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# Karyotypic analysis of adult pluripotent stem cells

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**Summary.** Three categories of precursor cells have been identified in postnatal mammals: tissue-committed progenitor cells, germ layer lineage-committed stem cells and lineage-uncommitted pluripotent stem cells. Progenitor cells are the immediate precursors of differentiated tissues. Germ layer lineage stem cells can be induced to form multiple cell types belonging to their respective ectodermal, mesodermal, and endodermal embryological lineages. Pluripotent stem cells will form somatic cell types from all three primary germ layer lineages. Progenitor cells demonstrate a finite life span before replicative senescence and cell death occur. Both germ layer lineage stem cells and pluripotent stem cells are telomerase positive and display extensive capabilities for self-renewal. Stem cells which undergo such extensive replication have the potential for undergoing mutations that may subsequently alter cellular functions. Gross mutations in the genome may be visualized as chromosomal aneuploidy and/or chromosomes that appear aberrant. This study was designed to determine whether any gross genomic mutations occurred within the adult pluripotent stem cells. Karyotypic analysis was performed using pluripotent stem cells purified from adult male rats using established procedures. Giemsa Banding was used in conjuction with light microscopy to visualize metaphase chromosome spreads. To date over 800 metaphase spreads have been analyzed. We found that the metaphase spreads averaged 42 chromosomes and concluded that these pluripotent stem cells isolated from adult rats have a normal karyotype.

**Key words:** Karyotype, Stem cells, Pluripontent, Adult stem cell, Rat

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## Introduction

Previous studies (review, Young and Black, 2004) have revealed the existence of three categories of precursor cells located within adult mammals, including humans. The three categories of adult precursor cells are tissue-committed progenitor cells, germ layer lineage-committed (ectodermal, mesodermal, and endodermal) stem cells, and lineage-uncommitted pluripotent stem cells.

Progenitor cells are variable in size (10-200 μm), as assessed by flow cytometric analysis of living cells. In solid tissues they are located near their respective differentiated cell types. Their population doubling time during log phase growth is days to months. Progenitor cells can be preferentially flash frozen and stored in liquid nitrogen. Progenitor cells do not display phenotypic expression markers for embryonic stem cells, such as stage-specific embryonic antigen-4 or carcinoembryonic antigen cell adhesion molecule-1. Similarly, they do not display phenotypic expression markers for germ layer lineage stem cells, such as nestin for cells of the neural ectodermal lineage or alpha-fetoprotein for cells of the endodermal lineage. However, progenitor cells do express tissue specific phenotypic markers, such as neurofilament markers for neuroblasts and myogenin for myoblasts. Progenitor cells do not spontaneously differentiate in serum-free defined medium in the absence of a differentiation inhibitory agent, such as leukemia inhibitory factor or anti-differentiation factor. Thus, unlike embryonic stem cells which spontaneously differentiate under these particular culture conditions, progenitor cells remain in stasis unless acted upon by proliferative agents (such as platelet-derived growth factor) and/or progressive agents (such as insulin, insulin-like growth factor-I or insulin-like growth factor-II). Progenitor cells are unresponsive to inductive agents outside their tissue type. For example, skeletal muscle morphogenetic protein, a myogenic inductive agent specific for skeletal muscle, accelerates the differentiation of myoblasts into skeletal

muscle, but has no inductive effect on chondroblasts or osteoblasts, respectively. Progenitor cells are contact inhibited at confluence, forming a single layer of cells even in the presence of an agent that stimulates proliferation, such as platelet-derived growth factor. Progenitor cells have a finite life span before replicative senescence and cell death occur. This life span varies for different species. Thus, the replicative life span for rodents is 8-10 population doublings. For humans the replicative life span is 50-70 population doublings. Progenitor cells are committed to particular tissue types and will only form cells within that type. Thus, myoblasts will only form muscle, chondroblasts will only form cartilage, and osteoblasts will only form bone (Young et al., 1991, 1992a,b, 1993, 1995, 1998, 1999, 2001a,b, 2004a,b; Young, 2000, 2004; Young and Black, 2004).

Germ layer lineage-committed (ectodermal, mesodermal, and endodermal) stem cells are 10-20 μm in size, as assessed by flow cytometric analysis of living cells. They are located within connective tissue compartments throughout the body. Their population doubling time during log phase growth is 18-24 hours. They can be preferentially slow frozen and stored at -70±5°C. Germ layer lineage (ectodermal, mesodermal, and endodermal) stem cells do not display phenotypic expression markers for embryonic stem cells, such as stage-specific embryonic antigen-4 or carcinoembryonic antigen cell adhesion molecule-1. Similarly, germ layer lineage stem cells do not display phenotypic expression markers for progenitor or terminally differentiated cells, such as neurofilaments or dopamine decarboxylase for neurons, glial fibrillary acidic protein for glia, myogenin and myosin for muscle, type-II and type-IX collagen for cartilage, or glucagon, insulin and somatostatin for α-cells, ß-cells, and δ-cells, respectively, for pancreatic islets. However, germ layer lineage stem cells do express general and specific germ layer lineage markers. For example, CD13, CD90, and MHC-I are general markers for human germ layer lineage stem cells. Nestin is a specific lineage marker for both human and rat germ layer lineage ectodermal stem cells. And alpha-fetoprotein is a specific lineage marker for both human and rat germ layer lineage endodermal stem cells. Germ layer lineage stem cells do not spontaneously differentiate in serum-free defined medium in the absence of a differentiation inhibitory agent, such as leukemia inhibitory factor or anti-differentiation factor. Thus, unlike embryonic stem cells which spontaneously differentiate under these culture conditions, germ layer lineage stem cells remain in stasis unless acted upon by specific proliferative and/or lineage-specific inductive agents. Germ layer lineage stem cells are unaffected by progression factors, such as insulin, as long as they remain uncommitted to a particular tissue type. Similar to progenitor cells, germ layer lineage stem cells are contact inhibited at confluence, forming a single layer of cells even in the presence of an agent that stimulates proliferation. However unlike progenitor cells, germ layer lineage stem cells can propagate extensively, through multiple passages, under the influence of a proliferation agent as long as the stem cells remain uncommitted to a particular tissue type. In this tissue-uncommitted state, germ layer lineage stem cells are telomerase positive and maintain telomerase activity as long as they remain uncommitted to a specific tissue. Once they commit to a particular tissue type they lose telomerase activity and assume the replicative senescence and cell death profile for the progenitor cells of their respective species, i.e., 8-10 population doublings for rodents and 50-70 population doublings for humans. A clone of germ layer lineage mesodermal stem cells derived from adult rat skeletal muscle has surpassed 300 population doublings without losing its capability for differentiation. When exposed to general and specific inductive agents, germ layer lineage ectodermal stem cells have differentiated into neuronal progenitor cells, neurons, ganglia, oligodendrocytes, astrocytes, synaptic vesicles, radial glial cells, and keratinocytes; germ layer lineage mesodermal stem cells have differentiated into skeletal muscle, smooth muscle, cardiac muscle, white fat, brown fat, hyaline cartilage, articular cartilage, elastic cartilage, growth plate cartilage, fibrocartilage, endochondral bone, intramembranous bone, tendons, ligaments, dermis, fibrogenic scar tissue, endothelial cells, erythrocytes, lymphocytes, and macrophages; and germ layer lineage endodermal stem cells have differentiated into endodermal progenitor cells, enterocytes, hepatocytes, oval cells, biliary cells, canalicular cells, pancreatic progenitor cells, α-cells (glucagon), ß-cells (insulin), δ-cells (somatostatin), and three-dimensional insulin-secreting pancreatic islet-like structures. The potential for use of germ layer lineage ectodermal stem cells in the treatment of stroke has been demonstrated. Germ layer lineage mesodermal stem cells have shown promise in the repair of articular cartilage, bone, and skeletal muscle, and as a donor replacement tissue for the transplantation of bone marrow. Germ layer lineage endodermal stem cells have shown promise in the treatment of type-1 diabetes mellitus (Young et al., 1991, 1992a,b, 1993, 1995, 1998, 1999, 2001a,b, 2004a,b; Romero-Ramos et al., 2002; Young, 2000, 2004; Vrouc'h et al., 2004; Young and Black, 2004, 2005).

