CREATING A COLONIC ORGANOID MODEL FOR STUDYING COLORECTAL CANCER

An Undergraduate Research Scholars Thesis

by

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ABSTRACT

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Although significant research is currently being conducted to study human cancers, the use of *in vivo* models is time consuming and costly. Recent findings have documented the use of mouse intestinal crypt organoids as a model system using $Lgr5^{+}$ stem cells to express cancer related genes in a targeted manner. The objective of this research project is to successfully transform colonic mouse organoids with a mutant form of the K-*ras* oncogene. This is relevant because patients diagnosed with colon cancer often express a mutated copy of the K-*ras* gene within colonic tumors. Thus, the transduction of a mutant gene into colonic organoids will create an *ex vivo* model for studying colon cancer.

CHAPTER I

INTRODUCTION

Background

Colorectal cancer remains a serious problem in the United States, affecting 6% of the population. Advances have been made in the study of the disease and preventative measures are currently being investigated. In order to study cancerous and premalignant tissue, transgenic mice are used as a model system. Some disadvantages involving the use of mice include the long generation time and the high cost of breeding and raising mouse colonies. More recent findings have demonstrated that intestinal cells can be grown as three-dimensional organoid systems *ex vivo* (1). Organoids have been developed from many different types of tissue including muscle, stomach, and intestinal cells with each organoid culture reproducing the architecture of the *in vivo* organ (1, 2).

The viral transduction of genes is an established method to manipulate a genome both *ex vivo* and *in vitro*. This is noteworthy, because organoid models can be transformed through a retroviral transduction system (1). The use of organoid models will allow for a cost-effective evaluation of transformed animal genomes relevant to human disease.

Ras genes

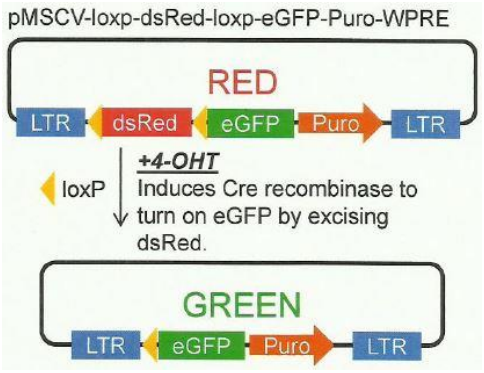
The mammalian *ras* gene family—consisting of H-*ras,* N-*ras,* K4A-*ras,* and K4B-*ras*—functions in cell proliferation and determination (3). In addition, the K-*ras* gene has a unique and essential role in mouse embryogenesis (3). It has been documented that *ras* genes are mutated in over 30%

of human tumors and a mutation in the 12th codon of K-*ras* is found in ~40% of colonic tumors (3, 4). Mutated K-*ras* is known to occur more frequently in patients with colorectal cancer as the tumor progresses (5). Due to the frequent mutation rate associated with K-*ras* and its function, this gene has been selected as a prime target for cancer therapy (6). Targeting dysregulated K-*ras* has led to antitumor activity, however, *ras* driven tumors are often refractory to conventional therapies and clinically effective targeted therapy has not yet been developed (6).

K-*ras* as a model for colorectal cancer

A mutant human K-*ras* gene will be inserted into a retroviral plasmid for the purpose of transforming colonic organoids using a tamoxifen (4-OHT) inducible cre-recombinase

Figure 1. Retroviral plasmid construct (1).



mechanism. As illustrated in Figure 1, transformed organoids will initially fluoresce red under ultraviolet (UV) illumination. Addition of 4-OHT to the culture media will induce the cre-recombinase reaction to excise the DNA between the loxp sites. After dsRed is excised, eGFP will be expressed and the transformed cells will fluoresce green in UV light. The mutated K-*ras* gene will be ligated into the plasmid between the second loxp site and the eGFP. In this manner, the transformed organoids, after excision of dsRed, will express the mutant K-*ras* gene.

This *ex vivo* model can be utilized to study the metabolism of oncogenic colonocytes vs isogenic (control) colonocytes.

CHAPTER II

METHODS

Mice

Lgr5^{+}-GFP mice between the ages of nine and twelve weeks were used. The handling and care of the mice were in accordance to the Animal Use Protocol 2010-285.

Colon crypt isolation

Primary colonic crypts were released in 20 mM EDTA at 37°C during a 30 minute incubation. After incubation, the crypts were pelleted at 500g for 5 min and counted. The isolated crypts were resuspended in Matrigel (BD Biosciences) at 750 crypts per 50 µL, then plated in a 24-well plate. The crypts were fed with 500 µL of crypt culture medium (50% Wnt3a conditioned medium, 47% Advanced DMEM/F12 (Invitrogen), 1% GlutaMax, 1% HEPES buffer, and 1% penicillin/streptomycin plus 50 ng/mL mouse epidermal growth factor (EGF), 100 ng/mL Noggin, 1:100 N2 supplement, 1:50 B27 supplement, 500 ng/mL human recombinant R-spondin1, and 1µM N-acetylcysteine) (7). Cultures were maintained for 6-8 days prior to viral transduction.

