The Detection of Immortal DNA Strand Co-segregation
as a Method of Adult Stem Cell Identification

By

Jennifer J. Cheng

B.E. Biomedical Engineering
Vanderbilt University, 2001

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Signature of Author: ________________________________
Division of Bioengineering
May 24, 2004

Certified by: ________________________________
James L. Sherley
Associate Professor
Division of Bioengineering
Thesis Supervisor

Accepted by: ________________________________
Alan J. Grodzinsky
Professor of Electrical, Mechanical, and Biological Engineering
Chair, BE Graduate Committee
Abstract

The study of stem cells is one of the most fascinating topics in biology. Adult stem cells (ASC), which play the prime role in the maintenance and restoration of tissues, are thought to hold great potential for the advancement of medicine. It has been postulated that adult stem cells are able to retain “immortal” DNA template strands over successive generations by non-random chromosome co-segregation, and in so doing, to protect the long-term genomic fidelity of whole tissue compartments. The investigation of this theory may yield insights into areas such as the development of cancer and the process of aging. In addition, it may lead to the discovery of an effective method for the unique identification of adult stem cells, the study of which has thus far suffered from the lack of unique identifiers. Thus, the goal of this research was to develop an assay for the detection of immortal DNA strand co-segregation that could be applied to the detection and analysis of adult stem cells. It is proposed that such an assay may in itself serve as a unique identification method for adult stem cells. In this thesis, the development of such an assay is described. This assay, referred to as the label release assay, has provided further evidence for the existence of immortal strand co-segregation in model cell lines, and will potentially be useful in the study of adult stem cells in tissues.
## Table of Contents

List of Figures ........................................................................................................................... 4

Chapter 1: Introduction ........................................................................................................... 5

  Stem Cell Definitions ........................................................................................................... 5
  Stem Cell Kinetics ............................................................................................................. 9
  Immortal Strand Co-segregation ...................................................................................... 12
  Purpose .............................................................................................................................. 17

Chapter 2: Assay Development ............................................................................................ 18

  Model Cell Systems ......................................................................................................... 18
  Label Retention Assay ..................................................................................................... 19
  Label Release Assay ........................................................................................................ 21

Chapter 3: Methods ............................................................................................................... 26

  Cell Culture ...................................................................................................................... 26
  Label Retention Assay ..................................................................................................... 26
  Label Release Assay ........................................................................................................ 27
  Detection .......................................................................................................................... 27

Chapter 4: Results and Discussion ....................................................................................... 28

  Label Retention Assay ..................................................................................................... 28
  Label Release Assay ........................................................................................................ 30

Chapter 5: Conclusions and Future Directions ................................................................... 34

References ............................................................................................................................... 35

Acknowledgements ................................................................................................................ 41
List of Figures

1. Stem Cell Division Kinetics ........................................................................................................... 9
2. Chromosome Segregation .............................................................................................................. 14
3. Label Retention Assay Schematic .............................................................................................. 19
4. Label Release Assay Schematic ................................................................................................ 21
5. Label Retention Assay Results .................................................................................................. 29
6. Label Release Assay Results ...................................................................................................... 30
Chapter 1: Introduction

Recently, there has been an explosion of interest in the field of stem cell research. The study of stem cells holds enormous potential both for the further development of medical therapies and for the advancement of our knowledge of the biological sciences. It is envisioned that stem cells, perhaps in conjunction with tissue engineering and gene therapy, will someday be used to regenerate and repair tissues and organs of every kind. The elucidation of the mechanisms by which stem cells function will provide further insight into our understanding of how humans develop, grow, function, heal, age, and develop cancer.

Stem Cell Definitions

Stem cells are cells that exhibit both self renewal and the ability to give rise to differentiated progeny. A stem cell can be classified as either an embryonic stem cell (ESC) or an adult stem cell (ASC). Embryonic stem cells are primordial, undifferentiated cells derived from the developing embryo. Specifically, they are isolated from the inner cell mass of an embryo in the blastocyst stage, at which point the embryo has not yet implanted in the uterine lining. The inner cell mass is the portion of the blastocyst that develops into the fetus. Thus embryonic stem cells are pluripotent – that is, they are able to generate all of the types of differentiated cells that make up the body. Significantly, they are able to generate progeny representing each of the three germ layers of the developing fetus – endoderm, mesoderm, and ectoderm.
Adult, or somatic stem cells, by contrast, are undifferentiated cells found in more mature tissues. Although their exact point of origin is unclear, it is presumed that adult stem cells arise at some point in fetal development from embryonic stem cells. Adult stem cells reside in different tissue compartments throughout the body, each filling its own stem cell “niche”. They are self-renewing and either unipotent or multipotent, giving rise to cell types specific to each tissue compartment. For instance, hematopoietic stem cells (HSCs), which reside in the bone marrow, produce all the different types of blood cells that make up the hematopoietic or blood system. There has been some controversy surrounding the plasticity of adult stem cells – that is, their ability to give rise to multiple cell lineages, including those lineages that have been thought to be distinctly divergent from the lineages to which the stem cells are seemingly committed to. There have been reports, for instance, of skin-derived stem cells generating cells such as neurons and adipocytes (fat cells), and of bone marrow-derived stem cells generating multiple non-hematopoietic cell types. However, it is still unclear whether these observations truly represent adult stem cell transdifferentiation events. As such, their significance – either as an indication of normal adult stem cell function or as a potential technology for the manipulation of adult stem cells – remains to be determined.

