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ORIGINAL ARTICLE

Anaplastic large cell lymphoma-propagating cells are detectable by side population analysis and possess an expression profile reflective of a primitive origin

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Cancer stem cells or tumour-propagating cells (TPCs) have been identified for a number of cancers, but data pertaining to their existence in lymphoma so far remain elusive. We show for the first time that a small subset of cells purified from human anaplastic lymphoma kinase (ALK)-positive and -negative, anaplastic large cell lymphoma cell lines and primary patient tumours using the side population (SP) technique have serial tumour-propagating capacity both *in vitro* and *in vivo*; they give rise to both themselves and the bulk tumour population as well as supporting growth of the latter through the production of soluble factors. *In vivo* serial dilution assays utilising a variety of model systems inclusive of human cell lines, primary human tumours and nucleophosmin (NPM)-ALK-induced murine tumours demonstrate the TPC frequency to vary from as many as 1/54 to 1/1336 tumour cells. In addition, the SP cells express higher levels of pluripotency-associated transcription factors and are enriched for a gene expression profile consistent with early thymic progenitors. Finally, our data show that the SP cells express higher levels of the NPM-ALK oncogene and are sensitive to an ALK inhibitor.

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INTRODUCTION

Cancer stem cells (CSCs) have been described for a number of cancers of both haematological and epithelial origin as being at the apex of a developmental hierarchy and possessing properties of tumour propagation and self-renewal.[1–4] This concept does not necessarily allude to the cell of origin, which may vary depending on the cellular context. Models describing the evolution of CSCs by hierarchical or stochastic processes are themselves the subject of much debate. This is particularly pertinent to the haematological system where cellular plasticity is especially evident.[5] Regardless of the mechanism, it may be more appropriate to define a cell which drives tumour growth *in vivo* as the tumour-propagating cell (TPC).[6] Data pertaining to the existence of a TPC in any human lymphoma to date has been sparse. This is in part due to the large array of lymphoma sub-types and limited availability of model systems.

A form of lymphoma for which many model systems do exist is nucleophosmin-anaplastic lymphoma kinase (NPM-ALK)-induced anaplastic large-cell lymphoma (ALCL).[7] NPM-ALK is produced as a consequence of the t(2;5) fusing the N-terminal region of NPM to the entire intracytoplasmic domain of ALK.[7,8] The oncogenic properties of NPM-ALK have been demonstrated many times; importantly, its ability to induce lymphoma *in vivo* has been proven unequivocally.[9–13]

We show that ALCL cell lines and primary patient samples contain a side population (SP) of cells, which express ABCG2 transporters. The SP cells cannot be uniquely defined by surface immunophenotype but do express a signature of genes associated with 'stemness' and pluripotency and are enriched for a gene expression profile associated with early thymic progenitors (ETPs). Furthermore, the SP cells not only propagate the bulk tumour but also support the growth of the bulk population. Furthermore, we show that the SP cells are resistant to therapy with etoposide yet are sensitive to the ALK inhibitor Crizotinib, suggesting the latter may protect to some extent against disease relapse.

RESULTS

Identification and characterisation of SP cells in ALCL cell lines and primary patient tumours

SP cells were first identified in murine bone marrow by virtue of their ability to efflux Hoechst 33342 dye via drug efflux pumps.[14] The SP fraction was then shown to be enriched for haemopoietic stem cells. Hence, we employed the SP technique as a crude method to identify cells within the bulk lymphoma population

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that may have stem-like properties. We first optimised the method to reveal SP cells in ALCL cell lines (SUDHL-1, DEL, Karpas-299, FEPD) using guidelines as described previously.[15] Each of the cell lines studied contained a proportion of SP cells accounting between 1 and 3.5% of the bulk cell population (Figure 1a). Furthermore, SP cells disappeared on blocking the ABC-transporter (ABCG2) responsible for Hoechst 33342 exclusion with Verapamil. Indeed, ABCG2 transcripts were detectable to a higher level in the SP cells in comparison to sorted main population cells (MP: SP-depleted cells; Figure 1b). Importantly, the presence of an SP population was also confirmed in primary tumour samples isolated from patients diagnosed with ALCL ($n = 7$; range = 0.4–3% SP cells; representative example shown in Figure 1c).

SP cells proliferate *in vitro* and *in vivo* and regenerate the bulk population

SP and MP populations were sorted from each of the ALCL cell lines into 96-well tissue culture plates and monitored for cell growth and differentiation (Figure 2, Supplementary Figure 1A). The SP cells isolated from each of the cell lines (representative example shown in Figure 2a, others in Supplementary Figure 1A) proliferated significantly more than the MP cells as determined by trypan blue exclusion (Figure 2a) and MTS assay (Figure 2b). In addition, 35 days after sorting the cells, analysis of the SP fraction was performed and showed that the SP cells had given rise to MP cells and furthermore the majority of this population were in either the S or the G_0/G_1 phase of the cell cycle (Figures 2c and d). Indeed, for all four of the cell lines examined, SP cells not only gave rise to MP cells but also more SP cells (Supplementary Figure 1B). By contrast, by 48 h following the initial cell sort, the majority of the MP cells were dead as assessed by positivity for Annexin V (Figure 2e), suggesting that in the absence of SP cells, the MP cells are unable to survive and proliferate. We also note that the isolated SP cells undergo a higher level of apoptosis than the whole-cell population, although as shown in Figures 2a and b the surviving cells continue to proliferate. Intriguingly, the whole

