Increased Expression of the ETS-related Transcription Factor FLI-1/ERGB Correlates with and Can Induce the Megakaryocytic Phenotype

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Abstract
The human leukemia cell line K562 can be induced by 12-O-tetradecanoylphorbol-13-acetate (TPA) to differentiate along the megakaryocytic pathway, generating morphological changes and increased expression of lineage-specific surface markers. We report that TPA-treated K562 cells also express higher levels of FLI-1/ERGB, a member of the ETS family of transcription factors. Furthermore, introduction of a retroviral construct expressing human FLI-1/ERGB into K562 cells induces changes similar to those seen following TPA treatment, including increased adherence to the surface of the culture vessel and altered size and morphology. Infected cells exhibit higher levels of the megakaryocyte marker CD41a and, to a lesser extent, CD41b. These markers, as well as virally encoded FLI-1/ERGB-specific RNA and protein, are expressed at the highest levels in the attached cell population, while the growth rate of adherent cells is reduced, and the fraction of cells in G0-G1 is increased. FLI-1/ERGB virus-infected cells also exhibit increased expression of hemoglobin, a marker of erythroid differentiation. Our results suggest FLI-1/ERGB plays a role in controlling differentiation and gene expression along the megakaryocytic/platelet pathway, and further implicate ETS-related genes in the control of multiple developmentally regulated hematopoietic genes.

Introduction
The ETS genes make up a family of transcription factors (1–3) which share a related DNA-binding domain and recognize the core... GGA/G. The ETS prototype gene was initially identified through analysis of the avian acute leukemia virus E26 (4, 5), which contains a fusion of the chicken homologue of the ETS gene to a part of c-myc (6, 7). V-ets was responsible for the capacity of the gag-myb-ets fusion oncogene of E26 to induce erythroid leukemia in birds (8, 9), and when expressed in a murine vector this fusion can also induce erythroid leukemia in mice (10, 11) and erythroid differentiation of murine cells in culture (12, 13) in an ets-dependent fashion. In animal models, ETS genes are most frequently associated with hematopoietic disease. The ETS-related gene fl1-1 is insertionaly activated in 75% of the erythroleukemias induced in mice by Friend MuLV (14, 15) while another family member, Pu-1/Spi-1, is insertionaly activated during spleen focus-forming virus-induced erythroleukemias (16, 17). Fl1-1 has also been shown to be insertionaly activated in primitive stem cell tumors induced by the 10A1 strain of MuLV (18), suggesting it may play a role in other types of hematopoietic oncogenesis. In humans, FLI-1, along with ERG and other ETS-related genes, have been shown to be involved in gene fusions associated with Ewing's sarcoma (19–23).

Studies have also linked ETS genes to normal hematopoietic functions, including T-cell, B-cell, monocyte, and granulocyte differentiation (24–26), and the regulation of T-cell receptor (27), immunoglobulin μ heavy chain (28), and interleukin 2 receptor (29, 30) gene expression. Analysis of both normal and abnormal cell functions has also suggested a role for ETS family members in megakaryocyte/platelet differentiation. Friend's murine erythroleukemia cells have been shown to be inducible for megakaryocytic marker expression (31), and the EBS, frequently associated with sites specific for the GATA-1 and SCL factors (32), has been identified in several megakaryocyte-specific genes (33–38). ETS1 has been shown to activate transcription driven by the megakaryocyte-specific membrane glycoprotein gpIIb promoter (33, 34), and the platelet glycoprotein Ibbα promoter (35) and the EBS in these promoters have been shown to be important for their tissue-specific expression. EBS sequences are also found in a number of other megakaryocytic-specific gene promoters, including c-mpl (36), rat platelet factor 4 (37), and platelet glycoproteins V and IX (38), supporting the hypothesis that one or more ETS transcription factors play a role in regulation transcription of megakaryocytic and platelet-specific genes.

