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Award Number: W81XWH-11-1-0327

TITLE: Inhibition of Embryonic Genes to Control Colorectal Cancer Metastasis

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REPORT DATE: September 2013

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
September 2013	Annual	1 September 2012 – 31 August 2013

4. TITLE AND SUBTITLE

Inhibition of Embryonic Genes to Control Colorectal Cancer Metastasis

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-11-1-0327

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Geneva Foundation
Lakewood, WA 98499-3976

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Embryonic core transcription factors (TFs), primarily the retrogene NanogP8, are the master regulators of cancer stem cells (CSC) in human colorectal carcinoma (CRC). The corollary is that inhibition of NanogP8 will inhibit neoplastic progression of CRC. During the second year of funding we confirmed that NANOG is expressed in the majority of primary human colon carcinomas and that its expression is a significant prognostic factor, especially when associated with expression of the cell adhesion scaffold protein NEDD9. We also elucidated that inhibition of NANOGP8 and/or NANOG induces apoptosis through inhibition of MCL-1 as well as proof of the principle that vector-delivered shRNA to NANOGP8 and/or NANOG complements inhibition of BCL-2 and BCL-XL as a means to inhibit and kill human colorectal cells. Finally we have demonstrated that intralesional injection of LV-delivered shRNA to NANOGP8 or NANOG transduces tumor cells within xenografts growing in vivo in NOD/SCID mice as well as the probable need to create a conditionally replicating adenovirus to achieve control of tumor growth in our human colorectal carcinoma preclinical models.

15. SUBJECT TERMS
None provided.

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
					USAMRMC
a. REPORT	b. ABSTRACT	c. THIS PAGE			19b. TELEPHONE NUMBER *(include area code)*
U	U	U	UU	25	

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Annual Report – Second Year of Funding

Hypothesis: Embryonic core transcription factors (TFs), primarily the retrogene NanogP8, are the master regulators of cancer stem cells (CSC) in human colorectal carcinoma (CRC). The corollary is that inhibition of NanogP8 will inhibit neoplastic progression of CRC.

Introduction

During the first year of funding we were successful in two of the three Tasks and identified new information that establishes the importance of the Nanogs, including NANOGP8, in maintaining the stemness of colorectal carcinoma (CRC) as well as the identification of two different pathways by which NANOG and NANOGP8 control pluripotency in CRC. We will first describe how we have established the importance of NANOGP8 in the maintenance of the stem cell characteristics of CRC and then what we have done to meet the specific aims.

Our manuscript entitled "NANOG Modulates Stemness in Human Colorectal Cancer" by Zhang et al. has been published by *Oncogene* that describes the effects of NANOGP8 on stemness of CRC. The findings that were not described in the initial IDEA application are:

- NANOGP8 is translated into protein in human CRC (Figure 2, Appendix I)
- Expression of NANOGP8 in CRC with knock down of both NANOGs recovers stemness as measured by increasing single cell spherogenicity when it was inhibited when both NANOGs are knocked down. The recovery of stemness by NANOGP8 is stronger than that caused by NANOG (Figure 3, Appendix I).
- Our allele-specific shRNA to NANOGP8 (shNp8-1) delivered by lentiviral vector (LV) inhibits the growth of CRC transduced ex vivo when implanted into NOD/SCID mice to a greater extent than the shRNA to NANOG (shNg-1) (Figure 6, Appendix I).
- Inhibition of the NANOGs inhibits WEE1 and other kinases associated with the G2/M part of the cell cycle (Figure 5, Appendix I).
- We have confirmed that NANOG binds Pin-1 (1) an enzyme that stabilizes AKT as a mechanism to promote cell survival (Figure 5, Appendix I) {inhibition of NANOG protein expression would decrease AKT signaling and enhance apoptosis}.

We have also identified two new approaches by which inhibition of the NANOGs induces apoptosis in CRC in support of Task 2. We have performed RNA-Seq in the CX-1 CRC line with cells transduced with shNp8-1 to inhibit NANOGP8 and with overexpression of NANOGP8. Analysis of the results of genes whose expression is decreased by shNp8-1 but increased by overexpression of NANOGP8 in the Ingenuity Pathway Analysis identified NEDD9 as an important intermediate that was associated with inhibition of CRC growth (Figure 1, Appendix I). NEDD9 is a scaffolding protein for adhesion plaques and associates c-src kinase with focal adhesion kinase (FAK) leading to activation of FAK by tyrosine phosphorylation (2). We have previously found that FAK appears to be constitutively activated in CRC (unpublished observation) and when activated it provides survival signals through AKT (3). We have found that NEDD9 transcript levels are associated with NANOG transcripts either decreased when NANOGs are inhibited and NEDD9 levels are increased when NANOGs are increased by overexpression (Figure 2, Appendix I). NEDD9 overexpression is an anti-apoptotic effector (4) and increases single cell spherogenicity (Figure 3, Appendix I). We have designed a shRNA to NEDD9 that partially inhibits NEDD9 but it does not entirely decrease NEDD9 protein expression (Figure 4, Appendix I) and it does not significantly decrease single cell spherogenicity (Figure 5, Appendix I). We have just obtained a commercial shRNA that is likely to shut down NEDD9 protect expression and if it does we will assess whether inhibition of NEDD9 causes loss of stemness as measured by spherogenicity. We have also performed ChIP and determined that NANOG binds to the NEDD9 promoter as predicted by Boyer et al. (5). Our current hypothesis is that NEDD9 is an anti-apoptotic molecule when intact whose inhibition as a result of a decrease in NANOG expression is one of the pathways by which NANOG mediates stemness. We are currently preparing a manuscript on the role of NEDD9 as a mediator of the effect of NANOG effect on stemness in CRC.

In addition to the research on the regulation of NEDD9 by modulation of NANOG described, we have identified a second pathway through which NANOG effects apoptosis. We have previously demonstrated that when CRC cells that normally grow attached to substrates are placed into suspension culture they die over the course of 96 hr through a form of apoptosis termed anoikis (6). The mechanism involves the clustering of TNFRSF10B (TRAIL receptor 2 or DR5) into the death signaling clusters and the activation of caspase 8 and the extrinsic pathway of apoptosis (6). There was essentially no activation of caspase 9 which would indicate activation of the mitochondrial or intrinsic pathway of apoptosis. These data set the stage for Task 2b.

Body

Specific Tasks:

Task 1. Confirm that NanogP8 and not Nanog is the important Nanog family member expressed in human CRC (timeframe months 1 -12). Task 1 is important because NanogP8 is a retrogene in the Nanog embryonic gene family that if specifically targeted in CRC without the need to inhibit Nanog would increase therapeutic safety because Nanog is essential for embryonic development. If our data indicate that inhibition of Nanog is necessary, we have designed specific shRNA to Nanog that may decrease toxicity.

1a. Measure Nanog gene product expression in primary CRC by quantitative immunofluorescence assay (qIFA) (timeframe months 1 – 8)

Nanog and NanogP8 only differ in 2 amino acids so detection of protein with commercial antibodies identifies both gene products. qIFA will be done with SAIC investigator Dr. Kinders in Frederick, MD. The Colon Tissue Microarray (TMA) is available through NCI (http://cdp.nci.nih.gov/colon/progression_tma.html) and contains 367 deidentified primary colon cancers from all stages along with normal colon, adenomas and CRC cell lines as controls and is statistically designed to test the expression of a protein with overall survival and stage of disease by qIFA.

During this second funding period we analyzed NANOG and NEDD9 protein expression in 367 primary colon carcinomas along with cell line and other controls. This TMA is important because it not only contains information about staging and first course of treatment but also overall survival. We measured NANOG expression by quantitative Immunofluorescence assay (qIFA) and NEDD9 by chromogenic immunohistochemistry (IHC) (Figure 1, Appendix I). In year 1, we demonstrated that the antibody used to detect NANOG did detect it because recombinant peptide blocked the antibody binding (7), Supplementary figure 1). NEDD9 was analyzed because NANOG controls the expression of NEDD9 and NEDD9 may mediate many of the effects of NANOG and NANOGP8 on pluripotency as described above in the **Introduction and later in other Task descriptions**. We have found that quantitation of NEDD9 was difficult because the cases are spread over 8 slides that have different levels of oxidation and hydrolysis that appear to effect NEDD9 expression more than NANOG. However, as shown last year qIFA is more useful because the image analysis software standardizes the dynamic range within individual cores. With my collaborators are Dr. Stephen Hewitt, the director of the Tissue Array Research Program (TARP), Laboratory of Pathology, CCR, NCI and Drs. Scott Lawrence and Robert Kinders of the pharmacodynamics assay development laboratory of SAIC-Frederick and DCTD, Frederick National Cancer Research Laboratory we found that ~75% of primary colon carcinoma expresses NANOG protein and 80% express NEDD9. Interestingly, while the expression of NANOG is similar within a small set of matched normal mucosas (N=21), mucosas with inflammation from diverticulitis (N=30), or adenomatous polyps (N=30), the expression of NEDD9 was more often absent in polyps than in primary cancers (Data not shown). The primary colon carcinomas were divided into a training set of 182 primaries and a test set of 184 primaries and then the analysis of NANOG and NEDD9 was optimized in the training set by our statistician Dr. Daekwan Seo as explained in the Legends to Tables 1 and 2 (Appendix I). We first demonstrated that NANOG and NEDD9 expression was significantly associated with each other in the training and test sets (Figure 2, Appendix I). The clinical outcome data show that while the expression of NANOG and NEDD9 are of borderline significance in univariate analysis in the Test set, combining the expression of NANOG and NEDD9 into a single variable increased the significance of this combined variable in univariate analysis (Table 1, Appendix I). While the combined expression of NANOG and NEDD9 was significantly associated with overall survival in the Test set, the individual expression of either NANOG or NEDD9 was not (Table 2, Appendix I).