Retroviral transduction

Platinum E cells (Cell Biolabs) were used to generate viral supernatant as detailed elsewhere (1). Organoids were harvested and dissociated using a 20-gauge needle five times. The fragments were incubated for 5 min at 37°C in TripLE (Invitrogen). Advanced DMEM/F12 (Invitrogen) media containing 5% serum was added before the samples were centrifuged at 1,000g for 5 min.

After removing the supernatant, the cells were resuspended in a small volume of infection media (50% Wnt3a conditioned medium, 47% Advanced DMEM/F12, 1% GlutaMax, 1% HEPES buffer, and 1% penicillin/streptomycin plus 10µM Y27632 (sigma), 8 µg/mL polybrene (sigma), 50 ng/mL EGF, 100 ng/mL Noggin, and 500 ng/mL R-spondin1). 250 µL of the cell suspension was combined with 250 µL of the retroviral supernatant and transferred to a 48-well culture plate. Each plate was centrifuged at 600g for 1 hr at 32°C, then incubated at 37°C for 6 hrs. After incubation, the samples were transferred to a 1.5 mL Eppendorf tube and spun down at 1,000g for 5 min. The pellet was resuspended in 100 µL of Matrigel and split into two wells of a 24-well culture plate. The cells were fed with 500 µL of crypt culture medium. After two days, the media was changed to crypt culture medium plus 2 µg/mL puromycin in order to select for organoids exhibiting incorporation of plasmid DNA.

CHAPTER III

RESULTS

Organoid growth

Colonic crypts were harvested and plated in Matrigel. After 2-3 days, the crypts grew as spherical structures that began to bud into 3-dimensional organoids around day 5.

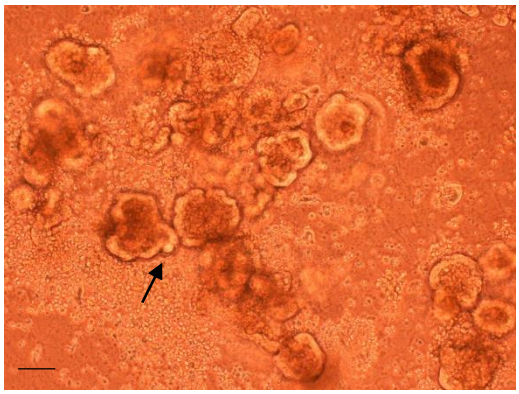


Figure 2. Budding organoids six days after isolation. Scale bar, 100 µm.

Figure 2 illustrates the growth of budding organoids six days following the initial isolation of crypts with the arrow indicating a bud. Organoids that were 6-8 days old displayed adequate growth and buds and were subsequently utilized for the viral transduction experiment.

Viral transduction

The organoids that were successfully transduced (approximately a 30% efficiency) expressed the dsRed gene under selection with puromycin. As shown in Figure 3, expression of the reporter gene (dsRed) increased as the organoids developed in culture. The non-transduced organoids did not express dsRed, but exhibited minimal autofluoresence over dead cells. Without the

expression of the puromycin resistant gene contained in the plasmid, the non-transduced organoids were not viable. This step was necessary to verify that the organoids were capable of being transduced through the use of the retroviral plasmid.