To analyze the potential role of specific ETS genes in hematopoietic differentiation, we have developed a series of
drug-selectable retroviral vectors expressing ETS1, ETS2, and FLI-1/ERGB to generate stable cell lines and measure the effect of overexpression on cell differentiation and growth. The human leukemia-derived cell line K562 (39) is a well-characterized cell line in which the potential to differentiate down multiple pathways (40–43) has led to its extensive use as an in vitro model, particularly for erythroid and megakaryocytic differentiation. Here, we report that TPA treatment of K562 cells, which is known to induce megakaryocytic differentiation, induces increased levels of FLI-1/ERGB RNA. Furthermore, the infection of K562 cells with a FLI-1-expressing virus induces dramatic morphological changes and changes in the levels of two megakaryocytic/platelet-specific surface markers [CD41a(gpib/IIia) and CD49b (an α2 integrin)]. FLI-1/ERGB-infected cells also exhibit increased benzidine staining, indicative of increased levels of hemoglobin expression, and exhibit growth arrest in G0-G1. These results support the hypothesis that FLI-1 plays a role in megakaryocyte/platelet and erythroid differentiation and in the control of genes associated with these lineages.

Results

TPA Treatment of Uninfected K562 Cells Induces Elevated Expression of Endogenous FLI-1 RNA. The differentiation-specific response of the pluripotent human erythroleukemia-derived cell line K562 to a variety of agents has been well characterized, and TPA-treated cells are known to exhibit many of the characteristics of cells differentiating along the megakaryocyte/platelet pathway (41, 42). To evaluate the possible involvement of known transcription factors in this process, we examined the effect of TPA treatment on expression of several members of the ETS gene family (ETS1, ETS2, and FLI-1). We observed that only FLI-1-specific RNA expression appeared to be reproducibly increased following TPA treatment. Although it had been reported that K562 cells do not express FLI-1 RNA as detected by Northern blot analysis (44, 45), we could detect extremely low levels of FLI-1-specific RNA in normal K562 cells (Fig. 1, Lane 1), and this level was significantly increased in cells induced to differentiate with TPA (Fig. 1, Lanes 2 and 3). TPA treatment of K562 cells rapidly induces a large fraction of the cell population to adhere to the surface of the culture vessel and to extend cellular processes and develop ruffled membranes. This adherent population expressed a higher level of FLI-1 RNA than the nonadherent fraction (Fig. 1, Lane 3 versus Lane 2). RNA levels in the three lanes were identical based on rehybridization of the filter with a G6PDH-specific probe (Fig. 1B) and on the level of staining of the transferred rRNA (data not shown). Analysis of FLI-1 expression as a function of time of exposure to TPA showed that the highest level of expression is seen after 1–2 days in the attached population of TPA-treated cells. This time course is similar to the time necessary for the appearance of attached, morphologically altered cells and is consistent with the hypothesis that elevated FLI-1 expression plays a role either causally or as a consequence of the induction of differentiation-associated markers, cell attachment, and morphological changes seen in TPA-induced K562 cells.

Infection of K562 Cells with FLI-1-expressing Retroviral Construct Induces Changes in Cell Morphology and Growth. We had previously observed that infection of murine hematopoietic cell lines with retroviral constructs expressing ETS oncogene fusions could induce a variety of phenotypic changes in growth and the expression of lineage-specific markers (10, 11, 13). When we infected K562 cells with ETS1-, ETS2-, and FLI-1/ERGB-expressing viruses, we observed that although viruses expressing ETS1 induced changes in the expression of erythroid-associated markers without any alteration in cell attachment or morphology, only FLI-1/ERGB induced dramatic morphological changes in K562 cells in culture. A significant fraction of the G418-resistant cells infected with the drug-selectable FLI-1-expressing virus attached to the surface of the culture dish and developed long, fibroblast-like processes (Fig. 2D), whereas vector-infected, drug-selected control cells (Fig. 2A) showed no visible changes. Many of the virus-infected attached cells assumed a flat morphology, were noticeably larger than control K562 cells, and contained multiple nuclei and a great many vacuole-like spaces within their cytoplasm (Fig. 2C). This change in cell morphology, with long branching processes and multiple nuclei, is especially apparent when normal and infected cells stained with Giemsa are compared (Fig. 3A versus C and D). These attached cells resembled cells seen in K562 cell cultures treated with TPA (Fig. 2B), but FLI-1-infected cells showed a much higher percentage of attached, process-forming cells, and the length of the processes and other morphological features were much more pronounced (Fig. 2D versus B).

