Investigation of Growth Factors and Cytokines that Suppress Adult Stem Cell Asymmetric Cell Kinetics

By

Michal Ganz

B.S., Biology
Massachusetts Institute of Technology, 2005

Submitted to the Division of Biological Engineering in partial fulfillment of the requirements for the degree of

Master of Science in Toxicology

at the

Massachusetts Institute of Technology

June 2005

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ABSTRACT

Adult stem cells are potentially useful in many biomedical applications that can save lives and increase the quality of a patient's life, such as tissue engineering, cell replacement, and gene therapy. However, these applications are limited because of the difficulty in isolating and expanding pure populations of adult stem cells (ASCs). A major barrier to ASC expansion in vitro is their property of asymmetric cell kinetics. Our lab has developed a method, Suppression of Asymmetric Cell Kinetics (SACK), to expand ASCs in vitro by shifting their cell kinetics program from asymmetric to symmetric. We have found that guanine nucleotide precursors can be used to convert the kinetics of adult stem cells from asymmetric to symmetric, which promotes their exponential expansion. Previously, we have used the SACK method to derive hepatic and cholangiocyte stem cell strains from adult rat livers in vitro. These cell strains provide an assay to evaluate whether growth factors and cytokines previously implicated in proliferation of progenitor cells act by converting the kinetics of the stem cells in the population from asymmetric to symmetric, and thus identify new SACK agents. We are evaluating three agents, Wnt, IGF-1, and Sonic hedgehog (Shh). Wnt has been found to cause self-renewal and proliferation of hematopoietic stem cells (HSCs) in vitro. IGF-1 also plays a role in

hematopoietic progenitor self-renewal in vivo as well as in tissue maturation. Shh has been implicated in the proliferation of primitive neural cells as well as in cellular proliferation during invertebrate development. Thus far, we have found that Wnt peptide shifts the cell kinetics from asymmetric to symmetric and may reduce the generation time, whereas IGF-1 appears only to affect generation time. Studies involving Shh are currently underway. We are also currently investigating whether Wnt acts additively or synergistically with guanine nucleotide precursors to shift cell kinetic symmetry. Discovering new SACK agents will allow us to obtain purer populations of ASCs that can be used to study properties unique to stem cells. Furthermore, the observation that Wnt shifts the kinetics of adult rat hepatic stem cells from asymmetric to symmetric implicates the involvement of similar cell kinetics symmetry mechanisms in the proliferation effect of Wnt on murine and human HSCs.

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Chapter 1 - Introduction

In the past several decades, stem cell research has come to the forefront of medical science internationally. Stem cell research has widespread potential for treating disease and studying a wide range of biological phenomena. One day, stem cell research could yield generation of tissues and organs thus alleviating the need for organ donors. Currently, the availability of organ donors is not increasing at a fast enough rate relative to the high increasing rate of patients in need of organs (Gridelli and Remuzzi, 2000). Using stem cells for organ and tissue transplants also overcomes the serious problem of transplant rejection. In addition to the significant impact that stem cells can have on organ transplants, stem cells are also a means of gene therapy. For example, hematopoietic stem cells are currently used in gene therapy because they can differentiate into numerous cell types (Wilson 1993, Brenner 1996). Furthermore, it has been determined that only a few stem cells are needed to give rise to thousands of differentiated progeny cells (Socolovsky et al. 1998). Despite the advances that have taken place in stem cell research, the molecular mechanisms of how stem cells function need to be further elucidated before the maximum potential of stem cells can be reached.

What are Stem Cells?

There are several criteria that a cell has to meet to be labeled as a stem cell: (1) Be undifferentiated, in other words lack specific markers characteristic of differentiation; (2) Able to proliferate; (3) Capable of self-renewal; (4) Able to give rise to many differentiated, functional progeny; (5) Capable of tissue regeneration following injury (Loeffler and Potten 1997). Cells that meet all of these criteria in a population are the stem cells. However, there are instances

where a cell may not meet all of the criteria, for example a stem cell can become quiescent and thus not proliferate. This cell has the potential to be a stem cell once it re-enters the cell cycle (Loeffler and Potten 1997).

Embryonic vs. Adult Stem Cells

Stem cells can be classified into two different categories, embryonic stem (ES) cells or adult stem cells (ASCs), based on the embryonic stage of development the cells are derived from. Embryonic stem cells, as the name suggests, are derived from an embryo. In humans, the embryonic stem cells are derived from the inner cell mass of the blastocyst, which develops four to five days following fertilization. These cells have the ability to give rise to any cells from any of the three germ layers in the developing embryo – mesoderm, endoderm, or ectoderm – thus, are called pluripotent.

On the other hand, adult stem cells are derived from adult tissues and are multipotent, meaning that they can only differentiate into a limited number of cell types. For example, hematopoietic stem cells (HSCs) can give rise to the various types of blood cells. Current studies have shown that stem cells from one tissue can be transplanted into a different tissue and give rise to differentiated cells characteristic of the transplanted tissue (Lagasse et al. 2000). For example, HSCs from mice have been shown to differentiate into muscle cells (Ferrari et al. 1998) and hepatocytes (Theise et al. 2000). This suggests that in certain environments adult stem cells can generate cells different than the cells from their original tissue. However, the adult stem cells could be producing differentiated cells due to fusion with cells in the transplanted tissue or because they truly are multipotent. Work done looking at how bone marrow-derived hepatocytes

repopulate the liver has strongly suggested that cell fusion between the transplanted HSCs and resident liver cells is what gives rise to hepatocytes derived from bone marrow (Wang et al. 2003, Vassilopoulos et al. 2003). Thus, it is believed that HSCs are only able to differentiate into blood cells.

Each stem cell category, embryonic and adult, provides certain advantages and disadvantages in research. There has been a lot of excitement regarding the study of human ES cells. As previously mentioned, these cells are pluripotent and their location within the developing embryo is known, making their isolation straightforward. Using well-developed culture techniques, ES cells can grow and divide indefinitely while remaining in their undifferentiated, pluripotent state (Draper et al. 2004). However, when ES cells are transplanted directly into adult tissue, they become tumorigenic (Thomson et al. 1998). Furthermore, the study of ES cells is no longer given federal funding in the United States due to debates on the ethical and societal risk of experiments with ES cells. Despite recent initiatives in some states to provide ES funding (Holden 2005), it is unclear how much progress can be made with ES cells without federal funds (Smaglik 2000).

On the other hand, ASCs do not face the restriction of federal funding. Furthermore, there are no ethical problems that arise in studying ASCs and their ability to differentiate into multiple cells types has a large implication in transplantation and gene therapy. However, certain limitations exist in studying ASCs. ASCs are multipotent, and thus cannot give rise to any cell type, unlike ES cells, which are pluripotent. Furthermore, isolating pure populations of ASCs from their resident tissues has been a major challenge. This challenge exists because ASCs are very rare in the cell population, for example, it is estimated that only 1 in 10,000 cells in the bone marrow are HSCs (Boggs et al. 1982, Spangrude et al. 1988), and due to the absence of a unique

stem cell marker (Hedrick and Daniels 2003). While purified populations of stem cells have been attained, such as with the hematopoietic system (Civin and Small 1995, Wognum et al. 2003), isolating and maintaining pure populations of stem cells has thus far eluded investigators. In most organ systems the physical niche of the ASCs are unknown, with the common exception of crypts in the small intestine (Potten and Morris 1988, Williams et al. 1992) and the hair follicle (Tumbar et al. 2004). A major barrier to expanding pure populations of stem cells in vitro is their trait of asymmetric cell kinetics (Sherley 2002), which is discussed in detail below.

Adult Stem Cell Differentiation

There are several theories that account for how stem cell cells differentiate. The first theory is the stochastic model (Loeffler and Potten, 1997; Metioli et al. 1970). This model states that the number of stem cells in the population remains stable and that each stem cell can undergo one of three types of division at random. The stem cell can divide to produce two stem cells, a stem cell and a differentiated cell, or two differentiated cells.

The second theory is a relatively recent idea that stem cells differentiate due to "transdifferentiation." Laboratories have reported that when stem cells are transferred to a different tissue than from where they were isolated, the stem cells fuse with cells residing in the tissue, giving rise to differentiated cells (Clarke and Frisen, 2001). The idea of transdifferentiation suggests that the stem cell itself changes when it is transferred to a different environment. This theory states that the stem cells differentiate after receiving signals in their local environment. As previously discussed, there is strong evidence that suggests that cell fusion

is responsible for the differentiation of adult stem cells into cells different than those found in their resident tissue.

Another theory regarding stem cell differentiation is that the stem cell itself does not actually differentiate, rather, the stem cell produces progeny cells, known as transit cells, that eventually differentiate into specialized cells (Loeffler and Potten 1997). Unlike the stem cell that it was derived from, these transit cells are not long lived in the tissue. The mechanism that leads to the formation of the transit cells is a process known as asymmetric cell kinetics, where a stem cell divides into another stem cell and a transit cell (Cairns 1975; Loeffler and Potten, 1997; Merok and Sherley, 2001).

