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13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*

To gain a better understanding of the deregulation of CDK4 activity *in vivo,* we created knock-in transgenic mice that expresses a tumor derived mutant form of CDK4. These mice are prone to develop tumors of different cellular origins, including breast tumors. Since the p53 tumor suppressor pathway is inactivated in a high percentage of mouse and human tumors, we decided to determine if inactivation of the p53 pathway is an important step in CDK4 mutant induced tumorigenesis. Our investigation revealed that MDM2 is overexpressed in 3 out of 10 breast tumors from these mice and have found no evidence that the p53 pathway is inactivated in tumors from these animals. MDM2 has oncogenic properties when overexpressed and high amounts of the protein are present in human cancers. However, studies indicate that MDM2 promotes cell proliferation by mechanisms that are independent of inactivating p53, including by inhibiting the anti proliferative activity of (TGF β1) in breast tumors. Considering we have found no evidence for loss of p53 function by p53 gene mutation or loss of $p19^{ARF}$ expression, the scope of this proposal is to address if MDM2 overexpression cooperates with mutant CDK4 in mammary cell transformation and tumorigenesis by affecting p53 independent proliferation control pathways.

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Introduction

In an effort to gain a better understanding of the consequence of deregulated CDK4 activity *in vivo,* we generated knock-in transgenic mice that expresses a tumor derived mutant form of CDK4 (r24c). Interestingly, these mice are very prone to develop tumors of different cellular origins, including breast tumors (1). Since the p53 tumor suppressor pathway is an inhibitor of oncogene induced transformation and is inactivated in a high percentage of mouse and human tumors, we decided to determine if inactivation of the p53 pathway is an important step in CDK4 mutant induced tumorigenesis (2 3). Our investigation revealed that MDM2 is overexpressed in 3 out of 10 breast tumors from these transgenic mice and we have found no evidence that the p53 pathway is inactivated in these tumors that arise in these animals. MDM2 has oncogenic properties when overexpressed and high amounts of the protein are present in human cancers(4-8). It is generally accepted that MDM2 promotes tumorigenesis by directly blocking the function of the p53 tumor suppressor. However, recent studies indicate that MDM2 promotes cell proliferation by mechanisms that are independent of inactivating p53, including by inhibiting the anti proliferative activity of Transforming Growth factor-β1 (TGF β1) in breast tumors (9 10). Considering that we have found no evidence for loss of p53 function by p53 gene mutation or loss of $p19^{ARF}$ expression, the scope of this proposal is to address if MDM2 overexpression cooperates with mutant CDK4 in mammary cell transformation and tumorigenesis by affecting p53 independent proliferation control pathways.

Body

In the first task outlined in the approved Statement of Work we set out to determine if there is a higher incidence of breast tumors in CDK4 mutant mice that overexpress MDM2 when compared to CDK4 mutant mice that lack p53. To address this hypothesis we obtained, genotyped and established mouse colonies of MDM2 transgenic mice as well as p53 knockout mice. Genomic DNA was isolated from the tail snips of mouse pups and MDM2 southern blots were performed to identify those mice that expressed the greatest copy number of MDM2 gene. Mice harboring the p53 knockout alleles were identified using a PCR approach. Numerous reports have demonstrated that different genetic backgrounds can have a profound influence on tumor spectra that arise in mouse models. This posed a significant concern for our tumor development studies since all three mice, MDM2, p53 and CDK4 r24C, were on three different genetic backgrounds. Therefore, before generating mice that 1) overexpress MDM2 and are homozygous for the CDK4R24C mutation 2) overexpress MDM2 and contain wild type CDK4 alleles, 3) contain normal levels of MDM2 and are homozygous for the CDK4 R24C mutation, 4) p53 null and homozygous for the CDK4R24C mutation and 5) p53 null we back crossed all three mouse strains for 6 successive generation onto the same BL6 genetic background. Currently, we are establishing mice colonies for each respective compound mouse combination.

