than normal control patients, and that these patients demonstrate a survival disadvantage at both 1 and 5 years. Further prospective and multicentre studies to investigate the potential applications of OPN would be worthwhile.

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Acknowledgement

This study was supported by grant NSC95-2320-B-002-073 from the National Science Council, Taiwan.

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Keywords: osteopontin, acute myeloid leukaemia, biomarker, prognosis.

First published online 7 April 2008
doi:10.1111/j.1365-2141.2008.07082.x

csf1 is required for early embryonic macrophage development: characterization of the *csf1^{op}/csf1^{op}* mutation in ES cell-derived macrophages

Macrophages are phagocytic cells that help to mediate the inflammatory response, and they also associate with tissue remodelling during mammalian development. Macrophages first develop in the yolk sac at day 10 of mouse embryogenesis, then from the aorta, gonads and mesonephros (AGM) region a day later. Both of these early haematopoietic centres contribute progenitor cells to the fetal liver; however, yolk sac-derived cells are not capable of definitive haematopoiesis. This difference raises the possibility that yolk sac macrophages have unique molecular requirements.

Colony-stimulating factor 1 (csf1), a cytokine required for the differentiation, proliferation and survival of most macrophages, signals through a high-affinity receptor, csf1r, that is expressed on macrophages and their precursors. Csf1-null mice (*csf1^{op}/csf1^{op}*) are viable but have multiple abnormalities that are thought to be direct or indirect effects of reduced macrophage numbers, since mutant mice have only 5–30% of normal macrophage numbers as adults (Wiktor-Jedrzejczak *et al*, 1990). Most aspects of the phenotype can be rescued by injection of exogenous csf1 or by inserting a *CSF1* transgene (Cecchini *et al*, 1994; Ryan *et al*, 2001).

Although the postnatal effects of the *csf1^{op}* mutation are well-understood, the effects on early embryonic development are less clear. Female reproductive defects hinder analysis of prenatal development in the absence of csf1, since mutant embryos generally develop in heterozygous females that

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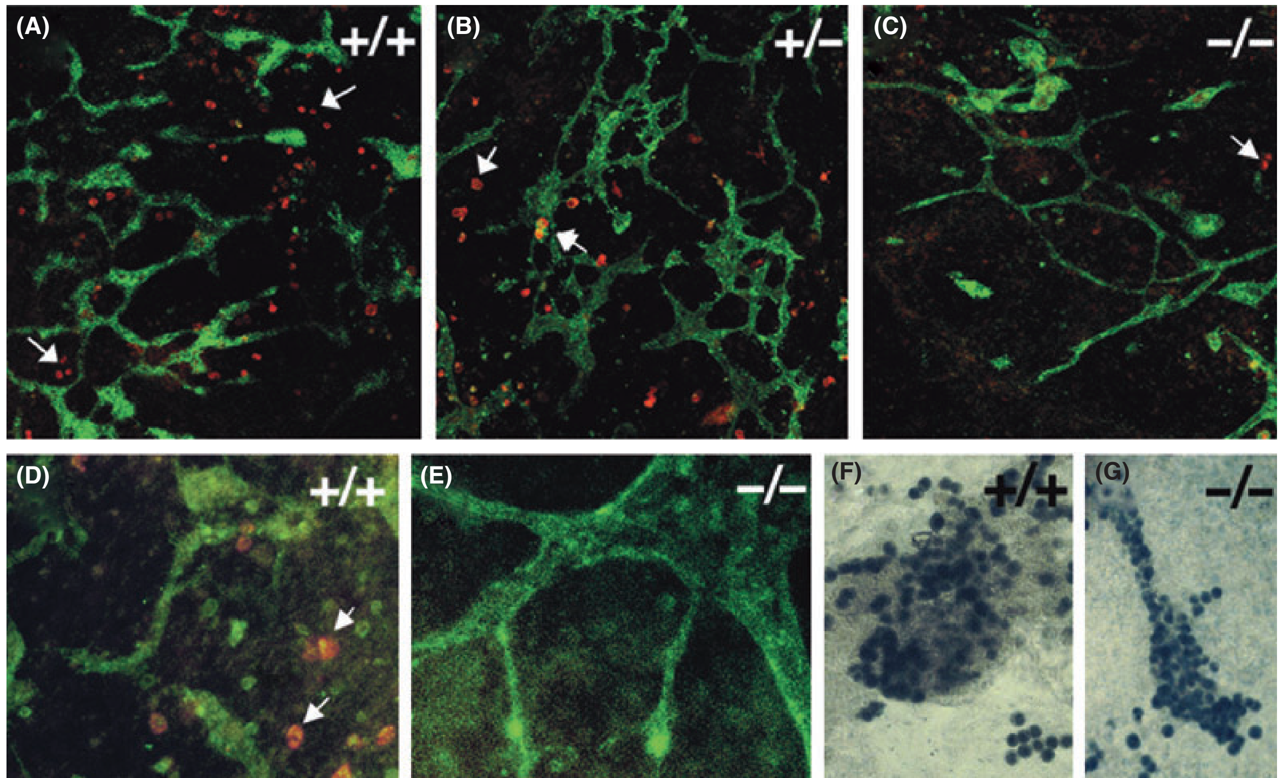

