2vJUN cells were seeded and cultivated for 5 days in the presence or absence of zinc. The number of viable cells was counted daily. We found that even the basal uninduced level of c-*jun* expression prevents an increase in the number of living BM2cJUN cells observed in controls (fig. 4). Slow growth was a common feature of all independent BM2cJUN clones. In contrast, uninduced BM2vJUN cells expressing low levels of v-Jun protein grew more rapidly than control BM2 or BM2CD4 cells. Zinc-induced c-*jun* expression further enforced growth suppression observed in uninduced BM2cJUN cells (fig. 4). v-*jun*-expressing BM2vJUN cells did not grow during the first 48 h of zinc treatment but later resumed proliferation. Initiation of BM2vJUN proliferation coincided with re-formation of spherical blastic cells (fig. 3). Cell

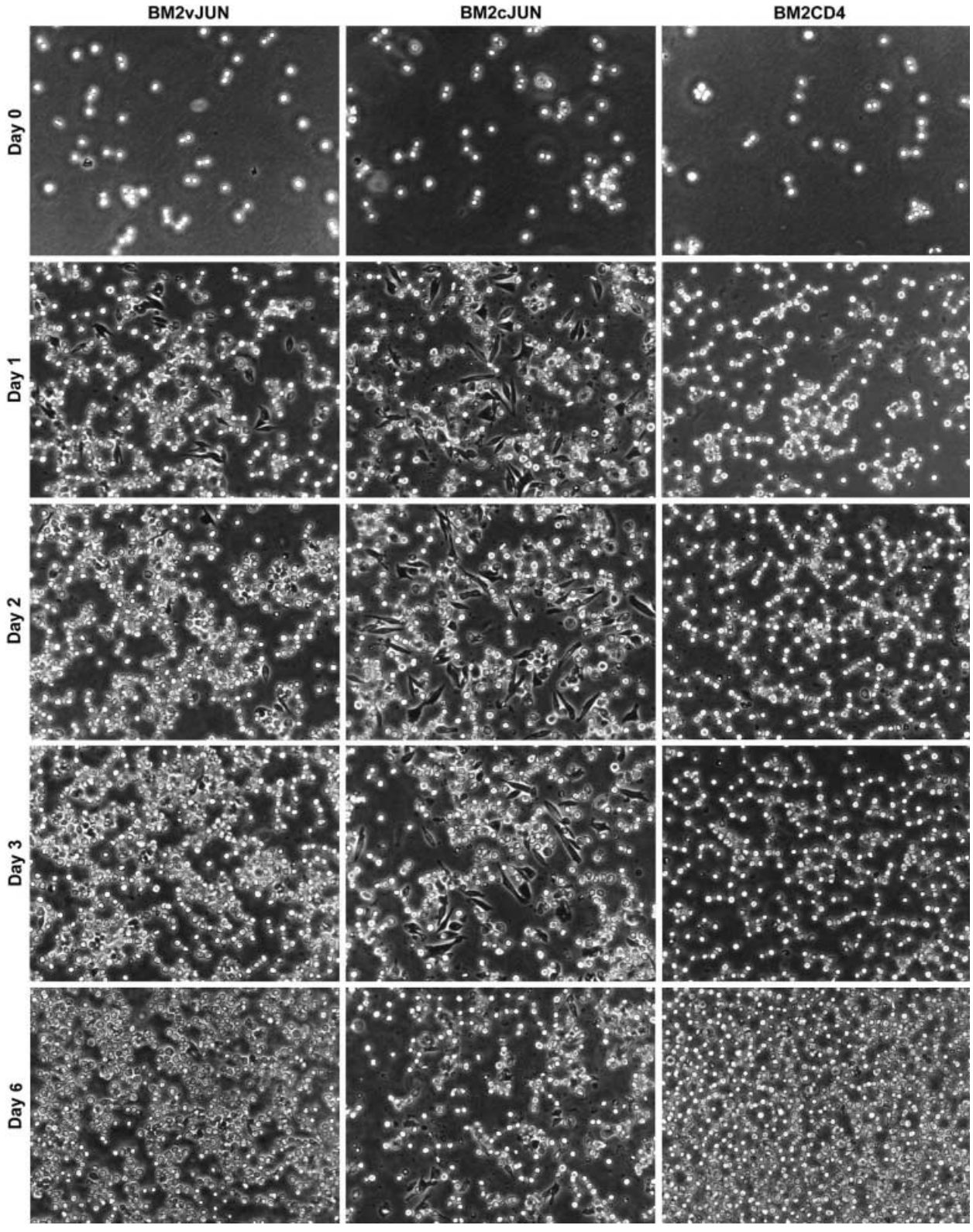

Figure 3. v-Jun and c-Jun induce changes in morphology of BM2 cells. A total of 1×10^6 BM2vJUN, BM2cJUN, and BM2CD4 cells were cultivated in the presence of zinc chloride (1.5×10^{-4} M) for 6 days. Cell morphology in day 0, 1, 2, 3, and 6 as representative phase-contrast photomicrographs is shown. The same magnification was used for each sample.

