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SHORT REPORT

p210 Bcr–Abl expression in a primitive multipotent haematopoietic cell line models the development of chronic myeloid leukaemia

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Chronic myeloid leukaemia (CML) is a clonal disorder of the pluripotent haemopoietic stem cell, the hallmark of which is the constitutively activated Bcr–Abl protein tyrosine kinase. During the initial chronic phase of CML the primitive multipotent leukaemic progenitor cells remain growth factor dependant and are capable of producing terminally differentiated cells. Although the available evidence suggests that Bcr–Abl directly affects signalling pathways involved in controlling the development of primitive haemopoietic progenitors the identification of the specific biological consequences of Bcr–Abl activity in these progenitors has been hampered by the lack of suitable systems modelling CML. By transfecting the multipotent haemopoietic cell line FDCP-Mix with a temperature sensitive mutant of Bcr–Abl we have developed the first working model that mirrors the chronic phase of CML. FDCP-Mix cells expressing Bcr–Abl tyrosine kinase activity remain growth factor dependent and retain their ability to differentiate. Normal neutrophilic cells are formed in response to G-CSF and GM-CSF. In addition, the transfected FDCP-Mix cells grown at the permissive temperature for Bcr–Abl tyrosine kinase activity display enhanced survival and proliferation in low concentrations of growth factor. These findings are consistent with the initial subtle changes seen in CML progenitor cells during the chronic phase and confirm that Bcr–Abl effects are context specific, i.e. they depend on the origin and developmental potential of the transfected cells. This questions the significance of studies in non-haemopoietic and differentiation blocked haemopoietic cells.

Keywords: Bcr–Abl; chronic myeloid leukaemia; stem cell line

Chronic myeloid leukaemia (CML) is a clonal haemopoietic stem cell disorder. In the majority of patients CML is characterised by the presence of the Philadelphia chromosome (Ph^+), the result of a reciprocal translocation between chromosome 9 and 22 (for reviews see Gotoh and Broxmeyer, 1997; Sawyers, 1993). This translocation results in the expression of a chimeric protein, Bcr–Abl. The Bcr–Abl protein contains several functional domains including most importantly, a constitutively activated form of the c-Abl protein tyrosine kinase (Konopka *et al*., 1984; Lugo *et al*., 1990).

CML is a biphasic disease with an initial chronic phase (which can last for several years) in which there is a marked expansion in the late myeloid cell population. CML progenitor cells possess only a subtle defect in maturation, whilst retaining their requirements for bone marrow stromal cells or cytokines to survive and proliferate (Gishizky and Witte, 1992; Wang, 1992). In the course of the human disease this stage is followed by a blast crisis in which the cells lose their differentiation capacity resulting in the accumulation of immature myeloid cells.

Attempts to establish cell lines from patients in the chronic phase of CML have been unsuccessful, therefore numerous attempts have been made to model CML using Bcr–Abl transfected Interleukin 3 (IL-3) dependent cell lines (Carlesso *et al*., 1994; Daley and Baltimore, 1988; Hariharan *et al*., 1988; Kabarowski *et al*., 1994; Laneuville *et al*., 1992). However, Ph^+ progenitor cells develop normally in culture whereas the majority of IL-3 dependent cell lines expressing Bcr–Abl display growth factor independent proliferation and cannot undergo maturation and development. Many of the studies on Bcr–Abl protein tyrosine kinase (PTK) induced events have therefore been performed within the context of what is an inappropriate biological response elicited by this oncogene. This suggests that these models of Bcr–Abl induced leukaemogenesis are of limited value. Studies on primary cells show that Bcr–Abl enhances the growth potential of the cells in the presence of limiting growth factor concentrations and that initially they retain their ability to differentiate (Daley and Ben Neriah, 1991; Gishizky and Witte, 1992; McLaughlin *et al*., 1987; Scherle *et al*., 1990; Young and Witte, 1988). However, insufficient early progenitor cells can be prepared to perform any meaningful biochemical analysis of the effects of Bcr–Abl on these cells.