Fig 1. *csf1^{op}/csf1^{op}* ES cell cultures have few macrophages. ES cells were differentiated *in vitro* to day 10 under non-supplemented culture conditions as described (Bautch, 2001). Paraformaldehyde-fixed cultures were reacted with antibodies as described previously (Inamdar *et al*, 1997; Bautch, 2001). Panels A–C, double label confocal images with Mac-1 (red) and pecam (green); panels D-E, double label epifluorescence images with F4/80 (red) and pecam (green); panels F and G were reacted with benzidine to reveal primitive erythrocytes (blue). A, D, F Wild-type cultures; B, *csf1^{op}/+* culture; C, E, G *csf1^{op}/csf1^{op}* cultures. Note the Mac-1+ cells (arrows A–C), and the F4/80+ cells (arrows D) that are rare or absent in the *csf1^{op}/csf1^{op}* mutant background.

provide maternal csf1 (Pollard *et al*, 1991). However, analysis of the *csf1^{op}* mutation in late gestation and early postnatal mouse development from csf1-null mothers confirmed that some macrophages develop in the absence of csf1(Roth *et al*, 1998). The importance of csf1 signalling at the first site of embryonic macrophage development, the yolk sac, has not been investigated. To study the effects of loss of csf1 on embryonic macrophages without the complicating effects of maternally-derived csf1, embryonic stem (ES) cell lines were isolated from blastocysts of *csf1^{op}/+* heterozygous mice intercrosses. One wild-type line, ES 4·5, one heterozygous line (*csf1^{op}/+*), ES 3·9, and two homozygous (*csf1^{op}/csf1^{op}*) lines, ES 3·4–5 and 3·6, were generated.

Differentiation of mouse ES cells recapitulates aspects of early yolk sac development (Bautch, 2001). During ES cell differentiation, a primitive vasculature and several haematopoietic lineages form. Specifically, primitive (nucleated) erythrocytes and embryonic macrophages differentiate from blood islands under non-supplemented ES cell culture conditions. ES cell-derived embryonic macrophages appear identical to yolk sac-derived embryonic macrophages (Inamdar *et al*, 1997). The ES cell lines were differentiated to day 10, which is the peak of macrophage production. Wild-type, heterozygous and homozygous mutant ES cells cultures were fixed and stained with antibodies to Mac-1 or F4/80 to visualize macrophages and platelet/endothelial cell adhesion molecule 1 (pecam1) to visualize the vasculature, or reacted with benzidine to visualize haemoglobinized erythrocytes (Fig 1). The number of Mac-1 positive cells was dramatically decreased in the homozygous mutant lines compared to wild-type or heterozygous lines (compare Fig 1C to panels A and B). Cultures stained with a second macrophage-specific antibody, F4/80, showed a similar reduction of F4/80 positive cells in the mutant ES lines (Fig 1D and E). The quantitative reduction in macrophages was estimated by fluorescence-activated cell sorting analysis of day 10 cultures stained with Mac-1. On average, 3% of the total cell counts from wild-type cultures and 5% of heterozygous (*csf1^{op}/+*) cultures were Mac-1 positive. Both *csf1^{op}/csf1^{op}* mutant ES cell lines had significantly fewer Mac-1 positive cells than heterozygous or wild-type cultures, with each line containing $<0·5\%$ Mac-1 positive cells. These results establish that embryonic macrophages require csf1 for expansion and survival from their earliest development in the yolk sac. This finding is consistent with the expression pattern of the only known csf1receptor (csf1r), which is expressed on early embryonic macrophages (unpublished observations).

