



THE TRANSFORMING POTENTIAL AND FUNCTIONAL ANALYSIS OF THE  
c-KIT RECEPTOR TYROSINE KINASE AND ITS NATURAL OCCURRING  
ISOFORMS.

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A degree submitted for the degree of Doctor of Philosophy, Faculty of Science at  
The University of Adelaide  
March, 1996

Awarded 1996

## ABSTRACT

In this study, the function of the receptor tyrosine kinase, c-Kit, was examined in relation to the role of receptor levels in factor dependence and cell transformation. In addition, the functions of several naturally occurring isoforms of the human c-Kit receptor were analysed by expressing cDNA encoding these isoforms in murine cells.

Cells of the murine factor-dependent cell line, FDC-P1, require the presence of granulocyte-macrophage colony-stimulating factor (GM-CSF) or interleukin-3 (IL-3) for cell growth. FDC-P1 cells also express c-Kit, however when cultured in the presence of its ligand, *Steel* factor (SLF), they fail to proliferate. Co-culture of FDC-P1 cells on wild-type (+/+) fibroblastoid-3T3 lines, in the absence of exogenously added factors, enabled contact-dependent support of the FDC-P1 cells in the presence of a neutralising antiserum to GM-CSF. +/+ fibroblasts are able to produce both membrane-bound and soluble forms of SLF. The lack of support of FDC-P1 cells by *Sl/Sl<sup>d</sup>*-3T3 and *Sl/Sl*-3T3 fibroblasts, which produce only soluble SLF or completely lack SLF production, respectively, in the presence of GM-CSF antiserum, demonstrated that membrane-bound SLF was supporting FDC-P1 cells on +/+ fibroblast feeder layers.

Although FDC-P1 cells, which had been previously cultured in GM-CSF or IL-3, failed to proliferate in high concentrations of soluble murine SLF, the cells displayed synergistic proliferative responses to SLF when combined with sub-optimal levels of GM-CSF or IL-3. FDC-P1 cells previously grown in GM-CSF were adapted to grow in SLF alone by gradual substitution of SLF for GM-CSF over a period of 3 weeks. The resulting population, FDC-P1(SLF) cells, were responsive to SLF alone and maintained the ability to grow in GM-CSF or IL-3. mRNA analysis demonstrated that *c-kit* had been downregulated approximately 7-fold or 2.5-fold in the cells grown in GM-CSF or IL-3, respectively, as opposed to the cells grown in SLF alone (FDC-P1(SLF)). Downregulation of surface protein was also seen in FDC-P1 cells grown in GM-CSF or IL-3 compared with cells grown in SLF. Conversely, proliferation assays suggested that the GM-CSF and IL-3 receptors were not functionally downregulated by SLF, since FDC-P1(SLF) cells were able to proliferate just as well in GM-CSF or IL-3 as the cells originally grown in the latter factors. The differential

proliferative response of FDC-P1 cells to soluble SLF may be a consequence of the level of *c-kit* expressed by these cells.

Elevated receptor levels have been implicated in carcinogenesis. Overexpression of the receptor tyrosine kinase encoded by the *Her2/neu* proto-oncogene has been shown to be a prognostic indicator in a subset of breast and ovarian cancers. Similarly, Ashman *et al.*, (1988) demonstrated that high levels of the c-Kit receptor in a sub-group of acute myeloid leukaemic (AML) patients at presentation also correlated with poor prognosis. The remainder of this study therefore focussed on the potential role of the wild type c-Kit receptor in transformation, with respect to c-Kit receptor levels and their effect on factor dependence.