In order to study the molecular mechanisms activated by Bcr–Abl in primitive haemopoietic cells we have expressed a temperature sensitive mutant of Bcr–Abl in the pluripotent primitive haemopoietic cell line, FDCP-Mix. FDCP-Mix cells are cytokine dependent and respond to stromal cells and growth factors to undergo multilineage differentiation that leads to clonal extinction and the production of functionally mature myeloid cells (Heyworth *et al*., 1990; Spooncer *et al*., 1986). The FDCP-Mix cells

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therefore provide a closer analogy to the biological characteristics of CML progenitor cells than other cell lines which lack this differentiation capacity. Thus expression of Bcr–Abl in this system should allow the subtle effects of Bcr–Abl on the balance between self renewal, differentiation and survival of haemopoietic stem cells to be assessed.

The multipotent FDCP-Mix cells were transfected with a myeloproliferative sarcoma virus based defective retroviral vector pM5 (Laker *et al.*, 1987), carrying either the neomycin phosphotransferase gene or the neomycin phosphotransferase gene and a temperature sensitive (ts) p210 Bcr–Abl cDNA (Kabarowski *et al.*, 1994) essentially as described in Spooncer *et al.* (1994). The pM5-neo and pM5 Bcr–Abl plasmids were transfected into the GP-env AM12 retroviral packaging cells (Markowitz *et al.*, 1988) by lipofection and transfection of the FDCP-Mix cells achieved by co-cultivation. The level of Bcr–Abl expression is unaffected by temperature but its tyrosine kinase activity is inactive at 39°C and active at 32°C. The cells were cultured at 39°C throughout the transfection procedure to ensure no exposure to Bcr–Abl PTK activity.

Following selection and cloning in semi-solid media, at 39°C under conditions which maintain the multipotency of this cell line, p210 tsBcr–Abl expressing clones were identified by Western blotting (Figure 1a). The parental FDCP-Mix cells and the pM5-neo transfected clones express only the normal cellular protein, p145 c-Abl, whilst the infected cells express both c-Abl and p210 Bcr–Abl. A direct comparison of c-Abl and Bcr–Abl expression levels was not possible as p210 protein degradation results in the appearance of a band at approximately 145 kDa. We have