Adult Stem Cell Kinetics

In vivo under homeostatic conditions, it has been proposed that ASCs divide with what is known as asymmetric cell kinetics (ACK) (Cairns 1975, Merok and Sherley 2001) (Fig. 1). In ACK, an ASC will divide giving rise to a daughter stem cell and a transit cell. As mentioned above, it is the transit cells that eventually divide into differentiated cells. ACK has been shown to occur in neural stem cells in vitro by using retroviral markers (Morshead et al. 1998, Wodarz and Huttner 2003). Also, by analyzing daughter and granddaughter pairs of hematopoietic stem cells (HSCs) in vitro, only one of the daughters have been found to have multiple differentiation potential, while the other daughter is differentiated (Ho 2005, Takano et al. 2004), suggesting that ACK is taking place.

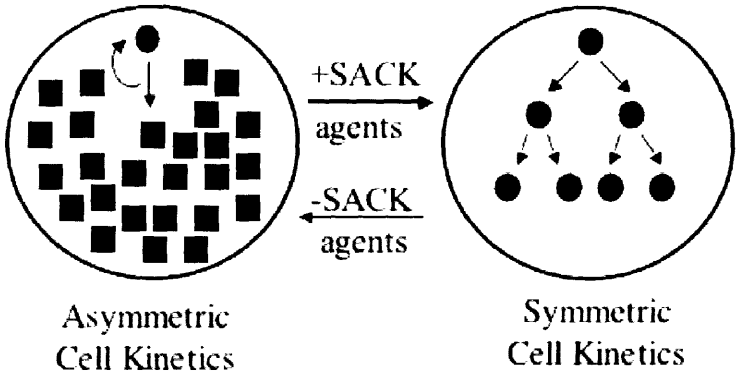

Figure 1. *Cell kinetic symmetry states of adult stem cell strain Lig-8.* In vitro, ASCs divide with asymmetric cell kinetics, resulting in the dilution of the ASC in the population by differentiated cells. Supplementing the medium with SACK agents, such as xanthosine (Xs), results in the conversion of the kinetics of the ASCs from asymmetric to symmetric, resulting in exponential growth of the ASCs. When the SACK agents are removed from the medium, the kinetics of the ASCs revert back to their asymmetric state.

ACK is an important characteristic of ASCs in vivo. By dividing with ACK in vivo, the ASCs are able to maintain a constant number, while the transit cells produce a large number of differentiated cells that repopulate the tissue. However, in vitro, ACK leads to the dilution of the number of ASCs in the population due to the large number of transit and differentiated cells produced. Thus, a loss of purity of the ASC population results. By converting the kinetics of the ASCs from asymmetric to symmetric, where two daughter stem cells are produced, the production of transit cells will be halted resulting in a more pure stem cell population.

Regulation of Adult Stem Cell Kinetics

Role of p53 in asymmetric cell kinetics

Previous work in our laboratory has shown that asymmetric cell kinetics are regulated by p53 via the guanine ribonucleotide pathway (Sherley 1991, Sherley et al. 1995, Rambhatla et al.

2001). Our lab has derived immortalized cell lines that exhibit ACK in response to controlled expression of the wild-type p53 gene (Sherley et al. 1995). Inducing p53 expression in these immortalized cell lines results in linear growth kinetics as a result of the accumulation of quiescent cells. The linear kinetics demonstrate that these cell lines are dividing asymmetrically, with each dividing cell giving rise to one daughter cell that continues to divide and one that is terminally differentiated (Sherley et al. 1995).

Our lab has found that p53 inhibits inosine-5'-monophosphate dehydrogenase (IMPDH), the rate limiting enzyme in guanine ribonucleotide synthesis (Liu et al. 1998). This inhibition by p53 causes a decrease in the guanine ribonucleotide pool resulting in asymmetric cell kinetics (Fig. 2). IMPD gene transfer into model cell lines that are p53-inducible prevents p53-dependent growth suppression (Liu et al. 1998).

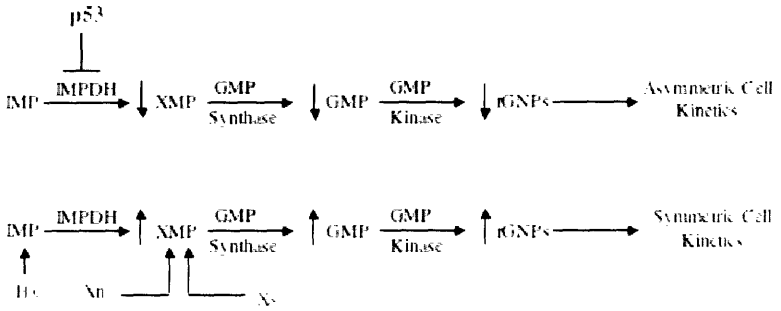

Figure 2. *Guanine ribonucleotide biosynthesis pathway responsible for asymmetric and symmetric cell kinetics.* The well-known tumor suppressor gene, p53, has been found to play a role in asymmetric kinetics of stem cells via the guanine ribonucleotide pathway. p53 acts by down-regulating expression of inosine monophosphate dehydrogenase, IMPDH, the rate-limiting enzyme for guanine nucleotide biosynthesis, resulting in a decrease in the guanine ribonucleotide (rGNP) pool. Supplementation with guanine nucleotide precursors, such as xanthine (Xn), xanthosine (Xs), and hypoxanthine (Hx), bypasses or over-rides, respectively, the regulation by p53, thus increasing the guanine ribonucleotide pools and shifting the kinetics of the ASCs from asymmetric to symmetric.

Using the SACK (Suppression of Asymmetric Cell Kinetics) method to derive model stem cell lines

Previously, our lab has developed a way to reversibly convert the kinetics of ASCs from asymmetric to symmetric, resulting in exponential expansion of the ASCs (Sherley et al. 1995, Liu et al. 1998). This method, the Suppression of Asymmetric Cell Kinetics (SACK), involves manipulation of the guanine nucleotide pathway to shift the kinetics of the cells from asymmetric to symmetric (Fig. 2). We have shown that compounds that circumvent IMPDH downregulation by p53, such as the guanine nucleotide precursor xanthosine (Xs), suppresses the p53 dependent ACK in a reversible manner (Lee et al. 2003).

We have used the SACK method to clonally expand hepatic (Lig-8) and cholangiocyte (Lig-13) cell strains from an adult rat liver (Lee et al. 2003). These strains were derived in the presence of the SACK agent Xs, and were found to be Xs-dependent for their growth. This dependence on Xs shows that the cell clones did not arise due to a growth-activating mutation, but rather that the cellular kinetics revert back to ACK when Xs is removed (Lee et al. 2003). Even though these SACK-derived strains exhibit exponential growth and a shift from asymmetric to symmetric cell kinetics, the cell culture is still heterogeneous due to a lack of complete SACK. Some transit cells are still produced, which divide and give rise to terminally differentiated cells (Lee et al. 2003).

Potential Role of Wnt, Shh, and IGF-1 in Regulation of ASC Kinetics

In addition to the p53 pathway, other pathways could also be involved in the regulation of ASC kinetics. Furthermore, other cellular factors could be part of the p53 pathway and addition of these factors could result in a change in the level of guanine nucleotides, thus changing the cellular kinetics. Several growth factors and cytokines previously implicated in the proliferation and expansion of progenitor cells could be acting to expand the population of cells by shifting the ASC kinetics from asymmetric to symmetric. If these agents do indeed have an effect on cell kinetics, they could be part of the p53 pathway or behave through a different pathway that plays a role in regulating cell kinetics.

Wnt has been reported to play a role in the self-renewal and proliferation of progenitor cells both *in vivo* and *in vitro* (Wang and Wynshaw-Boris 2004). *In vivo* studies have shown Wnt signaling is required for expansion of the progenitor cell population and the regulation of the levels of progenitors in the central nervous system of mice (Zechner et al. 2003). Furthermore, Wnt signaling has been implicated to have a critical role in proliferation and maintenance of intestinal stem cells in mice (Pinto et al. 2003, van de Wetering et al. 2002). *In vitro,* Wnt signaling has been shown to have a role in the proliferation and self-renewal of HSCs (Reya et al. 2003, Willert et al. 2003). Specifically, purified Wnt3a, the active peptide of fragment of the full-length Wnt protein, was shown to promote the proliferation and maintenance of HSCs (Willert et al. 2003). Our hypothesis is that Wnt acts to expand the progenitor pool by converting the kinetics of the cells from asymmetric to symmetric.

Sonic hedgehog (Shh), a secreted protein that was first described for its role in cell-fate determination and body-segment polarity, has also been implicated in expansion of human epithelial cells and in the proliferation of neural stem cells in the vertebrate nervous system (Fan and Khavari 1999, Wechsler-Reya and Scott 1999, Ho and Scott 2002). IGF-1, the downstream signaling molecule of Growth Hormone (GH), is known to play a role in cellular growth and proliferation throughout embryogenesis and development. For example, IGF-1 has been implicated in muscle stem cell proliferation and expansion (Deasy et al. 2002), as well as in the proliferation of hematopoietic progenitor cells (Tian et al. 1998, Kelley et al. 1996). Perhaps both Shh and IGF-1 function to expand progenitor cells by shifting cellular kinetics from asymmetric to symmetric.