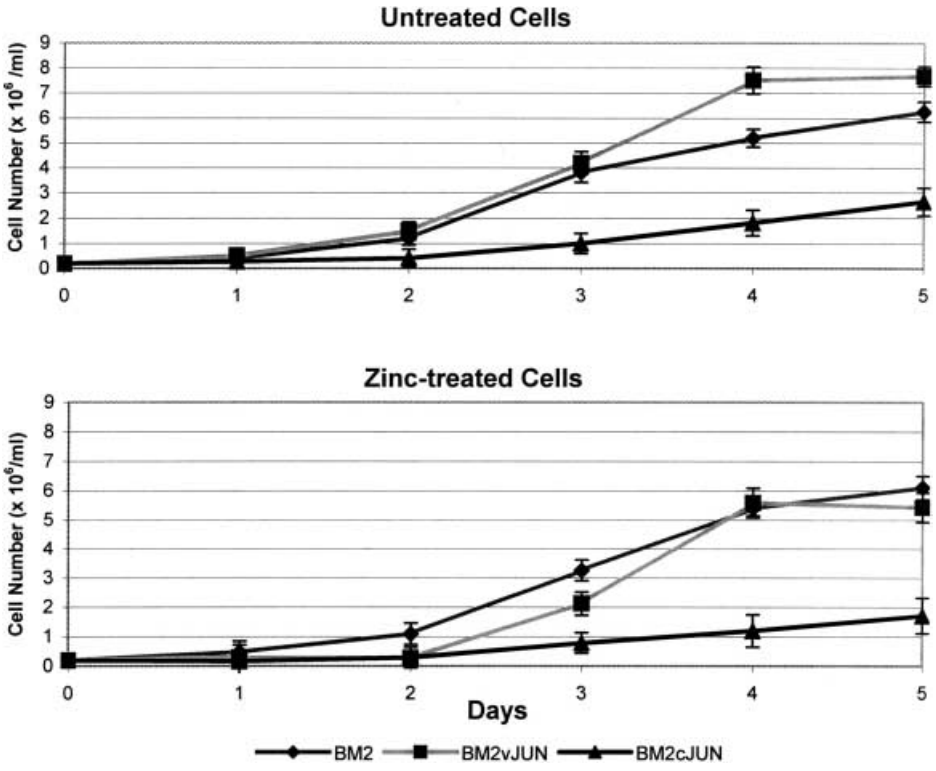

Figure 4. c-Jun but not v-Jun blocks proliferation of BM2 cells. A total of 1×10^6 BM2vJUN, BM2cJUN, and BM2CD4 cells were cultivated in the presence or absence of zinc chloride (1.5×10^{-4} M) for 5 days. The number of viable cells determined by eosin dye exclusion was counted daily using a hemocytometer. The data represent mean values from four independent experiments. Error bars indicate standard deviations.

cycle analysis of BM2vJUN, BM2cJUN, and control BM2 cells treated and not treated with zinc for 24 h revealed G0G1 phase arrest in BM2cJUN cells (fig. 5). This effect was independent of zinc induction, suggesting that low uninduced levels of c-Jun are sufficient to cause the cell cycle block. An increase in the frequency of BM2cJUN cells in the G0G1 phase was compensated by an adequate decrease in frequency of S phase and G2/M phase cells. Zinc-treated BM2vJUN cells also increased their fraction of G0G1 phase cells but much less efficiently than BM2cJUN cells. No apoptotic cells with a sub-diploid DNA content were observed. The shoulder shown in BM2cJUN histograms (fig. 5) corresponds to cellular debris. No significant clone-specific variations in growth of BM2vJUN and BM2cJUN cells were observed.

To determine the toxicity of over-expressed Jun proteins, we measured the viability of propidium iodide-stained BM2vJUN and BM2cJUN cells treated with zinc for 1, 2, 3, 4, 5 and 6 days by flow cytometry. We found that BM2cJUN cells were less viable than BM2vJUN and control BM2 cells cultivated both in the absence and presence of zinc (fig 6). The difference between the viability of BM2cJUN and BM2vJUN cells increased in samples cultivated for longer periods. These results imply that v-Jun and c-Jun proteins differentially regulate the viability of BM2 cells. In contrast to the v-Jun oncoprotein that does not significantly affect the viability of BM2 cells within 5 days of cultivation, c-Jun decreases it.

BM2 monoblasts can be induced to differentiate toward macrophages by treatment with certain differentiation inducers such as phorbol esters that at least partially target Jun proteins [47, 50]. Therefore, we wished to test whether Jun proteins can induce differentiation in BM2cJUN and BM2vJUN cells. First, we determined adherence of *jun*-expressing BM2 cells by counting adhesive and non-adhesive cells and found clear differences from control BM2 cells. While only about 1% of BM2 cells adhered to the surface of cultivation dishes in the presence or absence of zinc inducer, more than 76% of BM2vJUN and 74% of BM2cJUN cells treated with zinc for 24 h became adherent (fig. 7). We found that a significant fraction of BM2cJUN cells (54%) was adhesive even in the absence of zinc inducer in the medium, while only about 7% of BM2vJUN cells were found to be adhesive under these conditions. However, during extended cultivation, BM2cJUN and BM2vJUN cells loose adherence. The fraction of adherent BM2cJUN cells decreased to only 2% in untreated samples and to 20% in zinc-treated samples cultivated for 6 days (fig. 7). Simi-

