K-ras Proto-Oncogene Exhibits Tumor Suppressor Activity As Its Absence Promotes Tumorigenesis in Murine Teratomas

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Abstract

Ras proteins transduce signals from membrane-bound receptors via multiple downstream effector pathways and thereby affect fundamental cellular processes, including proliferation, apoptosis, and differentiation. K-ras activating mutations play a key role in neoplastic progression and are particularly prevalent in colorectal, pancreatic, and lung cancers. The present study addressed whether the K-ras proto-oncogene displays a tumor suppressor function by comparative analysis of mouse teratomas derived from wild-type embryonic stem (ES) cells, K-ras null (K-ras−/−) ES cells, and K-ras+/− ES cells that stably reexpress either wild-type K-rasgly12 or oncogenic K-rasval12. K-ras−/− and K-rasval12 teratomas were significantly larger than teratomas that expressed wild-type K-ras, contained significantly higher proportions of undifferentiated embryonal carcinoma-like cells, and showed significantly increased mitotic activity. However, K-rasval12 but not K-ras−/− teratomas exhibited significantly higher levels of apoptosis than wild-type teratomas. K-ras−/− and K-rasval12 ES cells showed a higher capacity for stem cell self-renewal in vitro compared with wild-type ES cells, and reexpression of K-rasgly12 in K-ras−/− ES cells restored the K-ras−/− phenotype to wild-type values. Thus, in view of evidence that tumors can derive from tissue stem cells and that tumors harbor “cancer stem cells,” aberrant K-ras expression could promote neoplastic progression by increasing their capacity for self-renewal.

Introduction

The three classical mammalian ras genes, K-, H-, and N-ras, encode membrane-associated, guanine nucleotide-binding proteins, which act as molecular switches in signal transduction pathways for a variety of hormones, growth factors, and cytokine receptors (1). By alternating between inactive GDP-bound and active GTP-bound states, Ras proteins affect multiple effector pathways, including the Raf/mitogen-activated protein (MAP) kinase and phosphoinositide 3-kinase/protein kinase B (PKB/Akt) pathways, and affect diverse biological processes, including proliferation, differentiation, and apoptosis (2–5). Ras activating mutations are found in ~30% of human malignancies, with K-ras mutations particularly prevalent in lung (~30%), colorectal (~50%), and pancreatic (~90%) cancers (6, 7). Ras mutations, which primarily affect codons 12, 13, and 61, lead to a constitutively active GTP-bound state and promote oncogenic activity by continually up-regulating downstream effector pathways in the absence of external stimuli.

The dogma that oncogenic ras plays a dominant role in cell transformation and carcinogenesis has recently been challenged by reports that the K-ras and N-ras proto-oncogenes have tumor suppressor activity. Diaz et al. (8) reported that absence of N-ras in mice promotes the development of thymic lymphomas, and overexpression of wild-type N-ras protects against lymphomagenesis in the presence or absence of its oncogenic allele. In the case of K-ras, Zhang et al. (9) investigated lung cancer induced by carcinogens that cause K-ras activating mutations and found that tumors in heterozygous K-ras null mice (that express oncogenic K-ras only) were more abundant, larger, and were predominantly undifferentiated malignant adenocarcinomas, whereas tumors in wild-type mice (that express wild-type and oncogenic K-ras) were smaller adenomas. Reexpression of wild-type K-ras in tumor cell lines (that express oncogenic K-ras) was also shown to inhibit their growth in vitro and their capacity to develop tumors in nude mice (9). The protective effect of the wild-type K-ras allele suggests that the K-ras proto-oncogene has tumor suppressor activity and is consistent with the conclusions of earlier studies that the dominant nature of ras oncogenes results from either overexpression of the mutant ras allele or loss of the wild-type allele (10, 11). Thus, K-ras appears to exert a dual function in that it promotes cancer development as a gain-of-function oncogene when mutated and inhibits cancer by loss of tumor suppressor activity when wild type (proto-oncogene).

