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CTCF Regulates Growth and Erythroid Differentiation of Human Myeloid Leukemia Cells*

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CTCF is a transcription factor and a candidate tumor suppressor that contains a DNA-binding domain composed of 11 zinc fingers. We reported previously that CTCF is differentially regulated during differentiation of human myeloid leukemia cells. In this study we aimed to investigate the role of CTCF in myeloid cell differentiation. A human cell line, K562, that can be chemically induced to differentiate into various hematopoietic lineages was chosen as a model system for this study. Several K562 cell lines with constitutive and conditional expression of CTCF have been generated. By using these model systems we demonstrated that: (i) ectopic expression of CTCF in K562 cells led to growth retardation and promotion of differentiation into the erythroid lineage; (ii) CTCF knock-down significantly inhibited differentiation of K562 cells into erythroid lineage; (iii) differentiation of K562 into the megakaryocytic lineage was not significantly affected; and (iv) down-regulation of MYC has been identified as one of the mechanisms by which CTCF promotes erythroid differentiation. Taken together our results demonstrate that CTCF is involved in the control of myeloid cell growth and differentiation.

CTCF is a transcription factor, which contains a DNA-binding domain composed of 11 zinc fingers (1, 2). CTCF is localized in the nucleus; it is ubiquitous and highly conserved (3). Genes regulated by CTCF include avian and mammalian *MYC* genes (3, 4), the chicken lysozyme gene (5), β-amyloid precursor protein (*APP*) gene (6), interleukin 1 receptor-associated kinase 2 gene (7), and others (1, 8). CTCF controls a number of insulators in vertebrate genomes (9), including the β-globin gene insulator (10), the *MYC*-N/TRE site, positioned 2 kb upstream of the human *MYC* gene (11), another MYC insulator element, MINE (12), and the F1/F2 silencer element in the chicken lysozyme gene (11). CTCF is also involved in the regulation of CTG/CAG repeats in the DM1 locus (13) and in the process of X-chromosome inactivation (14). The DNA target sequences recognized by CTCF are fairly long (~50 bp) and strikingly diverse. It is believed that to achieve this kind of multiple specificity CTCF employs different combinations of individual zinc fingers (1, 2). CTCF protein undergoes post-translational modifications. It can be phosphorylated by the protein kinase CK2 (formerly known as casein kinase II) (15, 16) as well as poly(ADP-ribosyl)ated, and this latter modification regulates its activity as a chromatin insulator (17, 18).

Our previous studies have revealed growth suppressive features of CTCF (19, 20). Furthermore, CTCF was found to be localized at human chromosome 16q22; this locus is associated with chromosomal abnormalities, loss of heterozygosity, or aberrant expression in different malignancies (21–23). A number of functionally significant, tumor-specific CTCF zinc finger mutations have also been identified in various cancers (24). Altogether, these findings suggest a role of CTCF as a tumor suppressor.

The importance of CTCF in the regulation of cell proliferation has been previously documented in several cellular systems (19, 20), however, much less is known about the possible role of CTCF in the regulation of cell differentiation. Human cell lines derived from myeloid leukemias have been widely used as models to study the molecular control of hematopoietic cell proliferation and differentiation. They can be induced into erythroid, megakaryocytic, monocytic-macrophagic, or granulocytic lineages in response to specific differentiation inducers (25). CTCF expression and post-translational modification of CTCF during differentiation of several human myeloid cells, such as K562, HL60, U937, and THP1 into different lineages have been investigated in our previous studies (26). We found that CTCF was differentially expressed and post-translationally modified depending on the particular differentiation pathway. These results indicated that CTCF may be important in the regulation of growth and differentiation in human myeloid cells. The aim of the present study was to gain further insight into CTCF function in myeloid cell differentiation. For this purpose, we developed cellular models with constitutive and conditional overexpression of CTCF as well as "knock-down" of CTCF in the multipotent cell line K562 derived from a chronic myeloid leukemia in blast crisis. K562 can be differentiated into the erythroid lineage with cytosine arabinoside (Ara-C)[1]

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[1] The abbreviations used are: Ara-C, 1-β-arabinofuranosylcytosine; 5-aza-dC, 5-aza-2′-deoxycytidine; ODN, oligodeoxyribonucleotides; tet, tetracycline; GFP, green fluorescent protein; RT, reverse transcriptase; TIG-1, tarazotene-induced gene-1.

(27) and into the megakaryocytic lineage with staurosporine (28). K562 cells therefore provide a useful model where the biological functions of CTCF can be studied during differentiation into two separate hematopoietic pathways. Here we report that CTCF overexpression in K562 cells leads to growth retardation and promotes induced differentiation into the erythroid lineage. CTCF down-regulation, on the other hand, significantly inhibits differentiation into the erythroid lineage. Possible molecular mechanisms involved in these processes will be discussed.

EXPERIMENTAL PROCEDURES

Antisense Oligodeoxynucleotides to CTCF mRNA and Expression Vectors—Phosphorothioate oligodeoxyribonucleotide (ODN) antisense targeting of different regions of CTCF mRNA were designed and analyzed by the S1 nuclease protection assay as described previously (29). Two of them (AS1 and AS6) were found to show the best ability to knock-down CTCF expression *in vivo*. For each antisense ODN, the complementary sense oligonucleotides (S1 and S6) were used as control. The sequences were as follows (5′-3′): AS1, TTCTCCGCGCCACACCCCCC; S1, GGGGGGTGTGGCGCCGAGAA; AS6, GTTGGGGGCATCTGTGGCAG; S6, CTGCCACAGATGCCCCCAAC.

To generate a vector for constitutive expression of CTCF, the full-length human CTCF cDNA was cloned into the pcDNA3 vector (Invitrogen) in the "sense" orientation. Constructs for inducible expression of CTCF were based on the tetracycline (tet)-inducible (pBIG2i, "tet-on") or tet-repressible (pBIG2r, "tet-off") vectors (30). Human CTCF cDNA was cloned in each orientation into pBIG2i and pBIG2r (cloning procedures and maps of these recombinant vectors are available on request). A GFP-CTCF construct carrying the entire human CTCF cDNA with GFP fused to the CTCF N terminus was also used.[2]

Cell Culture and Transfection—The K562 cell line was obtained from the American Type Culture Collection. Cells were grown in RPMI 1640 medium (Invitrogen) supplemented with 8% fetal calf serum (Biochrome) and 80 μg/ml of gentamycin at 37 °C and 5% CO_2. For transient transfection of ODN, exponentially growing K562 cells (10^5) were plated in 24-well plates in 0.5 ml of serum-free Opti-MEM medium (Invitrogen). Antisense or sense ODN (final concentration of 5 μM) were transfected with Lipofectamine (Invitrogen) following the manufacturer's instructions. After a 4-h incubation at 37 °C, 0.5 ml of Opti-MEM supplemented with 20% fetal calf serum was added. For stable transfections, exponentially growing K562 cells (5×10^6) were resuspended in 0.8 ml of RPMI-8% fetal calf serum containing 30 μg of the different plasmids and electroporated at 260 V and 1 millifarad with a Bio-Rad electroporator. 48 h after electroporation, G418 (500 μg/ml) for pcDNA-derived vectors or hygromycin B (100 μg/ml) for tet-inducible vectors were added. Individual cell clones were isolated by a limiting dilution method (31), expanded, and analyzed for CTCF expression. For the tet-inducible systems, cells where cultured in medium containing tet-free fetal calf serum (Clontech). Cells transfected with vectors based on pBIG2r were grown continuously in media supplemented with 2 μg/ml doxycycline (Sigma); CTCF gene expression in these cells was induced by removal of doxycycline. CTCF gene expression in cells transfected with vectors based on pBIG2i was induced by doxycycline added to media at 2 μg/ml.

5-Aza-2′-deoxycytidine (5-Aza-dC) Treatment—K562-derived cell lines were treated with freshly made 5-aza-dC (1 or 5 μM; Sigma) for up to 4 days. The RNA was extracted and tested for CTCF expression by RT-PCR. Protein expression was analyzed by Western and immunostaining assays.

Assessment of Cell Growth and Differentiation—Cells were counted using a hemocytometer and viability was assessed by the dye exclusion test with trypan blue (Sigma) according to the manufacturer's protocol. The WST-1 reduction assay, which measures metabolic activity of cells (32), was used according to the manufacturer's instructions (Roche) to assess cell proliferation and viability. To induce erythroid differentiation, exponentially growing K562 cells (2×10^5 cells/ml) were treated with 1 μM 1-β-D-arabinofuranosylcytosine (Ara-C, Upjohn). The erythroid differentiation was assessed by scoring the hemoglobin-containing cells after benzidine staining and monitoring the expression of the ϵ-globin gene as described earlier (33). Megakaryocytic differentiation was induced with 100 nM staurosporine (Roche) and assessed by the expression of the vimentin gene as previously described (28). Morphological differentiation was monitored in cytospin preparations stained with May-Grünwald Giemsa using established cytological criteria.