Although there has also been debate concerning the relative merits of studying adult and embryonic stem cells, both are thought to have great potential for the illumination of our biological understanding. Embryonic stem cells appear to have the greater potential to generate different types of cells than do adult stem cells. They also seem to contain
virtually unlimited self renewal capacity. However, it is not yet clear whether these self renewal and differentiation properties can be effectively controlled such that ESCs can safely be used therapeutically. Embryonic stem cells have been shown to form tumor-like masses called teratomas when injected into mice. Because the more mature, post-natal tissues of the body do not represent normal embryonic stem cell environments, ESCs may need to be properly differentiated or perhaps converted into adult stem cells prior to their clinical use in vivo. Moreover, the use of human embryonic stem cells is laden with ethical concerns regarding their derivation from human embryos. At present, they have been most commonly obtained from unused embryos from in vitro fertilization clinics or from electively aborted fetuses (embryonic germ cells). There has also been a recent report of human embryonic stem cells being derived from an embryo created by the nuclear-transfer cloning of a somatic cell. This report has in itself generated even more controversy because it involves the issue of human cloning. Regardless, the use of either pre-existing or newly created human embryos for the purpose of scientific research, even if it leads to beneficial therapies, will continue to encounter strong opposition from many.

The research discussed herein has been focused solely upon the study of adult stem cells. The use of adult stem cells avoids many of the ethical issues raised above concerning the use of ESCs, because ASCs can be directly obtained from adult tissues. In addition, adult stem cells may be used in autologous therapy – that is, a patient may be treated with ASCs obtained from his or her own body. Because these cells are recognized as “self”, autologous therapy avoids provoking immune reactions that occur when cells from donor,
non-self sources are introduced into the body. Adult stem cells are an attractive source for cell therapy, as they occur naturally in the various tissue compartments of the body, and they already function to maintain and replenish those tissues. Thus in contrast to embryonic stem cells, adult stem cells already have the precise self-renewal and differentiation properties that are needed in vivo. However, the use of adult stem cells presents a different set of barriers - namely, their identification, isolation, and expansion. Adult stem cells are very rare in the body. For instance, it has been estimated that only 1 in every 10,000 cells in the bone marrow is a hematopoietic stem cell.\textsuperscript{22,23} Methods have been devised to enrich the proportion of HSCs in a given cell population,\textsuperscript{24-26} but no set of markers has been found that uniquely identifies an adult stem cell and enables the perfect purification of a stem cell population.\textsuperscript{27,28} Some types of adult stem cells, such as those found in the small intestine, have a precisely defined physical location within the tissue architecture.\textsuperscript{29} For other tissues, the location or characteristics of the adult stem cell niche have yet to be definitively established. For these reasons, adult stem cells are difficult to identify and isolate. The barrier to adult stem cell expansion is a direct consequence of an adult stem cell characteristic termed asymmetric cell kinetics (ACK), which is discussed below.
Stem Cell Kinetics

It has been proposed that adult stem cells accomplish their dual tasks of self-renewal and generating differentiating progeny under the paradigm of asymmetric cell kinetics. That is, when an adult stem cell divides, it produces an identical adult stem cell, and a transit amplifying cell (Figure 1). This division is asymmetric because the two resulting cells have unequal kinetic and differentiating potentials. The adult stem cell daughter will continue to self-renew, whereas the transit amplifying daughter undergoes expansion and differentiation. Eventually it will terminally differentiate into the specialized cell types that make up that particular tissue compartment. In this way, a relatively small and constant number of stem cells can generate the multitude of cells needed to maintain a tissue.