In the present study, we examined whether absence of K-ras expression is sufficient to promote tumorigenesis and whether K-ras can exhibit tumor suppressor activity in the absence of its
Results

K-ras<sup>−/−</sup> and K-ras<sup>val12</sup> Teratomas Are Heavier Than Wild-Type Teratomas

A total of 30 teratomas was generated (15 wild type, 6 K-ras<sup>−/−</sup>, 4 K-ras<sup>gly12</sup>, and 5 K-ras<sup>val12</sup>), of which 22 were analyzed histopathologically, and these included 7 wild-type, 6 K-ras<sup>−/−</sup>, 4 K-ras<sup>gly12</sup>, and 5 K-ras<sup>val12</sup> teratomas (Table 1). There were significant differences between the four genotypes of teratoma by weight (<i>P</i> < 0.0001, Kruskal-Wallis test), and Mann-Whitney U tests showed that K-ras<sup>−/−</sup> (<i>P</i> = 0.0005), K-ras<sup>gly12</sup> (<i>P</i> = 0.0316), and K-ras<sup>val12</sup> (<i>P</i> = 0.0164) teratomas were significantly heavier than wild-type teratomas. K-ras<sup>−/−</sup> teratomas were also heavier than K-ras<sup>val12</sup> (<i>P</i> = 0.0446) and K-ras<sup>gly12</sup> (<i>P</i> = 0.0142) teratomas. Although K-ras<sup>val12</sup> tumors were on average heavier than K-ras<sup>gly12</sup> tumors, the variation was high and the difference was not significant. Importantly, the finding that K-ras<sup>gly12</sup> teratomas were significantly lighter than K-ras<sup>−/−</sup> teratomas indicated that wild-type K-ras expression can partially rescue this phenotypic feature of K-ras<sup>−/−</sup> cells.

K-ras<sup>−/−</sup> and K-ras<sup>val12</sup> Teratomas Contain a Higher Proportion of Undifferentiated EC-Like Cells

Teratomas from all four genotypes contained representative tissues of all three germ layers (Fig. 1, A and B). Standard point counting found that the proportion of undifferentiated EC-like cells in teratomas was related to genotype (Table 1; Fig. 2), and Mann-Whitney U tests showed that the mean number of points overlying undifferentiated EC-like cells in sections from wild-type teratomas was significantly less than the other genotypes (K-ras<sup>−/−</sup> < 0.0001, K-ras<sup>gly12</sup> < 0.0001, K-ras<sup>val12</sup> < 0.0001). K-ras<sup>−/−</sup> teratomas contained a significantly higher proportion of EC-like cells than K-ras<sup>gly12</sup> and K-ras<sup>val12</sup> teratomas (<i>P</i> < 0.0001 and < 0.0002, respectively), and K-ras<sup>val12</sup> teratomas contained significantly more EC-like cells than K-ras<sup>gly12</sup> teratomas (<i>P</i> = 0.0144). The mean teratoma weight was positively correlated with the proportion of undifferentiated EC-like cells (<i>r</i> = 0.821, Spearman’s ranked correlation <i>P</i> < 0.001; Fig. 3). Importantly, the observation that K-ras<sup>gly12</sup> teratomas contained significantly lower proportions of EC-like cells than K-ras<sup>−/−</sup> teratomas showed that wild-type K-ras expression can partially rescue this phenotypic feature of K-ras<sup>−/−</sup> cells.

K-ras<sup>−/−</sup> and K-ras<sup>val12</sup> Teratomas Show Higher Mitotic Activity

There were significant differences between the numbers of mitotic figures among the four genotypes of teratomas (tied <i>P</i> values = 0.0046 Kruskal-Wallis test; Table 1). K-ras<sup>−/−</sup> and K-ras<sup>val12</sup> teratomas contained significantly higher numbers of mitotic figures than wild-type teratomas (<i>P</i> = 0.0194 and < 0.0020, respectively, Mann-Whitney U test). While the number of mitotic figures was not significantly different between K-ras<sup>−/−</sup>, K-ras<sup>gly12</sup>, and K-ras<sup>val12</sup> teratomas, K-ras<sup>val12</sup> teratomas contained the most mitotic figures.

K-ras<sup>val12</sup> Teratomas Show a Higher Level of Apoptosis

There were significant differences between the numbers of apoptotic bodies in EC-like cells among the four groups (tied <i>P</i> values = 0.0083, Kruskal-Wallis test; Table 1). K-ras<sup>−/−</sup> and wild-type teratomas contained similar numbers of apoptotic bodies, which were not significantly different (<i>P</i> = 0.1489, Mann-Whitney U test), K-ras<sup>gly12</sup> and K-ras<sup>val12</sup> teratomas contained significantly more apoptotic bodies than K-ras<sup>−/−</sup> (<i>P</i> = 0.0202 and < 0.0090, respectively, Mann-Whitney U test) and wild-type teratomas (<i>P</i> = 0.0284 and < 0.0090, respectively, Mann-Whitney U test). K-ras<sup>val12</sup> teratomas contained the most apoptotic bodies. Thus, the larger EC cell-like components in K-ras<sup>−/−</sup> and K-ras<sup>val12</sup> teratomas do not reflect reduced levels of apoptosis relative to wild-type teratomas.