RNA Extraction and Northern Analysis—Total RNA was isolated from cells using the RNeasy kit (Qiagen) following the manufacturer's instructions. For Northern analysis, RNA (20 μg/lane) was separated by electrophoresis through a 1% agarose-formaldehyde gel and transferred onto nitrocellulose membrane (Millipore). The blots were hybridized with probes labeled with [α-^{32}P]dCTP, using a random-primed labeling kit (Amersham Biosciences). Filters were washed at a final stringency of 0.5× SSC (1× SSC: 0.15 M NaCl, 0.015 M sodium citrate), 0.1% SDS at 65 °C and autoradiographed. mRNA levels were quantified by measuring the radioactivity of the signal bands using a Molecular Imager apparatus (Bio-Rad). The probes for human ϵ-globin and vimentin mRNA were described in a previous report (34). The 2-kb PvuII fragment from the pCi7.1 CTCF construct (3) containing the *CTCF* cDNA was used to detect the human CTCF mRNA.

Semi-quantitative and Real-time RT-PCR Analysis—2 μg of total RNA were reverse transcribed with the iScript™ cDNA synthesis kit (Bio-Rad) in a 20-μl reaction for 30 min at 42 °C. cDNA (2 μl) was amplified by semiquantitative PCR (Sigma REDTaq™ PCR mixture) or real-time PCR (Bio-Rad iQ™ SYBR Green supermix). The following primers (5′-3′) were used at the indicated annealing temperature: CTCF sense, TTACACGTGTCCACGGCGTTC, and antisense, GCTTGTATGTGTCCCTGCTGGCA, at 59 °C; S14 ribosomal protein sense, TCCTGCGAGTGCTGTCAGAG, and antisense, TCACCGCCCTACACATCAAAC, at 59 °C. Primers for the TIG-1 (tarazotene-induced gene-1) were described previously (35), the annealing temperature used for these primers was 65 °C. The PCR products were resolved on 2% agarose gels.

Western Analysis and Immunostaining—For Western analysis, cell pellets were lysed in a buffer containing 100 mM Tris (pH 6.8), 10% β-mercaptoethanol, 4% SDS, and 7 M urea. Samples with 80 μg of protein per lane were separated on a 8% SDS-PAGE gel, transferred onto a nitrocellulose membrane (Millipore), and probed. Antibodies used were: anti-CTCF polyclonal antibody (Abcam), anti-CTCF monoclonal antibody (BD Biosciences), anti-MYC polyclonal antibody (Santa Cruz Biotechnology N262), and anti-α-tubulin rabbit polyclonal antibody (a gift from N. Cowan, New York University, New York). Immunocomplexes were detected by a chemiluminescent method (Bio-Rad). For immunofluorescence assays, cells were fixed with methanol for 10 min at −20 °C, and immunostaining was performed by standard procedures (36). Nuclei of the cells were visualized by staining with 4′,6-diamidino-2-phenylindole.

RESULTS

Inhibition of CTCF Expression with the Antisense ODN Enhances Proliferation of K562 Cells—We previously demonstrated that CTCF is differentially expressed and post-translationally modified, depending on the differentiation lineage of human myeloid cells (26). These data suggested a potential functional role for CTCF in leukemia cells. To address this issue, we first decided to use the antisense ODN approach to assess the effects of CTCF knock-down on myeloid cell proliferation. We designed multiple antisense ODN and analyzed them in the nuclease S1 protection assay to select the ODN with highest affinity to the CTCF mRNA (see "Experimental Procedures"). As shown in Fig. 1*A*, antisense ODN AS1 and AS6 can form stable complexes with the CTCF mRNA.

We then assessed the effects of antisense ODN on CTCF expression by Western analysis. A notable reduction of CTCF was observed in K562 cells exposed to antisense AS1 or AS6 for 3 days, compared with the untreated cells or cells treated with sense S1 and S6 ODN (Fig. 1*B* and data not shown). In this assay, AS6 ODN had a more pronounced effect than AS1 ODN.

The consequences of CTCF inhibition on cell proliferation were analyzed using the WST-1 assay. Cells exposed to antisense AS6 for 3 days had a growth rate (measured in relation to metabolic activity) markedly higher than the growth rate of cells exposed to the control sense S6 oligonucleotide (Fig. 1*C*), or cells not exposed to ODN (data not shown). Of note, antisense AS1 enhanced cell growth to a lesser extent than AS6 (data not shown), in good agreement with its lower efficacy in inhibiting CTCF expression (Fig. 1*B*). The correlation between

[2] L. Burke and R. Renkawitz, unpublished data.

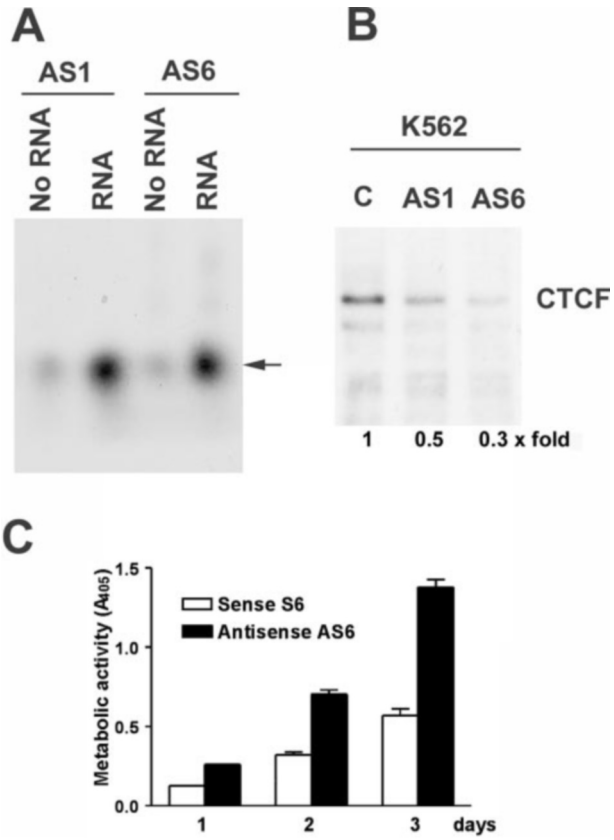

FIG. 1. **Inhibition of CTCF expression by antisense CTCF oligodeoxynucleotides leads to increased proliferation of K562 cells.** *A*, analysis of antisense ODN AS1 and AS6 by SI nuclease protection assay. Complex formation efficiency between antisense ODN and RNA is assessed by the appearance of the protected fragments (indicated by an *arrow*). This band can only be seen when the antisense ODN were incubated in the presence of RNA. *B*, Western analysis of K562 cells showing the effects of antisense ODN on CTCF expression. K562 cells were left untreated (*C*, control) or treated with AS1 or AS6 as indicated, lysed, and analyzed by Western assay with the monoclonal anti-CTCF antibody. *Numbers below* indicate relative levels of CTCF expression compared with the control designated as *1*. *C*, metabolic activity, as determined by the WST-1 assay in K562 cells exposed to sense or antisense ODN for 3 days. *Bars* indicate mean ± S.E. of three separate experiments.

antisense ODN affinity for CTCF RNA and its ability to increase cell growth argues that CTCF inhibition enhances the growth rate of K562 cells.

Constitutive Expression of CTCF in K562 Cells Inhibits Cell Proliferation and Enhances Erythroid Differentiation—The results of our transient transfection studies with ODN suggested that CTCF down-regulation promotes myeloid cell proliferation. We then asked whether CTCF overexpression would have an opposite effect and inhibit cell growth. For this purpose, K562 cells were stably transfected with the constitutive pcDNA3-CTCF expression vector. Two recombinant clones (termed KCTCF-D11 and KCTCF-G1) were selected for their higher CTCF mRNA expression (2–3-fold) compared with parental K562 cells or K562 transfected with the empty vector (termed KpCDNA cells) (Fig. 2*A*). In agreement with CTCF mRNA overexpression, KCTCF-D11 and KCTCF-G1 cells revealed higher amounts of CTCF protein as assessed by Western analysis (Fig. 2*B*). Immunostaining of KCTCF-D11 and KCTCF-G1 cells confirmed higher levels of CTCF in the nuclei of these cells (Fig. 2*C*), although CTCF expression was heterogeneous in the population of transfected cells. To obtain a more homogeneous cell population overexpressing CTCF, we subcloned the transfectants by a limiting dilution method. However, the individual subclones selected remained heterogeneous with respect to CTCF expression (data not shown). This heterogeneity could be because of inactivation of the *CTCF* gene followed by hypermethylation of its promoter that could occur randomly among cells in the population. We decided to explore this possibility further and asked whether CTCF levels would be increased in the heterogeneous population of K562 cells after treatment with a DNA demethylating agent, 5-aza-2′-deoxycytidine. In this experiment, KpcDNA, KCTCF-G1, and KCTCF-D11 cells were treated with 1 or 5 μM 5-aza-dC for up to 4 days, and CTCF expression was monitored. To verify the effects from 5-aza-dC on CTCF, we analyzed expression of the *TIG-1* gene known to be sensitive to 5-aza-dC in K562 (35). As illustrated in Fig. 2*D*, treatment with 5-aza-dC at 1 (data not shown) or 5 μM did not significantly alter the levels of CTCF mRNA in the constitutive transfectants, as assessed by semiquantitative RT-PCR. These results were further confirmed by real-time RT-PCR (data not shown). No visible changes in CTCF protein expression were observed in these experiments as shown by Western (Fig. 2*E*) or immunostaining (Fig. 2*F*) assays. In contrast, the expression of TIG-1 mRNA was dramatically induced following treatment with 5-aza-dC (Fig. 2*D*).