A significant fraction of FLI-1-infected K562 cells remained in suspension, but this nonadherent population also appeared to be morphologically altered in comparison to control cells. Analysis of stained cytopsins indicated that in comparison to vector-infected, drug-selected controls (Fig. 3A), FLI-infected cells, which continued to proliferate in suspension, were larger, and many of the cells contained vacuoles as well as morphologically aberrant or multiple nuclei (Fig. 3B).

The dramatic morphological changes seen in FLI-1-infected cells was not a property of a subset of the K562 cell population, since 12 of 12 individual K562 clones gave identical responses following FLI-1 virus infection (data not shown). Each infected clone generated the array of morphologies seen in the uncloned parental cell stock. Furthermore, when infected G418-resistant cells were plated in 96-well plates at approximately 1 cell/well, each well that contained growing cells generated cultures containing all of the morphological types of cells seen in mass culture. Attached cells, detached from the substrate with EDTA, would reattach and continue to proliferate slowly, again generating a mixed culture of attached and floating cells. Although attached cells grew slowly and were difficult to propagate as a pure population, floating cell populations proliferated normally and continued to generate attached colonies even after 3 months in culture, although the fraction of the population exhibiting the attached phenotype decreases with continued culture.

Although cells infected with the FLI-1-expressing virus resembled K562 cells treated with TPA, in the latter case the morphological changes were less pronounced. Nevertheless, the TPA-treated cells were larger than their untreated controls and exhibited multiple or irregular nuclei, vacuoles, and increased blebbing. The similarities between TPA-treated and FLI-1-infected K562 cells suggested that common molecular pathways might be activated in the two cell populations.

**FLI-1-infected Cells Show Elevated Levels of Cell Surface Markers Associated with Megakaryocytic/Platelet Differentiation.** Since TPA-induced and FLI-1-infected K562 cells exhibited similar morphological responses, we asked whether FLI-1 expression could induce analogous
molecular changes. Phorbol esters are known to induce K562 and other established human cells to express elevated levels of megakaryocytic/platelet surface markers (46). Flow cytometric analysis of control vector and FLI-1-expressing K562 cells showed that although the expression of CD14, a monocytic marker, is not altered, expression of CD49b and particularly CD41a is significantly increased (Table 1). We also see similar effects on the levels of CD61 (gpIIb) in infected cells (data not shown). The level of CD41a and CD49b markers is even higher in the attached FLI-1-expressing population, correlating with the higher expression of FLI-1 seen in attached cells. Consistent with previous reports, TPA treatment of K562 cells induced increased expression of both markers, but the level seen in FLI-1 virus-infected adherent cells is at least 2–4-fold higher than that seen in the adherent TPA-treated population (Table 1).

**Cell Attachment Correlates with Higher Expression of FLI-specific RNA and Protein.** Since FLI-expressing virus infection induced a fraction of the normally nonadherent K562 cells to attached to the substrate, we compared the properties of the attached and nonattached cells in detail. As can be seen in Fig. 4A, the level of expression of viral RNA was 2–3-fold higher in the attached than in the nonattached cell population in comparison to the levels of β-actin RNA probed on the same blot (Fig. 4B) and stained levels of rRNA seen by direct staining of the filters after transfer (data not shown). The higher level of FLI-specific RNA was consistent with a similar higher level of FLI protein observed in Western blot analysis of cell extracts from the two populations of cells (Fig. 4C). No FLI-1 protein could be detected in control K562 cells infected with the empty vector construct (Fig. 4C, Lane 3). In contrast, FLI-1 protein was easily detectable in both the nonadherent and adherent cell populations (Fig. 4C, Lanes 4 and 5), but the levels of FLI-1 protein in the infected adherent cell population were higher than the nonadherent and was similar to that seen in KG1 cells (Fig. 4C, Lane 1), a human

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*Representative result from six independent analyses.
myeloid cell line known to express high levels of FLI-1 mRNA (45) and protein. The levels seen in attached cells raised the possibility that attachment and the high level of FLI protein could be causally related.