K-ras<sup>−/−</sup> and K-ras<sup>val12</sup> ES Cells Show a Higher Capacity for Stem Cell Renewal In Vitro

The lower proportions of differentiated cell types in K-ras<sup>−/−</sup> and K-ras<sup>val12</sup> teratomas could indicate that K-ras proto-oncogene promotes differentiation. This was investigated further by comparing the capacity of wild-type, K-ras<sup>−/−</sup>,

Table 1. Mean Weight, Number of Points Overlying Undifferentiated EC-Like Cells, Mitotic Index, and Number of Apoptotic Bodies Scored in the Four Classes of Teratoma (±SE)

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Weight (g)</th>
<th>EC-Like Cells</th>
<th>Mitotic Index</th>
<th>Apoptotic Bodies</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>0.364 ± 0.127 (15)</td>
<td>15.1 ± 6.112 (7)</td>
<td>12.0 ± 1.563 (4)</td>
<td>38.5 ± 7.810 (4)</td>
</tr>
<tr>
<td>K-ras&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>6.991 ± 1.224 (6)</td>
<td>177.5 ± 2.654 (6)</td>
<td>19.2 ± 3.841 (4)</td>
<td>27.8 ± 6.135 (4)</td>
</tr>
<tr>
<td>K-ras&lt;sup&gt;gly12&lt;/sup&gt;</td>
<td>1.082 ± 0.414 (4)</td>
<td>123.8 ± 3.312 (4)</td>
<td>16.5 ± 1.374 (4)</td>
<td>57.0 ± 0.943 (4)</td>
</tr>
<tr>
<td>K-ras&lt;sup&gt;val12&lt;/sup&gt;</td>
<td>2.501 ± 1.719 (5)</td>
<td>147.0 ± 6.892 (5)</td>
<td>25.8 ± 3.508 (4)</td>
<td>108.0 ± 8.832 (4)</td>
</tr>
</tbody>
</table>

Note: The number of teratomas analyzed is indicated in parentheses.
K-ras\textsuperscript{gly12}, and K-ras\textsuperscript{val12} ES cells to self-renew following growth in the absence of LIF by staining for alkaline phosphatase activity, which identifies undifferentiated ES cell colonies. In the presence of LIF, the mean percentage of alkaline phosphatase positive colonies did not differ significantly between the four genotypes (\(P = 0.082\), Kruskal-Wallis test; Fig. 2). However, in the absence of LIF, K-ras\textsuperscript{+/+} and K-ras\textsuperscript{val12} ES cells showed significantly higher capacities for self-renewal with reduced commitment to differentiation than wild-type ES cells, with 52% and 37% of colonies staining for alkaline phosphatase activity, respectively (\(P = 0.0050\) for comparison of both genotypes with wild type, Mann-Whitney \(U\) test). The number of alkaline phosphatase positive K-ras\textsuperscript{+/+} colonies was also significantly greater than

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**FIGURE 1.** A. Schematic representation of differentiated features in teratomas of the four different genotypes (wild type, K-ras\textsuperscript{val12}, K-ras\textsuperscript{gly12}, and K-ras\textsuperscript{+/+}). Each class of teratoma is ranked in descending order of weight. B. H&E-stained sections of the four genotypes of teratoma. a. Wild-type teratoma comprised of differentiated features, including glands of endodermal lineage (g), squamous pearls of ectodermal lineage (s), and lymphoid tissue in a fibrous tissue background of mesodermal lineage (l). b. K-ras\textsuperscript{+/+} tumor with features more representative of an undifferentiated embryonal teratocarcinoma and composed mainly of embryonal carcinoma (EC)-like cells with a small proportion of differentiated elements, including glands of endodermal lineage (g). c. EC-like cells from a K-ras\textsuperscript{+/+} teratoma at high magnification. d and e. K-ras\textsuperscript{gly12} and K-ras\textsuperscript{val12} teratomas respectively showing EC-like cells and differentiated elements. f. EC-like cells in a K-ras\textsuperscript{val12} teratoma at high magnification. Scale bar, 400 \(\mu m\) in e; 200 \(\mu m\) in a, b, and d; and 50 \(\mu m\) in c and f.
permits comparison of the effects on tumorigenesis and primary only, this strategy enables examination of the roles of K-
syngenic mice, and the ES cells used harbor K-
K-
cancer-related mutations. Wild-type and K-
tumor development and differentiation in the absence of other
K-
P
similarly low capacity for self-renewal, with only 16% and 15% of colonies staining for alkaline phosphatase, respectively (\(P = 0.2971\), Mann-Whitney \(U\) test). The finding that K-
val12 ES cells showed a similarly low capacity for self-
renewal without differentiation as wild-type ES cells shows that K-
expression can rescue fully this K-
phenotypic feature. Importantly, a strong correlation was noted between the proportion of undifferentiated EC-like cells in teratomas and undifferentiated ES cells in self-
renewal assays in vitro (Fig. 2).

