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Inhibition of hematopoietic development from embryonic stem cells by antisense vav RNA

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The vav proto-oncogene is universally and specifically expressed in hematopoietic cells. vav contains a unique array of motifs allowing the protein to function as a signal transducer and possibly as a transcription factor. Under certain *in vitro* culture conditions murine embryonic stem cells develop into colonies containing multiple hematopoietic lineages. In embryonic stem cell lines, constitutively expressing high levels of antisense vav transcripts through a stably integrated transgene, differentiation into hematopoietic cells is disrupted. This observation presents the first evidence that vav has a critical role in the development of hematopoietic cells from totipotent cells.

Key words: antisense RNA/embryonic stem cells/hematopoiesis/vav proto-oncogene

Introduction

The vav proto-oncogene is restricted in its expression to the hematopoietic system (Katzav *et al.*, 1989). Within this system it is expressed in all lineages regardless of the stage of differentiation.

Structurally vav contains features of both a transcriptional factor and a signal transduction molecule. It has a helix-loop-helix domain in the N-terminus, two SH2 and one SH3 domain in the C-terminus, a leucine zipper-like region also in the C-terminus, and an internal dbl-homologous sequence (Katzav, 1992; Puil and Pawson, 1992). Deletion of the helix-loop-helix domain converts vav into a transforming oncogene (Coppola *et al.*, 1991). vav is also rapidly phosphorylated on tyrosine residues in response to various ligands (Bustelo *et al.*, 1992; Margolis *et al.*, 1992) and has GDP/GTP exchange activity on ras in activated T cells (Gulbins *et al.*, 1993). Therefore it has been suggested that vav is a member of a new class of mammalian signal transduction molecules (Puil and Pawson, 1992). However, nothing is known yet about the significance of vav for the development of hematopoietic stem and progenitor cells. Murine embryonic stem (ES) cells are totipotent cells capable of developing into all tissues, including hematopoietic cells. *In vitro* culture techniques have been developed which permit differentiation of hematopoietic cells from ES cells (Schmitt *et al.*, 1991; Wiles and Keller, 1991; Chen, 1992; Keller *et al.*, 1993; Lelias *et al.*, 1993; McClanahan *et al.*, 1993). Murine ES cells cultured in semi-solid methyl cellulose medium give rise to discrete colonies containing mixed hematopoietic lineages by day 8-10 of culture. Erythroid and myeloid cells are easily recognized by inverted light microscopy in at least 70-90% of the colonies. Replating these colonies in secondary methyl cellulose cultures shows that the development of erythroid, multilineage, macrophage and mast cell progenitors occurs in a well-defined temporal order (Schmitt *et al.*, 1991; Wiles and Keller, 1991; Keller *et al.*, 1993). The expression of genes related to hematopoietic cells is also activated sequentially, similar to *in vivo* ontogenesis (Lindenbaum and Grosveld, 1990; Whitelaw *et al.*, 1990; Keller *et al.*, 1993). Simon *et al.* (1992) were able to demonstrate that in colonies derived *in vitro* from ES cells with a GATA-1 null mutation, obtained by homologous recombination, erythropoiesis is abrogated. The same effect is observed in chimera generated by the ES lines harboring the null mutation of the GATA-1 gene (Pevny *et al.*, 1991). In this paper we have sought to utilize the *in vitro* ES cell differentiation system to examine the consequences of disrupting vav expression through antisense blocking. Blocking of vav was achieved by stable transfection of a transgene expressing antisense vav transcripts from the constitutively active phosphoglycerate kinase (pgk) promoter (Adra *et al.*, 1987) in ES cells.

Results

Expression of vav in ES-derived colonies

The expression of vav was studied over time in the developing embryoid bodies by Northern blot analysis of total RNA derived from pooled colonies. Figure 1 relates the expression of vav to that of other hematopoietic genes. vav can be detected from day 10 onwards, earlier than the myeloid antigen MAC-1 (day 12), but later than the erythroid proteins embryonic and adult globin. However, because only a subset of the cells in the developing embryoid bodies is hematopoietic, a small number of cells expressing vav or a low level of vav expression might go undetected by Northern blot analysis. Therefore, the RNA was analyzed for vav by the more sensitive reverse transcriptase PCR (RT-PCR) method. Figure 2 shows that the vav transcript is already present at a low level in undifferentiated ES cells. A detectable transient upregulation is observed consistently in as early as day 2 colonies. A second marked and sustained increase is seen around day 9 (Figure 2), corresponding closely to the initial detection by Northern analysis.

Derivation of ES lines expressing antisense vav

The pgk promoter has proven to be effective in achieving high level expression of transgenes in a wide range of mammalian cells, including embryonic cells and hematopoietic stem cells (Lim *et al.*, 1989). Therefore an antisense expression vector was constructed by replacing the neo cDNA cassette in the pgk-neo vector (Adra *et al.*, 1987) with two-thirds of the 5′ end of the murine vav cDNA in reverse orientation downstream of the pgk promoter. Vector DNA was cotransfected with pgk-neo into ES cells (CCE

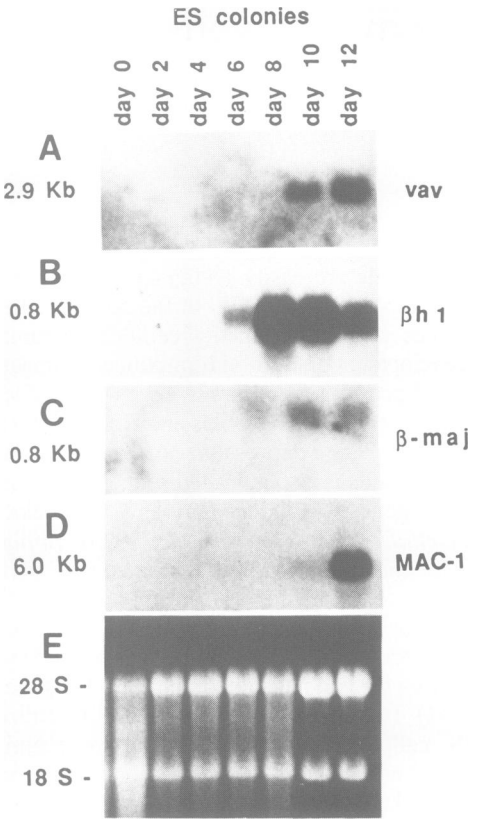

Fig. 1. Expression of vav in relation to other hematopoietic genes during ES cell differentiation. Total RNA was isolated from the developing embryoid bodies at the time in culture indicated. Northern blot analysis was performed for vav (**A**), embryonic (**B**) and adult (**C**) globin and MAC-1 (**D**). (**E**) shows the corresponding ethidium bromide picture.