Purpose

Our goal is to determine whether growth factors and cytokines previously implicated in proliferation of progenitor cells, specifically Shh, IGF-1, and Wnt3a, shift the cell kinetics from asymmetric to symmetric. We will use one of the cell strains derived by the SACK method, Lig-8, as a model to test this hypothesis. Adding these agents to Lig-8 cell media will allow us to determine what effect the compounds have on shifting the cell kinetics and on cellular proliferation. Agents that are able to change the cell kinetics from asymmetric to symmetric would be classified as new SACK compounds. Having more SACK compounds would result in more efficient exponential expansion of ASCs, and thus in the production of purer stem cell populations. These populations will allow researchers to study properties that are unique to stem cells. The SACK compounds could also explain how populations of progenitor cells are

expanded *in vivo*. If Shh, IGF-1, or Wnt3a are found to shift the kinetics of Lig-8 cells from asymmetric to symmetric, we would hypothesize that they act to expand progenitor cells by shifting their cell kinetics program.

Chapter 2 - Methods

Cell Culture

The derivation of the hepatic ASC cell strain Lig-8 has been described in detail previously (Lee et al. 2003). Lig-8 cells were maintained in DMEM (high glucose, 4500 mg/L) supplemented with 10% dialyzed fetal bovine serum (DFBS; JRH Biosciences) and 400 μM xanthosine (Xs) (Sigma Chemical Co., St. Louis, MO). The cells were maintained in 37°C humidified incubators with 5% CO_2.

Growth Curve Assay

Lig-8 cells were grown to 1/4 confluency in DMEM + 10% DFBS + 400 μM Xs, then had their medium replenished to allow for logarithmic growth. 24 hours later when the flask was approximately 1/2 confluent, the cells were trypsinized and seeded into 25 cm^2 flasks. The following day, the media was changed to contain varying concentrations of either Xs or recombinant mouse sonic hedgehog (Shh) (R&D Systems, Minneapolis, MN). The cells were then cultured for another 48 hours. At the end of the 48-hour period, adherent cells were removed with trypsin, combined with Coulter counting solution to a total volume of 20.5 ml, and counted using an electronic cell counter (Coulter Electronics, model Zl).

Microcolony Progression Analyses

Lig-8 cells growing in logarithmic phase in standard culture medium (DMEM + 10% DFBS + 400 μM Xs) were plated at the density of 3560 cells / 8.6 cm^2 in 4 ml of medium. After 2 hours for cell attachment, the culture medium was replaced in all slides with control (Xs-free) medium or medium supplemented with Xs, Shh, recombinant mouse IGF-1 (R&D Systems, Minneapolis, MN), or recombinant mouse Wnt3a (R&D Systems). Medium-replenished cells were cultured for 20 hours, equivalent to the Lig-8 generation time. At the end of the 20-hour period, 5-Bromo-2'-deoxyuridine (BrdU) (Sigma Chemical Co.) was added to 5 μM by adding 10 μl of a stock of 1 mM BrdU made up in PBS directly to the slides, and cells were cultured for either an additional 4 or 24 hours.

Following the BrdU incubation period, cells were fixed in 70% ethanol on ice for 30 minutes and stored at -20°C in a dark, foiled box for no more than one week. Thereafter, immunofluorescence detection procedures were performed at room temperature. Slides were washed for one minute in coplin jars with phosphate buffered saline, pH 7.4 (PBS), followed by denaturation with 2M HCl at room temperature for 10 minutes. Fixed, DNA-denatured cells were blocked with PBS containing 0.5% bovine serum albumin and 0.05% Tween-20 (= blocking solution) for 10 minutes. To detect incorporated BrdU, blocked cells were incubated for 2 hours at room temperature with mouse anti-BrdU monoclonal antibody (MAB 3424; Chemicon International, Temecula, CA) diluted 1:100 in blocking solution. Slides were then washed three times for five minutes each time in blocking solution. The wash was followed by a 45 minute incubation with FITC-conjugated rabbit anti-mouse IgG antibody (F0232; Dako, Carpinteria,

CA) diluted 1:200 in blocking solution. Slides were then washed three times for five minutes each time in blocking solution, then three times for three minutes each time in PBS. Thereafter, slides were stained for 10 minutes with 5 μg/ml propidium iodide (PI) in PBS to detect nuclei. Slides were stored in a dark, foiled box in -20°C overnight before image analysis.

A laser scanning cytometer (LSC; CompuCyte Model 090-0017-001, Cambridge, MA) equipped with a 480 nm Argon-Ion Laser (Cyonics Uniphase 2014A-20SL, San Jose, CA) and WinCyte software (Cambridge, MA) was used to detect cells with FITC (incorporated BrdU) and PI (nuclear DNA) fluorescence. Fluorescent images were captured using a Zeiss microscope, a Zeiss AxioCam CCD camera, and Openlab software (Improvision; Lexington, MA). Quantitative analyses for cells labeled with BrdU for 4-hours were performed for microcolonies containing at least one BrdU-labeled cell.

Chapter 3 - Results and Discussion

Optimization of SACK in Population Growth Kinetics Studies

The adult rat hepatocyte stem cell strain Lig-8 provided an approach for improving the SACK method. These cells could be used to develop assays for determining the optimal concentration of known SACK agents (e.g., Xs) and to identify other classes of compounds or cellular factors with SACK activity. As a first assay, we evaluated population cell kinetics of Lig-8 cells. Shifts from asymmetric cell kinetics to symmetric cell kinetics can be detected and quantified by determining the growth rate of cultured cell populations. If there is a shift from ACK to SCK, a reduction in population doubling time is observed. Population cell kinetics assays were performed to determine the optimum concentration of Xs for SACK and to evaluate the SACK activity of the cellular cytokine sonic hedgehog (Shh).

Xs, a SACK agent, was used to derive a hepatic, Lig-8, stem cell strain from an adult rat liver. This strain retained the ability to divide asymmetrically when Xs was removed (Lee et al. 2003). The concentrations of 200 μM and 400 μM Xs used to derive the strain were chosen in an arbitrary manner and were not optimized to obtain the greatest shift of cell kinetics from asymmetric to symmetric. To determine the concentration of Xs that resulted in the greatest cell number, we performed a population growth kinetics study. We found that 1 mM Xs maximizes the exponential growth of Lig-8 cells (Fig. 3A). The change in cell number in the presence of 1 mM Xs cannot be explained by a decrease in generation time, because Xs has been shown to have no effect on changing the cell cycle time of Lig-8 cells (unpublished data). Thus, we

conclude that 1 mM Xs acts to maximize the shift from asymmetric to symmetric kinetics. Increasing the Xs concentration did not appear to change the cell number relative to the 400 μM that was used to derive the Lig-8 cells (Fig. 3A). At the end of the growth period, almost all of the cells were adherent, thus very few cells were not counted in the analysis due to the procedure.

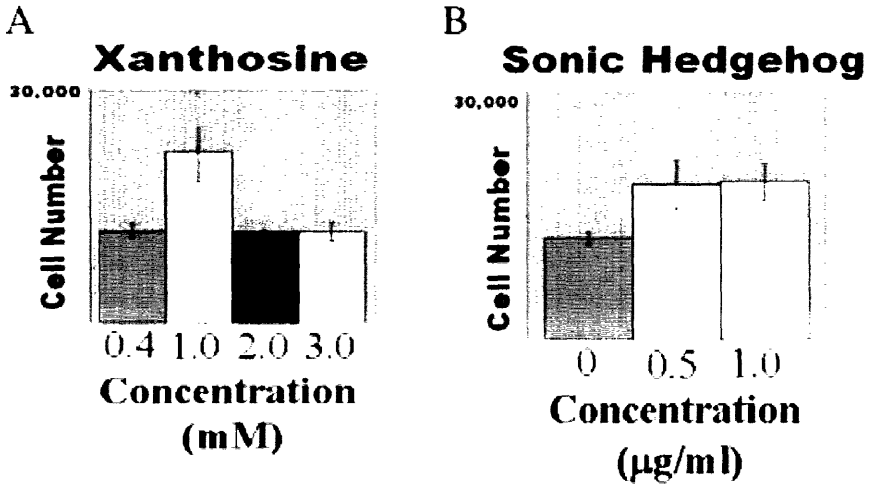

Figure 3. *Cell kinetics analyses of Xs and Shh with adult stem cell strain Lig-8.* Cell cultures were established as described in the *Methods* section. Twenty-four hours after the cells were plated, the medium was changed to contain the indicated concentration of Xs **(A)** or Shh **(B)**, and the cultures were allowed to grow for another 24 hours. Experiments were performed in triplicate, and the mean cell numbers at each tested concentration are shown. Error bars represent the standard deviation of triplicate data.

