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13. ABSTRACT *(Maximum 200 words)*

The objective ot this study is to characterize cellular and molecular events of chemically induced rat mammary carcinogenesis by asking why is selective activation of the Harvey ras gene, but not other similar members of the ras gene family, associated with this model. This study is divided into three main objectives. First, the ability of the activated and wild-type forms of Harvey ras and Kirsten ras gene products was evaluated. Both oncogenes produced rat mammary tumors (therefore, excluding the possibility that Kirsten ras was not tumorigenic in the rat mammary gland), although the Harvey ras gene product was more tumorigenic. Second, the relative mutation frequency of Harvey ras and Kirsten ras genes in nitromethylurea exposed rat mammary clonogenic growths are currently being determined. Third, a transgenic rat model has been developed to evaluate the role of Harvey ras gene regulation. Five founder animals expressing multiple copies of the Harvey ras gene have been produced. In one group, the transgene has been observed to reduce tumor multiplicity following carcinogen exposure. Three founder transgenic animals expressing the Kirsten ras gene under Harvey ras regulatory elements have been produced and are under investigation.

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PI - Signature 8/28/15 Date

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Introduction

Mammary carcinogenesis is a multistep process (1). Currently, several genes that may participate in breast cancer development are under investigation. The ras family of genes have been implicated in many different cancers and have also been observed to be overexpressed in human breast cancer (2,3,4,5). Interestingly, many cancers are associated with a specific member of the ras gene family (i.e. Harvey ras, Kirsten ras, or N-ras) (6). For example, in humans, activation of the Harvey ras gene is found in bladder cancer (7), Kirsten ras gene activation is found in colon (8,9) and pancreatic cancer (10), and N-ras gene activation is found in hematopoietic tumors (11). Therefore, ras genes play a central role in many different cancers. The goal of this research is to further characterize the role of the ras genes in mammary carcinogenesis.

The rat mammary carcinogenesis model is commonly used for investigations in breast cancer (12). Previous studies have found that the Harvey ras gene is frequently mutated in mammary tumors resulting from the administration of chemical carcinogens, such as nitroso-methylurea (NMU) or dimethylbenzanthracene (DMBA), to rats (13,14). This high frequency of activation implicates ras as an initiation lesion in mammary carcinogenesis (13). We have found that the direct introduction of an activated ras gene into the mammary gland results in tumor formation (15). In addition, tumor development was observed following the introduction of the normal ras gene into the mammary gland (16). This may be more analogous to the overexpression of a non-mutant Ras as found in human breast cancer. Yet, the steps and mechanisms that lead to the aberrant function of ras genes in cancer are poorly defined. The purpose of this work is to further define steps important to ras activation in the rat mammary carcinogenesis model. This will be done by comparing two closely related ras family members; Harvey ras, which is associated with mammary tumor development in this model, and Kirsten ras, which has not been found to play a role in rat mammary carcinogenesis.

Although the Harvey ras and Kirsten ras gene products are highly homologous, there are regions of variation in the two proteins (17). To study the tumorigenic potential of these genes, replication-defective retroviral vectors were used to introduce normal and activated Harvey ras or Kirsten ras genes into the mammary gland via the central lacteal into the rat mammary gland. The tumorigenic potential of the expressed gene is proportional to the titer of virus that is instilled into the mammary gland. Therefore, the potency of the Harvey ras and Kirsten ras genes to form tumors can be compared. Tumor development is quantitated and resultant tumors are removed for histological examination. Tumors are also analyzed for both the presence and the expression level of the introduced gene using polymerase chain reaction (PCR) based techniques, Southern blots, and Northern blots (and/or RNase protection assays).

Another step that may contribute to the tissue selective association of an oncogene is the relative action of a carcinogen on the genes themselves. Previous studies have looked at differences in DNA methylation patterns between the Harvey ras and Kirsten ras genes in rat mammary tissue following NMU administration (18). Both genes showed alterations in methylation patterns although the Harvey ras gene was more sensitive to these effects in the mammary gland. Using a clonogenic transplantation assay developed in our lab (19), we are further investigating the interaction of the carcinogen with the target gene. DNA is extracted from growths that result from the introduction of mammary clonogens (from rats administered carcinogen) into the intrascapular fat pad of hormonally primed recipient rats. A variation of the PCR-based method developed by Kumar (20) is used to determine whether or not DNA

from the growths contains an activated Harvey ras or Kirsten ras gene. This will allow the determination of the relative frequency of Harvey ras and Kirsten ras gene mutation in the mammary gland. This information will also provide an estimate of the relative penetrance of the Harvey ras gene for tumor formation versus mutation frequency.