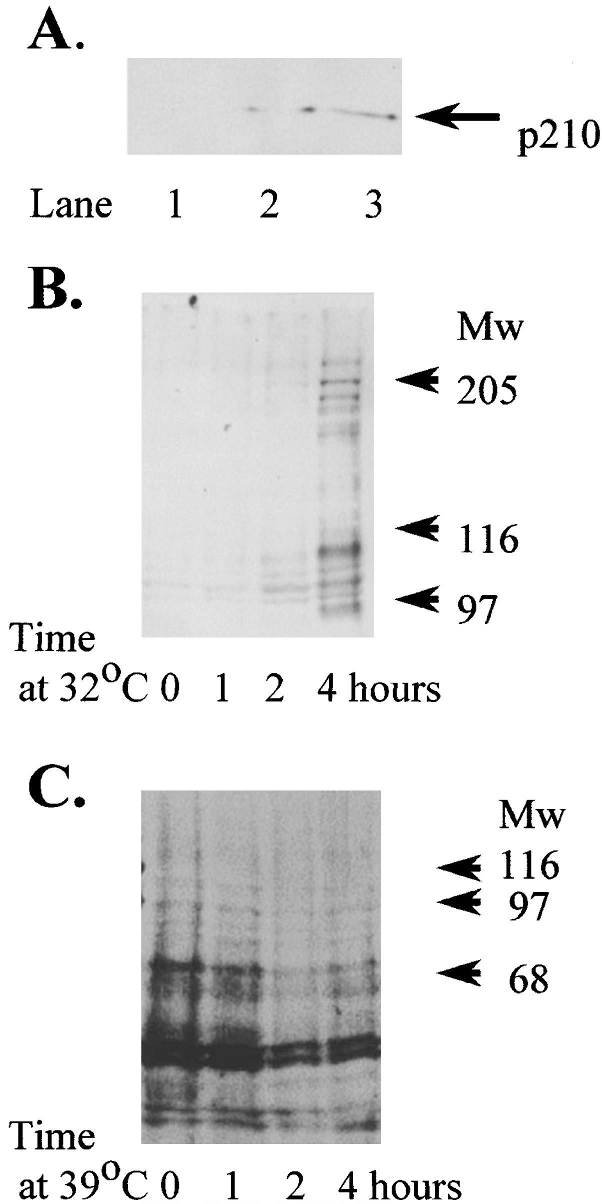

Figure 1 The expression and activity of p210 tsBcr–Abl in transfected FDCP-Mix cells. The expression of p210 Bcr–Abl and the level of tyrosine phosphorylation of intracellular proteins were analysed by Western blotting of total cell lysates as previously described (Spooncer *et al.*, 1994). The antibodies used were a polyclonal antibody to c-Abl (Santa Cruz) (**a**) and a monoclonal antibody to phosphotyrosine (UBI) (**b** and **c**). (**a**) illustrates the expression of p210 Bcr–Abl in FDCP-Mix clones 2 (lane 2) and 16 (lane 3) at 39°C. Lane 1 is the parental FDCP-Mix which can be seen not to express Bcr–Abl. (**b** and **c**) illustrate the time course of total phosphotyrosine protein content of tsBcr–Abl clones on switching the cells from 39°C to 32°C (clone 16) and 32°C to 39°C (clone 2) respectively

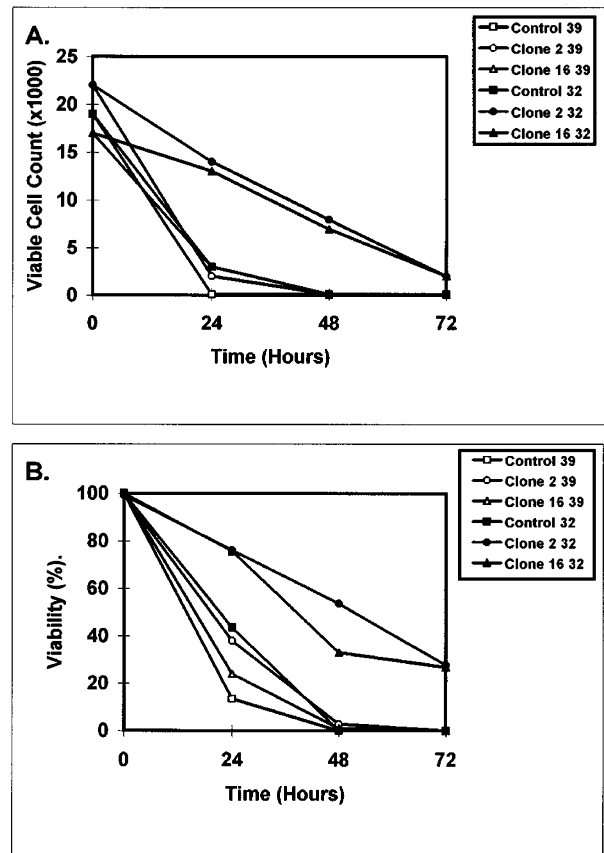

Figure 2 FDCP-Mix cells expressing p210 tsBcr–Abl remain growth factor dependent but have an enhanced survival potential in the absence of IL-3. TsBcr–Abl FDCP-Mix clones 2 and 16 and parental FDCP-Mix cells were washed free of growth factor and seeded at 2×10^5 cells/ml in Fischer's medium containing 20% (v/v) horse serum. Following incubation at 39°C or 32°C total viable cell count (**a**) and the percentage of viable cells in the culture (**b**) were determined at 24 h intervals by trypan blue exclusion. The data shown is from a representative experiment of 6 in **a** and the mean of 6 experiments in **b** (s.e.m. is less than 10% for all data points shown)

previously observed such a phenomenon with recombinant baculovirus p210 Bcr–Abl. The expression of the Bcr–Abl protein was independent of temperature and was seen at both the restrictive and permissive temperatures for Bcr–Abl PTK activity (data not shown).

To confirm that the activity of the Bcr–Abl PTK in FDCP-Mix cells was temperature dependent the level of tyrosine phosphorylation in total cellular protein at 39°C (restrictive temperature) and 32°C (permissive temperature) was investigated using antiphosphotyrosine antibody immunoblots. Switching the FDCP-Mix tsBcr–Abl clones from the restrictive to the permissive temperature resulted in an increase in phosphotyrosine content of numerous cellular proteins over a wide range of molecular size (Figure 1b). However, no increase was seen in the control parental cell line FDCP-Mix or in pM5-neo transfected cell populations (data not shown). The wide range of molecular weight proteins involved is illustrated by the use of a 7.5% gel, to show high molecular weight proteins, in Figure 1b and a 10% gel, Figure 1c, to show the low molecular weight proteins. The changes in cellular phosphotyrosine content are relatively rapid and can be seen within 2 h of temperature switch, and are maximal after 4 h. The reverse is also true, a switch from the permissive to the restrictive temperature results in a rapid decline in tyrosine phosphorylation of cellular proteins indicating that the activity of Bcr–Abl PTK is reversible (Figure 1c). Having maintained the FDCP-Mix cells at the restrictive temperature throughout the transfection and selection process several p210 tsBcr–Abl positive clones were then taken for subsequent determination of the primary effects of Bcr–Abl PTK activity on the characteristics of these multipotent myeloid cells.

