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Targeted overexpression of an activated N-ras gene results in B- and plasma cell lymphoproliferation and cooperates with c-myc to induce fatal B-cell neoplasia

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Abstract

Multiple myeloma (MM) is an incurable malignant expansion of plasma cells in the bone marrow. Although there is no pathognomonic genetic lesion among MM patients, activation of the *ras* gene has been identified as a common mutation. We have previously described the use of the 3' kappa immunoglobulin light chain enhancer (3'KE) to target transgenic expression in murine B- and plasma cells, resulting in *bcl-X_L* and *c-myc* driven murine models of MM. In this report, we characterize the role of activated mutant N-*ras* in B- and plasma cells in transgenic mice. We constructed transgenic mice that use the 3'KE to direct expression of a mutant activated N-*ras*. We also crossed the N-*ras* mice to mice bearing a *c-myc* transgene to study the cooperative effects of the transgenic constructs. Mice were sacrificed when moribund or at specific time intervals and characterized by serology, light microscopy, and flow cytometry. The transgenic N-*ras* animals develop B- and plasma cell lymphoproliferation, and aged mice develop immunoglobulinemia, renal hyaline tubular casts, and microscopic foci of abnormal plasma cells in extramedullary sites, including the liver and kidney. Bitransgenic 3'KE/N-Ras V12 x Eμ-c-Myc mice develop fatal B-cell neoplasia with a median survival of 10 weeks. These data indicate that activated N-*ras* can play a role in B- and plasma cell homeostasis and that activated N-Ras and c-Myc can cooperate to induce B-cell neoplasia.

Keywords

Multiple Myeloma; Lymphoma; N-Ras; oncogenes

Multiple myeloma is an incurable expansion of malignant plasma cells in the bone marrow (1). Unlike other hematological malignancies that have common genetic abnormalities, a pathognomonic genetic lesion has not been described for this disease (1). Regardless,

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common genetic themes, such as activation of growth-promoting oncogenes, have been implicated in disease initiation, progression, and therapeutic response (1).

The oncogene *ras* plays an important role in myeloma, and the Ras protein is transiently activated in the myeloma cell upon growth-promoting IL-6 stimulation (2). While not a universal genetic lesion, activating *ras* mutations have been described in 23–100% of myeloma patients (3–5) and 50% of human myeloma cell lines (HMCLs) (6). Most of these mutations involve K- and N-*ras* at codons 12, 13, and 61, but there has been evidence of a rare H-*ras* activating mutation in a fraction of cells from one HMCL (7). *Ras* mutations appear to be rare in monoclonal gammopathy of undetermined significance (MGUS), a putative precursor of myeloma, with only 12.5% of patients demonstrating evidence of such mutations (4, 8). Given the higher incidence of activating *ras* mutations in myeloma compared to MGUS, the current models of myelomagenesis suggest that activating *ras* mutations are involved in progression of MGUS to myeloma, or later stages of myeloma (9).

Although there has been a fairly extensive analysis of activating *ras* mutations in myeloma patients, there have been few reports that have focused on modeling the biology of an activated *ras* mutation in the context of B- and plasma cell development and tumorigenesis. In this report, we use the 3' kappa immunoglobulin enhancer (3'KE) to target transgenic expression of a mutant activated N-*ras* gene in B- and plasma cells of transgenic mice. We show that the presence of the activated N-*ras* transgene can lead to abnormal B- and plasma cell biology and to B-cell neoplasia.

Materials and Methods

Transgenic Construct

The transgenic cassette was constructed in a pBluescript (Stratagene, La Jolla, CA) backbone. R. Perlmutter's human growth hormone (hGH) minigene cassette (10) was excised from the 3'KE/KP/Bcl-X_L vector (11) by cutting with Bam HI and Eco RI and ligating the insert into a Bluescript vector cut with the same enzymes. A human activated N-Ras V12 cDNA was excised from pN-Ras V12 EE (12) using Bam HI and was ligated into the Bam HI-cut vector containing the hGH minigene. We confirmed the orientation of the N-Ras V12 insert by PCR. The 3'KE and kappa promoter (KP) were excised from pK3'E.KP.LUC.IM (13, 14) by cutting with Sac I and Hind III. The vector containing the N-Ras V12 and hGH minigene was cut with Xba I. Both the vector and insert were blunt-ended and ligated, and PCR confirmed correct orientation. We digested the plasmid with Not I and Ase I to liberate the transgenic cassette from its plasmid backbone. The linear transgenic construct was purified by CsCl ultracentrifugation and subsequent dialysis. We sent the construct to the University of Minnesota Mouse Genetics Laboratory for pronuclear injection into FVB/N embryos.