Discussion

Recent studies have reported that the N- and K-
proto-oncogenes exhibit tumor suppressor activity (8, 9). In the case of K-
, it was found that the wild-type allele can protect against the progression of lung tumorigenesis in mice that harbor activating K-
mutations and that reexpression of wild-type K-
in cell lines (which express oncogenic K-
) can inhibit their growth in vitro and their capacity for tumor formation in vivo (9). In the present study, we addressed the converse scenario: whether absence of wild-type K-
is sufficient to promote tumorigenesis. Because this cannot be examined directly in mice, as K-
mice die in utero (12, 13), we used K-
val12 ES cells and, for comparison, wild-type ES cells and K-
val12 ES cells in which K-
expression was reconstructed by stable expression of mini-genes encoding either wild-type (K-
val12 cells) or mutant (K-
val12 cells) K-
(14). Because ES cells form tumors spontaneously in syngenic mice, and the ES cells used harbor K-
mutations only, this strategy enables examination of the roles of K-
in tumor development and differentiation in the absence of other cancer-related mutations. Wild-type and K-
ES cells permitted comparison of the effects on tumorigenesis and differentiation between cells that either do, or do not, express K-
proto-oncogene. K-
ES cells were included to address whether expression of wild-type K-
can rescue some of the K-
phenotypic features. K-
val12 cells were examined to determine whether tumors that express oncogenic K-
only, as occurs in vivo (9, 16), share phenotypic similarities with tumors that do not express K-
at all. Importantly, the study of ES cells is very relevant to understanding the pathogenesis of cancer because of analogies between normal stem cells and tumorigenic cells and because tumors can originate in stem cell compartments and may contain “cancer stem cells” that drive neoplastic progression by their indefinite potential for self-renewal (17).

In the present study, it was found that K-
and K-
val12 teratomas were heavier than wild-type teratomas and contained higher proportions of undifferentiated EC-
like cells which showed higher levels of mitotic activity. In vitro, K-
and K-
val12 ES cells showed higher capacities for stem cell self-renewal (in the absence of LIF) compared with wild-type and K-
val12 ES cells. Although selected ES cell clones were analyzed, the differences between wild-type, K-
, K-
val12 and K-
val12 cells very probably reflect the K-
genotype rather than clonal variation for several reasons. Firstly, ES cells of all four genotypes were capable of differentiation into ectoderm, mesoderm, and endoderm lineages in teratomas. Secondly, reexpression of wild-type K-
in K-
ES cells reduced tumor weight and cell proliferation in teratomas and promoted their capacity for differentiation in vitro and in vivo. While expression of the K-
transgene could only partially rescue the phenotype of K-
teratomas, this is not unexpected given that transgenes often show a variegated pattern of expression in vivo (18). Thirdly, we reported previously that all 10 K-
val12 ES clones generated showed similar alterations in apoptosis in response to DNA damage (14), suggesting a lack of significant clonal variance. Finally, when we attempted to use Western blot analysis (data not shown), it was not possible to quantify the levels of K-
protein in the four genotypes of ES cells due to their low levels. However, using reverse transcription-PCR, we

![FIGURE 2. Differentiation of ES cells in vitro and teratomas. The primary Y axis (left) refers to the histogram of the mean percentages (±SE) of alkaline phosphatase (AP) positive colonies for the four genotypes of ES cells after growth for 5 days in the presence or absence of leukemia inhibitory factor (LIF); (-----) 250 units LIF, (.) 0 unit LIF). The secondary Y axis (right) refers to the line graph (---, Undifferentiated % score) of the mean percentages (±SE) of points overlying undifferentiated EC-like cells in the four genotypes of teratomas.](Image 57x560 to 278x695)

