

## ADULT STEM CELLS: PROSPECTS FOR REPROGRAMMING BONE MARROW DERIVATIVES IN STABLE HETEROKARYONS

HELEN M. BLAU

### *Introduction*

Stem cells have stimulated tremendous excitement because they provide hope that cellular therapy can aid in the treatment of diseases that have been refractory to other treatments. There are a range of stem cell types (for review see Chapter by Prof. Le Douarin in this volume or Blau, Brazelton *et al.*, 2001). The ultimate goal for each type is the same: to induce the nucleus of the cell to perform functions needed to maintain, replace, or rescue a particular tissue. Thus, the essence of stem cell-mediated therapy is nuclear reprogramming, which entails the induction of gene expression patterns unique to cell types in diverse tissues and organs. Consequently all stem cell types should be studied in parallel, as one may be better suited to the treatment of a given disease than another and all will yield findings fundamental to nuclear programming, critical to using any stem cell to replace or rescue damaged tissues.

Research in my laboratory and in that of others working on adult stem cells has focused on nuclear reprogramming in relation to adult bone-marrow-derived cells (BMDC), and in particular, the well characterized hematopoietic stem cells (HSC) and their derivatives. There is now ample evidence that these cells contribute naturally to adult tissues during life and that damage (traumatic or genetic) enhances that contribution. It therefore behooves us to better understand the mechanisms by which this contribution is achieved in the hope that it can be amplified and enlisted therapeutically. Adult cells (HSC and their derivatives) are advantageous in that they are not subjected to growth in tissue culture and they derive from the patient, thereby overcoming potential immunological obstacles. Ultimately

the goal of adult stem cell research is to enlist the hematopoietic stem cell derivatives of an individual to treat his or her own disease by delivering the appropriate chemical factors.

The finding that BMDC, and even HSC derivatives, can contribute to non-hematopoietic tissues that suffer from injury or are genetically defective has drawn worldwide attention, as it may have significant therapeutic applications for tissue repair due to trauma or disease. However, major challenges must be overcome before these cells can be considered as a potential therapy for non-hematopoietic tissues such as brain, skeletal muscle, and liver. The efficiency with which they contribute to tissues must be substantially augmented and their efficacy in treating disease must be clearly demonstrated in several animal models of human disease. To achieve these goals, the relevant HSC derivative must be identified and the cellular and molecular mechanisms leading to the contribution of adult bone marrow derived cells to specific tissues *in vivo* must be elucidated.

Two mechanisms have been reported. The bone marrow derived cells may fuse and then be reprogrammed in response to *intracellular* cytoplasmic factors, e.g., as stable binucleate heterokaryons. Alternatively, reprogramming may occur in response to *extracellular* signals in the microenvironment. Finally, the two mechanisms may act in combination in a given tissue. Regardless of which of these two mechanisms is used, reprogramming of the BMDC nucleus is of central importance. It has only recently been recognized that the contribution of BMDC constitutes a natural repair mechanism in adult muscle. Enhancement of such an existing mechanism may have a major impact on regenerative medicine.

In this article, I speculate that the different mechanisms for reprogramming nuclei described above depend on their inherent mitotic activity and tissue context. For example, a tissue such as epidermis, intestine or skeletal muscle that has a relatively simple morphology and is undergoing constant renewal through cell division (Janes, Lowell *et al.* 2002; Alonso and Fuchs 2003) might be expected to be replenished by the provision of cells with the capacity to proliferate and augment cell number. In response to environmental signals, tissues such as intestine and skeletal muscle are known to generate new cells throughout life and one source could be BMDC. By contrast, a highly elaborate cell such as a Purkinje neuron, which has more than one million synaptic connections to other cells and is not known to divide or be made anew in adulthood, would be hard to replace. In this case, 'rescue' by cell fusion and subsequent reprogramming of an incorporated nucleus may constitute a more plausible scenario than *de novo* cell production.

The growing excitement regarding the potential of BMDC and hematopoietic stem cells (HSC) to contribute to adult tissues has also stimulated growing controversy and attention and questions regarding its potential impact on normal life. As described in this article, although the quality of recent published reports varies, there is sufficient evidence to support the existence of bone marrow derived cells that can assume new functions in adulthood. Gene expression patterns can change, differentiation can be manipulated, and cells can incorporate into tissues from which they did not originate. The current state of the field is described. That BMDC contribute to tissues in adults now seems indisputable. Whether this phenomenon can be readily augmented will be determined by future research. The ultimate goal is to employ endogenous cellular components (directly or after brief *ex vivo* manipulation by genetic engineering) to repair the body using a combination of cell and gene therapy.

