

CHARACTERIZATION AND PROTOCOL DEVELOPMENT FOR POSTNATAL
PLURIPOTENT EPIBLAST-LIKE STEM CELLS USED IN THERAPEUTIC
APPLICATIONS

by

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ABSTRACT

STEPHANIE R. JACKSON

Characterization And Protocol Development For Postnatal Pluripotent Epiblast-Like Stem Cells Used In Therapeutic Applications

(Under the direction of DR. HENRY E. YOUNG and DR. EDWARD O'BRIEN)

The past decade has been characterized by a dramatic shift in the focus of medical research: from developing artificial and biocompatible tissues and organs to investigating methods of triggering the natural repair processes of the human body. This shift has been facilitated by the discovery of stem cells and an improved understanding of their capabilities. This paper presents the reserve precursor cell tissue restoration theory as a probable explanation for the ability of stem cells to maintain and restore tissues following injury.

Precursor cells are present in the connective tissue compartments and granulation tissue of postnatal mammals, including humans. These cells provide the cellular building blocks to maintain the tissues and organs of the body throughout the life span of an individual and repair and replace tissues following injury. There are three basic categories of precursor cells: progenitor cells, germ layer lineage cells, and pluripotent stem cells. These three categories are based upon the ability of precursor cells to form various differentiated cell types and their programmed developmental lineage patterns.

Progenitor cells and germ layer lineage stem cells have already been extensively characterized by our lab. We have utilized various techniques to determine the identity

and capabilities of these cells, including assays for phenotypic expression markers, gene expression and functional activity. We have examined the response of these stem cells to various endogenous and exogenous bioactive factors and developed media that induce proliferation, lineage progression, and lineage commitment in these cells. In addition, we have assessed the ability of these cells to serve in therapeutic treatments for diseased tissues of all three germ layers. This paper discusses the use of these techniques to characterize the third type of stem cells isolated from postnatal tissues, pluripotent epiblast-like stem cells.

CHAPTER 1

INTRODUCTION

The past decade has been characterized by a substantial shift in the focus of medical research: from developing artificial and biocompatible tissues and organs to investigating methods of triggering the natural repair processes of the human body. This shift, which has transformed the possibility of repairing and regenerating damaged and diseased tissue in the human body from an unlikely proposition to a virtual certainty, has been facilitated by the discovery of stem cells and an improved understanding of their capabilities.

Broadly speaking, a stem cell is a cell that is capable of both reproducing itself (through cell division) and maturing into a more specialized or differentiated form. From a biological perspective, the stem cell with the greatest potential is the fertilized egg cell, the zygote, which has the ability to form all parts of an organism. This cell, with totipotential capabilities, is one of the principal reasons that embryonic stem cells are considered the gold standard for stem cell research. However, the use of embryonic stem cells has raised many moral and ethical questions and incited debates. The discovery of endogenous stem cells capable of forming multiple cell and tissue types in adult organisms and the development of protocols for their isolation, culture, and induction have made available research opportunities that do not involve the moral and ethical dilemmas involved in research on embryonic stem cells.

To date, the scientific community remains divided over the use of embryonic stem cells versus the use of adult stem cells, because many questions about the origin, diversity, abilities of adult stem cells and the mechanisms that govern their behavior remain to be answered. The research presented herein responds to these concerns and presents protocols developed to characterize pluripotent epiblast-like stem cells, one of the most primitive of the stem cells types found in adult tissues. These studies are part of the ongoing efforts to understand the therapeutic potential of these miraculous cells in the laboratory of Dr. Henry E. Young at the Mercer University School of Medicine.

CHAPTER 2
POSTNATAL STEM CELLS

Introduction

The term “adult stem cell” is somewhat misleading, because these cells are actually present in an organism at all stages of development. Thus, the term postnatal stem cell has been coined to accurately reflect that adult stem cells are actually found in all postnatal organisms throughout their life span. In order to understand the theories about the origin of postnatal stem cells, it is necessary to review the basic concepts of development and lineage-commitment.

Development and Lineage-Commitment

According to a traditional theory of development, the fertilized egg is thought to retain its totipotential capabilities up to the stage of division into eight cells, at which time the cells are thought to differentiate (specialize) into specific tissue lineages within the organism. The cells then continue specializing through a series of programmed genomic events to perform each of the well-defined responsibilities that sustain the life of an organism. It has been traditionally believed that precursor cells are capable of forming only the distinct tissues in which they are found, not the entire organism. In accordance with this developmental theory, traditional postnatal cell theories presume that the cells of an organism are increasingly limited to specific functions as they differentiate. Thus,

they lose their unlimited potential as they undergo lineage-commitment to the tissue in which they are found.

Tissue Restoration: Transdifferentiation and Dedifferentiation

However, recently researchers claim to have derived neurons and neural supportive tissues, hepatic oval cells, and muscle cells from bone marrow stem cells. Similarly, other investigators report that precursor cells derived from neuronal tissues form blood elements and muscle cells, and precursor cells from skeletal muscle form blood. These researchers explain their results by stating that differentiated cells can revert to a more primitive undifferentiated state (through the process of dedifferentiation) or by proposing that tissue specific, lineage-committed progenitor cells involved in tissue repair can be 'reprogrammed' to form tissues of another organ (through the process of transdifferentiation). Thus, these theories challenge the theory of linear development and lineage-commitment (Young 2004a).

In fact, until the discoveries of the germ layer lineage stem cells and the lineage-uncommitted pluripotent epiblast-like stem cells, it was thought that the only precursor cells that existed in the human body were those of the lineage-committed progenitor cells. (Totipotent blastomere-like stem cells, BLSCs, were discovered following my involvement in the laboratory [Young and Black, 2005]). Thus, the discovery of stem cells at various stages of differentiation and located in tissues other than that for which they are programmed, or that possess the ability to cross lineage boundaries, also challenges the traditional developmental paradigm.

Tissue Restoration: Reserve Precursor Cell Theory

It is believed by our group and several other researchers that the majority of cells pass through the developmental sequence described by the traditional developmental paradigm, but that a few cells leave the developmental continuum at each stage of differentiation to become reserve precursor cells. Thus, the organism retains a bank of cells capable of forming all cell types (totipotent blastomere-like stem cells, BLSCs), all somatic cell types (pluripotent epiblast-like stem cells, ELSCs), cells within discrete germ layer lineages (germ layer lineage stem cells, GLSCs), and others capable of forming specific cell types (lineage-committed progenitor cells, PCs). These cells are held in reserve and can be involved in the maintenance of tissues and organs throughout the life of the organism or in repair following injury. Figure I depicts the cells identified in postnatal tissues by our group with respect to the proposed hierarchy of differentiation. Cells at each stage of differentiation are held in reserve in the connective tissues. They can be activated as required to serve in tissue maintenance or repair.

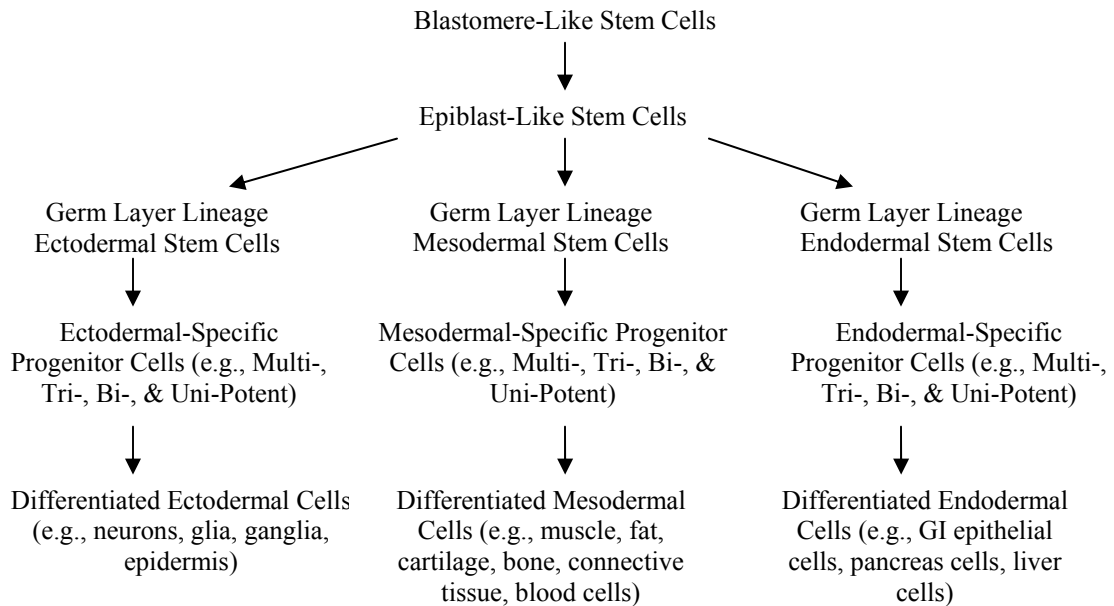


Figure I. Differentiation Hierarchy of Precursor Stem Cells (Young 2005)

Our group has been unable to repeat transdifferentiation results with clonal populations of precursor cells derived from single cells. The reserve precursor theory may offer an alternate explanation for the experiments involving stem cells cited on page 3. Studies of the reserve stem cell hypothesis (Young et al., 1998a,b, 2001b, 2004a; Young 2000, 2004; Reyes and Verfaillie, 2001; Jiang et al., 2002) reveal that the connective tissue compartments of many organs and tissues contain a variety of different reserve precursor cells. According to our research, approximately 50% of the precursor cells residing in a specific connective tissue compartment are lineage-committed unipotent, bipotent, tri-potent, or multipotent tissue progenitor cells for specific for cell types within that compartment (e.g., myosatellite cells in skeletal muscle). Approximately 40% are lineage-committed unipotent, bipotent, tri-potent, or multipotent tissue progenitor cells for other tissue types outside that compartment (e.g., fibroblasts,

adipoblasts, etc. within skeletal muscle). Approximately 9% are germ layer lineage stem cells, approximately 0.9% are pluripotent epiblast-like stem cells capable of forming any somatic cell type. And approximately 0.1% are totipotent blastomere-like stem cells capable of forming any cell type. Thus, the results of experiments purporting to support cell reprogramming through ‘transdifferentiation,’ or ‘dedifferentiation’ could instead be explained by the presence of totipotent, pluripotent, germ layer lineage, or lineage-committed progenitor cells for other tissue types within the tissue of origin (Young et al., 1998b; Young and Black, 2005).

CHAPTER 3

STEM CELL ISOLATION AND SEPARATION

Introduction

Because cell populations in postnatal organisms are mixed, precursor cells must be isolated from native tissue constructs. After isolation, separation protocols are used to segregate the precursor cells into each of the three stem cell lines identified by our group. Then, serial dilution clonogenic analysis is performed to identify each clonal population of cells and ensure that each is derived from a single cell.

Stem Cell Isolation

The technique used for isolation of the cells is detailed in studies by Young and colleagues (Young et al., 1991, 1992, 1995, 1998, 2001a, 2004a,b; Young, 2000, 2004). Briefly, a solid tissue biopsy specimen at least 5 mm³ in size is mechanically disrupted and enzymatically digested. Then, the tissue constituents are centrifuged, reconstituted for plating into T-25 culture flasks coated with 1% gelatin, and supplied with stem cell propagation medium. Stem cell propagation medium consists of 89% (v/v) Opti-MEM, 0.01 mM β -mercaptoethanol, 1% (v/v) antibiotic antimycotic solution (10,000 units/ml penicillin, 10,000 μ g/ml streptomycin, 25 μ g/ml Amphotericin-B), and 10% selected serum 12 (SS-12). SS-12 contains anti-differentiation factor (ADF), an endogenous inhibitor of differentiation (Young et al., 2004a). Thus, inclusion of SS-12 encourages

incubator with 95% air/ 5% carbon dioxide at 37°C until they became confluent. Once cells reach confluence, separation is performed to selectively preserve each of the three stem cell types (Fig. II) (Young et al., 2001a, b, 2004a, b).

Stem Cell Separation

Depending on the desired cell type, Dr. Young's group has developed two distinct freezing procedures to separate the cell types within the biopsy specimen. The first is used for isolation of lineage-committed progenitor cells and differentiated cells. These cells can be preferentially flash frozen and stored in liquid nitrogen (-196° C). The second is selective cryopreservation, which removes the lineage committed progenitor cells and differentiated cells from the cell cultures and preferentially preserves adult stem cells (Young et al., 2004 a, b). In this procedure, the cells are placed in a low temperature freezer at $-75 \pm 10^{\circ}\text{C}$ for a minimum of 36 hours to allow a slow drop in temperature of approximately 1°C per minute (Young et al., 1991, 2004b). Upon thawing and plating, only the postnatal stem cells, e.g., germ layer lineage stem cells (GLSCs), pluripotent epiblast-like stem cells (ELSCs), and totipotent blastomere-like stem cells (BLSCs), will survive. To separate the germ layer lineage stem cells from the pluripotent epiblast-like stem cells and totipotent blastomere-like stem cells, cell sorting and serial dilution clonogenic analysis are performed.