1b. Identify the relative transcript expression of Nanog, NanogP8, other family members in CRC by quantitative Reverse Transcriptase-Polymerase Chain reaction (qRT-PCR) and RE Assay (Timeframe months 1-12).

Identification of NanogP8 and the other members of the Nanog gene family requires specific qRT-PCR, our Restriction Endonuclease Assay (RE Assay) and direct sequencing. This will be on up to 20 deidentified primary or metastatic CRC undergoing surgery at NIH through collaboration with Dr. Avital, Surgery Branch, NCI. This subtask will establish the prevalence of NanogP8 expression in clinical tumors. Our data suggest that 70% of clinical mets express NanogP8 transcripts and NanogP8 protein is present in CRC cells.

This was completed in Year 1.

For Milestone 1: This milestone is met as we have shown that NANOG gene and protein protein is expressed in more than 40% of primary colon carcinomas. The expression is closer to 70% of primary carcinomas.

Task 2a. Determine whether inhibition of NanogP8 or other embryonic TFs by LV shRNA inhibits cell proliferation in CRC in vitro (Timeframe Months 6 -12)

This was completed in the first 12 months by the demonstration that inhibition of NANOGP8 by shNp8-1 is more active against the CRC lines than inhibition of NANOG alone by shNg-1 as reported last year.

Task 2b. Establish whether transduction of shRNA targeting NanogP8 induces apoptosis in CRC lines (Timeframe Months 6 - 24)

In the first 12 months we demonstrated that shRNA targeting NANOG or NANOGP8 decreased the mass of colorectal carcinoma cells growing in suspension in 3-D culture in serum free medium. This is important because it is a transition to growth in vivo away from the 2-D growth in monolayer that is important for the initial discovery of molecular mechanisms but those mechanisms often are blunted in 3-D growth either in vitro (eg, our suspension culture) or in vivo (eg, in preclinical models or in patients(reference PSOC)). In the first funding period we demonstrated that inhibition of NANOGP8 and to a lesser extent NANOG by LV shRNA inhibited growth in 3-D suspension culture in 3 cell lines and that this was associated with activation of the executioner Caspase 3 by activation of the indirect pathway of apoptosis through Caspase 9 activation instead of Caspase 8. Our group had previously shown that Caspase 8 was activated in CRC subjected to 3-D Suspension culture through activation of Caspase 8. In fact, LV shRNA appeared to decrease Caspase 8 activation as it increased Caspase 9 activity. This year we went back to explore mechanisms that might be used to stimulate caspase-dependent cell death that are enhanced by through inhibition of NANOG and perhaps NEDD9.

We studied the role of MCL-1 and the Bcl-2 family in inhibiting apoptosis in CRC cells because 1) caspase-dependent cell death is controlled by these two anti-apoptotic molecules, 2) NANOG, and perhaps NANOGP8, may regulate expression of Caspase 9 (5) and 3) MCL-1 expression is controlled by AKT which is indirectly regulated by NANOGs through NEDD9 (through its ability to control FAK activity that in turn activates AKT (8)) and PIN-1 that we confirmed forms complexes with NANOG while stabilizing AKT (9). Bcl-2 can be inhibited by several cell permeant peptidomimetics such as ABT-737 (10), but Mcl-1 inhibitors have not yet been optimized. Therefore, we found that the expression of Bcl-2 and its close family member Bcl-XL we quite high in our cell lines but that Clone A and CX-1 had relatively low levels of MCL-1 while LS 174T expressed a lot of MCL-1 protein (Figure 3, Appendix I). This is important because a Bcl-2 inhibitor would be expected to cause more apoptosis in Clone A and CX-1 than in LS 174T that would still have a significant amount of Mcl-1 expression to inhibit the induction of apoptosis. This is true because the IC50's for ABT-737 in these 3 lines is 2.1 μM, 7.5 μM and 25.6 μM for CX-1, Clone A, and LS 174T, respectively.

We then determined the effect of shRNA to NANOG or NANOGP8 on CRC MCL-1 protein expression. LV-delivered shRNA to either NANOG or NANOGP8 inhibits Nanog and MCL-1 protein expression whereas the control shRNA (shNEG) does not in all three CRC lines (Figure 4A, Appendix I). When CRC cells were cultured with 1 μM ABT-737 after first being treated with LV shRNAs , apoptosis was induced in Clone A and CX-1 cells by shRNA to either NANOG or NANOGP8 as measured by a nearly 6-fold or greater activation of Caspase 3 or 7 activity when ABT-737 was added to LV shRNA (Figure 4B, Appendix I). The Caspase 3/7 activity in LS 14T cells was lower in LS 174T cells but still the combination was at least two-fold greater than the untreated control. LV delivered shRNA alone in all three CRC cells did not increase caspase 3/7 activity (Figure 4B, Appendix I). In contrast, ABT-737 alone at 1 μM which was essentially IC 10 for Clone A and IC30 for CX-1 increased caspase 3/7 activity but still was significantly less than the combination of ABT-737 and an anti-NANOG or NANOGP8 shRNA. The ABT-737 used for these synergism studies was ~at an IC5 so that it may have been too low to detect synergism. Nonetheless, the combination of shRNA to either NANOG with ABT-737 stimulated significantly more Caspase 3/7 activity than either control or ABT-737 in LS 174T (Figure 4B, Appendix I). We then addressed the effect of the timing of ABT-737 and shRNA on the response to of CRC cells to the combination. The goal of this work was to determine whether inhibition of a NANOG should occur before, after, or during inhibition of BCL-2. Here CRC cells were exposed to the low dose of ABT-737 used above for at least 3 days while exposure to LV shRNA was for at least 5 days. Inhibition of growth is indicated by a reduction in the % of Control that was measured by WST-1 metabolism: fewer cells are associated with a lower amount of WST-1 (Figure 5, Appendix I). The data indicate that inhibition of BCL-2 by even a low concentration of ABT-737 ($IC_{10} - IC_{25}$) is synergistic with LV shRNA anti-NANOG or NANOGP8 treatment, especially if the ABT-737 is given before or continuously with the LV shRNA (Figure 5, Appendix I). Through controls that involve siRNA to MCL-1, we have been able to determine that the loss of CRC by apoptosis is due to inhibition of MCL-1 by inhibition of NANOG or NANOGP8 (data not shown). Thus, inhibition of either NANOG or NANOGP8 inhibits the expression of MCL-1 and that this is synergistic with agents that target Bcl-2 and Bcl-XL. Furthermore, the ABT-737 should be present continuously or after the LV shRNA has been administered.

Task 2 Milestones: A. Determine the MOI to cause 50% inhibition of NanogP8 in CX-1 and LS 174T cells that stably express LUC2. This was shown last year to be 10:1 and that has been used for the experiments described in the report. **B. Determine whether inhibition of NanogP8 gene expression by at least 50% compared to untreated parental cell line causes either inhibition of cell proliferation or induction of apoptosis in 3 CRC lines in monolayer cultures**. We have now completed these milestones by demonstrating that LV-delivered shNp8-1 inhibits tumor growth in 3-D suspension culture as well as in monolayer by 50% at a MOI of 10 in 3 CRC lines, including the 2 to be used in Task 3. In addition, we have demonstrated a mechanism of action for viral delivered by shRNA that involves inhibition of the expression of MCL-1 that complements and is synergistic with inhibition of the Bcl-2 family members. Finally, it should be clear from the data that inhibition of NANOG in these tumors has effects that very similar to but generally a little less than the effects of inhibiting NANOGP8. When we have examined CRC samples and cell lines, we generally find that both genes are present in tumors with NANOGP8 more common than NANOG. This is helpful because it suggests that either the shRNAs are not as specific as intended or if they are then inhibiting either will have the intended effect on inhibiting tumor growth.