Pluripotent stem cells are 6-8 μm in size, as assessed by flow cytometric analysis of living cells. They are located within connective tissue compartments throughout the body. Their population doubling time during log phase growth is 12-14 hours. They can be preferentially slow frozen and stored at -80±5°C. Pluripotent stem cells in the undifferentiated state do not express either general or specific markers for germ layer lineage stem cells or progenitor cells. Rather, adult pluripotent stem cells express the Oct-3/4 gene that is characteristic of embryonic stem cells. Adult rat pluripotent stem cells display cell surface markers for embryonic stem cells, such as stage-specific embryonic antigen-4 and carcinoembryonic antigen cell adhesion

molecule-1. Adult human pluripotent stem cells also display cell surface epitopes for CD10 (neutral endopeptidase), CD66e (carcinoembryonic antigen), stage-specific embryonic antigen-1 and stage-specific embryonic antigen-3. Pluripotent stem cells do not spontaneously differentiate in serum-free defined medium in the absence of differentiation inhibitory factors. Embryonic stem cells spontaneously differentiate under these conditions. However, germ layer lineage stem cells and pluripotent stem cells remain in stasis unless acted upon by specific proliferative and/or inductive agents. Pluripotent stem cells are unaffected by progression factors as long as they remain uncommitted to a particular tissue type. Pluripotent stem cells are telomerase positive and propagate extensively under the influence of a proliferative agent. Unlike progenitor cells or germ layer linage stem cells, pluripotent stem cells are not contact inhibited at confluence, but rather form multiple confluent layers of cells post confluence. Pluripotent stem cells show extensive capabilities for self-renewal as long as they remain uncommitted. However, once they commit to a particular tissue type they lose telomerase activity and assume the replicative senescence and cell death profile for the progenitor cells of their respective species, i.e., 8-10 population doublings for rodents and 50-70 population doublings for humans. A clone of pluripotent stem cells derived from adult rat skeletal muscle has surpassed 270 population doublings without losing its capability for differentiation. Daughter cells of this clone expressed phenotypic markers for more than 40 discrete cell types of ectodermal, mesodermal, and endodermal origin when exposed to general and specific induction agents. Subsequent in vivo studies utilizing a genomically-labeled clone of adult pluripotent stem cells demonstrated the potential of these cells for the treatment of Parkinson's disease (with the formation in vivo of dopaminergic neurons containing tyrosine hydroxylase in substantia nigra which had previously been ablated with 6-hydroxydopamine), vascular ischemia (with the formation in vivo of capillaries), myocardial infarction (with the incorporation of cells in vivo into myocardium, vasculature, and connective tissues that were undergoing repair following myocardial cryoinjury) and type-1 diabetes mellitus (chemically induced pancreatic islets from this stem cell clone that secreted insulin in response to a glucose challenge) (Young, 2004; Young and Black, 2004, 2005; Young et al., 2004a,b).

As discussed above, adult pluripotent stem cells are normally quiescent unless acted upon by exogenous agents. This is in direct contrast to embryonic stem cells which spontaneously differentiate unless acted upon by agents that inhibit differentiation. In addition, adult pluripotent stem cells have a relatively short doubling time (12-14 hours) during log phase growth. Adult pluripotent stem cells are telomerase positive. And adult pluripotent stem cells exhibit extensive capabilities for self-renewal without loss of the potential for differentiation. The capacity for extended self-renewal in adult pluripotent stem cells far exceeds the programmed replicative senescence established for rodent progenitor cells (8-10 population doublings) (Rohme, 1981) or the programmed replicative senescence established for embryonic lineage-committed and differentiated human fibroblasts (50-70 population doublings) (Hayflick and Moorehead, 1961), respectively. This raises the question of whether these pluripotent stem cells isolated from adults are true stem cells or whether they are aberrant cells that were either inherently altered genomically or were altered genomically during their isolation and subsequent purification ex vivo. One would postulate that any alteration affecting cellular function would occur within the genome of the cell. Therefore, the current study was undertaken to ascertain whether adult-derived pluripotent stem cells contained an abnormal genome, as demonstrated by grossly aberrant numbers of chromosomes.

## Materials and methods

The use of animals in this study complied with the guidelines of Mercer University. These guidelines reflect the criteria for humane animal care of the National Research Council as outlined in "Guide for the Care and Use of Laboratory Animals" prepared by the Institute of Laboratory Animal Resources and published by the National Institutes of Health (National Academy Press, 1996).

### *Purification of pluripotent stem cells*

#### Cell Isolation

The hind limb musculature of five adult rats was processed for isolation and purification of pluripotent stem cells following established procedures (Young et al., 1991, 1992, 1995, 1998, 2001a, 2004a,b; Young, 2000, 2004). Ten cell lines (one cell line from each hind limb) from 5 separate animals were generated for this study. In brief, male Wistar Furth rats, weighing 220-250 grams, were euthanized using carbon dioxide inhalation. The hind limbs were washed with Betadine solution and incised. The thigh and leg muscle from each hind limb was removed under aseptic conditions and processed separately. The musculature from each hind limb was placed in a 50 ml conical polypropylene tube (Falcon, Becton Dickinson Labware, Franklin Lakes, NJ) containing 40 ml of sterile transport solution, and transferred to a sterile hood. The musculature was removed, washed in sterile Dulbecco's phosphate buffered saline pH 7.4 (DPBS), and transferred to an individual 60 mm glass Petri dish containing 10 ml of serum-free defined stem cell basal medium. The muscle was minced to the consistency of orange marmalade and the tissue suspension transferred to a 50 ml conical tube for enzymatic digestion. The muscle tissue was digested, centrifuged, and reconstituted for plating as described

(Young et al., 1992, 1995, 2004b).

Cultivation

Cell isolates from each muscle mass were plated in toto in stem cell propagation medium-1 using T-75 culture flasks (Falcon) coated with 1% gelatin (EM Sciences, Gibbstown, NJ). The cells were incubated in a humidified incubator containing 95% air/5% carbon dioxide at 37°C until they became confluent. The confluent cells were harvested following established procedures (Young et al., 2001a,b, 2004b). The stem cell propagation medium-1 consisted of serum-free defined stem cell basal medium for pluripotent stem cells containing 10 ng/ml of platelet-derived growth factor-BB (R&D Systems, Minneapolis, MN).

Selective Cryopreservation

The harvested cells were cryopreserved to -80±5°C for a minimum of 36 hours (Young et al., 1991, 2004b). The cryopreservation step served to remove the lineage-committed progenitor cells and differentiated cells from the cell cultures and to preferentially preserve adult pluripotent stem cells (Young et al., 2004a,b).

Differential cultivation

Cells were initially flash thawed to ambient temperature (Young et al., 2004a) and plated at high density, $1.0x10^6$ cells per T-25 culture flask (Falcon), in stem cell propagation medium-1 using flasks coated with 1% gelatin (EM Sciences). Viable cell recovery from initial cryopreservation ranged from 5-10%, as assessed by Trypan Blue exclusion. The cells were propagated past confluence in a humidified incubator at 37°C, released, and cryopreserved to -80±5°C for a minimum of 36 hours. Viable cell recovery from subsequent cryopreservations ranged from 95-98%, as assessed by Trypan Blue exclusion. The procedure of flash thawing to ambient temperature, plating at high density, propagation past confluence, release and cryopreservation was repeated until the number of 6-8 mm cells generated was sufficient for analysis of pluripotency and karyotype. The cells in each line were slow frozen and stored at -80± 5°C until used.

### *Pluripotential analysis*

Pluripotential analysis was undertaken to ascertain the identity of the cells lines prior to karyotypic analysis. Pluripotential analysis consisted of dividing each cell line into two groups and incubating each group separately in progression medium or general induction medium. After incubation, the cells were analyzed for their expression of phenotypic markers characteristic of pluripotent stem cells, germ layer lineage stem cells, progenitor cells, and differentiated cells using an antibody microarray enzyme-linked immuno-culture assay (ELICA) (Young et al., 1992c, 2004b). Since progression medium contains no induction agents, it allows the cells to remain in their respective differentiative states. However, progression medium contains an agent that accelerates the terminal differentiation of progenitor cells. Thus, pluripotent stem cells remain as pluripotent stem cells, germ layer lineage stem cells remain as germ layer lineage stem cells, and progenitor cells progress to terminally differentiated cell types, with each group expressing their respective phenotypic markers. In contrast, the general induction/progression medium contains a non-specific induction agent, e.g., dexamethasone, a battery of ectodermal, mesodermal, and endodermal induction agents, and a general progression agent. The general induction/progression medium will induce pluripotent stem cells to form cells that express phenotypic markers from all three germ layer lineages. It will induce germ layer lineage stem cells to form cells that express phenotypic markers for each individual germ layer lineage. And it will accelerate progenitor cells to differentiate into their terminal cell types (Young et al., 2004b).

Progression Medium

Each cell line was plated at $1.0x10^3$ cells per well in stasis medium using 96-well tissue culture plates (Corning, Corning, NY) coated with 1% gelatin (EM Sciences). Stasis medium consisted of 99% (v/v) Opti-MEM, 0.01 mM ß-mercaptoethanol (ßME, Sigma, St. Louis, MO), 1% (v/v) antibiotic-antimycotic solution (10,000 units/ml penicillin, 10,000 µg/ml streptomycin, 25 µg/ml Amphotericin-B, GIBCO, Grand Island, NY), 2 µg/ml insulin (Sigma), at pH 7.4 (Young et al., 2004a,b). The cell lines were incubated in a 37°C humidified environment for 8 days, washed with DPBS and then processed as described below for antibody microarray ELICA (Young et al., 2004b).