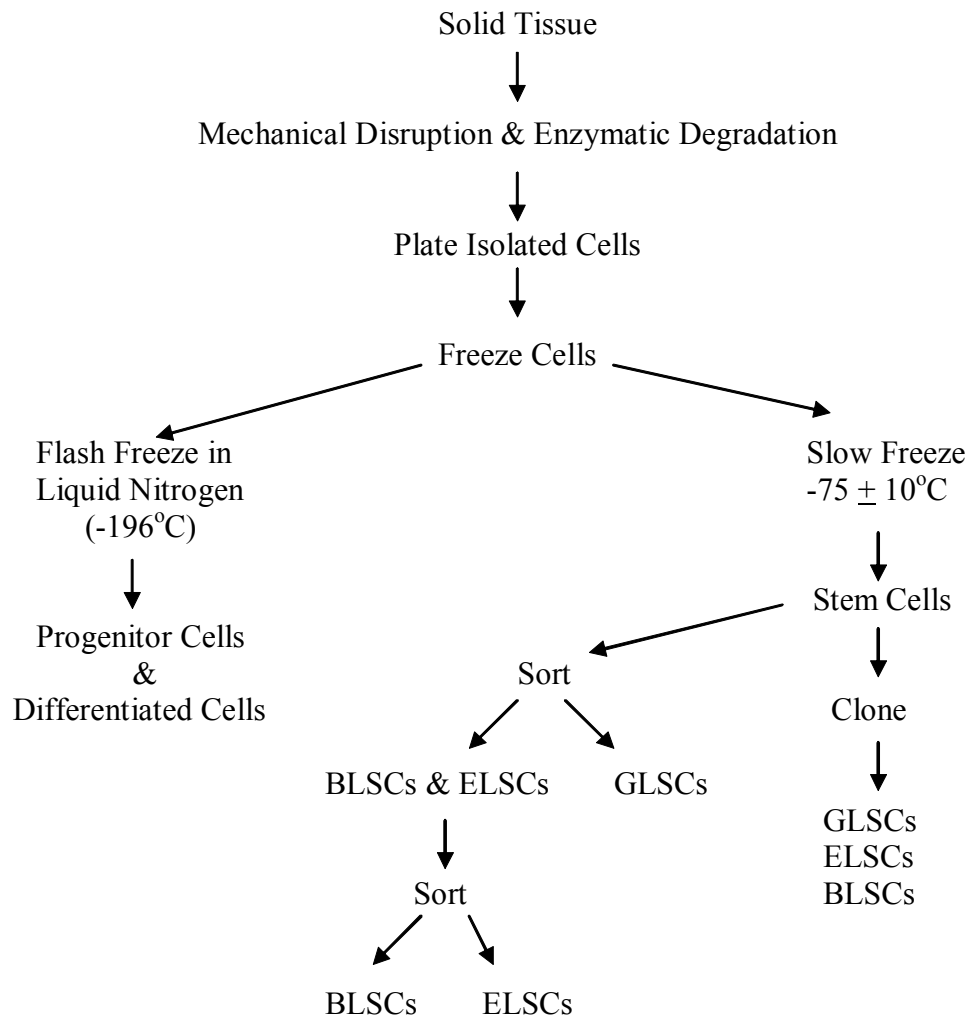


Figure II. Cloning and Cell Sorting Procedures (Young, 2005)

Cell Sorting

Segregation of the germ layer lineage stem cells, epiblast-like stem cells, and blastomere-like stem cells relies on cell surface markers unique for each stem cell line (Table 1, Figure II). Initially, germ layer lineage stem cells are separated from epiblast-like stem cells and blastomere-like stem cells using germ layer lineage-specific cell surface markers (e.g., CD13 and CD90 for human cells). Next, epiblast-like stem cell

surface-specific marker (e.g., CD10 for human cells) is used to segregate epiblast-like stem cells and blastomere-like stem cells. Sorting yields a relative purity for stem cell segregation of approximately 95% (Young and Black, 2005).

Serial Dilution Clonogenic Analysis

In serial dilution clonogenic analysis, one cell is plated per well in a 96 well plate. Then, the cells are proliferated in stem cell propagation medium until confluence and are tested for specific surface markers to determine their identity. The cells in each line are then placed in separate flasks, and aliquots are slow frozen and stored at $-80 \pm 5^{\circ}\text{C}$ (Young et al., 2004, 2001b).

CHAPTER 4

CULTURE, MAINTENANCE, AND CRYOPRESERVATION OF STEM CELLS

Cell Culture

Once a desired cell line has been specified for a study, frozen aliquots are thawed, reconstituted, counted and then plated in complete medium and incubated for 24 hours at 37°C in a humidified environment consisting of 95% air/ 5% carbon dioxide, to allow time for cell attachment to the substratum to occur. Our thawing procedure allows frozen cryovials containing cells to flash thaw to ambient temperature by immersion in a 37°C water bath or by rubbing the hands together to allow the body heat to thaw the cells. Complete medium is composed of 84% (v/v) Opti-MEM, 0.01 mM β -mercaptoethanol, 1% (v/v) antibiotic-antimycotic solution, and 15% selected serum-12 (SS-12) at pH 7.4. The medium provides all of the nutrients required for cell survival. As mentioned previously, SS-12 encourages cell proliferation, but contains an endogenous inhibitor of differentiation, anti-differentiation factor (ADF) (Young et al., 2004a). After attachment, the cells either continue to proliferate in complete medium, or they are fed a testing medium as dictated by the study in which they are being used. Once the cells reach confluence, they are harvested and counted.

Cell Harvest

The cells are attached to the gelatinous coating of the culture flask by two binding mechanisms, a calcium-dependent binding site and a trypsin-sensitive RGD binding site.

Therefore, cell harvest is performed via a two-step standard trypsin release including procedures for elimination of extracellular calcium and addition of trypsin.

Standard Trypsin Release

To remove serum proteins, which are competitive inhibitors for the enzyme trypsin, the medium is removed, and the cultures are washed twice with Dulbecco's phosphate buffered saline with Ca^{2+} and Mg^{2+} at pH 7.4. Inclusion of calcium and magnesium in the buffer allows the release of serum proteins from the cells without loss of the cells from the substratum. The cultures are then washed with 10 ml DPBS containing ethylenediamine tetraacetic acid (EDTA). EDTA is a chelator of divalent cations (e.g., Ca^{2+} , Mg^{2+} , Zn^{2+} , etc.) and is utilized to remove the extracellular Ca^{2+} from the cells. Removal of extracellular calcium releases the cells from their calcium-dependent binding sites. The EDTA solution is removed and replaced with a solution containing trypsin plus EDTA. The trypsin is added to the flask to release the cells from the RGD binding site and concurrently from the gelatinous substrate.

Once the cells have lifted off from the bottom of the flask, the cellular suspension is transferred from the flask to a 15 ml conical tube containing serum proteins. At this point, the serum proteins are added to competitively inhibit trypsin from destroying trypsin-sensitive cell membranes. The conical tube is topped off with 14 ml of complete medium and then centrifuged for 5 minutes at 500 x g. The cells are subsequently re-suspended in 1-5 ml fresh medium. Cell counting is performed under the microscope by placing 15 μl of a mixture of 15 μl of the cellular suspension and 15 μl of 0.4% trypan blue on a hemocytometer. The cells are re-plated, cryopreserved, or characterized after harvest and cell count.

Re-plating

Re-plating is done by diluting reconstituted cells with additional medium (if necessary) and placing them in T-25 flasks coated with 1% gelatin. Additional medium is added to the flask and the flask is rocked to ensure even distribution. The cells are then incubated in a 95% air/5% carbon dioxide humidified environment at 37°C.

Cryopreservation

To cryopreserve harvested cells, the reconstituted cells are pipetted into cryopreservation vials. 75µl 99.99% DMSO is added per 1 ml of cell suspension. The cryopreservation vial is inverted twice, placed in a cryo-chamber and slow frozen to and stored at $-80 \pm 5^{\circ}\text{C}$.

CHAPTER 5

CHARACTERIZATION OF PLURIPOTENT EPIBLAST-LIKE STEM CELLS

Introduction

Three basic categories of precursor cells: progenitor cells, germ layer lineage (ectodermal, mesodermal, and endodermal) stem cells, and pluripotent epiblast-like stem cells had been identified when I worked in the lab. Previous studies by Dr. Young and collaborators had extensively characterized germ layer lineage stem cells and progenitor cells. Thus, particular emphasis was placed on characterizing the pluripotent epiblast-like stem cells at the time in which I joined the laboratory.

Understanding the function and abilities of stem cells are important prior to analysis of their therapeutic applicability. Since stem cells by definition have not yet taken on a specific differentiated function, their identification and characterization relies primarily on use of cell surface (phenotypic expression) markers, expressed genes, and measurements of functional activity. The characteristics observed from our studies of pluripotent epiblast-like stem cells are summarized in this chapter. Those of progenitor cells, germ layer stem cells, and blastomere-like stem cells are provided for comparison in Appendix B. Table I provides a comparative summary of the characteristics of differentiated cells and the four types of precursor cells identified to date in Dr. Young's laboratory.

Table I. Summary of Postnatal Cell Characteristics

Characteristics	DCs ¹	PCs ²	GLSCs ³	ELSCs ⁴	BLSCs ⁵
Size	Variable	Variable	10-20 μm	6-8 μm	< 1 μm
Chromosome number					
- Rat	42 ⁶	42	42	42	42
- Human	46 ⁶	46	46	46	nyd ⁷
Viability within tissue post mortem					
- Rat	3-5 hours	24 hours	3 days	5 days	7+ days
- Human	3-5 hours	24 hours	3 days	5 days	7+ days
Cell Release from solid tissue					
- Explant	-	+/-	+	+	+
- Mincing	-	+	+	+	+
- Enzymatic	+	+	+	+	+
Presence in					
- blood	+	+	+	+	+
- bone marrow	+	+	+	+	+
Percentage within tissues ⁸	50	45	4	0.95	0.05
Location within solid tissues ⁹	Through-out tissue	Through-out tissue	Within connective tissue compartments	Within connective tissue compartments	Within connective tissue compartments
Positive CD marker profile (Human)	Cell Specific ⁶	Cell Specific ⁶	CD10, CD13, CD90, MHC-I ¹⁰	CD10, CD66e	CD66e

Table I - continued

Negative CD marker profile (Human)	Cell Specific ⁶	Cell Specific ⁶	CD1a, CD2, CD3, CD4, CD5, CD7, CD8, CD9, CD11b, CD11c, CD14, CD15, CD16, CD18, CD19, CD20, CD22, CD23, CD24, CD25, CD31, CD33, CD34 ^{+/-} , CD36, CD38, CD41, CD42b, CD45, CD49d, CD55, CD56 ^{+/-} , CD57, CD59, CD61, CD62E, CD65, CD68, CD69, CD71, CD79, CD83, CD95, CD105, CD117, CD123, CD135, CD166, Glycophorin-A, HLA-DRII, FMC-7, Annexin-V, and LIN	CD1a, CD2, CD3, CD4, CD5, CD7, CD8, CD9, CD11b, CD11c, CD13, CD14, CD15, CD16, CD18, CD19, CD20, CD22, CD23, CD24, CD25, CD31, CD33, CD34, CD36, CD38, CD41, CD42b, CD45, CD49d, CD55, CD56, CD57, CD59, CD61, CD62E, CD65, CD68, CD69, CD71, CD79, CD83, CD90, CD95, CD105, CD117, CD123, CD135, CD166, Glycophorin-A, MHC-I, HLA-DRII, FMC-7, Annexin-V, and LIN	CD1a, CD2, CD3, CD4, CD5, CD7, CD8, CD9, CD10, CD11b, CD11c, CD13, CD14, CD15, CD16, CD18, CD19, CD20, CD22, CD23, CD24, CD25, CD31, CD33, CD34, CD36, CD38, CD41, CD42b, CD45, CD49d, CD55, CD56, CD57, CD59, CD61, CD62E, CD65, CD68, CD69, CD71, CD79, CD83, CD90, CD95, CD105, CD117, CD123, CD135, CD166, Glycophorin-A, MHC-I, HLA-DRII, FMC-7, Annexin-V, and LIN
Cloned from single cell (Rat)	na ¹¹	+	A ₂ A ₂ β	Scl-40β	Scl-9β