Tasks 3. Determine the efficacy of intralesional injection of lentivirus shRNA to NanogP8 or other embryonic genes on CRC tumors in the livers of NOD/SCID mice (Timeframe Months 6 – 36).

There were three phases to this aim in the IDEA application. First phase was to assess the ability of intralesional therapy to transduce established nodules of CRC in vivo in the sub cutis to inhibit tumor growth. The second phase was to transduce small nodules of tumor growing in the liver while the third phase was to determine whether systemically delivered lentiviral shRNA could inhibit tumor growth in the liver and abdomen. We have reached an obstacle in the first phase. When we injected intralesionally ~3 - 7 mm diameter subcutaneous nodules with 0.10 ml of PBS containing ~5 x 10^6 lentiviral particles of shNg-1, shNp8-1 or the control shNeg we achieved a modest reduction in size of nodules. However, the negative control provided essentially as great a reduction as the anti-NANOGs shRNA (Figure 6, Appendix I). This suggested that the innate immune response or RNA interference caused by the lentivirus itself led to a nonspecific inhibition of tumor growth. This was confirmed when we tried on several occasions to then determine whether transduction of tumor cells within the nodules occurred. We developed a rapid isolation technique to create a single cell suspension and then evaluated GFP fluorescence under an inverted microscope. Since our lentiviral shRNA constructs all express Green Fluorescent Protein (GFP) as a reporter, the tumor cells should express GFP if they are transduced. As presented last year, we at that time could not identify transduction of tumor cells after intralesional therapy. We have done numerous experiments in small numbers of mice and have now come to the conclusion that one can obtain transduction with a MOI of 5 in small (3 – 5 mm diam tumors), but that the transduction is very spotty (Figure 7, Appendix I). However, as can be seen in the attached Figure 7, the transduction rate is neither high nor efficient enough to be useful as a preclinical therapy – especially when compared to cells within

suspension cultures transduced in vitro that clearly express functional GFP in 40 -75% of cells (Presented in Figure 8, Appendix I last year). However, the MOI for the in vivo experiments has been re-calculated as between 0.4 – 1. As a result, it is clear why the transduction in vivo is so low. As described below, we are lucky to have Dr. Korokhov join us who at one time oversaw the production of lentivirus for VIRxSYS and and has experience in increasing the production of lentivirus so that we can get to a MOI of 5-10 in vivo for small xenografts (this translates to $10^{10} – 10^{11}$ particles per mouse. This will enable us to complete Task 3 and its subparts in the next year as proof of principle.

Examination of the literature as well as data from the recent American Society of Gene Therapy meeting in Salt Lake City indicates that intralesional therapy with lentiviral shRNA is not an especially active area of investigation in cancer therapeutics, possibly because of this lack of transduction in vivo. Lentiviral therapy is excellent for ex vivo transduction with amplification in tissue culture and then transfer back to patients. This has been beautifully demonstrated by Carl June and collaborators at the University of Pennsylvania (11). Thus, while the biological rationale for targeting NANOGP8 by shRNA is amply supported, a better delivery vector is needed to move into the clinic ultimately. We propose to continue progress with Task 3a with higher numbers of lentiviral vector particles intralesionally into subcutaneous xenografts. However, we feel it is important during the last year of this application to develop a better vector as described below. We have brought in Dr. Korokhov an excellent virologist who has experience with lentiviral production as he was a production manager at VIRxSYS, the company that we were to contract with originally but went out of business as the project started. He also can help us develop quickly an alternative path that may be better in the long run.

Justification For Also Developing A Conditional Replicating Adenoviral (CRAd) Vector

We have learned during this grant that a MOI of at least 5 but more likely 10 viral particles to each tumor cell is necessary. Since a 1 cm tumor contains 10^9 cells (12) and most tumors are on average 2 cm at diagnosis, then one needs ~10^{10} viral particles to deliver enough shRNA to infect and transduce a 1-2 cm tumor in a patient. Patients with multiple metastases would need 10^{11-12} viral particles for treatment. Since large scale preparation of lentivirus generally yields ~10^{10} infectious particles total per run, it is not likely that a non-replicating vector will be able to inhibit the growth of cancers in patients with more than minimal disease that may be controlled by standard current therapies easily. I did not appreciate these facts when submitting the application and have learned them during the course of our work.

The solution to this problem is to create a vector that can selectively replicate within a cancer but not in normal tissue. There is a replicating lentivector that was originally described in the early 2000's (13), but this vector depends on the administration of doxycycline and has a potential for recombination to recreate wild type HIV. However, this potential to revert to a replicating HIV along with the fact that it cannot be easily pseudotyped to not infect immune cells make this conditional replicating virus of limited usefulness. We have been in contact with the developer of this vector, Prof. Berkhout in the Netherlands, but further modification of this vector is not an option because the lentiviral genome is highly complex with multiple genes interlaced within a stretch of viral genome RNA and with controls that still are not well understood. This means that one cannot change even the pseudotype without disrupting replication or control of the viral genome. As a result, we have decided to adopt technology that is currently showing clinical promise for gene therapy: the Conditionally Replicating Adenovirus vector that is not only oncolytic when it replicates within tumor cells but also can deliver a shRNA into neighboring cells that do not lyse. Conditional Replicating Adenoviruses (CRAds) can propagate within a neoplasm since each infected cell produces 1K – 10K's of selectively replicating virus. Also recent data suggest that these viruses may selectively target distant sites of tumor after systemic injection in preclinical models and therefore provide better targeting to tumor at distant sites. To accomplish this selective targeting a best approach appears to be to use an adenovirus of serotype 5 in which the knob of the fiber that distinguishes the type 5 virus is replaced with a type 3 knob to create a hybrid that is termed an Ad 5/3 viral vector. We are fortunate to have Dr. Korokhov (please see his Biosketch) join us who has worked with Dr. David Curiel who is a leader in this field and has created the basic tools for assembling CRAds that may be useful for our purpose. Our intent is to keep working with lentivirus but also to develop a CRAd that has a modified serotype with an Ad5/3 capsid fiber that decreases hepatic distribution but also allows for efficient targeting of systemic tumors (14). This is critical to enhancing safety as well as improving targeting of tumor after systemic injection.

A further improvement to safety for a conditionally replicating virus is to make the virus respond only to the transcription factors that drive the cancer. Since we focus on the activation and transcription of NANOGP8, it is logical to use the promoter for NANOGP8 to drive selective replication of the viral vector. There are two main strategies employed to restrict replication of adenoviruses to cancer cells; either expression of viral genes critical for viral replication are placed under the control of tumor-specific promoters or, viral genes required for replication in normal cells are modified to make them dependent on tumor gene expression. The first approach most often relies on a cancer-specific promoter to control transcription of the E1 region (for review see (15)). Since NANOGP8 appears to be expressed only in malignant cells and not in normal cells (except for 1 report in smooth muscle cells (16), its promoter is likely to be able to control viral genes necessary for replication and will constrain viral replication so that it is restricted to malignant cells in our preclinical studies. In addition, we have shave shown that shRNA to *NANOG* or *NANOGP8* inhibits stem cell function as well as tumor growth both in vitro and in vivo. All of these approaches are based on the original work of Graham et al. (17). The critical steps to making a CRAd that replicates within the colorectal carcinoma xenograft are: 1) to identify a promoter that is selectively active within the cancer but not in normal tissue and strong enough to drive adenoviral replication, 2) to insert this promoter into the adenoviral vector along with the shRNAs that we have developed, 3) to demonstrate that the CRAds amplify within 3-D suspension CRC cell cultures and 4) to use the CRAds to treat xenografts in vivo. We have the NANOGP8 promoter reporter construct of Jeter et al (18) that is active in both monolayer and spheroid cultures (Figure 8, Appendix I). Since this promoter construct is 3.8 Kb. we are currently testing whether shorter constructs that are closer to the 3' end may be active. Testing promoter activity is by simple transfection. Once an active construct is identified, then it will be inserted into the pShuttle vector AdEasy-1 as

diagramed in Figure 9 and, along with a rescue vector containing the H1 driven shRNAs CRAd genomes will be assembled by homologous recombination within the E. coli designed for this.

Dr. Korokhov has the expertise (please see Biosketch) to create the CRAd5/3 by January 2014 using the promoter for NANOGP8 that we have in hand along with the shRNAs that we have shown are active in vitro. We have the assays developed to assess the ability of a CRAd5/3 to grow in human CRC in spheroids as well as xenografts so that it should not be difficult to complete the milestones for task 3. Thus, we are confident that we can develop a vector by the end of this grant that is ready for further preclinical testing.