Finally, I would like to propose that the definition of a stem cell may benefit from revision and expansion based on the recent unexpected findings described in this report. The most well described stem cell, the hematopoietic stem cell (HSC), is a cell source of the donor nucleus that is reprogrammed to participate in foreign tissues, irrespective of mechanism. Whether an HSC derivative changes its gene expression program in response to the external microenvironment or in response to the internal milieu following cell fusion, the outcome is the same: nuclear reprogramming. If the nucleus derived from an HSC derivative is capable of self-renewal and has the potential to alter its genetic program in accordance with the diverse differentiated states typical of the tissue in which it resides, it has the attributes of a multipotential cell. These are the criteria for defining a cell as a stem cell as described in the introduction of this Symposium by Prof. Le Douarin. According to current definitions, whether the nuclear gene expression program changes as a result of exposure to different signals (extracellular vs. intracellular) does not dictate whether the cell harboring that nucleus is a stem cell. Since specific fusion of hematopoietic stem cell derivatives to other cells was never previously envisioned as a potential property of these cells, this novel feature presumably should not prohibit a cell from being designated as a stem cell. Specifically, if an HSC, the quintessential 'tissue specific stem cell' can give rise to progeny that naturally become reprogrammed to participate in the function of tissues other than blood such as skeletal muscle and Purkinje cells, then the definition of a stem cell may need to be changed. Taken together, these considerations suggest that stem cells may be better defined not as an entity but rather based on function (Blau, Brazelton *et al.* 2001).

Here, I report examples from my laboratory and that of others showing that BMDC and HSC contribute progeny to diverse tissues in adulthood. A historical perspective on mammalian cell fusion provides a context for these new findings. The goal of this report is to stimulate further investigation of adult stem cells, the mechanisms responsible for nuclear reprogramming and their potential therapeutic application.

### *Mammalian Cells Fused in Heterokaryons in Vitro*

In the 1980s it was still considered highly unlikely that specialized mammalian cells could be altered. Despite the ground-breaking experiments on nuclear transplantation into oocytes (cloning) in amphibians (Briggs 1952; Briggs 1959; Gurdon 1962; Gurdon and Uehlinger 1966; Di Berardino and King 1967), it was generally thought that once a cell had assumed a 'terminally differentiated' state in liver or skin, for example, that state was irreversible. To test whether this theory held true, experiments were performed in culture dishes in which cells were fused to one another with polyethylene glycol. In the first studies using synkaryons (cell hybrids that exhibit cell division and nuclear fusion), gene repression was the norm and only transient gene activation was observed. Interpretation of results was complicated by the multiple rounds of cell division that occurred, resulting in chromosome reduction and rearrangement (Ephrussi, Davidson *et al.* 1969; Harris, Wiener *et al.* 1971; Davidson 1974). However, some results of major importance were obtained with synkaryons: the transformed state was found to be recessive to normal and activation of genes by trans-acting factors was first shown. Nonetheless, such unstable fusion products made it difficult to determine whether the gene of interest or its regulator was lost or to derive information regarding the underlying mechanisms.

By contrast, in cell fusion products known as heterokaryons, each nucleus remained distinct and intact, as there was no cell division or accompanying chromosome loss (Fig. 1, see p. 140). Gene expression could therefore be monitored over time. The demonstration that specific genes were progressively activated or repressed following fusion of diverse cell types in heterokaryons provided evidence that differentiation in adult human cells is not irreversible, but instead, dynamic and continuously regulated by the stoichiometry of proteins present in the cells at any given time (Blau 1989; Blau and Baltimore 1991).

Again, a historical perspective is useful. In early work with heterokaryons, Ringertz and coworkers showed that fusion of rat myoblasts

and chick erythrocytes caused the nuclei of the red blood cells to swell and chromatin to become diffuse, a finding that was in agreement with the initial steps known to be involved in nuclear reprogramming (Ringertz 1976). Later, my laboratory showed that nuclear reprogramming could occur in heterokaryons. A means of inhibiting cell division to prevent aneuploidy and the use of cells of two different species that were not transformed were critical to the success of these experiments. When mouse muscle cells were fused in tissue culture with human primary diploid cells derived from all three embryonic lineages, endoderm (hepatocytes), ectoderm (keratinocytes) and mesoderm (fibroblasts), strikingly, nuclear reprogramming was observed in each of these cell types. In each case, human muscle gene expression was detected, providing evidence that the differentiated state could be altered in highly diverse specialized cells derived from human adults. More than ten previously silent muscle genes were activated; organelles, such as the Golgi, were redistributed; and certain cell surface proteins were shown to be localized in nuclear domains for the first time (Blau, Chiu *et al.* 1983; Chiu and Blau 1984; Blau, Chiu *et al.* 1985; Chiu and Blau 1985). In the ensuing years, these results were confirmed by others for muscle, hepatocyte and globin gene activation following fusion with other cell types in stable heterokaryons. As a result, the plasticity of the differentiated state was generally accepted as a property of normal, non-transformed specialized mammalian cell types (Wright 1984; Wright 1984; Baron and Maniatis 1986; Spear and Tilghman 1990). These *in vitro* heterokaryon studies showed that activation of previously silent genes could be achieved, resulted from the balance of cytoplasmic factors present in somatic cells at any given time, and did not require passage through the oocyte or embryogenesis.