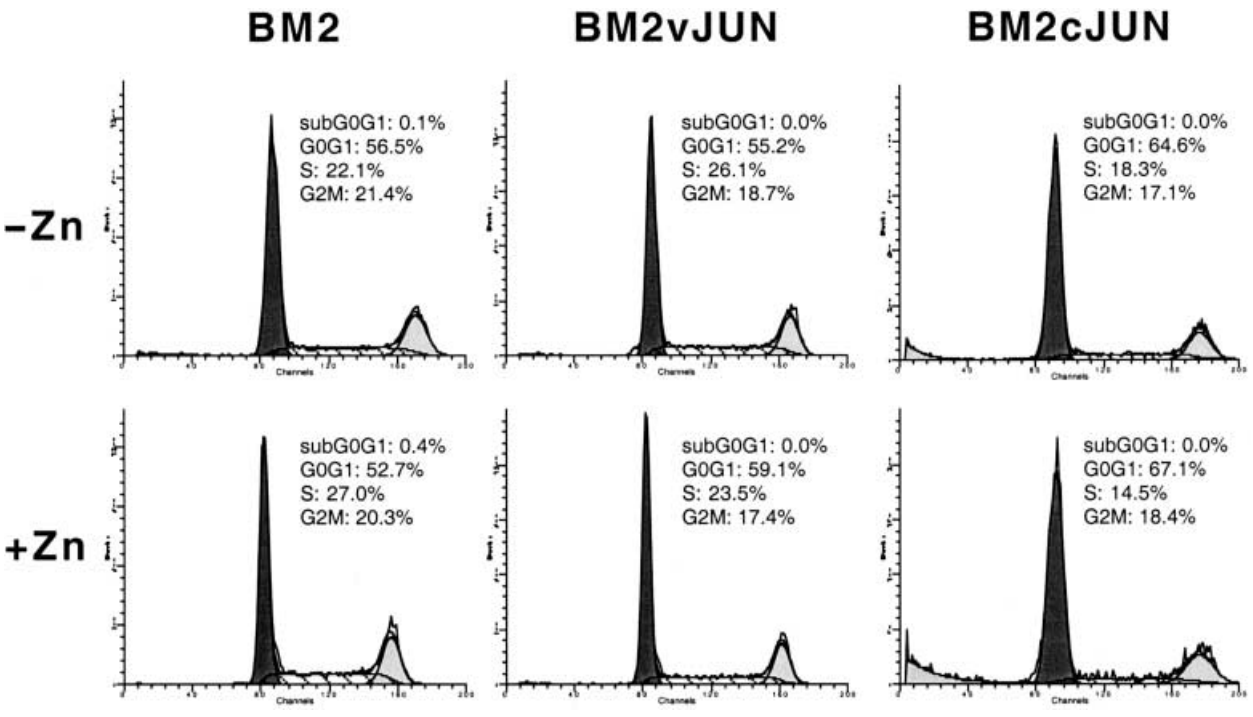

Figure 5. c-Jun but not v-Jun induces cell cycle arrest of BM2 cells. A total of 1×10^6 BM2vJUN, BM2cJUN, and BM2 cells were cultivated in the presence or absence of zinc chloride (1.5×10^{-4} M) for 1 day. The cells were fixed, stained with propidium iodide and the DNA content of at least 10,000 individual cells was analyzed by FACS.

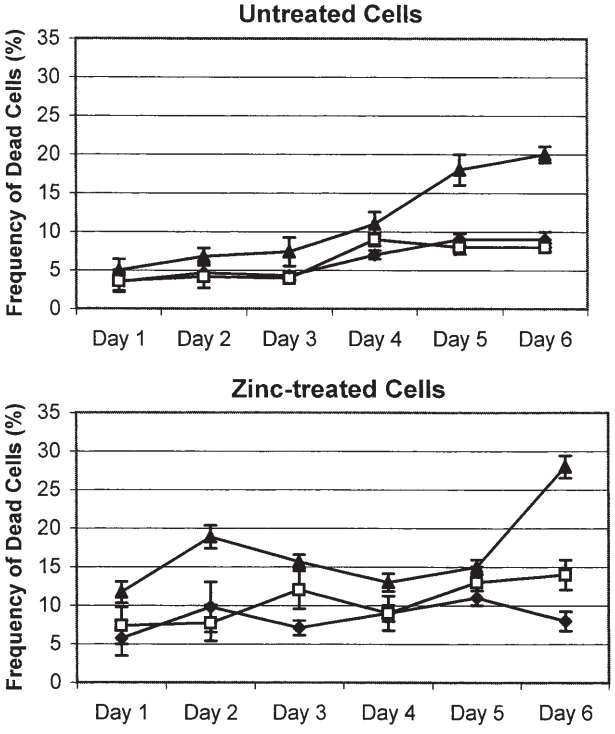

Figure 6. c-Jun decreases viability of BM2 cells. A total of 1×10^6 BM2vJUN, BM2cJUN, and BM2 cells were cultivated in the presence or absence of zinc chloride (1.5×10^{-4} M) for 6 days. The frequency of propidium iodide-positive (dead) cells within a population of at least 15,000 cells was determined daily by FACS. The lines represent mean values from four independent experiments. Error bars indicate standard deviations.

larly, adherence of BM2vJUN cells cultivated for 6 days in the absence or presence of zinc reached 2% and 56%, respectively. Expression of v-*jun* and c-*jun* was stable and did not change during the cultivation for 6 days (data not shown). This suggests that both v-Jun and c-Jun proteins efficiently promote fast but not permanent adherence of BM2 cells. While the adhesion-inducing capability of v-Jun is dependent on a 'high' concentration of v-Jun in BM2vJUN cells exposed to zinc inducer, a 'low' c-Jun concentration resulting from its leaking expression from the metallothionein promoter is sufficient to effectively induce adherence of BM2cJUN cells.

Maturing myeloid cells increase their phagocytic activity, their ability to produce oxygen radicals and the activity of their non-specific esterase. The ability of *jun*-expressing BM2 cells to produce reactive oxygen species (oxidative burst), which reflects the metabolic activity of phagocytes, was measured by chemiluminescence. We found that while v-*jun*-expressing BM2vJUN cells possess the same ability to produce oxygen radicals as control BM2 cells, BM2cJUN cells are clearly more active in oxygen radical production. After 5 days of cultivation, the oxidative burst of untreated BM2cJUN cells exceeded the BM2 control two fold and zinc treatment further increased this difference to more than three fold (fig. 8 A). These results indicate that c-Jun but not v-Jun protein can effectively induce oxygen radical production capabilities of BM2 cells. Since activation of cells by opsonized zymosan particles is dependent on binding of these particles on sur-

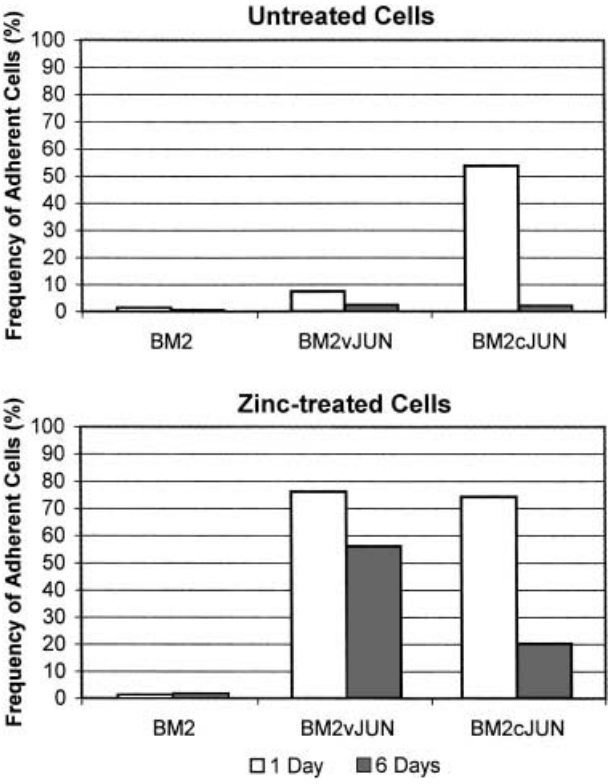

Figure 7. Jun proteins increase BM2 cell adhesion. A total of 1×10^6 BM2vJUN, BM2cJUN, and BM2 cells were cultivated in the presence or absence of zinc chloride (1.5×10^{-4} M) for 6 days. The number of adherent and non-adherent cells was counted on day 1 and 6 using a hemocytometer. The proportion (%) of adherent cells within the total cell population is shown in the graphs.