Several previous reports have shown that expression of Bcr–Abl in cell lines results in overt transformation to growth factor independence, a feature not found in cells from the bone marrow of patients with CML (Carlesso *et al*., 1994; Daley and Baltimore, 1988; Hariharan *et al*., 1988; Kabarowski *et al*., 1994; Laneuville *et al*., 1992). For this reason the ability of the tsBcr–Abl clones to survive and proliferate in the absence of any added growth factors was assessed.

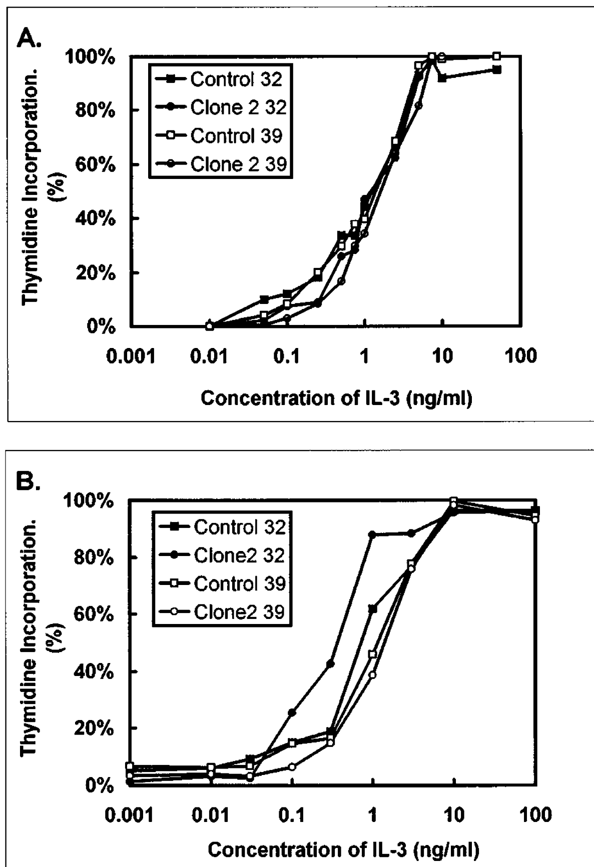

Figure 3 The effect of temperature switch on the response to IL-3 of FDCP-Mix cells expressing tsBcr–Abl. TsBcr–Abl FDCP-Mix clone 2 and parental FDCP-Mix cells were seeded at 2×10^5 cells/ml in Fischers medium containing 20% (v/v) horse serum with increasing concentrations of IL-3, and incubated at 39°C or 32°C for 24 h (**a**) or 3 days (**b**). Proliferation was assessed by [^{3}H]thymidine incorporation as previously described (Spooncer *et al*., 1994). Data is expressed as a percentage of that observed at maximal concentrations of IL-3 and is mean of 6 experiments (s.e.m. is less than 10% for all data points shown)