We then compared the growth rates of KCTCF-D11, KCTCF-G1 cells, and controls (KpCDNA cells transfected with the empty vector and non-transfected K562). As shown in Fig. 3*A*, KCTCF-D11 and KCTCF-G1 cells grow slower than the control K562 and KpCDNA cells. In agreement with this result, the metabolic activity of the cells overexpressing CTCF was considerably reduced in comparison with the control cells (Fig. 3*B*). This antiproliferative effect of CTCF was not related to an increase in programmed cell death because none of the morphological features of apoptosis such as chromatin condensation, nuclear fragmentation, and cytoplasmic shrinkage were observed in the growth-retarded cells (data not shown).

We next asked whether the growth arrest elicited by the higher levels of CTCF expression was affecting the process of K562 cell differentiation. For that purpose, differentiation of control cells and constitutive clones into the erythroid lineage were induced by treatment with Ara-C for 3 days. Using a benzidine test, we observed a higher percentage of hemoglobin-containing cells in KCTCF transfectants than in KpCDNA cells following treatment with Ara-C (Fig. 3*C*). Cells overexpressing CTCF (KCTCF D11 is shown as an example) showed a 30–40% increase in the fraction of cells expressing hemoglobin compared with control KpCDNA cells. This observation suggests that CTCF overexpression has a stimulating effect on differentiation into the erythroid lineage.

We then investigated whether CTCF was specifically involved in erythroid differentiation or if CTCF had a more general role in differentiation into other lineages. To answer this question, cell differentiation along the erythroid or megakaryocytic pathway was induced with Ara-C or staurosporine treatment, respectively, and following 1, 3, or 5 days of treatment, specific markers for each differentiation lineage were analyzed by Northern assay. In agreement with a previous report (33), treatment with Ara-C resulted in the accumulation of a specific erythroid marker, ϵ-globin mRNA, in control KpCDNA cells, whereas this marker was not expressed in megakaryocytic cells after staurosporine treatment (Fig. 3*D*). Notably, in CTCF overexpressing clones treated with Ara-C, the ϵ-globin mRNA expression was further increased in comparison with the induced KpCDNA cells (Fig. 3*D*). This observation is in agreement with the higher fraction of cells producing hemoglobin found in CTCF-expressing transfectants, as shown in Fig. 3*C*. These findings further confirm that CTCF promotes erythroid differentiation induced by Ara-C in K562.

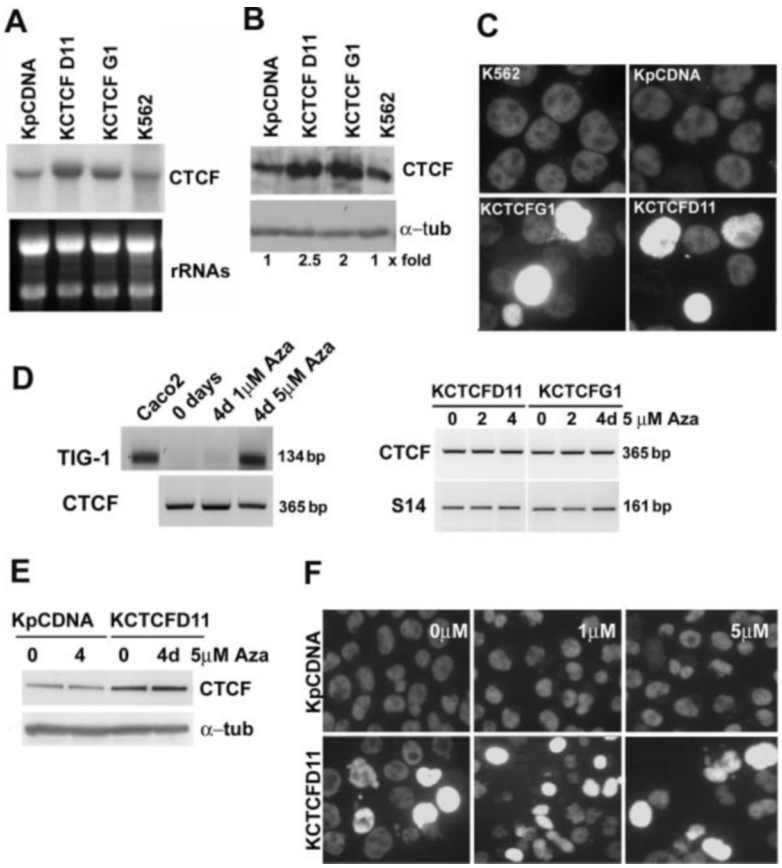

FIG. 2. **Constitutive overexpression of CTCF in stably transfected K562 cell lines; Effect of 5-aza-dC.** *A*, expression of CTCF mRNA is elevated in cells transfected with a vector for constitutive expression of CTCF (KCTCF-D11 and KCTCF-G1). Total RNA was prepared and analyzed by Northern assay using the human CTCF cDNA probe. Total RNA purified from K562 cells transfected with the empty vector (KpCDNA) and the parental K562 cells were used as control. The *lower panel* shows the rRNAs stained with ethidium bromide serving as loading control. *B*, CTCF protein expression in the indicated cell lines after Western analysis with the monoclonal anti-CTCF antibody. The membrane was further incubated with anti-α-tubulin antibody (*α-tub*) to serve as loading control. The developed films were scanned and quantified. The ratios of the intensity of the CTCF bands over the intensity of the corresponding α-tubulin bands were determined and expressed as -fold change relative to the lowest CTCF/α-tubulin ratio found in K562 (designated as *1*). *Numbers below* represent these results. *C*, immunofluorescence analysis of the indicated cell lines probed with an anti-CTCF polyclonal antibody. *D*, effect of 5-aza-dC on CTCF mRNA expression analyzed by RT-PCR. *Left panels*, no changes in CTCF expression can be detected in KpCDNA cells containing the empty vector pcDNA3, untreated or treated with 1 or 5 μM 5-aza-dC for 4 days. Expression of TIG-1 mRNA was induced in the same cells following treatment with 5-aza-dC. Caco2 cells were used as positive control for TIG-1 expression (35). *Right panels*, no changes in CTCF expression can be detected in KCTCF D11 and KCTCF G1 cells, untreated or treated with 5 μM 5-aza-dC for up to 4 days. The constitutive expression of the S14 ribosomal protein was used as internal control. *E*, treatment with 5-aza-dC does not alter CTCF protein expression. KpCDNA and KCTCF D11 cells were treated with 5-aza-dC, and Western analysis was performed with the monoclonal anti-CTCF antibody. The membrane was further incubated with the anti-α-tubulin antibody serving as loading control. *F*, immunofluorescence analysis of KpCDNA and KCTCF D11 cells treated for 4 days with the indicated doses of 5-aza-dC, and probed with an anti-CTCF monoclonal antibody.

As expected, expression of the vimentin mRNA used as a marker of K562 megakaryocytic differentiation (28) was increased after staurosporine treatment in KpCDNA cells (Fig. 3*D*). In cells overexpressing CTCF, levels of vimentin mRNA upon induction with staurosporine were similar to control cells. This observation suggests a specific role for CTCF in the regulation of K562 differentiation into particular lineages. In summary, our data indicate that constitutive expression of CTCF in K562 impairs cell growth and increases differentiation into the erythroid, but not the megakaryocytic lineage.