Using the same assay, we examined the effect of Shh on the growth of Lig-8 cells (Fig. 3B). We looked at two different concentrations of Shh, 0.5 μg/ml and 1.0 μg/ml. It appears that both concentrations act to increase the number of Lig-8 cells. This result suggests that Shh shifts the kinetics of the cells from asymmetric to symmetric or decreases the generation time of the cells. Both scenarios would explain the increase in cellular proliferation observed in the presence of Shh. Assays that are more specific in detecting changes in cell kinetics need to be performed to determine what is causing an increase in cellular proliferation.

Microcolony Progression Analyses

Performing microcolony progression analyses provide data not found by growth kinetic studies about both the proliferation and kinetics symmetry of Lig-8 cells in the presence of three growth factors, Wnt3a, IGF-1, and Shh, in addition to 1 mM Xs. Three different forms of the assay were used to study the behavior of the cells. First, a microcolony cell kinetics assay was used to infer asymmetric cell kinetics by examining the ratio of 3-cell to 4-cell microcolonies. Second, a microcolony labeling kinetics assay was performed where labeling statistics were used to determine whether asymmetric or symmetric cell kinetics are taking place. Third, a sister-pair labeling kinetics analyses was used as direct evidence for asymmetric cell kinetics taking place. All three forms of the assay allow us to detect both the cycling (BrdU-positive) and non-cycling (BrdU-negative) cells in the population, as well the size of the colonies present for each condition. Using the Laser Scanning Cytometer (LSC) we could detect the cells labeled with BrdU and perform quantitative analyses with the colonies containing at least one labeled cell. Examples of captured images by the LSC and epifluorescence microscopy are shown in Fig. 4.

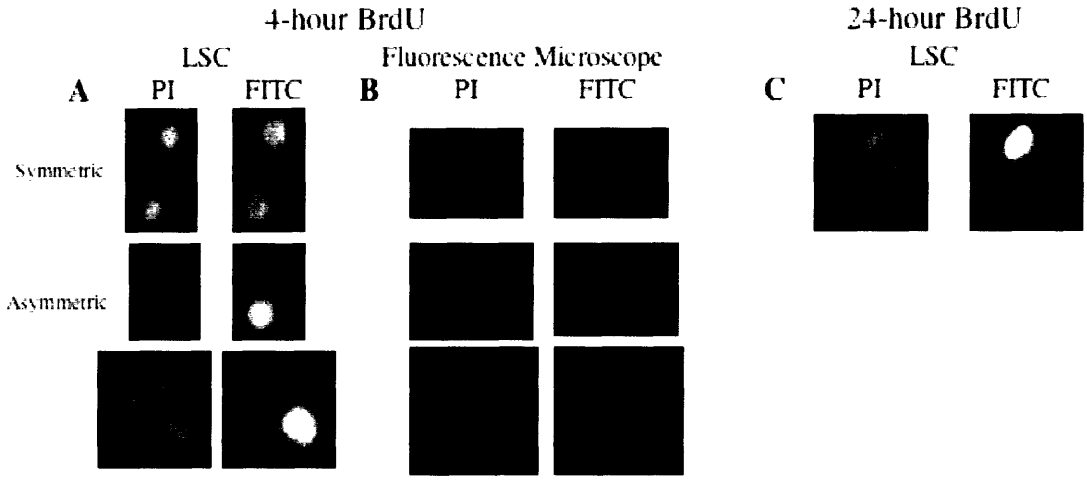

Figure 4. *Images of BrdU-labeled microcolonies captured by laser scanning cytometry (LSC) and fluorescence microscopy.* (A-B) 4-hour BrdU-labeling in a microcolony progression analysis detects BrdU-positive cycling S-phase cells. **(A)** Images captured by LSC, **(B)** Epifluorescence images. FITC: anti-BrdU immunofluorescence. PI: propidium iodide fluorescence to detect nuclear DNA. Symmetric refers to images indicative of cells dividing with symmetric cell kinetics, whereas asymmetric refers to images indicative of asymmetric cell kinetics. **(C)** Demonstration that after a 24-hour BrdU-labeling period stable non-cycling cells (unlabeled) are present in microcolonies (LSC images).

In order to determine what the unlabeled cells represent in the 4-hour BrdU-labeling study, a microcolony labeling kinetics assay was performed. In this assay, BrdU was added for 24 hours, more than a full generation period of Lig-8 cells. By determining the labeling statistics of Lig-8 colonies, conclusions can be drawn regarding whether cells that are unlabeled after a 4-hour BrdU labeling period remain unlabeled after a 24-hour BrdU labeling period. Under the conditions for these experiments, unlabeled cells correspond to the non-cycling sisters of asymmetric cell divisions. Thus, we can determine if there is asymmetric cell kinetics in the culture. We found that some of the non-cycling cells, detected as BrdU-negative after the 4-hour BrdU-labeling period, are stably arrested as can be seen in Fig. 4C. Furthermore, the percentage of non-dividing cells in colonies with at least one labeled cell was about 9% more for the control,

no-Xs condition than when cells were grown in the presence of Xs (1 mM). There were about 17% more 3 or 4-cell colonies in the control, no-Xs condition that had at least one unlabeled cell than in the presence of Xs, suggesting that more asymmetric divisions are taking place in Xs-free culture. The change in percentage of unlabeled cells is not as striking when looking at colonies of all sizes with at least one unlabeled cell because once colonies are greater than five cells, they would have had to arise due to symmetric divisions and thus all of the cells would be labeled (Table 1).

	No Xs	1 mM Xs
No label (Differentiated)	9.90%	0%
At least one cell unlabeled (All colonies)	37.80%	29.30%
At least one cell unlabeled (3 and 4 cell colonies)	47.20%	30.00%
% BrdU Positive of Total	82.8%	91.7%

Table 1. *24-hour BrdU-labeling kinetics analysis demonstrates that non-labeling cells produced in Lig-8 cell culture are stably arrested cells.* Two hours after Lig-8 cells were plated into slides, as detailed in *Methods*, the medium was changed to contain the indicated supplement, and culture continued for 20 hours. Cells were then labeled with BrdU for 24 hours. BrdU-labeling data were collected for microcolonies with greater than two cells. Cells that remain BrdU-negative during a 24-hour labeling period (equivalent to one cell generation time) are stably arrested in a non-S phase of the cell cycle.

Microcolony cell kinetics implicate Wnt3a and Shh as effectors of cell kinetics symmetry

To determine whether supplementation with growth factors or 1 mM Xs affected cellular proliferation, microcolonies with greater than two cells that had at least one BrdU-positive cell

were scored for their total of BrdU-positive cell percentage. This percentage reflects the S-phase fraction, and is an indicator of the cycling cell fraction. As a group, Xs, IGF-1, Wnt3a, Shh, and Xs+Wnt3a, show a significant increase in cellular proliferation by this measure (Fig. 5). To examine whether the increase in the cycling cell fraction was due to a decrease in generation time or a shift in cell kinetics from asymmetric to symmetric, we performed a microcolony cell kinetics analysis.

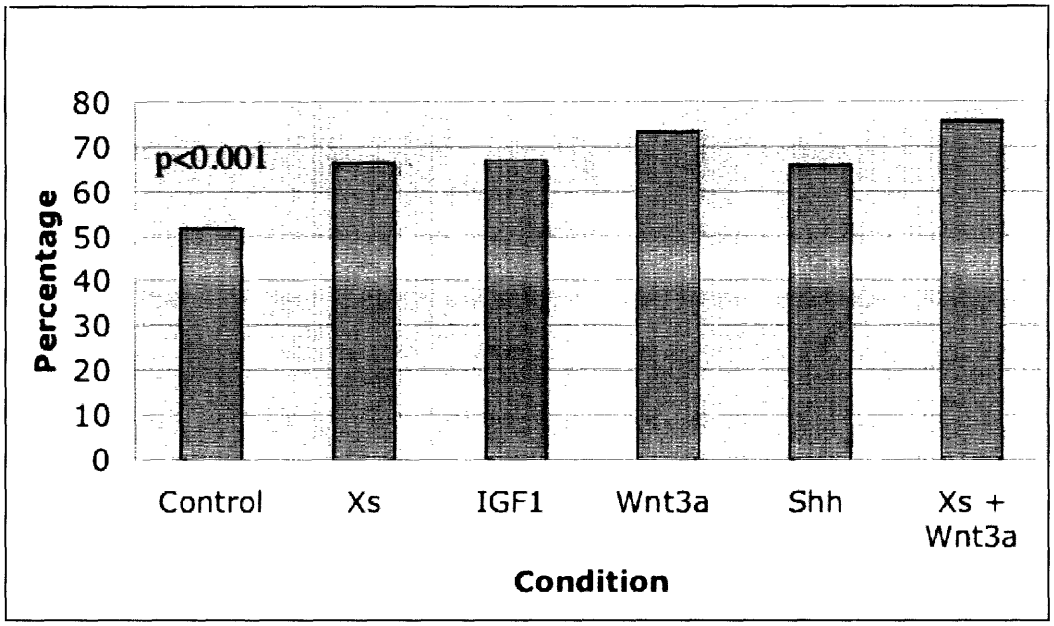

Figure 5. *Xs and the growth factors confer increased proliferation by Lig-8 cells.* Two hours after cells were plated into slides, as detailed in *Methods*, the medium was changed to medium supplemented with the indicated factors, and the cells were cultured for an additional 20 hours. BrdU was then added, and slides were cultured for 4 hours. Colonies with greater than two cells and at least one BrdU-labeled cell were scored for their percentage of BrdU-positive cells. The size of scored microcolonies range from 2 cells to 10 cells. Effects of supplementation with Xs (1 mM), IGF-1 (1 μg/ml), Wnt3a (25 ng/ml), Shh (0.5 μg/ml), and the combination of Xs (1 mM) and Wnt3a (25 ng/ml) on the percentage of BrdU-positive cells were compared to the control Xs-free condition. The p value indicates the significance of the increase in cellular proliferation of the growth factors and Xs as a group compared to the Xs-free control.