General Induction/Progression Medium

Each cell line was plated at $1.0 \times 10^3$ cells per well in general induction/progression medium using 96-well tissue culture plates coated with 1% gelatin. General induction/progression medium consisted of 84% (v/v) Opti-MEM, 0.01 mM ß-mercaptoethanol (ßME, Sigma, St. Louis, MO), 1% (v/v) antibiotic-antimycotic solution (10,000 units/ml penicillin, 10,000 µg/ml streptomycin, 25 µg/ml Amphotericin-B, GIBCO, Grand Island, NY), $10^{-8}$ M dexamethasone (Sigma), 2 µg/ml insulin (Sigma), 5% SS9, and 10% SS12 (MorphoGen Pharmaceuticals Inc., San Diego, CA), at pH 7.4. Five percent SS9 contained mesodermal induction activity. At a ten percent concentration, SS12 contained ectodermal and endodermal induction activities, but not enough ADF to inhibit those ectodermal and endodermal lineage-induction activities (Young, 2000, 2004; Young et al., 1998, 2004a,b). The cell lines were incubated in a 37°C humidified environment for 21 days with medium

changes every other day, washed with DPBS and then processed as described below for antibody microarray ELICA (Young et al., 2004b).

Antibody Microarray ELICA

Cells from each line were fixed for 10 minutes in 2% (v/v) formaldehyde (ChemPure, Curtin Matheson Scientific, Houston, TX), 0.2% (v/v) glutaraldehyde (Sigma, St. Louis, MO), and 97.8% (v/v) PBS. The fixative was removed and replaced with a 0.3% (g/v) sodium azide (Sigma) in PBS for 10 minutes to irreversibly inhibit endogenous peroxidases (Young et al., 1992b). Cells were rinsed with PBS and incubated for 10 minutes with blocking agent (Vecstatin ABC Reagent Kit, Vector Laboratories Inc., Burlingame, CA) in PBS. The blocking agent was removed. The cells were rinsed with PBS and incubated with primary antibody for 60 minutes at ambient temperature. The primary antibodies, diluted in PBS, consisted of the following. Potential pluripotent stem cells were identified with 0.005% (v/v) CEA-CAM-1 for carcinoembryonic antigen cell adhesion molecule-1 (Estrera et al., 1999; Young et al., 2004b) and 1 µg per well SSEA-4 (Developmental Studies Hybridoma Bank, DSHB, Iowa City, IA) for stage-specific embryonic antigen-4 (Lannagi et al., 1983; Young et al., 2004b).

Potential ectodermal lineage cells were identified utilizing 1.0 µg Rat-401 (DSHB) for nestin (Hockfield and McKay, 1985), 0.02 µg MAB353 (Chemicon, Temecula, CA) for nestin (Gritti et al., 1996), 1.0 µg FORSE-1 (DSHB) for nestin (Tole et al., 1995; Tole and Patterson, 1995), 1.0 µg 8A2 (DSHB) for neurons (Drazba et al., 1991), 0.5 µg RT-97 (DSHB) for neurofilaments (Wood and Anderton, 1981), 0.138 µg S-100 (Sigma) for neurons (Baudier et al., 1986; Barwick, 1990), 1.388 µg N-200 (Sigma) for neurofilament-200 (Debus et al., 1983; Franke, et al., 1991), 0.64% (v/v) T8660 (Sigma) for beta-III tubulin (Banerjee et al., 1988, 1990; Joshi and Cleveland, 1990; Young et al., 2004b), 1.0 µg TH (Sigma) for tyrosine hydroxylase (Lewis et al., 1993), 1.0 µg DOPA (Sigma) for dopamine decarboxylase (Zhu and Juorio, 1995), 1.0 µg SV2 (DSHB) for synaptic vesicles (Feany et al., 1992), 1.0 µg Rip (DSHB) for oligodendrocytes (Friedman et al., 1989), 0.357 µg CNPase (Sigma) for astroglia and oligodendrocytes (Sprinkle et al., 1987; Sprinkle, 1989; Reynolds et al., 1989), 1.0 µg GFAP (Sigma) for glial fibrillary acidic protein (Romero-Ramos, et al., 2002), 0.5 µg 40E-C (DSHB) for radial glial cells (Alvarez-Buylla et al., 1987), 1.0 µg VM-1 (DSHB) for keratinocytes (Oseroff et al., 1985; Young et al., 2004b).

Potential mesodermal lineage cells were identified using 1.0 µg F5D (DSHB) for myogenin (Wright et al., 1991), 0.064 µg MF-20 (DSHB) for sarcomeric myosin (Bader et al., 1982), 0.07 µg MY-32 (Sigma) for skeletal muscle fast myosin (Naumann and Pette, 1994), 0.125 µg ALD-58 (DSHB) for myosin heavy chain (Shafiq et al., 1984), 1.0 µg A4.74 (DSHB) for myosin fast chain (Webster et al., 1988), 0.028 µg IA4 (Sigma) for smooth muscle alpha-actin (Skalli et al., 1986), 0.25 µg MAB3252 (Chemicon) for cardiotin (Schaart et al., 1997), 1.0 µg MAB1548 (Chemicon) for myosin heavy chain type-b of cardiac muscle (Young et al., 2004a), 1.0 µg CIIC1 (DSHB) for type-II collagen (Holmdahl et al., 1986), 0.2 µg HC-II (ICN, Aurora, OH) for type-II collagen (Burgeson and Hollister, 1979; Kumagai et al., 1994), 1.0 µg D1-9 (DSHB) for type-IX collagen (Ye et al., 1991), 0.02 µg WV1D1 (DSHB) for bone sialoprotein II (Kasugai et al., 1992), and 0.04 µg MP111 (DSHB) for osteopontine (Gorski et al., 1990).

Potential endodermal lineage cells were identified using 0.2 µg R-AFP (Nordic, Tiburg, The Netherlands) for alpha-fetoprotein (Mujoo et al., 1983), 0.625 µg HESA (Sigma) for epithelial specific antigen (Young et al., 2004a,b), 1.0 µg YM-PS087 (Accurate, Westbury, NY) for glucagon-secreting α-cells (Young, 2004; Young et al., 2004b), 1.0 µg YM-PS5088 (Accurate) for insulin-secreting ß-cells (Young, 2004; Young et al., 2004b), and 0.5 µg 11180 (ICN, Aurora, OH) for somatostatin-secreting δ-cells (Young, 2004; Young et al., 2004b), 1.0 µg 151-IgG (DSHB) for liver epithelial growth factor receptor (Hubbard et al., 1985), 1.0 µg OC2 for hepatic oval cells, liver progenitor cells, and biliary epithelial cells (Faris et al., 1991; Gordon et al., 2000), 1.0 µg OC3 for hepatic 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), 1.0 µg OC4 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), 1.0 µg OC5 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), 1.0 µg OC10 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), 1.0 µg H-1 for hepatocyte cell surface marker (Walborg et al., 1985; Faris et al., 1991), 1.0 µg H-4 for hepatocyte cytoplasm (Walborg et al., 1985; Faris et al., 1991), 1.0 µg DPP-IV 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), 1.0 µg OV6 for oval cells, liver progenitor cells, and biliary epithelial cells (Faris et al., 1991; Gordon et al., 2000), and 1.0 µg HA4c19 (DSHB) for bile canalicular cells of liver (Hubbard et al., 1985).

The primary antibodies were removed. The cells were rinsed with PBS and incubated with secondary antibody for 20 minutes. The secondary antibodies consisted of 0.005% (v/v) biotinylated anti-goat IgM (Vector) in PBS for FORSE-1, 40E-C, OC2, OC3, OC5, OC10, and H4 (Young et al., 2004a,b), 0.9 µg biotinylated anti-sheep IgG (Vector) per 100 ml PBS for R-AFP (Young et al., 2004a,b), 0.9 µg biotinylated anti-

rabbit IgG (Vector) per 100 ml PBS for YM-PS087, YM-PS5088, and 11180, or 0.005% (v/v) biotintylated anti-mouse IgG (Vecstatin ABC Reagent IgG Kit) in PBS (Young et al., 2004a) for the remaining primary antibodies. The secondary antibody was removed. The cells were rinsed with PBS and then incubated with avidin-HRP for 20 minutes. The avidin-HRP consisted of 10 ml of 0.1% (v/v) Tween-20 (ChemPure) containing 2 drops reagent-A and 2 drops reagent-B (Vecstatin ABC Reagent Kit) in PBS. The avidin-HRP was removed. The cells were rinsed with PBS and incubated with 3, 3'-diaminobenzidine (DAB) substrate (Vecstatin ABC Reagent Kit) for 60 minutes. The DAB substrate was prepared as directed by the manufacturer (Vector Laboratories Inc.). The substrate solution was removed. The cells were rinsed with PBS and stored in 0.3% sodium azide.