Gene expression regulation largely defines the activity of a gene within a tissue type. Therefore, gene expression regulation may be a major contributor to the selective association of an oncogene with a particular tumor type. Previous studies have shown that the Kirsten ras gene is expressed at much lower levels than the Harvey ras gene in whole rat mammary tissue (18). Yet, due to the many cell types and the different developmental stages of a particular cell type in the mammary gland it cannot be concluded that the overall gene expression levels for the gland reflect expression levels for the cells important in tumor development.

To investigate the role of Harvey ras gene regulatory elements to the tissue specific association of the Harvey ras gene in rat mammary carcinogenesis, we have developed a transgenic animal model that uses Harvey ras gene regulatory elements to drive expression of the rat Kirsten ras gene. To validate this model, it was first necessary to develop a transgenic rat strain that contains a transgene with the Harvey ras gene under the same regulatory elements as those used to drive expression of the Kirsten ras transgene. For each strain produced, animals will be characterized for expression of the transgene, inheritance of the transgene in a Mendelian fashion, and determination of any anomalies that may arise from animals harboring the transgene, such as disease susceptibility, increased cancer incidence, and changes in lifespan. Once an acceptable strain is produced, animals will be administered carcinogen and resultant tumors will be assayed for mutation of the transgene and the normal Harvey ras gene. Effects of the Harvey ras transgene on tumor development will validate the model for further studies in transgenic animals that carry the Kirsten ras gene driven by Harvey ras gene regulatory elements. Identification of mutations in the Kirsten ras transgene in mammary tumors supports the hypothesis that Harvey ras gene regulatory elements contribute to the tissue specific association of Harvey ras gene activation in the rat mammary carcinogenesis model.

This proposal addresses a fundamental question of cancer biology that has direct value to breast cancer. The results of these studies will help in understanding factors important in controlling the association of oncogenes with a specific tumor type. This information will be useful for environmental risk estimation and provide insight into the development of organ specific prevention and therapeutic strategies.

Body

This study has three major objectives designed to further characterize factors important in the selective activation of the Harvey ras gene in the rat mammary gland following exposure to the chemical carcinogen NMU. This selectivity will be investigated by comparing the Kirsten ras gene, which is closely related but not found to be activated in rat mammary carcinogenesis, to the Harvey ras gene. The first set of experiments determine whether or not the ras gene products induce rat mammary tumors. The second objective is to determine the relative mutation frequency of the Harvey ras and Kirsten ras genes in rat mammary clonogens following carcinogen exposure. The final set of experiments look at the role of ras gene regulation in selective activation of the Harvey ras gene in chemically induced rat mammary carcinogenesis.

The first objective is to determine if Harvey ras and Kirsten ras, in their wild-type and activated forms, can induce rat mammary tumors. This is done using methods developed in our lab using replication-defective retroviral vectors to deliver the ras genes directly to the mammary parenchyma through the 12 teats of sexually mature female Wistar Furth rats (15). Tumor formation is assessed for 6 months following virus infusion. In our initial study, using viruses at titers ranging from $5x10^6$ to 10^7 colony forming units/ml, we found that the activated form of both Harvey ras and Kirsten ras genes resulted in tumor formation (Appendices A and B). Histological analysis of tumors resulting from the infusion of either vector showed that they were mammary carcinomas. PCR analysis was used to assay for the presence of viral DNA by amplifying the neomycin resistance gene, which is unique to the retroviral vectors, in the resultant tumors from both vectors. All tumors evaluated contained vector DNA and were, thus, likely to have resulted from vector expression of the oncogene. Although the viral titers were comparable for these vectors, the tumor multiplicity was greater for the Harvey ras gene. Therefore, both Harvey ras and Kirsten ras gene products, as found mutated in NMU-induced tumors, can lead to mammary tumor formation. Currently, studies are underway using vectors at titers of $5x10^6$, 10^7, and $5x10^7$ to determine the relative potency of the Harvey ras and Kirsten ras gene products to induce tumor formation.