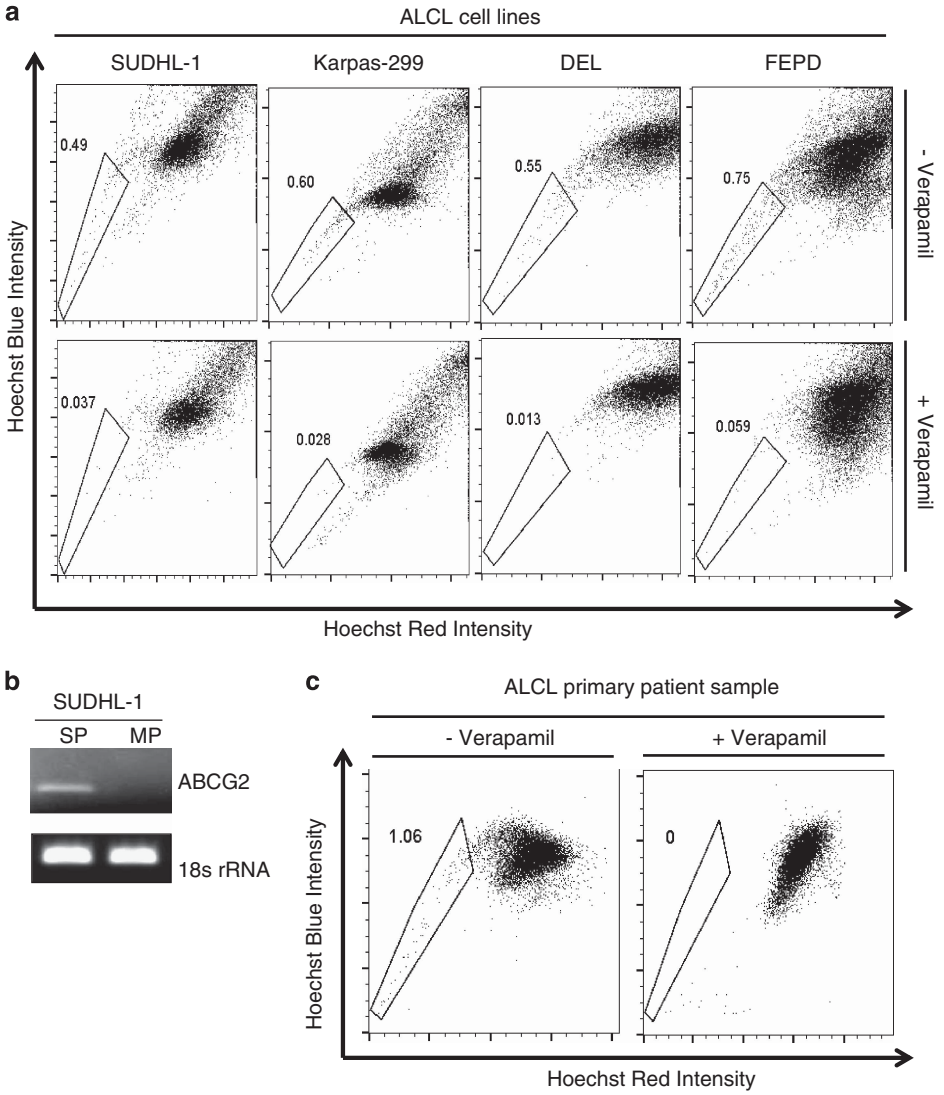

Figure 1. Human ALCL cell lines and primary patient tumours contain a small proportion of SP cells (< 3.5%). (**a**) Cells were incubated with Hoechst 33342 for 60 min in the absence or presence of 100 μM Verapamil in order to analyse the percentage of SP cells. (**b**) Reverse transcription–PCR detection of ABCG2 gene transcripts. (**c**) Primary patient tumour material contains a small percentage of SP cells (representative of seven individual patient specimens examined). Ho = Hoechst 33342; SP = side population. For all cell line experiments, the data shown in this figure are representative of those obtained from at least four independent cell lines, which in each scenario were analysed at least three times.