Positive and negative controls were included to assure validity of the staining procedures (Young et al., 2004a,b). The controls consisted of a clone of adult rat pluripotent stem cells (Scl-40ß, Young et al., 2004b) and procedural controls. The Scl-40ß clone was incubated in parallel in progression medium and general induction/progression medium and processed, as above, with the antibody microarray ELICA. The procedural controls consisted of the cells processed in the antibody microarray ELICA without primary antibodies (negative control), cells processed in the antibody microarray ELICA without secondary antibody (negative control), cells processed in the antibody microarray ELICA without primary and secondary antibodies (negative control), and cells processed in the antibody microarray ELICA without avidin-HRP (negative control) prior to incubation with the DAB substrate.

Visual Analysis

Stained cells were visualized using a Nikon TMS inverted phase/brightfield microscope with phase contrast and brightfield microscopy. Magnifications were as noted in the figure legends. Photographs were taken using a Nikon CoolPix 995 digital camera. Digital photographs were cropped and background color adjusted using Adobe Photoshop 7.0.

### *Karyotypic analysis*

Ten adult rat cell lines were generated. Only those cell lines demonstrating positive staining for pluripotent stem cells (CEA-CAM-1 and SSEA-4) and negative staining for germ layer lineage stem cells, progenitor cells, and differentiated cells after incubation in progression medium were processed for karyotypic analysis. The cell lines were flash thawed to ambient temperature and plated at $1.5x10^6$ cells in stem cell propagation medium-2 using T-75 culture flasks (Falcon) coated with 1% gelatin (EM Sciences). Viable cell recovery from cryopreservation ranged from 95-98%, as assessed by Trypan Blue exclusion. The cells were incubated at 37°C in a 5% $CO_2$/95% air humidified environment. Stem cell propagation medium-2 (SCPM-2) consisted of 84% (v/v) Opti-MEM, 0.01 mM ß-mercaptoethanol (ßME, Sigma, St. Louis, MO), 1% (v/v) antibiotic-antimycotic solution (10,000 units/ml penicillin, 10,000 µg/ml streptomycin, 25 µg/ml Amphotericin-B, GIBCO, Grand Island, NY), and 15% SS12 (MorphoGen Pharmaceuticals Inc., San Diego, CA), at pH 7.4 (Young et al., 2004a,b). Fifteen percent SS12 contained proliferative activity resembling that of 10 ng/ml platelet-derived growth factor and inductive/differentiation-inhibitory activity resembling that of 2 U/ml anti-differentiation factor. Two units per ml of anti-differentiation factor is of sufficient quantity to inhibit the activities of all serum-containing and most recombinant inductive agents (Young, 2000, 2004; Young et al., 1998, 2004a,b). Propagation in SCPM-2 generated a population doubling time of approximately 12 hours while maintaining the cells in a lineage-uncommitted, tissue-uncommitted state. Each stem cell line was allowed to propagate for a minimum of 12-14 population doublings after pluripotential analysis to generate cells for karyotypic analysis. We followed this regimen in order to surpass the 8-10 population doubling limit for replicative senescence characteristic of rodent progenitor cells (Rohme, 1981). The cell lines were then slow frozen and stored at -80±5°C.

Preparation of Chromosome Spreads

The cell lines were flash thawed to ambient temperature and plated at $0.5x10^6$ per flask in SCPM-2 using T-25 culture flasks (Falcon) coated with 1% gelatin. Viable cell recovery from cryopreservation ranged from 95-98%, as assessed by Trypan Blue exclusion. Twenty-four hours later the medium was removed and replaced with SCPM-2 containing 0.1 µg/ml colcemid (10 µl/ml KaryoMAX™ Colcemid, GIBCO). Cells were incubated in this propagation medium for 4 hours. Approximately 50% of the cells displayed mitotic profiles during this incubation period. Mitotic profiles were characterized by the loss of bipolar/stellate morphology, rounding up, and the appearance of two parallel lines of chromosomes (metaphase plate) within the area of the nucleus. After Colcemid incubation, the cells in each flask were harvested, centrifuged, and processed separately. Pelleted cells were resuspended in 200 µl of SCPM-2, then reconstituted in 10 ml of 0.075 M KCl and incubated for 15 min in a 37°C water bath. [Note that the KCL was added very slowly for the first few milliliters of solution.] Three to five drops of freshly prepared fixative (3:1 methanol: acetic acid, glacial) were added to stop the reaction. Cells were centrifuged at 500 x g for 5 min at 37°C. Following centrifugation the supernatant was discarded, the cells resuspended and 10 ml of freshly prepared fixative was added to the cells. This fixation regimen was performed three times in succession to ensure proper fixation of the cells.

Following the final centrifugation, the cells were resuspended in enough fixative to yield a slightly turbid mixture. [If slides were not prepared on the on the day of harvest from the culture flasks, the cells were reconstituted in 10 ml of fixative, resuspended by gentle trituration, and then stored at 4°C.]

Chromosome spreads were generated by applying one to two drops of cell suspension from a glass Pasteur pipette to a clean cold (~4°C) wet glass slide. The pipette was maintained approximately 6 inches above the surface of the slide. The slides were placed at a 30 degree angle to facilitate even distribution of the chromosomes.

Slides were stained utilizing "GTG" Banding (Giemsa Banding) protocol as per manufacturer's directions (GIBCO). In brief, slides were dried/aged at 60°C for a minimum of 18 hours. Slides were incubated in the trypsin solution for 30 to 60 seconds and then stained with KARYOMAX™ Giemsa stain (GIBCO) in Gurr's buffer, pH 6.8, for five minutes. After staining, slides were allowed to dry at ambient temperature and coverslipped with Cytoseal 60. Slides were allowed to dry under a fume hood for 24 hours prior to analysis.

### Visual Analysis

Chromosome spreads were visualized with a Nikon Fluorphot compound microscope using brightfield microscopy at 1000x. Photographs were taken with a Nikon CoolPix 995 digital camera. Digital photographs were cropped and color adjusted to optimize chromosome banding using Adobe Photoshop 7.0.

### Chromosome Counts

Over 800 chromosome spreads were generated for karyotypic analysis of these cells. One hundred randomly chosen spreads were analyzed for chromosome number using a double-blinded protocol. Each chromosome spread was independently counted by 12 individuals. Data from the counts were compiled and evaluated for statistical significance. The statistical analyses were performed using the ABSTAT computer program (Anderson Bell Corp., Arvada, CO).

### Karyotypic analysis

Chromosomes were paired based on banding patterns and position of their respective centromere. The chromosomes were arranged based on nomenclature rules for rat chromosome G-bands, as described by Levan (1974).

## Results

After incubation in progression medium for eight days, the cells used in this study remained small. All (100%) of these small cells demonstrated immunocytochemical staining for carcinoembryonic antigen-cell adhesion molecule-1 (Fig. 1A) and approximately 70% of these small cells demonstrated immunocytochemical staining for stage-specific embryonic antigen-4. The cells in each line, grown in progression medium, were negative for expression markers characteristic of germ layer lineage stem cells, progenitor cells, and differentiated cells. Representative examples of negative staining are shown for nestin using FORSE-1 (Fig. 1B), neurons using 8A2 (Fig. 1C) and S-100 (Fig. 1D), glia using CNPase (Fig. 1E) and 40E-C (Fig. 1F), synaptic vesicles using SV2 (Fig. 1G), keratinocytes using VM-1 (Fig. 1H), smooth muscle using IA4 (Fig. 1I), type-II collagen of cartilage using CIIC1 (Fig. 1J), osteopontine of bone using MPIII (Fig. 1K), and bone sialoprotein II using WV1D1 (Fig. 1L).