![FIGURE 3. Correlation between the weight and mean number of points overlying undifferentiated EC-like cells in the 22 teratomas analyzed (K-
, K-
val12, K-
val12 and wild type). Individual teratomas are shown (•) and the trend line was fitted to the graph using Excel (\(Y = 6.918\ln(X) + 28.218\)) to illustrate the positive correlation between weight and EC-cell-like content.](Image 308x107 to 527x242)
reported previously that the K-ras\(^{gly12}\) and K-ras\(^{val12}\) ES cells, as used in the present study, did express similar levels of K-ras mRNA (\(\times 1.6\) and \(\times 1.2\), respectively) to wild-type ES cells (14), and thus, their different phenotypic features in teratomas and stem cell renewal assays are unlikely to reflect differences in the levels of K-ras expressed.

The finding that wild-type teratomas were smaller and more differentiated than K-ras\(^{val12}\) teratomas suggests that the K-ras proto-oncogene can indeed exhibit tumor suppressor activity and can promote differentiation in ES cells. This is consistent with the study by Zhang et al. (9) of lung tumors induced by carcinogen treatment which found that tumors in heterozygous K-ras null mice (that express oncogenic K-ras only) are larger and poorly differentiated compared with those in wild-type mice (that express wild-type and oncogenic K-ras), and concluded that K-ras may promote differentiation of pulmonary cells. However, the present work based on K-ras\(^{-/-}\) ES cells derived by homologous recombination, and which therefore only harbor a K-ras mutation, showed that K-ras can exhibit tumor suppressor activity in the absence of its oncogenic allele and mutations in other genes. While wild-type K-ras can suppress lung tumorigenesis (9), its ability to suppress teratoma formation could be a phenotype specific to ES cells.

Tumor growth is regulated by mitosis, differentiation, and apoptosis. While we found no evidence that large teratoma size was linked with reduced apoptosis, the higher number of mitotic figures in K-ras\(^{-/-}\) and K-ras\(^{val12}\) teratomas suggests a role for increased cell proliferation. Importantly, the finding of strong correlations between (1) tumor size and proportion of undifferentiated EC-like cells (Fig. 3), and (2) proportion of undifferentiated EC-like cells in teratomas and undifferentiated ES cells in the stem cell renewal assay for each of the four genotypes (Fig. 2), suggests that the high proportion of EC-like cells in K-ras\(^{-/-}\) and K-ras\(^{val12}\) teratomas reflects an increased capacity for stem cell self-renewal. Therefore, in view of evidence that tumors can derive from tissue stem cells and that tumors may harbor “cancer stem cells” (17), the results suggest that either absence of K-ras proto-oncogene expression, or expression of oncogenic K-ras could promote neoplastic progression by increasing stem cell self-renewal. While oncogenic ras mutations play a crucial role in neoplastic progression, the current finding that the absence of K-ras proto-oncogene can promote tumorigenesis raises the possibility that this mechanism might also promote tumor development in vivo, either directly by homozygous deletions and/or inactivating mutations of K-ras, or indirectly by inactivating mutations of downstream genes in the Ras signal transduction pathways. However, the finding that K-ras\(^{val12}\) teratomas are significantly lighter and contain a lower proportion of undifferentiated EC-like cells than K-ras\(^{-/-}\) teratomas suggests that oncogenic K-ras\(^{val12}\) may exhibit, at least some activity similar to wild-type activity.

While the finding that both absence of K-ras expression and expression of oncogenic K-ras in teratomas can promote tumorigenesis of teratomas may appear somewhat contradictory, it could perhaps be explained by the differential activation of downstream Ras effector pathways by the K-ras proto-oncogene and oncogene. For example, because K-ras, as compared with H-ras, is a potent activator of the MAP kinase pathway (19) and because ES cell propagation is dependent on LIF which activates STAT3 (20) and MAP kinase (21) which promote self-renewal and differentiation, respectively (22), the disruption of the Ras-mediated activation of MAP kinase in K-ras\(^{-/-}\) ES cells could explain their reduced ability to differentiate in teratomas and higher capacity for stem cell renewal in vitro. In contrast, should oncogenic K-ras activate the PI3 kinase pathway preferentially, which regulates cyclin D1 levels and cell cycle progression in ES cells (23), and induces rapid tumor growth and proliferation of mammary epithelial cells in 3D collagen gels (24), its activation in K-ras\(^{val12}\) cells could explain the promotion of self-renewal rather than differentiation. Although it remains to be determined if ras proto-oncogenes and oncogenes preferentially affect different signaling pathways, this could explain the apparent anomaly that both absence of K-ras expression and expression of oncogenic K-ras can promote tumorigenesis. Importantly, the finding that the K-ras\(^{-/-}\) and K-ras\(^{val12}\) genotypes both promote tumorigenesis but only K-ras\(^{val12}\) teratomas showed high levels of apoptosis, which is consistent with our earlier report that K-ras\(^{val12}\) ES cells are more susceptible to apoptosis induced by etoposide than K-ras\(^{-/-}\) ES cells (14), suggests that wild-type and oncogenic K-ras have different phenotypic effects and, therefore, may indeed activate effector pathways differentially.