In the microcolony cell kinetics analysis, we compared the frequencies of 3 and 4-cell microcolonies to that of 2-cell microcolonies for each of the different conditions relative to the Xs-free control (Fig. 6).

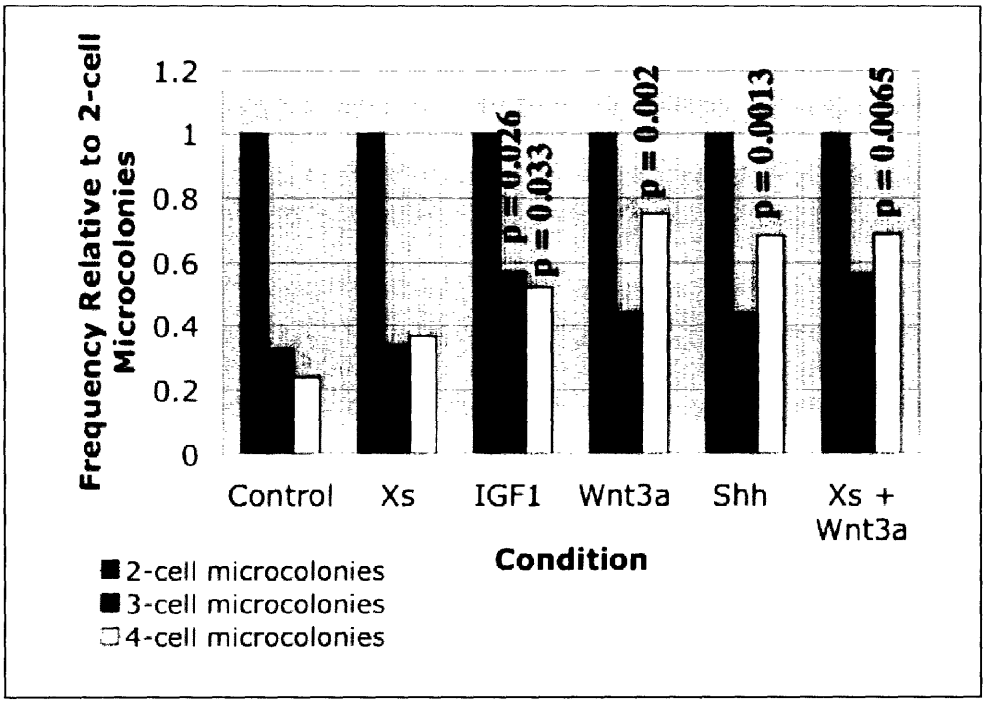

Figure 6. *Growth factors significantly change the relative frequencies of 2-cell, 3-cell, and 4-cell microcolonies of Lig-8 cells in microcolony cell kinetics analyses.* Two hours after cells were plated into slides, as detailed in *Methods*, the medium was changed to contain the indicated compounds, and cells were cultured for an additional 20 hours. Cells were then incubated with BrdU for 4 hours. The graph depicts the relative frequencies of 2-cell, 3-cell and 4-cell microcolonies produced for the compared medium supplementations. For each respective medium supplementation, tallied microcolony numbers were normalized to the 2-cell microcolony number. The different supplementations were Xs (1 mM), IGF-1 (1 μg/ml), Wnt3a (25 ng/ml), Shh (0.5 μg/ml), and the combination of Xs (1 mM) and Wnt3a (25 ng/ml). p values are reported for cases of statistically significant ($p < 0.05$) changes in the pattern of microcolony frequencies compared to the control pattern based on Fisher's exact test.

Quantification of cell kinetics symmetry effects of Xs, Wnt3a, and Shh by microcolony labeling kinetics analyses

We used microcolony labeling kinetics analyses to examine the labeling patterns of 3 and 4-cell microcolonies to give us a better understanding as to how each agent is affecting the proliferation and kinetics of Lig-8 cells. In this assay, the cells were labeled with BrdU for 4-hours and the labeling statistics of 3 and 4-cell microcolonies were evaluated. Analysis of BrdU-labeling in these microcolonies can provide information as to whether the microcolonies arose by ACK or SCK, and whether the growth factors had an effect on the cell kinetics symmetry.

First, we looked at 3-cell microcolonies, which can be made by either ACK or SCK (Fig. 7). The most likely way to obtain a 3-cell microcolony with only one BrdU-positive cell is by ACK. In ACK microcolonies, only one cell is cycling. When this cell is in S-phase during the 4-hour BrdU-labeling period, after two previous divisions, a single labeled cell with two unlabeled cells results (Fig. 7A). For SCK microcolonies, the 3-cell stage is infrequent because of the synchronous cell cycle transit of sister cells. In order to detect a 3-cell SCK microcolony with one cell labeled, the cell cycle composition must be two cells in G1 phase and one cell in late S/G2/M (Fig. 7A). This cell cycle composition will be highly transient. Thus, 3-cell SCK microcolonies with one BrdU-positive cell are predicted to be infrequent.

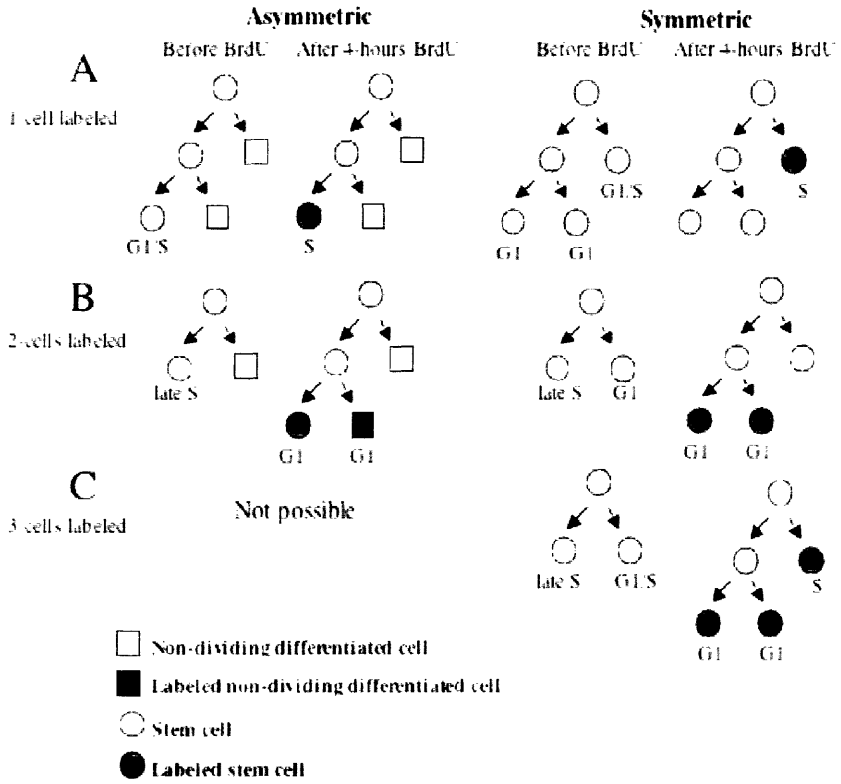

Figure 7. *BrdU-labeling kinetics modeling for 3-cell microcolonies generated by asymmetric cell kinetics (ACK) and symmetric cell kinetics (SCK) after 4 hours of BrdU-labeling.* Three-cell microcolonies obtained in the microcolony progression analyses can be generated by either ACK or SCK. Models are shown for how 3-cell microcolonies with **(A)** 1 BrdU-labeled cell, **(B)** 2 BrdU-labeled cells, and **(C)** 3 BrdU-labeled cells can be produced by ACK or SCK. At the time of inspection, BrdU-labeled cells are either in S phase or have arisen from a cell that was in late S-phase during the BrdU-labeling period. The latter case yields two BrdU-labeled daughter cells in G1. For each model, the types of cells in the microcolony and the place of each cell in the cell cycle, before and after the 4-hour BrdU-labeling period, are depicted. **Circles**, cycling adult stem cells; **squares**, cell cycle-arrested differentiating progeny cells; **filled symbols**, BrdU-positive.