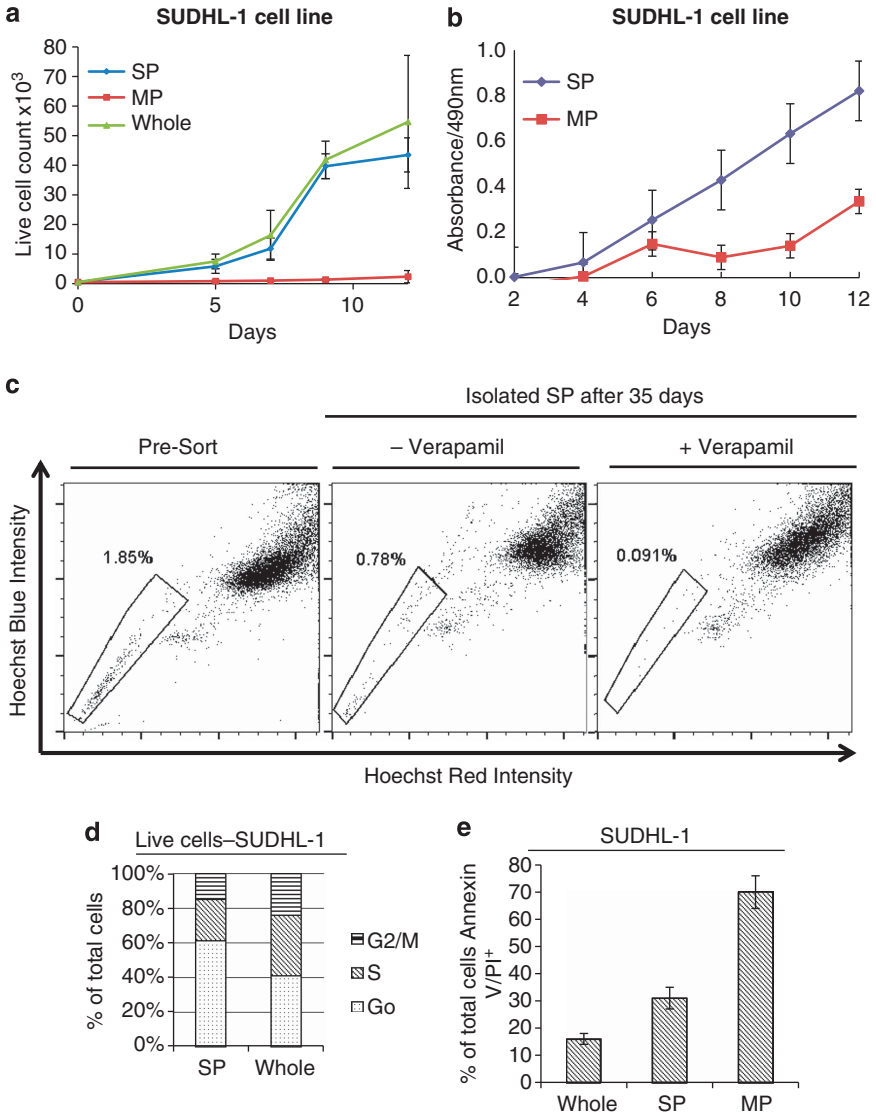

Figure 2. Isolated SP cells propagate *in vitro* and *in vivo* and give rise to MP cells. (**a**) The growth of SP cells isolated from cell lines by flow cytometry was assessed by trypan blue exclusion and data represent the increase in live cell number above the starting concentration of 500 cells/well. These data represent the SUDHL-1 cell line and results for the three other cell lines are shown in the Supplementary Information. (**b**) An MTS assay was performed to analyse the proliferative potential of SP in comparison to MP cells. (**c**) Isolated SP cells give rise to MP cells following 35 days in culture. These data are from the SUDHL-1 cell line and are representative of all four cell lines examined for which data are shown in the Supplementary Information. (**d**) Following 35 days in culture, the majority of the live cells produced from the isolated SP cells are in Go/G1 or S-phases of the cell cycle. (**e**) The majority of cells from the MP-initiated cell population are dead following 48 h in culture, as determined by positivity for propidium iodide (PI) and/or Annexin V. Error bars represent standard deviations of the mean of triplicate counts and these data are representative of at least five experiments.

population (SP and MP cells together), which had undergone the same procedures as the sorted cells, grew at a similar rate to the isolated SP, suggesting that the SP cells might provide proliferative and survival signals to the MP cells. Indeed, Transwell experiments suggest that the SP cells produce a soluble factor(s) that encourages the growth of MP cells (Figures 3a and b and Supplementary Figure 1C): when SP and MP cells were separated by a Transwell membrane that allows the free passage of soluble factors but not cells, MP cells cultured in shared media with SP cells proliferate, whereas those in the absence of SP cells did not. It was not possible to replicate this work on primary samples as none of the patient samples available propagated *in vitro* as has been found by others.

To further assess the tumorigenic capabilities of SP cells, NSG mice were injected subcutaneously under the flanks with either 5000 SP or MP cells isolated from the SUDHL-1 cell line suspended 1:1 in Matrigel. As shown in Figure 3c, the SP cells gave rise to tumours in 100% of cases and these tumours were significantly larger than those tumours that were produced from MP cells (SP cells were injected into the left flank of the mouse and MP cells to the right flank; given the same period of time (6 weeks), MP cells produced tumours in less than 50% of cases and were significantly smaller, $P < 0.001$). These data were reproducible with the Karpas-299 cell line (Figure 3d). Furthermore, on serial propagation of the SUDHL-1 cell line SP or MP-derived tumours in secondary recipients, 5/5 SP hosting mice developed tumours, whereas 5/5 MP-injected mice did not. Furthermore, with a primary patient tumour (isolated from a 55-year-old patient diagnosed with ALCL, ALK$^-$) we have been able to propagate tumours from 5000 SP cells (6/6 mice) and partially from 5000 MP cells (3/6 mice) when suspended in Matrigel in NSG mice (Table 1). Secondary transplantation of limiting dilutions of the tumours