In conclusion, the findings that wild-type teratomas are smaller, more differentiated, and show less mitotic activity than K-ras\(^{-/-}\) teratomas support the view that the K-ras proto-oncogene has tumor suppressor activity, and that K-ras, like N-ras (8), can exhibit this activity in the absence of its oncogenic allele. Importantly, the results suggest the possibility that lung, pancreatic, and colorectal tumor progression could be ameliorated by therapeutic expression of wild-type K-ras in tumor cells. Indeed, in view of the present finding that the absence of K-ras proto-oncogene promotes tumorigenesis, this approach could be preferable to inhibiting Ras protein function completely using anti-Ras agents, which is the focus of current therapeutic strategies (25).

Materials and Methods

ES Cells

The generation of K-ras\(^{-/-}\) ES cells and K-ras\(^{-/-}\) ES cells that reexpress wild-type K-ras\(^{gly12}\) or oncogenic K-ras\(^{val12}\) has been described previously (14). In brief, HM-1 ES cells (strain 129/Ola) that harbored a homozygous deletion of exons 1–3 of the K-ras gene (K-ras\(^{-/-}\)) were transfected with K-ras mini-genes that expressed either an activating mutation or wild-type sequence at codon 12 of K-ras (K-ras\(^{val12}\) and K-ras\(^{gly12}\) ES cells, respectively). Transfected clones from both genotypes expressed K-ras at comparable levels to that in wild-type ES cells, and all K-ras\(^{val12}\) and K-ras\(^{gly12}\) ES clones generated showed a similar altered apoptotic response following genotoxic damage compared with wild-type ES cells (14). In the present study, representative clones of each genotype were used, and the K-ras\(^{-/-}\) ES cells were capable of contributing to mesodermal, ectodermal, and endodermal lineages in mouse chimeras (data not shown).
Production and Analysis of Teratomas

Teratomas were produced by s.c. injection of \(10^5\) ES cells (in 100 µl of PBS) into syngenic (strain 129/Ola) female mice. In all studies, wild-type ES cells were injected into one flank and \(K^s\) mutant ES cells into the other. Recipient mice were sacrificed at 8 weeks and the teratomas excised, weighed, fixed in formalin, embedded in paraffin wax, and 5 µm sections cut and stained with H&E. Mitotic figures and apoptotic bodies were scored in two high-power fields \((\times400)\) and the ratio of EC-like cells and differentiated cell types was determined by a standard point counting method using 50 grid-based points placed randomly in each of four fields (randomly selected as one field in each of the four quadrants for each teratoma) with each grid point scored according to whether it was overlying an area of undifferentiated EC-like cells or differentiated cell types.

Self-Renewal Assay

The capacity of ES cells for self-renewal was determined as described previously (20). Briefly, ES cells were seeded in triplicate at \(10^5\) cells/well (in six-well plates), grown for 5 days in the presence (250 units) or absence of LIF, and the resulting colonies were stained for alkaline phosphatase activity (Alkaline Phosphatase Leukocyte Staining Kit, Sigma, Poole, Dorset, United Kingdom). The numbers of stained (undifferentiated) and unstained (differentiated) colonies were recorded.

Statistical Analysis

Graphs were drawn using Microsoft Excel 2000, v 9.0.2720 (Microsoft Corporation, Redmond, WA) and statistical analysis was performed using Minitab Release 13.1 (Minitab Inc., State College, PA). Because the data were not normally distributed, the following nonparametric tests were applied. The Kruskal-Wallis one-way ANOVA by ranks was used to compare the median weight, points overlying EC-like cells, mitotic index, and apoptotic bodies of the four genotypes of teratoma. The Mann-Whitney \(U\) test was used to compare the medians of pairs of sets of the above data with the null hypothesis that the medians do not differ. The Spearman rank-order correlation was used to investigate the association between teratoma weight and the proportion of points overlying undifferentiated EC-like cells.

References


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