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6. AUTHOR(S)
David T. Curiel, M.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
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We are developing methods to derive a gene transfer vector capable of accomplishing targeted gene delivery to metastatic breast cancer cells. In this regard, strategies were employed to modify adenoviral vectors by altering their binding tropism. Genetic methods undertaken allowed for the modification of the native adenoviral binding protein (fiber) to incorporate cancer-relevant cell-binding ligands. Immunologic methods yielded an antifiber antibody which specifically ablates native adenoviral tropism and provides a site for the subsequent addition of breast cancer-relevant ligands. Chemical methods demonstrated that biotin could be added to the virion exterior as an anchor for a streptavidin bridge to allow for incorporating of breast cancer relevant ligands. The results developed herein have allowed for the successful modification of the adenoviral vector to allow it to accomplish cell-specific gene delivery. This important first step will now allow the evaluation of this vector system in the context of in vitro and in vivo models of breast cancer. The utility of the vector in this context will allow the development of gene therapy strategies for disseminated breast cancer on this basis.

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PRINCIPAL INVESTIGATOR: David T. Curiel, M.D.

CONTRACTING ORGANIZATION: University of Alabama at Birmingham
Birmingham, Alabama 35294-2010

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D Curiel 9-17-97

PI - Signature Date

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5.) Introduction

The focus of this proposal is the development of a vector system capable of achieving specific delivery of therapeutic genes selectively to target breast cancer cells and to allow long-term expression of these delivered genes herein. The initial work has focused heretofore on the targeted delivery aspects of this development, as reported in previous progress reports. In the present interval, methods to achieve long-term expression of the delivered gene were explored. The results are reported herein.

6.) Body

1) Construction and characterization of adenoviral/retroviral chimeric vectors. We conceptualized a strategy to produce retroviral particles at target cell sites *in vivo*. It was hypothesized that the local production of retroviral particles would allow effective transduction of neighbor cells in the vicinity of the induced retroviral producer cells. To achieve the *in situ* induction of retroviral producer cells, we used adenoviral vectors to deliver retroviral vector and packaging functions. The use of adenovirus capitalizes on the high levels of *in situ* gene transfer that this vector can achieve *in vivo*. The depicted schema functionally couples the efficient *in vivo* gene delivery characteristics of the adenoviral vector with the integrative capacity of produced replication deficient retroviral vectors derived *in situ* (Figure 1).