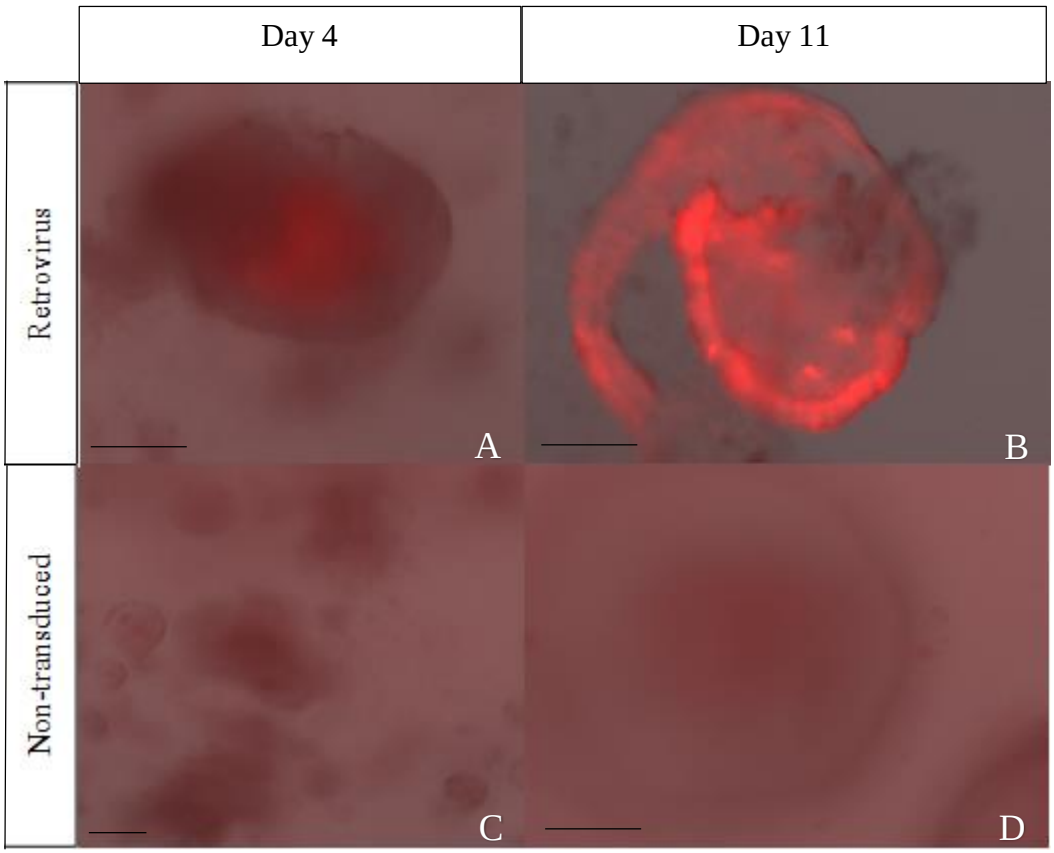


Figure 3. Expression of dsRed in transduced organoids. Figure 3A. Transduced organoid expressing limited dsRed four days post transduction. Figure 3B. Increased expression of dsRed in a transduced organoid eleven days after the viral transduction. Figure 3C. Non-transduced organoids (negative control) showing only minimal autofluorescence over dead cells on day four. Figure 3D. Limited autofluorescence in a non-transduced organoid on day eleven. Scale bars, 50 µm.

CHAPTER IV

CONCLUSIONS

As colorectal cancer continues to affect the population, new models and methods to study this disease are emerging. The current method of using mouse models to study human cancers is inefficient and time consuming. Thus utilizing primary cell cultures to create an *ex vivo* model will improve the efficiency of cancer research.

The stem cells isolated from the colon crypts developed into 3-dimensional organ like structures, called organoids. These organoids retained the architecture and integrity of the colon itself. Isolation of colonic crypts from a single mouse produced thousands of large intestinal organoids that were successfully passaged. Our findings indicate that wild-type organoids can be genetically manipulated by retroviral transduction. The viral transduction system can be modified with other genes in order to create an organoid model for various intestinal diseases, such as colitis. The organoid model is not limited to colonic tissue. Stem cells from other human tissues could produce an organoid model capable of studying other types of human cancers or diseases.

REFERENCES

1. Koo B, Stange DE, Sato T, Karthaus W, Farin HF, Huch M, et al. Controlled gene expression in primary Lgr5 organoid cultures. Nature methods. 2011;9(1):81-3.

2. Shansky J, Chromiak J, Del Tatto M, Vandenburgh H. A simplified method for tissue engineering skeletal muscle organoids in vitro. In Vitro Cellular & Developmental Biology-Animal. 1997;33(9):659-61.

3. Johnson L, Greenbaum D, Cichowski K, Mercer K, Murphy E, Schmitt E, et al. K-ras is an essential gene in the mouse with partial functional overlap with N-ras. Genes Dev. 1997 Oct 1;11(19):2468-81.

4. Forrester K, Almoguera C, Han K. Detection of high incidence of K-ras oncogenes during human colon tumorigenesis. Nature. 1987;327(6120):298-303.

5. Kinzler K, Vogelstein B. Lessons from hereditary colorectal cancer. Cell. 1996 OCT 18;87(2):159-70.

6. Friday BB, Adjei AA. K-ras as a target for cancer therapy. Biochimica et Biophysica Acta (BBA)-Reviews on Cancer. 2005;1756(2):127-44.

7. Sato T, Vries RG, Snippert HJ, van de Wetering M, Barker N, Stange DE, et al. Single Lgr5 stem cells build crypt villus structures in vitro without a mesenchymal niche. Nature. 2009;459(7244):262-5.