Inducible Overexpression of CTCF Enhances Erythroid Differentiation in K562 Cells—The results described above indicate that constitutive CTCF overexpression enhances erythroid differentiation of K562 cells. We chose to further substantiate these data by generating clones in which CTCF could be expressed in an inducible manner. For this purpose, K562 cells were stably transfected with the tetracycline-inducible vectors containing the human CTCF cDNA cloned in both orientations ("Experimental Procedures"). For each individual clone carrying CTCF plasmids in the sense orientation, CTCF exogenous expression was induced by addition (pBIG2i-derived cells) or removal (pBIG2r-derived cells) of doxycycline, and clones showing a substantial CTCF ectopic expression were selected for further experiments. A typical result of a Western analysis is shown in Fig. 4*A* for one of the selected clones, in which a 2.4-fold increase of CTCF levels was detected 24 h post-induction. This observation was also confirmed by immunofluorescence analysis; strong nuclear staining of CTCF was observed 24 h post-induction in all selected clones (Fig. 4*B* and data not shown). No difference in CTCF expression was found in parental K562 cells growing in the presence or absence of doxycycline, as assessed by Western and immunostaining (data not shown).

We then investigated the effects of CTCF overexpression on cell proliferation in two selected clones induced by addition (i-F11) or removal (r-C10) of doxycycline. As shown in Fig. 4*C*, induction of CTCF expression leads to inhibition of proliferation in both cell lines, in agreement with the results obtained with the constitutive transfectants. To investigate whether the enhancement of erythroid differentiation observed with constitutive CTCF-expressing clones could also be achieved with conditional CTCF expression, the latter clones were induced to overexpress CTCF protein, in the presence of Ara-C. Induction

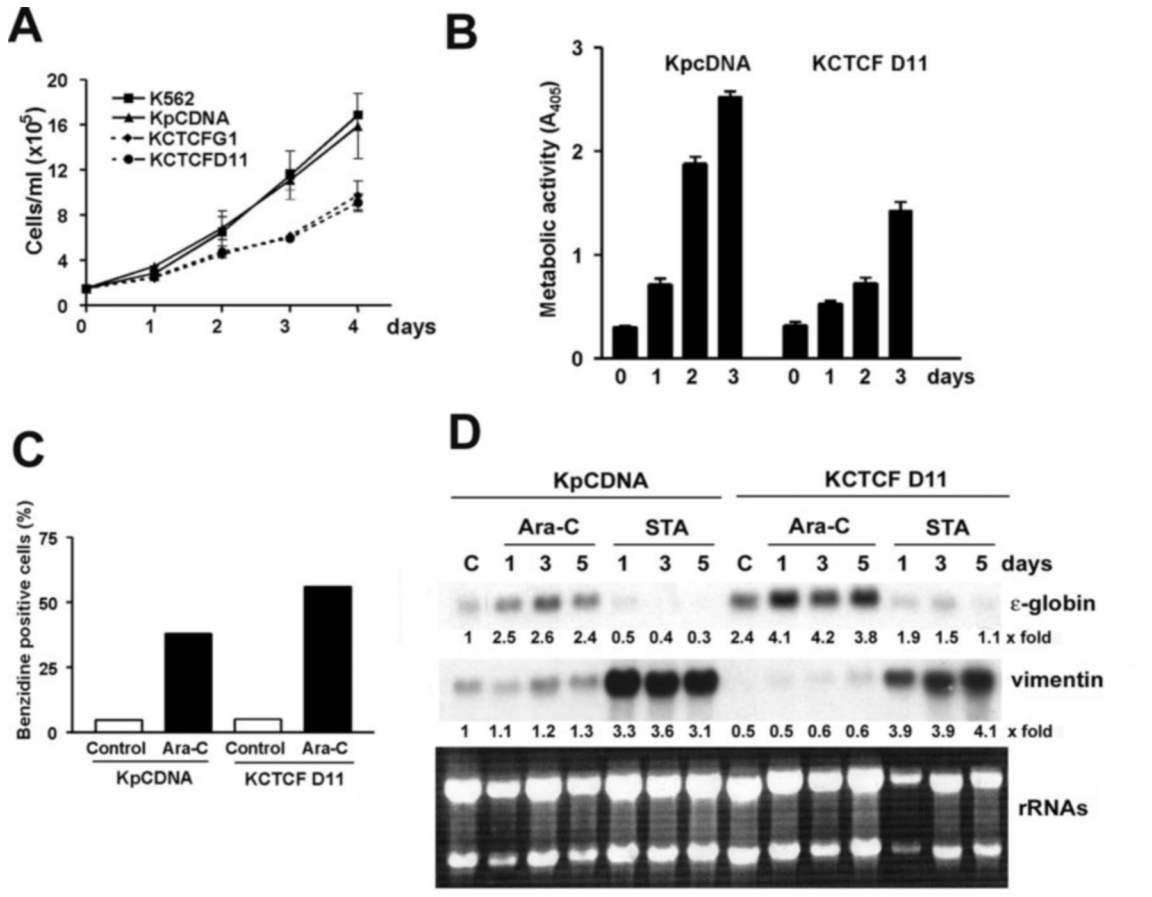

FIG. 3. **Constitutive CTCF overexpression leads to growth retardation and enhanced erythroid differentiation of K562 cells.** *A*, cell proliferation for parental K562, KpCDNA cells transfected with the empty vector pcDNA3 (*continuous lines*), and cell lines stably expressing CTCF (*dotted lines*). Cells were counted daily for 4 days. *Bars* indicate mean ± S.E. of three separate experiments. Cell viability, as determined by trypan blue staining, remained above 90% in all cases. *B*, metabolic activity of the indicated cell lines, as determined by the WST-1 assay. *Bars* indicate mean ± S.E. of three separate experiments. *C*, percentage of benzidine positive cells for the indicated cell lines after no treatment (control) or treatment with Ara-C for 3 days. In each experiment, a minimum of 200 cells were counted. A representative experiment, of three, is shown with essentially the same results. *D*, Northern blot analysis of K562 cells transfected with the empty vector (control) and constitutively expressing CTCF after induction of erythroid or megakaryocytic differentiation. Total RNAs were prepared from the indicated cell lines treated for 5 days with Ara-C or with staurosporine (*STA*) to induce the erythroid or megakaryocytic pathways, respectively. Untreated K562 cells were used as control (*C*). The same membrane was consecutively hybridized with the ϵ-globin and vimentin probes. The *lower panel* shows the rRNAs stained with ethidium bromide (loading control). The ratios of the intensity of the ϵ-globin and vimentin mRNA bands over the intensity of the corresponding 28 S rRNA bands were determined and expressed as -fold change relative to the ϵ-globin/28 S or vimentin/28 S in the control untreated K562 cells (designated as *1*). *Numbers in* the figure represent these results.

of differentiation into the erythroid lineage was analyzed by monitoring the hemoglobin content by staining the cells with benzidine. Parental K562 cells were subjected to the same treatment and used as control. We found that parental cells were differentiating with a similar efficiency, when incubated with Ara-C for 4 days, in the presence or the absence of doxycycline (Fig. 4*D*), thus ruling out any nonspecific effects caused by doxycycline on differentiation. On the other hand, when overexpression of CTCF was induced in K562 r-C10 cells following doxycycline removal, the number of cells containing hemoglobin rose sharply after Ara-C treatment compared with non-induced cells (Fig. 4*D*). This enhancement of erythroid differentiation elicited by CTCF overexpression was accompanied by a dramatic increase in the expression of ϵ-globin mRNA (a typical example is shown in Fig. 4*E*). Of note, the number of spontaneously appearing benzidine-positive cells detected in the K562 r-C10 clone, without Ara-C treatment, was found to be significantly increased compared with control parental K562 cells, independent of CTCF induction (Fig. 4*D*). This may be explained by a low level "leakage" from the promoter regulating transcription of the CTCF transgene in non-induced cells.

We then analyzed the megakaryocytic differentiation induced by staurosporine in K562 r-C10 cells. No significant difference was observed in the expression of vimentin mRNA after induction of CTCF compared with non-induced cells (Fig. 4*F*). Furthermore, ectopic expression of CTCF in these cells did not trigger morphological features of megakaryocytic differentiation (data not shown).

Similar results were obtained when we used another clone, K562 i-F11 (Fig. 4*A*), in the experiments described above (data not shown). Taken together, these results further substantiate the important role of CTCF in promoting erythroid maturation without affecting megakaryocytic differentiation in K562 cells.