Table I - continued

Cryopreservation ¹²⁻¹⁵ - DMSO - Conc. - Temp - Freeze Proc - Thaw Proc - % Recovery	Ultra-pure 10% Liq N ₂ Flash Fast to Ambient > 90%	Ultra-pure 10% Liq N ₂ Flash Fast to Ambient > 95%	Ultra-pure 7.5% -70° ± 5 °C Slow Fast to Ambient > 98%	Ultra-pure 7.5% -80° ± 5 °C Slow Fast to Ambient > 98%	Ultra-pure 7.5% -80° ± 5 °C Slow Fast to Ambient > 98%
Substrates: - Make their own - Adherent - Ca ⁺² dep ¹⁶ - RGD dep - Suspension cultures ¹⁷	+ + + +	+ + + +	- + + +	- + + +	- + + -
Cultivation using Serum -Free Defined Reagents (SFD): - SFD-PC - SFD-GLSC - SFD-ELSC - SFD-BLSC	+ + + +	+ + + +	- + + +	- - + +	- - - +
Response to a proliferation agent ¹⁸	+	+	+	+	+
Proliferation Rate (Rat & Human)	Days to Months	Days to Weeks	18-24 hr	12-14 hr	nyd
Growth at Confluence	Contact inhibited	Contact inhibited	Contact Inhibited	Multiple confluent layers	Multiple confluent layers
Replicative Potential - Rat - Human	8-10 50	8-10 50-70	Extensive Extensive	Extensive Extensive	Extensive nyd
Cell doublings to date w/o loss of differentia- tion potential - Rat (clones) - Human	na na	nyd 50	> 400 > 690	> 300 > 400	> 300 nyd

Table I - continued

Activity in SFD medium w/o inhibitory agents ¹⁹	Functional	Quiescent	Quiescent	Quiescent	Quiescent
LIF activity preconfluent ²⁰	na	Induction inhibition	Induction inhibition	Induction inhibition	nyd
LIF activity postconfluent ²¹	na	Na	Na	No effect	nyd
Dependent variable to LIF ²²	na	Absolute cell numbers	Absolute cell numbers	Absolute cell numbers	nyd
Concentration of LIF ²³	na	2000U/ml	2000 U/ml	2000 U/ml	nyd
ADF activity preconfluent ²⁴	na	Induction inhibition	Induction inhibition	Induction inhibition	No effect
ADF activity postconfluent ²⁵	na	Na	Na	Induction inhibition	No effect
Dependent variable to ADF ²⁶	na	Inductive factor concentration	Inductive factor concentration	Inductive factor concentration	No effect
Concentration of ADF ²⁷	na	2 U/ml	2 U/ml	2 U/ml	No effect
Response to progression agent(s) ²⁸	-	+	-	-	-
Response to inductive agent(s) ²⁹	-	-	+	+	+
Immediate Precursor Cell for:	na	DCs	PCs	GLLSCs	ELSCs
Cell types formed	na	Cell-specific	Ectodermal, mesodermal, & endodermal lineage cells	All somatic cells only, will NOT form gametes	All somatic cells and spermatogonia
Telomerase activity - Rat (clones) - Human	na na	na nyd	+ nyd	+ nyd	nyd nyd

Table I - continued

Oct ^{3/4} Expression - Rat (clones) - Human	na na	na nyd	- nyd	+ nyd	nyd nyd
Activity in vivo	Functional	Quiescent or reparative	Quiescent or reparative	Quiescent or reparative	Quiescent or reparative
Studies with repair models	na	na	Bone, Articular cart, Skeletal muscle, Bone marrow reconstitution	Parkinson's disease, Myocardial infarction, Type-I diabetes mellitus	Parkinson's disease, Myocardial infarction, Type-I diabetes mellitus, Bone marrow reconstitution, Infertility

DCs¹, differentiated cells; PCs², progenitor cells; GLLSCs³, germ layer lineage stem cells; ELSCs⁴, epiblast-like stem cells; BLSCs⁵, blastomere-like stem cells; ⁶, work performed by others; nyd⁷, not yet determined; Percentage within tissues⁸, percentages of cells recovered following first cryopreservation step after isolation from tissues by mechanical disruption and enzymatic digestion; Location within solid tissues⁹, based on cells isolated from tissues by mechanical disruption and enzymatic digestion; CD10, CD13, CD90, MHC-I¹⁰, shared lineage Cellular Differentiation (CD) markers for germ layer lineage (ectodermal, mesodermal, endodermal) stem cells; na¹¹, not applicable; Cryopreservation¹²⁻¹⁵: DMSO¹², dimethyl sulfoxide; Freezing Proc¹³, freezing process whereby cryovials containing cells are either directly immersed in liquid nitrogen for flash freezing or placed in a low temperature freezer at designated temperature to allow a slow drop in temperature of approximately one degree per minute; Thawing Proc¹⁴, thawing procedure whereby frozen cryovials containing cells are quickly brought to ambient temperature by either immersion in 37°C water bath or rubbing hands together to allow body heat to thaw cells; % Recovery¹⁵, the average percentage of cells recovered starting with the second time the cells are cryopreserved; dep¹⁶, dependent; Suspension cultures¹⁷, growth in suspension; Response to proliferation agent(s)¹⁸, response to proliferation agent(s) such as platelet-derived growth factors; Activity in SFD medium w/o inhibitory agents¹⁹, activity in serum-free defined medium in the absence of fibroblast feeder layer, recombinant-human leukemia inhibitory factor, recombinant-murine ESGRO, and/or recombinant-human anti-differentiation factor; LIF activity preconfluent²⁰, response of cells to leukemia inhibitory factor prior to confluence being reached in cell cultures containing serum-free defined medium + inductive factors; LIF activity postconfluent²¹, response of cells to leukemia inhibitory factor after confluence surpassed in cell cultures containing serum-free defined medium + inductive factors;

Table I - continued

Dependent variable to LIF²², the limiting variable to the induction inhibitory activity of leukemia inhibitory factor on cells; Concentration of LIF²³, concentration of LIF necessary to prevent induction of lineage commitment in cells; ADF activity preconfluent²⁴, response of cells to anti-differentiation factor prior to confluence being reached in cell cultures containing serum-free defined medium + inductive factors; ADF activity postconfluent²⁵, response of cells to anti-differentiation factor after confluence surpassed in cell cultures containing serum-free defined medium + inductive factors; Dependent variable to ADF²⁶, the limiting variable to the induction inhibitory activity of anti-differentiation factor on cells; Concentration of ADF²⁷, concentration of anti-differentiation factor necessary to prevent induction of lineage commitment in cells; Response to progression agent(s)²⁸, such as insulin, insulin-like growth factor-I, or insulin-like growth factor-II that accelerate the phenotypic expression of differentiated tissue and/or lineage-committed precursor cell types; Response to inductive agent(s)²⁹, such as general inductive agents (i.e., dexamethasone) and specific inductive agents (i.e., bone morphogenetic protein-4, basic-fibroblast growth factor, transforming growth factor-beta, skeletal muscle morphogenetic protein, etc.) that cause stem cells to commit to their respective downstream lineage-committed tissue and/or cell types. Data taken from (Young et al., 2004a; Young and Black, 2005)

Physical Attributes

Pluripotent epiblast-like stem cells are small cells, typically between 6-8 μm in size as determined by flow cytometric analysis of living cells. They are circular and have a high ratio of nucleus to cytoplasm. They also do not exhibit contact inhibition at confluence (Young et al., 2004a, b).

Population Doubling

The cell's life span is measured by observing the number of cell doublings prior to senescence and death or before the differentiation potential is affected. To determine population doubling capacity, cells are plated at a known density. They are propagated past confluence, harvested, and counted. After each harvest and count, cells are aliquoted at a specific density and excess cells are cryopreserved. The remaining cells are continuously cultured. At every other harvest, changes in phenotypic expression are

measured by the insulin-dexamethasone bioassay to assess effects on differentiation potential. We observed a greater than 300 population doublings in clonal populations of rat pluripotent epiblast-like stem cells and greater than 400 population doublings in adult human pluripotent epiblast-like stem cells without loss of differentiation potential (Young et al., 2004a, b, 2005; Henson et al., 2005; Young and Black, 2005b;). These capacities for population doubling far exceed the 8-10 characteristic of rodent differentiated cells and progenitor cells (Rohme, 1981) and the 50-70 characteristic of human cells (Hayflick, 1965).

Insulin-dexamethasone Bioassay

Cells being subjected to the insulin-dexamethasone bioassay are divided among multi-well plates. A series of the wells are treated with insulin and the other wells are treated with dexamethasone. Insulin is a progression factor that has no effect on the phenotypic expression of stem cells (e.g., germ layer lineage stem cells, pluripotent epiblast-like stem cells, or blastomere-like stem cells), but accelerates the time frame for phenotypic expression in cells already committed to particular cell and tissue lineages (e.g., lineage-committed progenitor cells). Dexamethasone is a general induction agent that causes induction of stem cells (germ layer lineage stem cells, pluripotent epiblast-like stem cells, and blastomere-like stem cells) into lineage committed progenitor cells, but does not alter the programmed phenotypic expression of progenitor cells.

Based on our understanding of these responses, we predict five potential different outcomes for the assay. Thus, if one well is treated with insulin and another well with dexamethasone and both express the same phenotypic markers in equal proportions, the cultures contain a population of progenitor cells. If the insulin-treated well exhibits no

change in phenotypic expression and the dexamethasone treated well expresses multiple phenotypes of the same germ layer lineage, the cells are a single germ layer lineage stem cell. If the insulin-treated well exhibits no phenotypic expression and the dexamethasone treated well expresses multiple phenotypes of two different germ layer lineages, the cells are a mixture of two germ layer lineage stem cells. If the insulin-treated well exhibits no phenotypic expression and the dexamethasone treated well expresses multiple phenotypes of all three germ layer lineages, the cells are either a mixture of germ layer lineage stem cells or pluripotent epiblast-like cells, or a mixture of both. If both the insulin-treated well and the dexamethasone-treated well exhibit phenotypic markers and the dexamethasone-treated well shows more phenotypic markers than the insulin-treated well then the cell population is a mixture of progenitor cells and either germ layer lineage stem cells and/or pluripotent epiblast-like stem cells (Young et al., 2004a, b). As long as the population's responses do not qualitatively or quantitatively change, the differentiation potential of the cells has not been affected.

Telomerase Activity

This assay tests for the presence of the telomerase enzyme, which plays a restorative role in chromosome replication. Repetitive telomere shortening during chromosomal replication without the restorative aid of the telomerase enzyme causes genetic instability. Genetic instability activates a DNA damage response pathway that arrests the cell cycle and ultimately causes cell death. Hence, repressed telomerase activity has been correlated with limited lifespan or “mitotic clock” of differentiated cells and lineage committed progenitor cells. Likewise, the presence of telomerase activity in cells showing unlimited proliferation potential, such as embryonic stem cells, adult

pluripotent stem cells, adult germ layer lineage stem cells, and a large majority of tumor cells is consistent with their extended capability for self-renewal (Harley et al., 1990, Campisi, 1997). Pluripotent epiblast-like stem cells processed for telomerase activity were found to be telomerase positive (Young et al., 2004b, Table I). This observation is consistent with the fact that they exceed the published limits for population doubling of differentiated cells.

Cell Surface Markers

Cell surface markers denote the expression of particular proteins associated with a specific differentiation state of the cell. These protein markers can be identified using Fluorescent Activated Cell Sorting (FACS) or with an Enzyme Linked Immuno-Culture Assay (ELICA) (Young 1992b).

Fluorescent Activated Cell Sorting (FACS) Analysis

In FACS analysis aliquots of live human cells are incubated with fluorescent-labeled antibodies to cell surface molecules, e.g., cluster of differentiation (CD) markers as a means to characterize the cells. Non-attached antibodies are removed and the cells are then processed through a machine that utilizes fluorescent light of a known wavelength to sort the cells into two groups - cells that have the fluorescent labeled antibody attached and cells that do not have the antibody attached. Utilizing FACS analysis with suspensions of live human pluripotent epiblast-like stem cells, these cells express CD10 and CD66e cell surface markers and are negative for CD1a, CD2, CD3, CD4, CD5, CD7, CD8, CD9, CD11b, CD11c, CD13, CD14, CD15, CD16, CD18, CD19, CD20, CD22, CD23, CD24, CD25, CD31, CD33, CD34, CD36, CD38, CD41, CD42b, CD45, CD49d, CD55, CD56, CD57, CD59, CD61, CD62E, CD65, CD68, CD69, CD71,

CD79, CD83, CD90, CD95, CD105, CD117, CD123, CD135, CD166, Glycophorin-A, MHC-I, HLA-DRII, FMC-7, Annexin-V, and LIN cell surface markers (Table I) (Young et al., 2004b, Young and Black, 2005b).