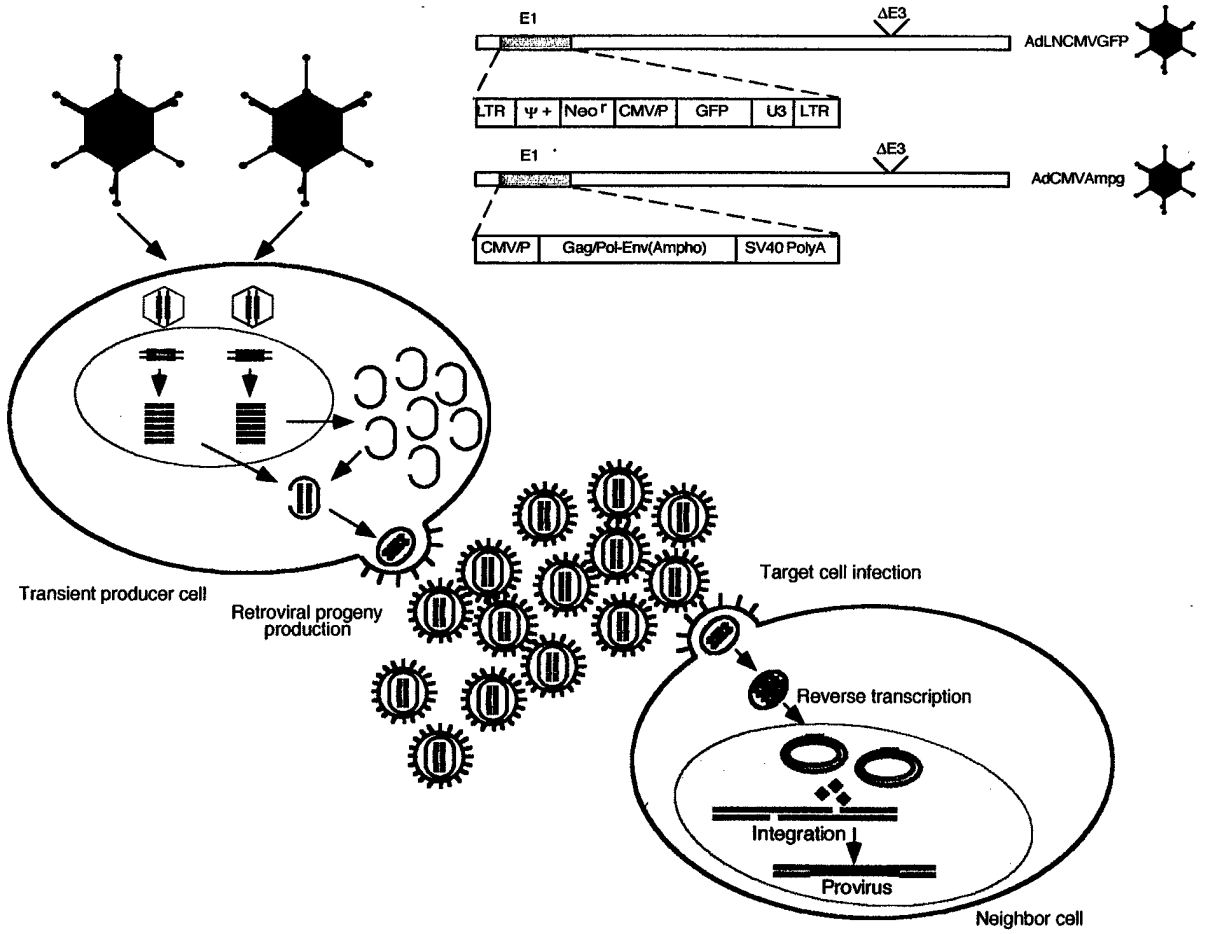
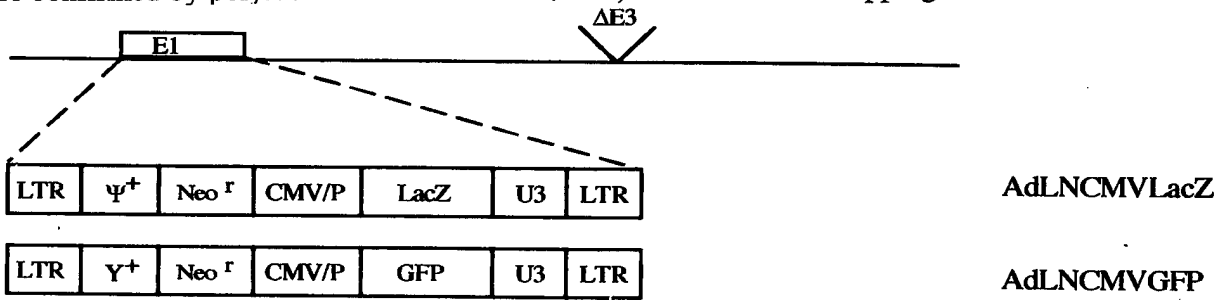


Figure 1: Schema of local generation of retroviral vectors at target organ site. Adenoviral vectors encoding retroviral vector and packaging functions accomplish *in vivo* gene transfer to target parenchymal cells at high efficiency, rendering them transient retroviral producer cells. The locally elaborated retroviral particles can thus directly infect niehbor cells.