Down-regulation of CTCF Inhibits Erythroid Differentiation of K562 Cells—We next asked whether down-regulation of CTCF in K562 would have the opposite effects to CTCF overexpression on the differentiation of K562 cells. For this purpose, we generated K562 cell lines in which CTCF expression could be inhibited in an inducible manner. After transfection with pBIG2r and pBIG2i vectors carrying CTCF cDNA in the antisense orientation, several K562 clones were selected based on CTCF protein expression after induction in the presence or absence of doxycycline. As shown in Fig. 5*A*, inducible down-regulation of CTCF was efficient in two representative clones,

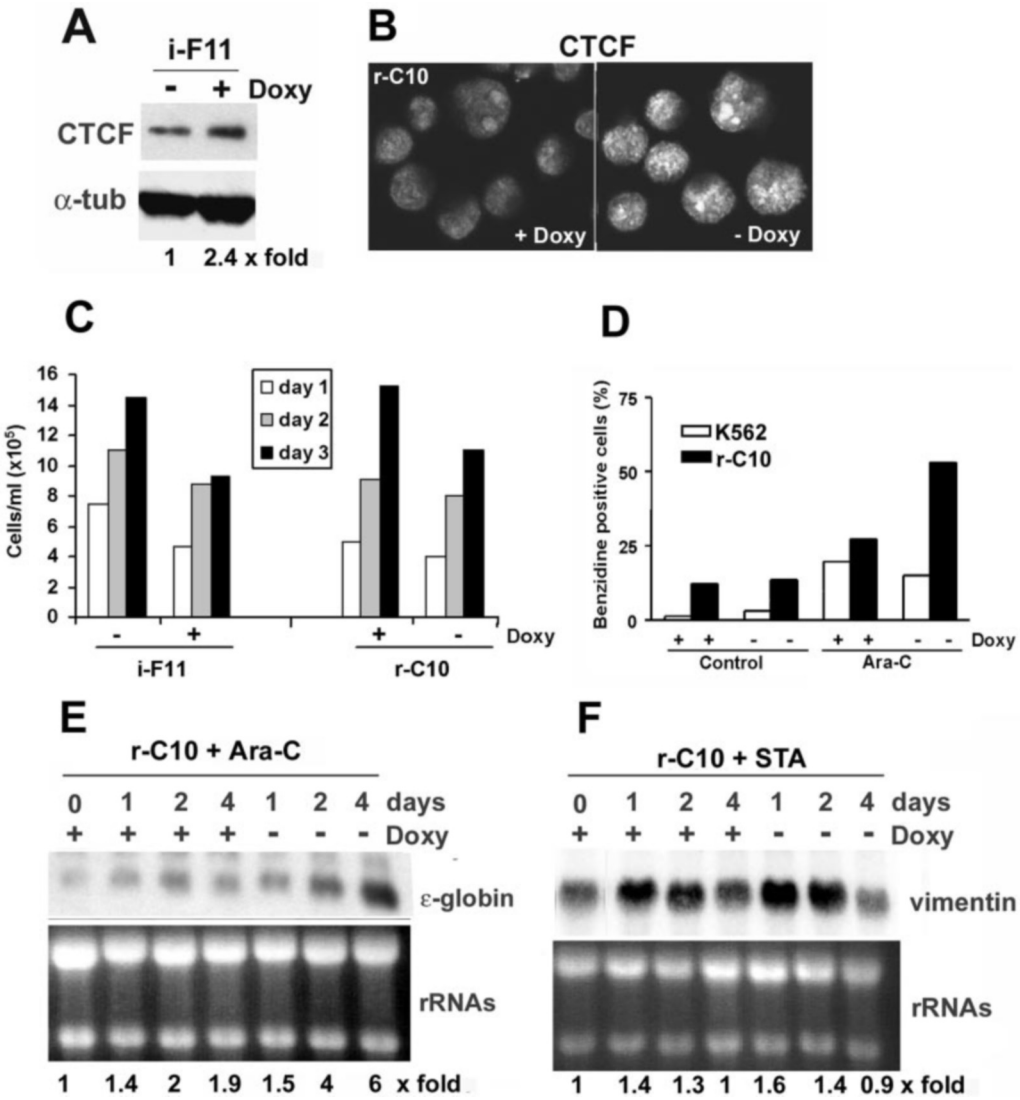

FIG. 4. **Inducible overexpression of CTCF results in growth retardation and enhanced erythroid differentiation of K562 cells.** *A*, Western analysis of CTCF expression in cell line K562 i-F11 (tet-on system) after induction with doxycycline for 24 h. The membrane was probed with the monoclonal anti-CTCF antibody and then with the anti-α-tubulin antibody (loading control). *Numbers below* indicate relative CTCF expression levels normalized to corresponding tubulin levels, after densitometric analysis. *B*, immunofluorescence analysis of cell line K562 r-C10 (tet-off system) after removal of doxycycline for 24 h. Cells were immunostained with the monoclonal anti-CTCF antibody. *C*, cell proliferation rates of K562 i-F11 and K562 r-C10 growing in the presence (tet-on) or absence (tet-off) of doxycycline, as indicated. *D*, percentage of benzidine positive cells for K562 and r-C10 (tet-off) cell lines, not induced or induced to overexpress CTCF, and subsequently treated or not treated (control) with Ara-C for 3 days. In each experiment, a minimum of 200 cells were counted. A representative experiment of three with essentially the same results is shown. *E* and *F*, Northern analyses of K562 r-C10 cells induced toward erythroid and megakaryocytic differentiation, respectively. Total RNAs were prepared from cells growing in the presence (+) or absence (−) of doxycycline and treated for up to 4 days with Ara-C or staurosporine (*STA*) as indicated. The membranes were hybridized with the ϵ-globin or vimentin probes. *Lower panels* show rRNAs stained with ethidium bromide to assess RNA loading. The ratios of the intensity of the ϵ-globin and vimentin mRNA bands over the intensity of the corresponding 28 S rRNA bands were determined and expressed as -fold change relative to the ϵ-globin/28 S or vimentin/28 S in the control untreated KpCDNA r-C10 cells (designated as *1*). *Numbers below* represent these results.

K562 iAS-G4 (tet-on) and K562 rAS-D6 (tet-off). Inhibition of CTCF expression was confirmed by immunofluorescence staining 24 h post-induction (Fig. 5*B*).

We then analyzed the effects of CTCF down-regulation on the differentiation of K562 cells. For this purpose, K562 (control) and tet-on K562 iAS-G4 cells were incubated in the presence or absence of doxycycline and treated with Ara-C for 4 days. After Ara-C treatment, the fraction of benzidine positive cells rose to similar levels in both non-induced K562 iAS-G4 and control K562 cells (Fig. 5*C*). However, upon CTCF down-regulation, the fraction of benzidine positive cells was reduced by ~60% (Fig. 5*C*). This result was confirmed by measuring the expression of ϵ-globin mRNA. As shown in Fig. 5*D*, the levels of this erythroid marker in K562 iAS-G4 cells gradually increased following treatment with Ara-C for 4 days, whereas the expression of ϵ-globin was dramatically reduced when CTCF inhibition was induced by doxycycline (Fig. 5*D*). On the other hand, down-regulation of CTCF had no significant effect on the expression of the marker of megakaryocytic differentiation, vimentin mRNA, after staurosporine treatment (Fig. 5*E*).

Similar results were obtained when we used another clone, K562 rAS-D6 (Fig. 5*A*), in the experiments described above (data not shown). Thus, results described in this section lend further support for a specific role for CTCF in promoting erythroid differentiation in K562 cells.

CTCF Is Involved in the Regulation of MYC in K562 Cells—CTCF has been shown to regulate several genes known to be important for control of cell proliferation, such as *MYC*, $p19^{ARF}/p14^{ARF}$, and others (see Introduction and Ref. 37). We have previously demonstrated that MYC is down-regulated