Tumors also resulted from infusion of vectors expressing wild-type Harvey ras, but not wild-type Kirsten ras (Appendices A and B). On histological analysis of these tumors, regions of keratinization were observed. This has not previously been found with tumors resulting from activated Harvey ras. Also, localized invasion of adjacent muscle was observed. Tumors resulting from the infusion of the wild-type Harvey ras gene were small and had a longer latency and compared to tumors resulting from the activated form of the Harvey ras gene (Appendix A). Currently, studies are underway to determine if the Harvey ras gene within these vectors became mutated following infusion of the vector into the mammary gland. This will be done by PCR amplification and sequencing the Harvey ras region within the vector. If the vector sequence has not been mutated to an activated form, this would support the hypothesis that ras gene regulation is important in ras-related carcinogenesis. Due to the small tumor size not all of the necessary evaluations could be completed from the initial study, therefore, a second study will be done to characterize tumorigenesis with wild-type Harvey ras.

The second objective is to determine the relative frequency of Harvey ras and Kirsten ras genes that are activated in those cells believed to lead to mammary tumor development (i.e. mammary clonogens). This technique uses methods developed in our lab (19). Donor rats are treated with 50mg/kg NMU. Twenty-four hours later, mammary glands are removed and a mammary cell suspension is prepared. Cells are counted and then instilled into the intrascapular fat pad of hormonally primed recipient animals. Mammary growths are then allowed to develop in the fat pad for 2 weeks to 1 month, at which time they are removed and stored at -80°C until DNA extraction is carried out. This DNA is then analyzed for a G to A transition mutation in the second basepair of the Harvey ras or Kirsten ras gene (as observed in NMU-induced tumors) using a variation of the method developed by Kumar (20).

Our initial studies indicated that there was potentially a problem with efficient clonogen growth when large numbers of cells are delivered to a single fat pad location. Therefore, cells expressing a unique marker (cells infected with a retroviral expression vector as described in the first objective) were instilled in the fat pad at varying numbers. Presence of the marker in resultant fat pad growths was determined using PCR to amplify sequences unique to vector DNA. Marker DNA was detected in all growth resulting from the instillation of up to 1000

clonogenic cells. Therefore, 1000 clonogenic cells are now being used per each of 3 instrascapular fat pad locations per a recipient animal. Currently, mammary fat pad growths are being collected and stored at -80°C for DNA extraction and determination of Harvey ras and Kirsten ras gene activation.

The final objective in this proposal is to evaluate the role of Harvey ras gene regulation in the selective association of Harvey ras gene activation in rat mammary carcinogenesis. To do this, a transgenic rat model was developed that expresses the rat Kirsten ras gene under the regulatory elements of the Harvey ras gene. To validate this model, it was first necessary to carry out a control study with transgenic animals that carry the normal Harvey ras gene under the same regulatory elements used to drive the Kirsten ras transgene (i.e. normal Harvey ras gene regulatory elements). To date, 5 founder animals carrying the Harvey ras transgene construct (designated HrHr) have been produced. The transgene is inherited in all animals in the expected Mendelian fashion, although, to date, no animals have been found that are homozygous for the transgene. HrHr animals heterozygous for the transgene do not develop any notable pathologies, they do not have an increased incidence of cancers, and live a normal lifespan compared to their non-transgenic littermates. The first HrHr founder animal (designated R8) has 15 to 20 copies of the HrHr transgene. The transgene is expressed at the same level as the wild-type gene in mammary, skeletal muscle, lung, liver, kidney, and colon tissues (as determined by reverse transcriptase PCR) in R8 transgenic rats.

In the first experiment, 25 female R8 descendents and 25 control littermates were treated with 50mg/kg NMU at between 42 and 50 days of age. Tumor development was assessed weekly for 6 months and any tumors reaching 5mm to 10mm in diameter were removed. Tumor samples were prepared for histological analysis and stored at -80°C until DNA extractions were prepared. DNA was PCR amplified using primers surrounding the codon 12 region of the Harvey ras gene. The PCR product was transferred to a nylon membrane and selective hybridization was carried out using a probe to screen for a G to A transition mutation in the second basepair of the Harvey ras gene (1). The latency of tumor development was slightly greater for HrHr transgenic animals (Appendix C). At 6 months, the multiplicity of tumor development in the HrHr transgenics was 39% that of non-transgenic controls. No tumor development was observed in rats not treated with the carcinogen. It was found that 68% of mammary tumors from non-transgenic animals contained the G to A transition mutation at the second basepair of codon 12 in the Harvey ras gene (Table 1).

