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14. ABSTRACT This project investigated the role of Bin1, a gene encoding a Myc-interacting adapter protein with features of a tumor suppressor, on the normal development or neoplastic transformation of the mouse prostate. In Aim 1, we determined the effect of deleting Bin1 on normal prostate development and tumorigenesis (tumor suppressor model). In Aim 2, we determined the effect of Bin1 deletion on neoplastic progression of lesions initiated by a prostate-specific c-Myc gene (negative modifier model). In Year 1, we showed that deletion of the conditional 'floxed' allele of Bin1 phenocopied deletion of the wild-type allele as anticipated. However, to overcome difficulties that arose in Year 2 with generating prostate-specific deletions we created mosaic animals for the study. Although studies are as yet incomplete, results collected to date in Year 3 suggest that Bin1 loss does not increase prostate cancer incidence nor does it drive progression of Myc-initiated cancers. Ongoing experiments test whether (i.) Bin1 loss limits castration-induced apoptosis in normal prostate cells and/or (ii.) Bin1 loss phenocopies Myc activation during progression of Ras-driven cancers, based on findings about the effects of Bin1 on Myc stability emerging from other projects in Year 3.					
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Introduction

In this project, we proposed to test the hypotheses that the Bin1 gene is required to promote normal development of the prostate and/or to restrain the initiation or progression of prostate cancer. These hypotheses were founded upon the following evidence. First, Bin1 encodes an adapter protein that inhibits the transforming activity of the Myc oncoprotein, the activation of which drives a large fraction of prostate cancers (1-8). Second, a considerable body of work indicates that Bin1 can facilitate differentiation, stress signaling, and immune control, and that it can restrain cancer development (9-18). Third, in certain neoplastically transformed cells it has been reported that Bin1 can facilitate a p53-independent mechanism of cell suicide which can be triggered by Myc and other proapoptotic stimuli (14, 15). Fourth, genetic studies in the mouse suggest that Bin1 can restrict immune escape of neoplastically transformed cells (20). Lastly, Bin1 has been observed to be attenuated frequently in human prostate cancers (12, 16) (McDonnell, T.J., Do, K.-A., Troncoso, P., Wang, X., Bueso-Ramos, C., Coombes, K., Brisbay, S., Lopez, R., Prendergast, G.C., and Logothetis, C. Application of data modeling strategies to prostate cancer tissue microarray: an analysis of selected cell cycle and cell death regulatory proteins. *Mod. Pathol.*, submitted.) Taken together, these studies supported the notion that Bin1 loss may facilitate the development or progression of prostate cancer.

While an unambiguous function for Bin1 has yet to be defined, the literature suggests that this gene encodes a scaffold that integrates signaling and trafficking processes in cells, perhaps critical for stress responses that suppress cancer. A key question is whether Bin1 attenuation in cancers is cause or consequence of tumor formation. 'Gene knockout' mice offer outstanding models to address such questions. In this project, we proposed to use a conditional Bin1 knockout model that uses well-established cre-lox technology. The reason to choose a conditional knockout model was based on evidence that complete ablation of Bin1 leads to an embryonic lethality (19). Briefly, we constructed a conditional allele of Bin1 that can be ablated in a generalized or tissue-specific manner by induction of a Cre recombinase transgene. Through this standard

genetic strategy, we sought to gauge the consequences of Bin1 loss on androgen-driven development and homeostasis of the prostate (Aim 1). If Bin1 loss is sufficient to promote tumor formation, then at later times one would expect an elevation in the formation of preneoplastic or neoplastic lesions (Aim 1). Alternately, if Bin1 loss did not affect tumor initiation, but was sufficient to drive tumor progression, then one would expect an increase in the progression of pre-neoplastic or neoplastic lesions that are initiated by a separate oncogenic event (Aim 2). These were posed as distinct aims, because it was conceivable that Bin1 may have no role in development, homeostasis, or tumor initiation, but still have a critical role in limiting tumor progression (e.g. by limiting survival or immune rejection after a tumor has developed). In support of the possibility, our studies in non-prostatic cells show that Bin1 can limit cancer progression by blocking the survival and immune evasion of neoplastically transformed cells (20).