In contrast, after incubation in general induction/progression medium for 21 days the cells within each line altered their morphological appearance, usually by increasing in size and/or shape. These apparently induced cells demonstrated absence of staining for carcinoembryonic antigen-cell adhesion molecule-1 (Fig. 2A). They also demonstrated a size-dependent decrease in staining for stage-specific embryonic antigen-4. Small cells exhibited the highest amount of staining. Cells intermediate in size demonstrated moderate staining. By contrast, large cells did not stain (Fig. 2B). These cells also exhibited immunocytochemical staining for phenotypic markers characteristic of germ layer lineage stem cells, progenitor cells, and differentiated cells. Representative examples of positive immunochemical staining are shown for ectodermal lineage cells, i.e., nestin using Rat-401 (Fig. 2C), MAB353 (Fig. 2D), and FORSE-1 (Fig. 2E); neurons using 8A2 (Fig. 2F), RT-97 (Fig. 2G), S-100 (Fig. 2H), TH (Fig. 2I), DOPA (Fig. 2J), and SV2 (Fig. 2K); glial cells using Rip (Fig. 2L), CNPase (Fig. 2M), GFAP (Fig. 2N), and 40E-C (Fig. 2O), and keratinocytes using VM-1 (Fig. 2P); mesodermal lineage cells, i.e., myogenin of skeletal muscle using F5D (Fig. 3A) and myosin of skeletal muscle using A4.74 (Fig. 3B), α-actin of smooth muscle using IA4 (Fig. 3C), cardiotin of cardiac muscle using MAB3252 (Fig. 3D), type-II collagen of cartilage using CIIC1 (Fig. 3E), type-IX collagen of cartilage using D1-9 (Fig. 3F), and osteopontine of bone using MPIII (Fig. 3G); and endodermal lineage cells, i.e., alpha-fetoprotein of endodermal lineage cells using R-AFP (Fig. 3H), epithelial cells derived from endodermal using HESA (Fig. 3I), glucagon-secreting pancreatic α-cells using YM-PS087 (Fig. 3J), insulin-secreting pancreatic ß-cells using YM-PS5088 (Fig. 3K), somatostatin-secreting pancreatic δ-cells using 11180 (Fig. 3L), liver epithelial growth factor receptor using 151-IgG (Fig. 3M), hepatic oval cells, liver progenitor cells, canalicular cells, and/or biliary epithelial cells using OC2 (Fig. 3N), OC4 (Fig. 3O), and OC10 (Fig. 3P), hepatocyte cell surface marker using H1 (Fig. 3Q), hepatocyte cytoplasm using H4 (Fig. 3R), DPPIV (Fig. 3S), and bile canalicular cells of liver using HA4c19 (Fig. 3T).

Scl-40ß incubated in progression medium demonstrated positive staining for CEA-CAM and SSEA-4 and negative staining for the phenotypic expression markers characteristic of germ layer lineage stem cells, progenitor cells, and differentiated cells (data not shown, but comparable to Young et al., 2004b). Scl-40ß incubated in general induction/progression medium demonstrated an increase in size with concomitant negative staining for CEA-CAM-1 and SSEA-4 and positive staining for the phenotypic expression markers characteristic of germ layer lineage stem cells, progenitor cells, and differentiated cells (data not shown, but comparable to Young et al., 2004b). Regardless of whether they were grown in progression medium or general induction/progression medium, the cells in each line and the Scl-40ß clone were negative for all of the procedural controls, i.e., without primary antibody,

**Table 1.** Karyotypic analysis of adult pluripotent stem cells

| BLINDED SCORERS | COUNT RANGE | MEDIAN COUNT | MEAN | STD DEV |
|---|---|---|---|---|
| 1 | 37-45 | 42 | 41.76 | ±0.97 |
| 2 | 39-43 | 42 | 41.74 | ±0.78 |
| 3 | 38-44 | 42 | 41.85 | ±0.71 |
| 4 | 38-43 | 42 | 41.84 | ±0.72 |
| 5 | 41-43 | 42 | 42.00 | ±0.24 |
| 6 | 38-44 | 42 | 41.57 | ±0.71 |
| 7 | 38-43 | 42 | 41.67 | ±0.97 |
| 8 | 41-43 | 42 | 41.98 | ±0.20 |
| 9 | 38-44 | 42 | 41.57 | ±0.71 |
| 10 | 38-43 | 42 | 41.67 | ±0.97 |
| 11 | 41-43 | 42 | 41.97 | ±0.26 |
| 12 | 37-43 | 42 | 41.70 | ±0.98 |
| Compiled Data | 37-45 | 42 | 41.86 | ±0.68 |

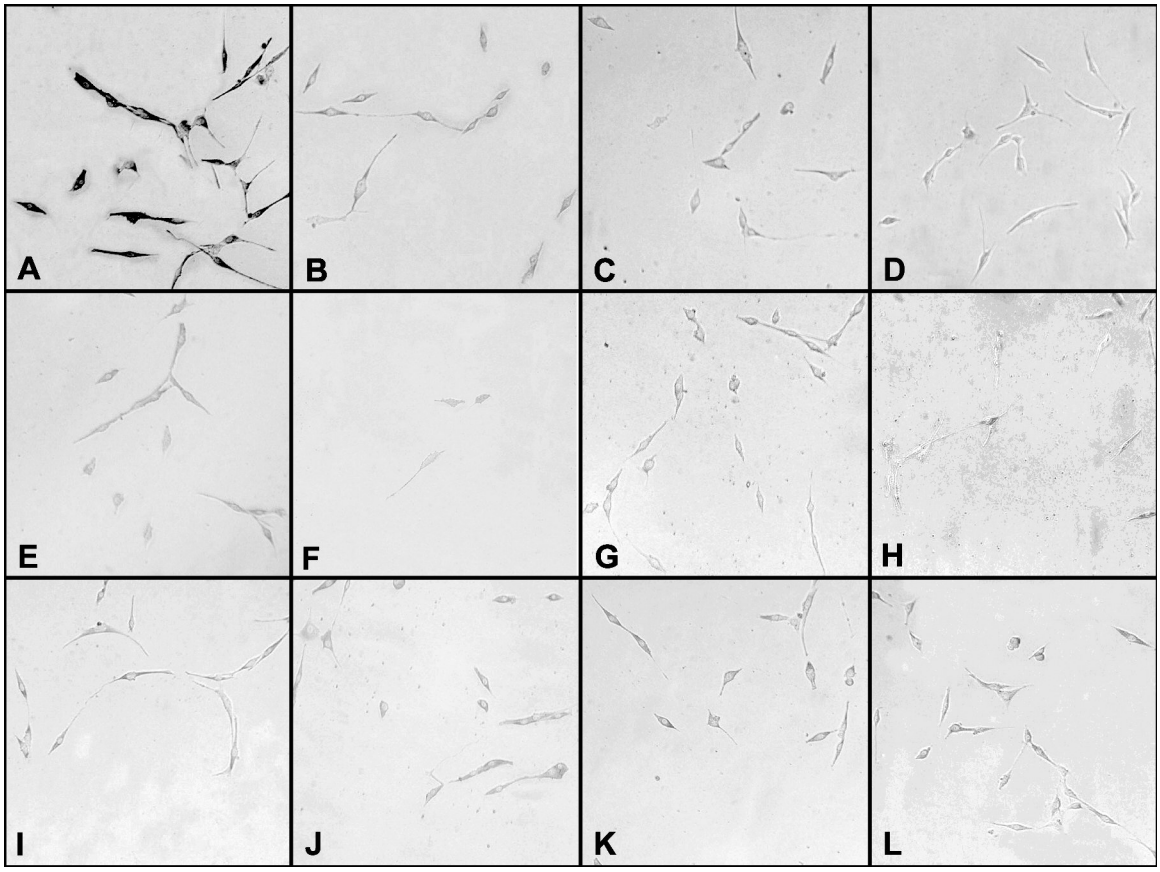


**Fig. 1.** Immunocytochemical staining of cells isolated from adult male Wistar Furth rat skeletal muscle. Cells were grown in progression medium for 8 days and then processed for antibody microarray ELICA. Note presence of very small cells with high nuclear to cytoplasmic ratios. Staining was either present **(A)** or absent **(B-L)**, depending on the particular antibody utilized, as noted. Cells were photographed with brightfield **(A)** or phase contrast **(B-L)** microscopy. **A.** CEA-CAM-1, carcinoembryonic antigen cell adhesion molecule-1, a marker expressed by adult pluripotent stem cells. **B.** FORSE-1, nestin, a marker expressed by neurogenic progenitor cells. **C.** 8A2, gangliosides, a marker expressed by neurons. **D.** S-100, neurofilament-100 ß-subunit, a marker expressed by neurons. **E.** CNPase, myelin, a marker expressed by glial cells. **F.** 40E-C, glial-specific vimentin, a marker expressed by radial glial cells. **G.** SV-2, synaptic vesicle marker, a marker expressed by neurons. **H.** VM-1, a marker expressed by keratinocytes. **I.** IA4, alpha-smooth muscle actin, a marker expressed by smooth muscle cells. **J.** CIIC1, type-II collagen, a marker expressed by cartilage. **K.** MPIII, osteopontine, a glycoprotein expressed by bone. **L.** WV1D1, bone sialoprotein II, a marker expressed by bone. Original magnifications, x 50

without secondary antibody, without primary and secondary antibodies, and without avidin-HRP (data not shown).

