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14. ABSTRACT
Here, we outline the progress we have made in the first year of the funding period. Our specific aims are to 1) quantitatively assess genomic instability in different mammary epithelial populations *in vivo* and *in vitro*, 2) determine how mutations in heritable breast cancer genes affect genomic stability in different mammary epithelial populations *in vivo* and *in vitro* and 3) determine the influence of estrogen and ionizing radiation on genomic stability in the mammary epithelium. Year 1 tasks in our Statement of Work exclusively relate to aim 1. We have made significant progress in subaim 1a, substantially improving the design of our proposed transgenic animal, the "deletion reporter mouse", and are finalizing cloning of necessary components. We expect to submit embryonic stem cells to the transgenic facility within the next few months. Furthermore, subaim 1b is nearing completion. As planned, we have successfully developed and validated an *in vitro* deletion reporter, which can now be used to assess the influence of genetic and exogenous factors on deletion rate in mammary epithelial cells.

15. SUBJECT TERMS
Breast cancer, genomic instability, mammary epithelium, DNA repair, estrogen, radiation

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1. Introduction

The goal of this project is to develop novel reagents to assess levels of genomic instability *in vivo* and *in vitro*, and to use these tools to enhance our understanding of breast cancer development. Since the start of this project in October 2015, we have made significant progress for the tasks associated with Specific Aim 1, as outlined in the Statement of Work.

Specific Aim 1. Quantitatively assess genomic instability in different mammary epithelial populations *in vivo* and *in vitro*.
Subaim 1a. Determine SCNA frequencies in different mammary epithelial cells in vivo. We will study whether subpopulations of mammary epithelial cells ($CD24^{+/low}Sca\text{-}1^{-}$ basal, $CD24^{+/high}Sca\text{-}1^{-}$ luminal ER-, and $CD24^{+/high}Sca\text{-}1^{+}$ luminal ER+ cells) show increased genomic instability. To that end, we propose to create a "deletion reporter mouse" to measure SCNA frequencies *in vivo*. In this animal model, two highly potent small hairpin RNAs (shRNAs) located on chromosome 19 suppress the expression of GFP-Luciferase (GFP-Luc) and RFP, respectively, located on chromosome 6. When loci harboring the shRNAs are deleted by a spontaneous mutation event, affected cells become GFP and/or RFP-positive. Fluorescent cells will be isolated by fluorescence activated cell sorting (FACS), and the percentage of cells with deletions will be confirmed by fluorescence in situ hybridization (FISH) and automated image analysis. The proposed experiments can also be accomplished by using an existing mouse strain with ubiquitous GFP expression (UBC-GFP or CAG-GFP); the success of aim 1a therefore does not depend on successful creation of the deletion reporter mouse.
Subaim 1b. Determine SCNA birth rates in human mammary epithelial cells in vitro. To complement aim 1a, we will study the rate of SCNA birth in human mammary epithelial cells *in vitro*. Many cell types found in the mammary gland *in vivo* cannot be stably propagated in culture. However, several fundamental questions about genomic stability in human cells can be answered in the *in vitro* setting. Using a deletion reporter assay adapted from the yeast genetics literature, we will determine whether baseline deletion rates in normal human mammary epithelial cells (HMECs) differ from rates in cancer cell lines.

Specific Aim 2. Determine how mutations in heritable breast cancer genes affect genomic stability in different mammary epithelial populations *in vivo* and *in vitro*. We will determine how deficiencies in DNA DSB repair affect genomic stability in different mammary epithelial subpopulations. We will breed the reporter mouse created in aim 1 (or the CAG/UBC-GFP mouse) with $BRCA1^{+/-}$ and $ATM^{+/-}$ mutant mice, and assess the changes in SCNA frequency, using the same methodology as in aim 1 (FACS and FISH). In a complementary approach, using CRISPR/Cas9, we will create human mammary epithelial and breast cancer cell lines that contain known pathogenic heterozygous and homozygous variants of BRCA1, BRCA2, CHEK2 and ATM, and quantify to what degree spontaneous deletion rates are affected by these mutations.