Enzyme Linked Immuno-Culture Assay (ELICA)

In the ELICA assay, cultured cells are fixed and incubated with a primary antibody that is specific for a particular protein expressed on the surface or within the cell at a certain differentiation state. The primary antibody is then removed and the cells are incubated with a secondary antibody which binds to an active site on the primary. The secondary antibody also has a molecule called biotin attached to it. Non-attached secondary antibody is removed and the cells are incubated with a tertiary probe containing avidin, a specific binding molecule to biotin. The avidin-containing tertiary probe has an enzyme attached to it. Non-attached tertiary probe is removed and a substrate for the enzyme, diaminobenzoic acid (DAB), is added which preferentially stains the cells to which the antibodies are attached. Those cells that stain positive for the antibodies are visualized using an inverted phase contrast/ brightfield microscope and photographed (Young et al., 1992b).

Pluripotent epiblast-like stem cells do not express general or specific markers for differentiated cells, or the other two types of postnatal cells, progenitor and germ layer lineage cells, identified by our group. Rather, they display cell surface markers similar to those expressed by embryonic stem cells. Pluripotent epiblast-like human stem cells in serum free medium without inhibitory agents express stage-specific embryonic antigen-3 (SSEA-3) and SSEA-4 (characteristic of undifferentiated human embryonic stem cells), and SSEA-1 (characteristic of differentiating human embryonic stem cells), as well as

carcinoembryonic antigens CEA and CEA-CAM-1, and human carcinoembryonic antigens HCEA and CD66e (Young, 2004; Young and Black, 2004; Young et al., 2004). In contrast, pluripotent epiblast-like rat stem cells demonstrate SSEA-4 (characteristic of differentiating rodent stem cells) and CEA-CAM-1 (Young, 2004; Young and Black, 2004; Young et al., 2004), but not SSEA-1 (characteristic of undifferentiated mouse embryonic stem cells) or SSEA-3 (characteristic of differentiating mouse embryonic cells) (Pera et al., 2000; Henderson et al., 2002; Cheng et al., 2003).

Oct-4 Gene Expression

Oct-4 is a POU transcription factor that serves as a regulatory gene in totipotent and pluripotent cells (Nichols et al., 1998; Niwa et al., 2000, 2002). This transcription factor can be identified by employing the oligonucleotide 5'-TGTCGAATGCAAATCACTAGA-3' containing the Oct-1 binding site in an electrophoretic mobility shift assay. Clonal populations of adult rat-derived pluripotent epiblast-like stem cells propagated and processed (Detn and Latchman, 1993; Young et al., 2004b) for the expression of the Oct 3/4 gene did in fact, display the gene, suggesting retention of an "embryonic-like" status.

Phenotypic Bioassay

A phenotypic bioassay was developed to explore lineage-commitment and cell differentiation potential. This assay uses testing medium and bioactive factors with known induction, progression, and/or proliferation abilities and assesses changes in phenotypic expression after 14-56 days of incubation with the cells. Examples of bioactive factors examined include insulin, platelet-derived growth factor, dexamethasone, and selected sera (SS) exhibiting known activities. The phenotypic

bioassay is performed with control medium and a variety of testing mediums. Complete medium consists of 84% (v/v) Opti-MEM, 0.01 mM β -mercaptoethanol, 1% (v/v) antibiotic-antimycotic solution, and 15% selected serum-12 at pH 7.4. Testing mediums consist of complete medium without SS12. Progression agent (insulin 2 μ g/ml), general induction agent (10^{-10} – 10^{-6} M dexamethasone), or progression and induction agent with 1-15% selected sera containing known inductive effects are added to the testing medium to assay alterations in phenotypic expression (Young et al., 2004b).

Cells incubated in testing medium without progression, induction, or proliferation agents do not proliferate or exhibit alterations in phenotypic expression. Thus, pluripotent epiblast-like stem cells do not proliferate or spontaneously differentiate unless acted on by exogenous agents. Cells incubated with SS-12 proliferative agent proliferate past confluence, but do not exhibit changes in phenotypic expression.

Pluripotent epiblast-like stem cells are lineage-uncommitted. Therefore, putative pluripotent epiblast-like stem cells incubated with progression agents do not alter phenotypic expression or proliferation. Pluripotent epiblast-like cells are responsive to general lineage induction agents, forming an assortment of cells from endodermal, mesodermal, and ectodermal lineages. The cells are also responsive to specific induction agents, such as those specific for any of the three primary germ layer lineages or progenitor cell types. We have induced pluripotent epiblast-like stem cells derived from rats and humans to form over 80 cell types. We have confirmed the identity of 46 cell types utilizing the phenotypic bioassay in conjunction with the ELICA procedure (Table II) (Young et al., 2004b).

Table II. Phenotypic Expression Markers by Cell Type

Phenotypic Markers	ELSCs ¹	EctoSCs ²	MesoSCs ³	EndoSCs ⁴	PanPCs ⁵	ILS ⁶
<u>Embryonic</u>						
SSEA-1 ⁷	+	-	-	-	-	-
SSEA-3 ⁸	+	-	-	-	-	-
SSEA-4 ⁹	+	-	-	-	-	-
CEA ¹⁰	+	-	-	-	-	-
HCEA ¹¹	+	-	-	-	-	-
CD66e ¹²	+	-	-	-	-	-
CEA-CAM-1 ¹³	+	-	-	-	-	-
Oct-4 ¹⁴	+	nd ¹⁵	nd	nd	Nd	nd
Telomerase	+	nd	+	nd	Nd	nd
<u>Ectoderm</u>						
Neuronal Progenitor Cells ¹⁶	+	+	-	-	-	-
Neurons ¹⁷	+	+	-	-	-	-
Ganglia ¹⁸	+	+	-	-	-	-
Oligodendrocytes ¹⁹	+	+	-	-	-	-
Astrocytes ²⁰	+	+	-	-	-	-
Synaptic Vesicles ²¹	+	+	-	-	-	-
Radial Glial Cells ²²	+	+	-	-	-	-
Keratinocytes ²³	+	+	-	-	-	-
<u>Mesoderm</u>						

Table II - continued

Skeletal Muscle ²⁴	+	-	+	-	-	-
Smooth Muscle ²⁵	+	-	+	-	-	-
Cardiac Muscle ²⁶	+	-	+	-	-	-
White Fat ²⁷	+	-	+	-	-	-
Brown Fat ²⁸	+	-	+	-	-	-
Hyaline Cartilage ²⁹	+	-	+	-	-	-
Articular Cartilage ³⁰	+	-	+	-	-	-
Elastic Cartilage ³¹	+	-	+	-	-	-
Growth Plate Cartilage ³²	+	-	+	-	-	-
Fibrocartilage ³³	+	-	+	-	-	-
Endochondral Bone ³⁴	+	-	+	-	-	-
Intramembranous Bone ³⁵	+	-	+	-	-	-
Tendon/Ligament ³⁶	+	-	+	-	-	-
Dermis ³⁷	+	-	+	-	-	-
Scar Tissue ³⁸	+	-	+	-	-	-
Endothelial Cells ³⁹	+	-	+	-	-	-
Hematopoietic Cells ⁴⁰	+	-	+	-	-	-
<u>Endoderm</u>						
Endodermal Progenitor Cells ⁴¹	+	-	-	+	-	-
GI Epithelium ⁴²	+	-	-	+	-	-

Table II - continued

Liver Oval Cells ⁴³	+	-	-	+	-	-
Liver Hepatocytes ⁴⁴	+	-	-	+	-	-
Liver Biliary Cells ⁴⁵	+	-	-	+	-	-
Liver Canalicular Cells ⁴⁶	+	-	-	+	-	-
Pancreatic Progenitor Cells ⁴⁷	+	-	-	+	+	-
Pancreas Ductal Cells ⁴⁸	+	-	-	+	+	+
Pancreatic β -Cells ⁴⁹	+	-	-	+	+	+
Pancreatic α -Cells ⁵⁰	+	-	-	+	+	+
Pancreatic δ -Cells ⁵¹	+	-	-	+	+	+

¹ELSCs, pluripotent epiblast-like stem cells (isolated and cloned) (Young, 2004; Young and Black, 2004, 2005a; Young et al., 2004).

²EctoSCs, germ layer lineage ectodermal stem cells (induced) (Romero-Ramos et al., 2002; Young, 2004; Young and Black, 2004; Young et al., 2004).

³MesoSCs, germ layer lineage mesodermal (pluripotent mesenchymal) stem cells (isolated and cloned) (Young et al., 1999, 2001a,b; Young, 2000, 2004).

⁴EndoSCs, germ layer lineage pluripotent endodermal stem cells (induced) (Young, 2004; Young and Black, 2004, 2005; Young et al., 2004).

⁵PanPCs, pancreatic progenitor cells induced from germ layer lineage pluripotent endodermal stem cells (Young and Black, 2005).

⁶ILS, islet-like structures induced from pancreatic progenitor stem cells (Young and Black, 2005).

Embryonic cells were identified as follows.

⁷SSEA-1, stage-specific embryonic antigen-1, MC480, Developmental Studies Hybridoma Bank (DSHB), Iowa City, IA (Solter and Knowles, 1978).

Table II - continued

⁸SSEA-3, stage-specific embryonic antigen-3, antibody MC631 (DSHB) (Damjanov et al., 1982).

⁹SSEA-4, stage-specific embryonic antigen-4, antibody MC-813-70 (DSHB) (Lannagi et al., 1983).

¹⁰CEA, carcinoembryonic antigen, (Hixson) (Estrera et al., 1999).

¹¹HCEA, human carcinoembryonic antigen (Sigma) (Young et al., 2003).

¹²CD66e, carcinoembryonic antigen (Vector) (Kishimoto et al., 1997).

¹³CEA-CAM1, carcinoembryonic antigen-cell adhesion molecule (Hixson) (Estrera et al., 1999).

¹⁴Oct-4, a gene directly involved in the capacity for self-renewal and pluripotency of mammalian embryonic stem cells (Pesce and Scholer, 2001; Young et al., 2003).

¹⁵nd, not as yet determined.

Ectodermal lineage cells were identified as follows.

¹⁶Neuronal Progenitor Cells, were identified using FORSE-1 (DSHB) for neural precursor cells (Tole et al., 1995; Tole and Patterson, 1995), RAT-401 (DSHB) for nestin (Hockfield and McKay, 1985), HNES (Chemicon, Temecula, CA) for nestin (Young et al., 2003), and MAB353 (Chemicon) for nestin (Gritti et al., 1996).

¹⁷Neurons, were identified using 8A2 (DSHB) for neurons (Drazba et al., 1991), S-100 (Sigma) for neurons (Baudier et al., 1986; Barwick, 1990), T8660 (Sigma) for beta-tubulin III (Banerjee et al., 1988, 1990; Joshi and Cleveland, 1990), RT-97 (DSHB) for neurofilaments (Wood and Anderton, 1981), N-200 (Sigma) for neurofilament-200 (Debus et al., 1983; Franke, et al., 1991), and SV2 (DSHB) for synaptic vesicles (Feany et al., 1992).

¹⁸Ganglia, were identified using TuAg1 (Hixson) for ganglion cells (Faris et al., 1990; Hixson et al., 1990).

¹⁹Oligodendrocytes, were identified using Rip (DSHB) for oligodendrocytes (Friedman et al., 1989) and CNPase (Sigma) for oligodendrocytes and astroglia (Sprinkle et al., 1987; Sprinkle, 1989; Reynolds et al., 1989).

²⁰Astrocytes, were identified using CNPase (Sigma) for astroglia and oligodendrocytes (Sprinkle et al., 1987; Sprinkle, 1989; Reynolds et al., 1989).

²¹Synaptic Vesicles, were identified using SV2 (DSHB) for synaptic vesicles (Feany et al., 1992).

Table II - continued

²²Radial Glial Cells, were identified using 40E-C (DSHB) for radial glial cells (Alvarez-Buylla et al., 1987).

²³Keratinocytes, were identified using VM-1 (DSHB) to keratinocyte cell surface protein (Oseroff et al., 1985; Morhenn, 2002).