Only those cell lines demonstrating positive staining for pluripotent stem cells (Fig. 1A) and negative staining for germ layer lineage stem cells, progenitor cells, and

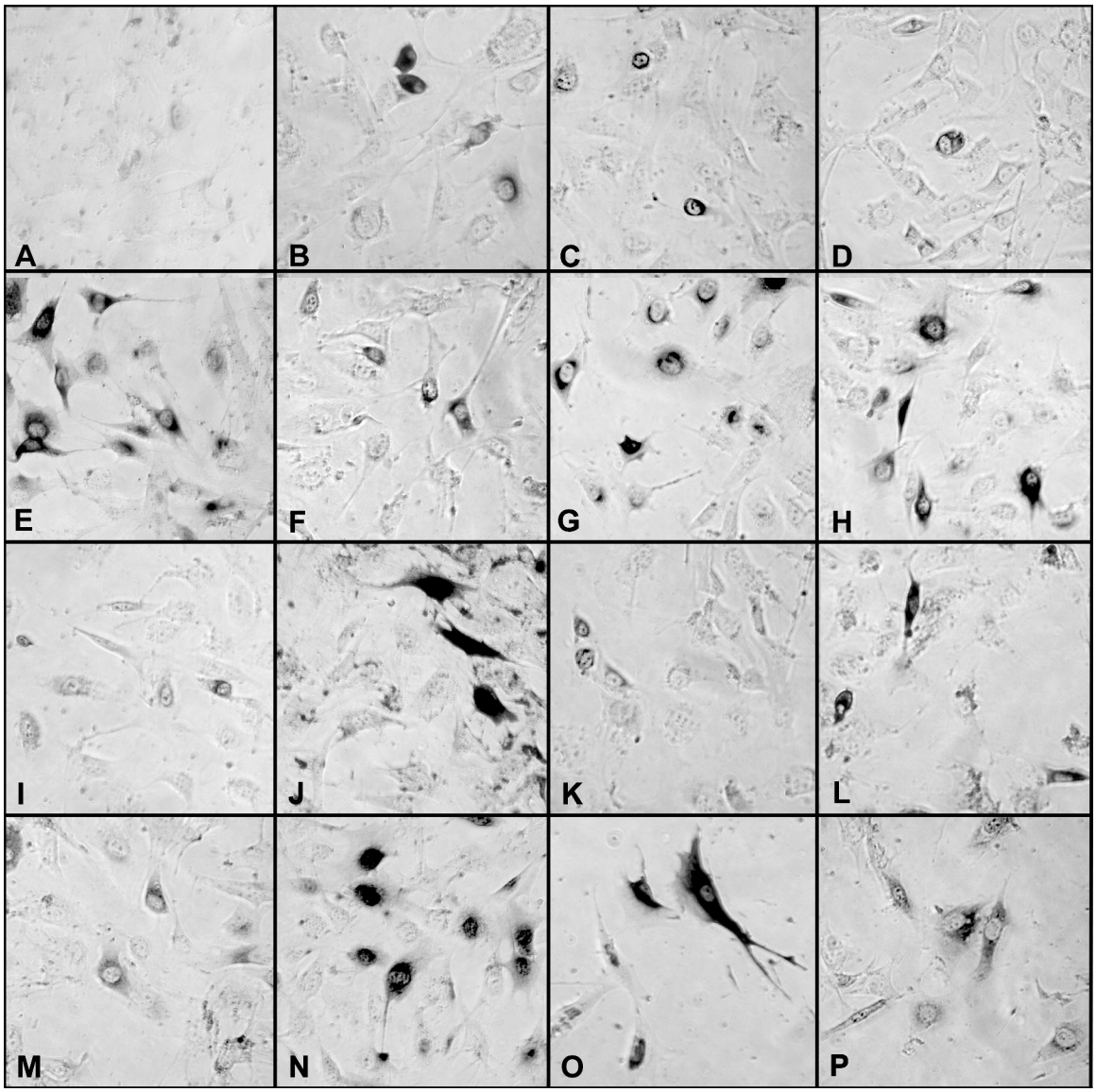


**Fig. 2.** Immunocytochemical staining of cells isolated from adult male Wistar Furth rat skeletal muscle. Cells were grown in general induction/progression medium for 21 days and then processed for antibody microarray ELICA. Note presence of larger cells, except where noted. Staining was either absent **(A)** or present **(B-P)**, depending on the particular antibody utilized, as noted. Cells were photographed with brightfield microscopy. **A.** CEA-CAM-1, carcinoembryonic antigen cell adhesion molecule-1, a marker expressed by adult pluripotent stem cells. **B.** SSEA-4, stage-specific embryonic antigen-4, a marker expressed by adult pluripotent stem cells. **C.** Rat-401, nestin, a marker expressed by neurogenic progenitor cells. **D**. MAB353, nestin, a marker expressed by neurogenic progenitor cells. **E.** FORSE-1, nestin, a marker expressed by neurogenic progenitor cells. **F.** 8A2, gangliosides, a marker expressed by neurons. **G.** RT-97, neurofilaments, a marker expressed by neurons. **H.** S-100, neurofilament-100 ß-subunit, a marker expressed by neurons. **I.** TH, tyrosine hydroxylase, an enzyme involved in the biosynthesis of catecholamines (dopamine, norepinephrine, and epinephrine) and expressed in catecholaminergic neurons. **J.** DOPA, dopamine decarboxylase, an enzyme involved in the decarboxylation of L-DOPA to form dopamine in both dopaminergic and noradrenergic neurons and involved in the decarboxylation of 5-hydroxytyrptophan to serotonin (5-HT, 5-hydroxytryptamine) in serotonergic neurons. **K.** SV2, synaptic vesicle marker, a marker expressed by neurons. **L.** Rip, a marker expressed by oligodendrocytes. **M.** CNPase, myelin, a marker expressed by glial cells. **N.** GFAP, glial fibrillary acid protein, a marker expressed by glial cells. **O.** 40E-C, glial-specific vimentin, a marker expressed by radial glial cells. **P.** VM-1, a marker expressed by keratinocytes. Original magnifications, x 100