Viral vector construction and amplification

All constructed viruses will be based on the Ad5 genome that still contains the adenovirus death protein (ADP) but with the rest of E3 deleted and the Ad 5 fiber gene replaced with a gene coding a Ad5/Ad3 fiber-knob chimera. This will enable binding to the prevalent DSG2 receptor rather than the Coxsackie Adenovirus Receptor (CAR or CXADR) whose expression is low in colorectal carcinoma. Prior RNA-seq analysis indicates that both Clone A and CX-1 express DSG2 so that they have the receptor for CRAd5. The following set of viruses will be generated (Figure 9C, Appendix I):

1. Ad5/3 – a control virus - nonreplicating
2. NP8-CRAd – Ad5/3 virus with E1 region expression driven by *NANOGP8* promoter selected first
3. Ad5/3_shNp8-1 – Ad5/3 virus containing shRNA shNp8-1 in place of the E3 region that targets NANOGP8
4. Ad5/3_shNG-1 – Ad5/3 virus containing shRNA shNG-1 in place of the E3 region that targets NANOG
5. Ad5/3_shNEG – Ad5/3 virus containing shRNA shNEG in place of the E3 region as a control shRNA that was designed to not bind any human sequence
6. NP8-CRAd_shNp8-1 – Ad5/3 virus containing *NANOGP8* promoter controlled E1 expression and shRNA shNp8-1 in place of the E3 region that targets NANOGP8
7. NP8-CRAd_shNG-1 – Ad5/3 virus containing *NANOGP8* promoter controlled E1 expression and shRNA shNG-1 in place of the E3 region that targets NANOG
8. NP8-CRAd_shNEG – Ad5/3 virus containing *NANOGP8* promoter controlled E1 expression and shRNA shNEG in place of the E3 region as a control shRNA that was designed to not bind any human sequence

The CRAd genomes will be constructed via homologous recombination in *E. coli (BJ5183 bacteria)* and viruses will be rescued in 293 cells as described in the manual of the AdEasy system (17). First, the DNA of interest will be cloned either into one of the pShuttle vectors (Figure 9) or a modified pAdEasy-1 essentially as described in (19-23). Shuttle vectors to generate viral genomes will be assembled using Clonetech's In-Fusion or similar techniques allowing directional, seamless cloning of any PCR fragment. Starting material for E1 and E3 shuttle vectors, and rescue vector were described previously (19). Once constructed, the shuttle vectors and the rescue vectors will be linearized and transformed into BJ5183 according to the instructions of the manufacturer Agilent (24). Transformants will be selected for kanamycin resistance, and recombinants will be subsequently identified by restriction digestion. Once a recombinant is identified, it will be produced in bulk using the recombination-deficient XL10-Gold strain to produce the purified Adenoviral plasmid DNA. Controls (transformation control and a Pme I-linearized pShuttle-CMV-lacZ recombination control) will be included as suggested by the manufacturer.

Purified recombinant Adenoviral plasmid DNA will be digested with restriction endonuclease to expose its inverted terminal repeats (ITR), and then used to transfect 293T17 cells that will be obtained from ATCC with transfection performed according to the manufacturer's (Agilent) instructions. Several viral plaques for each vector will be picked and viruses will be propagated by sequential passage in fresh 293T17 cells. The resulting viruses will be purified by either double CsCl density gradient ultracentrifugation or ion exchange chromatography. The titer of viral particles in each preparation will be determined by measuring the DNA and protein concentrations of the samples. Infectious titers will be determined by a spot assay on 293 cells. Overall yields and infectious unit/particle ratios will be used as measures of the viability of the viruses. The presence and correct configuration of new added components in the genomes of the rescued viruses will be confirmed by PCR and partial sequencing of the genomic DNA isolated from purified virions.

NANOGP8-promoter activity and specificity in the context of CRAds in vitro

Purified viruses will be tested in our cell lines, Clone A, CX-1 and LS 174T, with different level of NANOGP8 expression. Infected cells will be collected 24 hr later and E1 RNA level will be analyzed by qRT-PCR with one of the shuttle vectors employed to make references for quantification. The results will be normalized to GAPDH RNA level in analyzed samples.

In vitro analysis of cytocidal effect
The in vitro cytocidal effect of the CRAds will be analyzed by determining the viability of the cells with crystal violet staining after infection. Human CRC cells (Clone A and CX-1) growing in monolayer will be infected at MOI of 0.01vp/cell with CRAds or control vectors. Two hours later, the infection medium will be aspirated and replaced with an appropriate complete culture medium. Seven days later, viable cells are stained with crystal violet.

Analysis of viral replication
To analyze the viral replication and CRAd selectivity, Clone A and CX-1 cells will be infected with 10vp/cell of each virus and incubated for 3 days at which point cells and supernatant will be separately collected. Encapsulated viral DNA in the supernatant will be isolated following DNase treatment. The viral DNA isolated from cells or supernatant will be analyzed by qPCR with virus specific primers. Control cells will include PA-1, a human ovarian teratocarcinoma cell line that expresses only *NANOG* but not *NANOGP8* transcripts. CRAd replication should occur in the CRC line but not PA-1 cells.

Functionality of the shRNA expression cassette in viral genomes
After the creation of CRAd5 driven by the NANOGP8 promoter, the effect of *NANOGP8* shRNA delivered by Ad vectors will be evaluated in our human CRC cell lines that express the NANOGP8 gene. Cells will be expanded and, either infected with one of the viruses or transfected with plasmid carrying H1-NP8shRNA expression cassette. Total cellular RNA will be extracted 24hr later and NANOGP8 RNA level will be assessed by qRT-PCR.

Exigencies:
Potential problem:

1. As result of homologous recombination between integrated Ad sequences in 293 cells and Ad genomes we could expect generation of replication competent viruses (RCA) and contamination of adenovirus samples.
2. NANOGP8 promoter might be not strong enough to support potent CRAd replication and, as a result, inefficient cell lysis.

Solution:

1. Immediately following virus rescue its amplification will be performed in cells supporting NANOGP8 promoter activity.
2. To improve its potency the NANOGP8 promoter sequence may be tested in combination with minimal CMV or EF1-a promoters which by themselves are very weak if necessary.

Key Research Accomplishments

- Confirmation that NANOG is expressed in the majority of primary human colon carcinomas and that its expression is a significant prognostic factor, especially when associated with expression of the cell adhesion scaffold protein NEDD9.
- Elucidation of a molecular mechanism by which inhibition of NANOGP8 and/or NANOG induces apoptosis through inhibition of MCL-1 by Dr. Mattoo
- Proof of the principle that vector-delivered shRNA to NANOGP8 and/or NANOG complements inhibition of BCL-2 and BCL-XL as a means to inhibit and kill human colorectal cells.
- Confirmation that intralesional injection of LV-delivered shRNA to NANOGP8 or NANOG transduces tumor cells within xenografts growing in vivo in NOD/SCID mice.

Reportable Outcomes

Manuscripts

1. Zhang J, Espinoza LA, Kinders RJ, Lawrence SM, Pfister TD, Zhou M, Veenstra T, Tang DG, Jeter C, Thorgeirsson SS, Jessup JM. Nanog modulates stemness in human colorectal cancer. *Oncogene*. 2012. The final draft was submitted with last year's Annual Report.

Abstracts with presentations in 2013

1. John M Jessup JM, Jingyu Zhang J, Espinoza LA, Mattoo A, Thorgeirsson SS. Lentiviral shRNA Targeting NANOG/NANOGP8 Enhances Topotecan Chemotherapy In Human Colorectal Carcinomas. Presented at the Annual Meeting of the American Society of Gene and Cell Therapy. Salt Lake City, UT. May 16, 2013.

2. Zhang J, Lawrence S, Kinders RJ, Pfister T, Thorgeirsson SS, Jessup, JM. Is Every Cell a Potential Stem Cell in Human Colorectal Carcinoma: *NANOGP8* rescues Stemness and NANOG is a Prognostic Factor? Presented at the Keystone Symposium "Stem Cell Regulation in Homeostasis and Disease" at the Fairmont Banff Springs Hotel, Banff, Alberta, February 27, 2013.

3. Jessup, JM, Zhang J, Lawrence S, Kinders RJ, Pfister T, Hewitt SM, Seo D, Mattoo A, Thorgeirsson SS. *NANOGP8* Rescues Stemness While NANOG protein is a Prognostic Factor in Human Colorectal Carcinoma. Presented at the Annual Center for Cancer Research Meeting at the Ronald Reagan Building, Washington, DC, January, 2013.

These abstracts as well as the manuscript have been submitted to Geneva Research Foundation.