As a first step toward implementing this strategy, we constructed adenoviral vectors encoding the requisite retroviral functions for *in situ* generation of retroviral producer cells (Figure 2). DNA segments encoding the retrovirus packaging functions *gag*, *pol* and amphotropic *env* genes derived from the retroviral packaging plasmid pPAM3, were cloned into the adenoviral shuttle vector pCA13. The resultant plasmid, pCAAmpg, was constructed to allow expression of all retroviral packaging functions under the control of the CMV intermediate/early enhancer-promoter. In addition, an adenoviral shuttle plasmid, pΔE1LNCMVGFP, was designed to contain the retroviral vector components. In this instance, a unit containing the neomycin resistance gene and the green fluorescent protein (GFP) expression cassette, flanked by retroviral LTRs and containing the MoMLV packaging signal, was cloned into the polylinker of the adenoviral shuttle vector pΔE1SPIA. A similar construct containing the LacZ reporter, pΔE1LNCMVLacZ, was derived in the same manner. Prior to incorporation into adenoviral vectors, the structural and functional validity of each construct was confirmed by partial DNA sequencing, restriction analysis, and co-transduction experiments to transiently generate retroviral particles (data not shown). In addition, analysis was undertaken to determine retroviral titers obtainable with the retroviral vector and packaging functions in the adenoviral shuttle plasmid context compared to conventional plasmids. For these studies, 293 cells were transfected with $CaPO_4$ with subsequent infection of NIH-3T3 indicator cells with the derived supernatant for scoring of retroviral particle number. Control supernatants derived from transfections with the retroviral packaging plasmid, pPAM3, and the LacZ-encoding retroviral vector plasmid pLNCLZ, yielded retroviral titers of 1.8 X 10^5 virions/ml. A similar analysis was undertaken with the adenoviral shuttle plasmid involving the identical packaging function to pPAM3 (pCAAmgp) and the adenoviral shuttle plasmid containing the identical vector sequences to pLNCLZ (pΔE1LNCMVLacZ). This transfection yielded retroviral titers of 7.4 X 10^3 virions/ml. Based upon this analysis, it was clear that the key retroviral vector and packaging functions were operative in the context of an adenoviral vector shuttle plasmid, suggesting that they would likewise be functional when configured into a recombinant adenoviral vector. Adenoviral vectors were thus derived by co-transduction of the indicated shuttle vectors and the adenoviral rescue plasmid, pBGH11. The resulting adenoviral vectors were confirmed by polymerase chain reaction (PCR) and restriction mapping of viral DNA.

A.



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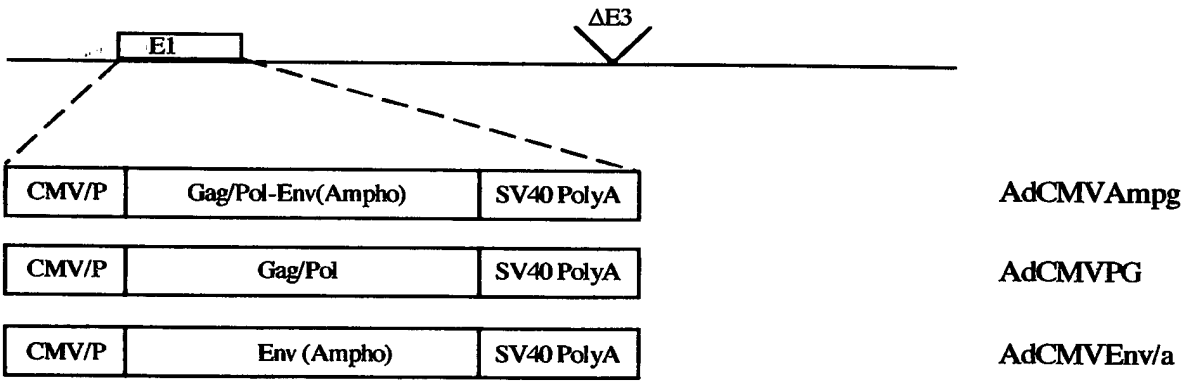

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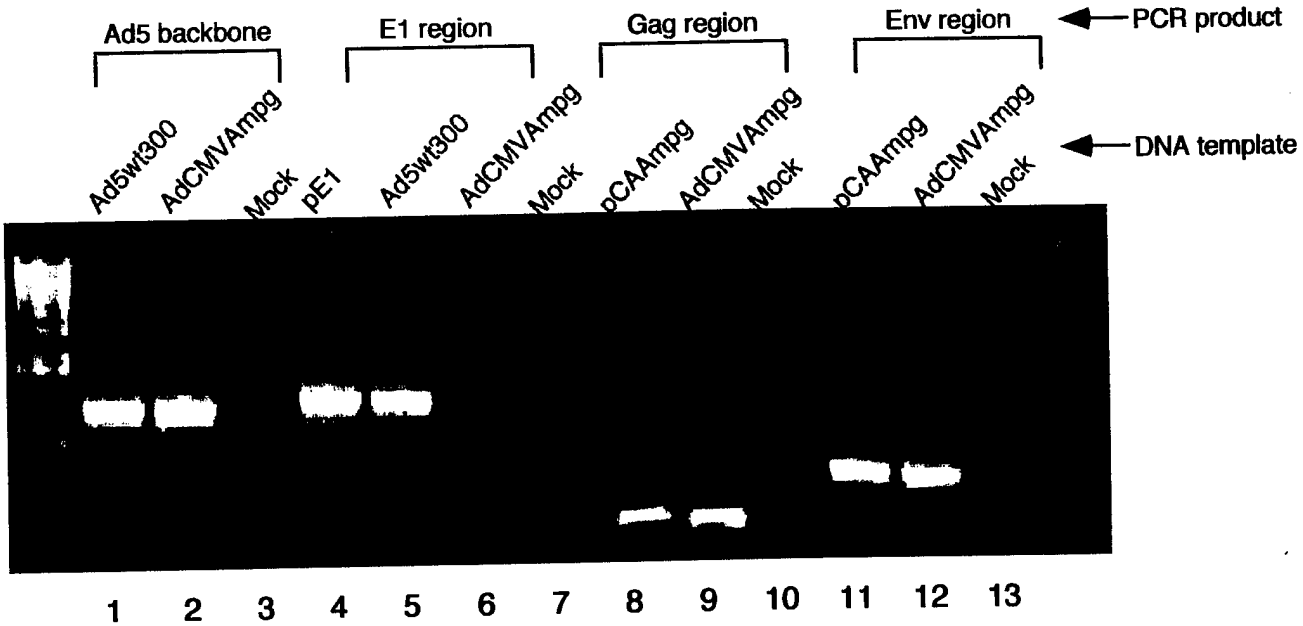