Body

Progress on the Original Aims (Years 1-3)

In Year 1 of the project, as documented in an earlier progress report, we performed a set of experiments to validate the expected operation of the conditional ‘floxed’ allele of Bin1 (termed Bin1flox). Using two different Cre transgenes, we showed that deletions in could be produced as desired in our transgenic mouse strain, altering the Bin1flox allele to the deleted Bin1flox Δ allele. Additionally, we showed that strains with two copies of the Bin1flox Δ allele phenocopied the cardiac defect produced in ‘straight’ knockout mice which had two copies of the Bin1 nullizygous allele (termed Bin1KO) (19). These mice were then used as a foundation to create a set of experimental strains that included a prostate-specific and tetracycline-regulatable Cre transgene that utilized the probascinc promoter for expression (termed PB-cre). The PB-cre transgenic animal used for breeding was provided by Dr. Tim Thompson (Baylor). For reasons that remain unclear, after creating the desired strains found that the PB-cre gene in the experimental strains did not function as anticipated. Specifically, we were unable to induce Cre expression by administration of tetracycline, either in drinking water or direct injection, in contrast to

successful induction in the parental strain as well as other mixed strains that included this PB-cre allele (T. Thompson, pers. comm.). We could not overcome this problem by varying the period, dose, or route of administration of tetracycline. To circumvent this problem, we turned to the use of mosaic knockout mice, a strategy which we correctly anticipated would allow us to produce sufficient numbers of mice that were viable but still predominantly composed of nullizygous, heterozygous, or homozygous wild-type cells as desired for comparative experiments.

In Years 1-2, we constructed and characterized these mosaic strains as described in detail in an earlier progress report. Briefly, the design for strain construction involved interbreeding mice which carried an E1a-cre transgene, the Bin1KO nullizygous allele, and/or the Bin1flox conditional allele. Following a published protocol (21), we used an E1a-cre transgenic mouse to create mosaic mice, based on the activity of the E1a promoter at very early times during embryonic development (E1-E4). By expressing Cre at such times, most of the Bin1flox alleles would be converted to Bin1flox Δ alleles that would be inherited by the cells making up the fully developed animal. Depending on the precise time of conversion of a Bin1flox to a Bin1flox Δ allele, a greater or lesser proportion of mosaicism in individual animals would result. In this way, we were able to obtain viable mosaic mice with sufficient Bin1 function to sustain cardiac development, bypassing the lethality caused by the complete loss of function (knockout) mutant (19). As part of the characterization of the mosaic model system, we confirmed our expectation that the degree of genetic mosaicism in any individual animal would be similar in different tissues throughout the individual, as would be predicted by very early expression of Cre during embryogenesis. Our design allowed the successful production of animals for experimental comparison that were functionally >90% nullizygous, heterozygous, or wild-type, on the basis of PCR genotyping results (an example of which was presented in the Year 2 progress report).

In Year 2, we obtained mosaic mice with the following desired genotypes, including KO/flox Δ (homozygous null), +/flox Δ (heterozygous), or +/flox (essentially wild-type). Through additional breeding steps, we also generated strains of animals that also

included a PB-c-myc transgene. Using these strains, we were now able to compare the effects of Bin1 deletion in normal prostate cells or prostate cells where Myc was activated at the beginning of sexual maturation (about 7 weeks in male mice).

In Year 3 (this year), we have begun to obtain results from the mosaic mouse model and we have also initiated additional experiments prompted by recent findings in our laboratory. To date, the observations to date have been uniformly negative in terms of the effect of Bin1 loss on prostate tumorigenesis.

Continuing long-term evaluations of control and experimental cohorts of mice

1. Mosaic Bin1KO/flox Δ mice (n=10). Mice that have reached the age of 20 months are euthanized and prostate tissue is biopsied and evaluated. While not all mice have been evaluated no prostate cancers have been detected.
2. Bin1+/flox mice serving as control for the above cohort (n=10). Mice are 8 months old as of January 2006. Based on historical information we do not expect prostate cancer to emerge in any animals in this cohort.
3. PB-c-myc; mosaic Bin1KO/flox Δ mice (n=10). Mice are 13 months old as of January 2006.
4. PB-c-myc; Bin1+/flox mice serving as control for the above cohort (n=10). Mice are 7 months old as of January 2006.