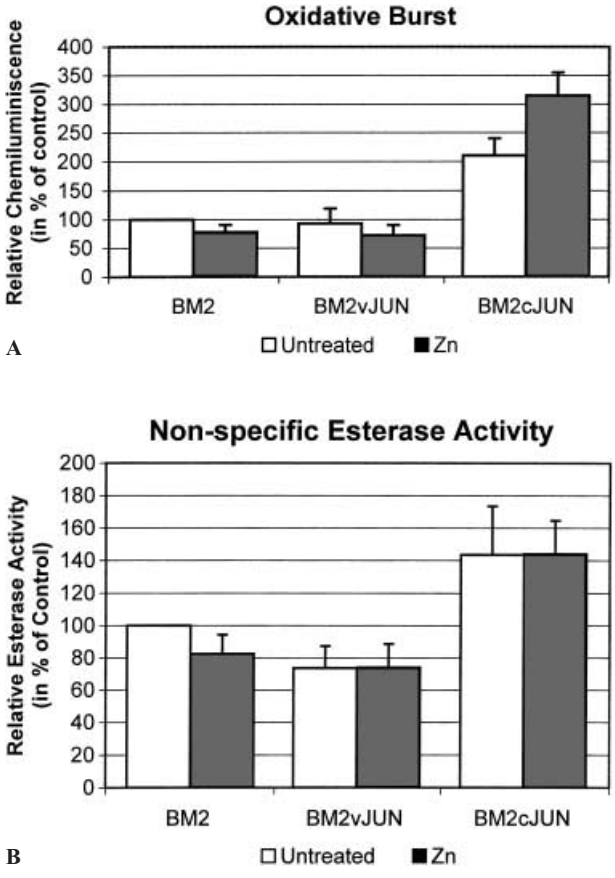

Figure 8. c-Jun but not v-Jun induces differentiation of BM2 cells. A total of 1×10^6 BM2vJUN, BM2cJUN, and BM2 cells were cultivated in the presence or absence of zinc chloride (1.5×10^{-4} M) for 5 days. (*A*) Luminol-dependent chemiluminiscence was measured in 10^6 cells in two parallels. Data represent the mean values of chemiluminiscence (as % of untreated control) from four independent experiments. Error bars represent standard deviations. (*B*) Non-specific esterase activity of at least 15,000 viable carboxyfluorescein diacetate-treated cells was measured by FACS. Data represent the mean values of non-specific esterase activity (as % of untreated cells) from four independent experiments. Error bars represent standard deviations.

face receptors of phagocytes with consequent phagocytosis [56], c-Jun may significantly potentiate the phagocytic activity of these cells.

Next, we wished to determine the effects of Jun proteins on the activity of the non-specific esterase of BM2 cells. The activity of this enzyme increases upon differentiation in monocytic cells [63]. Therefore, the same number of BM2, BM2vJUN, and BM2cJUN cells was cultivated in the presence or absence of zinc for 3 days and the activity of non-specific esterase in the cells was measured by flow cytometry. We found that v-Jun induced a slight decrease of non-specific esterase activity in BM2 cells, while c-Jun increased it by more than 40% (fig. 8B). These effects were not zinc dependent, suggesting that leaking *jun*-expression is sufficient to induce the same response as zinc-induced c-*jun* and v-*jun* expression in BM2cJUN and BM2vJUN cells. Similar results were obtained in all individual BM2vJUN and BM2cJUN clones that we tested. These results indicate that c-Jun but not v-Jun proteins can induce differentiation of BM2 cells.

Sensitivity of BM2vJUN and BM2cJUN cells to differentiation inducers

c-Jun protein is engaged in differentiation of various cell types induced by TPA and increases the sensitivity of WEHI-3B D^+ and U-937 cells to RA [21, 22]. To test whether over-expression of c-*jun* and v-*jun* affects the response of BM2 cells to these agents, we exposed v-*jun*- and c-*jun*-expressing BM2 cells to either TPA or RA. First, we found that the response of BM2cJUN cells to TPA was similar to the response of BM2 and BM2CD4 cells. No zinc was used to induce v-*jun* and c-*jun* expression in these experiments, because the metallothionein promoter is TPA inducible [42]. All of the BM2cJUN cells became enlarged and adhesive within 4 h of TPA treatment. They ceased proliferation and retained a macrophage morphology (fig. 9). BM2vJUN cells, how-

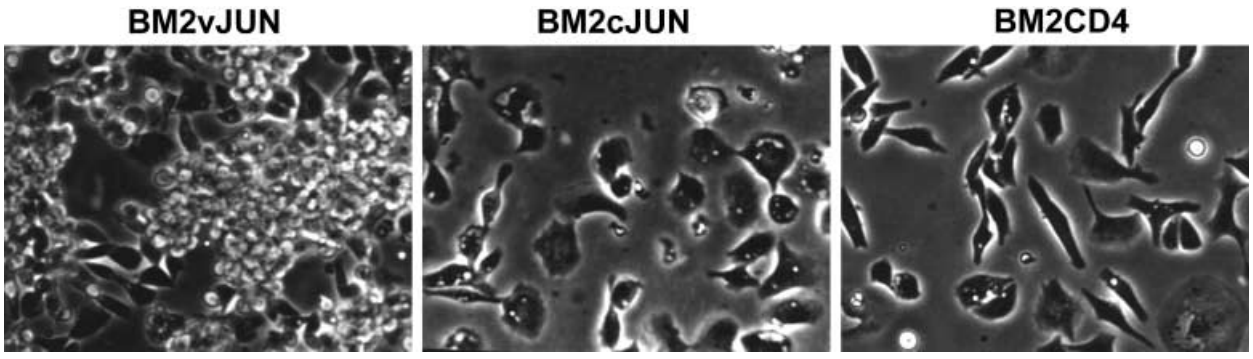

Figure 9. v-Jun blocks TPA-induced differentiation of BM2 cells. A total of 1×10^6 BM2vJUN, BM2cJUN, and BM2CD4 cells were cultivated in the presence of TPA (250 ng/ml) for 2 days. Cellular morphology was evaluated by phase-contrast microscopy. Representative fields taken under equal magnification are shown.