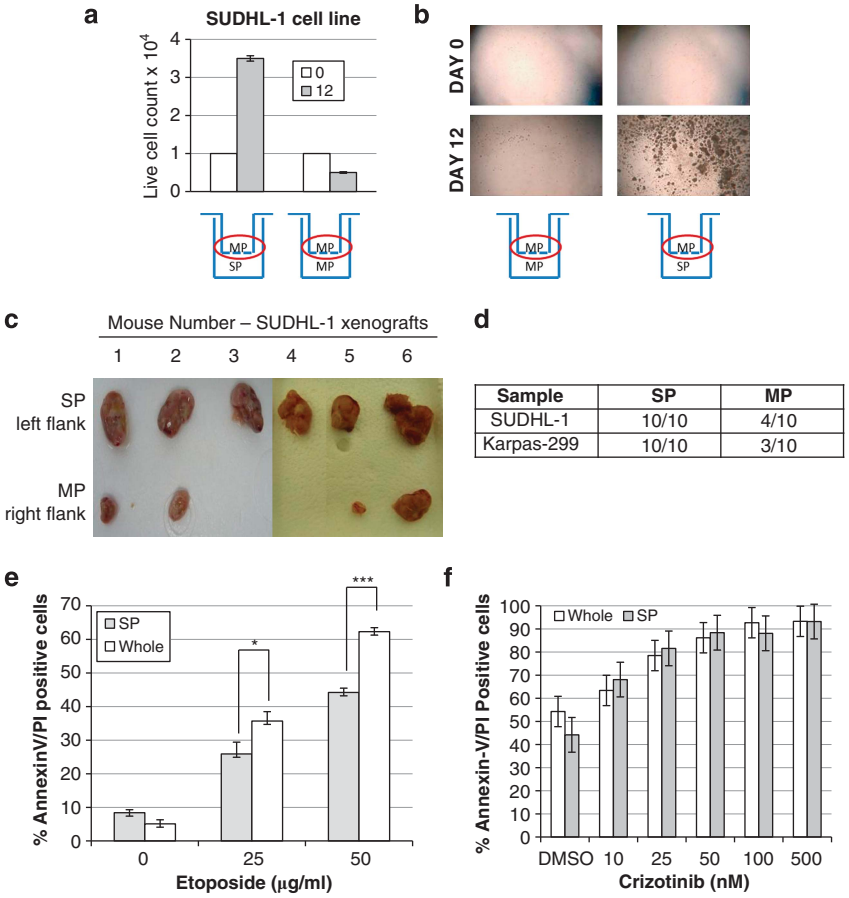

Figure 3. SP cells provide a soluble growth signal to MP cells and are sensitive to the ALK inhibitor Crizotinib. (**a**) SP and MP cells were cultured in Transwell plates separated by a membrane, which allows the free movement of media but restricts cell movement between compartments. Cell counts depicted in the graph represent those cells in the Transwell as indicated in the cartoon below the graph as do the images in the right panel (**b**). Data in this figure were obtained from the SUDHL-1 cell line; similar data for the Karpas-299 cell line are shown in the Supplementary Information. (**c**) SP cells produce large tumours *in vivo* when 5000 cells are injected in Matrigel into the flank of NSG mice. SP cells were injected into the left flank and MP cells into the right flank of the same mouse and tumour growth was monitored. These data are representative of 10 mice injected for each population. (**d**) Mice injected with SP cells isolated from ALCL cell lines as indicated produce tumours in NSG mice. NSG mice were injected under the flanks with either 5000 SP (left) or 5000 MP (right) cells suspended in Matrigel. (**e** and **f**) SP and whole-cell populations were incubated with the indicated concentrations of Etoposide (**e**) or Crizotinib (**f**) for 24 h and cell death monitored by positivity for Annexin V and/or PI as determined by fluorescence-activated cell-sorting analysis. $*P < 0.05$, $***P < 0.001$. These data are representative of triplicate experiments performed using either the SUDHL-1 or the Karpas-299 cell lines (data for the SUDHL-1 cell line are represented here; for Karpas-299 see Supplementary Information).

arising from the SP cells produced tumours in 100% of cases (9/9 mice), whereas for MP-derived tumours, no tumours resulted even following transplantation of 500 000 cells, suggesting that the original MP-derived tumours were the result of limited reproductive capabilities (Table 1).

Limiting dilution experiments were also carried out whereby a tumour derived from the SUDHL-1 cell line was isolated from an NSG mouse host, disaggregated and re-injected suspended in Matrigel in limiting dilutions into NSG mouse hosts (Table 2). When as few as 50 cells were injected into the recipient mice, 2/3 developed tumours, in contrast, none of the mice injected with five cells produced tumours indicating that at least 1 in 50 tumour cells have propagating capacity. Indeed following ELDA (Extreme Limiting Dilution Analysis) of the experimental data using a confidence interval of 95%, the predicted TPC frequency is 1/54 with a range of 1/14 to 1/215.[16] In a similar experiment, primary human tumour cells (the same patient sample as described above was employed) suspended in Matrigel were injected into the flanks of NSG mice. As shown in Table 2, at least 10^2–10^3 cells are required for tumour formation, suggesting that at least 0.1–1% of the patient tumour cells are capable of propagating the tumour *in vivo* in this model system consistent with the cell line xenograft. These data are also consistent with the 0.4–3% occurrence of SP cells in these patient specimens (Figure 1c). However, ELDA of these data indicate the ratio of TPC to be much lower at 1/1336 (95% confidence interval, 1/313 to 1/1336), which may be attributable to the difficulties of assessing TPC frequency with primary tumour tissue.[16] However, as has been previously shown, the model system employed to detect TPC can result in a considerable difference in both estimation of TPC frequency and even their presence.[17,18] Hence, we employed NPM-ALK transgenic mice using the CD4 enhancer promoter system, which in 100% of cases develop thymic lymphomas to investigate the presence of TPC in an immune-competent setting.[9] Tumours arising in the CD4/NPM-ALK transgenic mouse model were isolated and following injection in Matrigel, subcutaneously into immune-competent wild-type congenic C57BL/6 mice, developed tumours in 100% of cases when 10^3 cells were injected, 50% when 10^2 cells were present and at an incidence of 29% following implantation of 10 cells (Table 2). Applying ELDA software to analyse these data, the TPC frequency is estimated to be 1/105 (confidence interval 95%, 1/57 to 1/192), a value that sits closer to

Table 1. SP cells isolated from a primary patient tumour (isolated from a patient diagnosed at age 55 years with ALCL, ALK$^-$) propagate tumour growth *in vivo* in NSG mice, whereas MP cells have limited repopulating capacity

Origin of cells	*Number of cells injected*		*Number of mice injected*	*Number of mice developing tumours*
Primary patient tumour	SP	5000	6	6
	MP	5000	6	3
Secondary transplantation				
SP-derived tumour	Whole cells	5000	3	3
		50 000	3	3
		500 000	3	3
MP-derived tumour	Whole cells	5000	3	0
		50 000	3	0
		500 000	3	0

Abbreviations: ALCL, anaplastic large-cell lymphoma; AKL, anaplastic lymphoma kinase; SP, side population. SP and MP cells were sorted from a primary patient tumour before implantation into NSG mice at the quantities indicated above. A secondary transplantation using cells isolated from the resulting tumours was performed as indicated using limiting dilutions of cells. A final tertiary transplantation was performed with limiting dilutions of whole cells from originated from the SP-derived primary tumour.

Table 2. Limiting dilutions of cell line-derived tumour cells, a primary patient tumour and murine CD4/NPM-ALK tumours propagate tumour growth *in vivo*

Number of cells injected	*Number of mice injected*	*Number of mice developing tumours*
Human SUDHL-1 cell line		
5×10^3	4	4
5×10^2	4	4
50	3	2
5	3	0
Primary patient (human) tumour		
10^5	3	3
10^4	3	3
10^3	3	1
10^2	3	1
CD4/NPM-ALK murine tumours		
10^4	9	8
10^3	8	8
10^2	16	8
10	14	4

A tumour derived from the SUDHL-1 cell line was isolated from an NSG mouse, disaggregated and re-injected into recipient mice in limiting dilutions. Tumour growth was monitored over a 1-year period. Similarly, primary patient (ALK$^-$ ALCL) or murine tumour tissue (CD4/NPM-ALK transgenic mice; these data represent the combined results of three separate experiments) was disaggregated, suspended in Matrigel at the indicated concentrations and injected into the flanks of NSG (human tumour) or congenic C57Bl/6 (mouse tumour) mice. Tumour growth was again monitored over a 1-year period.

the TPC frequency determined from cell lines rather than from primary patient material.