Table 1

Number of tumors screened	Number of tumors with a H-ras mutation	Percent tumors with a H-ras mutation
50	34	68%

Due to the large number of Harvey ras transgenes in HrHr animals, the method described above could not be used to screen for Harvey ras gene mutations in mammary tumors from HrHr transgenic rats. Therefore, a PCR-based method is being developed that selectively amplifies the mutated gene. If a Harvey ras gene is found to be mutated, the PCR product can then be assayed to determine if it originated from the wild-type Harvey ras gene or the transgene.

To date, 5 transgenic founder animals carrying the Kirsten ras gene driven by Harvey ras gene regulatory elements have been produced (designated HrKr). Two of these animals have not successfully mated, so that it can not be determined if they are germline for the transgene. Two founder animals transfer the HrKr transgene in the expected Mendelian inheritance fashion. One animal has only recently produced offspring for HrKr transgene analysis. These HrKr transgenic animals are currently being bred to further characterize transgene expression and the effects of transgene expression on the animals health.

Conclusions

The first objective of this proposal was to determine the mammary tumorigenicity of the Kirsten ras gene product. It is well established that an activated Harvey ras gene product can induce tumor development in the rat mammary carcinogenesis model (13). Therefore, a retroviral vector expressing an activated form of the Harvey ras gene product was used as a positive control for mammary tumorigenesis in this model. Multiple mammary tumors developed from the infusion of the retroviral vector expressing the Harvey ras gene (Appendix A). Mammary tumors also developed from infusion of a Kirsten ras gene vector, but with a lower incidence than the Harvey ras gene expression vector (Appendix B). Therefore, the Kirsten ras gene product is capable of mammary tumor induction, although it may be less efficient than mammary tumorigenesis by the Harvey ras gene product. Thus, it is unlikely that the absence of Kirsten ras activation in mammary carcinogenesis is due to the inability of the Kirsten ras gene product to lead to mammary tumorigenesis. Currently, this experiment is being repeated with defined retroviral expression vector concentrations to more precisely determine the relative tumorigenic potency of the Harvey ras and Kirsten ras gene products. Also, we are continuing studies on tumorigenesis induced by the normal Harvey ras gene product.

The second objective is to determine the relative frequency of Harvey ras and Kirsten ras gene mutations induced by NMU in mammary clonogenic cells. Our initial study established that 1000 clonogens could efficiently be transplanted to a fat pad site. Currently, mammary fat pad growths are being collected and stored for DNA extraction and analysis of the relative frequency of Harvey ras and Kirsten ras gene activation. Therefore, no conclusions can be drawn from this objective at this time.

The final objective of this proposal is to evaluate the role of Harvey ras gene regulation in rat mammary carcinogenesis. Five founder animals have successfully been produced that express a Harvey ras transgene by Harvey ras gene regulatory elements (designated HrHr transgenics). The first founder group (designated R8) meets all the criterion necessary to use this strain in carcinogenesis studies. Surprisingly, tumor development in animals expressing the transgene was 39% that found in the non-transgenic control group (Appendix C). This was in opposition to our original hypothesis, that multiple copies of the Harvey ras gene would result in an increase in mammary tumor develop following carcinogen administration (due to the increased number of initiation lesion targets). Currently, we believe that expression of the Harvey ras transgene in the R8 strain is acting to suppress tumor formation in cells that would otherwise develop with an activated form of the Harvey ras gene. This hypothesis is supported by investigations showing that expression of the normal Harvey ras gene can suppress the tumorigenic phenotype of a mutated Harvey ras gene (21,22). Furthermore, the relative level of Harvey ras gene activation (i.e. 68% - Table 1) in mammary tumors that developed in non-transgenic animals is within reason for attenuation of tumor frequency if the Harvey ras

transgene is suppressing tumors that would develop due to Harvey ras activation. To establish this, activation of Harvey ras genes in mammary tumors that developed in animals carrying the HrHr transgene must be determined. This work is currently in progress. Importantly, this result demonstrates that the HrHr transgene does effect tumorigenesis in the rat mammary carcinogenesis model, therefore, HrKr transgenic rats are being set-up for a rat mammary carcinogenesis study.