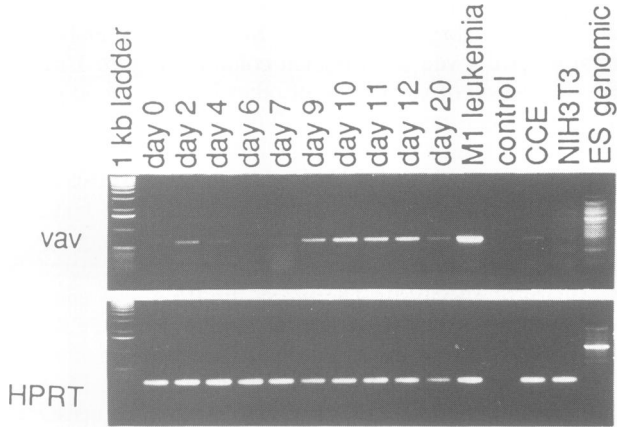

Fig. 2. Expression of vav during ES cell differentiation as analyzed by RT-PCR. The PCR was performed at 30 cycles in the linear range of amplification. The specificity of the PCR products was confirmed by Southern blot analysis, where the control (RT reaction without RNA), the NIH3T3 cells and the genomic ES cell DNA were negative. Phosphorimager analysis of the Southern filter confirmed an equal amount of the PCR product for HPRT as visible in the ethidium bromide stain.

line) and selected for G418 resistant clones. Several ES clones stably expressing antisense vav were derived. Figure 3 shows the Northern analysis of these lines, reflecting the range of high to low vav antisense transcript expression as compared with the endogenous vav mRNA

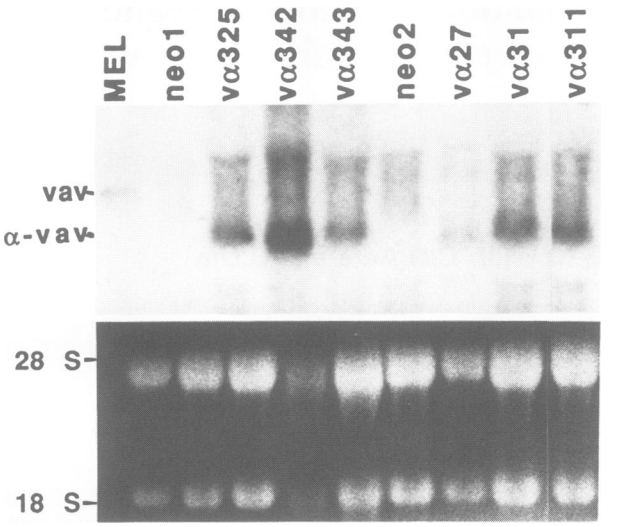

Fig. 3. vav antisense expression in single G418-resistant ES cell clones. ES cells were transfected with either the pgk-α-vav and pgk-neo (10:1 molar ratio) or the pgk-neo vector alone. Selection was performed with G418 at concentrations of 500 μg/ml for clones vα27, vα31, vα311 and vα325, and 2 g/ml for clones vα342 and vα343. A total of 70 single cell clones was analyzed. The figure is a composition of the positive clones. The level of vav mRNA in the hematopoietic cell line MEL was used as a comparison for levels of antisense transcripts generated in individual clones.

level in the hematopoietic cell line MEL (murine erythroleukemic line). Wild-type ES cells or G418 resistant lines transfected only with the pgk-neo DNA were used as controls.

Reduction in frequency of colonies with visible hematopoiesis

Maintained in the presence of leukemia inhibitory factor (LIF), antisense clones and those transfected only with the pgk-neo DNA (from now on referred to as neo control) remain as undifferentiated ES cells. Upon transfer into differentiation conditions in methyl cellulose, the development of ES colonies was monitored by inverted light microscopy. The total number of colonies and the number of colonies with visible erythropoiesis were counted on day 12 of culture. It was immediately evident that there is a morphological difference between colonies derived from neo control ES cells and those expressing antisense vav. Figure 4 contrasts the typical colonies seen in both instances. In normal ES or neo control colonies erythroid and non-erythroid hematopoietic cells were consistently detected in >70% of colonies. These colonies usually have a loose structure and easily disaggregate into single cells (Figure 4A). In contrast, colonies generated from all ES lines expressing antisense vav were mainly tight embryoid bodies with no visible hematopoiesis (from now on referred to as antisense colonies; Figure 4B). A quantitative assessment (Figure 5) demonstrates that the plating efficiency, i.e. the ability to develop into embryoid bodies, was similar for neo control and antisense ES lines. However, a significant reduction in the percentage of erythropoiesis positive colonies was found in cultures of all antisense-expressing ES lines, from 22% (clone vα325) to 3% (clone vα342) as opposed to 73% in the neo control. Figure 5 also shows that there is a positive correlation between the degree of suppression of hematopoiesis and the level of antisense vav transcripts expressed.

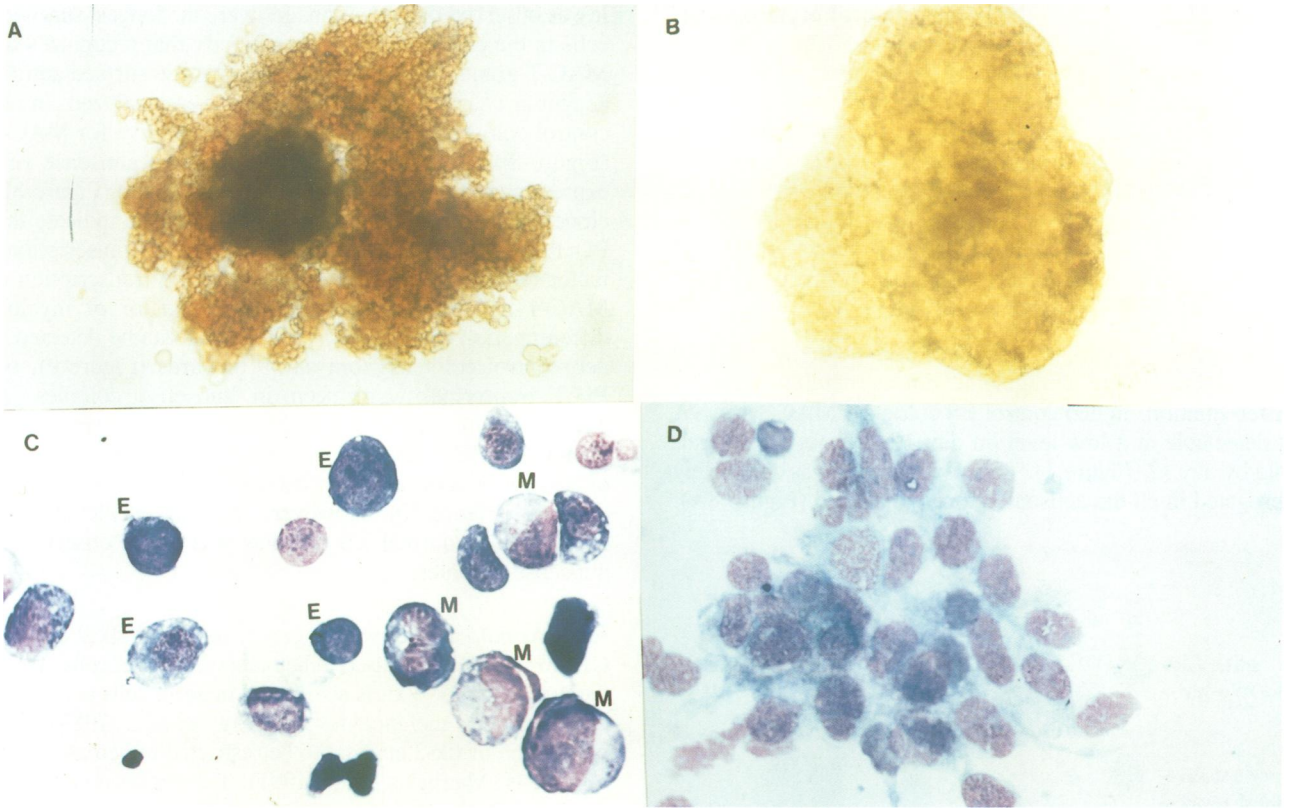

Fig. 4. Morphology and cytology of ES cell colonies derived from neo control ES cells (**A**, **C**) or clone vα342 (**B**, **D**). Pictures were taken on day 12 of the development of embryoid bodies either *in situ* under an inverted microscope (20× magnification) (**A**, **B**) or from a smear prepared from colonies and stained with Wright–Giemsa (63× magnification) (**C**, **D**). (**A**) Typical neo control colony with a mixture of erythroid and myeloid cells loosely proliferating beyond ill-defined boundaries of the colony. (**B**) Typical antisense colony, derived from clone vα342, with sharply defined borders and no evidence of hematopoiesis. (**C**) Wright–Giemsa stain of a smear prepared from a colony similar to (**A**) showing erythroid (labeled E) and myeloid cells (labeled M) at different stages of differentiation. (**D**) Wright–Giemsa stain of an antisense colony derived from clone vα342. The rather homogenous population of undefined epitheloid cells tends to remain in tight clumps.