ever, were more resistant to TPA than were BM2/BM2CD4 and BM2cJUN cells. A significant fraction of TPA-treated BM2vJUN cells growing in suspension retained the morphology of blasts and never became adherent. These cells were not growth-arrested and thus the number of viable cells increased in comparison with similarly treated BM2, BM2CD4, and BM2cJUN cells (not shown). This demonstrates that v-Jun enforces the transformed character of BM2 cells by increasing their resistance to differentiation induced by TPA.

Next, we exposed c-*jun*- and v-*jun*-expressing BM2 cells to RA for 6 days and observed their morphology by phase-contrast microscopy. We found that both c-Jun and v-Jun proteins increased the sensitivity of BM2vJUN and BM2cJUN cells to RA. In contrast to control BM2/BM2CD4 cells, most RA-treated BM2cJUN and BM2vJUN cells acquired the morphology of adhesive macrophage-like cells (fig. 10). The kinetics of RA-induced differentiation differed in BM2vJUN and BM2cJUN cells. While most zinc- and RA-treated BM2cJUN cells acquired the phenotype of macrophage-like cells within 3 days, BM2vJUN cells exhibited similar morphological features within 4–5 days of treatment. Viability of BM2vJUN cells treated with zinc and RA for 5 days was about threefold higher than the viability of similarly treated BM2cJUN cells and almost twofold higher than the viability of BM2/BM2CD4 cells (data not shown). The lower viability of BM2cJUN cells decreased the number of differentiated cells upon RA treatment for 5 and 6 days in comparison with the more viable BM2vJUN cells (fig. 10). Control BM2/BM2CD4 cells were significantly more resistant to RA treatment than both variants of *jun*-expressing BM2 cells. In controls, we detected almost no adherent cells even upon long-term treatment with RA, although some of the cells growing in suspension were enlarged.

To compare the phagocytic activity of zinc/RA-treated BM2/BM2CD4, BM2vJUN, and BM2cJUN cells, we cultivated these cells for 5 days, mixed them with Dynabeads M-270 EPOXY and enumerated the number of cells containing none, one, and two engulfed beads by light microscopy. We found that almost all v-*jun*- and c-*jun*-expressing BM2 cells treated with RA contained either one or two engulfed particles, while 99% of control BM2/BM2CD4 cells contained no particles (fig. 11). Interestingly, zinc/RA-treated BM2cJUN cells were more effective in phagocytosis than similarly treated BM2v JUN cells; we observed twice as many BM2cJUN cells that contained two particles than double particle-containing BM2vJUN cells. These results suggest that both v-Jun and c-Jun proteins are engaged in regulating the response of BM2 cells to RA. Both proteins return the capacity of BM2 cells to differentiate to phagocytic macrophage-like cells upon treatment with RA.

Jun proteins affect activity of the v-Myb oncoprotein

The amount of v-Myb protein found in BM2vJUN and BM2cJUN cells is the same as in BM2/BM2CD4 cells, excluding the option of direct control of v-*myb* expression by Jun proteins (fig. 12). To correlate the changes of phenotype induced by Jun proteins in BM2 cells with activity of v-Myb oncoprotein, we analyzed its transcription activation function. First, we transfected BM2vJUN, BM2cJUN, as well as control BM2CD4 cells with the reporter plasmid containing multiple Myb-binding sites upstream of the TATA box and the *luc* gene and determined luciferase activity in transfected cells. Transactivation by v-Myb was decreased by more than 60% in zinc-treated BM2vJUN cells in comparison with zinc-treated BM2CD4 cells (fig. 13). In contrast, v-Myb activity in c-*jun*-expressing BM2cJUN cells was significantly higher than in BM2CD4 controls. This suggests that the transforming viral form of the Jun protein lost the capacity of the cellular precursor to up-regulate v-Myb function. The artificial reporter systems are not optimal for exact determination of the transcription activation function of transcription regulators because they lack the natural chromatin structure. Therefore, we wished to determine the