Figure 2: Construction and validation of adenovirus/retrovirus chimeric vectors. **A.** Genome maps of adenoviral vectors containing retroviral vector sequences. Adenoviral vectors are replication-incompetent based on E1A deletions. These are also deleted in the non-essential E3 region. AdLNCMVLacZ contains a retroviral vector insert encoding the neomycin resistance gene (Neo^R) and the *E-Coli* β-galactosidase reporter gene (LacZ). AdLNCMVGFP contains Neo^R plus the green fluorescent protein (GFP) reporter gene. **B.** Genome maps of adenoviral vectors containing retroviral packaging functions. Expression of the encoded retroviral packaging functions is via the CMV intermediate/early promoter CMV/P. Vectors encode *gag/pol*/env (AdCMVAmpg), *gag/pol*(AdCMVPG), or amphotrophic env (AdCMVEnv/a). **C.** Molecular analysis of recombinant adenoviral vectors. Polymerase chain reaction (PCR) methods were employed to assay for specific incorporated vector components. Primers were specific for the E4 region of the adenoviral genome (lanes 1-3), the E1 region of adenoviral genome (lanes 5-7), the retroviral *Gag* sequence (lanes 8-10) and the retroviral Env region (11-13). The depicted analysis for AdCMVAmpg demonstrates that it is E1-deleted (lane G), that it contains incorporated retroviral *Gag* (lane 9) and Env regions (lane 12). A similar analysis was undertaken for each of the derived vectors. Additional confirmation was by restriction endonuclease analysis and direct sequencing.

2) **Long-term gene expression achieved *in vitro* via the adenoviral/retroviral chimeric vector**. We next demonstrated the ability of the adenoviral vectors to induce target cells to function as retroviral producers. Target cells were infected with a combination of the adenoviral vectors, AdCMVAmpg and AdLNCMVGFP, which we hypothesized would allow induction of retroviral particle production. As a parallel control, cells were also infected with AdLNCMVGFP only, which would not be predicted to generate retroviral particles. Target cells for this analysis were either the human fibroblast cell line WI62, the murine fibroblast cell line NIH- human ovarian cancer cell line $SKOV3._{ip1}$ (data not shown). Cells were infected with the appropriate adenoviral vectors for 3 hours and then washed to remove any free adenovirus. These cells were maintained in tissue culture for various time periods and then analyzed for the expression of the GFP reporter gene by fluorescence activated cell sorting (FACS) or by fluorescent microscopy. Analysis of adenoviral vector infected cells at day 2 post-infection

revealed that both groups, the control and the putative retroviral producers, demonstrated a high frequency of expression of the GFP reporter gene (Figure 3-1 and 3-4). This is consistent with a high initial frequency of AdLNCMVGFP infection having occurred in both groups, with subsequent GFP gene expression occuring in both groups as well. A second cohort of each group was passaged every 8 days and then analyzed at day 30. For GFP expression, FACS analysis of the cell group which had been exposed to both adenoviral vectors (AdCMVAmpg and AdLNCMVGFP) showed a substantially higher number of GFP positive cells compared to the cells which had received only AdLNCMVGFP. When visualized by fluorescent microscopy, it could be seen that the GFP positive cells from the cohort infected with both viruses were present in clustered outgrowths suggesting local retroviral spreading and/or clonal origin (Figure 3-5). A stable population of GFP expressing cells is shown at day 60 for the cells infected with both viruses (Figure 3-6), in marked contrast to the cells infected with AdLNCMVGFP alone, which had lost GFP expression, consistent with the known transient expression of gene delivered by the standard adenovirus approach (Figure 3-4). Confirmation of proviral integration by the demonstration of retroviral sequences in high molecular weight cellular DNA could be noted in the cells which received the two adenoviral vectors (see Appendix B).