Conclusion

- Inhibition of NANOGP8 or NANOG is an important adjunct to standard treatment because decreases cell proliferation and also induces apoptosis
- demonstrated that inhibition of NANOGP8 or NANOG complements standard approaches to inducing tumor cell death by targeting MCL-1 that facilitates approaches now being performed in the clinic to block the anti-apoptotic effects of BCL-2 family members.
- Accomplished transduction of xenografts in vivo but also clearly identified the limitations of a non-replicating vector delivery system.
- It is important to use a vector that conditionally replicates within a cancer and not in normal cells. Current results suggest that the CRAd 5/3 viral vector is such a vector that may replicate within human tumor cells and lyse the majority of those cells or for cells not lysed cause cell death through episomal expression of a shRNA to NANOGP8 or NANOG.

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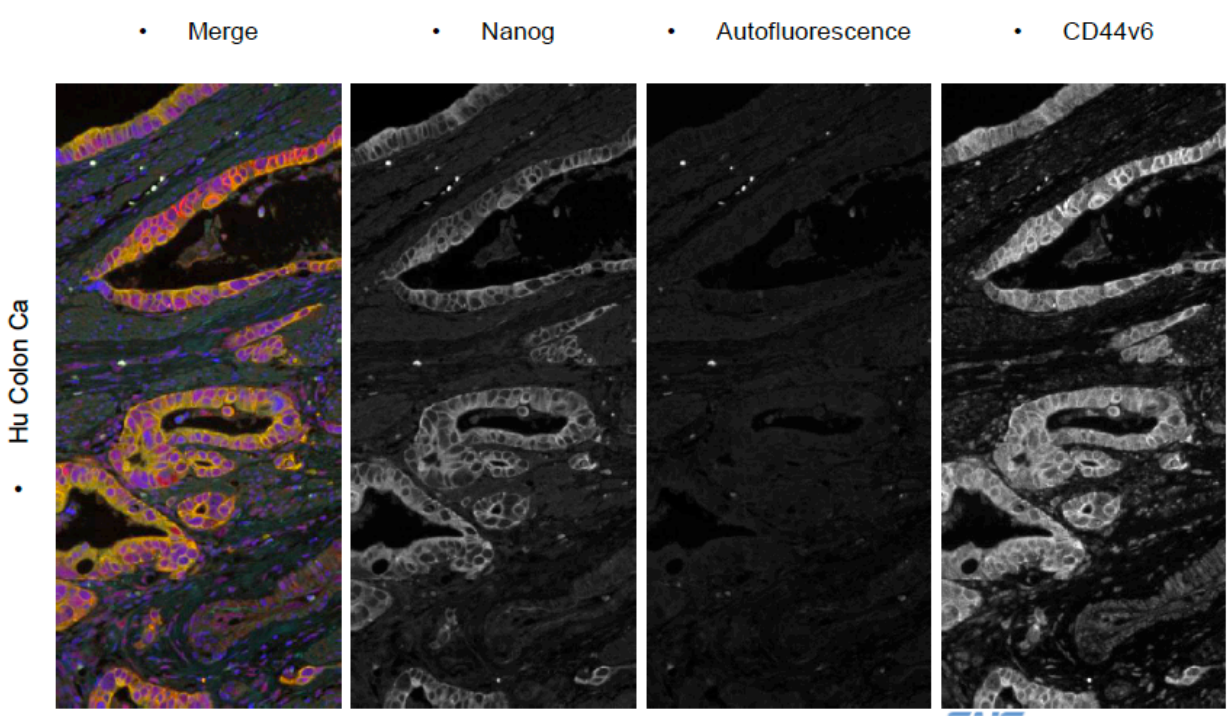
Appendix I
Data for Year II



. analysis of ession in colon carcinoma using CD44v6 Expression to identify carcinoma cells. Quantitative

Immunofluorescence Assay (qIFA) was performed on formalin-fixed paraffin embedded primary colon carcinoma samples using a R&D antibody as described in Zhang et al (2012). The images were exported to image analysis software (Definens Tissue Studio) where individual samples were analyzed for NANOG (green), CD44v6 (Red) and nuclei (DAPI, Blue). Autofluorescence was due to stromal elements and erythrocytes and was identified and subtracted from NANOG-expression by the software. Then, as shown in the left composite image, the fraction of CD44v6 positive areas that were also NANOG positive was calculated along with several intensity measurements of NANOG positivity. Subsequent analysis indicated that the important feature was the fraction of carcinoma that expressed NANOG. This variable is continuous. Magnification was 200x.

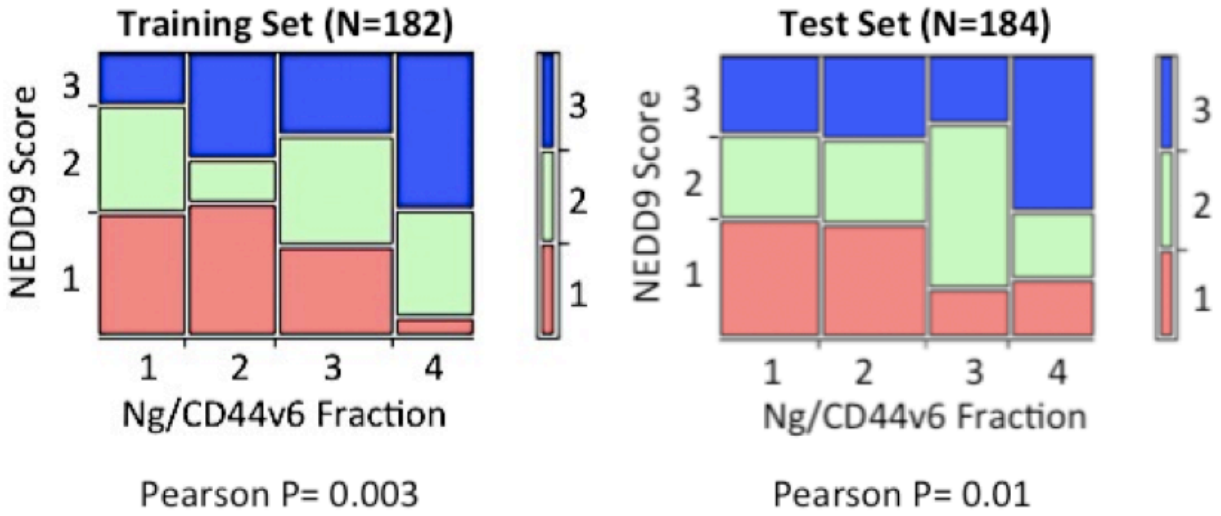


Figure 2. NEDD9 Expression is Associated with NANOG Expression in Human Colon Cancers. A colon cancer TMA was stained for NANOG and NEDD9 expression as described in the text. The 366 evaluable primary cancers were divided randomly into a training set of 182 patients and a test set of 184 patients while NEDD9 Scores were divided into tertiles while the fraction of CD44v6 positive cells (tumor cells) that expressed NANOG (Ng/CD44v6 Fraction) was divided into quartiles. The two groups were then tested for association by contingency table analysis and found to significantly associated in the two independent cohorts at the indicated Pearson P values.

Table 1. Univariate Analysis of the Association of Clinical, Pathologic and Biologic Variables in the Training Set of 182 primary colon carcinoma patients with Overall Survival.

		Kaplan-Meier Analysis			Univariate Proportional Hazards		
Variable	Category	N	% Failed	Wilcoxon P	Risk Ratio	95% CI	P
Tumor Stage	Low (I-II)	80	23%	<0.0001	0.49	0.36-0.63	0.0000
	High (III-IV)	102	61%				
Age	Young (< 70 yo)	92	43%	0.11	0.91	0.72-1.13	0.39
	Old	90	42%				
Race	White	171	42%	0.28	0.89	0.49-2.42	0.29
	AA	4	25%		0.59	0.09-1.68	
	Other	7	70%				
Gender	Male	87	44%	0.73	1.02	0.81-1.27	0.89
	Female	95	42%				
Grade	Low (I – II)	96	40%	0.01	0.81	0.64-1.01	0.06
	High (III-IV)	86	49%				
Vascular Invasion	Absent	168	39%	0.0001	0.47	0.35-0.67	0.0001
	Present	13	85%				
Nanog Fraction 10%	Low	13	15%	0.07	0.51	0.21-0.91	0.02
	High	140	44%				
NEDD9 Score 10%	Low	24	29%	0.04	0.71	0.45-1.01	0.06
	High	121	48%				
Nanog NEDD9 Score Combined	Low (0 or only 1+)	29	24%	0.01	0.62	0.40-0.89	0.008
	High (both positive)	105	50%				

Legend: The data for the colon TMA was divided into a training set of 182 primaries and a test set of 184 primaries based on odd-even samples numbers. Analyses of NANOG and NEDD9 expression were performed independent of any knowledge of diagnosis or any clinical or pathologic data. When the expression data were given to NCI, maps and clinical information were provided that identified the stage of disease (Tumor Stage), Age, Race, Gender Histologic Grade, Vascular Invasion (absent or Present for either lymphatic or venous invasion). All of these clinicopathologic variables are important for estimating prognosis in patients. Also it should be noted that clinical and pathologic information was available on all of these patients but that in the analysis of protein expression not all specimens could be used because some cores were not evaluable either to damage or loss of cores during processing.