Cytology of neo control and antisense-derived ES colonies

The genesis of hematopoietic cells during the development of the embryoid bodies was followed by examination of cytospin preparations. Figure 4C shows the typical presence of a heterogeneous population of erythroid and myeloid cells in day 12 neo control colonies at different stages of maturation. By contrast, antisense colonies at day 12 contained largely undefined fibroblastoid cells (Figure 4D).

Evidence for the suppression of vav expression during in vitro differentiation

Colonies at different days after initiation of culture were harvested to obtain total RNA for Northern analysis. This time course experiment was performed for the clones vα325, vα342 and vα343. Northern blot analysis confirmed that expression of antisense transcripts from the transgene was maintained throughout the entire culture period (Figure 6). In contrast to normal colonies, where vav transcripts are clearly seen from day 10 onwards (Figure 1), little or no vav transcripts were observed in antisense colonies.

Suppression of hematopoiesis as measured by lineage markers

Erythropoiesis. The embryonic globin, βh1 (Hill *et al.*, 1984), first detectable by Northern analysis in day 6 colonies, peaks around day 7–8 and decreases afterwards (Figure 1). In some experiments, erythroid cells begin to disintegrate earlier and βh1 transcripts disappear more rapidly. In colonies derived from clones expressing high antisense vav, no βh1 signal was detected during colony growth (Figure 7B,

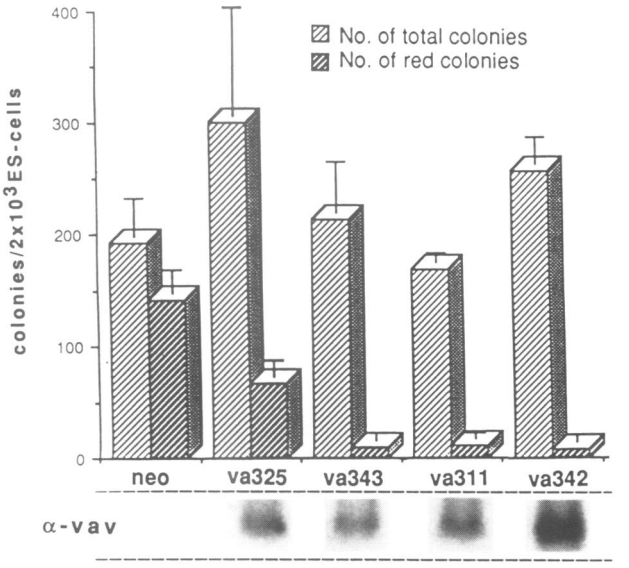

Fig. 5. Total and hematopoietic colony formation on day 12 of differentiation from neo control or vav antisense-expressing ES cells. Cells were plated at a density of 2 × 10^3 cells/ml in methyl cellulose and the developing colonies were counted under an inverted microscope. The values represent the mean ± SEM of three independent experiments, each of which was set up in duplicate.

clone vα342). To trace the perturbation of erythropoiesis to earlier stages, the expression of the GATA-1 transcriptional activator known to be absolutely required for erythropoiesis was examined (Pevny *et al.*, 1991). In neo control ES colonies, GATA-1 mRNA is first detected in day 6 colonies and parallels the time course for βh1 (Figure 7A). No GATA-1 mRNA was detected in colonies from clones expressing high levels of antisense vav (Figure 7B, clone vα342). The same difference between neo control and antisense vav-expressing ES cells was found in RT-PCR analysis of GATA-1 expression (Figure 10).

Myelopoiesis. The MAC-1 (murine homologue for CD11b) antigen is strongly upregulated during myeloid terminal differentiation. In neo control ES colonies, MAC-1 mRNA is detectable at a low level on day 10, increasing several-fold by day 12 (Figure 1). This development was completely abrogated in all the antisense clones examined (Figure 8A).

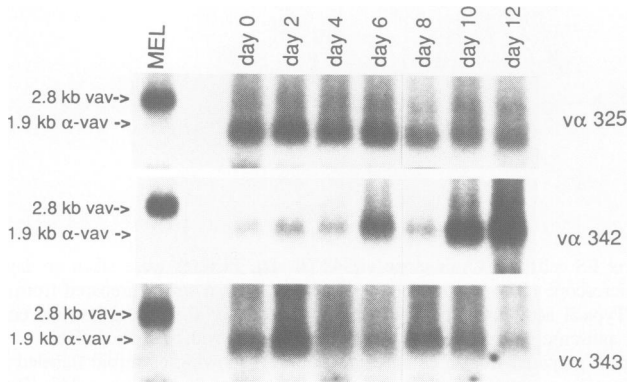

Fig. 6. Inhibition of vav sense transcripts in developing antisense colonies. Total RNA from the developing embryoid bodies was isolated at the time intervals indicated and analyzed by the Northern technique. Expression of antisense vav (1.9 kb) transcript in colonies was maintained throughout the entire time in culture. The longer 2.8 kb vav sense transcript is marked by the mRNA from the MEL cell line. Little or no vav transcripts were visible in the antisense colonies compared with sense colonies in Figure 1.

It was observed that macrophages were the longest surviving cells in the colonies. Using an antibody that recognizes the MAC-1 granulocyte–macrophage-specific surface antigen (Zager *et al.*, 1990), day 20 colonies were analyzed. In neo control colonies 10% of the cells were positive for MAC-1 (Figure 8B). In contrast, colonies from all antisense vav-expressing clones were negative for this antigen (Figure 8B, clone vα342 as an example). Recent findings indicate that PU.1, a macrophage- and B cell-specific transcriptional factor (Klemsz *et al.*, 1990), activates the transcription of MAC-1 and may be an important regulator of myeloid differentiation (Pahl *et al.*, 1993). PU.1 can be detected in neo control colonies from day 9 onwards (Figure 9). No PU.1 transcripts were seen in antisense colonies, as exemplified in clone vα342 by Northern analysis (Figure 9). Using RT-PCR analysis, a barely detectable transient expression was occasionally seen around day 2 in antisense colonies (Figure 10). However, the marked elevation of expression in normal ES colonies was never observed in antisense colonies.

Early hematopoietic marker genes. c-kit and flk-2 are two tyrosine kinases expressed in hematopoietic stem cells. While the expression of c-kit is also found in germ cells and neural crest-derived melanocytes (Nocka *et al.*, 1989), the expression of flk-2 appears to be restricted to hematopoietic stem cells (Matthews *et al.*, 1991). The expression of flk-2 was transiently, but consistently, detected at a low level around day 4 during normal ES cell development (Figure 10). No flk-2 mRNA was detectable in antisense colonies. In contrast, c-kit is expressed already in undifferentiated ES cells from the control and the antisense lines (Figure 10). During culture of normal ES cells its expression first decreases and then increases again from day 4 onwards in the control (Figure 10). In the antisense clones, c-kit expression was not suppressed and remained at a constant level throughout the entire culture period.