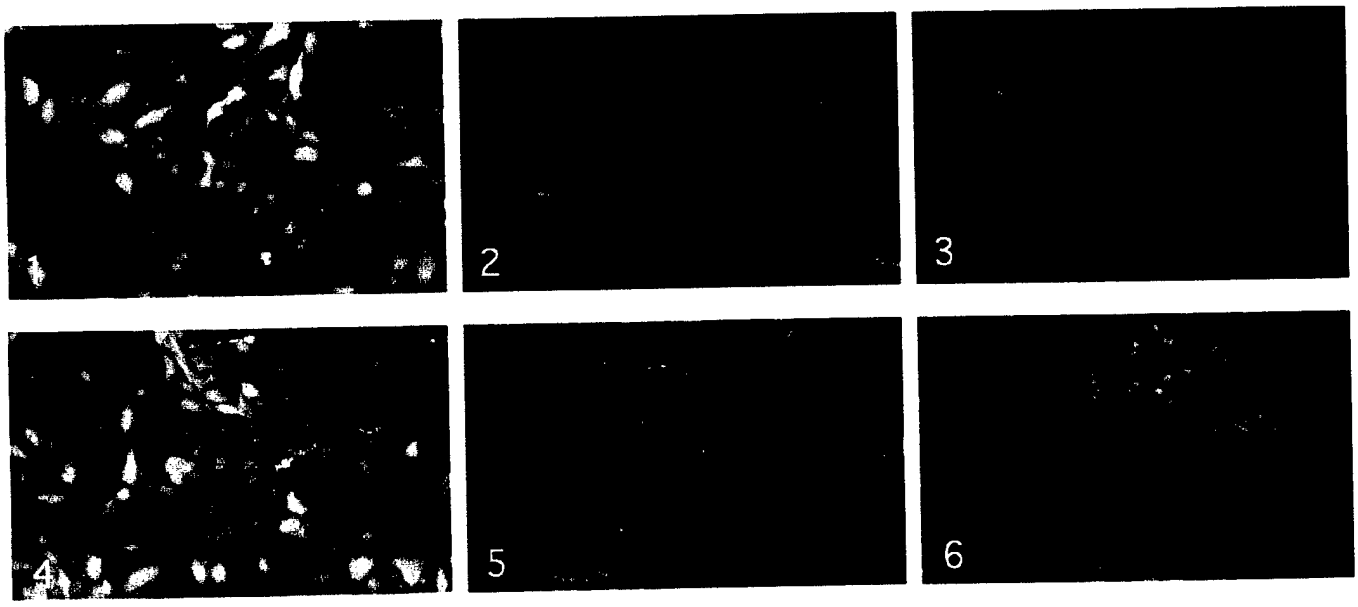

Figure 3: Persistent gene expression achieved via the adenoviral/retroviral chimeric vector system. Target cells were infected with AdLNCMVGFP and AdCMVAmpg (4, 5, 6) or AdLNCMVGFP (1, 2, 3) and then analyzed for stable genetic transduction. Analysis of GFP expression in WI62 cells by fluorescent microscopy was at day 2 (1, 4); day 30 (2, 5); and day 60 (3, 6). Magnification is 500X for 1, 2, and 4. Magnification is 250X for 3, 5, and 6.