Specific Aim 3. Determine the influence of estrogen and ionizing radiation on genomic stability in the mammary epithelium. We will determine what effect E2 has on genomic stability in the mammary epithelium of wild type, $BRCA1^{+/-}$ and $ATM^{+/-}$

mutant reporter mice. We will ovariectomize animals, implant pellets containing different doses of E2, and evaluate SCNA frequencies in mammary epithelial subpopulations. We will then superimpose on this experimental design ionizing radiation corresponding to doses delivered during mammography, X-ray and CT imaging, to determine whether E2 and radiation have synergistic effects on genome instability. As in previous aims, we will recapitulate these experiments *in vitro* by exposing HMECs, ER-positive and ER-negative breast cancer cells to E2 alone, radiation alone and E2 and radiation combined. Furthermore, we will study how interference with ER expression affects deletion rates.

2. Keywords

Breast cancer	Genomic deletion	Chromosomal instability	Mammary epithelium
Transgenic animal	DNA repair	shRNA	Homologous recombination
BRCA1	ATM	Estrogen	Radiation

3. Accomplishments

3.1 Research accomplishment pertaining to Subaim 1a (Determine SCNA frequencies in different mammary epithelial cells in vivo, months 1-18): Development of a new and improved design for the "deletion reporter" mouse.

Our plan, as proposed in the original SOW, was to create a transgenic animal, the "deletion reporter mouse", in which two highly potent shRNAs (J6/J9) located on chromosome 19 knock down the expression of a GFP-Luciferase fusion protein and RFP, respectively, both integrated into the Rosa26 locus on chromosome 6 (Figure 1). Loss of J6 would lead to RFP expression, loss of J9 to GFP-Luciferase expression, and whole chromosome loss would activate both GFP-Luciferase and RFP. After detailed discussions with the Partners Healthcare Transgenic Core and the Ragon Institute Flow Cytometry Facility, who pointed out some limitation of the proposed approach, we worked on improving the design of the deletion reporter mouse in order to ensure the success of our experimental strategy.

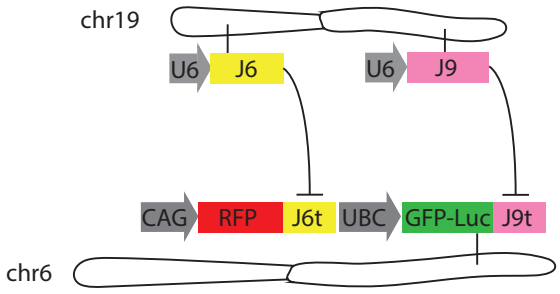

Figure 1. Original deletion reporter mouse design

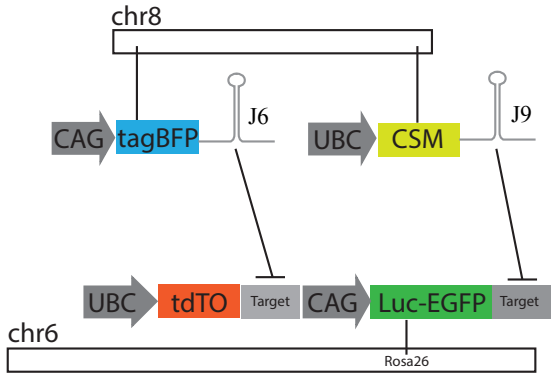

Figure 2. Improved deletion reporter mouse design

Improvement A: Expression of shRNAs from RNA Polymerase II promoters along with two marker proteins. To be able to visualize cells in which deletion reporter shRNAs are present and robustly expressed, we decided to exchange RNA Polymerase III promoters (U6) for RNA Polymerase II promoters, specifically the strong synthetic CAG promoter and the ubiquitin C (UBC) promoter (Figure 2). In this new design, hairpins are located in the 3' UTR of two marker proteins (described in more detail in the next section), thus enabling fluorescence activated sorting of hairpin-expressing cells. This design allows for a higher level of specificity when evaluating deletion events. For example, upon loss of the J6 shRNA, we not only expect activation of tdTomato expression, but also loss of the tagBFP signal.