The murine CD34 antigen is expressed in hematopoietic stem cells in the brain and in embryonic fibroblasts (Brown *et al.*, 1991). In the neo control cultures CD34 expression,

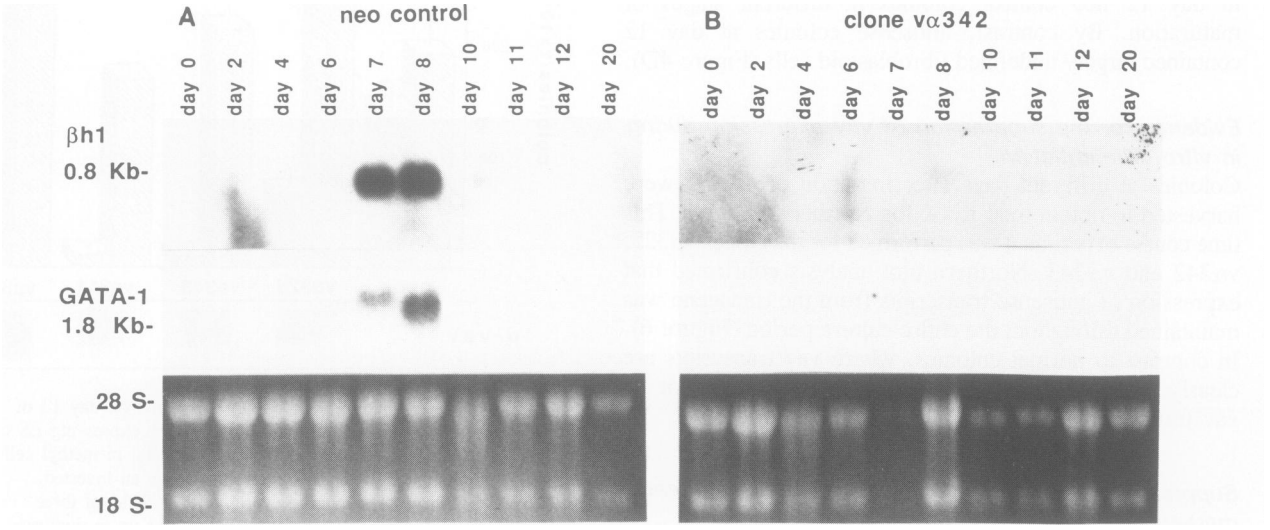

Fig. 7. Expression of GATA-1 and embryonic βh1-globin in colonies derived from neo control (**A**) or vav antisense-expressing ES cells (**B**). Total RNA from the developing embryoid bodies was isolated at the time intervals indicated and analyzed using the Northern technique. A similar absence or markedly reduced level of GATA-1 and βh1-globin was also found in embryoid bodies derived from clones vα325 and vα343 analyzed in the same way.

detectable at a low level in undifferentiated ES cells, continued to increase from day 2 to day 8 and declined on day 12 (Figure 10). Similar to c-kit, the expression of CD34 was not suppressed in the antisense clones. An initial increase on day 2, decrease or absence on day 4 and a second increase on days 8–12 was observed constantly (Figure 10). Northern blot analysis confirmed that the expression of c-kit and CD34 is not suppressed in the embryoid bodies derived from the antisense clones (data not shown).

Non-hematopoietic cells

To see if the developmental disturbance caused by the blocking of vav is specific for the hematopoietic system, the colonies for the expression of non-hematopoietic genes that are activated early in developing embryoid bodies were examined. GATA-4, a new member of the GATA family, is expressed in heart endothelium and is suspected to be important in cardiogenesis (Arceci *et al.*, 1993). GATA-4 was detected in embryoid bodies from day 6 onwards. ES colonies derived from antisense vav clones express a similar level of the GATA-4 transcript (Figure 11). 'flk-1' is a receptor tyrosine kinase expressed early during ontogenesis and later on in vascular endothelium and the postnatal brain (Millauer *et al.*, 1993). In contrast to flk-2, flk-1 is not expressed in hematopoietic cells. flk-1 expression is detected from day 8 onwards in both neo control and antisense vav-expressing cells by RT-PCR analysis (Figure 10).

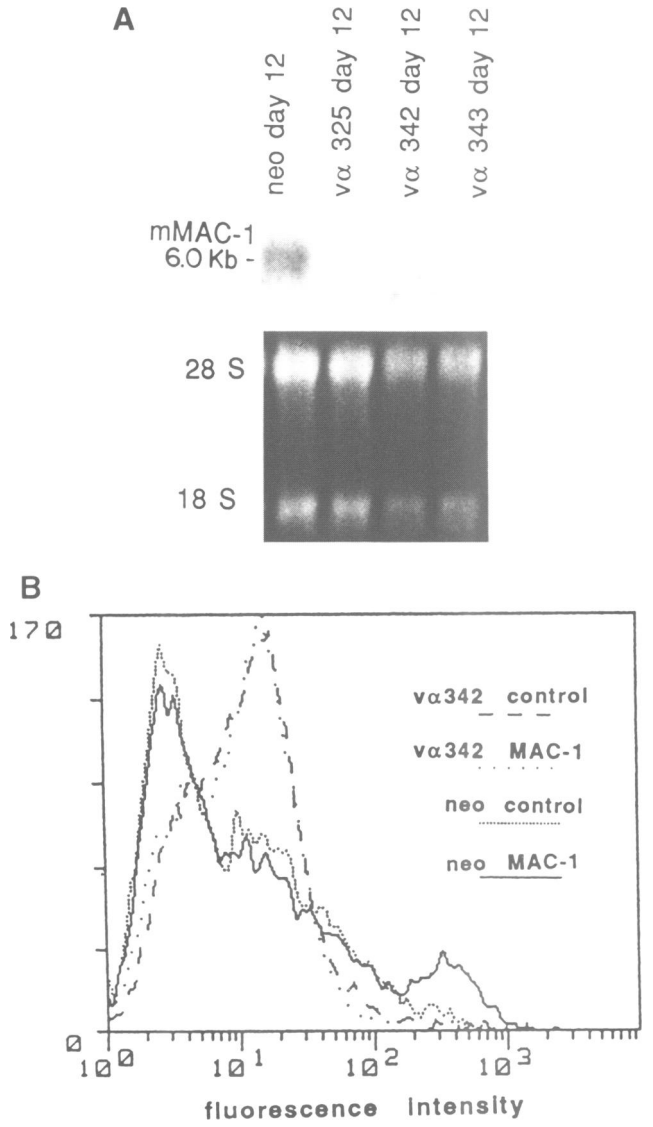

Fig. 8. (**A**) Northern analysis of MAC-1 mRNA in day 12 colonies derived from neo control (neo) or vav antisense-expressing ES cells (clones vα325, vα342 or vα343). Total RNA from the developing embryoid bodies was isolated on day 12 and analyzed by Northern technique. (**B**) MAC-1 expression in day 20 colonies derived from neo control (neo) or clone vα342 ES cells as detected by flow cytometry. Rabbit IgG1-PE served as a negative control. In the neo control ES cells, 10.4% were MAC-1 positive as compared with background level staining in colonies from clone vα342.

Assay for progenitor cells in primary ES colonies

At different times after culture, beginning at day 6, embryoid colonies harvested and disrupted into single cells were recultured in methyl cellulose in the presence of various combinations of cytokines. Neo control embryoid colonies revealed the presence of large numbers of progenitor cells. As early as day 6, erythroid precursors gave rise to small tight red colonies of ~20–30 cells. These precursors were observed at the same frequency in cultures with erythropoietin (Epo) only, Epo and stem cell factor (SCF) or with multiple cytokines (see Materials and methods) to maximize the growth of multilineage precursors. Some colonies were larger, resembling the burst forming unit (BFU-E)-derived colonies from the bone marrow. The frequency of erythroid precursors decreases rapidly and no erythroid colonies develop after day 13 (Figure 12A). Macrophage colonies could be seen from day 6 in culture conditions, which include GM-CSF and M-CSF. A surge in the increase of macrophage precursors was found in day 8–10 colonies, with a subsequent fall in later colonies (Figure 12B). In culture conditions with multiple cytokines, a small number of multilineage colonies, containing erythroid cells with at least another myeloid lineage, was detected at day 6 with a modest but recognizable increase and fall between day 8 and 18 (Figure 12C). The latest class of progenitor cells to emerge were mast cell precursors. They were first detectable in day 9–10 colonies, with a tremendous spurt of increase between days 13 and 21.