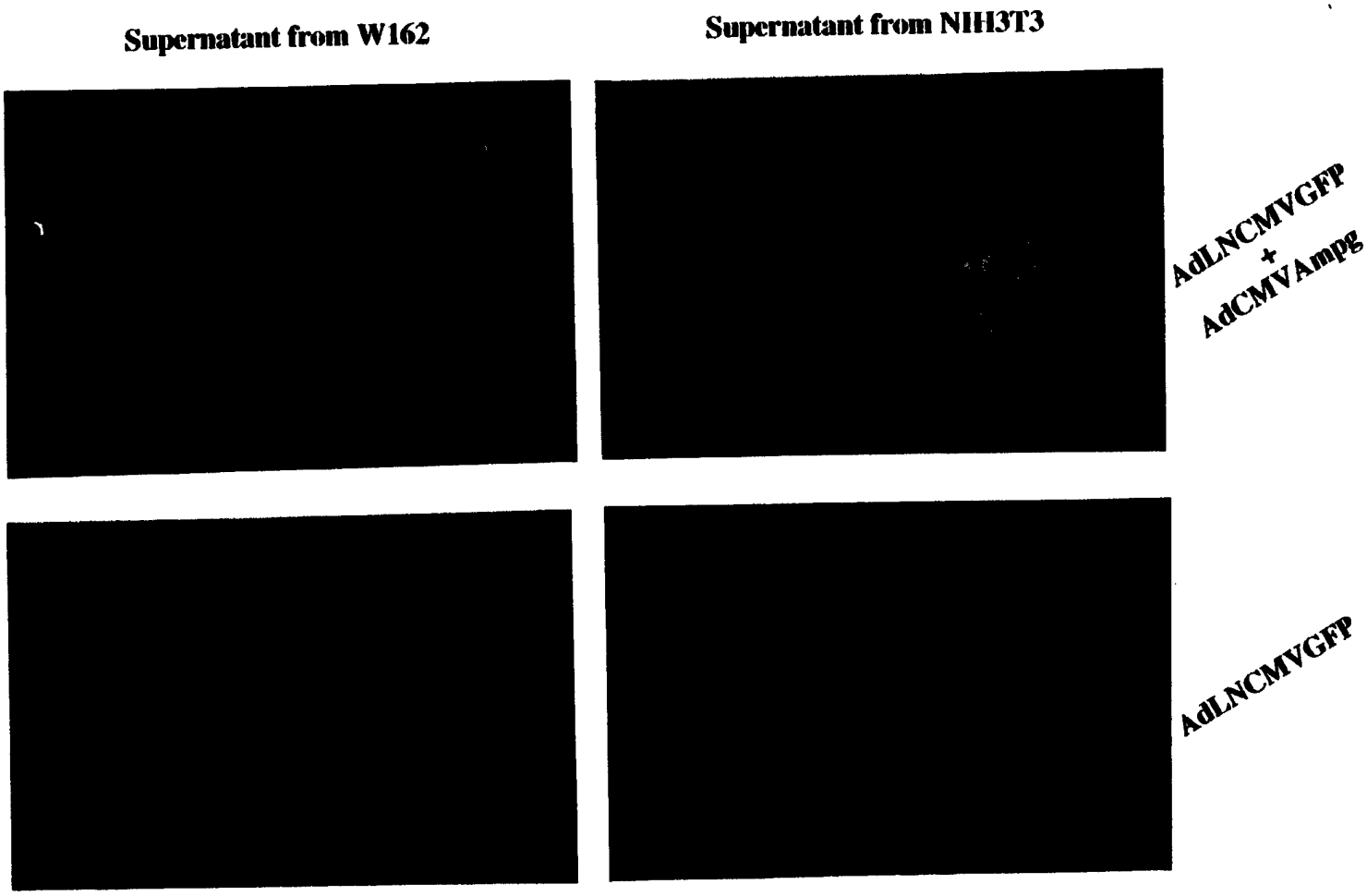

Figure 4: Derivation of transducing retroviral particles via the adenoviral/retroviral chimeric vector. Indicated cells were treated with AdLNCMVGFP only or AdLNCMVGFP plus AdCMVAmpg and supernatants analyzed for retroviral vector particles by titering on NIH-3T3 cells. Supernatant treated cells were analyzed at day twenty by fluorescent microscopy. Magnification is 500X.

3) **Generation of retroviral vector transducing particles via the adenoviral/retroviral chimeric vector.** As a next step, we sought to directly demonstrate the production of transducing retroviral particles based on the process of retroviral producer cell induction. NIH-3T3 or WI62 cells were infected with either a combination of AdCMVAmpg and AdLNCMVGFP, or AdLNCMVGFP only, then subsequently washed as before. The supernatants were harvested at 48 hours post-infection and then employed to infect NIH-3T3 cells to determine retroviral titers. The supernatant-infected cells were maintained in culture for 20 days and analyzed as before for GFP expression. In this study, the supernatant derived from the AdLNCMVGFP virus infected cells was not capable of inducing long-term GFP expression in target cells (Figure 4). In contrast, cells infected with AdCMVAmpg plus AdLNCMVGFP supernatant demonstrated a high rate of GFP positivity at day 20.