**FLI-1 Overexpression also Induces Expression of Erythroid-specific Genes.** The erythroid and megakaryocyte/platelet lineages are thought to share a common precursor cell (47), and K562 cells are known to be pluripotent and to respond to specific inducers to express either erythroid, macrophage, or megakaryocytic markers (40-42). Although FLI-1 overexpression clearly induces strong morphological and surface marker changes characteristic of a megakaryocytic/platelet response, we also observed that both the adherent and nonadherent populations exhibited an increase in the fraction staining positively with benzidine, indicative of hemoglobin production (Fig. 5). Strongly positive cells of all morphological types can be seen in the FLI-1-expressing populations, whereas vector and uninfected K562 cells show low background levels of positive cells. In the experiment shown in Fig. 5, 88% of the cells were positive for CD41a, whereas 20% were positive for benzidine staining, suggesting that at least some of the cells may express both megakaryocytic- and erythroid-specific markers.

**G₀-G₁ is Extended in FLI-1-expressing Adherent Cells.** Evidence has suggested that alteration of the cell cycle induces, or is associated with, differentiation of K562 cells down specific hematopoietic pathways (46). Analysis of nonattached FLI-1-infected, vector-infected, and uninfected control cells indicated that the rate of cell division of these cell populations was identical (data not shown). Consistent with this, fluorescence-activated cell sorting analysis of propidium iodide-labeled cells was also identical in uninfected (data not shown), vector, and nonattached FLI-1-infected populations (Fig. 6, A and B). In contrast, attached FLI-1-infected cells showed a decrease in the number of cells in the S-phase and a corresponding increase in the G₀-G₁ population (Fig. 6C). The change represented a 2.5-fold increase in the fraction of cells in G₀-G₁ (see "Materials and Methods") and was consistent with the apparent slow rate of proliferation in the attached cell population. Thus, cells expressing the highest levels of FLI-1 and exhibiting the most differentiated phenotype also exhibited the highest proportion of cells in G₀-G₁.

**Discussion**

Our results demonstrate a strong association between expression of the FLI-1/ERGB oncogene and expression of the megakaryocytic phenotype. Treatment of the pluripotent human leukemia-derived K562 cell line with TPA, a known inducer of megakaryocytic differentiation, induces increased levels of FLI-1/ERGB expression. Furthermore, infection of K562 cells with a retroviral construct expressing the human FLI-1 gene induces similar morphological and lineage-specific responses. These include increased adherence of infected cells to the plastic culture dishes and increased expression of two megakaryocytic surface markers, CD41a (the gp11b/IIa complex) and CD49b (α integrin). These results support previous work suggesting a role for members of the ETS family in regulating the expression of megakaryocyte-specific genes (33, 35). In addition, FLI-1 virus-infected cells, unlike TPA-treated cells, show an increased frequency of benzidine-positive cells, indicating an increase in hemoglobin, an erythroid-specific marker. ETS family members, particularly ETS1, have been linked to the regulation of erythroid differentiation (48), and our observation in K562 cells expressing high levels of FLI-1/ERGB are consistent with the known linkage between the erythroid and megakaryocytic lineages as well as the common features of the ETS family of transcription factors.

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The morphological changes, increased cell adherence, and increased CD41a levels seen in FLI-1 virus-infected cells resemble the response of K562 cells to the phorbol ester TPA and are consistent with activation of a megakaryocytic- or platelet-specific response. The fact that endogenous FLI-1 expression is increased when K562 cells are induced with TPA further supports a link between the megakaryocytic phenotype and FLI-1. This is also consistent with the obser-
vation that in virus-infected or TPA-treated cells the highest expression levels of FLI-1 are seen in the attached population.