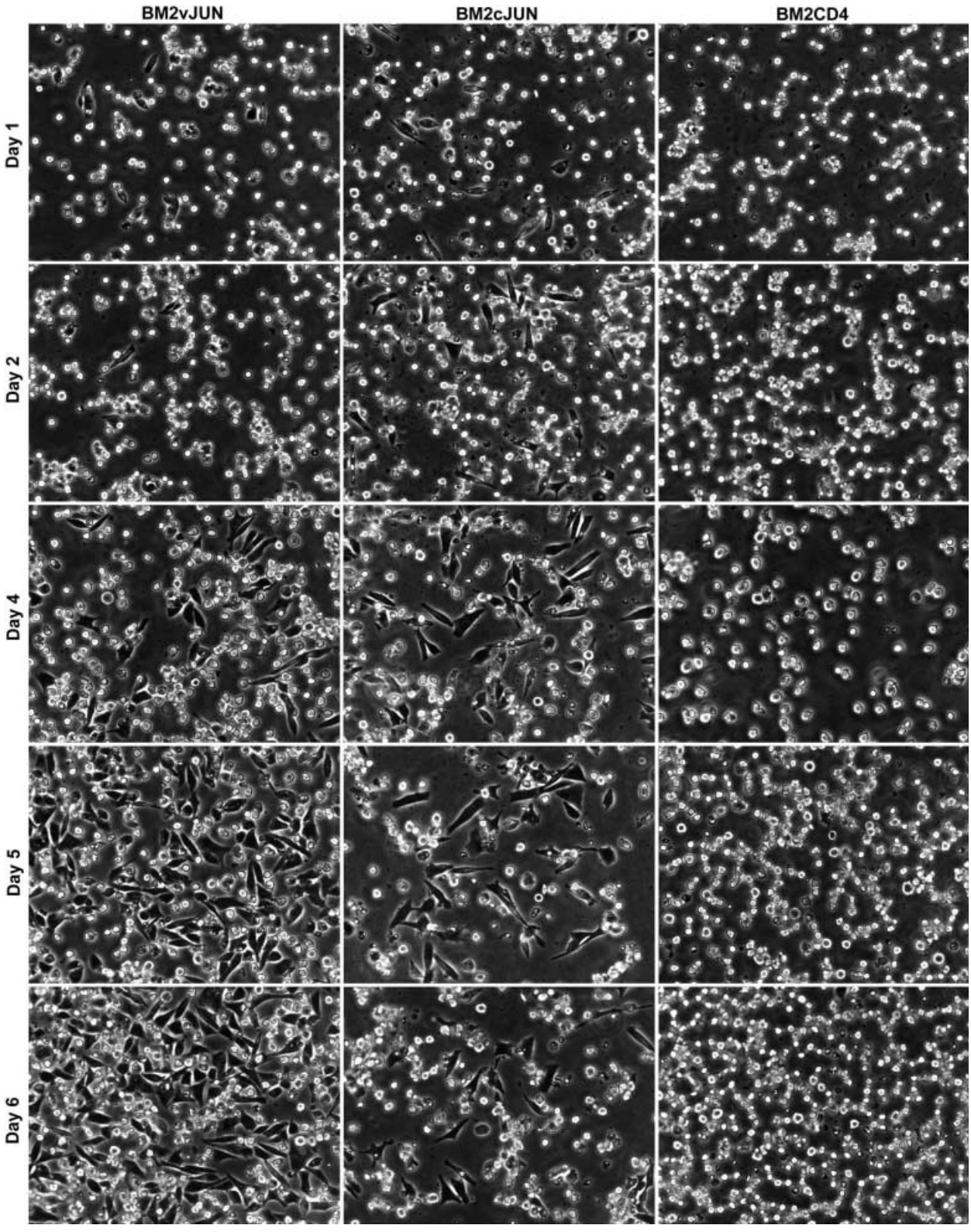

Figure 10. v-Jun and c-Jun increase the sensitivity of BM2 cells to RA. A total of 1×10^6 BM2vJUN, BM2cJUN, and BM2CD4 cells were cultivated in the presence of zinc chloride (1.5×10^{-4} M) and all-*trans* RA (1×10^{-6} M) for 6 days. Phase-contrast photomicrographs of representative fields were taken under equal magnification at the indicated times.

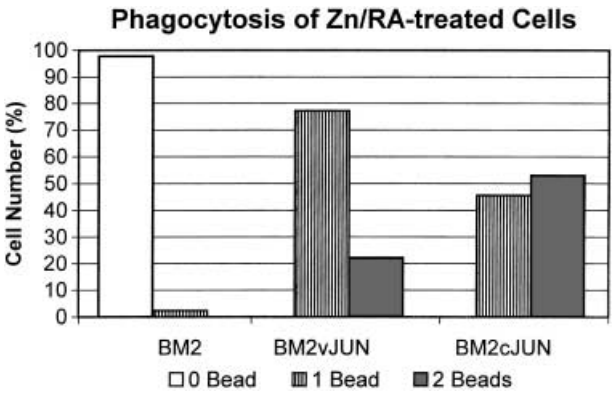

Figure 11. RA-treated BM2 cells expressing v-*jun* and c-*jun* are active in phagocytosis. A total of 1×10^6 BM2vJUN, BM2cJUN, and BM2 cells were cultivated in the presence of zinc chloride (1.5×10^{-4} M) and all-*trans* RA (1×10^{-6} M) for 5 days and incubated with Dynabeads M-270 Epoxy for 18 h. Number of cells that engulfed 0, 1, and 2 particles was determined on pre-stained slides by light microscopy. At least 300 cells were analyzed in each sample. Data represent the proportion (%) of cells containing 1 or 2 particles within the total cell population.

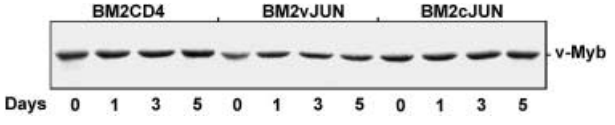

Figure 12. v-Myb expression is not affected by v-Jun and c-Jun proteins in BM2 cells. A total of 1×10^6 BM2vJUN, BM2cJUN, and BM2CD4 cells were cultivated in the presence or absence of zinc chloride (1.5×10^{-4} M) for 5 days. The level of v-Myb expression at indicated times was determined by SDS-PAGE and immunoblotting with the 4E3 and 10A3 (anti-Myb) monoclonal antibody mixture.

level of expression of the endogenous Myb-targeted gene *mim*-1 [53] in BM2CD4, BM2vJUN, and BM2cJUN cells. The cells were treated with zinc chloride for 24 h, and the amount of Mim-1 protein in cell extracts was determined by immunoblotting. We found that Mim-1 protein was completely absent from BM2vJUN cells, while it was present in BM2CD4 and BM2cJUN cells (fig. 14). However, Mim-1 was not detected even in BM2vJUN cells that were not treated with zinc, suggesting that low level of v-*jun* expression resulting from a leaky metallothionein promoter are sufficient to abrogate v-Myb transactivation function. This confirms our earlier observation that the transcription activation function of v-Myb is down-regulated by v-Jun but not by c-Jun (fig. 13).