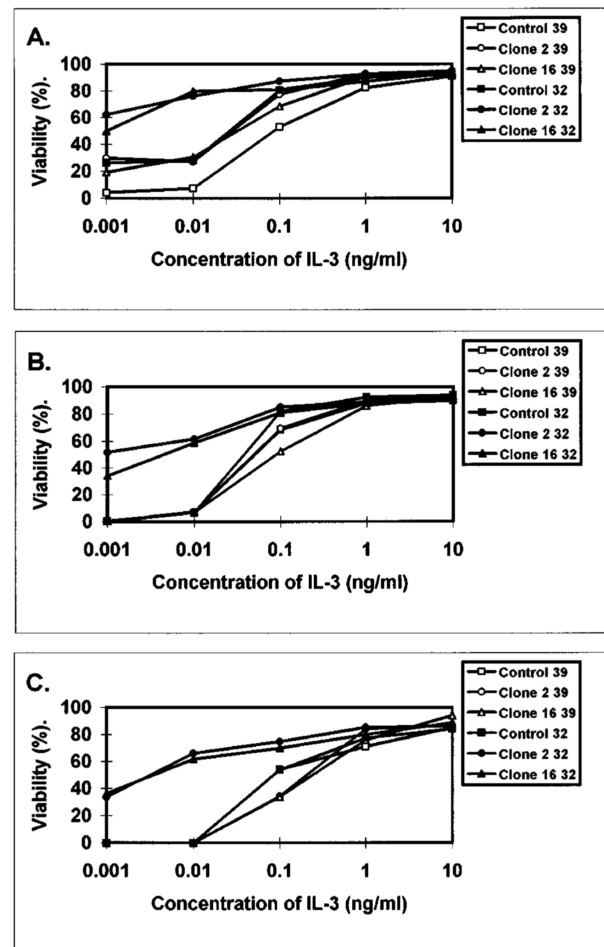

Figure 4 Effect of temperature switch on the time course and IL-3 dependency of cellular viability in tsBcr–Abl FDCP-Mix cells. TsBcr–Abl FDCP-Mix clones 2, 16 and parental FDCP-Mix cells were seeded at 2×10^5 cells/ml in Fischers medium containing 20% (v/v) horse serum with increasing concentrations of IL-3 shown and incubated at 39°C or 32°C. Viable and non-viable cell counts were determined by trypan blue exclusion at 24 h intervals, 24 (**a**), 48 (**b**) or 72 (**c**) h. The data is expressed as the percentage of viable cells in the culture. The data shown is the mean of 4–6 experiments (s.e.m. is less than 11% for all data points shown)

We observed no growth factor independent proliferation of either control or tsBcr–Abl transfected cells at either temperature (Figure 2a). In the absence of growth factor, FDCP-Mix cells (at 39°C or 32°C) and FDCP-Mix tsBcr–Abl clones 2 and 16 at the restrictive temperature for PTK activity die within 48 h. However, cultures of tsBcr–Abl clones 2 and 16 at the permissive temperature contain viable cells for up to 72 h after growth factor removal (Figure 2a) with cell viability being enhanced at all time points up to 72 h (Figure 2b). Significantly, no tsBcr–Abl FDCP-Mix cells survived beyond 96 h even at the permissive temperature i.e. apoptosis was delayed but not totally suppressed.

Having shown that activation of the Bcr–Abl PTK enhances short term survival in the absence of IL-3, a more detailed investigation of the effects of this PTK on cell viability and proliferation was carried out.

[^{3}H]thymidine incorporation assays showed no difference in the IL-3 dose response between control FDCP-Mix cells and the tsBcr–Abl expressing clones at 32°C or 39°C after 24 h (Figure 3a). However in longer term assays, over 72 h, distinct differences were observed (Figure 3b). The dose response curve for IL-3 showed significantly ($P<0.05$) enhanced rates of proliferation at lower doses (0.1–1.0 ng/ml) of IL-3 for tsBcr–Abl FDCP-Mix cell lines at the permissive temperature compared to the control cell line. The maximum proliferative response obtained, however, was equivalent for all of the cell lines shown. Thus Bcr–Abl synergises with sub-optimal concentrations of growth factor over a prolonged period in culture, as has been suggested to occur in Ph$^+$ cells (Eaves and Eaves, 1994; Laneuville *et al*., 1991; Matulonis *et al*., 1993).

A reduced dependence on growth factor for survival has been proposed as a mechanism by which Ph$^+$ cells acquire a survival advantage *in vivo*. (Bedi *et al*., 1994; Cotter, 1995; Daley *et al*., 1991; Eaves *et al*., 1985) We therefore assessed the possible synergy between IL-3 and Bcr–Abl PTK on survival. The results are shown in Figure 4. At the permissive temperature the Bcr–Abl expressing clones survive, but do not proliferate, at 0.01 ng/ml of IL-3 over a 72 h period. In contrast no viable cells persist at the restrictive temperature with the tsBcr–Abl clones or in the control cells at either 32°C or 39°C. Thus at very low concentrations of IL-3, where no enhanced proliferative response was observed (Figure 3), Bcr–Abl PTK synergistically promotes survival.