Animal housing and husbandry

All mice were housed in a specific pathogen free environment under the University of Minnesota Institutional Animal Care and Use Committee Protocol #0306A48493. The 3'KE/Bcl-X_L transgenic mice were bred and genotyped as previously described (11). The 3'KE/N-RasV12 mice were bred in a similar fashion, and we genotyped the mice by PCR using the following primers: 5NRAS, 5'-ATGACTGAGTACAACTGGTGGTGGTTGGAGCA-3'; 3NRAS, 5'-CATCACCACACATGGCAATCCCATACAACCCTG-3'. The Eμ-c-Myc (15) mice were bred and genotyped as previously described (11). The 3'KE/Bcl-X_L and 3'KE/N-RasV12 mice were of the FVB/N strain, and the Eμ-c-Myc were of the C57BL/6 strain. The Igh-Myc^{Ca} mice were received from Siegfried Janz, were of a mixed C57BL/6 x FVB/N strain, were reared in conventional housing, and were genotyped as previously described

(16). When transgenic mice were crossed with one another for experimental purposes, all comparisons were made among the F1 progeny. All mice were euthanized by carbon dioxide asphyxiation as per standard protocol of the University of Minnesota Institutional Animal Care and Use Committee.

DNA Purification and Southern blotting

DNA purification and Southern blotting were performed as previously described (11, 17). To generate a probe to confirm transgenic founder animals, we amplified the N-Ras cDNA from p3'KE/N-RasV12 using the 5NRAS and 3NRAS primers used for PCR genotyping. To determine transgene copy number, image analysis of the scanned Southern blot image was performed using Adobe Photoshop 6.0 software (Adobe Systems, San Jose, CA), adapted from a previously described technique (11, 18). To evaluate for clonal B-cell populations, we used a kappa light chain gene probe (17).

Western blotting

Peripheral blood was collected with glass capillary tubes by retro-orbital eye bleeds. Serum was separated from suspended blood components by routine centrifugation. Total protein concentration of each serum sample was calculated using a Bradford assay (Bio-Rad Protein Assay, Bio-Rad, Hercules, CA), and the protein concentration did not significantly vary among the animals from the control and experimental groups used for Western blotting. Western blotting of serum immunoglobulin proteins was performed as previously described (11). 0.5 uL of serum was loaded into each well of a polyacrylamide gel, proteins were transferred to a nylon membrane, and an anti-mouse Ig (H+L) HRP-conjugated antibody was used to detect murine immunoglobulin proteins (Amersham, Cleveland, OH). To detect N-Ras proteins in extracts, we used an anti-N-Ras antibody (Oncogene Research Products, San Diego, CA). Extracts from the pN-RasV12 transfected ANBL-6 myeloma cell line were used as a positive control (12).

Flow cytometry

We performed flow cytometry as previously described (11). The following antibodies were used for cell staining: anti-kappa, anti-CD138, and anti-B220 (all antibodies from BD Pharmingen, San Diego, CA). Standard flow cytometric analysis was performed on a FACSCalibur (BD Pharmingen) at the University of Minnesota Cancer Center Flow Cytometry Core, and data were collected and analyzed using Cellquest Pro (BD Pharmingen).

Radiology

Post-mortem whole-body radiographs were taken using a Faxitron cabinet x-ray machine (Faxitron, Wheeling, IL).

Histology

Histology was performed as previously described (11). After euthanasia, thoracic and abdominal cavities were opened and the carcasses were submerged in 10% neutral-buffered formalin. Before trimming, bone samples were decalcified in 10% EDTA. Tissue samples included sternal bone marrow, peripheral and intestinal lymph nodes, liver, kidneys, spleen and all organs with observed gross lesions. Samples were dehydrated, embedded in paraffin, sectioned at 3–5 µm and stained with hematoxylin and eosin (H&E). All sections were reviewed by NK and/or CSC, both ACVP board-certified veterinary pathologists and a subset were independently reviewed by ML, an ABP board-certified human hematopathologist. Tissue samples from litter mate controls were available for comparison.

Results

Generation of the 3'KE/N-Ras V12 transgenic mice

To examine the role of activated N-Ras expression in B- and plasma cells, we constructed the vector p3'KE/N-Ras V12 in which the 3'KE and kappa Vκ21 promoter (KP) were paired with human N-Ras V12 cDNA containing an EE epitope placed at the N-terminus (Figure 1A). We previously described the use of the 3'KE in mouse transgenesis and have shown that its activity is restricted to mature B-, and plasma cells (11). The human growth hormone (hGH) minigene sequence (19) was placed at the 3' end of the construct to promote RNA splicing and processing. Linearized p3'KE/N-Ras V12 was injected into the male pronuclei of FVB/N mouse embryos. Southern blotting identified 6 transgenic founders with low copy numbers; the 6079 and 6076 founders had two copies of the transgene and the remaining founders had one copy of the transgene (Figure 1B). All six founders demonstrated germline transmission. Transgenic 3'KE/N-Ras V12 mice demonstrated similar survival to littermate control (LMC) animals (data not shown). Data presented in this report are mainly from the 6079 founder line; we have noted the founder lineage and age of each mouse for each experiment.