Analysis of the promoter sequences of megakaryocyte/platelet-associated genes has indicated that an EBS is frequently present. ETS-binding sequences are found in the promoters of GpIIb (33), murine c-mpl (36), rat platelet factor 4 (32), and platelet glycoproteins V and IX, two components of the von Willebrand factor receptor (38). It has been shown that these binding sequences are required for the maximum expression of GpIIb, which is specific for platelets and mature megakaryocytes and serves as a model of megakaryocyte-specific gene expression (33, 34). Interestingly, FLI-1 has been shown to bind to this site more efficiently than ETS1 (49), consistent with our data that FLI-1 is a more effective inducer of GpIIb/IIIa. We observed that the retroviral vector induced overexpression of ETS1 in K562 cells had minimal effects on the level of the CD41a marker and did not alter cell morphology. Our data show that ectopic expression of FLI-1 is a potent inducer of the GpIIb/IIIa complex (CD41a) and suggest that FLI-1 may be the ETS gene involved in the transcriptional up-regulation of certain genes in megakaryocytes and platelets. A repressor domain in the promoter of the GPIIb/IIIa gene has been identified (50, 51), and it has been suggested that this domain plays a critical role in lineage-specific, differentiation-dependent expression. Our data suggest that FLI-1 overexpression must be able to overcome the effects of this repressor in K562 cells in the absence of other inducers of megakaryocytic differentiation. Alternatively, high levels of FLI-1 in infected cells may induce down-regulation of the repressor itself or induce factors which block its action and allow the elevated expression of the GPIIb/IIIa complex. Interestingly, overexpression of FLI-1 in transgenic mice, under control of a H-2Kβ promoter, alters normal lymphoid cell function and apoptosis but does not appear to affect the erythroid lineage (52). The effects of FLI-1 will likely depend on both the site and the timing of expression, as well as the level; therefore, it will be of interest to analyze mice in which FLI-1 is controlled by a megakaryocyte-specific promoter.

Although our data support a role for FLI-1 in the megakaryocyte/platelet lineage, we also observed an increase in the fraction of benzidine-positive cells in the FLI-1-expressing population, reflecting an increased erythroid character. This is consistent with the fact that activation of FLI-1 expression in vitro is associated with erythroid diseases in animal model systems (15, 49). ETS genes are known to act either positively or negatively on the cell cycle (13, 53–56), and FLI overexpressing adherent cells exhibit evidence of a block in normal cell cycle progression, whereas nonadherent cells show no evidence of cell cycle alterations, despite a similar level of FLI-1 expression. Since both populations exhibit elevated levels of benzidine staining, it is unlikely that induction of the erythroid phenotype is exclusively due to changes in the cell cycle, and the different effects on the cell cycle observed in adherent and nonadherent cells could be due to the different levels of FLI-1 expression observed in the two populations. Since evidence has linked ETS1 to the regulation of the erythroid lineage (48), it is possible that the induction of erythroid markers by FLI-1 also occurs through the ETS1 pathway as the result of abnormally high levels of FLI-1 in these cells. The level of FLI-1 is lower in TPA-treated cells, and no increase in benzidine staining is seen in TPA-treated K562 cells. Furthermore, TPA treatment of FLI-expressing cells reduced the level of benzidine staining to control levels. The mechanism by which TPA prevents the FLI-induced increase in benzidine staining is unknown, but could suggest novel interactions between the megakaryocyte and erythroid pathways. Early erythroid precursors express the GPIIb/IIIa antigen, and a number of leukemic cell lines such as HEL express markers of both lineages (57), suggesting that common factors may control or allow the expression of markers of both lineages in some circumstances.

Although a number of leukemia-derived cell lines are known to be inducible by TPA to differentiate along the megakaryocyte/platelet lineage (58–62), the association of FLI-1 expression in K562 with the induction of megakaryocytes differentiation could also be a specific property of the erythroleukemia-derived K562 cell line itself. We have pre-

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**Fig. 6.** Flow cytometric profiles of DNA of vector and FLI-1 virus-infected K562 cells. The fraction of the total cells in various compartments of the cell cycle in each cell population was calculated from the profiles and are shown in each panel.
liminary results indicating that increased FLI-1 expression in HEL cells can also increase the expression of megakaryocytic markers. The response of K562 cells to TPA and to retrovirally induced overexpression of FLI-1 is consistent with the hypothesis that FLI-1 induces the expression of specific genes during megakaryocyte/platelet differentiation, but at this time we cannot rule out the possibility that some other unidentified member of the ETS family gene functions during normal differentiation. Analysis of other megakaryocytic cell lines, primary cells, and further studies utilizing either regulatable or mutant forms of FLI-1 should help to further define the role of FLI-1 in both megakaryocyte and erythroid differentiation.

Materials and Methods

Cells, Viruses, and Induction of Differentiation. K562 cells were purchased from American Type Culture Collection and maintained in RPMI 1640 supplemented with 10% FCS. Differentiation induction experiments were performed by culturing K562 cells in the presence of 25 nM TPA for 48 h. The construction of the drug-selectable retroviral construct expressing FLI-1/ERGB (44) will be described elsewhere.5 Virus stocks were prepared from PA317 packaging cells (63) infected with supernatant from ecotropic CRE packaging cells (64) transiently transfected with the retroviral constructs. K562 cells infected with PA317-grown stocks were selected by growth in media containing 800 μg/ml Geneticin (G314; Life Technologies) starting 24–48 h after infection.