![Figure 1. Stem cell division kinetics.](image)

The term “symmetric cell kinetics”, on the other hand, denotes situations in which a cell divides to produce two functionally identical daughter cells with equal proliferative potential (Figure 1). Embryonic stem cells divide with symmetric cell kinetics, whereas
adult stem cells normally divide with asymmetric cell kinetics. However, adult stem cells may reversibly switch to symmetric cell kinetics during times in which the stem cell pool needs to be expanded or replenished, such as during body growth or wound healing. Asymmetric cell kinetics, combined with symmetric cell kinetics induction when needed, allow adult stem cells to maintain homeostasis within a tissue unit throughout the lifetime of an organism. In tissues that experience high cell turnover rates, such as the intestinal epithelium, terminally differentiated cells are continually lost from the system, and they are continually replenished by adult stem cells cycling asymmetrically.\textsuperscript{30}

Evidence that adult stem cells divide with asymmetric cell kinetics has been demonstrated in many different contexts. Through the use of retroviral marking to conduct clonal lineage analyses in mice, neural stem cells (NSCs) have been found to divide asymmetrically in order to self renew and generate differentiating progeny \textit{in vivo}.\textsuperscript{31} It has also been inferred from the analysis of methylation patterns in human colon crypts that intestinal stem cells exhibit ACK \textit{in vivo}.\textsuperscript{32} Several studies have provided evidence that explanted hematopoietic stem cells have ACK, using various methods including time-lapse microscopy, cell growth analysis, and functional analyses including repopulation and colony formation assays.\textsuperscript{33-38} Explanted rat liver stem cells have also been demonstrated to have ACK through time-lapse microscopy, colony formation, and daughter pair analyses.\textsuperscript{39}

From \textit{in vitro} investigations of model cell systems, it has been found that asymmetric cell kinetics is dependent upon intracellular guanine nucleotide levels.\textsuperscript{40-42} Specifically, it
was shown that ACK can be activated in symmetrically cycling cells by the induction of the tumor suppressor gene p53. This occurs through p53’s down regulation of inosine-5’-monophosphate dehydrogenase (IMPDH), an enzyme that is rate-limiting for the formation of guanine ribonucleotides. Conversely, it was found that constitutive over-expression of IMPDH induces symmetric cell kinetics, even when p53 is expressed.40-44

As mentioned above, asymmetric cell kinetics also create a barrier to the expansion of adult stem cells.45,46 Because adult stem cells occur rarely in the body and may be difficult to obtain in many tissues, a small number of ASCs may need to be expanded in order to be useful for tissue and organ replacement therapies. Moreover, in order to simply study adult stem cells in vitro, the need arises to expand and propagate them over long periods of time. However, adult stem cells cycling with asymmetric cell kinetics will become progressively diluted in a cell population due to the continuous production of transit amplifying cells and terminally differentiated cells. While the total number of adult stem cells remains constant, the number of non-stem cell progeny produced increases. Over successive passages of a cell culture, the ASC fraction steadily decreases, until eventually the culture undergoes senescence, appearing to stop dividing.45,46 Thus, in order to effectively culture, study and utilize adult stem cells, it will be necessary to devise ASC expansion methods which can overcome the barrier of asymmetric cell kinetics.
Immortal Strand Co-segregation

As discussed, adult stem cells are self-renewing, capable of generating differentiating progeny, and cycle with asymmetric cell kinetics. It has been proposed that adult stem cells also possess a fourth characteristic – namely, immortal DNA strand co-segregation. This theory is described below.

Assuming that asymmetrically cycling adult stem cells are responsible for replenishing the tissues of the body throughout the lifetime of an organism, the issue of maintaining genomic fidelity arises. It has been estimated that for the intestinal epithelia alone, normal tissue maintenance requires roughly $10^{13}$ cell divisions in the lifespan of a rat, and $10^{16}$ cell divisions in a human.\(^4\)\(^7\) Spontaneous mutations are known to occur at a rate of at least $10^{-8}$ per nucleotide per generation in humans.\(^4\)\(^8\)\(^9\) Given these figures, it is expected that at least $10^{8}$ spontaneous mutations will occur over the lifetime of a human being, again in intestinal epithelial tissues alone. Similar numbers of mutations are expected to occur in other tissues with similar turnover rates. One might further predict that this overwhelming number of spontaneous mutations would inevitably lead to the development of cancer, and the serious malfunction of the cells of the body. However, a correspondingly high incidence of cancer has not been empirically observed. For instance, in the United States, the lifetime risk for the development of stomach cancer is around 1%, and under 1% for esophageal cancers.\(^5\)\(^0\) Cancers of the small intestine are even less prevalent, being diagnosed 20 times less frequently than stomach cancer in the US.\(^5\)\(^1\)
In 1975, John Cairns formulated the immortal strand co-segregation theory to explain why this high rate of spontaneous mutation does not lead to a correspondingly high incidence of cancer. He proposed that adult stem cells protect their genomic code by selectively retaining chromosomes containing “immortal” DNA template strands over successive generations.\textsuperscript{47} This nonrandom segregation of chromosomes ensures that newly synthesized DNA strands are always passed onto transit amplifying daughter cells within two divisions, while the original templates, or immortal DNA strands, remain perpetually in the stem cell. By using this mechanism, adult stem cells avoid the accumulation of spontaneous mutations arising from unrepaired replication errors.\textsuperscript{52} Instead, the spontaneous mutations, which occur on newly synthesized strands, are passed onto transit amplifying daughter cells, and eventually lost from the system due to terminal differentiation and turnover. Thus, by maintaining the genomic fidelity of adult stem cells, the overall integrity of the tissue compartment is protected.