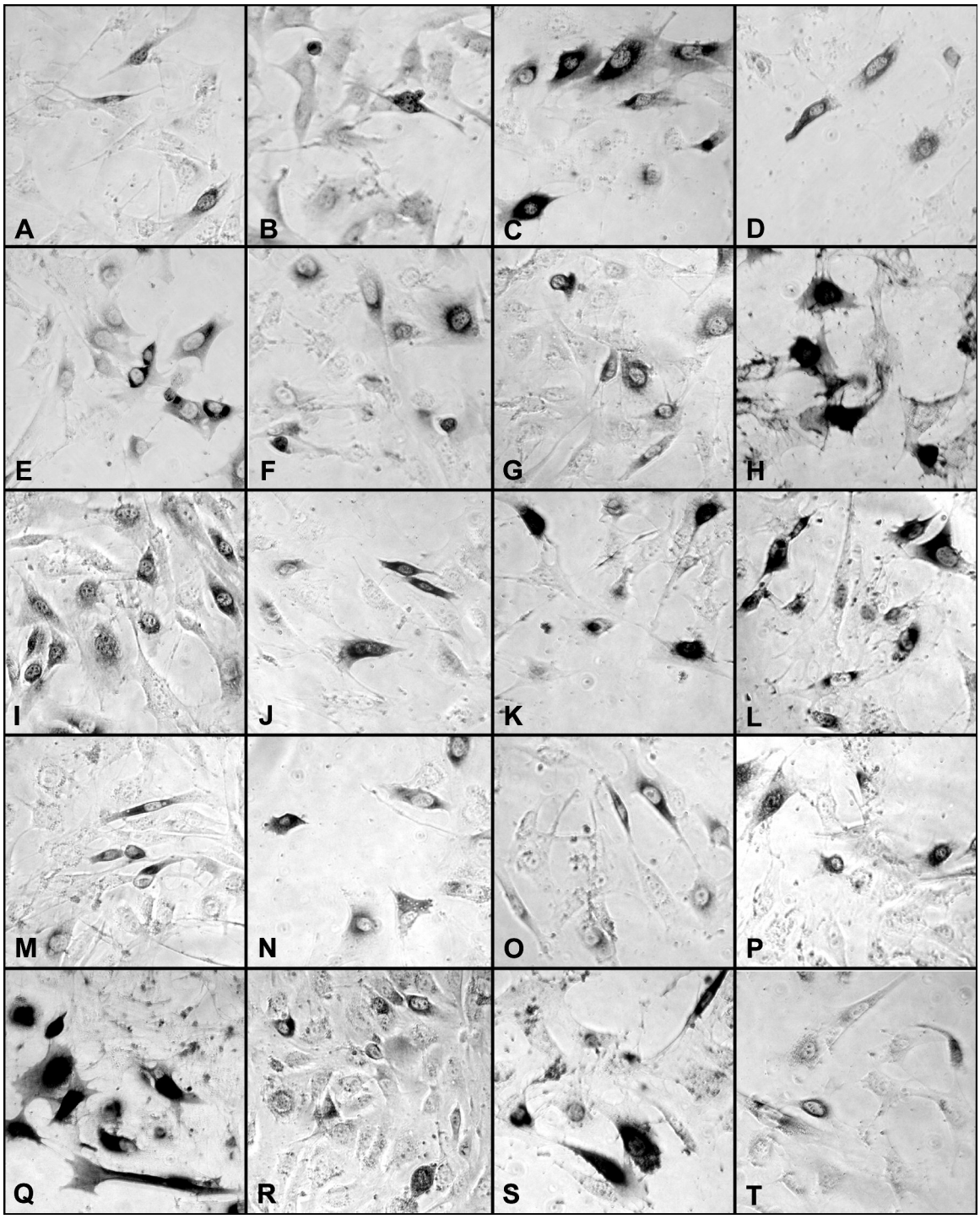


**Fig. 3.** Immunocytochemical staining of cells isolated from adult male Wistar Furth rat skeletal muscle. Cells were grown in general induction/progression medium for 21 days and then processed for antibody microarray ELICA. Note presence of larger cells. Staining was present **(A-T)**, depending on the particular antibody utilized, as noted. Cells were photographed with brightfield microscopy. **A.** F5D, myogenin, a marker expressed by myogenic progenitor cells. **B.** A4.74, adult myosin, a marker expressed by skeletal muscle. **C.** IA4, alpha-smooth muscle actin, a marker expressed by smooth muscle cells. **D.** MAB3252, cardiotin present in cardiac myocytes. **E.** CIIC1, type-II collagen, a marker expressed by cartilage. **F.** D1-9, type-IX collagen, a marker expressed by cartilage. **G.** MPIII, osteopontine, a glycoprotein expressed by bone. **H.** RAFP, rat alpha-fetoprotein, a marker expressed by endodermal progenitor. **I.** HESA, epithelial specific antigen. **J.** Glucagon, synthesized and secreted by $\alpha$-cells of pancreatic islets. **K.** Insulin, synthesized and secreted by ß-cells of pancreatic islets. **L.** Somatostatin, synthesized and secreted by $\delta$-cells of pancreatic islets. **M.** 151-IgG, epidermal growth factor receptor on surface of GI epithelial cells. **N.** OC2, liver progenitor cells, oval cells, and biliary epithelial cells. **O.** OC4, liver progenitor cells, oval cells, canalicular cells and biliary epithelial cells. **P.** OC10, liver progenitor cells, oval cells, canalicular cells and biliary epithelial cells. **Q.** H1, hepatocyte cell surface marker. **R.** H4, hepatocyte cytoplasm. **S.** DPPIV, liver progenitor cells, oval cells, canalicular cells and biliary epithelial cells. **T.** HA4c19, bile canalicular cells. Original magnifications, x 100

differentiated cells (Figs. 1B-1L) in the presence of progression medium were processed further for karyotypic analysis. The range, median and mean chromosome number ± standard deviation were determined for each independent and blinded scorer (Table 1). Counts for the 100 spreads by the twelve independent scorers ranged from 37 to 45 chromosomes. The median chromosome number was 42 for each scorer. There was a total combined mean of 41.86±0.68 chromosomes (Table 1). A randomly chosen chromosome spread is shown in Figure 4A with its karyotypic analysis shown in Figure 4B.

## Discussion

Young et al. (2004a,b) reported the existence of pluripotent stem cells within the skeletal muscle of adult rats. The cells used in this study were purified from the skeletal muscle of adult male rats following the protocol for isolation and preferential cultivation for pluripotent stem cells as described by Young et al. (2004a,b). Ten cell lines were generated. The cells in each line were extensively propagated, both before and after analysis of pluripotency to ensure that their respective population doubling numbers were above the 8-10 population doublings for replicative senescence in rodent cells, as described by Rohme (1981). While exact numbers of population doublings before analysis of pluripotency were not recorded, there was a minimum of 12-14 population doublings for each cell line after analysis of pluripotency. This suggested that the cells used in this study were neither differentiated cells nor progenitor cells, since both display 8-10 population doublings before replicative senescence and cell death occur (Young et al., 2004b).

Differentiated cells and progenitor cells preferentially survive flash freezing and storage in liquid nitrogen (-196°C) (Young et al., 1991, 2004a). In contrast, germ layer lineage stem cells preferentially survive slow freezing and storage at -70±5°C (Young et al., 1991, 2001a,b), while pluripotent stem cells preferentially survive slow freezing and storage at -80±5°C (Young et al., 2004a,b). The cells used in this study were cryopreserved (frozen and stored) multiple times at -80±5°C. The initial cryopreservation step after cell harvest at -80±5°C resulted in cell recoveries of 5-10%. This low number of recovered cells suggested that differentiated cells and progenitor cells were lost during the freezing and storage procedure. Subsequent cryopreservation steps at -80±5°C yielded cell recoveries of 95-98%. Since both germ layer lineage stem cells and pluripotent stem cells survive cryopreservation at -75±10°C, we postulate that the cells used in this study were neither differentiated cells nor progenitor cells, since neither type of cell survives cryopreservation at -75±10°C (Young et al., 1991, 2001a,b, 2004a,b).

The cells used in this study were selected for their ability to grow past confluence to form multiple confluent layers of cells, both before and after the analysis for pluripotency. This unique attribute has been

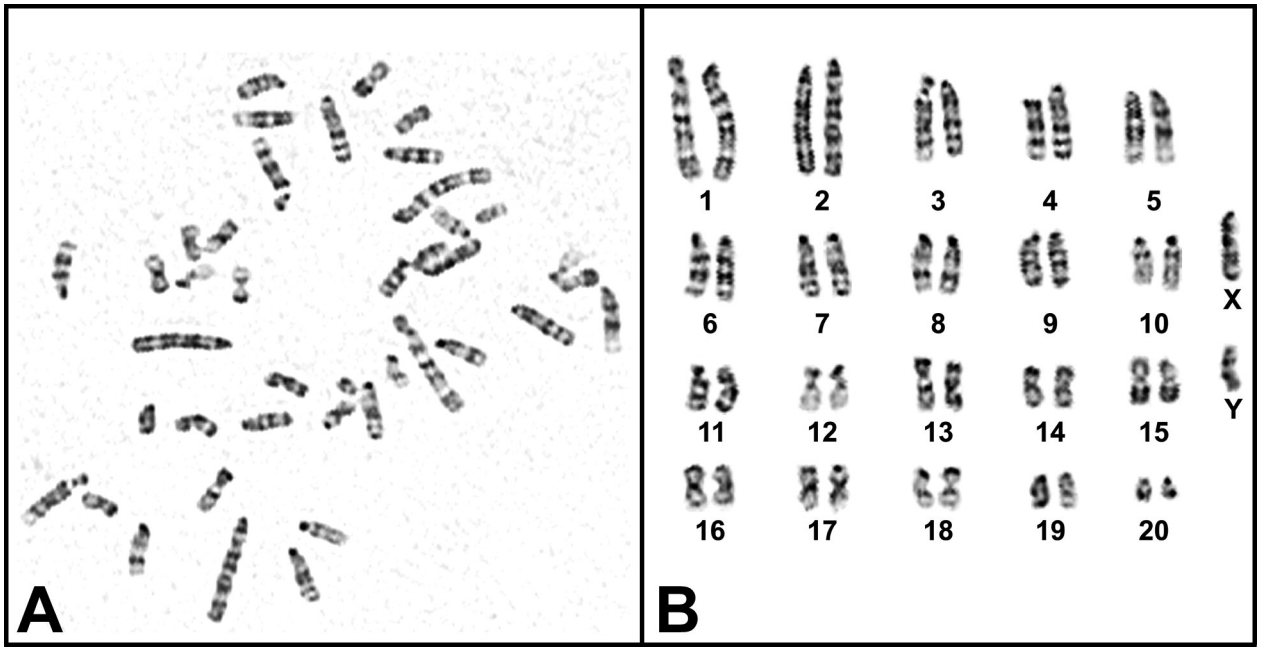


**Fig. 4.** Chromosome spread and karyotypic analysis of a representative adult pluripotent stem cell isolated from an adult male Wistar Furth rat. **A.** Chromosome spread. **B.** Karyotypic analysis. Twenty pairs of somatic chromosomes are shown, as well as one X chromosome and one Y chromosome, for a total of 42 chromosomes.

seen previously in the pluripotent stem cell clone Scl-40ß derived from the adult rat (Young et al., 2004b) as well as in fresh isolates of adult-derived pluripotent stem cells (Young et al., 2004a). In contrast, clones or fresh isolates of differentiated cells, progenitor cells and germ layer lineage stem cells are contact-inhibited at confluence. Thus, they will only form a single confluent layer of cells, even in the presence of a proliferation agent (Young et al., 1999, 2001a,b, 2004a,b). The lack of contact inhibition at confluence suggests that the cells used in this study were not differentiated cells, progenitor cells, or germ layer lineage stem cells.