Aim 1. Bin1 deletion does not appear to affect prostate development in mosaic null mice, which do not exhibit any prostate deficiencies in either structure or function, on the basis of successful mating. We can not rule out the possibility that this result reflects the lack of a complete nullizygous condition in all of the cells of the prostate. Nevertheless, the presence of many null cells in the tissue does not seem to compromise development of the organ. Second, Bin1 deletion does not appear to increase the incidence of prostate cancer. None of the mosaic null mice sacrificed to date at 19-20 months of age have presented evidence of prostate malignancy. While

the control wild-type cohort for this experiment is still under one year old, we do not expect to see prostate malignancy in this control group. We have yet to complete evaluation of the hypothesized role for Bin1 in castration-induced apoptosis of prostate cells (a subaim of Aim 1), although anecdotal observations would seem to argue against any role.

Aim 2. Bin1 loss also does not appear to cooperate with deregulated PB-c-myc to increase the incidence or progression of prostate cancer (Aim 2). Evaluation of this group is ongoing however.

Aim 2: Additional studies to assess a role for Bin1 in prostate cancer progression

During Year 3, new findings that emerged in our laboratory from another project have prompted us to alter our model for Bin1-Myc function and to perform additional experiments in the prostate to test this model.

Recent findings from other studies have altered our thinking about how Bin1 may affect Myc. At the start of this project, we had hypothesized that Bin1 played an effector role for Myc in triggering apoptosis. To test this hypothesis, we asked whether Bin1 loss could inhibit apoptosis and whether Bin1 loss could drive the incidence or progression of prostate cancer, including that driven by c-Myc. While the most recent findings in the laboratory have not falsified this hypothesis, they have corroborated an alternate hypothesis in which Bin1 may have a regulatory role for Myc. Specifically, the findings suggested that Bin1 loss may stabilize Myc protein, effectively increasing its steady-state accumulation in cells. Based on the present understanding of Myc regulation in the field, events that stabilize Myc would activate its oncogenicity. For example, mutational events that stabilize Myc blunt its apoptotic prowess and license its ability to phenocopy SV40 small t antigen in human cell transformation (22, 23). We therefore reasoned that if the loss of Bin1 stabilizes Myc, then the loss of Bin1 should *phenocopy* Myc activation, not cooperate with it. The implication of the new model to our prostate cancer work was that the design of our experiments was not appropriate to testing the

consequences of Bin1 deletion on cancer progression: activating Myc by ectopic overexpression would merely bypass the benefit that Bin1 loss could confer to Myc. (Here it is worth noting that a regulatory role for Bin1 does not rule out an effector role for Bin1, e.g. as illustrated by RasGAP, which has both regulatory and effector functions for Ras.).

Initial Model: Myc → Bin1 → apoptosis

Newer Model: Bin1 --| Myc

Our observations in the prostate experiments are consistent with the newer model, which would *not* predict that Bin1 loss cooperates with ectopically overexpressed Myc. Instead, the new model predicts that Bin1 loss may cooperate with activated Ras, based on the ability of activated Ras to cooperate with overexpressed Myc to drive malignant transformation (cancer).

In essence, the new model predicts:

Bin1 loss = Myc activation

and

If activated Ras + activated Myc = cancer, then activated Ras + Bin1 loss = cancer

To test this prediction in the setting of the prostate, we extended the scope of Aim 2 to investigate whether Bin1 loss promotes progression of Ras-initiated tumors. The experimental design employed a standard rodent carcinogenesis protocol that has been described in the literature (detailed below). The reason we chose this protocol is that it offers the quickest and most straightforward method to introduce cancer-inducing Ras mutations into the prostate of a rodent. Two criticisms of this design are the following. First, less than 10% of human prostate cancers display Ras mutation, arguably limiting the pathological significance of the design. This criticism does not address the larger number of prostate cancers where Ras is activated as a result of an upstream oncogenic lesion. On this basis, the model can be defended. Second, in the design Ras

signaling is activated indirectly with a carcinogen, rather than directly with an activated Ras or receptor transgene. While valid, this criticism does not invalidate the model. In fact, positive results could increase impact by demonstrating that loss of Bin1 can contribute to cancer progression in a genetic background that may be *more* complex than that solely initiated by Ras activation.