Under the same culture conditions, the frequency of all classes of secondary progenitors detected in antisense-derived ES clones was markedly reduced (Figure 12). The highest number of progenitors was seen in clone vα325 which showed already a breakthrough of 22% hematopoiesis positive embryoid colonies in the primary plating (Figure 5). In clone vα342, with the highest level of antisense vav

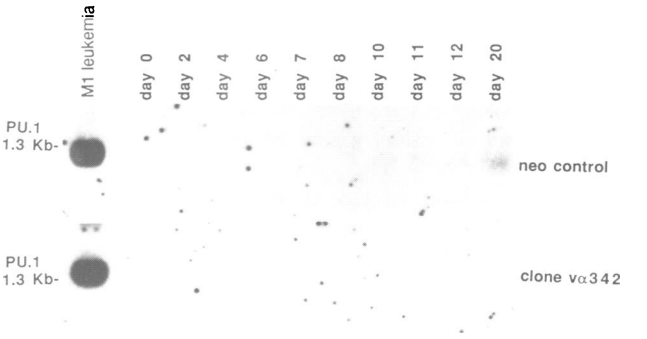

Fig. 9. Northern analysis of PU.1 mRNA in colonies derived from neo control or vav antisense-expressing ES cells (clone vα342). The time course analysis was performed as described in Figure 6.

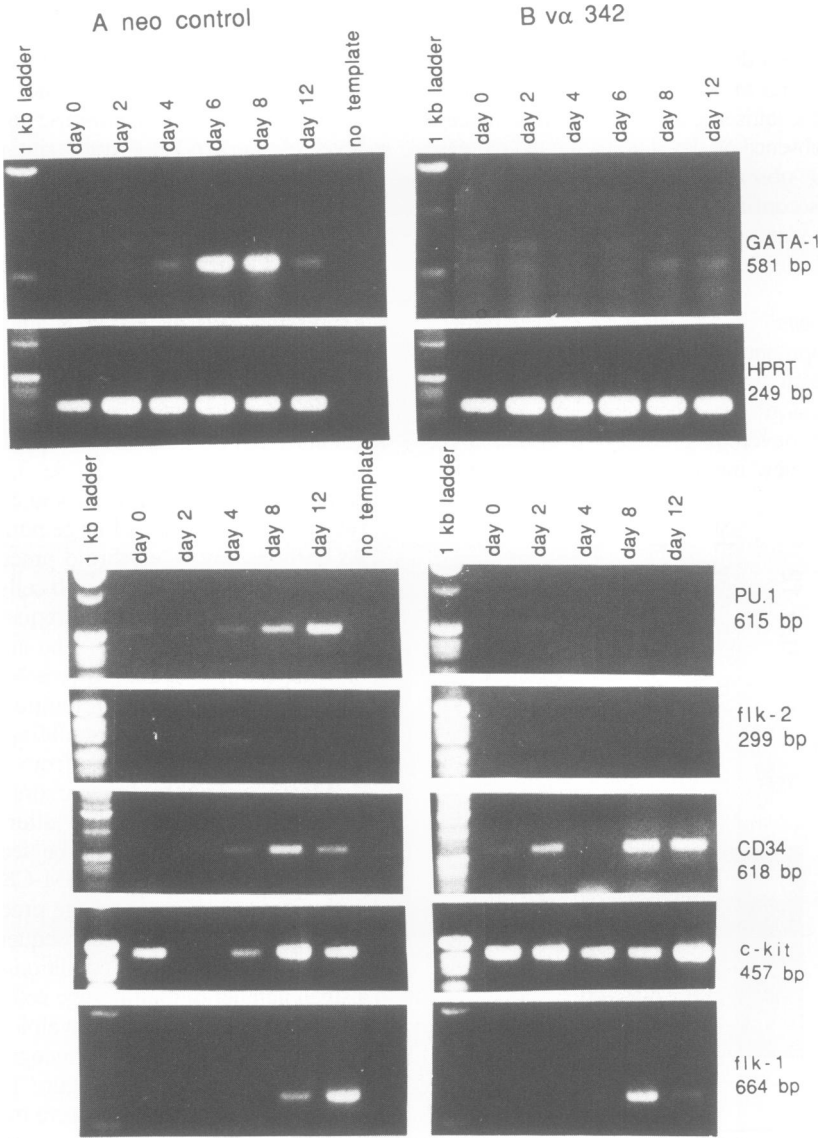

Fig. 10. Comparison of gene expression during the time course of *in vitro* differentiation of ES cells from either neo control (**A**) or clone vα342 (**B**), analyzed by RT-PCR. Ethidium bromide gel of the PCR products for GATA-1, HPRT, PU.1, flk-2, c-kit, CD34 and flk-1 are shown.

expression, there was essentially no progenitor cells detected at all time points. As observed in primary ES colonies, there was a direct correlation between the level of antisense vav expression and the severity of suppression of progenitors in the colonies.

Discussion

vav is expressed at a low level already in undifferentiated ES cells. During *in vitro* differentiation there is an increase in expression consistently detectable by RT-PCR analysis around day 2, followed by a marked upregulation from day 9 onwards. Therefore vav expression in developing ES colonies follows a pattern similar to c-kit, kit ligand and Thy1, and precedes the appearance of lineage-restricted molecules such as GATA-1, globin, CD45 and receptors for IL-3, CSF-1 and G-CSF (Schmitt *et al.*, 1991; Keller *et al.*, 1993). These kinetics suggest that vav may be important for the early development of hematopoietic cells. To test this hypothesis, the consequences of antisense RNA-mediated blocking of vav mRNA was examined.

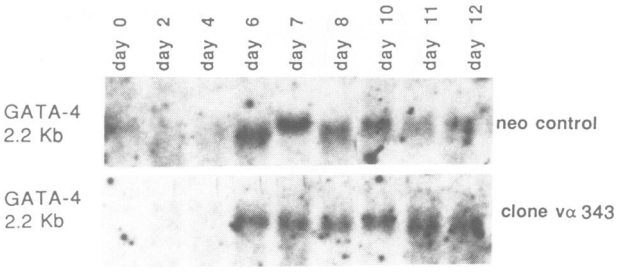

Fig. 11. Northern analysis of GATA-4 mRNA in colonies derived from neo control or vav antisense-expressing ES cells (clone vα343). The time course analysis was performed as described in Figure 6.

Several embryonic stem cell lines stably transfected with, and expressing, the vav antisense transcript were studied. The vav antisense transcript did not impair the growth of the derived cell lines as undifferentiated ES cells in the presence of LIF, suggesting that the baseline expression of vav observed by RT-PCR is not physiologically significant for the growth of ES cells. However, once transferred into