This synergistic effect on both proliferation and survival between Bcr–Abl PTK and IL-3 at low cytokine doses is in keeping with the apparent proliferative advantage that Ph$^+$ progenitor cells exhibit over their normal counterparts *in vivo* (Raskind and Fialkow, 1987).

One of the major advantages of the use of FDCP-Mix cells is their ability to undergo multilineage differentiation in response to cytokines (Heyworth *et al*., 1990; Spooncer *et al*., 1986). This allows the effects

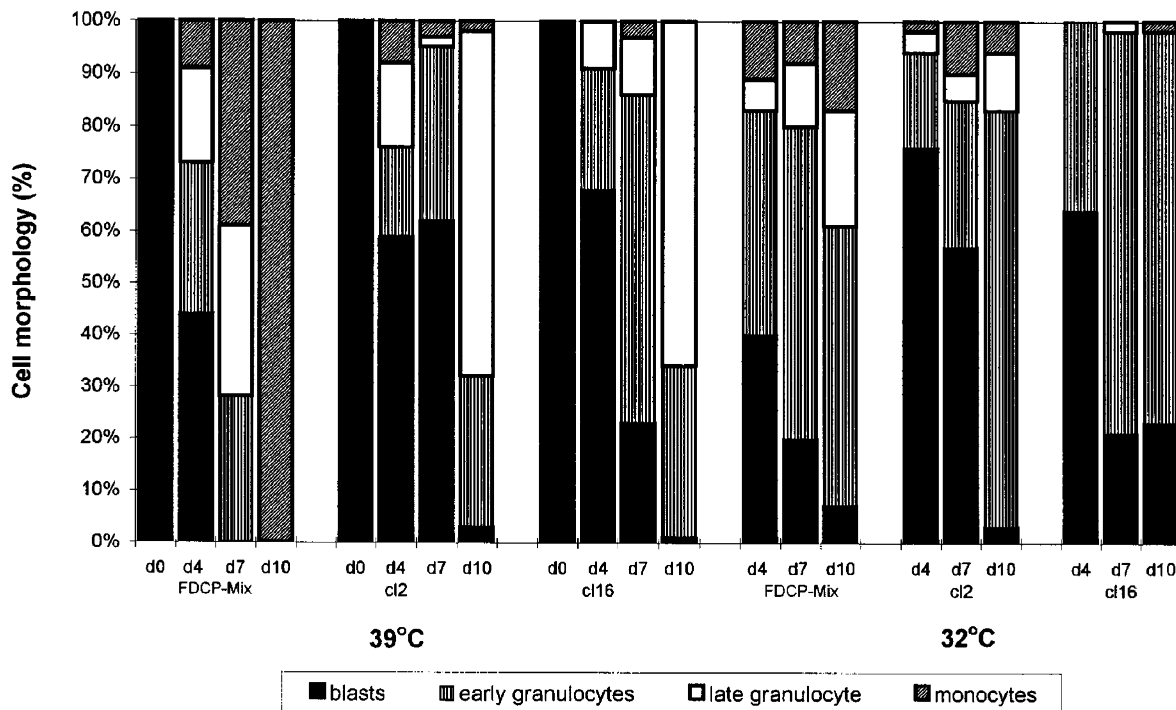

Figure 5 FDCP-Mix cells expressing p210 tsBcr–Abl retain their ability to develop into mature myeloid cells. TsBcr–Abl FDCP-Mix clones 2 and 16 and the parental FDCP-Mix cells were washed twice in Fischers medium (800 *g*, 10 min) and resuspended at 6×10^4 cells/ml in medium designed to support granulocytic differentiation and incubated at either 32°C or 39°C in an atmosphere of 5% CO_2 in air. Granulocyte differentiation medium consists of Iscoves Modified Dulbeccos Medium supplemented with preselected fetal calf serum (20% v/v), recombinant (r) murine GM-CSF (50 U/ml, Biogen, Geneva, Switzerland), r-human G-CSF (3000 U/ml, Amgen, Thousand Oaks, USA) and r-murine IL-3 (1.5 U/ml, Calbiochem, Nottingham, UK). Viable cell counts and levels of primitive clonogenic cells were monitored over a 10 day period. Cytospins were prepared using a Shandon cytospin 2 ($<5 \times 10^4$ cells/slide at 1000 r.p.m. for 5 min) after 4, 7 and 10 days, stained with May-Grunwald-Giemsa and differential morphology scored for greater than 100 cells per slide. Data shown is from a representative experiment of three

of Bcr–Abl on self renewal and differentiation to be assessed. When cultured in maximal stimulating concentrations of IL-3 the FDCP-Mix cells retain a primitive phenotype. However when cultured in lower concentrations of IL-3 supplemented with G-CSF and GM-CSF they differentiate into mature myeloid cells over a period of 7–10 days. The transfection procedure and temperature had no effect on the morphology of the cells as all the cells retained a blast cell morphology (Figures 5 and 6).