SP cells are sensitive to ALK inhibition

Having established that SP cells propagate tumour growth both *in vitro* (cell lines) and *in vivo* (cell lines and a primary human tumour), we set out to establish the chemotherapeutic sensitivity of these cells. As MP cells when cultured on their own undergo apoptosis, they were excluded from these studies (Supplementary Figure 2A). Initially, the SP cells isolated from the SUDHL-1 cell line were incubated in the presence of etoposide, a standard chemotherapeutic agent, and apoptosis monitored by Annexin V positivity. In comparison to the whole-cell population, SP cells were significantly more resistant to etoposide exposure (Figure 3e; 44% SP cells apoptotic following treatment with 50 μg/ml etoposide vs 62% of the whole-cell population, $P < 0.001$, and Supplementary Figure 2B for the Karpas-299 cell line). Given that ALCL is associated with aberrant expression of the ALK protein, which has previously been demonstrated as a valid therapeutic target, we also assessed the chemotherapeutic sensitivity of SP and whole-cell populations to Crizotinib, the ALK/c-met inhibitor (Figure 3f).[19] Initially, we confirmed the dose range of ALK-sensitivity to Crizotinib in whole-cell populations (Supplementary Figure 2C) and expression of ALK (Supplementary Figure 2D) in the SP cells isolated from the Karpas-299 and SUDHL-1 cell lines. SP cells were found to express higher levels of NPM-ALK transcripts than the MP perhaps due to a higher dependency on aberrant ALK-induced signalling pathways for cellular survival (Supplementary Figure 2D). Regardless, following 24 h of treatment with 10 nM Crizotinib, 63.4% and 68.1% ($P>0.5$) of SP and whole-cell population, respectively, had undergone apoptosis as demonstrated by positivity for Annexin V and this trend was maintained as the doses of Crizotinib were increased (Figure 3f, a similar trend was observed for the Karpas-299 cell line—Supplementary Figure 2E). By 48 h of treatment, both SP and whole-cell populations displayed 100% cell death following Crizotinib treatment (data not shown). These data suggest that while etoposide might not be optimal for the eradication of SP cells, the specific ALK inhibitor Crizotinib may well be effective in their elimination, although these data require corroboration in patient tumour samples and xenografts as well as with other chemotherapeutic regimens.

SP cells express a primitive gene expression profile

To further define the SP and MP cellular populations, gene expression profiling was performed with RNA isolated from sorted SP and MP cells isolated from the ALCL cell lines using Affymetrix ST arrays (genes significantly altered in the SP in comparison to the MP cells common to all cell lines are shown in Supplementary Tables 3 and 4). Despite SP and MP populations having derived from the same culture, the gene expression profiles clustered separately for each of the individual cell lines (unsupervised analysis) indicating distinct differences in the expression signature between them, although the combined SP did not cluster separately from the MP for all cell lines together (Supplementary Figure 3A). However, following supervised clustering, the SP and MP for all of the cell lines analysed clustered into two distinct branches clearly separating the SP from the MP populations (Figures 4a and b). These data suggest that differences in gene