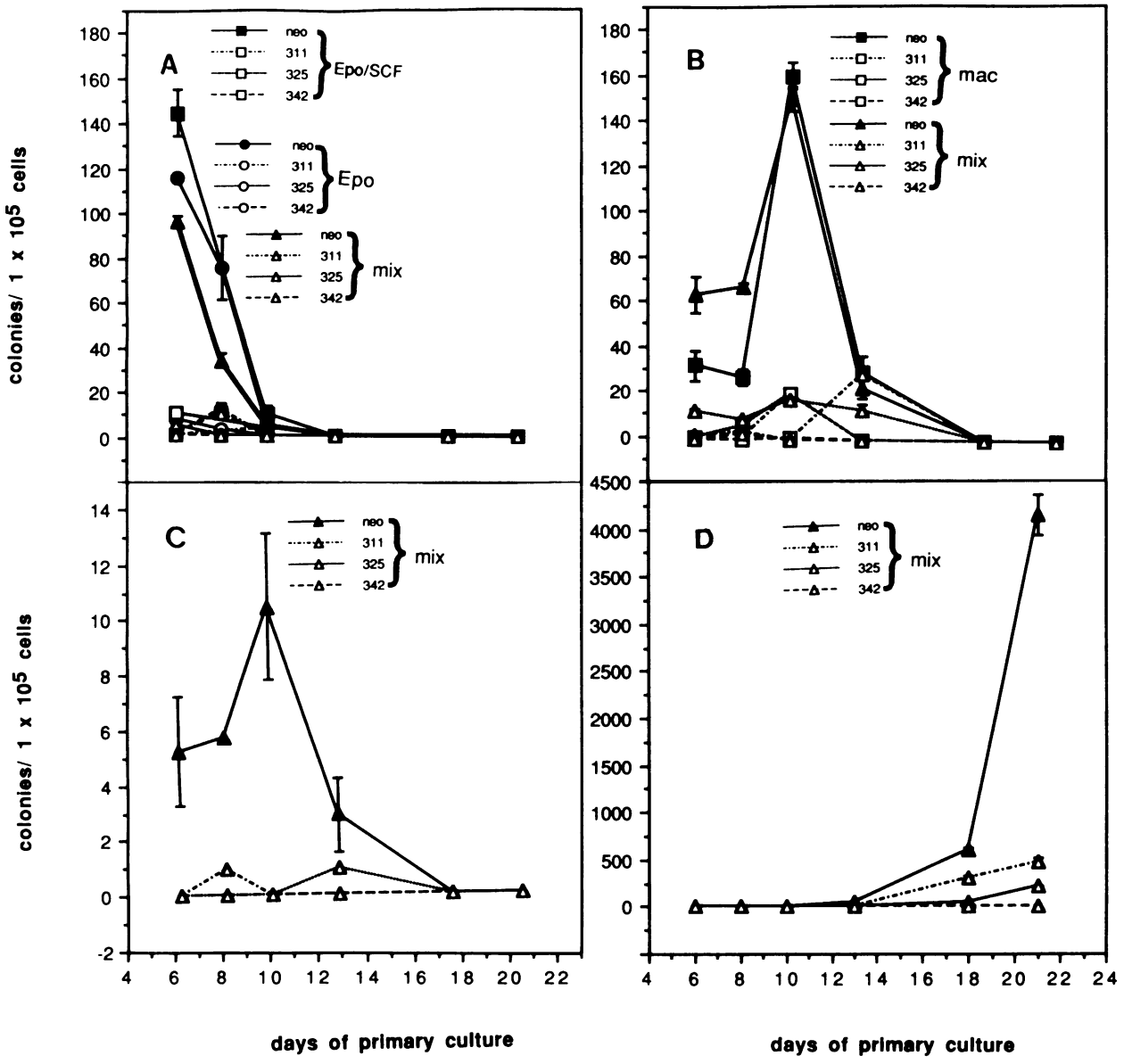

Fig. 12. Comparison of the kinetics of progenitor cell development in embryoid bodies derived from neo control (closed symbols) or antisense clones (open symbols). Embryoid colonies were harvested at the indicated times of primary culture, disrupted and replated as single cells in the presence of different cytokines or cytokine combinations [Epo, Epo/SCF, mac (M-CSF, IL-3, IL-1) and mixed (Epo, SCF, IL-1, IL-3, GM-CSF and M-CSF)]. The cultures were evaluated on day 7 of secondary plating for erythroid (**A**), macrophage (**B**), mixed erythroid/myeloid (**C**) or mast cell (**D**) colonies. The values of the mean and SD of duplicate cultures are plotted.

the *in vitro* differentiation system, the blocking of vav abrogated the development of hematopoietic cells from ES cells. Moreover, a correlation was found between the amount of vav antisense expression and the suppression of hematopoiesis. Because our system favors the development of erythroid and myeloid cells, the absence of these two lineages could be well documented by cytology, immunophenotyping and RNA analysis (Figures 4, 5 and 7–10).

It was expected that the blocking by antisense transcript might be incomplete and that some residual vav protein synthesis would occur. Therefore clones were selected expressing the vav antisense RNA at a level as high as, or even higher than, the vav mRNA in the hematopoietic cell line MEL. To ensure that we were not misled by clone to clone variations, we analyzed six individual clones transfected with and expressing the pgk-neo transgene and many untransfected ES clones derived from the CCE line by limiting dilution. In no instance was a clone encountered where hematopoiesis was seen in $<70\%$ of the colonies; this is to be contrasted with four out of four clones expressing different levels of antisense vav where only 3% to, at most, 22% hematopoiesis positive colonies were seen. The effective suppression of hematopoiesis by antisense vav is reflected in the absence or markedly reduced level of vav transcripts normally seen in Northern blot analysis of developing colonies from day 9–10 onwards.

To find out whether the embryoid bodies from the antisense clones contained progenitor cells with developmental potential, hematopoietic colony formation was studied in secondary methyl cellulose cultures of cells from primary embryoid colonies. Our cell culture conditions allowed the detection of at least five classes of progenitor cells, giving rise to pure erythroid, pure macrophage, pure

mast cells and mixed macrophage and granulocytic or erythroid and myeloid mixed colonies. A well defined temporal pattern was observed in the frequencies of progenitors, with precursors of pure erythroid colonies being generated earliest and mast cell progenitors latest. Our results are very similar to those described by Keller *et al.* (1993), although the overall frequencies of colonies obtained in our study were lower and the sustained level of erythroid and macrophage progenitors reported by them were not observed by us. Replating experiments with primary embryoid colonies derived from antisense clones demonstrated a clear and significant reduction in the frequency of progenitors. Similar to what was observed in primary cultures, the degree of suppression of the progenitor cell content correlates with the level of antisense vav expression. Furthermore, the suppression of hematopoiesis by antisense vav appears to be even more marked when evaluated by secondary progenitor cell assay, rather than by the morphology of primary embryoid colonies. Finally, the reduction or absence of secondary colonies indicates either a failure of the development to progenitor cell stage or an inability of progenitor cells to undergo further growth and differentiation. It has not been possible thus far to assay directly for more primitive progenitors in primary ES colonies, such as CFU-S-derived colonies or stem cells capable of reconstituting the hematopoietic system. Therefore the consequences of blocking vav on the development of this population of hematopoietic precursors cannot be evaluated directly.

At the RNA level the absence of the terminal-differentiated markers for erythropoiesis (globin) and myelopoiesis (MAC-1) is demonstrated. Evidence for perturbation of hematopoiesis at earlier stages was obtained by analyzing the expression of transcriptional activators that govern the differentiation of these lineages. The GATA-1 protein appears to be required absolutely for erythropoiesis, acting like a master transcriptional activator (Pevny *et al.*, 1991; Simon *et al.*, 1992). One of the transcriptional factors possibly governing myeloid differentiation is PU.1. PU.1 is expressed specifically in macrophages and B cells (Klemsz *et al.*, 1990). PU.1 probably has a role in regulating the transcriptional activation of the CD11b gene (Pahl *et al.*, 1993). The absence of GATA-1 and PU.1 transcripts in antisense colonies indicates that blocking of vav disrupts the developmental progression and survival of precursors in early stages when transcriptional factors are critical for erythro-myelopoiesis. To trace hematopoietic development even earlier, before the stage of lineage commitment, the expression of flk-2, CD34 and c-kit was investigated. flk-2 is a receptor tyrosine kinase expressed in hematopoietic stem cells but not in non-hematopoietic cells. The highest level of flk-2 expression is in the fetal liver, while a very low level is detected in bone marrow cells of the adult animal. In embryoid colonies from the neo control, a transient flk-2 expression was detected on day 4. In colonies from antisense vav clones, no flk-2 expression was observed at any time point. Because the flk-2 population of cells is capable of reconstituting the hematopoietic system (Matthews *et al.*, 1991), our result suggests that the development towards early hematopoietic stem cells is inhibited by the blocking of vav. This observation also indicates that the suppression observed in the secondary progenitor assays is caused by an absence or reduction of progenitors, rather than by a failure of progenitors to proliferate and form colonies. Other early stem cell markers are CD34 (Brown *et al.*, 1991) and c-kit (Nocka *et al.*, 1989). The developmental relationship between the CD34 and c-kit positive cells and the flk-2 positive population is unclear. However, unlike flk-2, CD34 and c-kit are expressed also in non-hematopoietic tissues (Nocka *et al.*, 1989; Brown *et al.*, 1991) and in undifferentiated ES cells (Schmitt *et al.*, 1991; McClanahan *et al.*, 1993; Figure 10). In contrast to all other hematopoietic-specific genes, the expression of c-kit and CD34 was not suppressed in vav antisense-expressing cells (Figure 10). This result can be interpreted in two different ways. (i) It is possible that the CD34 and c-kit positive cells in the antisense colonies include early stem cells that have undergone commitment to hematopoiesis but are unable to undergo further development, including differentiation, into lineage-restricted progenitor cells. (ii) It is also possible that amongst the CD34 and c-kit positive cells are totipotent cells with a potential to develop into different tissues, including hematopoietic stem cells, and that the progression of commitment to hematopoiesis is blocked by antisense vav.