Improvement B: Selection of suitable marker proteins for specific detection of deletion events. Our new design depends on highly accurate distinction between the four marker proteins to identify specific deletion events (Table 1). We chose three colors (tagBFP,

tdTomato, EGFP) with practically no spectral overlap, but were not easily able to identify a fourth fluorescent protein that would be distinguishable from the others in all instances.

	Chr. 8 intact	Left end of Chr.8 deleted	Right end of Chr.8 deleted	Chr. 8 deleted
tagBFP	ON	OFF	ON	OFF
CSM	ON	ON	OFF	OFF
tdTomato	OFF	ON	ON	ON
EGFP/Luc	OFF	OFF	OFF	ON

Table 1. Identification of specific deletion events through unique combinations of marker protein expression.

Therefore, we decided to link expression of J9 shRNA to an artificial cell surface marker (CSM) which can be identified through staining with a specific antibody (conjugated to a fluorochrome with a unique spectrum). Our lab has developed two such surface markers: pQCXIN-FLAG-FOSx2-CD8tm, a type I transmembrane protein which consists of a N-terminal FLAG followed by two leucine zipper domains of c-fos separated by a flexible linker and another flexible linker connecting to the CD8 transmembrane domain, and pQCXIN-ANPEPtm-JUNx2-HA, a type II transmembrane protein which consists of an HA-tag, followed by two leucine zippers of c-jun separated by the same linker as above, then another linker linking it to the transmembrane domain of ANPEP. We are currently evaluating both constructs and will choose the one with more robust cell surface localization in our deletion reporter mouse.

Improvement C. Bioinformatic analysis of suitable integration loci on chromosome 8.

We further improved our strategy by selecting a new chromosome for deletion reporter shRNA knock-in. Accurate measurement of deletion frequencies will depend on the ability of somatic cells to tolerate chromosome loss. We searched the literature for experimental evidence that a given chromosome could be somatically deleted without leading to cell death. Dan et al. showed that mouse kidney cells spontaneously lose chromosome 8 at a relatively high frequency, approximately 5×10^{-5} (REF). These mutants can easily be expanded in culture, demonstrating that mouse cells that have lost chromosome 8 are viable and able to proliferate. We therefore chose chromosome 8 as the new target for our deletion reporter shRNA knock-in.

Our assay depends on robust and consistent expression of deletion reporter shRNAs. Since transgenes can get silenced if they are located in regions that become heterochromatic during differentiation of different tissues, we wanted to select an integration locus that would remain euchromatic in all mouse cells. To determine such "obligatory euchromatic" regions on chromosome

Figure 3. Consistently highly expressed genes on chromosome 8.

8, we downloaded expression data from the GNF Mouse GeneAtlas V3, which consists of whole transcriptome microarrays of 182 mouse tissues. We ranked genes by their expression intensity and found 148 genes that were in the 95th expression percentile in all 182 tissue samples. At least 7 of these genes were located on chromosome 8 (Figure 3). To avoid increased frequency of mitotic recombination, we skipped genes closest to the telomeres and selected Vdac3 and Cox4i1 as target integration sites for deletion reporter shRNAs.

In summary, we believe that these alterations in deletion reporter design will significantly improve the likelihood that experiments in the new transgenic animal will be successful. The plan presented in our original SOW has been somewhat delayed by these conceptual changes, but we are now finalizing the cloning of these constructs and expect that we will be able to submit mouse embryonic stem cells to the Partners Healthcare Transgenic Core within the next few months.

3.2 Research accomplishment pertaining to Subaim 1b (Determine SCNA birth rates in human mammary epithelial cells *in vitro*, months 1-14): *In vitro* deletion reporter successfully developed.