3'KE/N-Ras V12 mice make transgenic N-Ras protein

We isolated mononuclear cells from the spleens of 18-week-old N-Ras V12 transgenic (6079 founder line) and littermate control mice. Whole cell lysates were prepared and separated on a polyacrylamide gel, and we transferred the electrophoresed proteins onto a nylon membrane. We then used an anti N-Ras antibody to detect endogenous and transgenic N-Ras protein. A series of controls were run on the gel, including whole cell lysates from the N-*ras*-V12-EE transfected ANBL-6 human myeloma cell line (12) and littermate control mononuclear splenocytes. The transgenic N-Ras protein contains an EE-epitope tag, and it migrates with a slower electrophoretic mobility than endogenous N-Ras. Murine endogenous N-Ras could be detected in the littermate and transgenic mouse samples (Figure 1C). Transgenic N-Ras (human EE-tagged N-Ras V12) could be detected in the transfected ANBL-6 cell line and in the transgenic N-*ras* animal (Figure 1C).

Flow cytometry profiles of 3'KE/N-Ras V12 B-lymphocyte populations

We used flow cytometry to profile B-lymphocyte populations in representative 3'KE/N-Ras V12 animals. We hypothesized that the presence of the mutant *ras* transgene would affect normal B- and plasma cell homeostasis, as using the 3'KE to drive a *bcl-X_L* transgene leads to a significant expansion of immature and mature B-cells in young transgenic animals and abnormal plasma cell foci in aged mice (11). Samples of mononuclear splenocytes from 4-week-old littermate and 3'KE/N-Ras V12 mice from the 6079 founder line were stained with anti-B220, anti-CD138, and anti-kappa antibodies, and samples of mononuclear bone marrow cells from 4-week-old littermate and 3'KE/N-Ras V12 mice from the 6080 founder line were stained using the same antibody panel (Figure 2). The absolute number of cells in each population was calculated by multiplying the percentage of cells in a gated flow cytometry plot times the total number of cells harvested from each animal. While there are similar percentages of B220⁺kappa⁺ immature/mature B-cells in the spleens of representative LMC and 3'KE/N-Ras V12 mice, there is a 74% increase in the absolute number of this population of cells in spleen of the representative transgenic animal. When we profiled the plasmacytoid B220⁺CD138⁺ populations, we noted a 256% increase in the absolute number of plasmacytoid cells comprising the B220⁺CD138⁺ population in the spleen of the representative transgenic animal. Bone marrow B-cell populations were similar between these mice. While these findings are subtle and are representative of only a few mice in this study, they suggest that the splenic B-cell compartment of the transgenic animals is different from that of the littermate controls.

Aged 3'KE/N-Ras V12 mice develop immunoglobulinemia

We next took peripheral blood samples from aged 3'KE/N-Ras V12 mice. Samples from all six founder mice and one LMC mouse, aged 35 weeks, were analyzed on a Hemavet automated cell counter (CDC Technologies, Oxford, Connecticut). The 3'KE/N-Ras V12 mice had normal peripheral lymphocyte counts with an average value of $4.3 \pm 0.2 \times 10^3$ lymphocytes per microliter, and the LMC mouse had a lymphocyte count of 5.6×10^3 lymphocytes per microliter, all within normal limits for mice ($0.9 - 9.3 \times 10^3$ lymphocytes per microliter).

We then stained peripheral mononuclear cells with anti-B220, anti-kappa, and anti-CD138 antibodies and analyzed the samples by flow cytometry. We also included peripheral lymphocytes from an age-matched 3'KE/Bcl-X_L mouse in our analysis. While we did see an increase in peripheral B220⁺kappa⁺ lymphocytes in the 3'KE/Bcl-X_L mice, similar to our previous studies (unpublished results), the peripheral lymphocyte flow cytometry profiles of the 3'KE/N-Ras V12 mice were similar to LMC mice (data not shown).

We previously demonstrated that the 3'KE/Bcl-X_L mice demonstrate an immunoglobulinemia (11). We hypothesized that the expression of the activated Ras protein in the B- and plasma cells of the 3'KE/N-Ras V12 mice would lead to abnormal plasma cell homeostasis, as activated Ras would promote proliferation and survival. We determined that serum protein concentrations were similar among samples using a Bradford assay, and we loaded 5 μ L of serum from age-matched LMC, 3'KE/Bcl-X_L, and 3'KE/N-Ras V12 mice onto a polyacrylamide gel, transferred the proteins to a nylon membrane, and probed the samples for immunoglobulin proteins. The serum from the 3'KE/Bcl-X_L mice demonstrated an immunoglobulinemia as previously described (11). The sera from 3'KE/N-Ras V12 mice contained excess immunoglobulin proteins compared to the LMC mouse (Figure 3).