Figure 2 shows a schematic example of immortal strand co-segregation for an adult stem cell with three chromosomes. For each chromosome, one strand, depicted in gray, is the immortal template strand. Following S phase, each of the original strands has been replicated and is paired with a newly synthesized strand. During mitosis, the theory dictates that the chromosomes containing immortal strands are non-randomly co-segregated to the adult stem cell daughter, whereas the other chromosomes go to the transit amplifying daughter. Note that in the next asymmetric cell division, the stem cell daughter will lose the rest of the strands that were synthesized in the previous cycle.
Immortal strand co-segregation

Random strand segregation

**Figure 2.** Chromosome segregation. Immortal strands are depicted in gray. Squares represent transit amplifying daughters.

Also shown in Figure 2 is one possible result of random chromosome segregation between the two daughter cells. The conventional view is that chromosomes are randomly segregated when cells undergo mitosis. We and others propose that this is not true for adult stem cells. When considering the existing evidence that immortal strand co-segregation does occur, it is useful to note the following. There are only 8 possible ways in which the three chromosomes can be divided amongst the two cells. There is a 1:4 probability that the three immortal strand-containing chromosomes will co-segregate by chance alone, and a 1:8 probability that they will co-segregate specifically to the adult stem cell daughter. Similarly, the probability that a cohort of $n$ chromosomes will specifically co-segregate by chance in a given cell division is $1:2^n$, or about 1 in $7 \times 10^{13}$ for the 46 chromosomes in a human cell. Consider that the probability that this would occur by chance repeatedly over $x$ successive divisions, decreases to $1:(2^n)^x$. Clearly, evidence for immortal strand co-segregation cannot be explained by chance.
Nonrandom chromosome segregation has been observed in various forms and contexts, since before Cairns’ formulation of the immortal strand hypothesis. Some of the first reports of nonrandom chromosome segregation came from studies of bacteria and plants, as well as mammalian cells in vitro.\textsuperscript{53, 54} It has been suggested that some mutant strains of yeast, as well as certain wild-type strains, also exhibit nonrandom chromosome segregation.\textsuperscript{55, 56} Others have searched for immortal strand co-segregation in vivo, in studies of tissues ranging from the intestinal crypt to the tongue papilla.\textsuperscript{57-61} Some of the clearest evidence for immortal strand co-segregation has been found in the study of model cell lines with inducible asymmetric cell kinetics, as described above.\textsuperscript{62}

All of the studies mentioned are similar in that they entail the labeling of DNA according to specific regimens, followed by the retrospective analysis of the cells or tissues involved. Generally, they have attempted to label immortal DNA strands, in order to use this label to observe whether the immortal DNA strands are retained in adult stem cells. The most common method of permanently labeling DNA involves the incorporation of a detectable thymidine analogue such as bromodeoxyuridine (BrdU) or tritiated thymidine (\textsuperscript{3}H-thymidine) into new strands during DNA synthesis. However, by definition, “new” immortal DNA template strands are not synthesized during normal asymmetric adult stem cell cycling. In order to observe labeled immortal DNA strands, one must first label the DNA during symmetric cell division, and then study the cells as they begin to cycle asymmetrically. When the cells switch from symmetric cell kinetics to ACK, they will begin to retain “immortal” strands, some of which will already be labeled. In the model
cell systems, this assay is made possible through the precise control of cell kinetics, as will be discussed further.\textsuperscript{62} However, \textit{in vivo}, adult stem cells should only cycle symmetrically when the stem cell pool needs to be expanded – namely, during development and wound repair. Thus in order to capture immortal strand co-segregation \textit{in vivo} using this method, scientists have either subjected animals to injury first, or studied young, developing animals.\textsuperscript{57,61} It is both desirable and fundamental, however, to also be able to study adult stem cell function during normal tissue maintenance, without causing these perturbations, and in mature tissue compartments as well as \textit{ex vivo} cell preparations.
Purpose

The purpose of this research is to develop a new assay which can be used to detect immortal strand co-segregation without necessitating the precise control of cell kinetics. This would enable the further investigation of this mechanism in systems such as adult stem cell lines, ex vivo tissue explants, and ultimately, adult stem cells in vivo in their unperturbed, asymmetrically cycling state. Such an assay would serve to substantiate and illuminate the role of immortal DNA strand co-segregation in adult stem cell biology. It would also provide further evidence to support the claim that deterministic asymmetric cell kinetics is the correct model of adult stem cell function. This derives from the fact that immortal strand co-segregation relies upon deterministic asymmetric cell kinetics in order to effectively prevent the accumulation of spontaneous mutations in adult stem cells. An assay for the detection of immortal strand co-segregation could be utilized as a new, unique method of identification of adult stem cells. Not only would this aid in the identification of stem cell niches and further definition of tissue architecture, but it may also enable the more precise isolation and purification of adult stem cell populations. The development of this assay is discussed herein.
Chapter 2 – Assay Development

