Stem Cells in Regenerative Medicine

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Stem Cells in Regenerative Medicine

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Preface

Regenerative Medicine is devoted to replacing diseased cells, tissues, or organs, or repairing tissues in vivo by augmenting natural or inducing latent regenerative processes. Underlying these goals is the manipulation – both expansion and directed differentiation – of stem cells, which are the primary source of de novo tissue regeneration and maintenance of organ homeostasis. In this book, *Stem Cells in Regenerative Medicine*, we aim to provide biomedical researchers, clinicians and biomedical engineers an updated representation of the landscape of stem cell-based therapies in a wide spectrum of tissue systems and ontogenic stages, starting from the isolation and culture of stem cells to their actual use in vivo. In this first edition, we have attempted to compile a foundation of protocols which can be refined in subsequent publications. We hope that this series of protocols will contribute to the definition of standardized procedures for the manipulation of somatic and embryonic stem cells in research and clinical applications.

William L. Stanford, Ph.D.
Julie Audet, Ph.D.

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Color Plates

Color Plate 1 — Tetraploid blastocyst injection. This procedure can be used in order to decrease the time required to generate mice from targeted ES cells. Two-cell embryos are fused as described in **Section 3.11** and injected with the diploid-targeted ES cells (here shown as GFP-positive ES cells). The tetraploid cells of the blastocysts will only give rise to extra-embryonic tissues such as the placenta, whereas injected diploid ES cells will give rise to the embryo *(17)*. This procedure takes only a few weeks to generate completely ES-derived mice, in contrast to first generating chimeras and then breeding to obtain germline transmission. (*See* discussion on p. 8)

Color Plate 2 — Analysis of patterned H9 hESC colonies (D = 400 μm, P = 500 μm). (**A**) Immunohistochemistry to identifying Oct4+ cells (*green*), all nuclei by Hoechst 33342 (*blue*). Individual Oct4+ cells (*light green*) and Oct4- cells (*dark blue*) are identified by the Target Activation algorithm of the Cellomics^{TM} Arrayscan V^{TI} software by drawing overlay masks around the nuclei. (**B**) Representative flow cytometry plots of H9 hESCs before and after patterning by immunocytochemistry. Data demonstrating how the Morphology Explorer algorithm of the Cellomics^{TM} Arrayscan V^{TI} software can be used to identify cells and colonies, (**C**) calculate their area, (**D**) diameter, and (**E**) the number of Oct4+ cells in the colony. Error bars represent the standard deviation over 150 colonies. (*See* discussion on p. 31)

Color Plate 3 — Infection of hESCs with GFP marked lentiviral RNAi vectors. (**A**) hESC colony infected with a GFP marked lentivirus displays a patchwork of infected cells. (**B**) hESC colony after enrichment of GFP+ cells by FACS. (*See* discussion on p. 41)

Color Plate 4 — The iliac crest with a line through where to remove the flat triangular piece of cartilage (**A**) and where the acetabular notch is removed ready to be flushed (**B**). (*See* discussion on p. 97)

Color Plate 5 — Transplanted LSK cells detected using a spatial distribution assay. Cells are designated as either central (**C**) or endosteal (**E**). (*See* discussion on p. 103)

Color Plate 6 — Donor cells migrate normally following engraftment. Parasagittal fluorescence montage through the brain of an adult mouse host following transplantation of GFP+ neurogenic astrocytes into the lateral ventricle. Sagittal sections were immunostained with

antibodies against GFP (*green*) and NeuN (*red*) 28 days following engraftment. A large number of GFP+ donor cells can be seen within the rostral migratory stream (RMS) and olfactory bulb (OB). A few donor cells are also present along the roof of the lateral ventricle (LV), and in the septum (S). CC: corpus callosum. (*See* discussion on p. 187)

Color Plate 7 Higher magnification of GFP+ donor cells within the RMS and olfactory bulb. (**A**) Parasagittal section of a host brain immunolabeled for GFP (*green*) and β-III tubulin (*red*). Donor cells are distributed with in the SEZ–RMS–OB system, as well as lining the wall of lateral ventricle (LV). (**B**) Parasagittal confocal image of a donor cell that migrated into the olfactory bulb and differentiated into a granule interneuron. (*See* discussion on p. 187)

Color Plate 8 Donor-derived cells are capable of differentiating into neurons and astrocytes. (**A**) and (**B**): Confocal Z-series of GFP+ donor cells (*green*) located in the granular cell layer of olfactory bulb that are double immunolabeled with the neuron markers (*red*) NeuN (A) and β-III tubulin (B). (**C**) A GFP+ (*green*) donor cell that has differentiated into a cortical astrocyte, and is immunolabeled with the astrocyte marker GFAP (*red*). (*See* discussion on p. 188)

Color Plate 9 Grafted cells survive, migrate, and differentiate upon transplantation into the cerebellum of both wild type and weaver transgenic mouse pups. (**A**) Donor GFP+ cells (*green*) are seen migrating and differentiating within the cerebellum 3 weeks post-engraftment in the wild-type mouse. (**B**) High magnification confocal z-series of a donor cell transplanted into the cerebellum of a weaver mutant mouse. P$\epsilon\delta$= β-III tubulin in A, and NeuN in B. (*See* discussion on p. 188)