Aged 3'KE/N-Ras V12 mice exhibit abnormal peripheral plasma cell accumulation

As the 3'KE/N-Ras V12 mice aged, they did not demonstrate any apparent morbidity or mortality that differed significantly from age-matched littermate controls. Thus, to evaluate potential gross and microscopic lesions, we euthanized and necropsied 17 3'KE/N-Ras V12 mice (average age 339 days) representing all six founder lines, along with multiple littermate controls. Gross and histologic findings from these animals are summarized in Figure 4 and Table 1. The bone marrow of the transgenic animals was highly cellular with normal trilineage hematopoiesis, but without lymphoid aggregates (Figure 4A). Splenic architecture was maintained, but intense extramedullary hematopoiesis, hemosiderosis, and increased numbers of plasma cells were noted (Figure 4B). Livers contained centrilobular and periportal, lymphoplasmacytic foci that exhibited frequent mitotic figures and demonstrated some degree of atypia (variation in nuclear as well as cell size) (Figure 4C). Similarly, foci of plasmacytoid cells with abnormal morphology were found in the renal pelvis (Figure 4D). Abnormal plasma cell foci were not noted in any of the littermate controls. Finally, consistent with a history of immunoglobulinemia (demonstrated in Figure 3), one Ras animal developed eosinophilic casts in the renal tubules. (Figure 4D).

Targeted overexpression of N-Ras V12 and Bcl-X_L do not cooperate in our system

While the aged 3'KE/N-Ras V12 mice developed abnormal lymphoproliferative foci, they did not demonstrate any evidence of neoplasia or exhibit any difference in morbidity or mortality compared to LMC mice. We hypothesized that the rate of stochastic mutations was too slow to allow for clonal outgrowth of N-Ras V12-expressing B-cells and plasma cells. Thus, we crossed the 3'KE/N-Ras V12 mouse to the 3'KE/Bcl-X_L mouse (11). We used both the 6080 and 6088 3'KE/N-Ras V12 transgenic founder lines for the crosses to generate a group of over 40 mice comprised of LMC, single, and bitransgenic mice, all of the FVB/N

strain. When we compared flow cytometry profiles of anti-CD138, anti-B220, and anti-kappa stained bone marrow and spleen mononuclear cells from all four genotypes, the bitransgenic progeny demonstrated a phenotype very similar to the single transgenic 3'KE/N-Ras V12 mice (data not shown). Although we allowed the cohort of mice to age to over thirty weeks, we did not see any evidence of altered morbidity or mortality.

Activated N-Ras V12 and elevated c-Myc cooperate to induce B-cell neoplasia

Our previous studies have demonstrated that c-Myc can cooperate with Bcl-X_L to form B- and plasma cell tumors (11, 16). Thus, we decided to study the role of elevated c-Myc expression in the context of elevated mutant N-Ras expression. Others have shown that Ras and Myc can cooperate in neoplasia in general; however, we hypothesized that our targeted system would specifically result in B-cell neoplasia affecting cells late in their developmental stage, including plasma cells. We crossed the 3'KE/N-Ras V12 mice to the Eμ-c-Myc transgenic mice (15), which were purchased from Jackson Lab (Bar Harbor, ME) under academic license from E. I. du Pont de Nemours and Company. Because the 3'KE/N-Ras V12 mice were of the FVB/N strain and the Eμ-c-Myc mice were of the C57BL/6 strain, we made all comparisons among the syngeneic F1 progeny.

The 3'KE/N-Ras V12 x Eμ-c-Myc bitransgenic mice demonstrated a significantly diminished survival of 10 weeks compared to the single transgenic mice and littermate control mice in the group [$P = 0.02$, Student's T-test, 3'KE/N-Ras V12 x Eμ-c-Myc vs. Eμ-c-Myc] (Figure 5). Some deaths occurred among the Eμ-c-Myc single transgenic group, consistent with their previously reported median survival of 20 weeks (15). Bitransgenic progeny demonstrated marked lymphadenopathy, splenomegaly, lethargy, and morbidity. Mice were sacrificed when they became moribund and were fixed intact in formalin after the thoracic and abdominal cavities were opened. Faxitron® radiographs of the animals revealed that the bitransgenic animals were radiographically similar to those of LMC and single transgenic animals, with no evidence of focal bone lesions or demineralization.

Microscopic analysis of multiple tissues demonstrated effacement by monomorphic cell population composed of atypical lymphocytes that were, characterized by high nuclear:cytoplasmic ratios and multiple, abnormal mitotic figures (Figure 6). The bone marrow was replaced by similar atypical cells, with extension of cells to the cortical/periosteal surface (Figure 6A). Compared to the normal reactive follicular splenic architecture of the wild type littermate controls (Figure 6B, top panel), bitransgenic mice developed splenomegaly with effacement of splenic tissue by abnormal lymphocytes (Figure 6B, bottom two panels). Large areas of the renal parenchyma were effaced by similar abnormal cells (Figure 6C, bottom two panels). Thymomegaly, with loss of normal architecture due to effacement by abnormal lymphocytes, also was noted (Figure 6D, bottom two panels). Finally, lymph nodes demonstrated effacement by abnormal lymphocytes, with macrophages scattered among the sheets of lymphocytes (Figure 6E).