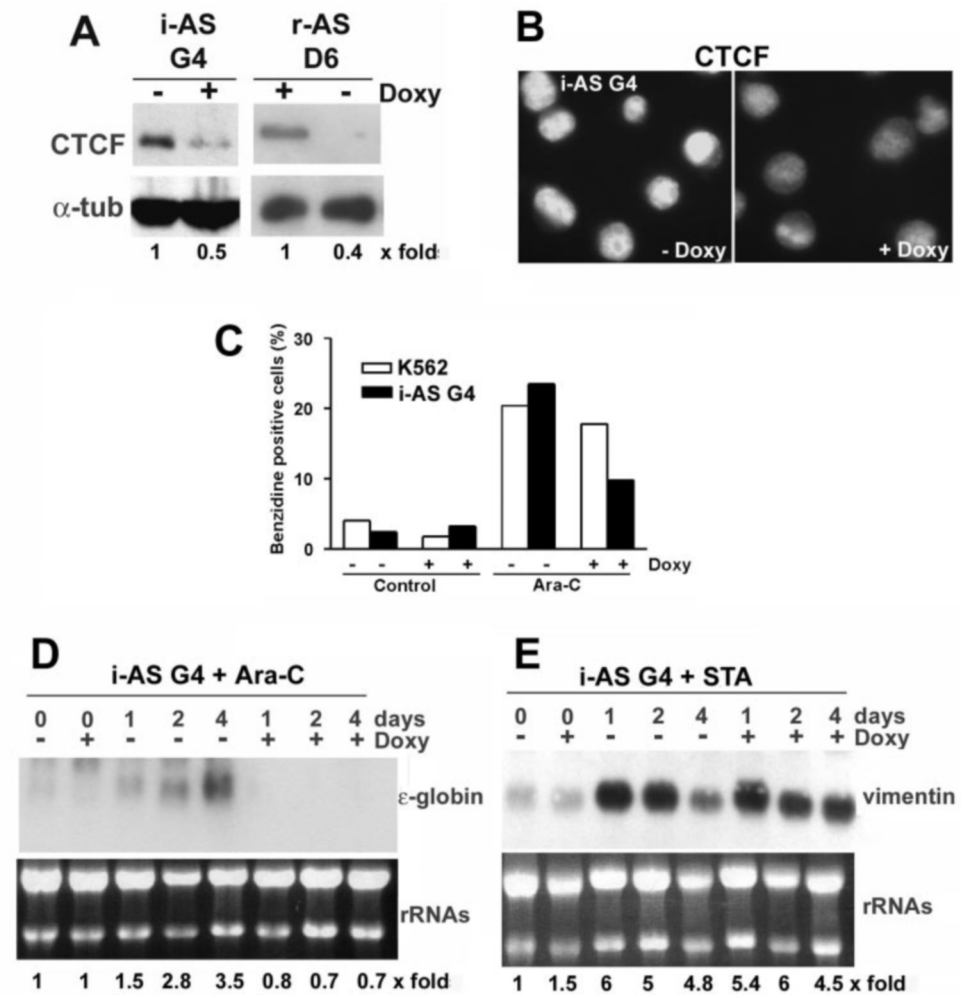

FIG. 5. **Inducible down-regulation of CTCF results in inhibition of erythroid differentiation of K562 cells.** *A*, Western analysis of CTCF protein expression with a monoclonal anti-CTCF antibody. CTCF down-regulation was induced by addition of doxycycline (+) to cell line K562 i-AS G4 (tet-on system), and by removal of doxycycline (−) in the cell line K562 r-AS D6 (tet-off system). The membranes were subsequently incubated with an anti-α-tubulin antibody (loading control). *Numbers below* indicate relative CTCF expression levels normalized to levels of tubulin after densitometric analysis. *B*, immunofluorescence analysis with the monoclonal anti-CTCF antibody of the K562 i-AS G4 cells growing in the absence or presence of doxycycline for 24 h. *C*, fraction of benzidine positive cells from K562 clones non-induced or induced to down-regulate CTCF, and subsequently treated or not treated (control) with Ara-C for 3 days. In each experiment, a minimum of 200 cells were counted. A representative experiment of three with essentially the same result is shown. *D* and *E*, Northern analyses of K562 i-AS G4 cells induced into erythroid and megakaryocytic differentiation, respectively. Total RNAs were prepared from cells growing in the absence (−) or presence (+) of doxycycline and treated for up to 4 days with Ara-C or staurosporine (*STA*) as indicated. The membranes were hybridized with the ϵ-globin or vimentin probes. *Lower panels* show rRNAs stained with ethidium bromide to assess RNA loading. The ratios of the intensity of the ϵ-globin and vimentin mRNA bands over the intensity of the corresponding 28 S rRNA bands were determined and expressed as -fold change relative to the ϵ-globin/28 S or vimentin/28 S in the control untreated K562 i-AS G4 cells (designated as *1*). *Numbers below* represent these results.

during erythroid differentiation (38). Moreover, enforced expression of MYC inhibits Ara-C-mediated erythroid differentiation of K562 cells (33), and inhibition of MYC activity enhances erythroid differentiation (39). Because *MYC* is a known target for transcriptional repression by CTCF (4), we asked whether CTCF effects on proliferation and erythroid differentiation in K562 could be mediated through down-regulation of MYC. To investigate this, we analyzed *MYC* expression in the inducible K562 cell models, upon activation or repression of CTCF. As shown in Fig. 6*A*, in a representative experiment, MYC protein levels were found to be decreased following CTCF overexpression by doxycycline (*middle panel*). In agreement with this result, an increase in MYC protein levels was observed when CTCF expression was down-regulated in both AS-CTCF recombinant clones (*right panels*). No change in MYC expression was observed in parental K562 growing in the presence or absence of doxycycline (*left panel*). Thus, the induction of MYC expression observed in the conditional K562 cell models correlates with CTCF down-regulation. Conversely, inhibition of MYC correlates with CTCF overexpression.

To further confirm these observations, we also investigated the effect of CTCF protein on MYC expression in transient transfection experiments using a GFP-CTCF expression vector (see "Experimental Procedures"). As shown in Fig. 6*B*, immunostaining of transfected cells with anti-MYC antibody revealed a dramatic down-regulation of MYC following CTCF overexpression, thus corroborating the results obtained with the inducible systems.

DISCUSSION

Although the importance of CTCF in the regulation of cell growth has been previously reported for several cellular systems (15, 19, 20), the role of CTCF in cell differentiation has not been investigated. In particular, very little is known about the possible function of CTCF in the regulation of hematopoietic cell differentiation. Our previous work (26) demonstrated a differential expression and post-translational modification of CTCF that were dependent on cellular differentiation path-

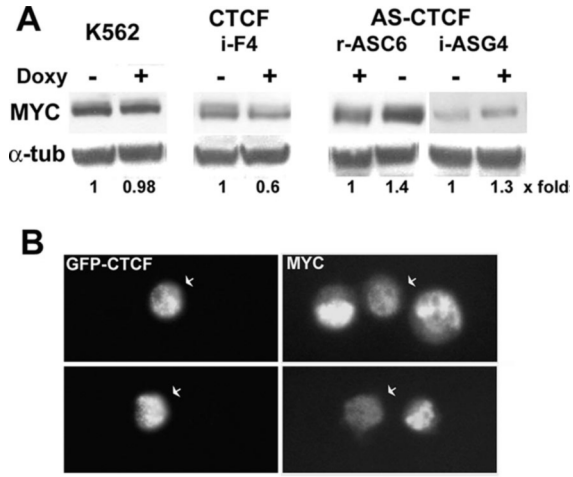

FIG. 6. **CTCF protein regulates MYC expression.** *A*, Western analysis of MYC in K562 cells that over- or underexpress CTCF. As indicated, cells were treated with doxycycline for 24 h to induce expression of sense (*i-F4*) or antisense (*i-ASG4*) CTCF. Antisense CTCF was induced by doxycycline removal in K562 r-AS-C6 cells. The membranes were incubated with the polyclonal anti-MYC antibody and then with an anti-α-tubulin antibody (loading control). *Numbers below* show relative MYC expression levels normalized to tubulin levels, after densitometric analysis. *B*, MYC protein expression analyzed by immunofluorescence in K562 overexpressing CTCF. K562 cells were transfected with a GFP-CTCF construct and immunostained with anti-MYC antibody 24 h later. *Left panels*, GFP expression indicating CTCF-transfected cells. *Right panels*, MYC expression. CTCF-transfected cells (*arrows*) show lower MYC levels than neighboring, non-transfected cells.

ways, suggesting that CTCF could be important for growth and differentiation of myeloid cells.

The aim of the present study was to provide further evidence that CTCF indeed has a functional role in the regulation of cell growth and differentiation in human myeloid cells. To pursue this investigation, we developed several approaches: 1) inhibition of CTCF expression in K562 cells by targeting CTCF mRNA with the antisense ODN; 2) generation of K562 recombinant cells with constitutive overexpression of CTCF; and 3) generation of K562 recombinant cells with the regulated expression of CTCF. In these models, CTCF up- and down-regulation was verified by several methods (Northern, Western, and immunofluorescence analyses). Expression of the CTCF protein in these systems was reconfirmed with the different antibody (anti-CTCF polyclonal antibody (Abcam) (data not shown); these results were identical to the data obtained using the anti-CTCF monoclonal antibody (BD Biosciences) (Figs. 1–6).

Effects of CTCF on Cell Growth—The present study indicates that K562 cells overexpressing CTCF exhibit a reduced growth ability in liquid medium, in agreement with the previously reported findings (19). We also observed that enforced expression of CTCF leads to reduction in the clonogenic capacity of K562 (data not shown). Transient inhibition of CTCF with antisense oligodeoxynucleotides enhanced the growth of K562 cells, thus further substantiating the importance of CTCF in the regulation of proliferation of these cells. On the other hand, we noted that in recombinant cells expressing CTCF, both in a stable and inducible manner, only moderate levels of ectopic CTCF can be achieved (2–4-fold), suggesting a natural selective pressure against high CTCF levels. For example, in the population of K562 cells constitutively expressing CTCF, the levels of CTCF in individual cells varied significantly (Fig. 2*C*) and the mixed population remained even after subcloning. This may be explained by the fact that CTCF-positive stably transfected clones are selected against during propagation. These observations are in line with the hypothesis that significant overexpression of CTCF may not be compatible with cell growth (19).