This study provides confirmation that GFP expression resulted from infection with retroviruses derived from the original adenovirus-infected target cells. These long-term GFP expression studies were designed to distinguish carry-over adenoviral transient gene expression (< 2 weeks)

from stable transduction mediated by retrovirus production. The results suggested that transducing retroviral particles had indeed been generated by adenoviral vector delivered genes in target cells. In addition to this analysis, we sought to determine whether this methodology was associated with significant production of replication-competent retrovirus (RCR). For this study, we compared RCR generation derived via plasmid based transfection methods versus the employment of the adenoviral/retroviral chimeric vector. HeLa cells were thus transfected with either pPAM3 plus pLNCLZ, or infected with AdCMVAmpg or AdLNCMVLacZ, or AdCMVAmpg plus AdLNCMVLacZ. These supernants were then analyzed for the presence of RCR by a widely employed transformation assay. In this analysis no RCR was noted with employment of an adenoviral/retroviral chimera component (data not shown). Thus, the generation of RCR by this method does not appear to be in excess of conventional methods.

4) **Efficient and long-term gene expression *in vivo* achieved via the adenoviral/retroviral chimeric vector**. Next we sought to exploit this process *in vivo*. For these studies, the ovarian carcinoma cell line SKOV3.ip1 was infected *in vitro* with either AdCMVAmpg plus AdLNCMVGFP, or AdLNCMVGFP alone. To confirm the in vivo generation of infected retroviral particles and infection of neighboring cells we then mixed infected cells with uninfected cells at a ratio of 25% adenoviral vector infected cells with 75% untreated SKOV3.ip1 cells and implanted subcutaneously in athymic nude mice to allow tumor formation. Twenty days after implantation, both animal groups had palpable tumors that were harvested for analysis of GFP reporter gene persistence and expression. The group infected with AdLNCMVGFP only had rare, isolated fluorescent cells (Figure 5B). In contrast, the tumors derived from the two virus group had large expansive clusters of GFP positive cells (Figure 5C). Counting of positive cells in multiple fields allowed an estimate of transduced cells such that the one virus group had 15-20% positive cells whereas the two virus group had >80% GFP positive cells, thus in this group the number of positive cells was substantially greater than the proportion of adenovirally infected cells in the original implanted mixture. The extensive distribution of GFP positive cells in the two virus group suggested stable genetic modification of neighboring cells via *in situ* retroviral vectors.

As a more stringent test of our concept, we explored the potential to link *in vivo* adenoviral vector transduction to *in situ* retroviral producer generation. For these experiments, athymic nude mice were orthotopically transplanted with the human ovarian cancer cell line SKOV3.ip1. Five days post-implantation, animals were treated intraperitoneally with either AdLNCMVGFP only, or AdLNCMVGFP plus AdCMVAmpg. Sixteen days post adenovirus infection, the animals were sacrificed and tumors analyzed as before. In this analysis, no GFP positive cells could be demonstrated in the one virus group (Figure 5E). In contrast, islands of GFP-positive cells could readily be identified in the group which received both adenoviral vectors (Figure 5F). Again, analysis of multiple microscopic fields demonstrated an overall transduction rate of <1% for the one virus group and 10-15% for the two virus group. The relative paucity of GFP expression in the animals given the AdLNCMVGFP is consistent with the temporal pattern of extinction of adenoviral vector-mediated gene transfer in this context. The persistence of GFP expression *in vivo* in the group receiving the two adenoviral vectors, which allow full induction of retroviral packaging, is consistent with our *in vitro* findings whereby stable transduction had occurred based on secondarily elaborated retroviral vectors.