Mesodermal lineage cells were identified as follows.

²⁴Skeletal Muscle, was identified as mononucleated myoblasts staining with OP137 (Calbiochem, San Diego, CA) for MyoD (Thulasi et al., 1996), F5D (DSHB) for myogenin (Wright et al., 1991), and DEU-10 (Sigma) for desmin (Debus et al., 1983), and as multinucleated spontaneously contracting structures staining with MF-20 (DSHB) for sarcomeric myosin (Bader et al., 1982), MY-32 (Sigma) for skeletal muscle fast myosin (Naumann and Pette, 1994), ALD-58 (DSHB) for myosin heavy chain (Shafiq et al., 1984), and A4.74 (DSHB) for myosin fast chain (Webster et al., 1988).

²⁵Smooth Muscle, was identified as mononucleated cells staining with antibodies IA4 (Sigma) for smooth muscle alpha-actin (Skalli et al., 1986) and Calp (Sigma) for calponin (Frid et al., 1992; Lazard et al., 1993).

²⁶Cardiac Muscle, was identified as binucleated cells co-staining with MF-20 (DSHB) + IA4 (Sigma) for sarcomeric myosin and smooth muscle alpha actin (Eisenberg and Markwald, 1997; Eisenberg et al., 1997), MAB3252 (Chemicon) for cardiotin (Schaart et al., 1997) and MAB1548 for cardiac muscle (Chemicon).

²⁷White Fat, also known as unilocular adipose tissue, was identified as a mononucleated cell with a peripherally-located nucleus and containing a large central intracellular vacuole filled with refractile lipid and stained histochemically for saturated neutral lipid using Oil Red-O (Sigma) and Sudan Black-B (Chroma-Gesellschaft, Roboz Surgical Co, Washington, DC) (Young et al., 2001a).

²⁸Brown Fat, also known as multi-ocular adipose tissue was identified as a mononucleated cell with a centrally-located nucleus containing multiple small intracellular vacuoles filled with refractile lipid and stained histochemically for saturated neutral lipid using Oil Red-O (Sigma) and Sudan Black-B (Chroma-Gesellschaft) (Young, 2000; Young et al., 2001b).

²⁹⁻³³Cartilage: structures thought to be cartilage nodules were tentatively identified as aggregates of rounded cells containing pericellular matrix halos. Cartilage nodules were

confirmed both by both histochemical and immunochemical staining. Histochemically, cartilage nodules were visualized by staining the pericellular matrix halos for proteoglycans containing glycosaminoglycan side chains with chondroitin sulfate and keratan sulfate moieties. This was accomplished using Alcian Blue (Alcian Blau 8GS, Chroma-Gesellschaft), Safranin-O (Chroma-Gesellschaft) at pH 1.0, and Perfix/Alcec Blue. Verification of glycosaminoglycans specific for cartilage was confirmed by loss of

Table II – continued
 extracellular matrix staining following digestion of the material with chondroitinase-AC (ICN Biomedicals, Cleveland, OH) and keratanase (ICN Biomedicals) (Young et al., 1989a,b, 2001a,b) prior to staining (negative staining control). Immunochemically, the chondrogenic phenotype was confirmed by initial intracellular staining followed by subsequent staining of the pericellular and extracellular matrices with CIIC1 (DSHB) for type-II collagen (Holmdahl et al., 1986), HC-II (ICN Biomedicals, Aurora, OH) for type-II collagen (Burgeson and Hollister, 1979; Kumagai et al., 1994), D1-9 (DSHB) for type-IX collagen (Ye et al., 1991), 9/30/8A4 (DSHB) for link protein (Caterson et al., 1985), 12/21/1C6 (DSHB) for proteoglycan-hyaluronate binding region (Caterson, 2001), and 12C5 (DSHB) for versican (Asher et al., 1995). Types of cartilage were segregated based on additional attributes.

²⁹Hyaline Cartilage, was identified by a perichondrial-like connective tissue surrounding the above stained cartilage nodule and histochemical co-staining for type-I collagen (Young et al., 1989c).

³⁰Articular Cartilage, was identified as the above stained cartilage nodule without a perichondrial-like connective tissue covering (Young et al., 1993).

³¹Elastic Cartilage, was identified by nodular staining for elastin fibers and a perichondrial-like connective tissue surrounding the above stained cartilage nodule and histochemical co-staining for type-I collagen (Young et al., 1989c).

³²Growth Plate Cartilage, was identified by nodular staining for cartilage phenotypic markers (see above) and co-staining for calcium phosphate using the von Kossa procedure (Young et al., 1999, 2001a,b).

³³Fibrocartilage, was identified as three-dimensional nodules demonstrating extracellular histochemical staining for type-I collagen (Young et al., 1989c) and co-staining for pericellular matrices rich in chondroitin sulfates A and C. The latter were assessed by Alcian Blue pH 1.0 staining. Negative staining controls were digested prior to staining with chondroitinase-ABC or chondroitinase-AC (Young et al., 1989a,b, 2001a,b).

³⁴Endochondral Bone, was identified as the formation of a three-dimensional structure with progressional staining from one displaying chondrogenic phenotypic markers, i.e., pericellular type-II collagen, type-IX collagen, chondroitin sulfate/keratan sulfate glycosaminoglycans (see above) to three-dimensional nodules displaying osteogenic phenotypic markers, i.e., WV1D1(9C5) (DSHB) for bone sialoprotein II (Kasugai et al.,

1992), MPIII (DSHB) for osteopontine (Gorski et al., 1990), and the von Kossa procedure (Silber Protein, Chroma-Gesellschaft) for calcium phosphate. In the von Kossa procedure, negative staining controls were pre-incubated in EGTA, a specific chelator for calcium (Sigma) (Young et al., 1993, 1999, 2001a,b).

Table II – continued

³⁵Intramembranous Bone, was identified as a direct transition from stellate-shaped stem cells to three-dimensional nodules displaying only osteogenic phenotypic markers, i.e., WV1D1(9C5) (DSHB) for bone sialoprotein II (Kasugai et al., 1992), MPIII (DSHB) for osteopontine (Gorski et al., 1990), and the von Kossa procedure (Silber Protein, Chroma-Gesellschaft) for calcium phosphate. In the von Kossa procedure, negative staining controls were pre-incubated in EGTA, a specific chelator for calcium (Sigma) (Young et al., 1993, 1999, 2001a,b).

³⁶Tendon/Ligament, was identified as linear structures with cellular staining for fibroblast specific protein IB10 (Sigma) (Ronnov-Jessen et al., 1992) and displaying extracellular histochemical staining for type-I collagen (Young et al., 1989c).

³⁷Dermis, was identified by the presence of interwoven type-I collagen fibers (Young et al., 1989c) interspersed with spindle-shaped cells staining for fibroblast specific protein IB10 (Sigma) (Ronnov-Jessen et al., 1992) with an extracellular matrix rich in chondroitin sulfate and dermatan sulfate glycosaminoglycans as assessed by Alcian Blue pH 1.0 staining. In the latter procedure negative staining controls were digested with chondroitinase-ABC or chondroitinase-AC prior to staining (Young et al., 1989a,b, 2001a,b).

³⁸Scar Tissue, was identified as interwoven type-I collagen fibers (Young et al., 1989c) interspersed with spindle-shaped cells staining for fibroblast specific protein IB10 (Sigma) (Ronnov-Jessen et al., 1992) with an extracellular matrix rich in chondroitin sulfate glycosaminoglycans as assessed by Alcian Blue pH 1.0 staining. In the latter procedure negative staining controls were digested with chondroitinase-ABC or chondroitinase-AC prior to staining (Young et al., 1989a,b, 2001a,b).

³⁹Endothelial Cells, were identified by staining with antibodies P2B1 (DSHB) for CD31-PECAM (Young et al., 2001b), H-Endo (Chemicon) for CD146 (Solovey et al., 1997; St. Croix et al., 2000), P8B1 (DSHB) for VCAM (Dittel et al., 1993; Young et al., 2001b), and P2H3 (DSHB) for CD62e selectin-E (Young et al., 2001b);

⁴⁰Hematopoietic Cells, were identified using H-CD34 (Vector) for sialomucin-containing hematopoietic cells (Kishimoto et al., 1997; Young et al., 2001b); Hermes-1 (DSHB) for CD44 – hyaluronate receptor (Picker et al., 1989; Lewinsohn et al., 1990; Butcher, 2002); and H5A4 (DSHB) for CD11b- granulocytes, monocytes; and Natural Killer cells, H5H5 (DSHB) for CD43 – leukocytes, H4C4 (DSHB) for CD44 – hyaluronate receptor, H5A5

(DSHB) for CD45 – all leukocytes, and H5C6 (DSHB) for CD63 – macrophages, monocytes, and platelets (Hildreth and August, 1985; August and Hildreth, 2002).

Endodermal lineage cells were identified as follows.

⁴¹Endodermal Progenitor Cells, were identified with H-AFP (Vector) and R-AFP (NORDIC) for alpha-fetoprotein (Mujoo et al., 1983).

Table II - continued

⁴²GI Epithelium, was identified with HESA (Sigma) for GI-epithelium (Young, 2004; Young and Black, 2004; Young et al., 2004).

⁴³Liver Oval Cells, were identified with OC2 and OV6 (Hixson) for oval cells, liver progenitor cells, and biliary epithelial cells (Faris et al., 1991; Gordon et al., 2000).

⁴⁴Liver Hepatocytes, were identified with H-1 and H-4 (Hixson) for hepatocyte cell surface marker and hepatocyte cytoplasm, respectively (Walborg et al., 1985; Faris et al., 1991) and 151-IgG for liver epithelial growth factor receptor (Hubbard et al., 1985).

⁴⁵Liver Biliary Cells, were identified with OC2, OC3, OC4, OC5, OC10, DPP-IV, and OV6 (Hixson) for biliary epithelial cells, liver progenitor cells, oval cells, and canalicular cells (Hixson et al., 1984, 1990, 2000; Walborg et al., 1985; Faris et al., 1991; Gordon et al., 2000).

⁴⁶Liver Canalicular Cells, were identified with antibodies H4Ac19 (DSHB), DPP-IV, OV6, and LAP (Hixson) for bile canalicular cells, liver progenitor cells, biliary epithelial cells, and canalicular cell surface protein (Hixson et al., 1984, 1990, 2000; Hubbard et al., 1985; Walborg et al., 1985; Faris et al., 1991; Gordon et al., 2000).

⁴⁷Pancreatic Progenitor Cells, were tentatively identified as three-dimensional structures void of chondrogenic or osteogenic phenotypic markers. This identity was confirmed by the presence phenotypic markers for pancreatic ductal cells, β -Cells, α -Cells, and δ -Cells (Young, 2004; Young and Black, 2004; Young et al., 2004).

⁴⁸Pancreatic Ductal Cells, were identified with cytokeratin-19 (Chemicon) to pancreatic ductal cells (Young, 2004; Young and Black, 2004; Young et al., 2004).

⁴⁹Pancreatic β -Cells, were identified with YM-PS5088 (Accurate) an antibody to insulin (Young, 2004; Young and Black, 2004; Young et al., 2004).

⁵⁰Pancreatic α -Cells, were identified with YM-PS087 (Accurate) an antibody to glucagon (Young, 2004; Young and Black, 2004; Young et al., 2004).

⁵¹Pancreatic δ -Cells, were identified with 11180 (ICN) an antibody to somatostatin (Young, 2004; Young and Black, 2004; Young et al., 2004).

Potential for Induction and Lineage-Commitment

Once induced to commit to a particular germ layer lineage, pluripotent epiblast-like stem cells will continue along their directed differentiation pathway and eventually become unidirectional lineage-committed progenitor cells. At this point, they will lose telomerase activity and adhere to the limit of 50-70 population doublings for humans or 8-10 population doublings for rodents which characteristic of their respective mitotic clock before cellular senescence and death occur (Young et al., 2004b, 2005).