qIFA of NANOG expression yielded continuously distributed values for the fraction of NANOG-expressing cells in regions that expressed CD44v6 that identified carcinoma cells. Intensity of the region was also assessed but the best discriminator appeared to be at a cut-off with the lowest 10% of the fraction of NANOG expressing cells (NANOG Fraction 10%). NEDD9 analysis by IHC used a five step categorical scoring system for both % of cells positive and intensity and these were multiplied to yield a NEDD9 Score that was again binarized at the lowest 10% of the score distribution (NEDD9 Score 10%). The NANOG NEDD9 Score Combined was then derived by adding the NANOG Fraction 10% and NEDD9 Score 10% and considering those that were both positive for NANOG and NEDD9 expression as High while negatives or those with only high NANOG or NEDD9 expression were low. The data were then analyzed with univariate Kaplan-Meier or Proportional Hazards methods and suggested while both NANOG and NEDD9 expression were of borderline significance in the univariate analyses, their significance incresed when their expression was considered as a combined variable.

Table 2. Univariate Analysis of Association of Clinical, Pathologic and Biologic Variables in the Test Set of 184 primary colon carcinomas with Overall Survival

		Kaplan-Meier Analysis			Univariate Proportional Hazards		
Variable	Category	N	% Failed	Wilcoxon P	Risk Ratio	95% CI	P
Tumor Stage	Low (I-II)	91	18%	<0.0001	0.44	0.33-0.57	0.0000
	High (III-IV)	93	61%		-		
Age	Young (< 70 yo)	80	43%	0.64	0.99	0.79-1.25	0.95
	Old	104	38%		-		
Race	White	177	40%	0.15	0.73	0.33-2.44	0.24
	AA	5	20%		0.32	0.04-1.24	
	Other	2	50%		-		
Gender	Male	88	40%	0.75	0.97	0.77-1.23	0.82
	Female	96	40%				
Grade	Low (I – II)	95	38%	0.41	0.94	0.74-1.18	0.58
	High (III-IV)	89	42%				
Vascular Invasion	Absent	156	35%	0.0007	0.61	0.46-0.84	0.004
	Present	24	58%				
Nanog Fraction 10%	Low	13	23%	0.42	0.73	0.36-1.21	0.25
	High	135	43%				
NEDD9 Score 10%	Low	22	27%	0.13	0.70	0.44-1.03	0.08
	High	119	45%				
NANOG NEDD9 Score Combined	Low (0 or only 1+)	27	22%	0.05	0.63	0.39-0.93	0.019
	High (both positive)	105	46%				

Legend: The same as for Table 1 except that the data demonstrate that the combined expression of NANOG or NEDD9 significant in univariate analysis but not when they are analyzed independently. Both Tumor Stage and Vascular Invasion are also highly significantly associated with overall survival in the test set.

Table 3. Multivariate Cox Proportional Hazards in the Training Set

Model	-LogLikelihood	ChiSquare	DF	P
Difference	19.5543	39.1086	8	<.0001
Full	237.1072			
Reduced	256.6615			

Variable	Risk Ratio	95% CI	P<
NANOG NEDD9 Score Combined (Low vs High)	0.60	0.38-0.87	0.006
Age (Young (<70 yo) vs Old)	0.93	0.71-1.22	0.61
Race [Caucasian vs Other]	0.80	0.40-2.27	0.58
Race [African American vs Other]	0.78	0.11-2.47	
Gender [Male vs Female]	0.93	0.70-1.23	0.59
TNM Stage (Low vs High)	0.50	0.35-0.69	0.0000
Grade (Low vs High)	0.86	0.65-1.13	0.27
Vasc Invasion (Absent vs Present)	0.56	0.38-0.89	0.018

Legend: The training set contains 182 patients but the multivariate analysis requires that all variables be available for the analysis of each patient. All variables were available in 133 of the 182 patients with 58 patients dying of disease and 75 patients surviving for at least 12 years without disease. The Variables are as described in Tables 1 and 2.
The data demonstrate that the combined NANOG and NEDD9 expression is highly significant covariate associated with overall survival in the Test Set independent of Tumor Stage and Vascular Invasion.

Table 4. Multivariate Cox Proportional Hazards in the Test Set

Model	-LogLikelihood	ChiSquare	DF	Prob>Chisq
Difference	22.6458	45.2916	8	<.0001
Full	210.4830			
Reduced	233.1288			

Variable	Risk Ratio	95% CI	P<
NANOG NEDD9 Score Combined (Low vs High)	0.58	0.36-0.86	0.006
Age (Young (<70 yo) vs Old)	1.38	1.03-1.84	0.03
Race [Caucasian vs Other]	0.73	0.31-2.51	0.63
Race [African American vs Other]	0.59	0.08-2.41	
Gender [Male vs Female]	0.87	0.65-1.16	0.34
TNM Stage (Low vs High)	0.46	0.32-0.64	0.0000
Grade (Low vs Hi	1.03	0.78-1.38	0.82
Vasc Invasion (Absent vs Present)	0.66	0.48-0.95	0.03

Legend: Same variables as for Tables 1-3. All variables were available in 130 of the 184 patients with 52 patients dying of disease and 78 patients surviving for at least 12 years without disease. In this analysis age is associated with overall survival. Data confirm that the combined expression of NANOG and NEDD9 is a significant independent prognostic factor.

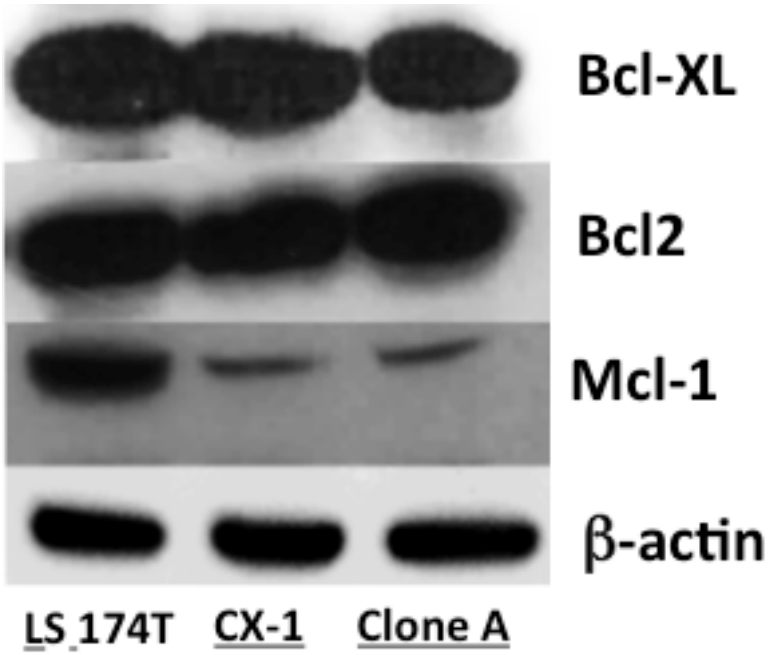


Figure 3. Expression of the Anti-Apoptotic Proteins MCL-1 in Three CRC lines. The CRC cells were grown in monolayer culture under exponential growth conditions, lysates prepared and western blots probed for the indicated proteins. Clone A and CX-1 express high levels the BCL-2 and BclXL proteins but little MCL-1 while LS-174T highly expresses all three proteins that block apoptosis.