No significant difficulties have occurred in this proposal during the past year. Therefore, no modifications to the original proposal are requested at this time. This research should prove valuable in further understanding the molecular and cellular events that lead to breast cancer. It is hoped that the results from this study will assist in the development of modes for breast cancer prevention, diagnosis, and treatment.

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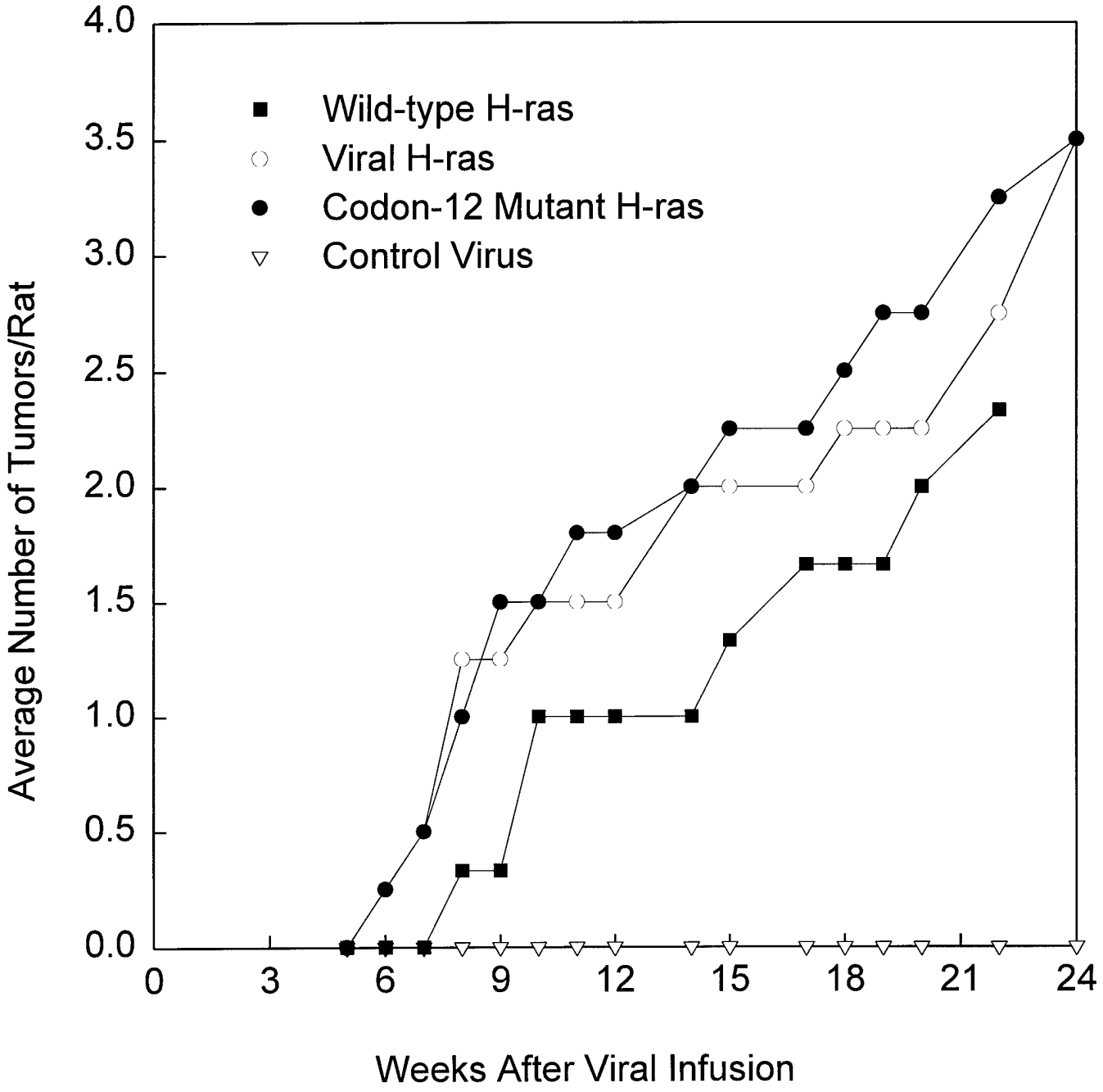
APPENDIX A