To determine whether the expression of Bcr–Abl PTK activity could perturb the development of the FDCP-Mix cells the cells expressing tsBcr–Abl and control cells were cultured at both 32°C and 39°C over a ten day period in the conditions which induce myeloid development. After 7 days in culture the blast cell morphology was lost and more mature cells were formed in all the control and tsBcr–Abl transfected cells at both temperatures. Quantitative assessment of the differential morphology of the cells in these conditions is shown in Figure 5.

In control FDCP-Mix cells after 4 days in culture the proportion of blast cells had diminished from 100% to approximately 40% at both the permissive and the restrictive temperature (Figure 5). After 7 days in culture the population consisted of a mixture of early granulocytic cells, neutrophils and macrophages at both 39°C and 32°C. After 10 days in culture at 39°C FDCP-Mix cells consisted principally of macrophages, whilst at 32°C macrophages, neutrophils and immature granulocytes were present in the culture. The delay in cell maturation at 32°C appears to be a temperature dependent effect. The change, at 39°C, from neutrophils and macrophage (at day 7) to principally macrophage (at day 10) reflects the very short life span of neutrophils. Indeed, in the day 10 cultures apoptotic neutrophils could be seen. The process of terminal maturation is thus occurring in FDCP-Mix cells at both 39°C and 32°C. Micrographs of the typical cells formed under these conditions are illustrated in Figure 6. We next investigated the effect of Bcr–Abl PTK activity on the development observed.

In both FDCP-Mix tsBcr–Abl clones neutrophils, macrophages and immature granulocytes could be seen in cultures at both the permissive and the restrictive temperature, after 4 days in the combination of cytokines that lead to myeloid development. By day 10 the cultures at 39°C consisted mainly of neutrophils and immature granulocytes (Figure 5). Cells grown at the permissive temperature also consisted predominantly of immature granulocytes with a large reduction