Model Cell Systems

Two previously developed model cell systems, in which cell division kinetics can be controlled, \(^{40-42}\) were used to develop and test the assay for the detection of immortal strand co-segregation. These were derived from spontaneously immortalized, p53-null murine embryonic fibroblasts, which cycle with symmetric cell kinetics. The first system consists of the Zn-dependent, p53-inducible Ind-8 cell line, and its control cell line Con-3, which lacks the inducible p53 gene. When exposed to zinc, the Ind-8 cell line expresses p53, and begins to cycle with asymmetric cell kinetics. Under the same conditions, the Con-3 cell line, lacking p53, continues to cycle with symmetric cell kinetics.\(^{40,41}\) The second model system was derived from the first, by transfection of the p53-inducible cell lines with IMPDH. This resulted in the Zn-dependent, p53-inducible, constitutive IMPDH expressing tI-3 cell line, and its control, the tC-2 cell line, which lacks the constitutively expressed IMPDH gene. When p53 is induced, the tI-3 cell line continues to cycle symmetrically, due to constitutive IMPDH expression, whereas the tC-2 cell line, lacking constitutive IMPDH expression, begins to cycle asymmetrically.\(^{42}\) It is important to note that when these model cell lines cycle asymmetrically, each asymmetric division produces one stem-like daughter cell which has the potential to continue dividing, and one “transit amplifying”-like daughter, which in this system undergoes terminal arrest either immediately or after one more division, as determined by time-lapse microscopy.\(^{41}\)
Label Retention Assay

As discussed in the introduction, the method previously used to demonstrate immortal strand co-segregation required the labeling of the immortal strand, followed by the observation that the labeled immortal strands were retained in adult stem cells. This assay, referred to as the label retention assay, can be implemented in the model cell systems as described below (Figure 3).

In the label retention assay, symmetrically cycling cells are labeled with BrdU for one generation time (GT), producing cells with hemi-labeled DNA – that is, for each chromosome, one of the strands is now labeled (depicted in gray). Zinc is then added, inducing p53 expression and asymmetric cell kinetics in the Ind-8 and tC-2 cell lines (pathway A). Simultaneously, the BrdU is removed, and the cells continue to cycle for 5GT. At the point of induction of asymmetric cell kinetics, one of the two strands for
each chromosome is selected to be the immortal strand. We are currently investigating whether this initial selection of the immortal strand is random, or is always the more recently synthesized (and in this case, labeled) strand. As depicted in Figure 3, it has been shown that at least some of the strands chosen as immortal strands are labeled. Both labeled and unlabeled immortal strands are retained in the stem-like cell over the 5 successive generations of asymmetric cell division kinetics. Finally, the cells are arrested by the addition of cytochalasin D, an actin inhibitor which prevents cytokinesis, trapping the two daughter cell nuclei in a single cytoplasm. This binucleate will have unequal fluorescence, because the nucleus that was destined to become the stem cell-like daughter continues to retain some or all of the labeled immortal strands. The other nucleus contains only strands that were synthesized within the last two divisions, and are thus unlabeled.

Pathway B shows the outcome for cells that continue to cycle symmetrically even when zinc is added – such as Con-3 cells, which lack p53, and tI-3 cells, which constitutively express IMPDH. Since these cells segregate their chromosomes randomly, the label is geometrically diluted with each successive division. After 6 generations, the cells show a uniformly decreased labeling intensity across the total population, therefore no asymmetric binucleates are seen.
Label Release Assay

As discussed, in order to study immortal strand co-segregation in adult stem cells in their native state, we perceived the need for a detection method in which it is not necessary to change or manipulate the cell division kinetics. Instead of trying to label immortal strands and look for their retention, a new strategy was conceived in which non-immortal strands are labeled and observed. In this case, the captured event is the release of these non-immortal strands from the stem cell in the following cycle. This method, termed the label release assay, is shown in Figure 4.