While the results of this experiment are not yet available, the design of the proposed carcinogenesis trial using N-methyl-N-nitrosourea (MNU) is provided for consideration below. The design is adapted from a standard rat protocol described in the literature (24). Briefly, surgically castrated mice are injected i.p. daily for 3 days with 100 mg/kg testosterone propionate, to induce prostate cell proliferation. On day 4, mice are injected i.v. with a single bolus dose of 50 mg/kg MNU to induce prostate tumor formation. In a variant of this protocol reported to increase the incidence of prostate cancer, mice are treated similarly except that after MNU injection they are also administered testosterone as a subcutaneous 20 mg time-release pellet. The response of functionally null or wild-type mice are compared with or without the presence of an activated PB-c-myc transgene, to generate a background of activated Ras or activated Ras plus Myc. In summary, there are a total of 2 cohorts of mice in the experiment with each cohort including 4 different transgenic strains for evaluation (powered at 10 mice/experiment). The full protocol was approved by IACUC review.

Cohort A. Castration + Testosterone propionate s.c. + MNU i.v.

Cohort B. Castration + Testosterone propionate s.c. + MNU i.v. + testosterone pellet

Strain 1. mosaic Bin1 flox)/KO

Strain 2. Bin1 flox/+ (control for strain 1)

Strain 3. PB-c-myc, mosaic Bin1 (flox)/KO

Strain 4. PB-c-myc, Bin1 flox/+ (control for strain 3)

Key Research Accomplishments (Entire Project)

1. Validation of the conditional Bin1 mouse model created, namely, that the Bin1^{flox} allele has wild-type function before Cre-mediated recombination and that Cre-mediated conversion of the Bin1^{flox} allele to the Bin1^{flox} Δ allele creates a functional knockout of the gene.
2. Discovery of a pitfall in the use of a tet-regulatable PB-cre transgene to elicit prostate-specific knockout of a floxed Bin1 allele (in a mixed PB-cre;Bin1 KO/flox strain).
3. Generation of E11a-cre;Bin1^{flox} and E11a-cre;PB-myc;Bin1^{flox} mouse strains that are mosaic for Bin1 knockout. The viable mice used are +/flox, +/KO, KO/flox Δ which are closely related to wild-type, heterozygous, and nullizygous genotypes.
4. Negative evidence that Bin1 loss increases prostate cancer incidence in mice.
5. Negative evidence to date that Bin1 loss increases the progression of prostate cancers initiated by Myc. Experiments are still ongoing to evaluate this question and an hypothesized role for Bin1 in castration-induced apoptosis of prostate cells. Additionally, ongoing experiments will test the new hypothesis that Bin1 loss may phenocopy rather than cooperate with Myc activation, in the setting of Ras-initiated prostate cancer.

Reportable Outcomes (Entire Project)

1. A novel method to produce a 'floxed' allele in mice is reported. This method employs a variant loxP site in the most distal position of the targeting construct which lies downstream of the targeted exon(s). The method facilitates the generation of the desired ES cell line without compromising in vivo utility of the cre-lox system.
2. A method is reported to generate highly mosaic mice for ubiquitously expressed genes that are essential in particular tissues. Bin1^{KO/+} and Bin1^{KO/flox} Δ mosaic mice have been generated that offer a reduction to practice of the method. Such mice are useful to test hypothesized roles for a gene as a tumor suppressor or negative modifier gene in cancer.

Conclusions

Results from transgenic mice collected to date suggest that Bin1 loss does not increase the incidence of prostate cancer nor does it appear to drive progression of cancers initiated by Myc activation. Ongoing experiments are testing the hypotheses that (i.) Bin1 loss may limit castration-induced apoptosis in normal prostate cells and that (ii.) Bin1 loss may phenocopy Myc activation during progression of Ras-driven cancers. Studies were conducted in a mosaic Bin1 knockout model that was created to circumvent a defect in the original experimental design that emerged in Year 2. Using the alternate design, new strains of mice were created that addressed the central questions of the proposal and that will be useful for continued analysis of the role of Bin1 in prostate cancer progression.

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List of Personnel receiving pay from this project:

<u>Name</u>	<u>Degree</u>	<u>Role</u>
George Prendergast	Ph.D.	Principal Investigator
Mee Young Chang	Ph.D.	Postdoctoral Trainee
Alexander Muller	Ph.D.	Consultant/Collaborator
James DuHadaway	B.S.	Research Lab Associate