It was found that macrophage development from the AGM region of day 11·5 *csf1^{op}/csf1^{op}* mutant embryos was

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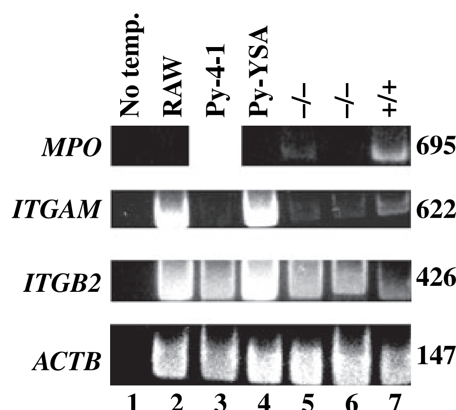

Fig 2. Reverse transcription-polymerase chain reaction (RT-PCR) analysis of day 10 ES cultures and controls. Total RNA was isolated from each line and RT-PCR was performed as described (Olson *et al*, 1995) for 35 cycles, and the products were separated on an 8% acrylamide gel and visualized using ethidium bromide. The genes analysed are on the left, and the size of the PCR products is on the right. Lane 1, no DNA; Lane 2, RAW 264·7 cells; Lane 3, Py-4-1 cells; Lane 4, Py-YSA cells; Lane 5, day 10 line 3.6 ES cells (*csf1^{op}/csf1^{op}*); Lane 6, day 10 line 3·4–5 ES cells (*csf1^{op}/csf1^{op}*); Lane 7, day 10 line RI (+/+) ES cells. *MPO*, myeloperoxidase; *ITGAM*, integrin alpha M; *ITGB2*, integrin beta 2; *ACTB*, beta-actin.

compromised (Minehata *et al*, 2002), so it is likely that both yolk sac-derived and AGM-derived macrophages require csf1 for their development. However, it was also found that precursors of other haematopoietic cells and endothelial differentiation were affected by csf1. To determine whether loss of csf1 led to reduction of multiple haematopoietic lineages, the ES cultures were reacted with benzidine to visualize primitive erythrocytes. Benzidine staining of homozygous mutant cultures was similar to that of wild-type cultures, indicating that primitive erythrocytes develop in the absence of csf1 (Fig 1F and G). Close visual inspection also revealed no significant difference in the vasculature among the different genotypes (Fig 1A–E). This apparent discrepancy in results could reflect intrinsic differences between yolk sac- and AGM-derived cells, or it could reflect different culture and assay conditions. However, our results correlate with the restriction of csf1r expression to individual cells with macrophage-like morphology in the ES cell cultures.

To determine when embryonic macrophage development was compromised by the lack of csf1 in ES cell cultures, RNA expression of macrophage markers was analysed using reverse transcription polymerase chain reaction (Fig 2). This semi-quantitative assay showed that the early marker myeloperoxidase and the later marker integrin alpha M (also known as CD11b) both had weak or absent amplification signals in mutant cultures. In contrast, the expression of integrin beta 2 (also known as CD18), the beta subunit of Mac-1 that is also associated with other integrin ligands, was not down-regulated in the *csf1^{op}/csf1^{op}*mutant ES cell cultures. These results suggest that embryonic macrophage development is affected at relatively early stages of differentiation and maturation by the lack of csf1. SFFV proviral integration 1 (Sfpi1, also known as PU.1) is a transcription factor required for the maturation of several haematopoietic lineages including macrophages, and targeted mutation of *SFPI1* also aborts embryonic macrophage development at a similar developmental stage (Olson *et al*, 1995). This is expected given that sfpi1 is thought to regulate *CSF1R* expression. However, unlike the *csf1^{op}/csf1^{op}* ES cell cultures that have rare Mac-1 positive cells (Fig 2C), differentiated *SFPI1*–/– ES cells completely lack Mac-1- and F4/80-expressing macrophages [(Olson *et al*, 1995) and unpublished observations]. This result suggests that csf1 mediates only a subset of the effects of sfpi1 in the embryonic macrophage lineage.