To further characterize the nature of the expanded lymphocyte population, we used flow cytometry to confirm that the cells were of B-cell origin and to evaluate for possible expansion of plasma cell population, although these cells did not have plasma cell features histologically. We analyzed mononuclear cells from the spleen and marrow of LMC and bitransgenic progeny by staining with anti-B220 and anti-CD138 antibodies. Bitransgenic progeny demonstrated significant expansions of B220⁺CD138⁻ cells (Figure 7A). We also profiled cells harvested from a superficial inguinal lymph node of a bitransgenic animal and B cells comprised a majority cells in this enlarged node, although the significance of this is unclear, as the non-enlarged lymph nodes from littermate controls were not simultaneously analyzed (Figure 7A);.

To evaluate whether the lymphoproliferative process was poly- or monoclonal, we evaluated for populations of cells with identical kappa immunoglobulin gene rearrangements. Samples of genomic DNA harvested from unsorted mononuclear cells from bone marrow, lymph nodes, spleen, and peripheral blood were used for Southern blot hybridization analysis. Tailsnip DNA was used as an unrearranged control. As seen in Figure 7B, multiple tumor-bearing tissues in one representative bitransgenic animal demonstrated populations with identical kappa rearrangements. Thus, the ability of the abnormal lymphocytes to proliferate in the lymphoreticular tissues and to infiltrate the surrounding soft tissues together with their clonal nature support that these B-cells comprise a malignant neoplastic population in this mouse.

Discussion

The 3'KE has been studied extensively by our laboratory, and our initial *in vitro* studies demonstrated that, while the 3'KE is weakly active in non-secreting B-cells, it much more active in plasma cells (13). In a prior report, we used this genetic element as a tool to drive Bcl-X_L expression in a transgenic mouse (11). Indeed, *bcl-X_L* transgene transcripts were identified in the B-cell compartment of the transgenic mice, and the mice displayed a phenotype consistent with apoptosis-resistant B- and plasma cells (11). When co-expressed with c-Myc in a transgenic or knock-in system, malignant plasma cell populations were frequently identified in the bone marrow, associated with lytic bone lesions (Table 2), and these models have many features of human MM (11, 16, 20).

Given the utility of the 3'KE to direct overexpression of a gene of interest to the B-cell and plasma cell compartments, we sought to use this genetic element to target expression of a mutant activated N-*ras*. We hypothesized that, similar to our 3'KE/Bcl-X_L mice and c-Myc crosses, the 3'KE/N-Ras V12 mice and c-Myc crosses would develop B- and plasma cell neoplasms and possibly implicate a role of activating *ras* mutations in plasmacytomagenesis.

We first created the 3'KE/N-RasV12 mice. Similar to the 3'KE/Bcl-X_L mice, albeit less robust, the aged 3'KE/N-RasV12 mice developed immunoglobulinemia, renal tubular casts, and foci of abnormal plasma cells, sequalae consistent with B- and plasma compartments with an oncogenic drive and prolonged survival. When we crossed the 3'KE/N-RasV12 mice to the 3'KE/Bcl-X_L mice, however, we did not see overt cooperation of aberrant expression of N-Ras and Bcl-X_L, as defined by gross pathology and/or shortened lifespan. We propose that the N-Ras V12 and Bcl-X_L proteins did not cooperate in our system due to the biologic redundancy of the anti-apoptotic signals up-regulated by the mutant activated N-Ras protein. Alternatively, it is possible that these transgenic proteins would cooperate in B- or plasma cell tumor formation if the experiment were lengthened to more than 30 weeks.

Our next step was to use a series of c-Myc mice to see if the Myc and N-Ras V12 proteins could cooperate in our system. When these proteins were co-expressed under the control of the 3'k and E μ enhancers, respectively, the oncogenic proteins cooperated to induce rapid fatal B-cell neoplasia. However, the B-cell developmental phenotype reflected lymphocytes that were at an earlier developmental stage than plasma cells, unlike coexpression of Bcl-X_L and c-Myc using the same genetic elements (Table 2). Thus, in the context of an E μ -c-Myc-driven model with concomitant mutant Ras expression, the neoplasms represent the B-cell stage at which the 3'KE first becomes active. Similar features were previously identified when a retrovirus bearing v-H-Ras or v-raf was used to infect E μ -myc transgenic mice (21) – neoplasms were of B-cell origin, but without plasmacytoid differentiation. Additionally, the E μ -c-myc mice, have been shown to bear spontaneous *ras* mutations in some B-cell lymphoma clones (22). Given these findings, we propose that coexpression of the Myc and

Ras proteins causes a transformation in the developmental stage at which both the 3'κ and Eμ enhancers are active and impairs plasmacytoid differentiation; in contrast, coexpression of Myc and Bcl-X_L results in neoplasms with both B- and plasmacytoid differentiation.