Color Plate 10 Monkey ES cells. Undifferentiated ES cells on STO feeder (**A**), and neural progenitors induced from monkey ES cells. Detached ES cell colonies formed spheres similar to those of neural progenitor cells (**B**). These spheres give rise to Tuj1- (green, neuronal marker) and TH-positive (red, DA neuron marker) cells in vitro. Bar=100 μm (A, B), 100 μm (**C**). (B, C: Reproduced from ref. *(6)* with permission). (*See* discussion on p. 205)

Color Plate 11 Parkinson's disease model monkey induced by MPTP. Neurological scores of MPTP-treated monkeys (**A**, n = 34). Immunofluorescence study revealed that neuro-terminals of the nigro-striatal tract, which is composed of DA neuron fibers and immunoreactive for TH, are severely reduced (**B, C**). (*See* discussion on p. 210)

Color Plate 12 Function of ES cell-derived neurospheres in MPTP-treated monkeys. Neurological scores of ES cell-transplanted ($n = 6$) and sham-operated animals ($n = 4$) are plotted. All values are the mean ± SD. *$p<0.05$. (*See* discussion on p. 211)

Color Plate 13 HUCPVCs expressing α-actin (**a**), vimentin (**b**), and desmin (**c**). (*See* discussion on p. 275)

Color Plate 14 HUCPVCs reduce lymphocyte proliferation, even if added 3 and 5 days into a 6 day culture. Addition of HUCPVCs showed a significant decrease in lymphocyte cell number compared to control (no HUCPVCs) over 6 days in a two-way MLC. There is no significant difference among HUCPVCs added on day 0, 3, or 5 ($n = 6$). This figure shows the average cell numbers, + standard deviations. (*See* discussion on p. 276)

Color Plate 15 Transduction efficiency by fluorescent microscopy. (**A**) Phase contrast microphotograph of P5 bone marrow-derived MSCs. (**B**) The same microscopic field observed under fluorescent light shows that the majority of the cells express GFP, meaning that they were successfully transduced. In our hands, this protocol allows a transduction efficiency higher than 80%, as confirmed also by FACS analysis. (*See* discussion on p. 288)

Color Plate 16 Conditioned media production and different in vitro and in vivo assays. (**A**) Stem cells are expanded in normal conditions until they are 90% confluent. The growth medium is then exchanged with medium not containing serum and the cells are left for 24 h in a CO_2 incubator. The medium is then collected and tested either in vitro or in vivo. (**B**) For the in vitro experiments, the conditioned medium is transferred into culture dishes containing different kind of cells according to the goal of the specific experiment. Several different properties of the medium can be tested in vitro. For example, the cytoprotective effects exerted by the conditioned medium can be tested on murine cardiomyocytes. After exposing the cardiomyocytes to hypoxia in the presence of control medium or conditioned medium, apoptosis and necrosis assays are performed and the results compared. To verify if the conditioned medium contains chemotactic factors, a specific cell type (i.e., endothelial cell or cardiac stem cell) is seeded on the membrane of the upper chamber of a dual chamber dish. The number of cells migrating into the lower chamber, containing either conditioned medium or control medium, is then counted.

To test the pro-angiogenetic properties of conditioned medium, endothelial cells are seeded on a matrigel and the number of capillaries is quantified after exposure to conditioned medium or control medium. To verify if cell metabolism is influenced by factors present in the medium, the cell type of interest (i.e., murine cardiomyocytes) is exposed to the conditioned and to the control medium and then collected to perform metabolic assays. Another example: cardiomyocyte contractility may be assessed in the presence of control or conditioned medium; if inotropic factors are present, then cell contractility will be increased in the presence of conditioned medium. Proliferation assay may also be performed. Finally, proteomic analysis of conditioned medium may allow the discovery of new therapeutic molecules and targets. (**C**) Conditioned medium may

be tested also in vivo using different experimental disease models. For example, the effects of conditioned medium on ischemic myocardium may be assessed in a murine model of myocardial infarction. Small volumes of concentrated conditioned medium obtained by ultrafiltration are injected at the infarct border zone after left coronary ligation. At established time points, heart function is analyzed by echo (an M-mode image of the left ventricle is depicted in the figure) or other methods. The heart may also be collected for histology to determine, for example, the infarct size (a cross-section of mouse heart stained with Masson Trichrome is depicted in the figure). Furthermore, immunohistochemistry staining may performed to determine different parameters, such as cardiac regeneration, neoangiogenesis, apoptosis. (*See* discussion on p. 289)