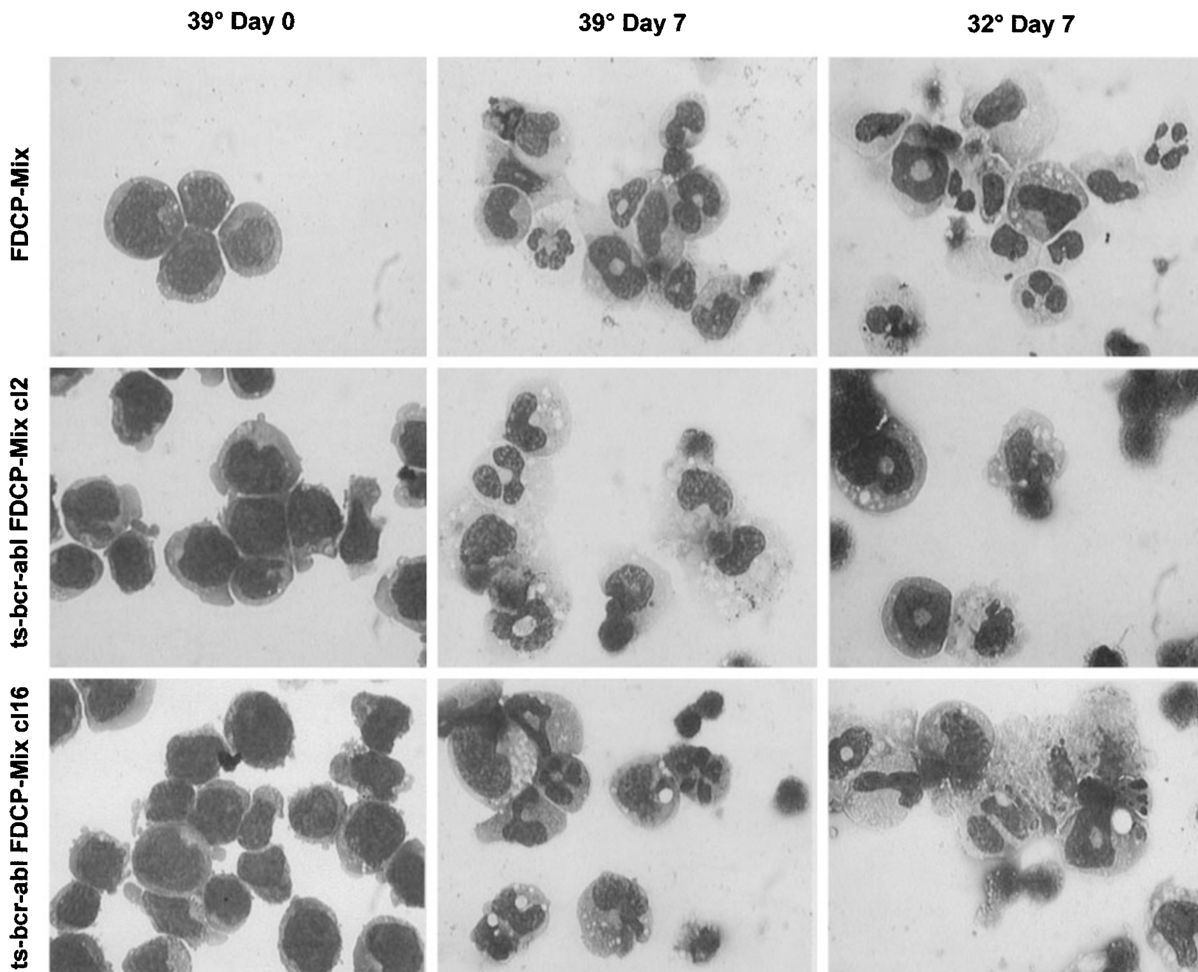

Figure 6 Morphology of control and p210 tsBcr–Abl expressing FDCP-Mix cells under conditions which induce myeloid differentiation. Micrographs of the parental FDCP-Mix and tsBcr–Abl transfected cells, clones 2 and 16, at both 32°C and 39°C under conditions which induce myeloid differentiation (see Figure 5). The Micrographs are of May–Grunwald–Giemsa stained cytospins prepared during the differentiation assay illustrating the typical morphology of the cells formed on days 0 and 7

in the number of blast cells. Micrographs of typical cells formed under these conditions are illustrated in Figure 6. Following differentiation the mature cells exhibited no further proliferative potential, in other words differentiation was accompanied by clonal extinction of the FDCP-Mix cells as has been previously described (Heyworth *et al*., 1990; Spooncer *et al*., 1986). Hence the immediate effect of expression of Bcr–Abl PTK activity in these multipotent cells is not to induce a differentiation blockade or 'immortalise' the FDCP-Mix cells. This reflects the situation seen in progenitor cells obtained from the bone marrow or peripheral blood of patients with CML in the chronic phase (Clarkson and Strife, 1993; Wetzler *et al*., 1993).

The Bcr–Abl transfected FDCP-Mix cells thus display many of the characteristics of Ph^+ progenitor cells from patients with CML which are not seen in other model systems. The initial effects of Bcr–Abl expression are subtle, the transfected cells retain their ability to differentiate into mature myeloid cells, as do CML progenitor cells during the initial chronic phase. They also retain their absolute requirement for growth factors to survive and proliferate. However, in the absence of added growth factor the Bcr–Abl transfected FDCP-Mix cells exhibit enhanced survival over a 24–48 h period. In the presence of concentrations of IL-3, where the parental cells exhibit no growth factor mediated survival (0.01 ng/ml) or proliferation (0.3 ng/ml), the transfected cells show increased survival and display an enhanced ability to proliferate. This synergistic proliferative effect between Bcr–Abl and IL-3 at low doses is in keeping with observations on Ph^+ progenitor cells which are capable of outcompeting the normal healthy progenitor cells *in vivo* (Raskind and Fialkow, 1987).

These results demonstrate that a closer analogy exists between the Bcr–Abl transfected FDCP-Mix cells and CML chronic phase progenitor cells than any other available model. These cells will therefore allow a systematic analysis of the consequences of Bcr–Abl expression on multipotent cells. Our present work is aimed at exploiting this model system to identify the key molecular mechanisms by which Bcr–Abl may contribute to the progression of the disease to the blast crisis.

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