Cell Cycle Analysis. Cells (106) were washed with PBS and analyzed using the Thorsenwaite procedure (65). Briefly, 106 cells were suspended in 0.5 ml of detergent buffer and stained in the presence of 50 μg/ml propidium iodide + 10 μg/ml RNase for 2 h at room temperature in the dark.

Surface Marker Analysis. Cell surface antigens were examined by the indirect immunofluorescence methods using flow cytometry. FITC-conjugated monoclonal antibodies specific for the cell surface markers CD14, CD41a, and CD49b were obtained from Pharmingen. Cells (106) were washed with PBS + 2% calf serum and incubated for 45 min at 4°C in the dark with the antibodies. After washing three times with PBS + serum, they were analyzed using flow cytometry at 510–550 nm.

RNA Preparation and Analysis. Total RNA was purified using RNazol [acid-guanidinium thiocyanate-phenol (86)] according to the manufacturer's specifications. RNAs (10 μg/lane) were separated electrophoretically on 1.2% agarose gels containing 2.2 μM formaldehyde, transferred to nylon membranes (New England Nuclear), fixed by UV cross-linking (Life Technologies), and analyzed by hybridization using specific probes prepared by random priming (Life Technologies) using [32P]dCTP (New England Nuclear). Hybridizations were performed at 65°C overnight in 0.5 x SSC buffer (pH 7) containing 7% SDS, 5 mM EDTA, and 1% BSA. Filters were washed three times (20 min each) at room temperature in a solution containing 1% SDS, 40 mM NaOH, and 1 mM EDTA that was prewarmed at 65°C. Probes were removed before rehybridization by boiling the filters for 10 min in 0.1% SDS, 10 mM NaOH, and 1 mM EDTA. Probes were prepared from fragments from recombinant clones containing sequences specific for β-actin, G6PDH, or the Aval-Hindll fragment of FLI-1/ERGB (44).

Western Blotting. Cells were lysed in RIPA buffer in the presence of 1% phenylmethylsulfonyl fluoride, 10 μM pepstatin, and 100 units/ml aprotinin. Lysates were sonicated for 10 s and clarified by centrifugation (13,000 rpm for 30 min at 4°C). The protein concentration of supernatants was determined with the Bio-Rad assay (Bio-Rad Laboratories). Twenty μg of each protein extract were separated on 8% Tris-glycine gel (Novex) and transferred to nitrocellulose paper (Schleicher and Schuell) using an electrophoretic transfer apparatus ( Hoefer). Membranes were incubated for 2 h with FLI-1/ERGB-specific antibody (87) and then with horseradish peroxidase-labeled secondary antibody (Amersham). This antibody detects a single species of FLI-1 by Western blot, but multiple species can be detected by immunoprecipitation of labeled extracts.7 The antibody was detected by using the Enhanced Chemiluminescence Detection System (Amersham). Lysates from KG-1 and NIH3T3 cells were used as a positive and negative control for the expression of the FLI-1/ERGB protein. The blots were stripped by incubating at 50°C for 30 min in a buffer containing 100 μM β-mercaptoethanol, 2% SDS, and 62.5 mM Tris-HCl (pH 6.7) and reprobed with RFA1-specific antibody to confirm that the levels of total protein in each lane were equivalent.

Cytochemical Assays. For assessment of morphology, cells grown on Lab-Tek chamber slides (Nunc) or transfected onto slides by cyto centrifugation were dried, fixed with methanol, and stained with Wright-Giemsa stain (Sigma).

Hemoglobin expression was detected using acid benzidine staining (67, 68). Three μl of 30% hydrogen peroxide were mixed with 500 μl of benzidine dihydrochloride (Sigma), and 100 μl of this benzidine solution were added to attached cells or to an equal volume of cell suspension. The mixture was incubated at room temperature for 3 to 5 min, and the percentage of benzidine-positive cells was determined by counting a minimum of 500 cells.

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References


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7 X. Zhang, unpublished observations.