While the disruption of vav blocks hematopoiesis within the embryoid bodies, the formation and development of the embryoid bodies did not appear to be affected (Figure 5). Therefore the expression of two other genes that regulate early embryonic development, GATA-4 and flk-1, was studied. GATA-4 is a newly described transcription factor of the GATA family that is expressed in heart endothelium and endoderm-derived tissues. In contrast to GATA-1, -2 and -3 it is absent from hematopoietic cells (Arceci *et al.*, 1993). flk-1 is a receptor tyrosine kinase that is expressed in vascular endothelium. Early during ontogenesis flk-1 plays a key role in the development of blood vessels (Millauer *et al.*, 1993). It was found that the expression of GATA-4 and flk-1 in the vav-disrupted embryoid bodies is the same as the expression level in the neo control ES cells. Therefore it is concluded that the blocking of vav inhibits hematopoietic development but not the potential of the ES cells to differentiate into other tissues.

The phosphorylation of vav through activation of several different ligand-activated receptors (Bustelo *et al.*, 1992; Margolis *et al.*, 1992) suggests that vav is involved in several biochemical pathways. One consequence of vav activation, demonstrated by Gulbins *et al.*, is the recruiting of vav as a GDP exchange stimulator (GDS) for ras in T cells after activation of the TCR (Gulbins *et al.*, 1993). Similar GTP exchange activity of vav for other GTP binding proteins has not been ruled out. In particular, the dbl-homologous domain in vav (Adams *et al.*, 1992) suggests that it might function also as an exchange factor for the members of the rho family of GTP binding proteins which participate in the regulation of cytoskeletal organization (Ridley *et al.*, 1992). The special alignment of an SH2-SH3-SH2 motif in the C-terminus of vav is similar to that found in adaptor proteins typified by Grb2 (Sem-5), which in complex with the guanine exchange protein, Sos, directly couples receptor activation and exchange activity on downstream target GTP binding proteins such as ras (Egan *et al.*, 1993; Gale *et al.*, 1993; Li *et al.*, 1993; Rozakis-Adcock *et al.*, 1993).

Accumulating evidence therefore indicates that vav might be a protein of pleiotropic function mediating both mitogenic and differentiation signals. Our results indicate that vav plays a central role in the early events of hematopoietic

differentiation. Blocking of vav causes either an arrest of development into primitive hematopoietic stem cells or it affects the viability and expansion of cells committed to hematopoiesis. Therefore, vav may be involved in the commitment events during the transition of totipotent cells into hematopoietic stem cells. Alternatively, vav may be important for the viability or structural integrity of hematopoietic cells at all stages of differentiation. The *in vitro* differentiation ES system permits analysis of the earliest events of hematopoiesis and therefore provides a focal context for further investigations of the biochemical function of vav.

Materials and methods

Construction of the antisense vav vector

A human vav cDNA probe, generated by RT-PCR from bone marrow RNA, was used to obtain a full-length murine vav cDNA from a cDNA library, constructed as described previously (Lelias *et al.*, 1993) from the factor-dependent cell line FDCP (Spooncer *et al.*, 1986). The murine vav cDNA was cloned into the Bluescript KS vector (Stratagene). A 1.9 kb fragment containing the 5′ untranslated region and almost two-thirds of the 5′ end of the coding region was excised with restriction enzymes *Xho*I (5′ end) and *Bam*HI (3′ end). Using the pgk-neo vector (Adra *et al.*, 1987), the neo cDNA cassette with *Xho*I and *Bam*HI was removed and replaced with the truncated murine vav cDNA ligated in the antisense direction to yield the pgk-α-vav vector.

ES cell culture and transfection

The ES cell line (CCE) was routinely maintained as described (Robertson, 1987) in Dulbecco's modified medium (DMEM, Gibco) supplemented with 10% FCS (Hyclone), 1.3×10^{-4} M β-mercaptoethanol and LIF (Genetics Institute, Cambridge, MA) on gelatinized tissue culture dishes. Under these conditions the cells remain undifferentiated as determined by phase contrast microscopy. The ES cells were cotransfected with pgk-α-vav and pgk-neo DNA at a 10:1 molar ratio using the calcium phosphate transfection kit (Promega). Two days after transfection, the growth medium was replaced with fresh medium containing different doses of G418 above that lethal for unprotected cells. After 7–10 days, single G418-resistant colonies were picked, expanded and analyzed for antisense vav expression using the standard Northern blot technique. Clones vα311 and vα325 were selected at 0.5 mg/ml G418 and clones vα342 and vα343 at 2 mg/ml. MEL or the murine myeloid leukemic cell line M1 were used as controls.

ES cell differentiation and secondary progenitor assay conditions

In vitro culture of ES cells in methyl cellulose was carried out as described (Lelias *et al.*, 1993). Briefly, a single cell suspension of ES cells was cultured in non-tissue culture 35 mm dishes with a mixture consisting of 0.9% methyl cellulose, 20% FCS, 1% BSA, 400 U/ml of IL-1, 150 ng/ml of stem cell factor (Amgen Biologicals), 2 U/ml of erythropoietin (D.Worchowski, Pennsylvania State University, PA) at a final cell concentration of 1000–2000 cells/ml. Incubation was maintained in a humidified atmosphere at 37°C. Colonies were scored for visibly hemoglobinized erythroid cells by light microscopy and differentiation, confirmed by staining with Wright–Giemsa staining of cytopreparation of individual or pooled colonies. For secondary plating, embryoid bodies were harvested at the indicated time intervals, disrupted by trypsinization and replated at a density of 1×10^5 cells/ml in 0.9% methyl cellulose, 10% FCS, 10% human plasma, 1% BSA and 0.13 mM β-mercaptoethanol (Sigma). Growth factors or factor combinations used were (Figure 12): Epo, 2 U/ml of erythropoietin; Epo/SCF, 150 ng/ml of stem cell factor, 2 U/ml of erythropoietin; mac, 400 U/ml IL-1, 50 U/ml IL-3, 100 U/ml M-CSF; mix, 150 ng/ml of stem cell factor, 2 U/ml of erythropoietin, 400 U/ml IL-1, 50 U/ml IL-3, 100 U/ml M-CSF, 50 U/ml GM-CSF. The colonies were evaluated under inverted light microscopy on day 7 of secondary plating.