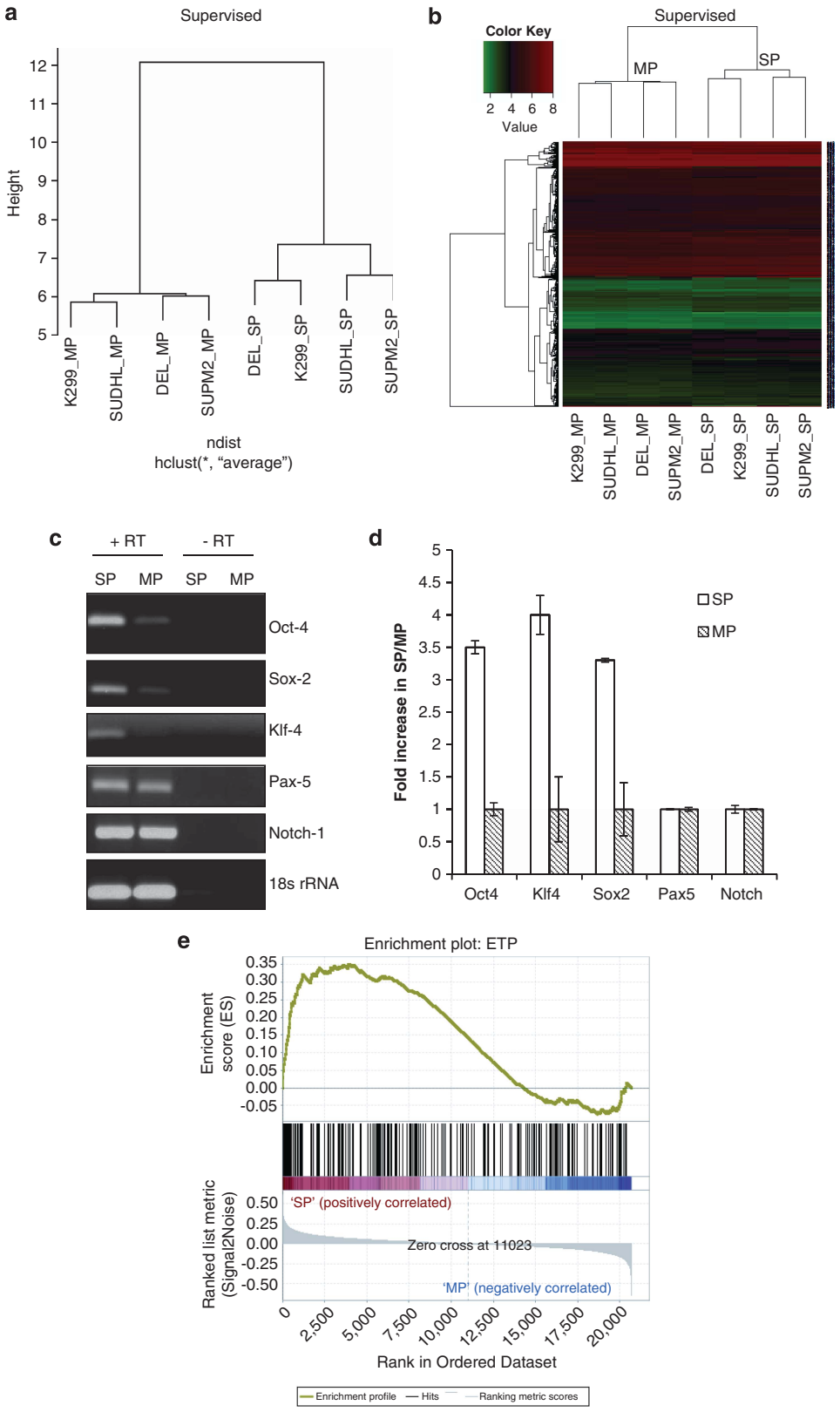

Figure 4. SP and MP cells isolated from cell lines express a 'primitive' expression signature. (**a**) Supervised clustering of gene expression array data obtained from isolated SP and MP of the ALCL cell lines as indicated. (**b**) Heat map corresponding to data shown in '**a**'. (**c**) Reverse transcriptase PCR demonstrates the elevated expression level of Oct4, Sox2 and Klf4 in SP cells. (**d**) Data are represented as fold increase in transcript expression in SP cells as compared with MP cells as detected by real-time quantitative PCR. (**e**) Gene set-enrichment analysis plot of ETP (early thymic progenitor; CD34+ CD1a- from neonatal thymus) signatures in SP vs MP gene expression profiles ($P = 0.0062$, FDR = 0.044). In **c** and **d,** data are shown for the SUDHL-1 cell line, although at least three other cell lines were also analysed. These data showed the same overall results but are not shown here. In **a**, **b** and **e**, combined data are shown for all of the cell lines examined including SUDHL-1, SUP-M2, Karpas-299 and DEL.

expression signatures between the individual cell lines outweigh those that differentiate the SP from the MP, and therefore we also conducted gene set-enrichment analysis as detailed below. Genes upregulated in the SP as opposed to the MP compartment were enriched for functions such as transcription/chromatin organisation (31%), development (32%) and reproduction (32%) based on biological process annotations (GO analysis, Supplementary Figure 3B). Gene set-enrichment analysis of data from all of the cell lines together showed significant enrichment ($P = 0$, FDR = 0) within the SP compartment for HSC-associated marker genes (Supplementary Figure 3C).[20] Indeed, Oct4, Sox2 and Klf4 were observed at higher levels in SP as compared with isolated MP cells, whereas lineage defining transcription factors, Pax-5 and Notch 1 were equally expressed in SP and MP cellular compartments (Figures 4c and d). When the gene expression profile of SP cells (all cell lines) was compared with other gene expression signatures derived from recently defined normal human haematopoietic stem and progenitor subsets,[21,22] we observed significant enrichment ($P < 0.01$, FDR < 0.25) within the SP population for only the ETP transcriptional signature (the list of probes from the ETP data set detected with significance in the SP population are shown in Supplementary Figure 3D; there was no enrichment for the multi-potent progenitor, megakaryocyte-erythroid progenitor, myeloid-erythroid progenitor, B/NK precursor, Pro-B cell, common myeloid progenitor or multi-lineage progenitor gene profiles), suggesting an origin in a primitive cellular compartment (Figure 4e).

CD117 is expressed on the surface of a small percentage human tumour cells but is not an exclusive marker of ALCL TPCs

As immature cell surface markers have previously been linked with TPC identification, we examined the cell lines and three of the patient samples (all ALK^+) for expression of a variety of cell surface markers typically associated with immature haemopoietic cells including CD34, CD90 and Flt3 (CD135) and these data are displayed in Supplementary Table 5. Although a small percentage of cells in each case express some of these proteins, we have thus far been unable to confirm that their expression correlates with the functional capabilities of SP cells. In particular, we found that a small percentage were positive for CD117 (Supplementary Figure 4, Supplementary Table 6). We therefore sorted $CD117^+$ and $CD117^-$ cells from the SUDHL-1 cell line and injected them into NSG mice. In all cases, mice developed tumours in the same time frame suggesting that although $CD117^+$ cells are able to propagate the tumour, they are not the exclusive host of this capacity (Supplementary Table 7).

DISCUSSION

The concept of CSCs is not new but has been evolving for well over 50 years.[23] Indeed, the definition of a CSC varies slightly from study to study and there is much debate over whether a CSC must arise from a tissue-specific stem cell or even if it can evolve from a more differentiated cell, which re-acquires self-renewal properties. Naturally, in the lymphoid system, self-renewal properties are re-acquired in the memory compartment presenting ideal candidates for CSC. In fact, the memory T-cell compartment has recently been shown to be the source of peripheral T-cell lymphoma in a mouse model.[24] In our study, we define the CSC as a TPC, which is the population of cells which not only gives rise to the bulk population but that also sustains its growth. In our study, we identified TPC based on their functional capacity to regenerate the bulk tumour (by SP analysis) rather than a primitive cell surface marker expression profile. Indeed, primitive (or any other) haemopoietic cell surface markers did not distinguish a population of tumour cells with a unique tumour-propagating capacity. At least one other study has also found that tumour-initiating activity is not restricted to distinct immunophenotypic cellular compartments.[25] Intriguingly, although the bulk tumour population depleted of SP cells is unable to propagate, the presence of SP-conditioned media reverses this phenotype supporting proliferation, although the identity of the soluble factors that mediate this activity remain to be determined. In addition, we show that the TPC population expresses transcripts typically associated with pluripotent potential including Oct4, Sox2 and Klf4 perhaps enabling self-renewal. Indeed, one other study has also indicated Sox2 expression in a subset of cell within ALCL cell lines, which following inhibition results in decreased tumour growth in a xenograft model.[26] In further evidence of the presence of TPC in human lymphoma, we show employing two model systems (human ALCL cell lines and primary patient tumours in immunodeficient mice) that TPC are present in all three scenarios albeit at different frequencies. In addition, a murine model of NPM-ALK-induced T-cell tumours in immunocompetent mice demonstrates the presence of TPC. Employing ELDA analysis, the TPC frequency is predicted to be as many as 1/54 to as few as 1/1336 cells depending on the model system assessed (cell lines >murine tumours >primary patient tumours). Indeed, it would be expected that a healthy cell line adapted to growth *in vitro* would have a higher percentage of detectable TPC than a primary patient tumour as would a murine tumour in a congenic immune-competent recipient. Underestimation of TPC frequency has previously been attributed to the robustness of the model system used, most notably in the case of melanoma.[17] In addition, the presence of TPC in lymphoma has previously been discounted or at least been described as highly prevalent using murine model systems.[18] In order to address the caveats associated with xenograft systems raised by these previous publications, we have employed the NSG mouse and have resuspended human tumour cells in Matrigel in order to enrich their environment *in vivo*. Furthermore, we have assessed a model of NPM-ALK-induced tumour growth for the presence of TPC employing an immune-competent congenic host to discount immune-mediated destruction of transplanted cells and hence an underestimation of TPC frequency. Although none of these model systems alone are perfect representations of the presence of TPC, the combined data lend support to their existence.