Ectopic expression of murine c-Kit in NIH/3T3 cells was achieved by infection with the defective retroviral vector, pZenneo, containing *c-kit* cDNA. Overexpression of the receptor demonstrated that the pool population of infectants, NIH(*mukit*), grew in an anchorage independent, though factor-dependent manner in semi-solid agar, with a plating efficiency of 11% in 200 ng/ml of murine SLF. In the absence of exogenous SLF colonies appeared at a plating efficiency of 1%, possibly due to autocrine production of SLF by NIH/3T3 cells. Attempts to block endogenously produced SLF from binding to surface c-Kit, using the antagonistic anti-c-Kit monoclonal antibody, ACK2, or by neutralising SLF production with an anti-SLF antiserum revealed that the colonies were resistant to inhibition, implying that an intracellular interaction was occurring between c-Kit and SLF. The cells giving rise to these colonies expressed functional c-Kit receptors, demonstrated by their ability to reproduce SLF-dependent anchorage independent colonies upon replating in semi-solid agar. Analysis of the transformation potential of 24 clones spanning a 16.8 fold range of *c-kit* mRNA expression revealed a correlation between *c-kit* expression and anchorage-independence in the presence of 100 ng/ml SLF ( $R = 0.53$ ;  $p < 0.01$ ). Cells expressing low levels of *c-kit* mRNA were mostly unable to produce anchorage independent colonies, in contrast to clones expressing medium to high levels of *c-kit* mRNA relative to the pool of NIH(*mukit*) infectants. In the absence of exogenously added SLF only two clones, which also expressed high levels of *c-kit*, demonstrated significant anchorage independent

colony production. An increase in murine *c-kit* expression in early passage (ep) NIH/3T3 cells also resulted in enhanced transformation and tumorigenicity.

Similar studies were performed with the human c-Kit receptor and several of its natural occurring isoforms. One pair of isoforms differ in the deletion/insertion of 12 base pairs ( $12^-/12^+$ ), encoding the amino acids Gly-Asn-Asn-Lys (GNNK), located in the extracellular domain adjacent to the transmembrane domain as a result of mRNA alternative splicing. A second set of mRNA splice variants exist, resulting in c-Kit isoforms which differ in the deletion/insertion of a serine residue ( $S^-/S^+$ ), at position 715 located within the interkinase domain. Four *c-kit* cDNAs encoding the following isoforms were generated: *Kii(GNNK<sup>+</sup>S<sup>+</sup>)*, *Kii(GNNK<sup>-</sup>S<sup>+</sup>)*, *Kii(GNNK<sup>+</sup>S<sup>-</sup>)*, *Kii(GNNK<sup>-</sup>S<sup>-</sup>)* and cloned into the defective retroviral vector, pRUFMC1*neo*. Infection of epNIH/3T3 cells with these constructs and subsequent analysis of the ability of the c-Kit isoforms, expressed at comparable levels, to transform these cells, revealed discernible differences in their transforming potentials. c-Kit(GNNK-S<sup>+</sup>) was the most potent able to produce focus-formation, anchorage independent growth and induce tumours in *nude* mice. c-Kit(GNNK<sup>+</sup>S<sup>+</sup>) displayed the ability to produce focus-formation and anchorage independent growth, while c-Kit(GNNK<sup>+</sup>S<sup>-</sup>) was only able to induce focus-formation. Unfortunately, c-Kit(GNNK-S<sup>-</sup>) protein expression was not detected and as a result its transforming potential was not analysed. Analysis of cells expressing increasing levels of the isoforms demonstrated that focus-formation increased with an increase in c-Kit expression in a factor-dependent manner. epNIH/3T3 cells expressing c-Kit(GNNK-S<sup>+</sup>) at all levels and cells expressing c-Kit(GNNK<sup>+</sup>S<sup>-/+</sup>) at high levels were also able to induce focus-formation in the absence of exogenously added human SLF. In contrast to the focus-formation results cells expressing increasing levels of the human c-Kit isoforms demonstrated that, surprisingly, as the level of c-Kit increased, the number of anchorage independent colonies decreased in the presence and absence of human SLF. The latter was also demonstrated in an independent system in which the *c-kit(GNNK<sup>+</sup>S<sup>+</sup>)* cDNA was cloned in a mammalian expression vector pRSV009/A<sup>+</sup>. This vector encodes a dihydrofolate reductase gene, allowing sequential amplification of the linked *c-kit* gene upon increasing concentrations of methotrexate selection in NIH/3T3 cells. It appeared that a

'window' of c-Kit receptor level expression was required for maximal induction of transformation.

Constructs encoding the c-Kit isoforms were also introduced into murine factor-dependent cell lines. Cells expressing comparable levels of the c-Kit receptor were analysed for their responsiveness to SLF, and the ability of a range of monoclonal antibodies to c-Kit, to block SLF-driven proliferation.

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