Our most refined model of plasma cell neoplasia utilizes the 3'KE/Bcl-X_L transgene to make the B- and plasma cell compartments resistant to apoptosis and uses the Ig switch region enhancers to drive a *c-myc* gene which is knocked-into that region (Table 2, (16)). The localization of the *c-myc* gene near the Ig switch region enhancer is thought to more tightly restrict aberrant c-Myc expression to the plasma cell compartment in comparison to Eμ. Eμ is active at relatively constant levels throughout B-cell development, but becomes weakly active at the plasma cell stage(23). The activity of the 3'-Ca enhancer varies considerably during B-cell development: it is inactive in pre-B cells but is able to stimulate Vh promoters in mature B- and plasma cells to levels that cannot be matched by Eμ (23). However, when N-Ras V12 and c-Myc were co-expressed under the control of the 3'KE and the Ig switch region enhancers, respectively, the oncogenes did not appear to cooperate, and the phenotype was governed exclusively by the *c-myc* knock-in (data not shown, summary in Table 2). We hypothesize that this observation is related to differences in the absolute amount of c-Myc protein in the systems, since the transgenic *c-myc* mouse likely had multiple copies of the *c-myc* transgene, while the *c-myc* knock-in had one copy per animal. Indeed, a previous group looking at cooperation of *c-myc* and *ras* genes in a cell culture system demonstrated the striking finding that a relatively modest 50% reduction in c-Myc expression resulted in a greater than 10-fold reduction in susceptibility to transformation by oncogenic Ras or Raf proteins (24).

Previous work has demonstrated that the *ras* oncogene is influential in B- and plasma cell tumorigenesis. One report demonstrated that immortalized lymphoblastoid cells can be transformed by activated *ras* transfection and that transformed cells develop a plasmacytoid phenotype (16). Furthermore, these *ras*-transfected EBV-immortalized cell lines have been shown to be effective tools in the production of monoclonal antibodies (25). Another report demonstrated that murine plasmacytomas could be rapidly induced in mice by infection with a novel retrovirus that uses the immunoglobulin heavy chain promoter and enhancer to drive *c-myc* and v-Ha-*ras* (26). When a mutant *ras* gene is expressed under the control of the Eμ, however, transgenic mice develop T-cell lymphomas (27).

The role and prevalence of *ras* mutations in myeloma has been widely debated. In the largest reported study, *ras* mutations were identified in 39% of the tumors at diagnosis (3). Indeed, reported prevalence of *ras* mutations varies in numerous studies, and likely is influenced by the population studied, as well as the technique used to assess *ras* mutational status. For example, a study from Spain found 0% of myeloma patients have *ras* mutations (28); a study from China, 13%(29); a study from Brazil 21%(30), and a study from the United Kingdom, 100% (31). Regardless, the natural history of myeloma is thought to be related to *ras* mutational activation, since these mutations mark, if not cause, the MGUS to myeloma transition in some cases, since patient MGUS samples almost never have *ras* mutations (9, 32). Ras mutations are also thought to be involved in the transition of intramedullary MM to extramedullary MM (33). These data are further supported by microarray studies that demonstrate differential expression of RAS family members in MGUS and myeloma samples (34). Thus, in these examples of human myelomagenesis, *ras* mutations occur once aberrant clones have already reached plasmacytoid differentiation

The role and relevance of aberrant *c-myc* expression has been implicated in most varieties of plasma cell tumors in mice and is frequently an initiating genetic event (23). Indeed, many genetically-induced murine models of plasmacytomagenesis which recapitulate features of myeloma are *c-myc* driven (11, 16, 35). Humans, however, differ in that aberrant c-myc

expression seldom arises in plasma cell malignancies due to a primary translocation (33). While translocations of the *c-myc* gene are implicated as primary events in B-cell lymphomas such as Burkitt, *myc* translocations are thought to be a paradigm for secondary translocations in MM (33). Translocations that involve a *myc* gene are rare or absent in MGUS, but occur in 15% of MM tumors, 44% of advanced tumors, and nearly 90% of human myeloma cell lines (HMCLs) (33). *Myc* rearrangements are thought to represent a very late progression event that occurs at a time when MM tumors are becoming less stromal-cell-dependent and/or more proliferative (33).

The results of the data in this study demonstrate that targeted overexpression of activated N-Ras V12 can lead to abnormal plasma cell homeostasis and lymphoproliferation, and that N-Ras V12 and c-Myc can cooperate to induce B-cell, but not plasma cell, neoplasia in our transgenic system. Thus, we propose that in a *c-myc* driven murine model of plasmacytoma/multiple myeloma, activating *ras* mutations act as secondary (late) mutations, for if the *ras* mutation occurs as an early event, neoplastic differentiation is arrested at the B-cell developmental stage, and the neoplastic phenotype represents a B-cell lymphoma.

Similarly, in human multiple myeloma, primary translocations of the IgH and recurrent partners (CCND1, CCND3, MAF, FGFR3, and WHSC1) are thought to occur in germinal center B-cells, and in conjunction with other genetic events, lead to a clonal expansion of plasma cells, such as MGUS (1, 33). Similar to our findings with murine *myc*-driven plasmacytoma/MM models, we propose that, if *ras* mutations arose in human B-cells that bore translocations of the IgH with growth promoting-oncogenes before those clones had differentiated to the plasma cell stage, that genetic cooperation would lead to a B-cell lymphoma and/or leukemia.