Wild-type H-ras
Viral H-ras
Codon-12 Mutant H-ras
Control Virus
Average Number of Tumors/Rat
0.0
0.5
1.0
1.5
2.0
2.5
3.0
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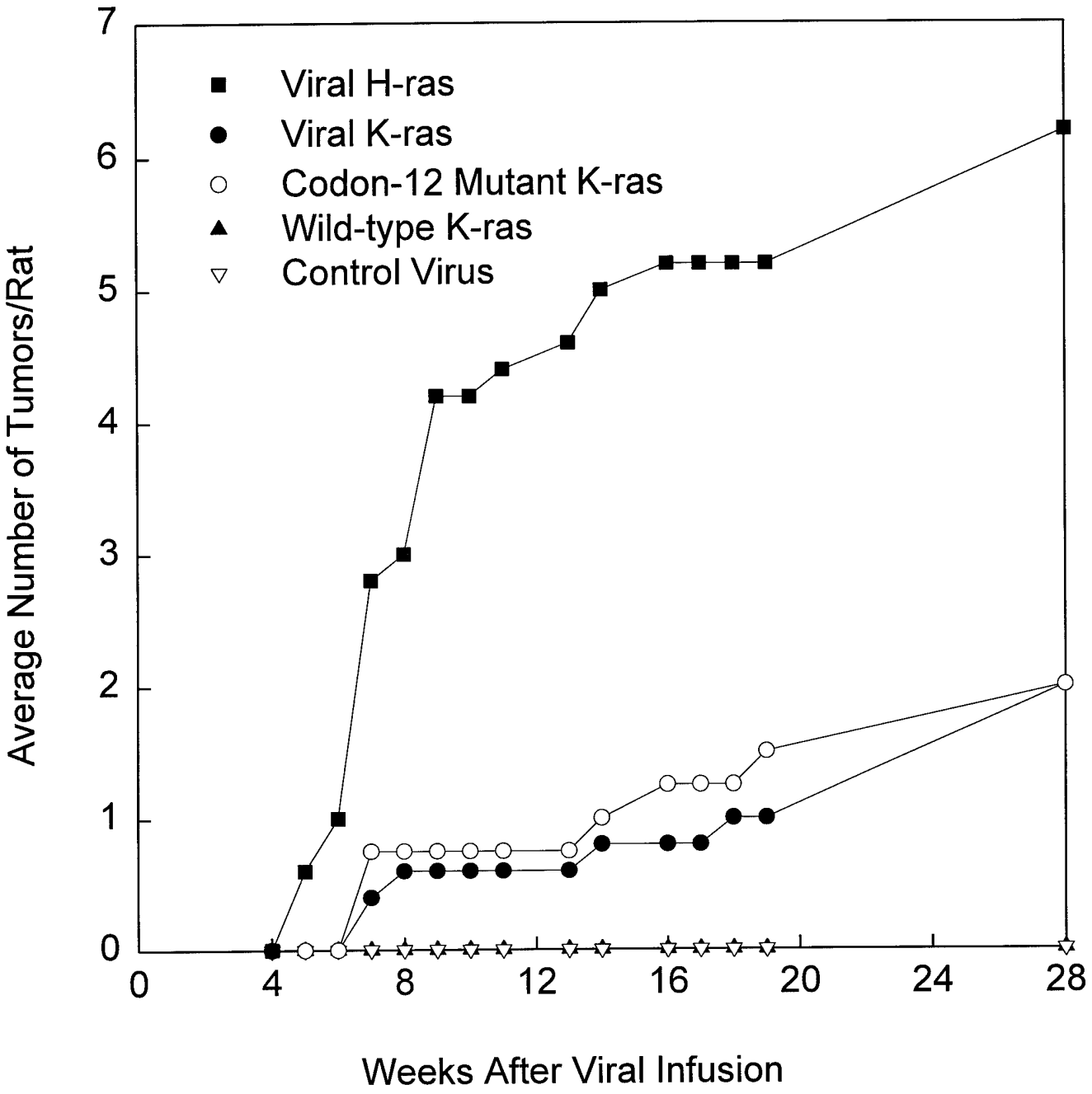
0
3
6
9
12
15
18
21
24
Weeks After Viral Infusion