Furthermore, we have shown that the TPC population resembles an ETP at the transcript level suggestive of an origin in a primitive haemopoietic cell with T-lineage potential. Our proposed origin in an ETP, the resultant tumour phenotype and the route between them can be postulated (Supplementary Figure 5). It is feasible that a primitive haemopoietic cell, most likely in this scenario a cell harbouring T-lineage potential, hosted the initiating event, in this case the t(2;5). This cell may well reside in the thymus as the result of a developmental defect in keeping with the paediatric presentation of the disease, which is then sufficiently long-lived to acquire further mutations that confer the hallmarks of cancer.[27] This cell may have then undergone stepwise progression through lineage differentiation to evolve into a TPC, a process possibly aided by VDJ (variable, diverse and joining) recombination. It is also likely that multiple clones of TPCs exist accounting for inter-patient variability, although it will be difficult to dissect these populations given their small frequency and the rarity of fresh patient material for this disease. However, we cannot rule out the hypothesis that an ETP-like signature is switched on in mature T cells transformed as a result of NPM-ALK activity, an activity that confers stem-like properties to these mature cells. Indeed, the presumed cell of origin of ALCL is considered a mature cytotoxic T cell owing to expression of cell surface proteins such as CD4, and furthermore, as having an activated phenotype because of the expression of CD30 and the production of Perforin and Granzyme B.[28,29] Moreover, rearrangements of the T-cell receptor genes are common and detectable at

the molecular level even in tumour cells that do not express any obvious T-cell-associated cell surface markers, the so-called 'null' entities.[28] However, it is not clear whether these cancers arose from transformed activated T cells, indeed our data suggest otherwise and in support a recent publication has shown that expression of cytotoxic molecules is dependent on expression of the driving oncogene and hence may be an artefact of its activity rather than an indicator of the cell of origin.[30] In addition, it has been reported that NPM-ALK transcripts are detectable in 1.95% (2/103) of cord blood samples, a body fluid that is enriched for haemopoietic stem cells, and although the exact cellular compartment within cord blood that carries the translocation was not indicated, these data lend support to the hypothesis that the origins of ALCL might indeed lie in a primitive cellular compartment.[31] A screen of paediatric thymi for the presence of the t(2;5) may shed light on the true cell of origin but given the 1.2 per million incidence of ALCL in the paediatric population this may be a giant undertaking.[31]

Finally, it is possible that the TPC population accounts for the high rate of relapse (25–30%) and minimal residual disease observed for ALCL.[32–34] Indeed, quiescent leukaemic stem cells have been attributed to minimal residual disease in paediatric lymphoblastic leukaemia.[35] Our data hint that the ALK/c-met inhibitor Crizotinib may be efficacious in the elimination of the ALCL TPC and therefore may prevent at least some cases of disease relapse but of course this remains to be extensively determined.

MATERIALS AND METHODS

Primary patient samples

Primary patient samples were provided by the Leukaemia and Lymphoma Research lymph node bank (Glasgow, UK) with ethical approval (ref: 06/MRE04/90). A total of seven patient specimens were analysed for the presence of SP cells (all diagnosed with ALCL: four ALK^+ specimens, ages at diagnosis: 5, 12, 25 and 50 years, and three ALK^- specimens, ages at diagnosis: 37, 55 and 85 years). All samples were provided as freshly frozen, disaggregated viable tumour tissues for which flow cytometry and a full pathology report had been performed before freezing and stored in liquid nitrogen. Before use, tumour cells were thawed and cultured in RPMI 1640 (GE Healthcare Life Sciences, Little Chalfont, UK), 10% fetal bovine serum (FBS; Biosera, Ringmer, UK) and 1% penicillin-streptomycin (Life Technologies, Paisley, UK) at 37 °C, 5% CO_2 overnight.

Cell Lines

Human ALCL cell lines used were as described in Cui *et al*.,[36] except the FEPD cell line, which was kindly provided by Professor A Del Mistro, University of Padova, Italy.[37]

Animals

Adult female NOD.Cg-Prkdcscid Il2rgtm1Wjl/SzJ (NSG) mice (Jackson Labs, Bar Harbor, ME, USA) were housed in individually ventilated cages under sterile pathogen-free conditions in the animal facilities at the Gurdon Institute, University of Cambridge, UK. All animal procedures were performed in accordance with the directives of the Home Office, UK under PPLs 80/2169 and 80/2630.

CD4/NPM-ALK transgenic mice generated as previously described[9] were kindly provided by Professor G Inghirami, University of Turin, Italy, and were housed as detailed above. These mice were maintained on a C57BL/6 background.