Others have shown that csf1 is required for adult and late-gestational embryonic macrophage development. The reduction of early macrophage markers in mutant ES cell cultures indicates that the earliest stages of embryonic macrophage development also require csf1; therefore, we conclude that csf1 is critical for macrophage development and survival throughout life. Furthermore, with the increasing interest in *in vitro* stem cell systems, a better understanding of the requirements for macrophage development from ES cells can provide a basis for development of haematopoietic therapeutics.

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Acknowledgement

This work was supported by NIH grant HL43174 to V.L.B.

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Keywords: embryonic macrophage, colony-stimulating factor 1, yolk sac, Mac-1, embryonic stem cell differentiation.

First published online 14 April 2008
doi:10.1111/j.1365-2141.2008.07105.x

Chronic lymphocytic leukaemia and acute myeloid leukaemia are not associated with *AKT1* pleckstrin homology domain (E17K) mutations

The serine/threonine kinase AKT1 (v-akt murine thymoma viral oncogene homologue 1) has a central role in the signaling of growth factors and other stimuli, leading to diverse cellular functions including cell survival, proliferation, growth and metabolism (reviewed in Vivanco & Sawyers, 2002). Furthermore, the deregulation of the phosphoinositide-3-kinase (PI3K)/AKT pathway is a common mechanism for transformation and by far the most frequently mutated component of this pathway is the tumour suppressor *PTEN* (Manning & Cantley, 2007).

The original description of *AKT1* involved virally induced carcinogenesis resulting from a constitutively active form of v-akt (Manning & Cantley, 2007). A recent report identified a recurrent mutation in the pleckstrin homology (PH) domain of *AKT1* that results in a glutamic acid to lysine substitution at amino acid 17 (E17K) in breast cancer (8%), ovarian cancer (2%) and colorectal cancer (6%) (Carpten *et al*, 2007). The mutation was shown to activate AKT1 and lead to cellular transformation and the development of leukaemia in mice. In addition, the PI3K/AKT pathway plays an important role in both chronic and acute leukaemia pathogenesis, and a growing number of compounds have been developed to target the PI3K/AKT. Therefore, we investigated a possible role of *AKT1* E17K mutation in a large cohort of patients with chronic lymphocytic leukaemia (CLL) and acute myeloid leukaemia (AML).

We studied the *AKT1* E17K genotype in 106 CLL patients and 95 patients with AML (Table I). DNA was extracted using Trizol or Qiagen and the following primer sequences were used to generate the DNA product (*AKT1* x3 FWD ACATCTGTCCTGGCACAC; *AKT1* x3 REV GCCAGTGCTTGTTGCTTG) (Carpten *et al*, 2007). Polymerase chain reaction (PCR) amplifications were performed using the Gene Amp PCR core reagent kit (Applied Biosystems, Foster City, CA, USA). The final concentrations of the PCR reagents were 1× PCR Gold buffer, 2 mmol/l $MgCl_2$, 200 μmol/l of each dNTP, 400 nmol/l each forward and reverse primer, 1·25 U of AmpliTaq Gold DNA polymerase enzyme, and 200 ng of genomic DNA in a 50-μl reaction volume. Thermal cycler conditions were: 95°C for 5 min, 35 cycles of 94°C for 30 s, 60°C for 30 s, 72°C for 1 min, and a final extension at 72°C for 10 min. The PCR products were analysed by automated fluorescent sequencing using Big Dye Terminator Kit and ABI 3100 sequencer (Applied Biosystems). The sequences were analysed visually and with the Staden package (available at http://staden.sourceforge.net).

The *AKT1* E17K mutation was not found in any of the 204 leukaemia patients, suggesting that the mutation is unlikely to play a significant role in transformation or disease modification in CLL and AML. Similar to the results reported here, a very recent report did not identify the *AKT1* E17K mutation in 49

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