CHAPTER 6

TISSUE REPAIR MODEL SYSTEMS AND THERAPEUTIC APPLICATIONS

Introduction

The attributes discussed in the previous chapter suggest that postnatal pluripotent epiblast-like stem cells are unique cells specifically regulated to act in tissue repair. Since they can be isolated from a small biopsy of skeletal muscle or dermis or any other tissue or organ of the body (Young et al., 2004a) from newborn to geriatric individuals, use of postnatal stem cells in therapeutic applications avoids the prevailing moral objections to therapeutic use of embryonic stem cells. A large population of these stem cells can rapidly be obtained from a few harvested cells, facilitating autologous transplant and eliminating the need for immunosuppressant therapy for human leukocyte antigen (HLA) mismatches. Pluripotent epiblast-like stem cells can be induced to form cells from all three primary germ layer lineages, i.e., ectoderm, mesoderm, and endoderm, but remain quiescent in serum-free defined medium in the absence of inhibitory agents to induction or differentiation. Once chemically or naturally induced, the progenitor cells derived from pluripotent stem cells behave as native progenitor cells with respect to loss of differentiative potential and adherence to Hayflick's limit of 50-70 population doublings (human) or 8-10 population doublings (rodent) before programmed cell death occurs. These characteristics of pluripotent epiblast-like stem cells give them distinctive advantages over embryonic stem cells for

therapeutic applications (Young, 2004; Young and Black, 2004; Young et al., 2004, 2005).

In light of their potential for therapeutic applications, we devised both *in vivo* and *in vitro* repair model systems to investigate the ability of the pluripotent cells to repair diseased tissues from each germ layer. Type-1 diabetes was selected to model endodermal repair, myocardial infarction was selected to model mesodermal repair, and Parkinson's disease was selected to model ectodermal repair. Scl-40 β , an adult rat-derived pluripotent epiblast-like stem cell clone isolated and characterized as described in the previous chapters, was utilized in these studies. To aid in tracking the cells within *in vivo* model systems, the cells were genomically-labeled with Lac-Z to express the β -galactosidase gene.

Endodermal Repair Model: Type- I Diabetes

To measure the applicability for therapeutic Type-I Diabetes treatment, cells from the Scl-40 β clone were sequentially induced *in vitro* stepwise down their respective differentiation lineage pathway to form three-dimensional (3D) pancreatic islet-like structures. In this procedure, the pluripotent epiblast-like stem cell clone first underwent chemically induced differentiation to form germ layer lineage endodermal stem cells. These cells were then expanded in number prior to the next induction step. The second step involved chemical induction to form pancreatic progenitor cells. These cells were then expanded in number prior to the next induction step. The third step involved chemical induction to form 3D islet-like structures containing cells that expressed phenotypic expression markers for β -cells (insulin), α -cells (glucagon), and δ -cells (somatostatin). The induced 3D structures and isolated native rat islets were then assayed

for insulin secretion with a rat-specific insulin radioimmuno assay (RIA) following a glucose challenge (5 mM/24 hrs, 5 mM/1hr, 25 mM/ 1hr). The 3D structures secreted approximately 25-50% of the insulin secreted by native islets under the non-optimized conditions examined. Under similar glucose challenge conditions, embryonic stem cells that had been induced to form insulin-secreting β -cells secreted 5% of the insulin secreted by native islets (review, Young and Black, 2005a). These results are included in Figure III. Figure IV Compares native and induced structures (Young et al., 2004b; Young and Black, 2005a).

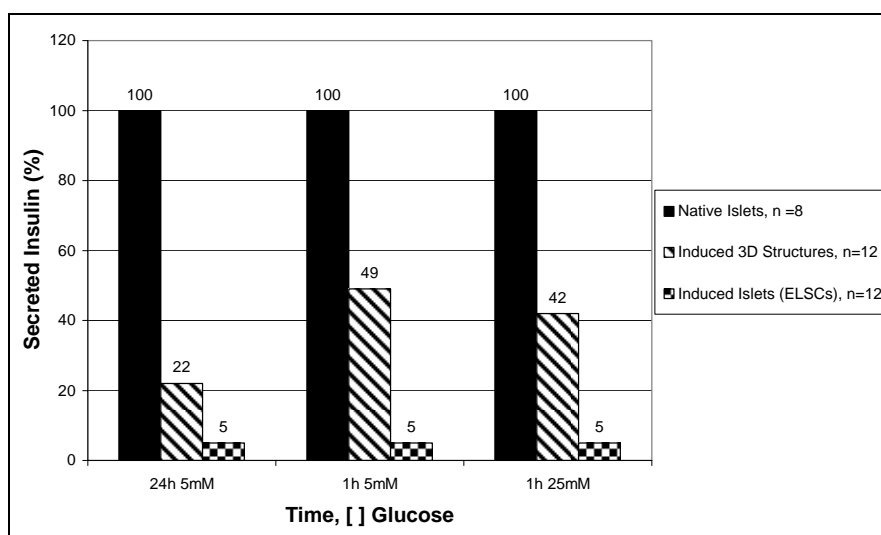


Figure III. Glucose Challenge Results: Native Islet versus Induced 3D Islet-Like Structures versus Induced Epiblast-Like Stem Cells

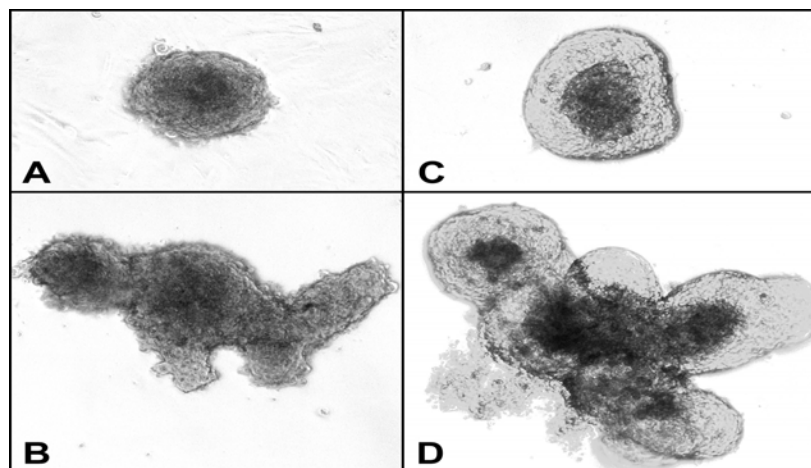


Figure IV. Comparison of Induced 3D Structures versus Native Pancreatic Islets
A. & B. Induced Structures C. & D. Native Structures

Mesodermal Repair Model: Myocardial Infarction

Cryo-injury to the heart muscle was utilized as a model system for myocardial infarction for this next series of studies. In this *in vivo* model the tip of the left ventricle was frozen with liquid nitrogen to kill the resident cells, e.g., cardiac myocytes, fibroblasts and endothelial cells and smooth muscle cells (blood vessels), and to generate infarcted myocardial tissue. Putative repair cells were then introduced into the area, either by direct injection or infusion through the existing vasculature, to determine their ability to repair the myocardium.

We utilized the Scl-40 β pluripotent epiblast-like stem cell clone for both routes of stem cell input. In the first series of experiments the Scl-40 β clone was injected directly into the infarcted adult rat myocardial tissue following cryo-injury. In the second series of experiments the Scl-40 β clone was injected into the tail vein following cryo-injury of adult rat myocardial tissue and allowed to migrate to the heart for incorporation into the infarcted tissue. The hearts from both experiments were harvested one day to four weeks

later. They were sectioned, fixed, stained and assessed for incorporation of stem cells into the tissues. In the first model, as early as one week after injection, the cells incorporated into all myocardial tissues undergoing repair, including the myocardium, vasculature, and connective tissue (Figure V). Interestingly, in the second, the labeled cells migrated from the site of insertion to the site of injury and incorporated into the myocardium and connective tissues (Figure VI) (Young et al., 2004b).

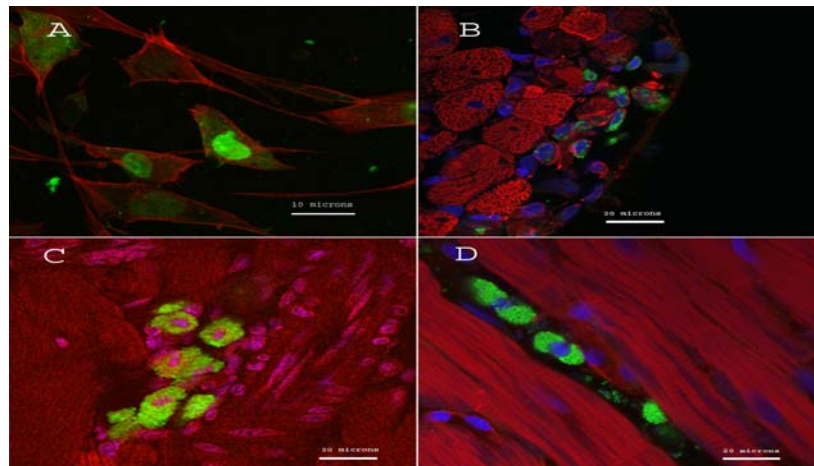


Figure V. Incorporation of Pluripotent Stem Cells in Damaged Myocardium via Direct Injection. A. In vitro B. Vasculature C. Myocardium D. Connective Tissues

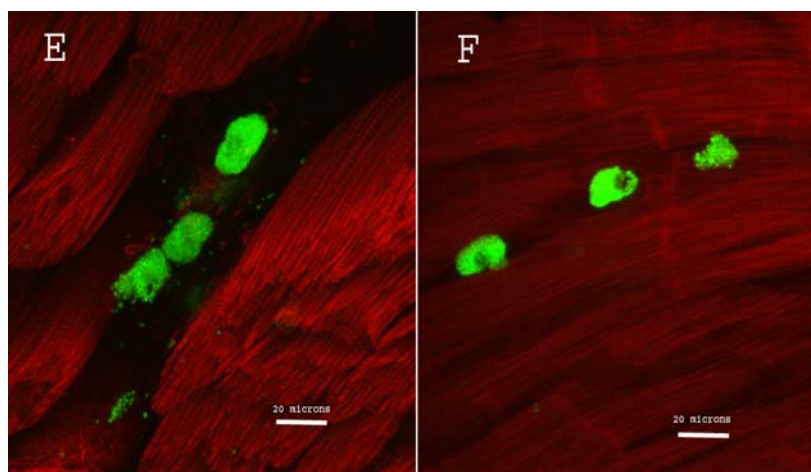


Figure VI. Incorporation of Pluripotent Stem Cells in Damaged Myocardium via Tail Injection. E. Connective Tissues F. Myocardium

Ectodermal Repair Model: Parkinson's Disease

A Parkinson's disease model was created in adult rats by destruction of dopaminergic neurons within the substantia nigra utilizing the neurotoxin 6-hydroxydopamine (6-OHDA). In this model the neurotoxin is stereotactically injected into the substantia nigra of the brain where it irreversibly binds to the dopaminergic neurons and kills the cells. To assess the extent of damage or repair, tissue samples were stained with antibodies for the enzyme tyrosine hydroxylase. Tyrosine hydroxylase is a critical rate limiting enzyme within the biosynthetic pathway for the formation of dopamine. The presence or absence of tyrosine hydroxylase denotes the presence or absence of neurons that synthesize dopamine, a key constituent in Parkinson's disease. To determine the ability of the pluripotent epiblast-like stem cells to restore dopaminergic neurons following ablation with 6-OHDA we compared stereotactic injection of the Scl-40 β clone suspended in saline ascorbate buffer (experimental) versus stereotactic

injection of the saline ascorbate buffer only (control). Tissue sections stained with antibodies to tyrosine hydroxylase were used to assess tissue recovery with respect to restoration of dopaminergic neurons.

The injected control was tyrosine hydroxylase negative, but the injected experimental was tyrosine hydroxylase positive (Figure VII). This study suggests that naïve pluripotent epiblast-like stem cells have the potential to restore dopaminergic neurons following ablation with 6-OHDA. Additional staining of tissue sections utilizing an antibody to β -galactosidase and visualization of the tissue sections demonstrated migration of the Scl-40 β cells away from the site of injection with subsequent formation of pyramidal neurons, glial cells, and capillary vasculature. This suggested the potential for the pluripotent epiblast-like stem cells to migrate to and repair areas of damaged tissue (Figure VIII) (Young et al., 2005).