![Label Release Assay Diagram](image)

**Figure 4.** The label release assay.

Pathway A illustrates the label release assay as it would occur in a cell that is cycling asymmetrically. For ease of reference, the immortal strands are depicted as striped bars,
but note that they are not in fact labeled. Again, the cells are labeled with BrdU for one generation, producing two hemi-labeled cells – the stem cell daughter, and the transit amplifying daughter (square). The newly synthesized labeled strands, depicted as gray bars, are not immortal. In the next generation, the BrdU is chased from the cell and cytochalasin D is added to arrest the cell. During this division, the labeled non-immortal strands generated in the previous cycle are non-randomly co-segregated to the transit amplifying daughter nucleus, while the immortal strands co-segregate to the stem cell daughter nucleus. Each of the chromosomes in either of the 2 cohorts also contains a new, unlabeled strand synthesized in the absence of BrdU during the current cycle. Thus, as depicted, an asymmetric binucleate is produced.

When subjected to the same regimen, cells cycling with symmetric cell kinetics behave in the manner depicted in Pathway B. Following the one generation BrdU labeling period, two equally hemi-labeled daughter cells are produced (only one of these is shown). When the BrdU is removed, and the cells arrested, the previously labeled strands are randomly segregated between the two daughters, producing binucleates with equally fluorescent nuclei.

In the development and optimization of the label release assay in the model cell systems, many variables were considered, including cell density, chase conditions, assay timing, and detection methods. Each of these factors can greatly influence our ability to detect immortal strand co-segregation. For instance, it is known that cell density affects cell division kinetics, both in the model cell systems, and in pre-senescence primary cell
cultures, which are likely to contain adult stem cells. In the model cell systems, the most efficient induction of asymmetric cell kinetics occurs at low cell densities. At higher cell densities, the cells become resistant to p53-induction and continue to divide exponentially. If the cell density is too low, however, the cells tend to arrest entirely. For the label release assay, the best results were observed at a density of 500 cells / 1.7 cm², the same density used for the label retention assay.

The next factor mentioned was the chase method. The goal of the chase is simply to halt the incorporation of BrdU into the DNA so that the strands synthesized during this period of the assay are unlabeled, thus allowing us to distinguish between chromosomes containing immortal strands (unlabeled) and those containing labeled non-immortal strands generated during the first cell cycle. Various chase regimens were attempted, including adding 100µM deoxythymidine (dT) to compete out the remaining BrdU; adding both 100µM dT and 100µM deoxycytidine (dC) in order to ensure that cell kinetics are not affected; and simply changing the media. Each of these methods was seen to be effective in preventing the continued incorporation of BrdU, as determined by in situ immunofluorescence. Changing the media is perhaps the simplest and most obvious method, and empirically, it enabled us to observe the most asymmetric binucleate events. However, other methods were tested because it has been observed in other contexts that changing the media, even in the continuing presence of zinc, can induce the asymmetrically cycling model cell lines to return to symmetric cell kinetics (J. Sherley and S. Ram-Mohan, unpublished observations).
Another important variable considered was the timing of the various phases of the assay. In developing the assay, we worked with unsynchronized cell populations because this most closely resembles the systems to be studied *in vivo*. However, because they are unsynchronized, not all of the cells in the population are expected to produce the same ideal outcome that is depicted in Figure 4. In the current assay configuration, the BrdU labeling period is 24 hours, immediately followed by the simultaneous chase and arrest. For example, cells in mid-S phase at the beginning of the labeling period could partially progress through a second S phase during the labeling period, thus labeling two successive generations of non-immortal strands and generating binucleate with symmetric fluorescence. In attempting to decrease the observation of such “false negative” symmetric binucleate events, the assay timing was changed in the following ways. The BrdU labeling period was shortened to 8 or 12 hours, followed by a distinct chase period of 12 or 8 hours, respectively, and finally a 16 hour arrest. In each of these attempts, however, the incidence of asymmetric binucleates detected was not increased.

The final experimental feature to be discussed here is the method of detection. The importance of this factor cannot be underestimated, given that one of the ultimate goals of this research is to develop an assay that can be used to identify adult stem cells in heterogeneous populations and tissues, in which they may occur at frequencies as low as 1 in 10,000. To accomplish this goal, the sensitivity of the detection method must be improved. Over the course of this research, different detection methods that have been investigated include the use of different BrdU-staining antibody systems, the use of vital DNA dyes as a replacement for BrdU incorporation, the use of flow cytometry to detect...
asymmetrically fluorescent binucleates, and the use of two-photon microscopy for automated image collection and data analysis. However at this stage, improved detection methods are still under development. The results presented here were collected as described in Chapter 3, Methods.
Chapter 3 – Methods

Cell Culture

The culture conditions and maintenance of the Zn-dependent, p53-inducible Ind-8 cell line and the control cell line Con-3 have been previously described. Cells were maintained as subconfluent monolayers in DMEM supplemented with 10% dialyzed fetal bovine serum (JRH Biosciences) and 5 μg/ml puromycin (Sigma), and kept at 37°C in humidified incubators with 5% CO₂. The p53-inducible, constitutive IMPDH-expressing tI-3 cell line and the control cell line tC-2 were maintained in the same way, except that the growth medium was also supplemented with 1 mg/ml geneticin (Sigma), and for the tI-3 cell line, 45 μM ZnCl₂.