In our original *in vitro* deletion reporter design, EGFP and a negative selectable marker, thymidine kinase, would both be expressed from two independent promoters (Figure 4). We engineered the construct as proposed in our original application and attempted to knock it into the neutral AAVS1 locus on chromosome 19 in human mammary epithelial cells (HMECs). Unfortunately, there was a very high level of background integration. After isolating more than 60 GFP+ puromycin-resistant clones, we could not find a single one in which the construct had integrated in the correct location. Therefore, we altered our design and cloned a new construct in which the promoter was removed from the puromycin resistance-thymidine kinase fusion gene and replaced with a splice acceptor site and a self-cleaving peptide (T2A). We flanked the reporter cassette with homology arms that would direct it to the intron of the PTTG1IP gene on chromosome 21. In this

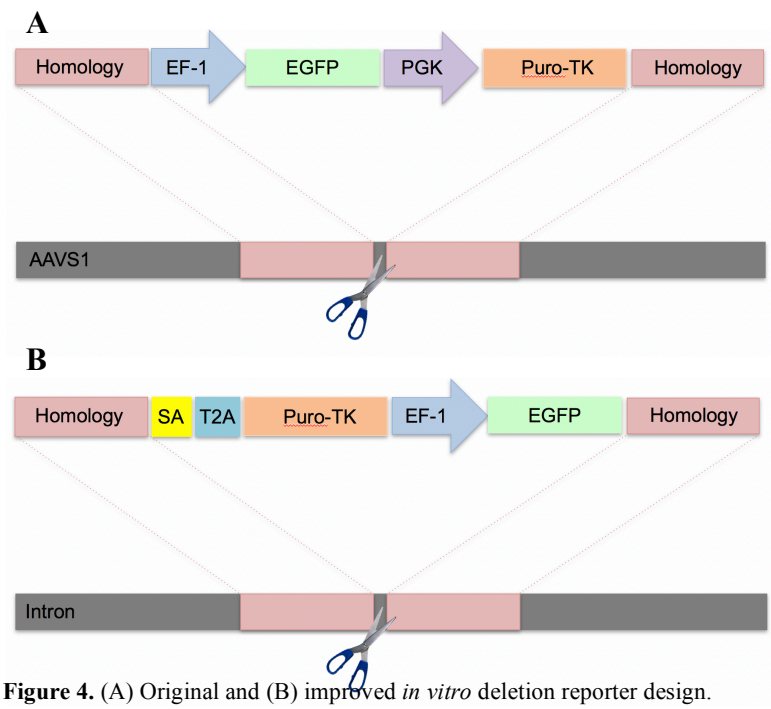

Figure 4. (A) Original and (B) improved *in vitro* deletion reporter design. Puro-TK: puromycin resistance-thymidine kinase fusion gene. SA: splice acceptor site.