While our experiments did not result in an N-Ras V12-driven model of plasma cell neoplasia, we enumerate the utility of a 3'KE driven *ras* transgenic system as follows. Since farnesyl- and geranylgeranyl transferase inhibitors (FTIs and GTIs) are being identified as possible candidate therapeutics for myeloma patients, a mutant N-*ras* driven *in vivo* B-cell neoplasia system could be used to test drug efficacy. Additionally, as combined targeted therapies of c-Myc and activated K-Ras in conditional models of lung adenocarcinoma and T-cell lymphoma are more effective than targeting a single oncogene (36), we propose that our system could be used to screen small molecule inhibitors of c-Myc and mutant activated Ras proteins in malignant B-cell neoplasms. Finally, we propose that the 3'KE/N-Ras V12 mice, which exhibit plasmacytosis and immunoglobulinemia with aging, may act as a useful system for the production of monoclonal antibodies.

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Figure 1. Construction and expression analysis of the N-Ras V12 transgenic mouse

(A) Diagram of the 3'KE/N-Ras V12 transgenic construct. (B) Southern blot analysis of tail-snip DNA to identify founders. "+" denotes lanes loaded with serially diluted p3'KE/N-Ras V12 added as a positive control. The blot was stripped and reprobed with kappa gene probe as a loading control. Densitometry was used to estimate copy number. Founders 6079 and 6062 have two transgene copies each, whereas the remaining founders have one transgene copy each. (C) Western blot analysis to confirm mutant N-Ras expression in the 6079 founder line. ANBL-6=human myeloma cell line transfected with pN-RasV12. LMC=Littermate control. 6079=3'KE/N-RasV12 mouse which expresses both endogenous and mutant transgenic N-Ras. The blot was stripped and probed with actin as a loading control. As seen in the blot, splenic mononuclear cells from the littermate control and transgenic mice both contain endogenous N-Ras protein. The transgenic mouse contains mononuclear cells that also express mutant transgenic N-ras, which has the same electrophoretic mobility as the mutant N-ras expressed in the transfected ANBL-6 human myeloma cell line. The mutant N-Ras protein has a slower electrophoretic mobility, as it includes a unique epitope tag.

Figure 2. Immunophenotypic analysis of lymphocytes from spleen and bone marrow in the transgenic and littermate controls

Results are reflected as a population percentage as well as an absolute number of cells. The absolute number of cells in each population was calculated by multiplying the percentage of cells in a gated flow cytometry plot times the total number of cells harvested from each animal (counted by Trypan blue exclusion dye and light microscopy with a hemacytometer). LMC=littermate control; S = spleen; M = bone marrow. 6079 and 6080 denote 3'KE/N-Ras V12 mice from separate founder lines.

Figure 3. Transgenic animals develop an immunoglobulinemia

LMC = Littermate control. Bcl-XL = serum sample from 3'KE/Bcl-X_L transgenic mouse. A = albumin (partially blocked). HC = heavy chain. LC = light chain. AU = arbitrary units. The sera from the 3'KE/N-Ras V12 were collected from each of the aged founders. The serum from the 3'KE/Bcl-X_L mice demonstrated an immunoglobulinemia as previously described. The sera from 3'KE/N-Ras V12 mice contained excess immunoglobulin proteins compared to the LMC mouse.

Figure 4. Aged transgenic animals develop atypical lymphoplasmacytoid populations

(A) Bone marrow, femur. The bone marrow is highly cellular, with trilineage hematopoiesis and without lymphoid aggregates. (B) Spleen. While the spleen has normal architecture, there is intense extramedullary hematopoiesis, hemosiderosis, and increased numbers of plasma cells. (C) Liver. Livers contained centrilobular and periportal lymphoplasmacytic foci that exhibited frequent mitotic figures and cellular and nuclear atypia. (D) Kidney. The “*” denotes atypical plasmacytoid foci in the renal pelvis. Renal tubular casts are present in the images captured using the 2X and 20X objectives. All photomicrographs are from aged 3’KE/N-Ras V12 mice from the 6079 founder line. All specimens were H&E stained. The objective used to capture the image is listed in the lower right-hand corner of each photomicrograph.

Figure 5. 3'KE/N-RasV12 x Eμ-c-Myc bitransgenic mice demonstrated significantly decreased survival compared to littermate monotransgenic or control animals

P=0.02, Student's T-test, 3'KE/N-RasV12 x Eμ-c-Myc vs Eμ-c-Myc. The median survival of the bitransgenic mice was 10 weeks. The solid line at the top of the Kaplan-Meier plot represents all four groups, except where the bitransgenic animals diverge at 45 days and the monotransgenic animals diverge at 100 days. The dotted line representing the 3'KE/N-RasV12 mice overlaps with the LMC mice, as both groups had 100% survival over the time period represent on the plot.