APPENDIX B


Viral H-ras
Viral K-ras
Codon-12 Mutant K-ras
Wild-type K-ras
Control Virus
Average Number of Tumors/Rat
Weeks After Viral Infusion
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1
2
3
4
5
6
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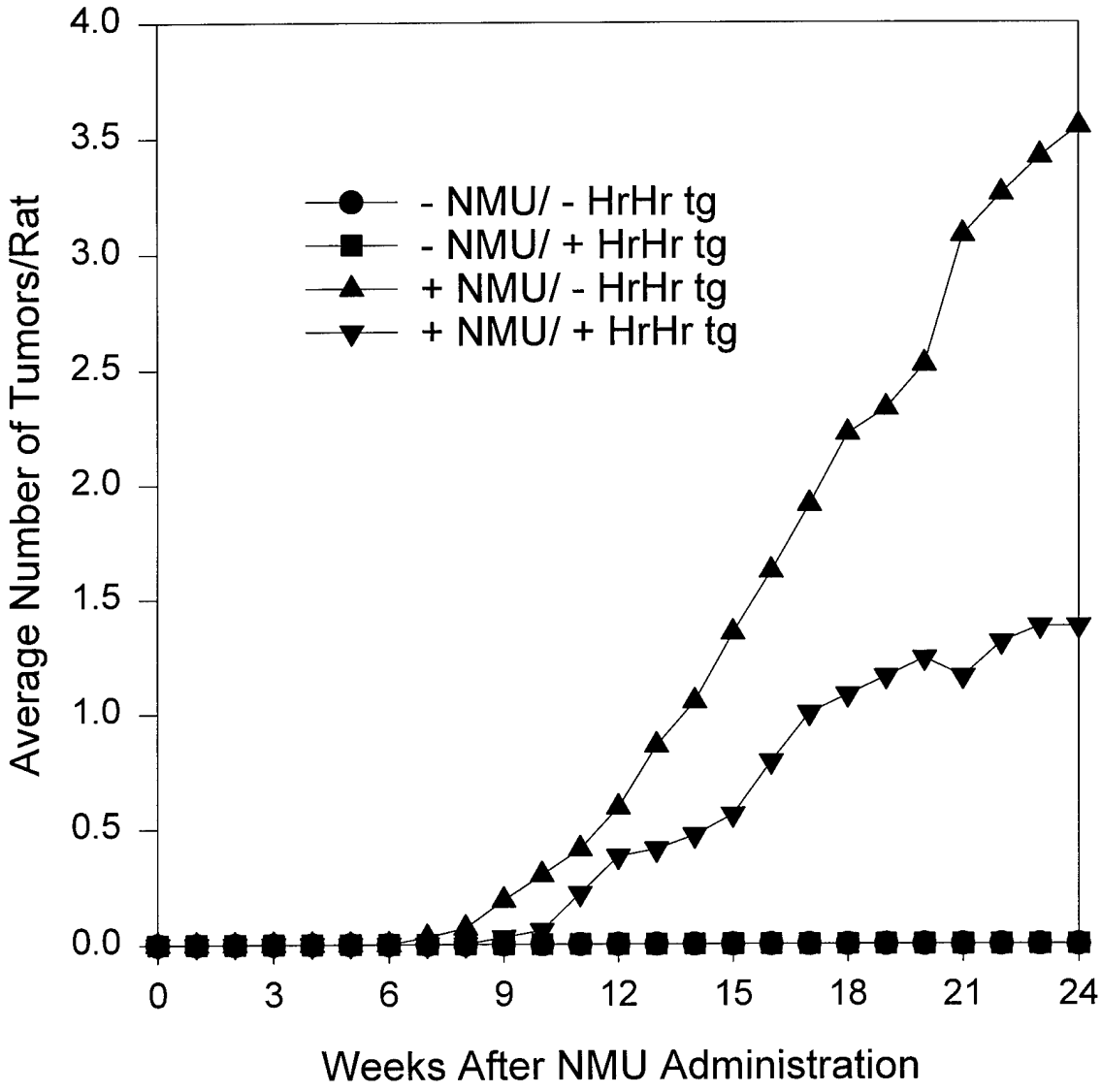
24
28

APPENDIX C


4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Average Number of Tumors/Rat
- NMU/ - HrHr tg
- NMU/ + HrHr tg
+ NMU/ - HrHr tg
+ NMU/ + HrHr tg
0
3
6
9
12
15
18
21
24
Weeks After NMU Administration