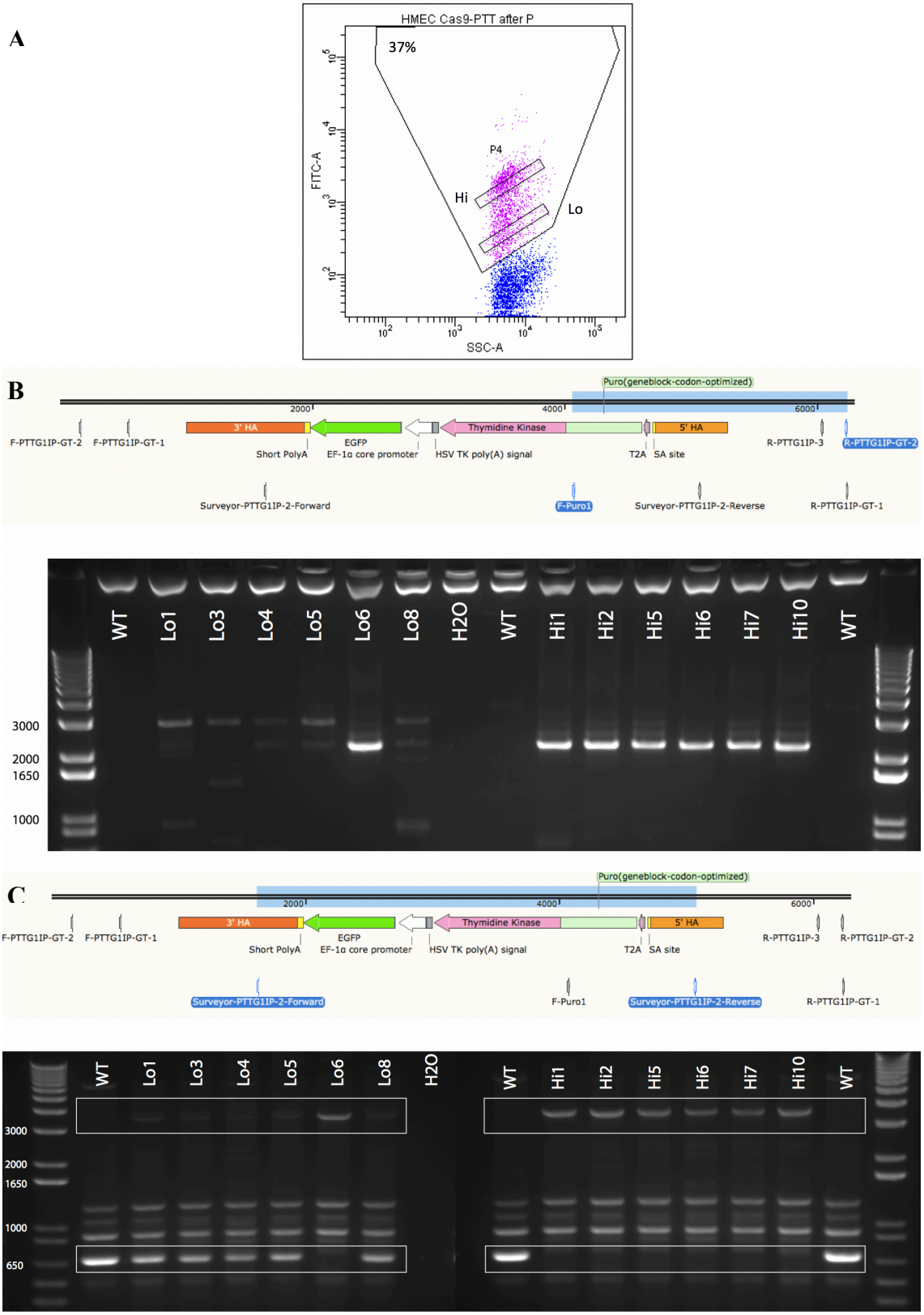

Figure 5. (A) Isolation of GFP+ Hi and Lo populations after puromycin selection. (B) Diagnostic PCR of individual Hi and Lo clones. Expected band after correct integration: 2175 bp. One primer hybridizes to Puro resistance, the other to flanking genomic DNA. (C) Diagnostic PCR of individual Hi and Lo clones. Expected band after correct integration: 673 bp for wilde type, 3461 bp for correctly integrated deletion reporter. Primers hybridize to homology arms.

new design, off-target integration in intergenic regions would not lead to expression of puromycin resistance or thymidine kinase, thus allowing us to select for clones with a correctly integrated construct (in which the endogenous PTTG1IP promoter drives Puro-TK expression) with higher efficiency. This strategy was successful. After puromycin selection, we isolated two GFP+ populations (bright GFP+ cells tagged "Hi" in Figure 5A and dim GFP+ cells tagged "Lo") and generated individual clones from each population. PCR confirmed that Hi cells almost exclusively consisted of cells in which the reporter cassette had integrated into both copies of the PTTG1IP gene, Lo cells contained one correctly integrated copy (Figures 5B and 5C).

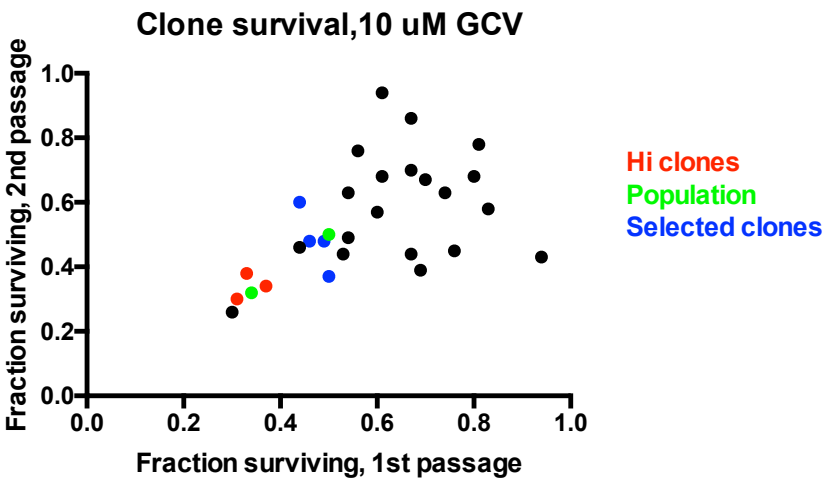

Figure 6. Lo clones with one copy of the deletion reporter (black circles) die when exposed to 10 uM ganciclovir.