Antibodies and chemicals

Directly conjugated antibodies for fluorescence-activated cell-sorting analysis were purchased from BD Biosciences (Oxford, UK) and used at dilutions of 1:100 (see Supplementary Table 1). Western blot antibodies including anti-pALK (Y1604) and anti-ALK (clone 4C5B8) were purchased from Cell Signalling Technology (Leiden, The Netherlands) and Life Technologies (Paisley, UK), respectively, and used at a dilution of 1:1000, anti-tubulin was purchased from Sigma (Gillingham, Dorset, UK) and used at a dilution of 1:50 000. Crizotinib was kindly provided by Pfizer (La Jolla, CA, USA) and was dissolved in DMSO before storage in aliquots at − 20 °C. Etoposide was purchased from Sigma and suspended in DMSO before use.

Fluorescence-activated cell sorting

Cells were analysed as described by Turner *et al*.[12] Nonspecific binding of antibody was assessed using isotype control stained samples (See Supplementary Table 1). Cells were analysed using the FACSCaliber or sorted into distinct populations with a MoFlo cytometer (BD Biosciences) and events captured using FlowJo Flow Cytometric Analysis Software (http://www.flowjo.com/).

Real-time quantitative PCR and reverse transcriptase PCR

RNA was isolated from cells using Qiagen RNeasy kits (Qiagen, Manchester, UK) according to the manufacturer's instructions. Both real-time quantitative PCR and reverse transcription PCR were carried out on unamplified RNA as described by Cui et *al*.[36] using primers described in Supplementary Table 2.

SP assay

Cell lines were cultured as indicated above and employed in the assay when in the log-phase of growth. The method used was previously described by Goodell *et al*. and employing the refinements indicated by Golebiewska *et al*.[14,15,38–40] Briefly, 1×10^6 cells/ml were washed then resuspended in 1x phosphate-buffered saline, 2% FBS, 10 mM HEPES (Life Technologies). For optimisation of the assay, the cells were incubated with a variety of Hoechst 33342 (Sigma-Aldrich, Dorset, UK) dye concentrations for different time intervals and incubated at 37 °C in a water bath for 60, 90 or 120 min with or without 100 μM Verapamil (Sigma-Aldrich). Tubes were mixed several times at regular intervals during the incubation step. After incubation, the samples were immediately put on ice and collected by centrifugation at 1200 r.p.m. at 4 °C for 5 min then maintained on ice to prevent further dye efflux until analysed. The cells were counterstained with 2 μg/ml of propidium iodide (Sigma-Aldrich) to discriminate dead cells.

The cells were analysed for dye efflux on a CyAn ADP UV Model Detector (DakoCytomation, Cambridgeshire, UK) and sorted using a MoFlo cell sorter (DakoCytomation). Cells were analysed by dual-wavelength analysis using 424/44 (Hoechst blue) and 585/42 (Hoechst red) band-pass filtres. Propidium iodide fluorescence was excited by a 488-nm laser and detected after passing through a 630/22 band-pass filtre. Cell events were collected within a defined forward and side scatter gate to exclude cellular debris. At least 10 000 events were collected.

The cells were sorted into RPMI 1640, 10% FBS culture medium for *in vivo* and *in vitro* functional analyses. The SP cells were collected in a defined gate drawn according to the cell events detected in the Verapamil-treated control cell samples. Hence, only cells that were Hoechst low or negative were selected as SP cells with the remainder defined as MP cells.

Cell viability/proliferation assays

The growth potential and viability of SP and MP cells were determined by trypan blue exclusion. Briefly, 500 SP or MP cells were sorted in triplicate (for each time point) into the wells of a 96-well plate and cultured in RPMI 1640, 10% FBS at 37 °C, 5% CO_2. Every other day, an aliquot of cells were stained with trypan blue solution (Sigma-Aldrich) and counted using a haemocytometer.

Proliferative capabilities of SP and MP cells were determined using the CellTiter 96 AQueous One Solution cell Proliferation Assay kit (Promega, UK) according to the manufacturer's protocol.

Cell cycle analysis

Cell cycle analysis was performed as described by Cui *et al*.[36] Data were analysed using FlowJo v7.6 Flow Cytometry Software (Treestar, Ashland, OR, USA).

Apoptosis assay

Cells were analysed as described by Cui *et al*.[36] Data were analysed using FlowJo v7.6 Flow Cytometric Analysis Software (Treestar).

Transwell assay

Isolated SP and MP cells were cultured on Polyester Transwell Permeable supports in 24-well plates (Becton Dickinson, Oxford, UK). Cells suspended in RPMI 1640/10% FBS (600 µl of 5×10^3/ml) were added to the wells of the 24-well plate at the same time as cells were plated in the Transwell (100 µl of 1×10^4/ml) before incubation at 37 °C, 5% CO_2 for 12 days. On day 12, live cell counts from each lower well and Transwell were taken following incubation with Trypan blue to allow dead cell discrimination. Images of cells in the Transwells 12 days following initial seeding were taken using a Leica D-LUX 3 camera mounted onto a Labovert microscope at x100 magnification (Leica, Milton Keynes, UK).

Gene expression array

RNA quality was assessed using the RNA Pico Kit (Agilent Technologies, Wokingham, UK) and analysed using a Bioanalyzer 2100 (Agilent Technologies). Samples with RNA Integrity Number values above 5 were amplified according to the study by Marko *et al*.[41] using Nugen Kits—Ovation Pico WTA system, WT-Ovation Exon Module and Encore Biotin Module (NuGen, Bemmel, The Netherlands), then hybridised on Human Gene ST 1.0 169 format cartridges (Affymetrix, High Wycombe, UK) using the GeneChip Hybridization Wash and Stain Kit (Affymetrix), washed using the Affymetrix GeneChip Fluidics Station 450 (Affymetrix) and finally scanned using the GeneScan 3000 (Affymetrix). Whole transcriptome analyses were performed using the R (Fred Hutchinson Cancer Center, Seattle, WA, USA) system software package (version 2.13.2) including Bioconductor (version 2.9) and in-house software written using R. Quality control analyses were performed on GeneSpring GX (Agilent Technologies) and in R (version 2.13.2). Supplementary Methods provide additional information on data analysis. Data have been deposited at: http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE38906.

Statistical analysis

Data were analysed using a Student's two-tailed *t*-test.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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