Discussion

There is considerable evidence documenting the association of Jun proteins with the terminal differentiation program of monocyte/macrophage lineages: (i) Jun proteins are induced stably during normal macrophage differentiation [64], (ii) *jun* mRNAs are induced upon macrophage differentiation of various leukemia cell lines such as M1, U-937, HL-60, and THP-1 [15, 65, 66], (iii) ectopic c-*jun* expression induces partial macrophage differentiation in U-937 and WEHI-3B D^+ cells [21, 22], and (iv) suppression of c-*jun* by antisense oligonucleotides inhibits cell adhesion during phorbol ester-induced differentiation of U-937 cells [67]. In this work, we compared the effects of c-Jun and v-Jun proteins on the line of v-*myb*-transformed BM2 chicken monoblasts. We show that ectopic

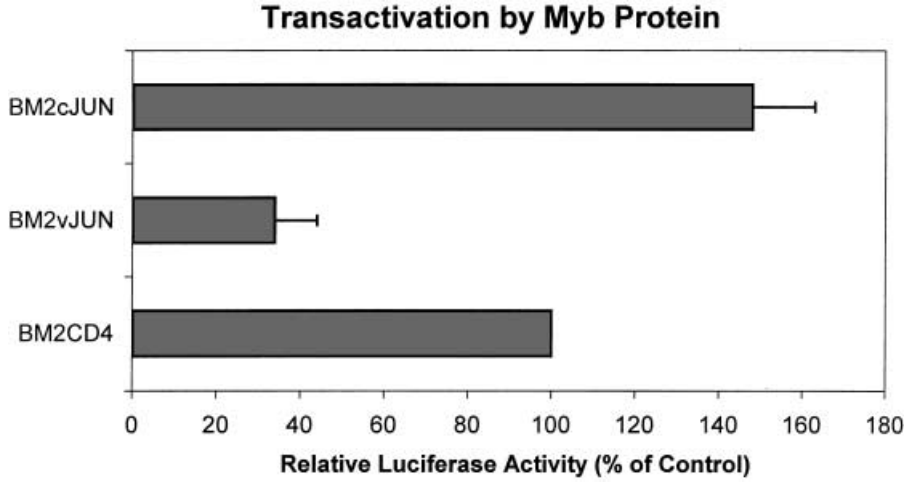

Figure 13. v-Jun and c-Jun proteins differentially regulate transcription activation by v-Myb protein in BM2vJUN and BM2cJUN cells. A total of 5×10^6 exponentially growing BM2vJUN, BM2cJUN, and BM2CD4 cells were transfected with 5 µg of EW5luc plasmid containing five copies of the Myb-binding site upstream of the TATA box and cDNA coding for luciferase [58] together with 5 µg of cmv-βgal internal control plasmid by electroporation. Electroporated cells were treated with zinc chloride (1.5×10^{-4} M) for 1 day. Luciferase and β-galactosidase activities in harvested cells were analyzed as described in Materials and methods. Data show the mean value of luciferase activity (as % of control BM2CD4 cells) from four independent experiments. Error bars indicate standard deviations.

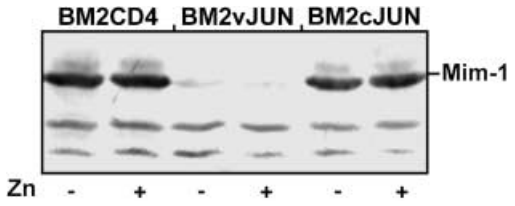

Figure 14. v-Jun suppresses expression of *mim*-1 in BM2 cells. A total of 1×10^6 BM2vJUN, BM2cJUN, and BM2CD4 cells were cultivated in the presence or absence of zinc chloride (1.5×10^{-4} M) for 1 day. The level of Mim-1 protein was determined by SDS-PAGE and immunoblotting with anti-Mim-1 polyclonal antibody.

c-*jun* but not v-*jun* expression can induce differentiation of these cells. Five main differences between the effects of c-Jun and v-Jun proteins on BM2 cells were found: (i) c-Jun but not v-Jun arrested the growth of BM2 cells, (ii) c-Jun induced differentiation of BM2 cells to macrophage-like cells more effectively than v-Jun, (iii) c-Jun was proficient in activating production of oxygen radicals and non-specific esterase activity in BM2 cells, while v-Jun lacked this ability, (iv) v-Jun but not c-Jun increased the resistance of BM2 cells to TPA-induced differentiation, (v) v-Jun down-regulated, while c-Jun up-regulated transcription activation by v-Myb. On the other hand, both v-Jun and c-Jun proteins shared the ability to induce BM2 cell adhesion and sensitivity to RA.

The human metallothionein IIA gene promoter was used to inducibly express v-*jun* and c-*jun* in BM2 cells. The basal level of c-*jun* expression was repeteadly detected in uninduced BM2cJUN cells. This presumably results from the fact that the human metallothionein promoter is leaky [21, 68]. In addition, this promoter contains AP-1 recognition sites [69], providing positive feedback for a further increase in leaky c-*jun* expression. In contrast, v-Jun would be expected to inhibit this promoter at the same sites via negative feedback. In our experiments, the uninduced level of v-*jun* expression was generally lower than c-*jun* but the basal amount of v-Jun protein in BM2vJUN cells was detectable. This suggests that the negative feedback was rather weak and could be efficiently overcome by metal ions in BM2vJUN cells.

c-Jun has been shown to decrease the growth and cloning efficiency of U-937 cells [22]. We showed that c-Jun also brings a growth disadvantage to BM2 cells by inducing an arrest in the G0G1 phase of the cell cycle and by accelerating the death rate. Dying BM2cJUN cells did not exhibit typical marks of apoptosis, such as DNA laddering and formation of apoptotic bodies (data not shown). This may result from the fact that over-expressed Myb protein directly transactivates anti-apoptotic *bcl*-2 gene in BM2cJUN cells [70]. In contrast, v-*jun* expression did not induce G0G1 phase cell cycle arrest in BM2vJUN cells, allowing their continuous proliferation. In contrast, a tendency to increase the proportion of S-phase BM2vJUN cells during extended cultivation was noted (data not shown). This phenomenon results from the reduced duration of the G1 phase and accelerated onset of the S phase and has been described for v-Jun-transformed fibroblasts [71]. In addition to growth arrest, c-Jun protein decreased the viability of BM2cJUN cells even in a 'low' uninduced, intracellular concentration by accelerating the death rate. In contrast, a 'high' level of the v-Jun protein obtained by zinc-induced v-*jun* expression from the metallothionein promoter was tolerated by all the BM2vJUN clones we tested. The observation that the viral form of the Jun protein is less cytostatic and cytotoxic than its cellular counterpart is compatible with the known transforming capabilities of this oncoprotein.