Label Retention Assay

Cells were cultured on chamber slides at the density of 500 cells / 1.7 cm². 16-24 hours post-seeding, BrdU (Sigma) was added to the medium at a final concentration of 20 μM for 24 hours. p53 expression was induced by changing the medium to medium containing 65 μM ZnCl₂. After a 96 hour incubation period, cells were arrested by the addition of cytochalasin D (Sigma) at a final concentration of 2 μM for 16 hours.
Label Release Assay

Cells were cultured on chamber slides at the density of 500 cells / 1.7cm². 16-24 hours post-seeding, p53 expression was induced by changing the medium to medium containing 65μM ZnCl₂. After 24 hours, BrdU (Sigma) was added to the medium at a final concentration of 20μM for another 24 hours. The cells were then chased and arrested by replacing the media with fresh media containing 65μM ZnCl₂ and 2μM cytochalasin D (Sigma) for 16 hours.

Detection

Cells were fixed in 70% ethanol on ice for 30 minutes and stored at -20°C. Staining was done at room temperature. Slides were washed with PBS, and denatured with 2N HCl at room temperature for 10 minutes prior to staining. Blocking was performed in PBS containing 0.5% bovine serum albumin and 0.05% Tween-20 for 5 minutes. BrdU incorporation was detected by staining with a FITC-conjugated, mouse monoclonal anti-BrdU antibody (BD Pharmingen) diluted 1:10 in blocking solution for 45 minutes. Cells were counterstained with Hoechst 33258 at the concentration of 0.5μg/ml in PBS for 5 minutes. Fluorescent images were captured using a Zeiss Axiovert 100 inverted microscope, a Zeiss AxioCam CCD camera, and Openlab software (Improvision).
Chapter 4 – Results and Discussion

The primary focus of this research has been the development of the label release assay, in which the release of labeled, non-immortal strands from stem cells is captured. This assay has now been successfully used to detect immortal strand co-segregation in our p53-inducible model cell lines. In addition, new results have been obtained from the label retention assay, in which the retention of labeled immortal strands in stem cells is observed. These results will now be presented and discussed in the context of our current state of understanding.

Label Retention Assay

The label retention assay described above was previously used to demonstrate immortal strand co-segregation in the Ind-8 cell line, which cycles with asymmetric cell kinetics when p53 is induced. In independent experiments, 10% (n=100, S. Ram-Mohan, unpublished observations) and 22% (n=262) respectively, of binucleates were observed to have clearly asymmetric BrdU fluorescence in asymmetrically cycling Ind-8 cells. The latter result may have been higher due to the selection of mitotic cells prior to the initiation of the label retention assay. We now report the results of a third independent label retention experiment, in which 13% (n=67) of binucleates were observed to be asymmetric, again in asymmetrically cycling Ind-8 cells (Figure 5). In contrast, clearly asymmetric binucleates were not found in the corresponding label retention experiments performed on symmetrically cycling Con-3 cells (n>400). In these experiments, the level
of BrdU fluorescence is homogeneous across the Con-3 cell populations, in contrast to the Ind-8 cell population, in which fluorescence is heterogeneous.

![Hoescht BrdU Phase](image)

**Figure 5.** Example asymmetric Ind-8 binucleate seen in the label retention assay.

In order to further interrogate the relationship between asymmetric cell kinetics and immortal strand co-segregation, the label retention assay was also previously used to study the constitutive IMPDH expressing tI-3 cell line. No clearly asymmetric binucleates were observed for the symmetrically cycling tI-3 cells, even under the induction of p53 (S. Ram-Moham, unpublished results). The same result has now been obtained in an independent tI-3 label retention experiment. The tI-3 cell line continues to cycle symmetrically under the induction of p53 due to constitutive expression of IMPDH, and it is therefore predicted that immortal strand co-segregation does not occur. By confirming this, we have provided further evidence that immortal strand co-segregation is dependent upon asymmetric cell kinetics, and is not merely an artifact of p53-induction.
Label Release Assay

The label release assay has now been tested on all four of the model cell lines discussed. When p53 was induced, asymmetric binucleates were observed in both the Ind-8 and tC-2 cell lines, at the frequencies of 8% (n=107) and 15% (n=26), respectively (Figure 6). Asymmetric binucleates were not observed, however, in the Con-3 (n=178) and p53-induced tI-3 cell lines (n=135), which cycle symmetrically. As may be noted in Figure 6A, in some instances, although asymmetry is clearly observed, some label also appears