RT PCR analysis

Total RNA was isolated from undifferentiated ES cells and from developing embryoid bodies using the RNAzol method (Biotecx). Oligo (dT) primed cDNAs were prepared from 1 μg total RNA using MMLV reverse transcriptase (Gibco BRL) under conditions recommended by the manufacturer in a 20 μl reaction volume; 1 μl was then used for PCR amplification. PCR conditions for vav, GATA-1 and HPRT were as described by Keller *et al.* (1993). The specificity of the PCR products was confirmed by Southern blot analysis. PCR conditions for PU.1 were 1 min annealing at 55°C, 1 min extension at 72°C, 30 cycles, 100 nM primers. The primers used were: PU.1, 5′-TGGAAGGGTTTTCCCTCACC-3′ and 5′-TGCTGTCCTTCATGTCGCCG-3′ (Klemsz *et al.*, 1990). RT-PCR for c-kit, CD34 and flk-2 was performed as described by McClanahan *et al.* (1993). PCR conditions for flk-1 were 1 min annealing at 55°C, 1.5 min extension at 72°C, 40 cycles, 100 nM primers. The primers used for flk-1 were 5′-GCCCGACTCCCTTTGAAGTGG-3′ and 5′-CCACTTCAAAG-GGAGTCGGGC-3′], resulting in a 664 bp fragment. Semiquantitative analysis of the relative amounts of transcripts expressed during the course of differentiation was done by comparing the expression of the respective gene with that of the housekeeping gene, HPRT.

FACS analysis

For flow cytometry, colonies were harvested on day 20 of differentiation, washed twice with PBS, disaggregated by repeated gentle pipetting and resuspended in PBS with 10% BSA. Immunostaining was performed with a PE-labeled rabbit anti-mouse MAC-1 mAb (Pharmingen). These were analyzed in comparison with an IgG1-PE matching control with a FACStar cell sorter.

General methods

Standard methods of Southern and Northern analysis were carried out as described by Sambrook *et al.* (1989).

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References

Adams,J.M., Houston,H., Allen,J., Lints,T. and Harvey,R. (1992) *Oncogene*, **7**, 611–618.
Heyworth,C.M., Dunn,A. and Dexter,T.M. (1986) *Differentiation*, **31**, 11–118.
Adra,C.N., Boer,P.H. and McBurney,M.V. (1987) *Gene*, **60**, 65–74.
Arceci,R.J., King,A.A.J., Simon,M.C., Orkin,S.H. and Wilson,D.B. (1993) *Mol. Cell. Biol.*, **13**, 2235–2246.
Brown,J., Greaves,M.F. and Molgaard,H.V. (1991) *Int. Immunol.*, **3**, 175–184.
Bustelo,X.R., Ledbetter,J.A. and Barbacid,M. (1992) *Nature*, **356**, 68–71.
Chen,U. (1992) *Devl Immunol.*, **2**, 29–50.
Coppola,J., Bryant,S., Koda,T., Conway,D. and Barbacid,M. (1991) *Cell Growth Differentiation*, **2**, 95–105.
Egan,S.E., Giddings,B.W., Brooks,M.W., Buday,L., Sizeland,A.M. and Weinberg,R.A. (1993) *Nature*, **363**, 45–51.
Gale,N.W., Kaplan,S., Lowenstein,E.J., Schlessinger,J. and Bar-Sagi,D. (1993) *Nature*, **363**, 88–92.
Gulbins,E., Coggeshall,K.M., Baier,G., Katzav,S., Burn,P. and Altman,A. (1993) *Science*, **260**, 822–825.
Hill,A., Hardies,S.C., Phillips,S.J., Davis,M.G., Hutchison,C.A. and Edgell,M.H. (1984) *J. Biol. Chem.*, **259**, 3739–3747.
Katzav,S. (1992) *Brit. J. Haematol.*, **81**, 141–144.
Katzav,S., Martin-Zanca,D. and Barbacid,M. (1989) *EMBO J.*, **8**, 2283–2290.
Keller,G., Kennedy,M., Papayannopoulou,T. and Wiles,M.V. (1993) *Mol. Cell. Biol.*, **13**, 473–486.
Klemsz,M.J., McKercher,S.R., Celada,A., Van Beveren,C. and Maki,R.A. (1990) *Cell*, **61**, 113–124.
Lelias,J.M., Adra,C.N., Wulf,G., Guillemot,J.C., Khagad,M., Caput,D. and Lim,B. (1993) *Proc. Natl Acad. Sci. USA*, **90**, 1479–1483.
Li,N., Batzer,A., Daly,R., Yajnik,V., Skolnik,E., Chardin,P., Bar-Sagi,D., Margolis,B. and Schlessinger,J. (1993) *Nature*, **363**, 85–87.
Lim,B., Apperley,J.F., Orkin,S.H. and Williams,D.A. (1989) *Proc. Natl Acad. Sci. USA*, **86**, 8892–8896.
Lindenbaum,M.H. and Grosveld,F. (1990) *Genes Dev.*, **4**, 2075–2095.
Margolis,B., Hu,P., Katzav,S., Li,W., Oliver,J.M., Ullrich,A., Weiss,A. and Schlessinger,J. (1992) *Nature*, **356**, 71–74.

Matthews,W., Jordan,C.T., Wieg,G.W., Pardoll,D. and Lemischka,I.R. (1991) *Cell*, **65**, 1143–1152.
McClanahan,T., Dalrymple,S., Barkett,M. and Lee,F. (1993) *Blood*, **81**, 2903–2915.
Millauer,B., Wizigmann-Voos,S., Schnuerch,H., Martinez,R., Moeller,N.P.H., Risau,W. and Ullrich,A. (1993) *Cell*, **72**, 835–846.
Nocka,K., Majumder,S., Chabot,B., Ray,P., Cervone,M., Bernstein,A. and Besmer,P. (1989) *Genes Dev.*, **3**, 816–826.
Pahl,H.L., Scheibe,R.J., Zhang,D.E., Chen,H.M., Gilson,D.L., Maki,R.A. and Tenen,D.G. (1993) *J. Biol. Chem.*, **268**, 5014–5020.
Pevny,L., Simon,M.C., Robertson,E., Klein,W.H., Tsai,S.F., D'Agati,V., Orkin,S.H. and Constantini,F. (1991) *Nature*, **349**, 257–260.
Puil,L. and Pawson,T. (1992) *Curr. Biol.*, **2**, 275–277.
Robertson,E.J. (1987) In Robertson,E.J. (ed.), *Teratocarcinomas and Embryonic Stem Cells: A Practical Approach*. IRL Press, Oxford, pp. 71–112.
Rozakis-Adcock,M., Fernley,R., Wade,J., Pawson,T. and Bowtell,D. (1993) *Nature*, **363**, 83–85.
Ridley,A.J., Paterson,H.F., Johnston,C.L., Diekmann,D. and Hall,A. (1992) *Cell*, **70**, 401–410.
Sambrook,J., Fritsch,E. and Maniatis,T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd edition. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
Schmitt,R.M., Bruyns,E. and Snodgrass,H.R. (1991) *Genes Dev.*, **5**, 728–740.
Simon,M.C., Pevny,L., Wiles,M.V., Keller,G., Constantini,F. and Orkin,S.H. (1992) *Nature Genet.*, **1**, 92–98.
Spooncer,E., Heyworth,C.M., Dunn,A. and Dexter,T.M. (1986) *Differentiation*, **31**, 111–118.
Whitelaw,E., Tsai,S.F., Hogben,P. and Orkin,S.H. (1990) *Mol. Cell. Biol.*, **10**, 6596–6606.
Wiles,M. and Keller,G. (1991) *Development*, **111**, 259–267.
Zager,D.C., Osman,N., Hennings,M., McKenzie,I.F., Sears,D.W. and Hogarth,P.M. (1990) *Immunogenetics*, **31**, 191–197.

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