A general explanation of the variegated expression of CTCF may be a high incidence of silencing in transgenes, which is believed to be the result of the influence of surrounding endogenous condensed chromatin. Such silencing involves changes in the chromatin structure and methylation status of the transgene (31, 40–42). Treatment with the DNA methylation inhibitor 5-aza-2′-deoxycytidine results in global DNA demethylation and in the reactivation of genes silenced by promoter methylation (43). However, in K562 transfectants, the CTCF mRNA or protein levels did not change upon 5-aza-dC treatment. In sharp contrast, the expression of the methylation-dependent *TIG-1* gene (35) was dramatically induced by 5-aza-dC. Therefore, DNA methylation does not seem to be the cause of the low CTCF levels observed in some K562 cells expressing ectopic CTCF. It remains to be investigated whether repressed chromatin structure (*e.g.* histone hypoacetylation, histone H3-Lys^9 methylation (44)) can have inhibitory effects on the expression from the exogenous CTCF in these cells. These experiments are currently being carried out in our laboratories.

CTCF may induce growth arrest through different molecular mechanisms. In WEHI 231B cells CTCF was found to induce growth arrest and apoptosis (20), and it was proposed that this effect was mediated through the enhanced expression of ARF, p53, p21, and p27, and inhibition of MYC (19, 20). In our case, growth arrest observed in stably transfected K562 cells was not accompanied by typical apoptosis. It is conceivable that cellular context may account for different effects of CTCF overexpression in different cell lines. As K562 cells are known to be p53-, ARF-, and p16INK4A-negative (45–47), our results argue against CTCF inhibition of cell growth through ARF, p16, or p53-dependent pathways. Although the molecular mechanism by which CTCF induces growth arrest in K562 is not fully resolved as yet, our observations suggest that MYC down-regulation by CTCF could be, at least partially, responsible for this effect.

Effects of CTCF on Cell Differentiation—Investigations of the growth and differentiation program of erythroid progenitor cells is of great contemporary interest because alteration of these programs can lead to malignant leukemias. K562 cells represent a model for immature hematopoietic precursors that can be differentiated into the erythroid lineage or into megakaryocytic lineage. To test whether CTCF was involved in the maturation pathway, we analyzed erythroid markers (hemoglobinization and expression of ϵ-globin mRNA) in K562 cells overexpressing CTCF in a constitutive or inducible manner. In both systems, we observed that ectopic expression of CTCF increased erythroid differentiation induced by Ara-C treatment. On the other hand, when CTCF expression was down-regulated, a clear inhibition of both erythroid markers was detected. No significant changes were observed regarding the levels of the megakaryocytic marker (vimentin) in K562 cells upon CTCF overexpression, thus indicating a specific role of CTCF in erythroid lineage differentiation. Interestingly, erythroid differentiation of K562 cells is accompanied by the appearance of two hyperphosphorylated forms of CTCF protein, whereas CTCF remains hypophosphorylated during megakaryocytic differentiation (26). Taken together, these data strongly suggest an important functional role for CTCF in differentiation along the erythroid pathway.

The signaling mechanisms underlying erythroid differentiation are not clearly understood, although altered expression of a number of genes involved in signaling pathways has been reported for K562 cells induced to erythroid differentiation (Ref. 48, and references therein). Several transcription factors

and cell cycle regulators that have a role in differentiation of K562 cells have been described. For example, the cyclin-dependent kinase inhibitor p27 promotes erythroid differentiation, whereas p21 triggers megakaryocytic maturation in K562 (49). Interestingly, the conditional expression of CTCF in WEHI231 cells enhanced the expression of p21 and p27 (20). However, no noticeable up-regulation of either protein by CTCF in our K562 constitutive or inducible systems has been observed (data not shown).

Another good candidate as a mediator for CTCF effects on erythroid differentiation in K562 is *MYC*. We previously reported that *MYC* enforced expression inhibits erythroid differentiation of K562 (33, 39). As *MYC* is a target gene repressed by CTCF (2–4), it is conceivable that down-regulation of *MYC* is one of the mechanisms by which CTCF promotes erythroid differentiation. Our results support this hypothesis, because MYC expression is reduced in K562 cells overexpressing CTCF and increased in K562 cells when CTCF is down-regulated. In agreement with our results, the conditional expression of CTCF-sense and CTCF-antisense in a murine B cell line (WEHI231) also induced reciprocal changes in MYC expression (20). Of note, in both models CTCF expression results in growth arrest, but it is accompanied by erythroid differentiation in K562, whereas CTCF overexpression induces apoptosis in WEHI231. Thus, the effect of CTCF on cell death or differentiation seems to be cell-type dependent, regardless of the mechanism involved. The complex behavior of CTCF has been further illustrated by the observation that elevated levels of CTCF in breast cancer cells are associated with the anti-apoptotic function of CTCF in these cells.[3]

Down-regulation of MYC by CTCF and subsequent growth inhibition may be important for erythroid differentiation, however, it is not sufficient. Prior treatment of K562 cells with Ara-C committing K562 to erythroid differentiation was required to reveal the effects of CTCF. It is likely that these effects are a combination of both direct and indirect influences of CTCF. For example, CTCF can directly regulate transcription of hematopoietic/erythroid transcription factors (GATA-1, FOG, and others) and/or have effects on erythroid-transcription-activation complexes. It is also possible that CTCF may directly control expression of erythroid genes (*e.g.* genes for synthesis of heme and globins and many others) (50–53).

Functions other than regulation by CTCF at the transcriptional level should also be considered in this context. In particular, specific protein-protein interactions regulating growth and differentiation of myeloid cells may occur between CTCF and its interacting partners depending on a particular localization of CTCF in the cells. Indeed, the mitotic localization of CTCF at centrosomes and midbodies has been previously suggested as another possible mechanism by which cell growth is controlled by CTCF (54). Such a mechanism could also be operational in K562 cells. Interestingly, translocation of CTCF from the nucleoplasm into nucleoli has been observed following erythroid differentiation of K562 cells.[4] The importance of this finding is currently being investigated.

A detailed study involving expression profiling, and also elucidation of DNA targets affected by CTCF in K562 cells upon erythroid differentiation promoted by CTCF, are currently being carried out in our laboratories. We believe this approach will provide further insight into molecular events underlying the regulation of myeloid differentiation by CTCF.