In the second task outlined in the Statement of Work we set out to determine if overexpression of a p53-binding deficient MDM2 mutant immortalizes and/or transforms primary mammary epithelial cells (MECs) harboring a CDK4 mutation at a higher efficiency when compared to MECs that contain wild type CDK4. To address this hypothesis we harvested MECs from six-week-old CDK4 r24c and wild type mice.

Early passage MECs from CDK4 mutant mice and wild type controls were transduced with recombinant retrovirus encoding wt MDM2, the p53-binding deficient MDM2 mutant g58d and an empty vector alone control. A retroviral packing cell line was transfected by calcium phosphate with the various recombinant retroviral constructs. After 48 hours, the retroviral supernatants were collected and placed on the MECs for 24 hours. The media was removed after the retroviral infection, cells were rinsed with PBS, and new growth media was added. Before starting the foci transformation assay or the 3T3 transformation assay we performed western immunoblotting assays to ensure that the transduced MECs were overexpressing the MDM2 transduced genes. Unfortunately, the western blots revealed no overexpression of the MDM2 transduced genes in the transduced MECs. However, we were able to detect overexpression of MDM2 in the transfected retroviral packing line. To determine where the technical deficiencies were occurring in our retroviral system we repeated the transfections and transductions in MECs and in wild type mouse embryo fibroblasts (MEFs), which are routinely transduced successfully by other members of our laboratory. We also included a retroviral B galactosidase control, which serves a colorimetric marker to determine the transfection and transduction efficiencies. Consistent with the last experiment, we were able to detect MDM2 overexpression in the transfected retroviral packing line. This same retroviral packing line when transfected with the B galactosidase control exhibited a high percentage of intensely stained blue cells after performing a B galactosidase *in situ* assay. Using supernatants from the same retroviral transfections we transduced both the MECs and the MEFs with wt MDM2, the p53-binding deficient MDM2 mutant g58d, empty vector alone and the B galactosidase control. The western blots against MDM2 combined with B galactosidase *in situ* assays revealed the MEFs are being transduced at a much higher efficiency when compared to the MECs, which still did not overexpress the retroviral, encoded genes. One possible explanation may be that retroviruses only infect cells that are activity dividing. Compared to the MEF controls, our early passage MECs display decreased growth characteristics and a substantial amount of cell death, which could account for the decreased transduction efficiencies. As a result, we are currently trying to improve the overall heath of our MECs by testing different isolation techniques and cell culture conditions. Also, we are currently investigating new approaches to successfully transduce MECs to complete the foci transformation assay and the 3T3 transformation experiments outlined in this task.

Key Research Accomplishments:

- Maintained and successfully genotyped MDM2 transgenic and p53 knockout mice
- Backcrossed MDM2, p53 and CDK4 r24c mice into the same genetic background
- The following compound mouse colonies are currently being generated:
    1) Mice that overexpress MDM2 and are homozygous for the CDK4R24C mutation
    2) Mice that overexpress MDM2 and contain wild type CDK4 alleles
    3) Mice that contain normal levels of MDM2 and are CDK4 r24c homozygous
    4) Mice that are p53 null and homozygous for the CDK4R24C mutation
    5) Mice that are p53 null

- Determined that our retroviral system is unable to transduce MECs at a high enough efficiency to overexpress wt MDM2 or the p53-binding deficient MDM2 mutant

Reportable Outcomes: None.

Conclusions:

With respect to the first Approved Statement of work we were successfully able to complete over half the objectives outlined under this task. Presently, we are generating colonies for each respective compound mouse combination stated in the proposal to complete the remaining objectives. With regard to the second Approved Statement of work we were unable to perform the foci transformation assay or the 3T3 transformation assay. Since retroviruses only infect cells that are activity dividing, we are currently trying to improve the overall heath of our MECs by testing different isolation techniques and cell culture conditions. Also, we are exploring other alternatives to the retroviral transduction system, to successfully transduce MECs at a high enough efficiency to overexpress the encoded retroviral genes. Some of these options presently being tested are a different retroviral cell packaging cell line and an adenoviral transduction system. Once we are able successfully transduce MECs at a high enough efficiencies, we will complete the foci transformation assay and the 3T3 transformation experiments outlined in this task.

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Appendices: None.