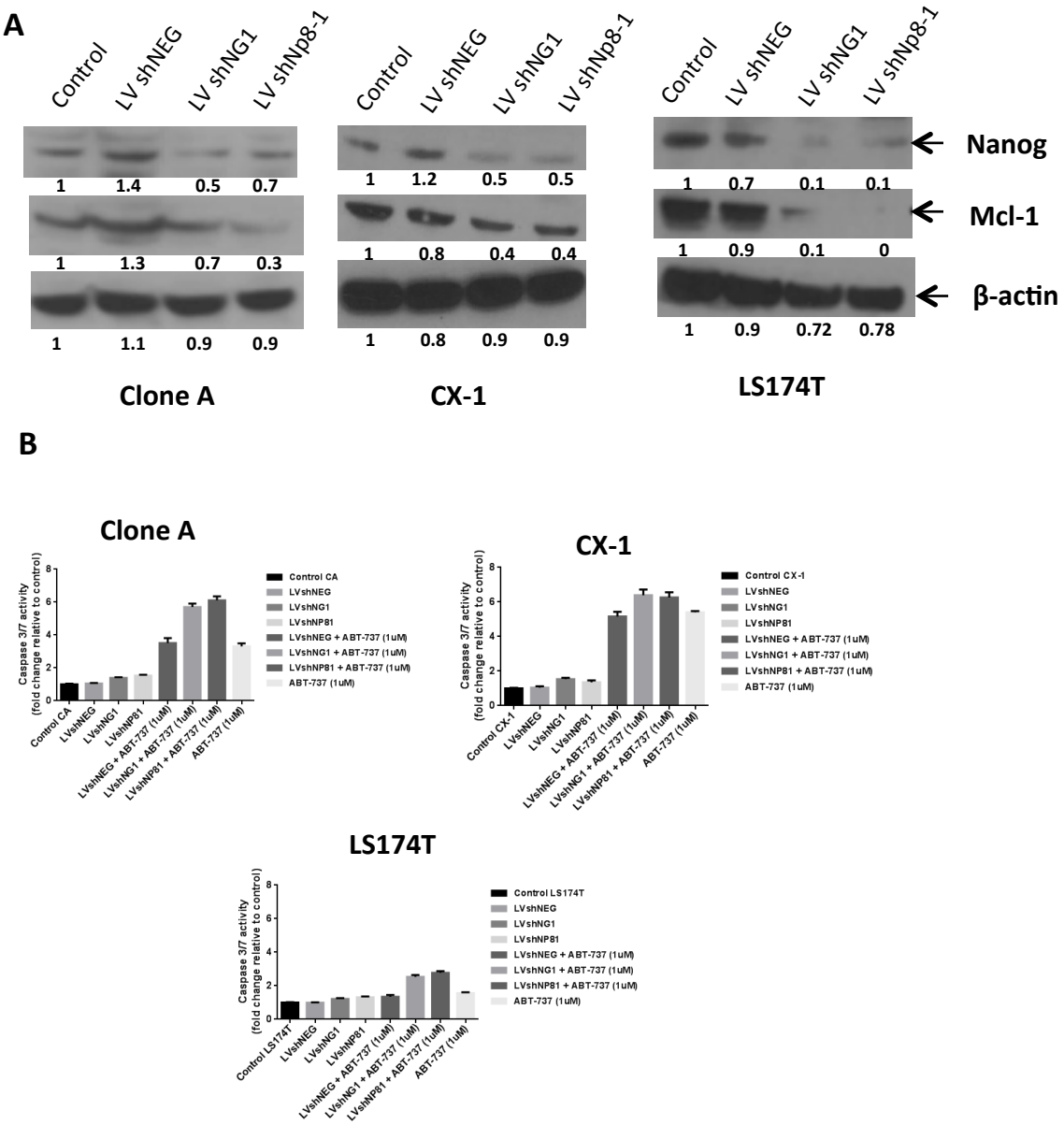


Figure 4. Lentiviral Delivered (LV) shRNA to NANOG or NANOGP8 Inhibits Mcl-1 and increases Caspase 3/7 Activity. In Panel A, the three cell lines were treated with the indicated LV shRNA for 6 days in monolayer culture. Cells were collected and lysates made. Western blots were probed for NANOG and Mcl-1 protein expression with the values below each blot determined by optical densitometry and compared to the control. In Panel B, each of the three cell lines was treated with LV shRNA and/or ABT-737 for 6 days together in 96 well microtiter plates in triplicate in complete medium. Casapse 3/7 activity was determined in the wells using the Promega Caspase-Glo kit according to the manufacturer's protocol. The results are presented as Mean ± SD of the activity normalized to the untreated control cells within each experiment. Caspase 3/7 activity was greatest in the combination therapy groups that received ABT-737 and LV shRNA to NANOG or NANOGP8.

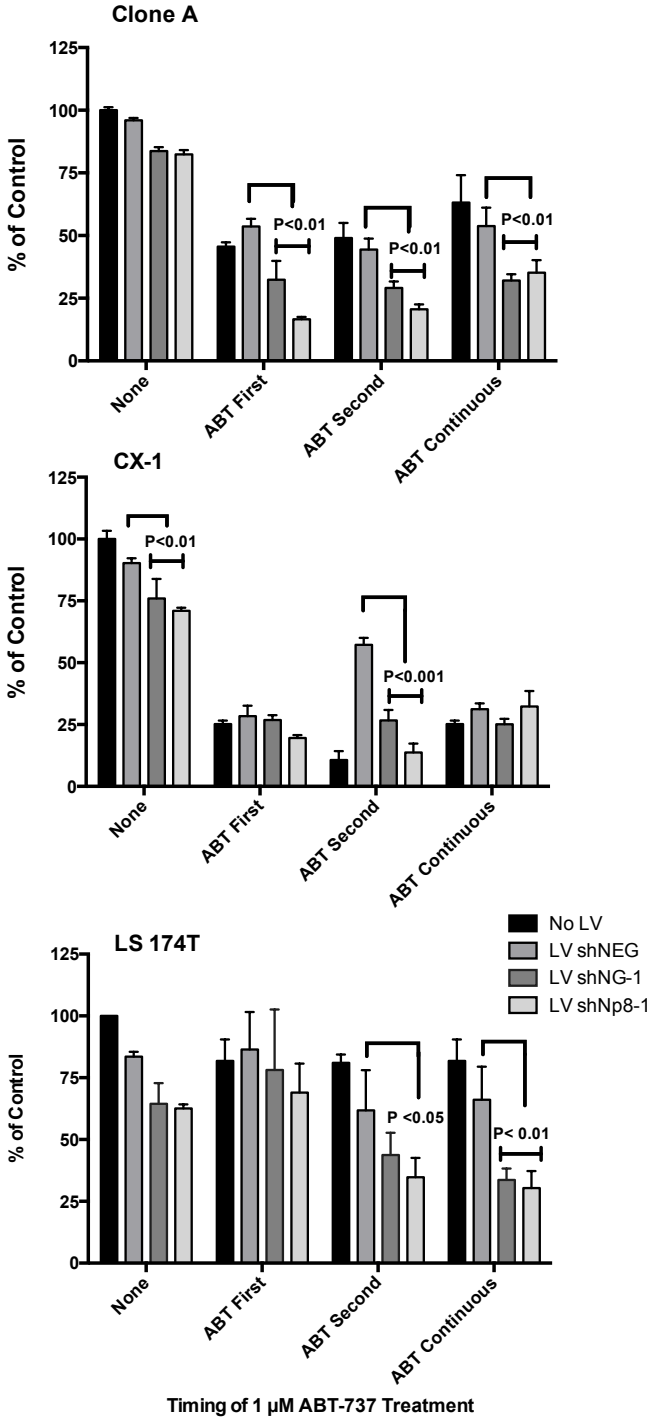


Figure 5. The Timing of ABT-737 and LV shRNA Combination Therapy Contributes to Increased Inhibition of CRC Growth. 5,000 CRC cells were cultured in monolayer culture in individual wells of a 96 well microtiter plate in complete medium and LV or ABT-737 or both added as indicated for a total of 8 days. Cells were treated with either LV shRNA for 5 days or 1 µM ABT-737 for 3 days or with both from the beginning of the culture. The "None" cells did not receive any ABT-737 whereas the "ABT First", ABT Second" or ABT Continuous" represent cells exposed to ABT-737 first and then LV, or ABT-737 after a 5 day exposure to LV shRNA or "ABT Continuous" where both agents were added at the beginning of the 8 day culture. P values are the significance of the indicated LV shNG-1 and/or shNp8-1 compared to the LV shNEG in the same treatment group as determined by 2-way ANOVA. Where there is a horizontal cupped bracket spanning across shNG-1 and shNp8-1, the P value of each compared to the corresponding shNEG control is the indicated value. Thus, for the three CRC lines either ABT-737 added after the LV-delivered shRNA or present for the whole 8 days suggests a more significant reduction in the survival of each cell line.