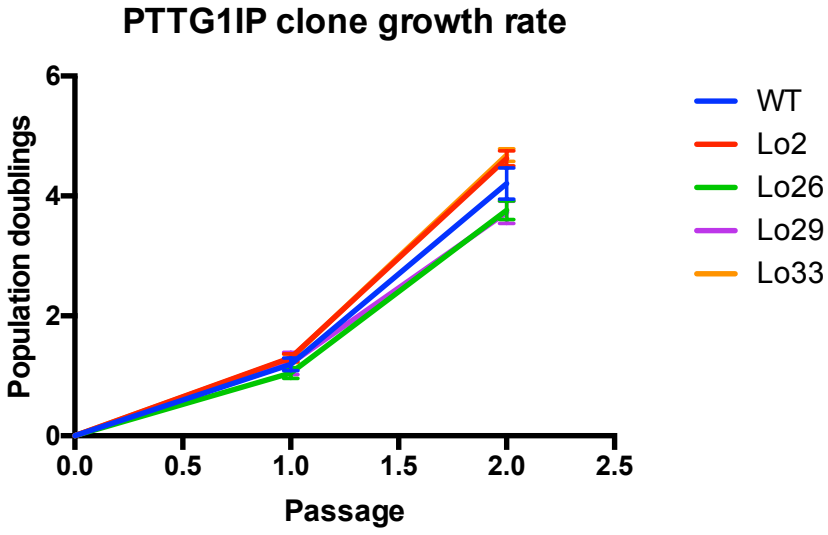

Figure 7. Growth rates of wild type cells and Lo clones with one copy of the deletion reporter are comparable.

In the next step, we wanted to determine the sensitivity of our deletion reporter cells (specifically, Lo clones with only one integrated copy) to ganciclovir, a drug that gets converted to a toxic compound by thymidine kinase, thus killing cells that express the gene. We found that at a 10 uM concentration of ganciclovir (which has no effect on wild type cells), Hi clones with two integrated copies died at a rapid rate (Figure 6). Lo clones showed a distribution of responses. We chose four clones (Lo2, Lo26, Lo29, Lo33) with relatively high sensitivity to ganciclovir for further study and determined that their growth rates in normal media did not significantly alter from wild type,

suggesting that loss of one copy of PTTG1IP has no detrimental effect (Figure 7).

Finally, we set out to test whether we could detect rare wild type cells that do not contain the deletion reporter in a large number of cells that express thymidine kinase. We mixed cells in the following proportions and exposed them to 10 uM ganciclovir:

- No wild type cells
- 1 wild type cell : 100 deletion reporter cells
- 1 wild type cell : 1000 deletion reporter cells
- 1 wild type cell : 10000 deletion reporter cells

Surprisingly, we found that in all conditions, all cells died. Investigation of older literature revealed that wild type cells that form gap junctions with thymidine kinase-expressing cells suffer from a "bystander effect" upon ganciclovir treatment (REF). We therefore reasoned that outgrowth of wild type clones could only be achieved under sparse plating conditions. When we plated only 10,000 cells Lo cells per 10 cm dish and treated with ganciclovir, we could observe a consistent number of clones that escaped TK-induced death (Figure 8). These are probably clones that lost or silenced the deletion reporter during clonal expansion. We conclude that the deletion reporter system works as expected. In the next step, we will use the deletion reporter cells to calculate deletion rate per cell division, using a formal Luria-Delbrück Fluctuation analysis. We are well within the intended timeframe for this project (according to the approved SOW, fluctuation analysis should be completed approximately 14 months into the funding period).