Color Plate 17 Lineage progression of an adult skeletal muscle satellite cell to a differentiated cell. (**A**) Isolated fibre from the Tibialis Anterior muscle of a *Myf5*$^{nlacZ/+}$ mouse showing nuclear β-galactosidase activity by X-gal staining. Hoechst staining reveals myonuclei inside the fibre as well as the nucleus of the satellite cell on the fibre. (**B**) Lineage progression of a quiescent to an activated satellite cell, hallmarked by MyoD expression, to a myoblast. Myoblasts fuse homotypically or to pre-existing differentiated fibres or myotubes after leaving the cell cycle. Differentiated fibres are characterised by the expression of myosin heavy chain (MyHC). The expression of different commonly used markers is indicated. (*See* discussion on p. 296)

Color Plate 18 Dissections of TA, EDL and Soleus muscles. The skin was removed from a 4-week-old mouse to reveal the underlying muscles. (**A–D**) Removal of TA and EDL muscles. (**E-F**) Removal of Soleus muscle. Lower tendon (1) is sectioned first, the muscles are lifted, then the upper Soleus tendon (2) is sectioned. Note in older mice, the soleus appears more red and distinguishable. G) Actual sizes of dissected muscles with associated tendons (white arrowheads). Muscle attachment proximal to the knee is at the top of the photo. See main text for details. Red arrowhead, cartilage bridge in foot under which tendons are transit. White arrowheads, tendons. (*See* discussion on p. 301)

Color Plate 19 Asymmetric segregation of Numb in non-differentiating satellite cell-derived myoblasts. (**A**) Two examples of asymmetric distribution of endogenous Numb protein in mitotic satellite cell-derived myoblasts after 4 days in culture; immunocytochemistry with anti-Numb and anti-Ki67 antibodies; (**B**) p66Numb-EGFP and H2B-mRFP fusion proteins were overexpressed in satellite cell-derived myoblasts using pCAG-p66Nb-EGFP and pCAG-H2B- mRFP plasmids (transfected at 96 h after plating of myofibres). At 4 days after plating, cells were harvested by mitotic shake-off, re-plated at low density and grown for several hours on poly-D-lysine-coated dishes. The cells were fixed and stained with anti-Numb antibody (green), and visualised using EGFP and mRFP epifluorescence. Phase

contrast on left; reconstituted confocal stack on right was rendered with Imaris software. Note asymmetric distribution of the majority of the Numb-GFP protein to one pole of this cell. (*See* discussion on p. 306)

Color Plate 20 Asymmetric segregation of template DNA strands in label retaining cells. (**A**) Asymmetric segregation of BrdU label to only one daughter satellite cell after mitosis, on a freshly isolated EDL fibre. $Myf5^{nlacZ/+}$ mice were pulsed with BrdU from P3-P7; chase for 7 days. (**B**) Clonal analysis of LRCs after 4 weeks chase in vivo, 72 h in culture. Antibody stainings for detecting Myo protein and BrdU in the nuclei of satellite cell derived myoblasts. The LRC in this case has a lower expression of MyoD. Scale bars: A, 15 μm; B, 40 μm. (*See* discussion on p. 311)

Color Plate 21 Time-lapse imaging of ES cell differentiation cultures. An ES cell line expressing endothelial cell-specific H2B-GFP was differentiated to day 8. It was imaged on a Perkin Elmer spinning disk confocal microscope. Culture is fixed right after imaging and stained for PECAM-1. (A) Frames of a time-lapse movie. Time is in minutes at the top right corner of each panel. (B) Last frame of the movie in green (**a**), the same field with PECAM-1 stain in red (**b**), and the overlay of the two images (**c**). (*See* discussion on p. 341)

Color Plate 22 Immunofluorescence for SMαA in intact EBs and puromycin purified SMCs. Immunostaining was carried out as described in 3.5 on intact day 28 EBs (**A**) and on cells following enzyme dispersal and overnight selection (**B**). A "sheet" of SMCs may be seen in an intact day 28 EB with adjacent non-staining non-SMC at the bottom of the image (**A**). Such groups of SMC may be seen to contract spontaneously under the microscope. Following puromycin selection, relatively pure populations of cells with a typical SMC morphology were seen (**B**). Red staining: SMαA immunofluorescence, Blue: DAPI nuclear staining. (*See* discussion on p. 356)

Color Plate 23 Transformation of embedded islets into DLS, and subsequent regeneration of ILS. **(A)** Immediately after embedding, islets are characterized by a solid-spheroid shape. **(B)** DLS formation appears to initiate in specific foci, **(C)** until a DLS replaces the islet. **(D)** Treatment with INGAP induces the budding of regenerating ILS from DLS (bar = 100 μm). (*See* discussion on p. 377)

Color Plate 24 Immunocytochemistry. Analysis of islets, DLS, and ILS demonstrates that while islets and ILS express endocrine hormones (insulin, glucagon, somatostatin, and pancreatic polypeptide) in approximately the same proportions, these hormone^{+} cells are absent in DLS. Conversely, staining for a ductal cell marker (pan-cytokeratin) is observed primarily in DLS. Furthermore, while few or no ductal cells are observed in islets, ILS can be observed to be "budding" from ductal structures (bar = 100 μm). (*See* discussion on p. 380)