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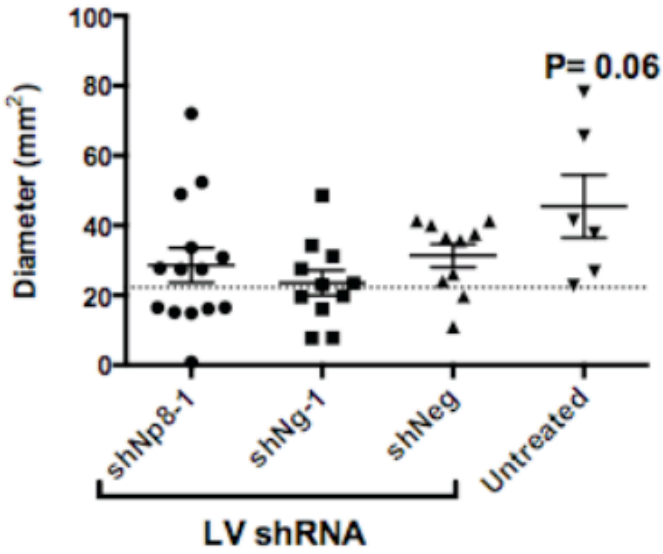


Figure 7. Limited Transduction of CX-1 Xenograft After Intralesional Therapy. CX-1 Xenografts were established subcutaneously in NOD/SCID mice and injected at a MOI of 3 when xenografts were 4 – 7 mm diameter with LV shRNA that contains a GFP reporter. At 7 days tumors were collected, minced into 1 mm or smaller pieces and then studied under an inverted epi-fluorescent microscope. White bar is 25µM. The left panel reveals a cluster of CX-1 cells that are expressing GFP and, as a result, are transduced. The middle panel indicates that this island of transduced cells is a minor component of the surrounding CX-1 cells. The right panel is the merged image.

This is the greatest amount of in vivo transduction that we have found in 16 experiments with any lentivirus shRNA preparation in this tumor model.

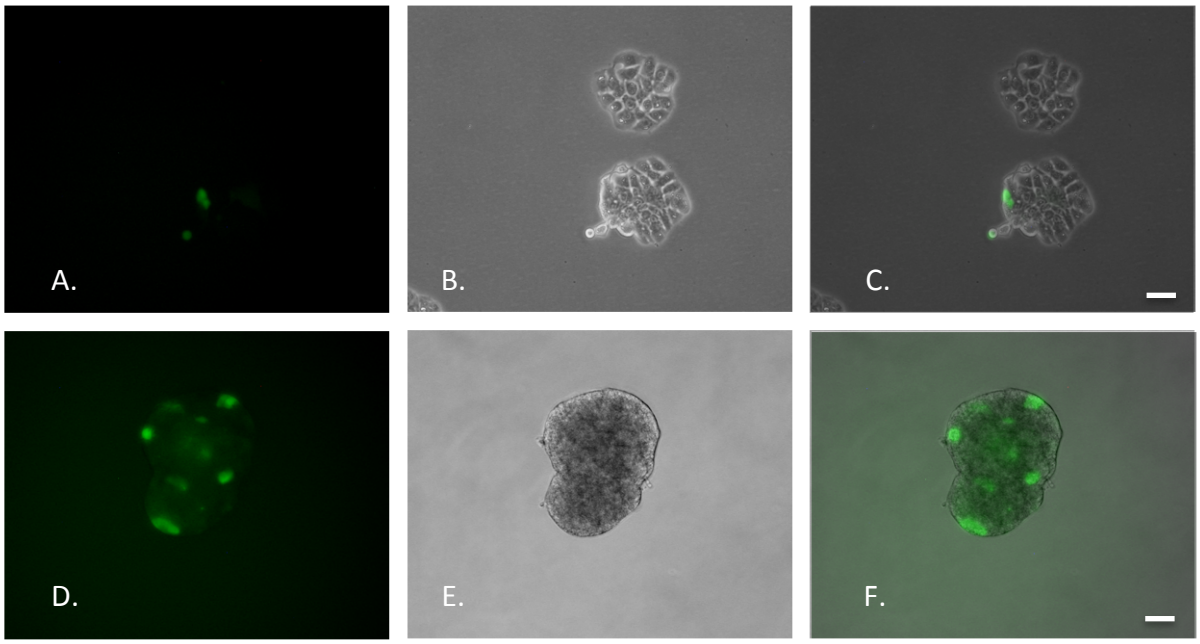
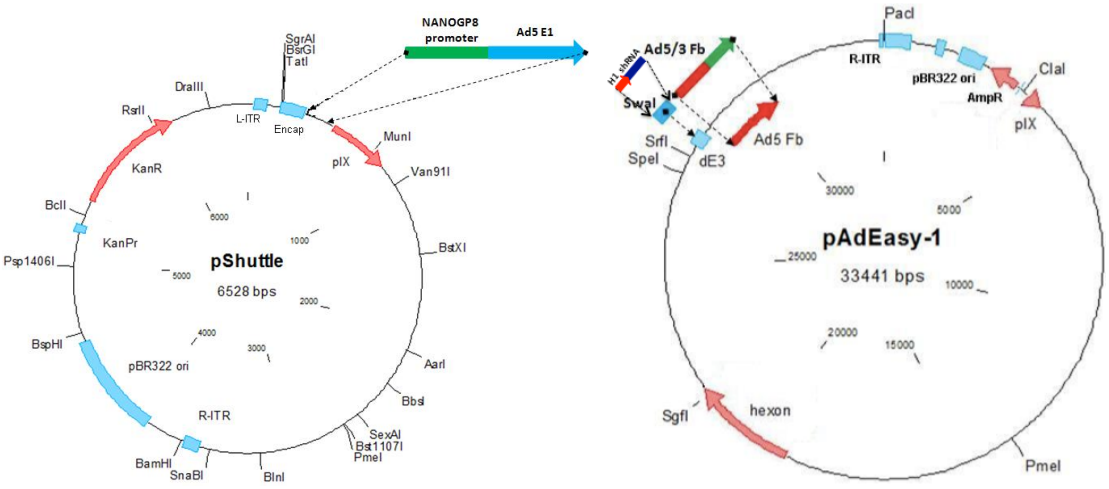
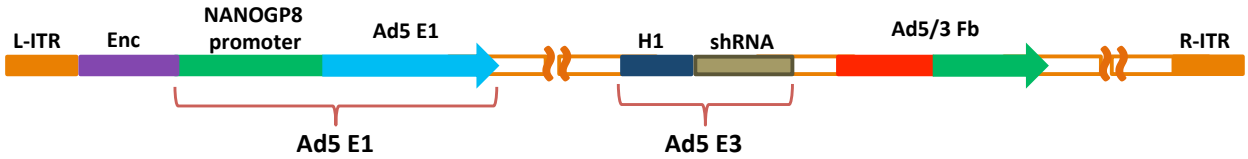


Figure 8. NANOGP8 Promoter Activity in CX-1 Cells in Monolayer and Spheroids. CX-1 cells cultured in monolayer for 3 days (Panels A – C) or in suspension (Panels D – F) for 5 days after transduction with lentivirus containing the 3.5 Kb NANOGP8 promoter reporter that expresses GFP. Panels A and D are epifluorescent images whereas panels B and E are transilluminated and panels C and F are the merged images. The white bars are 25 microns. The data suggest that the promoter is more active in spheroids than in monolayer culture.



A. Shuttle Vector **B. Rescue Vector**



C. CRAd Genome

Figure 9. Vectors For Creation of the CRAd5. Panel A is the schematic for the shuttle vector that leads to integration of a promoter construct into the shuttle plasmid. Panel B is the Rescue Vector that introduces the modification to the Ad5 fiber to create the recombinant AD5/3 fibritin that reduces hepatic targeting by increasing binding to DSG2 on cells.

Biographical Sketch

Provide the following information for each individual included in the Research & Related Senior/Key Person Profile (Expanded) Form.

NAME	POSITION TITLE
NIKOLAY KOROKHOV	**SPECIAL VOLUNTEER**

EDUCATION/TRAINING (Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training).

INSTITUTION AND LOCATION	DEGREE (IF APPLICABLE)	YEAR(S)	FIELD OF STUDY
Novosibirsk State University, Russia	B.S./M.S.	1982	Biology/Biochemistry
BIFIP, Russia	Ph.D.	1997	Biology/Biotechnology
UAB, Alabama	Postdoctoral training	1999-2000	Gene Therapy

RESEARCH AND PROFESSIONAL EXPERIENCE: Concluding with present position, list in chronological order, previous employment, experience, and honors.

1982-1984 Research Trainee
1984-1988 Junior Research Scientist
1988-1992 Research Scientist, Laboratory of Molecular Biology, Institute of Cytology and Genetics, Novosibirsk, Russia
1992-1996 Research Scientist
1996-1999 Senior Research Scientist, Laboratory of Molecular Biology, Institute of Physiology, Biochemistry and Nutrition of Farm Animals, Borovsk, Russia
1999-2000 Postdoctoral fellow, Gene Therapy Center, University of Alabama at Birmingham, Birmingham, AL
2000-2006 Senior Scientist, VectorLogics, Inc., Birmingham, AL
2006 Scientist II, OpenBiosystems, Birmingham, AL
2006-2008 Associate Director
2010 **Naturalized US Citizen**
2008-2013 Director, Lentiviral Vector Group, VIRxSYS Corporation, Gaithersburg, MD
2013-present Special Volunteer, Geneva Foundation for NCI-NIH

List in chronological order the titles, all authors, and complete references to all publications during the past 3 years and to representative earlier publications pertinent to this application.

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