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REFERENCES

1. Ohlsson, R., Renkawitz, R., and Lobanenkov, V. (2001) *Trends Genet.* **17,** 520–527
2. Klenova, E. M., Morse, H. C., 3rd, Ohlsson, R., and Lobanenkov, V. V. (2002) *Semin. Cancer Biol.* **12,** 399–414
3. Filippova, G. N., Fagerlie, S., Klenova, E. M., Myers, C., Dehner, Y., Goodwin, G., Neiman, P. E., Collins, S. J., and Lobanenkov, V. V. (1996) *Mol. Cell. Biol.* **16,** 2802–2813
4. Klenova, E. M., Nicolas, R. H., Paterson, H. F., Carne, A. F., Heath, C. M., Goodwin, G. H., Neiman, P. E., and Lobanenkov, V. V. (1993) *Mol. Cell. Biol.* **13,** 7612–7624
5. Burcin, M., Arnold, R., Lutz, M., Kaiser, B., Runge, D., Lottspeich, F., Filippova, G. N., Lobanenkov, V. V., and Renkawitz, R. (1997) *Mol. Cell. Biol.* **17,** 1281–1288
6. Vostrov, A. A., and Quitschke, W. W. (1997) *J. Biol. Chem.* **272,** 33353–33359
7. Kuzmin, I., Geil, L., Gibson, L., Cavinato, T., Loukinov, D., Lobanenkov, V., and Lerman, M. I. (2005) *J. Mol. Biol.* **346,** 411–422
8. Dunn, K. L., and Davie, J. R. (2003) *Biochem. Cell Biol.* **81,** 161–167
9. Bell, A. C., West, A. G., and Felsenfeld, G. (1999) *Cell* **98,** 387–396
10. Farrell, C. M., West, A. G., and Felsenfeld, G. (2002) *Mol. Cell. Biol.* **22,** 3820–3831
11. Lutz, M., Burke, L. J., LeFevre, P., Myers, F. A., Thorne, A. W., Crane-Robinson, C., Bonifer, C., Filippova, G. N., Lobanenkov, V., and Renkawitz, R. (2003) *EMBO J.* **22,** 1579–1587
12. Gombert, W. M., Farris, S. D., Rubio, E. D., Morey-Rosler, K. M., Schubach, W. H., and Krumm, A. (2003) *Mol. Cell. Biol.* **23,** 9338–9348
13. Filippova, G. N., Thienes, C. P., Penn, B. H., Cho, D. H., Hu, Y. J., Moore, J. M., Klesert, T. R., Lobanenkov, V. V., and Tapscott, S. J. (2001) *Nat. Genet.* **28,** 335–343
14. Chao, W., Huynh, K. D., Spencer, R. J., Davidow, L. S., and Lee, J. T. (2002) *Science* **295,** 345–347
15. Klenova, E. M., Chernukhin, I. V., El-Kady, A., Lee, R. E., Pugacheva, E. M., Loukinov, D. I., Goodwin, G. H., Delgado, D., Filippova, G. N., Leon, J., Morse, H. C., 3rd, Neiman, P. E., and Lobanenkov, V. V. (2001) *Mol. Cell. Biol.* **21,** 2221–2234
16. El-Kady, A., and Klenova, E. (2005) *FEBS Lett.* **579,** 1424–1434
17. Yu, W., Ginjala, V., Pant, V., Chernukhin, I., Whitehead, J., Docquier, F., Farrar, D., Tavoosidana, G., Mukhopadhyay, R., Kanduri, C., Oshimura, M., Feinberg, A. P., Lobanenkov, V., Klenova, E., and Ohlsson, R. (2004) *Nat. Genet.* **36,** 1105–1110
18. Klenova, E., and Ohlsson, R. (2005) *Cell Cycle* **4,** 96–101
19. Rasko, J. E., Klenova, E. M., Leon, J., Filippova, G. N., Loukinov, D. I., Vatolin, S., Robinson, A. F., Hu, Y. J., Ulmer, J., Ward, M. D., Pugacheva, E. M., Neiman, P. E., Morse, H. C., 3rd, Collins, S. J., and Lobanenkov, V. V. (2001) *Cancer Res.* **61,** 6002–6007
20. Qi, C. F., Martensson, A., Mattioli, M., Dalla-Favera, R., Lobanenkov, V. V., and Morse, H. C., 3rd (2003) *Proc. Natl. Acad. Sci. U. S. A.* **100,** 633–638
21. Filippova, G. N., Lindblom, A., Meincke, L. J., Klenova, E. M., Neiman, P. E., Collins, S. J., Doggett, N. A., and Lobanenkov, V. V. (1998) *Genes Chromosomes Cancer* **22,** 26–36
22. Yeh, A., Wei, M., Golub, S. B., Yamashiro, D. J., Murty, V. V., and Tycko, B. (2002) *Genes Chromosomes Cancer* **35,** 156–163
23. Rakha, E. A., Pinder, S. E., Paish, C. E., and Ellis, I. O. (2004) *Br. J. Cancer* **91,** 1591–1596
24. Filippova, G. N., Qi, C. F., Ulmer, J. E., Moore, J. M., Ward, M. D., Hu, Y. J., Loukinov, D. I., Pugacheva, E. M., Klenova, E. M., Grundy, P. E., Feinberg, A. P., Cleton-Jansen, A. M., Moerland, E. W., Cornelisse, C. J., Suzuki, H., Komiya, A., Lindblom, A., Dorion-Bonnet, F., Neiman, P. E., Morse, H. C., 3rd, Collins, S. J., and Lobanenkov, V. V. (2002) *Cancer Res.* **62,** 48–52
25. Drexler, H. G., Matsuo, A. Y., and MacLeod, R. A. (2000) *Leuk. Res.* **24,** 881–911
26. Delgado, M. D., Chernukhin, I. V., Bigas, A., Klenova, E. M., and Leon, J. (1999) *FEBS Lett.* **444,** 5–10
27. Rowley, P. T., Ohlsson-Wilhelm, B. M., Farley, B. A., and LaBella, S. (1981) *Exp. Hematol.* **9,** 32–37
28. Lerga, A., Crespo, P., Berciano, M., Delgado, M. D., Canelles, M., Cales, C., Richard, C., Ceballos, E., Gutierrez, P., Ajenjo, N., Gutkind, S., and Leon, J. (1999) *Cell Growth & Differ.* **10,** 639–654
29. Sambrook, J., and Russell, D. W. (2001) *Molecular Cloning: A Laboratory Manual*, 3rd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
30. Strathdee, C. A., McLeod, M. R., and Hall, J. R. (1999) *Gene (Amst.)* **229,** 21–29
31. Henry, C., Marbrook, J., Vann, D. C., Kodlin, D., and Wofsy, C. (1980) in *Selected Methods in Cellular Immunology* (Mishell, B. B., and Shiigi, S. M., eds) pp. 138–152, W. H. Freeman, New York
32. Tan, A. S., and Berridge, M. V. (2000) *J. Immunol. Methods* **238,** 59–68
33. Delgado, M. D., Lerga, A., Canelles, M., Gomez-Casares, M. T., and Leon, J. (1995) *Oncogene* **10,** 1659–1665
34. Delgado, M. D., Quincoces, A. F., Gomez-Casares, M. T., Martinez, C. A., Cuadrado, M. A., Richard, C., and Leon, J. (1992) *Cancer Res.* **52,** 5979–5984
35. Youssef, E. M., Chen, X. Q., Higuchi, E., Kondo, Y., Garcia-Manero, G., Lotan, R., and Issa, J. P. (2004) *Cancer Res.* **64,** 2411–2417
36. Harlow, E., and Lane, D. (1988) *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY

[3] Docquier, F., Farrar, D., D'Arcy, V., Chernukhin, I., Robinson, A. F., Loukinov, D., Vatolin, S., Pack, S., Mackay, A., Harris, R. A., Dorricott, H., O'hare, M. J., Lobanenkov, V., and Klenova, E. (2005) *Cancer Res.* **65,** 5112–5122.

[4] V. Torrano and F. Docquier, unpublished observations.

37. Mukhopadhyay, R., Yu, W., Whitehead, J., Xu, J., Lezcano, M., Pack, S., Kanduri, C., Kanduri, M., Ginjala, V., Vostrov, A., Quitschke, W., Chernukhin, I., Klenova, E., Lobanenkov, V., and Ohlsson, R. (2004) *Genome Res.* **14,** 1594–1602
38. Gomez-Casares, M. T., Delgado, M. D., Lerga, A., Crespo, P., Quincoces, A. F., Richard, C., and Leon, J. (1993) *Leukemia* **7,** 1824–1833
39. Canelles, M., Delgado, M. D., Hyland, K. M., Lerga, A., Richard, C., Dang, C. V., and Leon, J. (1997) *Oncogene* **14,** 1315–1327
40. Mutskov, V., and Felsenfeld, G. (2004) *EMBO J.* **23,** 138–149
41. Garrick, D., Sutherland, H., Robertson, G., and Whitelaw, E. (1996) *Nucleic Acids Res.* **24,** 4902–4909
42. Jones, L. K., and Saha, V. (2002) *Br. J. Haematol.* **118,** 714–727
43. Karpf, A. R., and Jones, D. A. (2002) *Oncogene* **21,** 5496–5503
44. Richards, E. J., and Elgin, S. C. (2002) *Cell* **108,** 489–500
45. Ceballos, E., Delgado, M. D., Gutierrez, P., Richard, C., Muller, D., Eilers, M., Ehinger, M., Gullberg, U., and Leon, J. (2000) *Oncogene* **19,** 2194–2204
46. Delgado, M. D., Vaque, J. P., Arozarena, I., Lopez-Ilasaca, M. A., Martinez, C., Crespo, P., and Leon, J. (2000) *Oncogene* **19,** 783–790
47. Lewis, J. L., Chinswangwatanakul, W., Zheng, B., Marley, S. B., Nguyen, D. X., Cross, N. C., Banerji, L., Glassford, J., Thomas, N. S., Goldman, J. M., Lam, E. W., and Gordon, M. Y. (2001) *Blood* **97,** 2604–2610
48. Kang, C. D., Do, I. R., Kim, K. W., Ahn, B. K., Kim, S. H., Chung, B. S., Jhun, B. H., and Yoo, M. A. (1999) *Exp. Mol. Med.* **31,** 76–82
49. Munoz-Alonso, M. J., Acosta, J. C., Richard, C., Delgado, M. D., Sedivy, J., and Leon, J. (2005) *J. Biol. Chem.* **280,** 18120–18129
50. Cantor, A. B., and Orkin, S. H. (2002) *Oncogene* **21,** 3368–3376
51. Perry, C., and Soreq, H. (2002) *Eur. J. Biochem.* **269,** 3607–3618
52. Ponka, P. (1999) *Am. J. Med. Sci.* **318,** 241–256
53. Bieker, J. J. (1998) *Curr. Opin. Hematol.* **5,** 145–150
54. Zhang, R., Burke, L. J., Rasko, J. E., Lobanenkov, V., and Renkawitz, R. (2004) *Exp. Cell Res.* **294,** 86–93