Three-cell microcolonies with two BrdU-positive cells are also predicted to occur more frequently as a result of ACK. For ACK, this occurs when, after a first division, a cycling cell is in late S-phase when BrdU was added. Its second division before analysis will yield two labeled cells and one unlabeled cell (Fig. 7B). This event will be significantly less frequent than detecting a single cycling cell in 3-cell ACK microcolonies. For SCK, during a 4-hour labeling

period, it is unlikely that one newly divided sister will label in late S-phase and divide, before its sister, to produce two labeled cells and one unlabeled cell (Fig. 7B). Similarly, it is unlikely that the two progeny of one divided sister will both be in S-phase before the other undivided sister.

For all of the cells in the 3-cell colony to be BrdU-positive, SCK must be responsible, because all of the cells retain the ability to cycle (Fig. 7C). In contrast, in the case of ACK at least two of the cells produced are non-cycling differentiating cells. Thus, 3-cell colonies with one or two cells labeled indicate primarily ACK, but are not exclusive of SCK; and 3-cell microcolonies with 3 BrdU-positive cells are a specific indicator of SCK.

Analysis of labeled 3-cell microcolonies for the number of BrdU-labeled cells showed that all supplements, except Xs+Wnt3a, showed a significant increase in the fraction of 3-cell microcolonies with three labeled cells compared to the fraction with one or two labeled cells (Fig. 8). Supplementations were 1 mM Xs, 1 μg/ml IGF-1, 25 ng/ml Wnt3a, 0.5 μg/ml Shh, and 1 mM Xs + 25 ng/ml Wnt3a. The largest increase was observed in the presence of Wnt3a and Shh. This shift in representation is consistent with a shift from ACK to SCK. However, it might also be due in part to a decrease in the cell cycle time of SCK or ACK microcolonies. Interestingly, Xs appeared to antagonize the effect of Wnt3a.

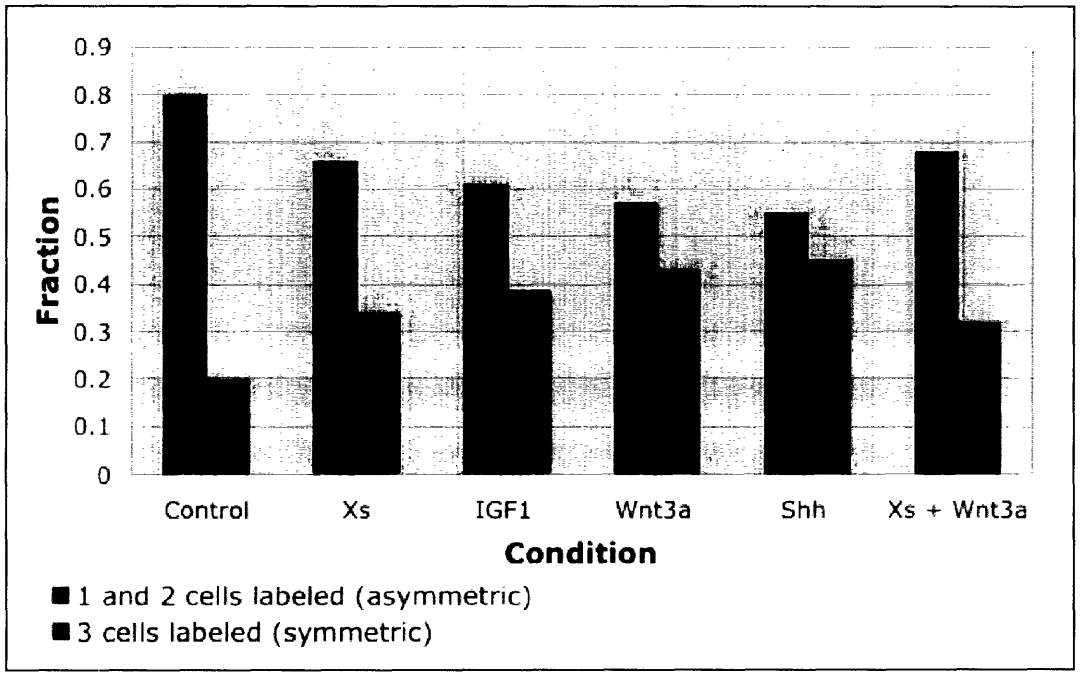

Figure 8. *BrdU-labeling kinetics of 3-cell microcolonies indicate factor-induced shifts by Lig-8 adult stem cells from asymmetric cell kinetics to symmetric cell kinetics.* Two hours after cells were plated into culture slides, the medium was changed to contain the indicated supplement, and culture was continued for 20 hours. Cells were then labeled with BrdU for 4 hours. BrdU-labeled 3-cell microcolonies were evaluated for the number of labeled cells. Three-cell microcolonies with 1 or 2 BrdU-labeled cells, indicating ACK, were compared to 3-cell microcolonies with 3-cells labeled, which represents SCK (explained in Figure 7 and text). The different supplementations were Xs (1 mM), IGF-1 (1 μg/ml), Wnt3a (25 ng/ml), Shh (0.5 μg/ml), and the combination of Xs (1 mM) and Wnt3a (25 ng/ml). The average number of microcolonies examined for each supplementation conditions was 33 (range from 25 to 53).

Similar analysis was peformed with 4-cell microcolonies, which can also alrise by ACK or SCK (Fig. 9). 4-cell microcolonies with only one BrdU-positive cell are most likely to be due to ACK. Such ACK microcolonies are composed of the products of 3 asymmetric divisions, 3 non-cycling cells and one cycling stem cell in S phase during the labeling period (Fig. 9A). It is also possible for such 4-cell microcolonies with a single labeled cell to be produced by SCK. However, this requires that only one of the four cycling products of two successive symmetric

cell divisions be in S phase during the 4 hour labeling period (Fig. 9A). This degree of symmetric sister asynchrony is unlikely. Thus, 4-cell microcolonies with only one labeled cell are highly indicative of ACK.