Figure 6. Atypical lymphoid populations infiltrate/involve multiple organ systems in the 3'KE/N-RasV12 x Eμ-c-Myc bitransgenic mice

(A) Bone marrow, femur. The top panel depicts normal marrow. In contrast, the bitransgenic mice have an expansion of abnormal lymphocytes with extension into the cortical/periosteal space. (B) Spleen. The top panel depicts normal spleen. In contrast, the bitransgenic mice have effacement of splenic tissue by abnormal lymphocytes. (C) Kidney. The top panel depicts normal kidney. In contrast, the bitransgenic mice have renal parenchyma effaced by abnormal lymphocytes. (D) Thymus. The top panel depicts normal thymus. In contrast, the bitransgenic mice have thymic effacement by abnormal lymphocytes. (E) Lymph node, popliteal. The bitransgenic mice have lymphadenopathy. The lymph node architecture has been effaced with abnormal lymphocytes that are admixed with macrophages. All specimens

were H&E stained. WT = Wild Type Control; RM = 3'KE/N-RasV12 x Eμ-c Myc bitransgenic mice. The objective used to capture the image is listed in the lower right-hand corner of each photomicrograph.

Tissue	%B220 ⁺ CD138 ⁻ (LMC)	%B220 ⁺ CD138 ⁻ (DP)
Spleen	47.5	89.4
Marrow	21.0	88.7
Lymph	-	95.6

Figure 7. The atypical lymphocytes are B-cells and the populations are clonal, as evidence by identical B-cell receptor rearrangements

(A) Flow cytometry of lymphocyte populations from control (LMC) and bitransgenic mice (DP). The “-” in the table denotes that flow cytometry was not performed on lymph node tissue from the LMC mice. (B) Southern blot analysis of genomic DNA from involved tissues of two bitransgenic mice (DP#1 and DP#2). Note that DP#1 has a dominant clonal population in the peripheral blood, spleen, and two of three lymph nodes sampled. Additional clones are identified in the bone marrow and one additional lymph node. Although DP#2 demonstrated lymphadenopathy, there were no distinct clones identified by this methodology. DP = double positive (bitransgenic); BM = bone marrow; LN = lymph

node; S = spleen; PB = peripheral blood; T = tailsnip DNA control; GL_{κ} = fragment length of unarranged kappa locus.

Table 1

Gross and histologic findings of aged 3'KE/N-Ras V12 transgenic animals.

FOUNDER LINEAGE (NO./AGE, DAYS)	
Gross findings (no. affected)	Histologic findings (no. affected)
Littermate Control (3/300)	
none	none
6080 (1/301)	
none	none
6076 (1/302)	
none	foci of plasma cells in renal pelvis (1)
6081 (1/301)	
none	foci of plasma cells in renal pelvis (1)
6082 (1/296)	
none	foci of plasma cells in renal pelvis (1)
6088 (1/300)	
none	none
6079 (12/359)	
Bladder - urinary bladder distension with proteinaceous material (2)	Bladder - not examined
Liver - hepatomegaly with hypopigmented foci (2)	Liver - multiple small foci of plasma cells (2) and one plasma cell focus with atypia (1)
Bone marrow (long bones and/or sternum) - none	Bone marrow (long bones and/or sternum) - hypercellular (8); mainly myeloid cells with maturation (7) or blasts (4); abundant plasma cells diffusely (5)
Spleen - none	Spleen - EMH (2), increased pigment-laden macrophages and plasma cells (8), with atypical features (4)
Lymph node - none	pale and prominent follicular architecture (2)
Kidney - none	granular casts (1), large basophilic tubular casts (1), renal pelvis with foci of plasma cells (5) with pleomorphism (2) and high mitotic index (1)
Intestine - none	Cystic structures of unknown origin (2)

Table 2

Phenotypic summary of 3'KE-controlled transgenic mice.

ANIMAL MODEL	PHENOTYPE	REFERENCE (PMID)
3'KE/Bcl-X _L	Non-malignant B- and plasma cell lymphoproliferation	14656874
3'KE/N-RasV12	Non-malignant B- and plasma cell lymphoproliferation	Present study
3'KE/Bcl-X _L x3'KE/N-RasV12	No gross evidence of disease up to 30 weeks	Present study
3'KE/Bcl-X _L xEμ-myc	Fatal extramedullary B-cell and medullary plasma cell neoplasms	14656874
3'KE/N-RasV12xEμ-myc	Fatal B-cell neoplasms in the bone marrow and periphery	Present study
3'KE/Bcl-X _L xIgh-Myc ^{Ca}	Fatal plasma cell neoplasms in bone marrow and lymph nodes	15199411
3'KE/N-RasV12 x Igh-Myc ^{Ca}	No cooperation of oncogenes; aged mice bear phenotype similar to Igh-Myc ^{Ca} mice	Data not shown