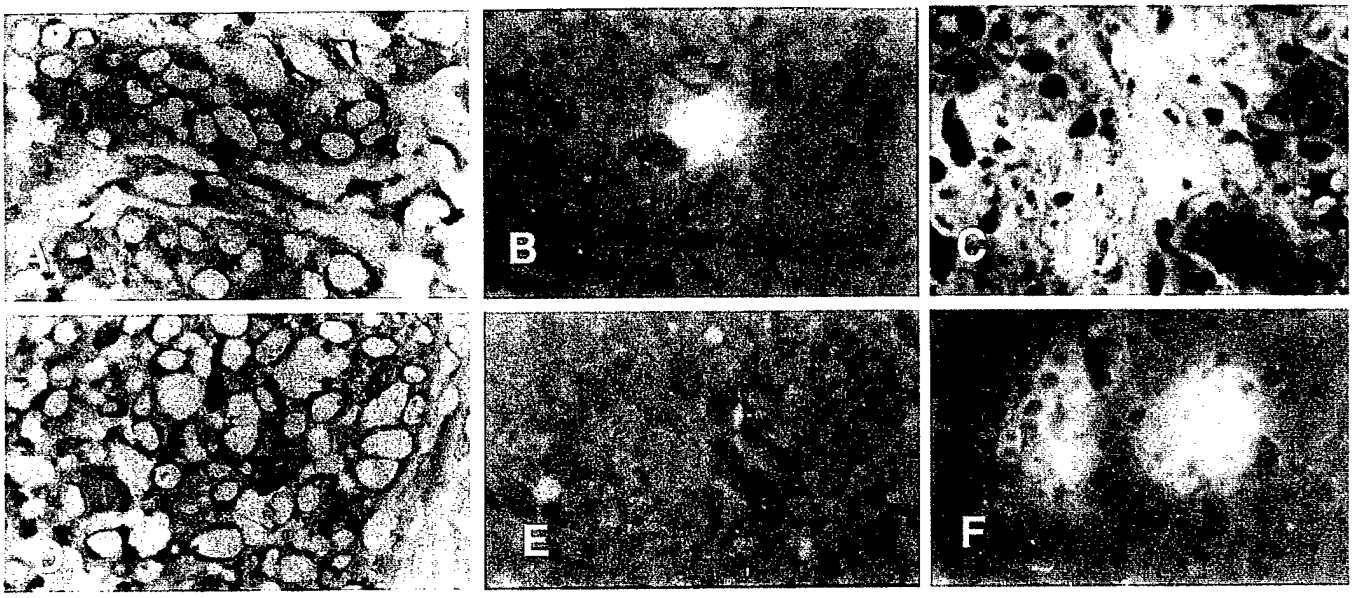

Figure 5: *In vivo* gene transfer via the adenoviral/retroviral chimeric vector. SKOV3.ip1 cells were infected with either AdLNCMVGFP (B) or AdLNCMVGFP plus AdCMVAmpg (C), mixed with virgin tumor cells and implanted subcutaneously in athymic nude mice. At day twenty, tumors were harvested and analyzed by fluorescent microscopy. A representative hematoxylin-stained section taken from a two virus, subcuteneous nodule is depicted in panel (A). SKOV3.ip1 cells were also implanted intraperitoneally and animals challenged with either AdLNCMVGFP alone (E) or AdLNCMVGFP plus AdCMVAmgp (F). Analysis was done by fluorescent microscopy for expression of the GFP reported at day sixteen. Panel (D) shows a hematoxylin stained intraperitoneal tumor nodule. Magnification is 250x.

Herein we have demonstrated a novel vector approach to achieve efficient and stable genetic modification of target cells *in vivo*. **Although this initial proof of concept was accomplished in a model system distinct from our proposed target organ, we have established the principles of a novel method for accomplishing high levels of long-term gene expression.** This was accomplished by using the adenovirus as a delivery system for retroviral vector and packaging components and therefore inducing target cells to function as transient producers of retroviral vectors *in situ*. This strategy thus allowed the generation of retroviral vector particles capable of infecting neighboring cells and accomplishing stable transduction. In this approach, two key factors allowed this level of stable transduction *in vivo*. Firstly, the local production of retroviral vectors at the site of the target cells circumvented the deleterious effects of exposure of the retroviral particles to humoral factors which would have resulted in their inactivation. Secondly, the efficient induction of retroviral producer cells *in situ* capitalized on the ability of adenoviral vectors to achieve effective *in vivo* delivery to target cells. This strategy thus represents a novel conceptual approach whereby desirable aspects of two component vector systems are combined to achieve a gene delivery goal.

7.) Conclusion

The first part of this project has allowed the development of methods to achievetargeted, cell-specific gene delivery via modified adenoviral vectors. The capacity will allow breast cancer

specific delivery of therapeutic genes. The present portion of the project has allowed the development of a novel adenoviral/retroviral chimeric vector for the achievement of integration of therapeutic genes. This capacity will allow long-term expression of therapeutic genes in breast cancer cells. The full exploitation of these capacity will be tested in *in vivo* model systems in the next phase of this work.

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