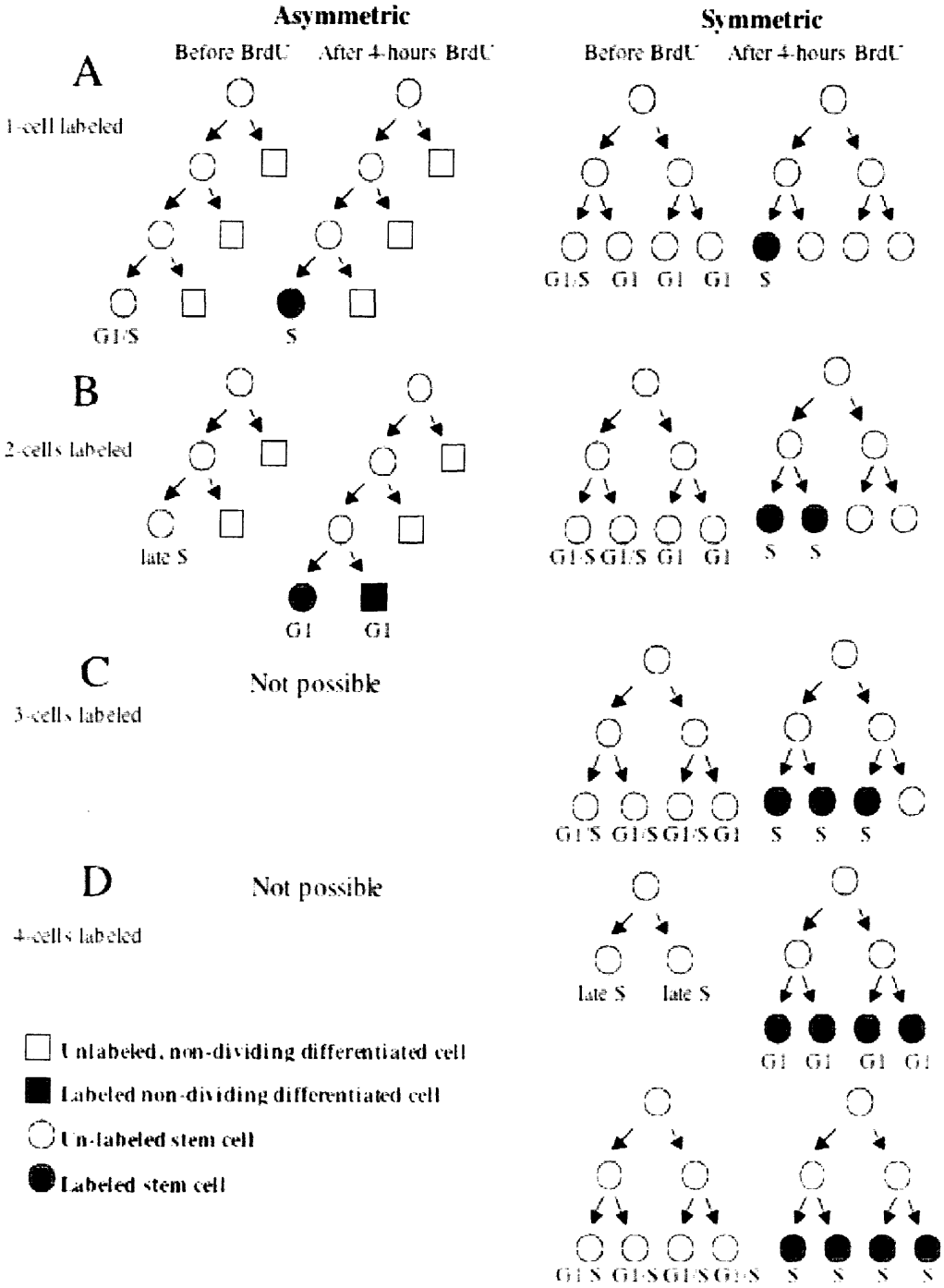

Figure 9. *BrdU-labeling kinetics modeling for 4-cell microcolonies generated by asymmetric cell kinetics (ACK) and symmetric cell kinetics (SCK) after 4 hours of BrdU-labeling.* Four-cell microcolonies obtained in the microcolony progression analyses can be generated by either ACK or SCK. Models are shown for how 4-cell microcolonies with **(A)** 1 BrdU-labeled cell, **(B)** 2 BrdU-labeled cells, **(C)** 3 BrdU-labeled cells, and **(D)** 4 BrdU-labeled cells. At the time of inspection, BrdU-labeled cells are either in S phase or have arisen from a cell that was in late S-phase during the BrdU-labeling period. The latter case yields two BrdU-labeled daughter cells in G1. For each model, the types of cells in the microcolony and the place of each cell in the cell cycle, before and after the 4-hour BrdU-labeling period, are depicted. **Circles**, cycling adult stem cells; **squares**, cell cycle-arrested differentiating progeny cells; **filled symbols**, BrdU-positive.

4-cell microcolonies with two BrdU-positive cells can be explained by either ACK or SCK. For ACK, this occurs when, after a second division, a cycling cell is in late S phase when BrdU was added. This cycling cell will divide before analysis and will yield two labeled cells, one that will continue to cycle and one that arrests, resulting in a 4-cell microcolony with two labeled and two unlabeled cells (Fig. 9B). For SCK, obtaining a 4-cell microcolony with two BrdU-positive cells occurs when, after a second division, one of the two sister pairs has reached S phase while the other pair is still in G1 (Fig. 9B). With a 4-hour labeling period this occurrence is minimized, but not completely avoided. Thus, this 4-cell microcolonies with only 2 cells labeled are not informative for distinguishing cell kinetics symmetry.

4-cell microcolonies with either three or four BrdU-positive cells can only arise by SCK. To observe three BrdU-positive cells in a 4-cell SCK microcolony, following two successive symmetric divisions, three out of the four cells must enter S phase, while the fourth cell is still in G1 (Fig. 9C). This event is predicted to be infrequent because of the synchronous cell cycle transit of symmetric sister cells, but it will occur occasionally. Four BrdU-positive cells will arise frequently when all four sisters of two successive symmetric cell divisions are simultaneously in S phase or when two sisters in late S phase both divide during the labeling period (Fig. 9D). In contrast, in all scenarios for ACK, at least two of the cells in the 4-cell microcolony must have

been stably arrested during the labeling period. Therefore, three labeled cells or four labeled cells are not possible for 4-cell ACK microcolonies. Thus, 4-cell microcolonies with three or four BrdU-positive cells are specific indicators of SCK.

Labeled 4-cell microcolonies for each of the indicated conditions were evaluated for the number of BrdU-positive cells. The average number of microcolonies examined for each condition was 43. Supplementations were the same as for 3-cell microcolonies. With the exception of IGF-1, all supplements showed a significant increase in the fraction of 4-cell microcolonies with three or four cells labeled, and a corresponding decrease in the fraction with a single labeled cell (Fig. 10). This shift is consistent with a shift from ACK to SCK. Although IGF-1 caused a decrease in the fraction of 4-cell microcolonies with a single labeled cell, it did not significantly increase the fraction with three or four labeled cells. This difference is consistent with IGF-1 having a primary effect of decreasing generation time. It is noteworthy that Xs and Wnt3a together showed evidence of synergy in this assay.

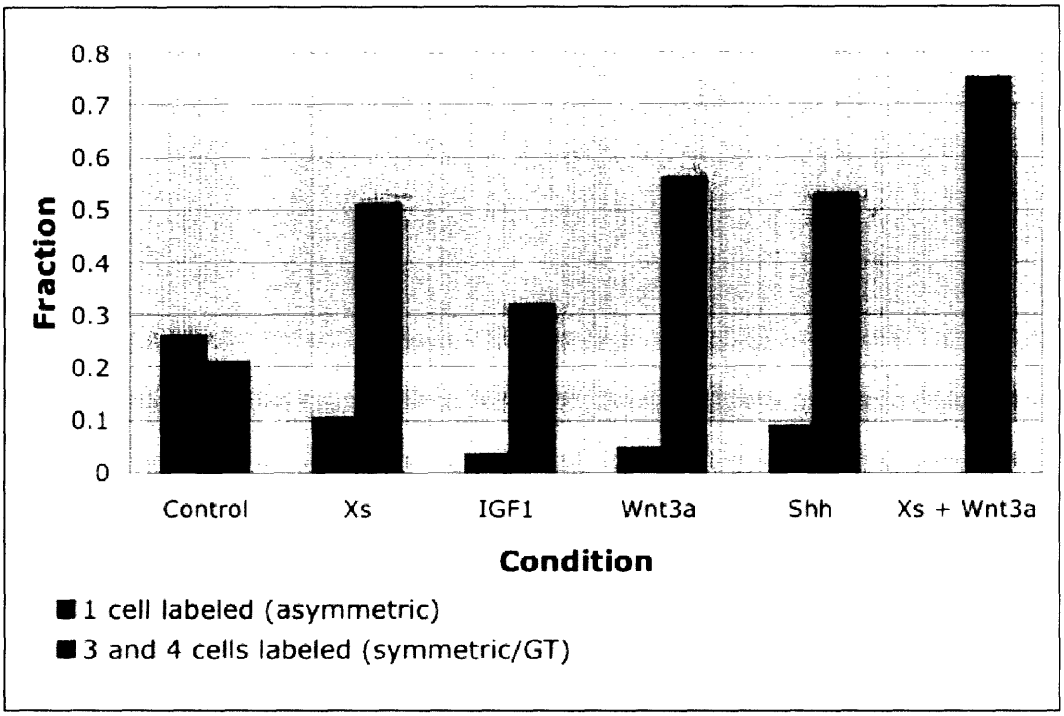

Figure 10. *BrdU-labeling kinetics of 4-cell microcolonies indicate factor-induced shifts by Lig-8 adult stem cells from asymmetric cell kinetics to symmetric cell kinetics.* Two hours after cells were plated into culture slides, the medium was changed to contain the indicated supplement, and culture was continued for 20 hours. Cells were then labeled with BrdU for 4 hours. BrdU-labeled 4-cell microcolonies were evaluated for the number of labeled cells. Four-cell microcolonies with 1 labeled cell, indicating ACK, were compared to 4-cell microcolonies with 3 or 4 labeled cells, indicating SCK (explained in Figure 9 and text). The different supplementations were Xs (1 mM), IGF-1 (1 μg/ml), Wnt3a (25 ng/ml), Shh (0.5 μg/ml), and the combination of Xs (1 mM) and Wnt3a (25 ng/ml). Four-cell microcolonies with 2 BrdU-labeled cells are not shown, because 2 labeled cells could be obtained by either SCK or ACK and thus do not provide a basis for kinetics symmetry discrimination. The average number of microcolonies evaluated was 43 (range 23 to 63) for each supplementation condition.

Direct evidence that Wnt3a is a potent SACK agent

The most specific measure for looking at ACK versus SCK is a sister-pair labeling kinetics assay. This assay provides direct evidence of asymmetric cell kinetics taking place in the culture. We found the number of double-positive sister-pairs, which represent primarily SACK,

as well as the number of single-positive sister-pairs, which indicate primarily ACK for each condition following a 4-hour BrdU labeling period. We then determined the ratio of double-positive to single-positive sister pairs for each condition (Fig. 11). An increase in the ratio of double-positive to single-positive sister pairs is indicative of a shift of cells from ACK to SCK, i.e., suppression of asymmetric cell kinetics (SACK). We find that Wnt3a or Xs+Wnt3a induced significant SACK. Under the conditions of this experiment, Xs+Wnt3a appear to act additively. This result contrasts the apparent antagonistic effect of Xs on Wnt3a in 3-cell microcolony labeling kinetics analyses (see Fig.8) and synergistic effect in 4-cell microcolony labeling kinetics analyses (see Fig. 10). In this sister-pair labeling kinetics analysis, Xs and Shh exhibit mild SACK effects. However, consistent with a primary effect on generation time, IGF-1 showed the smallest effect on the double-positive to single-positive sister pair ratio. Therefore, the effect of IGF-1 to increase the labeled 3-cell and 4-cell microcolonies (see Fig. 8 and Fig. 10) may be due to its induction of more rapidly cycling ACK and/or SCK microcolonies.