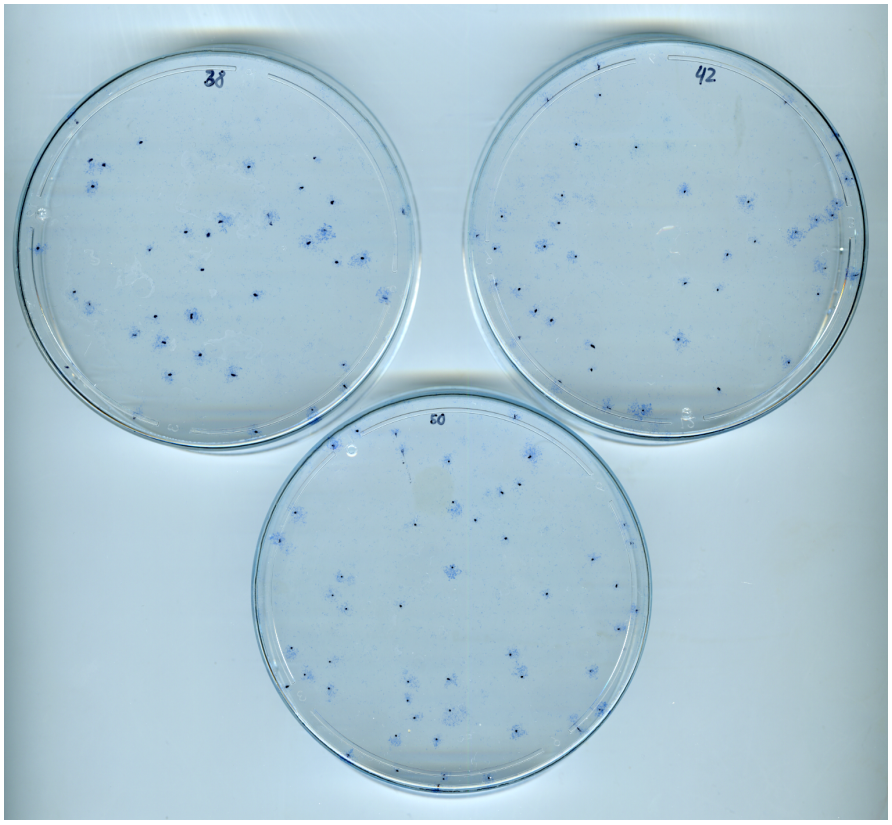

Figure 8. Outgrowth of ganciclovir-resistant clones under sparse plating conditions.

4. Impact

Progress made in year 1 has laid the foundation for successful completion of the remaining aims. A robust, well-thought out design for the deletion reporter mouse will ensure that the resulting animal can deliver the expected insights. For the first time, we now have access to an *in vitro* deletion reporter. Therefore, one very important goal of this proposal has already been accomplished. Years 2 and 3 will build upon these results and hopefully thereby enhance our understanding of breast cancer development.

5. Changes/Problems

All problems and subsequent changes in our experimental design have been described in detail in section 3, "Accomplishments". Here, we will only summarize these alterations as bullet points:

- Problem: no marker for deletion reporter shRNA expression in original design.
  Change: expression of marker proteins from RNA Pol II promoters, shRNAs are located in 3' UTR of these proteins
- Problem: Fluorescent proteins employed in the deletion reporter mouse need to be distinguishable with high accuracy.
  Change: Three fluorophores with completely distinct spectra have been selected, and an artificial cell surface marker has been developed as a fourth marker.
- Problem: loss of target chromosome for deletion reporter shRNAs (Chr. 19) may not be tolerated, some loci may get silenced during development
  Change: selection of a chromosome whose loss is tolerated in somatic tissues (Chr. 8), bioinformatic selection of loci that are highly expressed in all mouse tissues to identify target loci that are unlikely to get silenced
- Problem: high rates of background integration when using two strong independent promoters for expression of puromycin resistance-thymidine kinase and GFP.
  Change: Gene trap design in which Puro-TK is expressed from the endogenous PTTG1IP promoter
- Problem: Bystander effect kills wild type cells that are surrounded by deletion reporter expressing cells
  Change: Sparse plating protocol enables outgrowth of wild type cells.