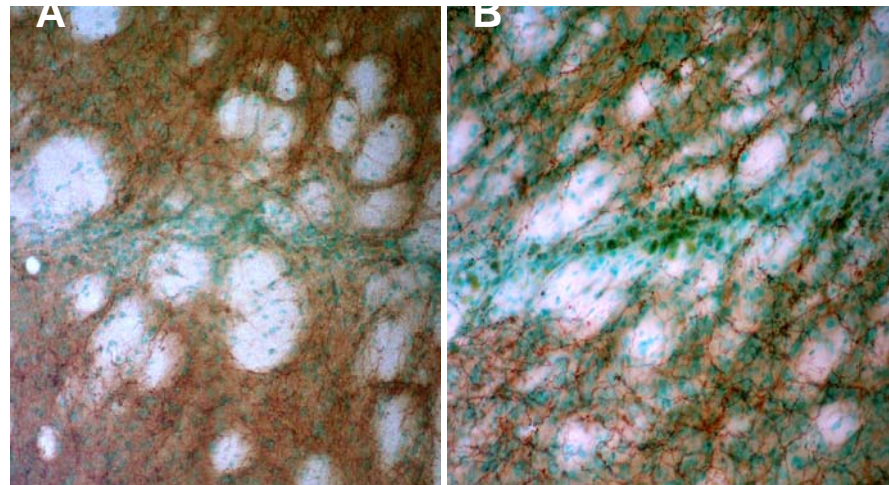


Figure VII. Tyrosine Hydroxylase Staining. A. Control Injection: Negative
B. Control plus Naïve Pluripotent Epiblast-Like Stem Cells: Positive

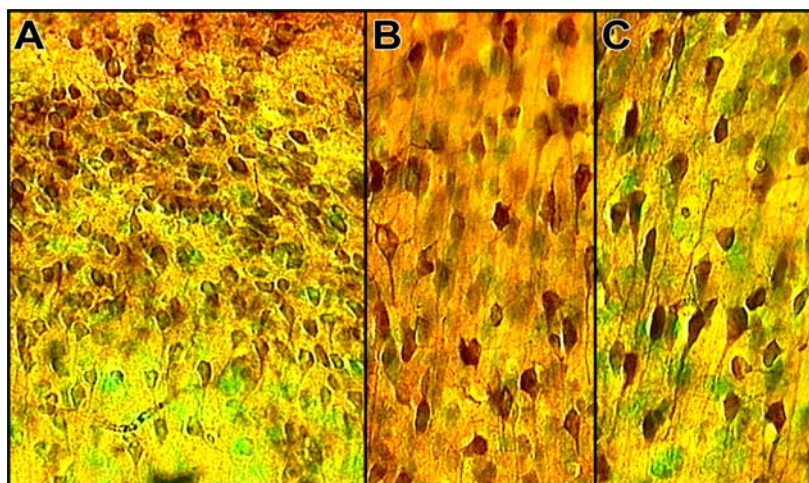


Figure VIII. β -Galactosidase Labeling of Scl-40 β Origin Dopaminergic Neurons

Additional neuronal studies by our collaborator, Marie-Francoise Chesselet and her colleagues (Romero-Ramos et al., 2002; V'rouch et al., 2004, 2005a, b; Mignon, 2005) have also demonstrated that adult-derived pluripotent epiblast-like stem cells are able to form immature neurons and glia *in vitro*, and form similar cell types when injected into the hippocampus *in vivo*, thus demonstrating their utility for autologous neuronal transplant.

Summary

These above results demonstrate that postnatal pluripotent stem cells have potential for use in tissue engineering and treatment of trauma and disease in tissues derived from all three germ layer lineages. The cells can be induced *in vitro* to desired cell types and implanted or injected directly into the body in the naïve state to permit the body to dictate the type of cell formed from the stem cell. The mobility of reserve stem cells within the

body suggests that stimulation of endogenous stem cells located within a tissue may be another method of stimulating tissue regeneration.

CHAPTER 7

CONCLUSION

Our characterization techniques make it possible to recognize, select, and induce stem cells to form mature cells. Results from our studies indicate that postnatal stem cells have significant capabilities for repairing and regenerating damaged cells and tissues. An added advantage of treatment with postnatal stem cells is the evasion of immunological rejection, because the cells can be isolated from a small tissue biopsy sample from the patient, proliferated and/or induced in culture, and used for subsequent autologous transplants. With continued investigation, these cells may help alleviate or heal many degenerative diseases, for which there are no effective therapies to date.

In spite of all of the potential of adult stem cells, there are still substantial challenges to overcome. The cells are relatively rare and are somewhat challenging to locate. They are also very sensitive to culture conditions *in vitro*. As researchers continue to grapple with the challenges posed by these miraculous cells, they will foster our understanding of the fundamentals of development and tissue differentiation.

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APPENDIX A

GLOSSARY

A

Antibody- An antibody is a protein that preferentially binds to a specific antigen, or a cell surface protein that identifies the cell type and differentiation state. The antibody-antigen identification system helps the organism's immune system identify foreign objects, but also has proven helpful for identifying cells in culture.

B

Beta-galactosidase gene- an insect gene with which the stem cells are transfected. Its presence can be identified with histochemical, enzymatic, and/or fluorescence assays to ensure that genetic material is not altered and to identify the therapeutic cells in tissue repair models.

Bioassay- shorthand term for biological assay. Generally, an experiment conducted to measure the biological response of a living organism to a substance.

Bioactive factors- substances that elicit some biological response from a cell; for example, causing it to proliferate, differentiate, or express a certain phenotype.

Bipotent- primitive cells capable of forming two differentiated cell types.

C

CD66e- a human carcinoembryonic antigen, present on the cell surface of embryonic stem cells, adult pluripotent stem cells, and adult totipotent stem cells.

CEA-CAM-1- a human carcinoembryonic antigen cell adhesion molecule-1, present on the cell surface of embryonic stem cells, adult pluripotent stem cells, and adult totipotent stem cells.

Complete medium- a solution composed of 84% (v/v) Opti-MEM, 0.01 mM β -mercaptoethanol, 1% (v/v) antibiotic-antimycotic solution, and 15% selected serum-12 (SS-12) at pH 7.4 that provides all of the nutrients required for cell survival. SS-12 encourages cell proliferation, but contains an endogenous inhibitor of differentiation, e.g., anti-differentiation factor (ADF).

Confluence- state in which cells have completely covered their respective substrate and come in contact with one another.

Contact inhibition- cellular behavior in which cells will not proliferate beyond confluence, or when they are in contact with other cells.

Cryopreservation- a process where cells or tissues are preserved by cooling to sub-zero temperatures, typically -80°C or -196°C (the boiling point of liquid nitrogen). All biological activity, including the biochemical reactions that would lead to cell death is effectively halted.

D

De-differentiation- a theory to explain the reparative phenomenon which states that a differentiated cell has the ability to be reprogrammed to a more primitive state.

Differentiation - the process by which cells become specialized and take on well-defined responsibilities to sustain the life of the organism. The cell changes phenotypically or morphologically, but not genetically.

Dexamethasone- a synthetic corticosteroid with ten times the functional activity of cortisone. With respect to precursor cells it functions as a general induction agent that causes induction in totipotent stem cells, pluripotent epiblast-like stem cells, and germ layer lineage stem cells, but does not effect phenotypic expression of lineage-committed progenitor cells.

Dopaminergic neurons- neurons utilizing tyrosine hydroxylase in their biosynthetic machinery to synthesize and secrete dopamine, a chemical messenger that functions in the brain.

E

Ectoderm- A germ layer or collection of cells, formed during early embryonic development that eventually gives rise to surface epidermal tissues (including epidermis, nails, hair, tooth enamel, cornea, sclera, sweat glands, sebaceous glands) and tissues within the peripheral and central nervous systems.

Endoderm- A germ layer or collection of cells formed during early embryonic development that eventually gives rise to the interior lining of the respiratory system, gastrointestinal system, and the parenchyma (functional portion) of the liver, gall bladder, and pancreas.

Enzyme Linked Immuno- Culture Assay (ELICA) - a biological assay used to quantify specific phenotypic or cell surface markers, to assess the location of the specific phenotypic or cell surface markers, and to quantify DNA content of the cells.

F

Flash freezing- process of rapidly freezing cells by directly immersing them in liquid nitrogen at its boiling point (-196°C); process used to isolate progenitor cells and differentiated cells from adult stem cells, e.g., germ layer lineage stem cells, pluripotent stem cells, and totipotent stem cells.

G

Germ layer- collection of cells formed during early embryonic development that are the forerunners of all adult tissues and organs; can be endodermal, ectodermal, or mesodermal.

Germ layer lineage stem cells- stem cells isolated from postnatal tissues that are capable of forming cells and tissues of one of the three primary germ layer lineages, e.g., ectoderm, mesoderm, or endoderm.

Glucose challenge- a biological assay used to determine the response of cells stimulated with varying concentrations of glucose. Specifically, it is designed to measure insulin secretion.

H

HCEA- human carcinoembryonic antigen, present on the cell surface of embryonic stem cells, adult pluripotent stem cells, and adult totipotent stem cells.

Human leukocyte antigen (HLA) - a protein expressed on the surface of leukocytes that can be recognized as foreign by the body during autologous transplantation. A key player in the rejection of transplanted organs or tissues.

I

Insulin- a hormone that regulates carbohydrate metabolism. Patients with type 1 diabetes mellitus depend on exogenous insulin for their survival because of an absolute deficiency of the hormone; patients with type 2 diabetes mellitus have either relatively low insulin production, insulin resistance, or both.

Insulin-dexamethasone bioassay- a biological assay used to induce or accelerate changes in phenotypic expression to assess effects on differentiation potential. The assay utilizes a factor that accelerates phenotypic expression in lineage-committed progenitor cells (insulin) and a factor that induces lineage commitment in uncommitted stem cells (dexamethasone).

L

Lineage-committed- description used for cells preprogrammed to form only specific cell types.

Lineage progression- expression of the phenotypic markers specific to a cell's lineage-commitment.

Lineage-uncommitted- description used for cells that are not committed to any particular tissue lineage and are capable of forming any cell or tissue type in the body.

M

Mesoderm- A germ layer or collection of cells, formed during early embryonic development that eventually gives rise to muscle, fat, cartilage, bone, connective tissues, scar tissue, blood cells, the immune system, and the urogenital tract.

Myocardial infarction- "heart attack"; a condition that occurs when a part of the heart muscle is injured or dies because of interruption of blood flow to the area.

Multipotent- stem cells capable of forming four or more cell types, but still incapable of forming all cell types of a particular germ layer, i.e., hematopoietic "stem" cells can form

all cell types of the hematopoietic lineage but can not form cell types outside the hematopoietic lineage.

N

Naïve Scl 40β- undifferentiated pluripotent epiblast-like stem cell clone genomically-labeled with Lac-Z to express the insect gene, β-galactosidase.

O

Oct-4- gene expressed in totipotent and pluripotent cells.

Oligonucleotide- short sequences of RNA or DNA nucleotides (< twenty base pairs) and used as probes for detecting complementary DNA or RNA sequences.

P

Pancreatic islet- a conglomerate of cells consisting of glucagon-secreting α-cells, insulin-secreting β-cells, somatostatin-secreting δ-cells, and polypeptide-P-secreting φ-cells of the pancreas.

Parkinson's disease - a neurodegenerative disease of part of the basal ganglia of the brain. The disease involves a progressive disorder of the system that controls communication between neurons in the brain and muscles in the human body.

Phenotypic expression markers- markers located on the cell surface or within the cell that denote characteristics inherent to the cells, e.g., CD66e (cell surface), smooth muscle alpha-actin (internal).

Platelet-derived growth factor- a bioactive factor synthesized and released by blood platelets that has a known proliferative effect on stem cells.

Pluripotent- a term used to describe stem cells capable of developing into any of the three primary tissue types of the body, e.g., endoderm, mesoderm, or ectoderm, but can not form the gametes sperm and/or ovum.

Pluripotent epiblast-like stem cells- term used to describe primitive lineage-uncommitted stem cells discovered within tissues of postnatal vertebrates, including humans. The cells are capable of forming any somatic cell type, but not gametes.

Postnatal stem cell- primal cells located within newborn to geriatric-aged individuals which retain the ability to differentiate into other cell types. This ability allows them to act as a repair system for the body, replenishing other cells as long as the organism is alive.

Progenitor cells- lineage-committed cells that are pre-programmed to commit to a particular cell lineage and are unidirectional in their ability to form differentiated cell types.

Proliferation- increasing the number of cells by cell division.

Q

Quiescent - the "resting" state of a cell, i.e., the state of a cell when it is not dividing, differentiating or dying.

R

Radioimmuno assay (RIA) - bioassay used to identify the synthesized and secreted product of a cell.

Re-plating- procedure used to continue culturing cells, generally performed after thawing, harvest or cell counting.

Reserve precursor cell theory- a theory describing the origin of postnatal stem cells which states that a few cells leave the developmental continuum at each stage in tissue differentiation to become reserve precursor cells that repair and regenerate damaged tissues.

S

Sc1-40 β - a clone of pluripotent epiblast-like stem cells derived from adult rat skeletal muscle and transfected with Lac-Z to express the insect gene, β -galactosidase. One of the most primitive stem cell types. It is capable of forming any somatic cell type, but not gametes.

Selected sera- a serum used in experimentation with known bioactive factor effects.