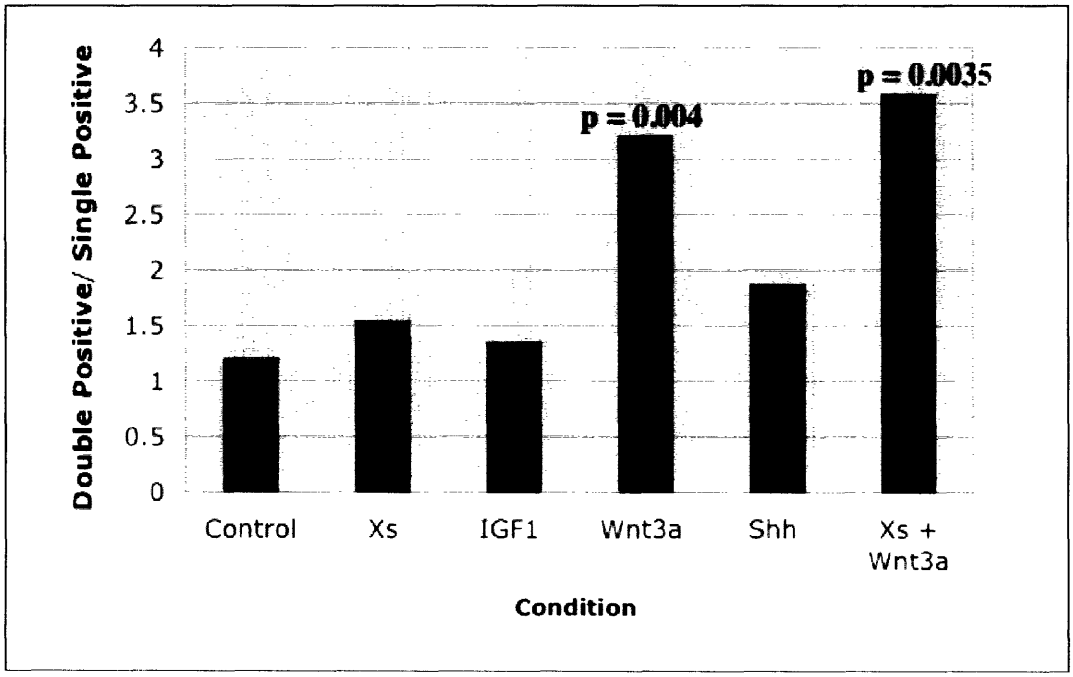

Figure 11. *Wnt3a shows significant shifts from asymmetric cell kinetics to symmetric cell kinetics in sister-pair BrdU-labeling kinetics analyses.* Two hours after Lig-8 cells were plated into slides, as detailed in *Methods*, the medium was changed to contain the indicated supplement, and culture continued for 20 hours. Cells were then labeled with BrdU for 4 hours. The different supplementations were Xs (1 mM), IGF-1 (1 μg/ml), Wnt3a (25 ng/ml), Shh (0.5 μg/ml), and the combination of Xs (1 mM) and Wnt3a (25 ng/ml). Shown is the ratio of double-positive (both sister cells BrdU-labeled) to single-positive (only one sister cell BrdU-labeled) sister pairs for each supplementation condition. Sister-pairs that are both BrdU-labeled result from symmetric cell kinetics, whereas sister-pairs with only one BrdU-labeled sister cell signify asymmetric cell kinetics. p values are reported for cases in which the double-positive to single-positive ratio was increased significantly ($p < 0.05$) compare to control ratio by Fischer's exact test.

Conclusions

We use microcolony progression analyses to determine whether growth factors and cytokines previously implicated in proliferation of progenitor cells behave by converting cell kinetics from asymmetric to symmetric. Using this analysis, we conclude that 1 mM Xs, Wnt3a, Shh, and IGF-1 increase the cellular proliferation of Lig-8 adult rat hepatic stem cells. It appears that Wnt3a increases proliferation primarily by shifting cell kinetics from asymmetric to symmetric, thus implicating Wnt3a as a new SACK agent. The combination of Xs and Wnt3a suggests interactive effects on the kinetics symmetry of 3-cell and 4-cell microcolonies, based on microcolony cell kinetics and labeling kinetics analyses, but not on 2-cell microcolonies, based on the sister-pair labeling kinetics analyses. The significance of this observation is currently under investigation. IGF-1 does not appear to affect cell kinetics symmetry. The data is more consistent with increasing generation time. Both 1 mM Xs and Shh have a moderate effect on shifting the cell kinetics symmetry from asymmetric to symmetric. Previous experiments with earlier passage Lig-8 cultures showed a significant shift in cell kinetics symmetry induced by Xs (Lee et al., 2003). The lack of complete concordance between conclusions with microcolony cell kinetics analyses and microcolony labeling kinetics analyses may arise from ambiguities

regarding the kinetics symmetry designations of larger microcolonies and/or intercellular effects in microcolonies.

We hypothesize that an interaction, either direct or indirect, may occur between Wnt and p53 to result in either ACK or SCK. Sadot et al. have found that activated p53 results in the down-regulation of β-catenin, the downstream signaling molecule of Wnt. They also suggest that an autoregulatory loop exists, where an excess of β-catenin induces p53 activation, which in turn results in the down-regulation of β-catenin levels. β-catenin in the Lig-8 cell strain may be unresponsive to p53 inhibition and thus if an up-regulation of β-catenin results in SCK, a shift in cell kinetics symmetry may occur. p53 expression results in ACK by decreasing the pool of guanine nucleotide precursors (GNPs) by inhibiting the rate limiting enzyme in this pathway, IMPDH. Thus, if Wnt acts directly to increase IMPDH activity or increase GNPs by a different mechanism, the cell kinetics would shift from asymmetric to symmetric. Further studies will have to be done to elucidate what the relationship is, if any, between p53 and Wnt.

Chapter 4 – Future Implications

Finding new SACK agents, such as Wnt3a, will allow us to obtain purer ASC populations to be used in studying properties unique to ASCs. The microcolony progression assay that was developed can be used to determine whether asymmetric cell kinetics is taking place in different populations of adult stem cells, such as adult liver cells and pancreatic cells. Using this assay we can determine what effect Wnt3a, or other agents, have on the kinetics of various cell types, and find new SACK agents.

Wnt signaling has been implicated in the self-renewal and proliferation of numerous types of adult stem cells (Reya and Clevers 2005). For example, Wnt3a has been found to promote self-renewal of murine and human HSCs in vitro (Reya et al. 2003). Wnt signaling has also been found to result in the increased cycling and expansion of neural progenitor cells (Chenn and Walsh 2002).

Inappropriate regulation of Wnt signaling has been found in many cancerous tissues. Mutations in this pathway in stem cells and progenitor cells is believed to result in constituitive renewal and expansion of the stem cell and progenitor pool, resulting in cancerous growth. For example, in the colon, inactivation of the APC gene results in the inappropriate stabilization of β-catenin, resulting in cancerous growth (Rubinfeld et al. 1996). This suggests that constituitive activation of the Wnt pathway results in uncontrollable growth and proliferation of mutated cells. Furthermore, oncogenic growth in leukemias have been found to contain activated Wnt signaling (Jamieson et al. 2004).

We suggest that the observation that Wnt3a shifts the kinetics of the adult rat hepatic stem cell strain Lig-8 supports the hypothesis that Wnt functions to expand HSCs and other stem cell populations by similar cell kinetics symmetry mechanisms. We also suggest that

overexpression of Wnt signaling results in symmetric cell kinetics leading to the unregulated exponential expansion that is observed in cancerous tissues. Furthermore, we hypothesize that an inhibition of Wnt signaling exists in some stem cell populations, such as bulge stem cells in the hair follicle, because these stem cells undergo self-maintenance via asymmetric cell kinetics.

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Acknowledgements

First and foremost, I would like to thank my advisor, Professor James Sherley, for his guidance and mentorship from the very first day I arrived at MIT as a freshman, up until graduating MIT with a Masters of Science degree. During this exciting period, Professor Sherley taught me how to conduct independent, meaningful research, as well as introduced me to research that has such a large potential in treating human disease. This knowledge will stay with me throughout my professional career.

I would also like to thank the past and present members of the Sherley lab, who helped me become familiar in lab techniques and procedures and always being available to answer my many questions. Specifically, thanks to Jennifer Cheng, Dr. Gracy Crane, Amy Nichols, Minsoo Noh, Krisha Panchalingam, Dr. Jean-François Paré, Sumati Ram-Mohan, Rouzbeh Taghizadeh, and Dr. Chris Utzat.

Many Thanks also to Lisiane Meira of the Samson lab for training me and giving me valuable advice on using the laser scanning cytometer.

I would like to give a big thanks my family and friends. In particular I would like to thank my mother, Aura, my father, Zvi, and my brothers Adi and Dan. They have been there with me and supported me during this exciting and sometimes difficult period at MIT.

I also would like to thank the MIT community, who during my Bachelor's and Master's years at MIT, provided me with a tremendous amount of academic and personal assets. These assets, which include knowledge on how to overcome challenges and become a better person, will definitely help me as I proceed into my medical and research career, as well as in my private life.