Figure 6. Examples of asymmetric binucleates seen in the label release assay. A,B: Ind-8 binucleates, C: tC-2 binucleate.
in the other daughter nucleus. It is hypothesized that these events may arise from cells which completed portions of two successive S phases during the BrdU labeling period, thus segregating some labeled strands with their immortal strands to the stem cell-like nucleus. An analogous feature, illustrated in Figure 6B, is that sometimes the label is not uniformly spread across the labeled nucleus, but is instead localized to a confined region. Similar labeling patterns were frequently observed in the label retention assays (J. Merok, and S. Ram-Mohan, unpublished observations), and they may have implications with regards to the mechanism by which immortal strand co-segregation is accomplished. For instance, this intranuclear co-localization or clustering of the labeled strands may imply that non-random strand co-segregation is achieved through the stable attachment of the strands to some cellular structure, such as microtubules. One must be careful to note, however that the localized staining patterns have appeared in both assays, indicating that the behavior, if it exists, may occur in both immortal and non-immortal strands.

The above results confirm that the label release assay may be used as a new method for the detection of immortal strand co-segregation without perturbing asymmetric cell kinetics. Using this novel approach, it has again been demonstrated that immortal strand co-segregation occurs in the Ind-8 cell line, and this observation has been extended to the independently-derived tC-2 cell line. To clarify, this cell line was transfected with an empty vector to serve as the control for the IMPDH-transfected tI-3 cell line. It has the same p-53 inducible asymmetric cell kinetics as the Ind-8 cell line. The results obtained from the tI-3 experiments also again reinforce the claim that immortal strand co-
segregation depends upon asymmetric cell kinetics, as distinct from p53 expression in general.

It must be noted, however, that the label release assay in its present form is not as robust as the label retention assay. Multiple label retention experiments conducted by multiple investigators have produced similar results. The results obtained from the label release assay, on the other hand, have not thus far been reproducible. Even when asymmetric binucleates have been observed, the frequency of detection has been quite low. Out of the many possible explanations for this, perhaps the most readily apparent one is that there are still technical shortcomings in the assay. Technical problems such as those involving staining and detection, the induction of asymmetric cell kinetics, BrdU incorporation, and cell arrest may be ruled out, since most of these are common elements shared with the label retention assay. The mostly likely culprits appear to be those of timing of the assay regimen, and chase conditions, two of the most important factors studied during assay development. It is still possible that chasing by changing the media disrupts the asymmetric cell kinetics, or that we have not yet found the optimal timing regimen for capturing the largest cohort of cells undergoing the label release event. Perhaps we only stumbled upon the original asymmetric binucleates events by the slimmest of margins, and the assay needs to reconfigured once again. This also suggests that the assay may require re-optimization for its use in other cell lines and systems.

Leaving technical difficulties aside, one must also consider the distinct possibility that the cells do not behave exactly as supposed. Even the results obtained from the label
retention analyses fall short of expected detection levels, if it is assumed that most cells are labeled during the labeling period and that the majority of the binucleates arise from asymmetrically cycling stem-like cells. Perhaps on the individual cellular level, asymmetric cell kinetics are not always uniformly preserved, or perhaps the immortal strand co-segregation itself is an imperfect process. Considering biology as a whole, it is quite likely that the process contains its own imperfections. However, the evidence obtained thus far from these and other analyses, including the direct physicochemical analysis of BrdU labeled immortal DNA strands (J. Lansita, unpublished observations), still strongly suggests that immortal strand co-segregation does occur, imperfections and all.
Chapter 5 – Conclusions and Future Directions

In summary, a new assay has been developed to study immortal strand co-segregation without necessitating the control of cell division kinetics. This label release assay has provided further evidence of immortal strand co-segregation in model cell systems, substantiating this in a novel way, and in an additional cell line. Although the label release assay has not yet achieved the desired robustness, it has already demonstrated that it is possible to study immortal strand co-segregation by capturing the release of labeled, non-immortal strands from stem like-cells. Now that the concept has been established, all that remains is the further optimization of the assay and its application to other systems. Indeed, preliminary investigations into the application of the label release assay to the study of pre-senescent cell cultures, adult stem cell lines, and tissues in vivo, have already begun. Specifically, the label release assay has already been applied to pre-senescent wild-type murine embryonic fibroblasts (wt MEFs), pre-senescent WI-38 human diploid fibroblasts, and early passage Lig 8 rat liver adult stem cells grown in the absence of xanthosine to stimulate asymmetric cell kinetics. Thus far, however, the wt MEFs and WI-38s have appeared to be highly sensitive to the assay conditions, and have not shown BrdU incorporation, perhaps due to growth arrest. In the Lig 8s, on the other hand, only symmetrically fluorescent binucleates have been observed (n=92). Again, further assay optimization is necessary before it can be determined whether these results are truly indicative of chromosome segregation behavior in these cell lines. Nevertheless, the label release assay is a promising candidate for a method of immortal strand co-segregation detection that can be used to study adult stem cells.
References


References


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