Previous studies have shown that c-Jun is capable of initiating differentiation of certain myelomonocytic leukemia cell lines [21, 22]. Several lines of evidence document that c-Jun protein can also induce the differentiation of v-*myb*-transformed BM2 monoblasts. First, c-*jun*-expressing BM2cJUN cells acquired the morphology of adherent macrophage-like cells, although during long-term cultivation the proportion of adherent cells decreased. Second, BM2cJUN cells were able to produce reactive oxygen species (oxidative burst) efficiently, as measured by luminol-dependent chemiluminiscence. This parameter reflects well the functional state of differentiation of myeloid cells and correlates with the widely used nitroblue tetrazolium reduction test [72]. Third, non-specific esterase activity which increases in differentiating monocytes [63] was up-regulated in BM2cJUN cells. Only cell adhesion was also induced in v-Jun transfectants, but neither oxidative burst nor non-specific esterase activity of BM2 cells was affected by v-Jun. This suggests that the v-*jun* oncogene has lost the capability of its cellular counterpart, c-Jun, to induce growth arrest and differentiation of monocytic cells. c-Jun promoted differentiation of BM2 cells despite the continued presence of the v-Myb oncoprotein, similarly to phorbol ester- or liganded RA receptor-induced differentiation of BM2 cells [50]. This suggests that the changes brought to transformed cells by AMV v-Myb are reversible by enhanced function of certain parts of cellular signaling networks, including AP-1.

The BM2 cell line is a suitable model for studies of v-Myb activity. It provides the correct cellular context for expression of high levels of Mim-1 despite the fact that AMV v-Myb does not usually activate the endogenous chromosomal *mim*-1 gene [73]. We found that expression of the endogenous *mim*-1 gene correlates well with expression of an exogenous luciferase reporter gene placed under the control of a promoter with multiple Myb-binding sites that was transferred into BM2 cells by transient transfection. Our data imply that c-Jun stimulates transactivation by v-Myb, while v-Jun represses it. These antagonistic effects of the cellular and viral forms of Jun

protein may partially result from repression of endogenous c-*jun* proto-oncogene expression by v-Jun [74]. Our results confirm that there is no strong correlation between transcriptional activation and strength of oncogenic transformation by v-Myb [75, 76]. We detected stronger transcriptional activity of v-Myb in differentiated macrophage-like cells derived from BM2 cells by c-*jun* expression than in original BM2 monoblasts, providing further evidence for the concept that repression rather than activation of v-Myb can correlate with transformation [35].

As observed in WEHI-3B D^+ cells [21], ectopic expression of c-*jun* promoted the capacity of RA to induce differentiation of BM2 cells. The differentiation-promoting effect of c-Jun on BM2 cells was further enhanced by RA that allowed terminal differentiation to stable, mature cells. Although v-Jun failed to induce differentiation of BM2 cells, it retained the same capacity possessed by c-Jun to strengthen the sensitivity of BM2 cells to RA. Since v-Jun increases the viability of BM2 cells, BM2vJUN cells were viable even after prolonged treatment with RA, causing the formation of a much higher number of mature cells than is found in BM2cJUN samples. Surprisingly, RA-induced differentiation of BM2cJUN and BM2vJUN cells was not dependent on the activity of the endogenous v-Myb. In contrast to BM2cJUN, BM2vJUN cells lack a functional v-Myb as documented by the absence of the endogenous Myb-targeted gene *mim*-1 product and by decreased transactivation of Myb-specific luciferase reporter. Nevertheless, both BM2cJUN and BM2vJUN cells can be induced to differentiate to macrophages by RA. This suggests that Jun proteins may regulate their intracellular targets that predispose BM2 cells to RA-induced differentiation independently of v-Myb activity.

Tumor promoter TPA induces the expression of c-*fos* and c-*jun* genes [8, 10] and thereby indirectly stimulates the expression of AP-1 target genes. Part of our study was to determine whether Jun can simulate the effects of TPA on BM2 cells. We found that over-expression of c-Jun induced similar effects in BM2 cells as TPA but with lower efficiency. This suggests that c-Jun may participate significantly in processes driven by TPA in BM2 cells but it requires the presence of additional factors to strengthen its effect. These may include partners for heterodimerization such as c-Fos, FosB, Fra1, and Fra2 or activating transcription factors such as ATF2, ATF3/LRF, and B-ATF [4, 77, 78]. v-Jun increased the resistance of BM2 cells to TPA suggesting that parts of the c-Jun protein that are different in the viral form are essential for TPA-mediated differentiation. This corresponds well with the observation that phorbol esters stimulate phosphorylation of c-Jun but not v-Jun [79]. We can hypothesize that this effect may result from dominant negative inhibition of endogenous c-Jun or its partner proteins by over-expressed v-Jun protein. Antagonistic effects of v-Jun and c-Jun proteins can cause suppression of c-Jun transactivation by v-Jun [34]. Alternatively, v-Jun and c-Jun are capable of mediating differentiation of BM2 cells by RA, suggesting that those parts of the Jun molecules that are conserved in v-Jun and c-Jun are required for RA-mediated differentiation. Positive and negative regulatory interactions between nuclear receptors and c-Jun/c-Fos have been reported [80]. In contrast to the interaction between AP-1 and other nuclear receptors, the interaction between AP-1 and the retinoid receptors is considered to be solely inhibitory [81]. c-Jun and c-Fos, either individually or together, have been shown to repress the transcriptional activity of RAR and/or RXR [82]. Conversely, both RAR/RXR heterodimers or homodimers can inhibit AP-1 transactivation of several AP-1-responsive promoters [83]. Jun synergizes, rather than antagonizes RAR in our system, allowing for RA-induced differentiation of BM2 cells. This suggests that Jun protein participates in the retinoid signal transduction pathway that controls maturation of myeloid cells. Since the Jun proteins did not affect the level of endogenous RAR and RXR genes (not shown), we hypothesize that Jun may up-regulate the activity of RAR and/or RXR proteins, presumably through interaction mediated by CBP/p300 co-activator. The results presented in this work also show that v-Jun and c-Jun proteins participate in deregulation of v-Myb function within the signaling network driven by RA, while only c-Jun but not v-Jun protein interferes with v-Myb transformation in BM2 cells on its own.

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