Prior to karyotypic analysis each cell line underwent analysis for pluripotency to ascertain the initial identity and subsequent differentiation capabilities of the cells. First, aliquots of cells were incubated in progression medium and then stained for phenotypic expression markers. Since progression medium contains no induction agents, it allows the cells to remain in their respective differentiative states. Thus, pluripotent stem cells remain pluripotent stem cells and germ layer lineage stem cells remain germ layer lineage stem cells, with each group expressing its respective phenotypic markers. In contrast, this medium contained a non-specific progression agent that accelerates the differentiation of progenitor cells, thereby accelerating the expression of their terminal phenotypic markers (Young et al., 1998, 2001a,b, 2004a,b). Next, sister aliquots were incubated in the general inductive/progression medium and then stained for phenotypic expression markers. In contrast to progression medium, the general induction/progression medium contains a non-specific induction agent, e.g., dexamethasone, a battery of ectodermal, mesodermal, and endodermal induction agents (contained within the sera) and a non-specific progression agent, e.g., insulin. This combination of induction and progression factors will induce native pluripotent stem cells to form cells that express phenotypic markers from all three germ layer lineages, it will induce native or induced germ layer lineage stem cells to form cells that express phenotypic markers for each individual germ layer lineage (ectoderm, mesoderm, and endoderm), and it will accelerate the progression of native or induced progenitor cells to differentiate and express phenotypic markers indicative of their terminal cell types (Young et al., 1998, 2001a,b, 2004a,b). The particular phenotypic expression markers characteristic of specific cell types were identified utilizing our antibody microarray enzyme-linked immuno-culture assay (Young et al., 1992b, 2004a,b). The antibodies used in the assay were directed against the following: epitopes for pluripotent stem cells (carcinoembryonic antigen cell adhesion molecule-1 and stage-specific antigen-4); epitopes for cells of the ectodermal lineage (nestin, neurons, neurofilaments, beta-III tubulin, tyrosine hydroxylase, dopamine decarboxylase, synaptic vesicles, oligodendrocytes, astrocytes, radial glial cells and keratinocytes); epitopes for cells of the mesodermal lineage (skeletal muscle, smooth muscle, cardiac muscle, cartilage and bone); and epitopes for cells of the endodermal lineage (alpha-fetoprotein, gastrointestinal epithelial cells, pancreatic islet (α-, ß- and δ-cells), and liver cells, including liver progenitor cells, oval cells, biliary cells, canalicular cells, and hepatocytes (Young et al., 2004a, b).

Procedural controls were utilized to determine if non-specific binding occurred between either the antibodies or substrate and the cells. All procedural controls were negative for both the cells lines and Scl-40ß. In addition, note in the composite figures (Figs. 2, 3) that not all the cells in each field exhibited positive staining for phenotypic expression markers. This data suggests indirectly that the antibodies used to generate the positive staining shown in these figures were specific for certain cell subtype(s) rather than exhibiting non-specific binding to all cell types in general.

As reported, the cells in progression medium demonstrated positive staining for the pluripotent stem cell markers carcinoembryonic antigen-cell adhesion molecule-1 (Fig. 1A) and stage-specific embryonic antigen-4 and demonstrated negative staining for germ layer lineage stem cell phenotypic expression markers, progenitor cell phenotypic expression markers, and differentiated cell phenotypic expression markers (Fig. 1B-L). A similar staining pattern of positive staining for carcinoembryonic antigen-cell adhesion molecule-1 and stage-specific embryonic antigen-4 and negative staining for germ layer lineage stem cell phenotypic expression markers, progenitor cell phenotypic expression markers, and differentiated cell phenotypic expression markers was seen with the pluripotent stem cell clone Scl-40ß during its original study (Young et al., 2004b) as well as Scl-40ß which was used as a positive control in this study (data not shown). This suggested that the cells used in this study could be pluripotent stem cells.

However, the presence of a stem cell marker profile characteristic of a pluripotent stem cell is only one piece of evidence for the identity of that cell. Pluripotent stem cells display characteristic features, such as the capability for extended self-renewal past replicative senescence (discussed above), cryopreservation at -80±5°C with recoveries greater than 90% (discussed above), non-contact inhibition at confluence (discussed above), staining for pluripotent stem cell markers (discussed above), and the capability for induction and differentiation into multiple cell types across all three primary germ layer lineages. As reported, the cell lines incubated in the general induction/ progression medium increased in size and lost their positive staining for the pluripotent stem cell markers (Fig. 2A,B). Progressive increases in the size of the cells suggested that they were undergoing progressive differentiation. As the cells increased progressively in size, they acquired positive staining for phenotypic expression markers characteristic of germ layer lineage stem cells, progenitor stem cells, and differentiated cells. Representative images for expressed phenotypic markers include nestin for neural

progenitor cells (Fig. 2C-E), neurofilaments for neurons (Fig. 2G,H), tyrosine hydroxylase (Fig. 2I) and dopamine decarboxylase (Fig. 2J) for neurons, synaptic vesicles (Fig. 2K), glial cells (Fig. 2L-O), keratinocytes (Fig. 2P), skeletal muscle (Fig. 3A,B), smooth muscle (Fig. 3C), cardiac muscle (Fig. 3D), cartilage (Fig. 3E,F), bone (Fig. 3G), endodermal progenitor cells (Fig. 3H), gastrointestinal epithelial cells (Fig. 3I,J), pancreatic islet cells (Fig. 3K-M), and liver cells (Fig. 3N-T).

Therefore, based on their relatively small size in progression medium (compare Fig. 1A-L and Fig. 2A to Fig. 2B-P and Fig. 3), bipolar to stellate morphology, capabilities for extended self-renewal past replicative senescence, cryopreservation at -80±5°C, non-contact inhibition at confluence, staining for pluripotent stem cell markers in progression medium, and the capability for induction, differentiation, and expression of multiple cell types across all three primary germ layer lineages after incubation in general induction/progression medium, we propose that the cell lines used in this study were composed of homogenous populations of adult pluripotent stem cells.

The cell lines were then analyzed karyotypically (Fig. 4A,B) to determine if they demonstrated a normal or abnormal chromosome number. Over 800 chromosome spreads were generated for analysis. Of the initial 800 chromosome spreads generated, 100 spreads were randomly chosen and analyzed for chromosome number using a double-blinded protocol. Each of the 100 chromosome spreads was independently counted by 12 individuals. The median chromosome number for each spread was 42, with a combined mean of 41.86±0.68 chromosomes (Table 1). This suggested that the chromosome number for Wistar Firth rat pluripotent stem cells was 42. Tjio and Levan (1956) reported that the postnatal rat contained diploid cells demonstrating a karyotype of 42 chromosomes. Subsequently, Vaithilingham et al. (1973) reported normal diploid cells with karyotypes of 42 chromosomes for the following strains of rats: Norway, Hooded Lister, Wistar, Wistar-King, Wistar white, AS, Fischer, Buffalo, and Sprague-Dawley. The data reported herein for karyotypes of adult pluripotent stem cells derived from the Wistar Furth rat are not significantly different from those reported by Tjio and Levan (1956) or Vaithilingham et al. (1973).

The counts ranged from 37 to 45 chromosomes by the twelve independent scorers for the 100 spreads counted. This suggested the potential that some cells with aberrant chromosome numbers might have been present within the population analyzed. However, analysis of the database of the individual counts revealed that each of the 100 chromosome spreads was counted as having 42 chromosomes by at least one or more of the 12 scorers and the median chromosome number for each scorer was 42 chromosomes. This observation suggests that the range of chromosome numbers reported (37-45) is more likely to reflect errors in scoring than aberrant numbers of chromosomes. We therefore conclude that these pluripotent stem cells derived from adult rats are diploid cells containing 42 chromosomes. Future studies will address micro changes within the genome that could explain the stem cell-like qualities that these cells appear to possess.

Freshly isolated stem cells have the potential for use as autologous transplants. Such transplants would not require the use of immunosuppressant drugs. In the past we have worked with a highly selected purified clone of pluripotent stem cells. Such clones are valuable as research tools. However, they require far too much time and expense for use in routine autologous transplantation therapies. Such clones could only be used as allogeneic transplants, which would require the use of immunosuppressant drugs to prevent tissue rejection.

This study was designed to demonstrate that freshly isolated pluripotent stem cells display a normal number of chromosomes. We derived two cell lines from each of five animals that were not littermates for a total of ten cell lines. Each cell line was derived using our standard protocol for pluripotent stem cells. Each cell line was examined using cultivation parameters for pluripotent stem cells, progression medium, general induction/progression medium, positive controls, negative controls, and the antibody microarray-ELICA. The cell lines were determined to consist of pluripotent stem cells. They were then propagated further to ensure that they had bypassed replicative senescence for rodent cells, and thus did not consist of progenitor cells. The cell lines were subjected to karyotypic analysis to determine the number of chromosomes present in the cells. The cells of each cell line displayed 42 chromosomes, the normal number for rat cells. The presence of a normal number of chromosomes suggests that fresh tissue isolates may be suitable for autologous tissue transplantation.

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