Selective cryopreservation- procedure which selectively preserves a population of stem cells based on the freezing temperature. For example, progenitor cells are selectively preserved by flash freezing to -196°C , while germ layer, pluripotent epiblast-like stem cells, and blastomere-like stem cells are preserved by slow freezing to -80°C .

Serial dilution clonogenic analysis- procedure used to clones of stem cells from single cells. In this procedure, one cell is plated per well in a 96 well plate. Then, the cells are proliferated in stem cell propagation medium until confluence and are tested for specific surface markers to determine their identity.

Serum- serum is blood plasma without clotting factors (such as fibrin). It is used in cell culture. Different sera can evoke different responses from primitive cells based on their unique content of bioactive factors.

Slow freezing- procedure whereby cryovials containing cells are placed in a low temperature freezer at a designated temperature, usually (-80°C) to allow a slow drop in temperature of about 1°C per minute.

Stage-specific embryonic antigens- proteins expressed on a primitive cell's surface that are specific to the differentiation state of the cell.

Stem Cell- a cell that is capable of both reproducing itself (through cell division) and maturing into a more specialized or differentiated form.

Stem cell propagation medium- a solution composed of 89% (v/v) Opti-MEM, 0.01 mM β -mercaptoethanol, 1% (v/v) antibiotic-antimycotic solution, and 10% selected serum-12 (SS-12) at pH 7.4 that provides all of the nutrients required for cell survival. Inclusion of SS-12 encourages cell proliferation, but contains anti-differentiation factor, an endogenous inhibitor of differentiation.

T

Telomerase- an enzyme that adds the DNA sequence repeat "TTAGGG" to the 3' end of DNA strands in all vertebrates, in the telomere regions at the ends of chromosomes. Telomerase activity is repressed in differentiated cell types.

Totipotentiality- the ability of a single cell to form all somatic (non-reproductive) cells of the organism, to form the reproductive cells (sperm and ova), and to form the cells and tissues comprising the extra-embryonic placental and fetal membranes.

Transcription factor- a protein that binds to DNA at a specific promoter site to regulate gene transcription.

Transdifferentiation- a theory which states that one type of differentiated cell can be reprogrammed to form another differentiated cell type.

Tripotent- stem cells capable of forming three differentiated cell types within a particular germ layer lineage.

Trypsin release- procedure used to release cells from adherence to the gelatinous substrate of the culture flask, utilizes the enzyme trypsin.

Type- I diabetes- type of diabetes characterized by autoimmune destruction of the body's β -cells in the Islets of Langerhans of the pancreas. The organism suffers from an absolute deficiency of insulin and the inability to store glucose in the body.

U

Unipotent- cells which are only capable of forming one differentiated cell type.

APPENDIX B
CHARACTERISTICS OF PROGENITOR CELLS,
GERM LAYER LINEAGE STEM CELLS, &
BLASTOMERE-LIKE STEM CELLS

Progenitor Cells

Progenitor cells are variable in size, ranging from 10-200 μ m. They are the immediate precursors of differentiated cells. They contain a normal chromosomal complement. Progenitor cells are pre-programmed to commit to a particular cell lineage and are unidirectional in their ability to form differentiated cell types. There are four subcategories of tissue-specific progenitor cells: unipotent, bipotent, tripotent, and multipotent stem cells. The myosatellite cell of skeletal muscle is an example of a unipotent progenitor cell. This cell is only able to form a single differentiated cell type. An adipofibroblast is a bipotent progenitor cell. It will form two differentiated cell types, adipocytes (fat cells) and/or fibroblasts. A tripotent progenitor cell has the ability to form three differentiated cell types. A chondro-osteo-adipoblast is a tripotent progenitor cell that will only form chondrocytes (cartilage), osteocytes (bone), and/or adipocytes (fat cells). The precursor cell residing in bone marrow, originally designated as a hematopoietic “stem” cell, is an example of a multipotent progenitor cell. It has the ability to form multiple different cell types within the hematopoietic cell lineage. For example, the bone marrow precursor cells will form red blood cells (erythrocytes) and all

white blood cells (leukocytes), including monocytes, macrophages, lymphocytes (T and B), neutrophils (PMNs), basophils, plasma cells, eosinophils, Natural Killer cells, mast cells, Langerhans cells, antigen presenting cells, and dendritic cells.

Progenitor cells do not exhibit phenotypic expression markers for germ layer lineage cells (nestin for cells of the ectodermal lineage; sarcomeric myosin, type-II collagen, or bone sialoprotein II for cells of the mesodermal lineage; or alpha-fetoprotein for cells of the endodermal lineage). They do not exhibit markers for embryonic stem cells (stage specific embryonic antigen-4 or carcinoembryonic antigen cell adhesion molecule-1). Instead, they express tissue specific phenotypic markers, such as myogenin for myoblasts or neurofilament for neuroblasts (Young et al., 1999, 2001a,b, 2004a; Young, 2000, 2004).

Progenitor cells are unresponsive to lineage-induction agents that have actions outside of their particular tissue lineage. Thus, myoblasts can be induced to form muscle, but cannot be induced to form cartilage or bone. However, progenitor cells are responsive to progression agents that accelerate the time frame of expression for tissue-specific phenotypic differentiation expression markers. They are also responsive to proliferation agents such as platelet-derived growth factors, but exhibit contact inhibition at confluence *in vitro*. Unlike embryonic stem cells which spontaneously differentiate, progenitor stem cells remain quiescent in a serum free environment lacking proliferation agents, lineage induction agents, progression agents, and inhibitory factors (review, Young et al., 1998, 2004a; Young and Black, 2004).

Like the differentiated cells for which they are the immediate precursors, progenitor cells have a finite life span. They are tissue specific in their differentiation capabilities

and have a “mitotic clock” of 50-70 population doublings (human) or 8-10 population doublings (rodent) before programmed cellular senescence and death occur. Consistent with this observation, telomerase activity is repressed in these cells. Thus, the mitotic clock for (human) progenitor cells begins at birth and exponentially increases until it reaches about 30 population doublings at about 20 years of age. From this point on, as long as the individual does not succumb to diseases or acute trauma leading to death, the progenitor cells will undergo the remaining 40 population doublings over a period of time that roughly equates to a total human life expectancy of approximately 120 years of age (Henson et al., 2005, Young and Black, 2005, Young et al., 2005). Their optimum temperature for cryopreservation is -196°C (liquid nitrogen). Additional characteristics for progenitor cells are listed in Table I.

Germ Layer Lineage Stem Cells

Germ layer lineage stem cells are between 10-20 µm in size. They are the immediate precursor cells for progenitor cells. They contain a normal chromosomal complement. There are three basic subcategories of germ layer lineage stem cells, paralleling the original embryonic germ layer lineages of ectoderm, mesoderm, and endoderm. They are named accordingly as germ layer lineage ectodermal stem cells, germ layer lineage mesodermal stem cells, and germ layer lineage endodermal stem cells.

Germ layer lineage stem cells are lineage-uncommitted with respect to their particular germ layer of origin, that is, they will form any cell within their respective germ layer of origin, but no cell of another germ layer. For example, germ layer lineage ectodermal stem cells will only form derivatives of the ectoderm e.g., epidermis, neurons, peripheral nerves, etc. Germ layer lineage mesodermal stem cells will form the structural

framework (stroma) of the organism e.g., vertebral column, thoracic cage, abdominal muscles, head and extremities, etc. as well as urinary, reproductive, gastrointestinal, vascular, lymphatic, hematopoietic, and immune systems. Germ layer lineage endodermal stem cells will form the parenchyma and/or the internal lining cells of the visceral organs, e.g., the nasopharynx, larynx, trachea, lungs, esophagus, stomach, small intestine, liver, gall bladder, large intestine, exocrine pancreas, pancreatic islets, etc.

Therefore, as a group, germ layer lineage stem cells have the potential to form any somatic cell type in the body. However, germ layer lineage stem cells are unidirectional in their commitment to tissues within their respective germ layers of origin and are unresponsive to lineage-induction agents that act outside their respective germ layer lineage. For example, mesodermal germ layer lineage stem cells are unresponsive to brain-derived neurotrophic factor (which acts on cells belonging to the ectodermal lineage).

Germ layer lineage stem cells are responsive to proliferation agents such as platelet derived growth factors, but exhibit contact inhibition at confluence *in vitro*. They are unresponsive to progression agents that accelerate the time frame of expression for tissue specific phenotypic differentiation expression markers, as long as they remain uncommitted to a particular tissue type. Germ layer lineage stem cells remain quiescent in a serum-free environment lacking proliferation agents, lineage-induction agents, progression agents, and inhibitory factors (review, Young et al., 1998, 2004a; Young 2000, 2004; Young and Black, 2004).

Germ layer cells do not exhibit phenotypic expression markers for embryonic, progenitor or differentiated cells. Instead, they express general and specific germ layer

lineage markers, such as the germ layer stem cell markers, CD13, CD90, and MHC-1 and markers for endodermal (alpha-fetoprotein, insulin), mesodermal (sarcomeric myosin, type-II collagen, or bone sialoprotein II), or ectodermal cells (nestin, synaptic vesicles, and oligodendrocytes) (Young et al., 1999, 2001a,b, 2004a; Young, 2000, 2004).

The life span of germ layer lineage stem cells is essentially unlimited as long as they do not commit to a progenitor cell lineage (i.e., they remain lineage-uncommitted). In the lineage-uncommitted state, they are telomerase positive and demonstrate capacities for population doublings that far exceed the 50-70 characteristic of differentiated human cells and progenitor cells and the 8-10 characteristic of differentiated rodent cells and progenitor cells. The mitotic clock for germ layer lineage stem cells begins only when these stem cells commit to a progenitor cell lineage. Once germ layer lineage stem cells commit to a particular tissue type, they become tissue-specific progenitor cells. They will then exhibit the characteristics described for progenitor cells described above and like all progenitor cells, their life span will be limited to 50-70 (human) or 8-10 (rodent) population doublings before programmed cellular senescence and death occur. Young and collaborators have induced germ layer stem cells derived from rat and human to over 20 ectodermal, 40 mesodermal, and 20 endodermal cell types. They confirmed the identity of 10, 25, and 11 of these cell types, respectively. (Young et al., 1999, 2001a,b, 2004a; Young, 2000, 2004). They have found the optimum temperature for cryopreservation to be $-70^{\circ} \pm 5^{\circ}\text{C}$.

Blastomere-Like Stem Cells

Blastomere-like stem cells are between 0.2 – 2.0 μm in size (review, Young and Black, 2005b). They are the immediate precursor cells for epiblast-like stem cells. They

contain a normal chromosomal complement. Blastomere-like stem cells are lineage-uncommitted and will form all somatic (non-reproductive) cells of the body. They will form the reproductive gametes sperm and/or ovum, and will form cells and tissues of the embryonic and fetal portions of the placenta.

Blastomere-like stem cells are responsive to lineage-induction agents, proliferation agents, and differentiation inhibitory agents. They are unresponsive to progression agents. Similar to epiblast-like stem cells they are not contact inhibited at confluence, but rather will form multiple confluent layers of cells as long as they are maintained with an adequate nutrient supply.

Blastomere-like stem cells do not express phenotypic expression markers for progenitor or differentiated cells, germ layer lineage stem cells, or epiblast-like stem cells. Instead, they express general and specific embryonic lineage markers, such as the embryonic stem cell markers CD66e, HCEA, CEA, and CEA-CAM-1 (Young and Black, 2005b).

The life span of blastomere-like stem cells is essentially unlimited as long as they do not commit to a progenitor cell lineage, e.g., they remain lineage-uncommitted. In the lineage-uncommitted state, they demonstrate capacities for population doublings that far exceed the 50-70 characteristic of differentiated human cells and progenitor cells and the 8-10 characteristic of differentiated rodent cells and progenitor cells. The mitotic clock for blastomere-like stem cells begins only when these stem cells commit to a progenitor cell lineage. Once blastomere-like stem cells commit to a particular tissue type, they become tissue-specific progenitor cells. They will then exhibit the characteristics described for progenitor cells described above and like all progenitor cells, their life span

will be limited to 50-70 (human) or 8-10 (rodent) population doublings before programmed cellular senescence and death occur. Young and collaborators have induced blastomere-like stem cells derived from rat and human to form more than 20 ectodermal, 40 mesodermal, 20 endodermal cell types, and one gamete. They have confirmed the identity of 10, 25, 11, and 1 of these cell types, respectively, including spermatogonia, utilizing insulin-dexamethasone bioassay coupled with the ELICA procedure (Young and Black, 2005b).