#### *BMDC and HSC Contribution to Non-Hematopoietic Tissues*

In the past few years a number of investigators have focused on BMDC in adults as a source of cells with potential for tissue regeneration, because they have access to all tissues of the body via the circulation. When the first remarkable report appeared demonstrating that bone marrow cells could contribute to muscle in mice (Ferrari, Cusella-De Angelis *et al.* 1998), it remained to be determined whether this was a rare and sporadic event or of fundamental physiologic significance. In these experiments, following transplantation of lethally irradiated mice with bone marrow from a transgenic mouse that expressed a myosin light chain

enhancer driving beta galactosidase, a few muscle fibers that were blue due to reporter gene expression were detected. There followed a number of reports of similar findings, using marrow cells genetically marked with b-galactosidase, Y-chromosome or green fluorescent protein (GFP). Such cells were reported to be present in the brain, the liver, heart, skeletal muscle, and epithelia of the kidney, lung and skin (Gussoni, Soneoka *et al.* 1999; Jackson, Mi *et al.* 1999; Petersen, Bowen *et al.* 1999; Brazelton, Rossi *et al.* 2000; Lagasse, Connors *et al.* 2000; Mezey, Chandross *et al.* 2000; Krause, Theise *et al.* 2001; Priller, Persons *et al.* 2001; LaBarge and Blau 2002; Wang, Montini *et al.* 2002; Camargo, Green *et al.* 2003; Corbel, Lee *et al.* 2003; Ianus, Holz *et al.* 2003; Kale, Karihaloo *et al.* 2003; Vassilopoulos, Wang *et al.* 2003; Weimann, Charlton *et al.* 2003) (Fig. 2, see p. 141). Although the incidence was generally low (0.01 to 0.1% of total cells) and the results were not always definitive due to differences in the experimental techniques employed and lack of replication, these studies provided the impetus for further investigation.

Of particular interest was the finding that the 'rare spontaneous' contribution of BMDC to non-hematopoietic tissues could be significantly increased in response to tissue stress or damage. Injection of toxins, exercise on a running wheel, or the use of an otherwise underutilized muscle caused a significantly increased contribution of BMDC to tissues by at least 20 fold, or a 4-5% of the total fiber within the tibialis exterior muscle. (LaBarge and Blau 2002; Camargo, Green *et al.* 2003; Corbel, Lee *et al.* 2003).

#### *BMDC Contribution to Skeletal Muscle and Purkinje Neurons in the Brain*

Experiments regarding the basis for BMDC incorporation into non-hematopoietic tissues are now well underway. There are two major questions of interest: (1) Which cell type in bone marrow is responsible? (2) By what mechanism does this cell type contribute to diverse tissues? Although clearly other excellent examples exist (see above), here I focus on two that have been extensively studied by my laboratory and are illustrative: skeletal muscle and Purkinje neurons in brain.

To understand and enhance the above findings, it is critical to identify the cell type with the capacity for contributing to these specialized cell types. Single cell transplants have now shown definitively that one very well characterized cell type within the bone marrow can be implicated. This cell is the hematopoietic stem cell (HSC), with characteristic mark-

ers ckit+, sca-1+, lin- (Spangrude, Heimfeld *et al.* 1988; Smith, Gasparetto *et al.* 1991; Uchida and Weissman 1992; Morrison and Weissman 1994; Osawa, Hanada *et al.* 1996). This cell, the HSC, is the quintessential tissue-specific stem cell capable of reconstituting all lineages of the blood in lethally irradiated mice. Definitive evidence that this cell or its derivatives could contribute to other tissues was provided when we and others showed that a *single* HSC yielded GFP-expressing cells that not only replenished the blood, but also were incorporated into mouse muscle fibers (Camargo, Green *et al.* 2003; Corbel, Lee *et al.* 2003). (Fig. 3, see p. 142). Key for eliciting contribution of such bone marrow-derived cells to muscle was injury, either caused by local toxin injection (Camargo, Green *et al.* 2003; Corbel, Lee *et al.* 2003) or caused by exercise (LaBarge and Blau 2002) (Figs. 4-6, see pp. 143-145). It had been postulated that the bone marrow contained determined precursors to cells such as neurons of the brain and skeletal muscle cells (Anderson, Gage *et al.* 2001; Korblyng and Estrov 2003). Although this may still be true, these findings suggested that HSCs could give rise not only to all of the cells of the blood, but also cross lineages to contribute to skeletal muscle, and neurons in the brain.

Bone marrow contribution to non-hematopoietic tissues also occurs in the cerebellum of the brain (Priller, Persons *et al.* 2001; Wagers, Sherwood *et al.* 2002; Alvarez-Dolado, Pardal *et al.* 2003; Weimann, Charlton *et al.* 2003; Weimann, Johansson *et al.* 2003) (Fig. 7, see p. 146). In both humans and mice, BMT-derived nuclei are present in Purkinje neurons. In mice, a low frequency is observed that increases over time after BMT. Essentially all GFP-expressing Purkinje neurons exist as binucleate heterokaryons (Alvarez-Dolado, Pardal *et al.* 2003; Weimann, Johansson *et al.* 2003) (Fig. 8, see p. 147). The use of transgenic mice harboring the Purkinje specific promoter, L7, first showed that BMDC nuclei that are present in heterokaryons are reprogrammed: their chromatin is dispersed and L7 promoter expression is induced (Weimann, Johansson *et al.* 2003) (Fig. 9, see p. 148). After transplantation of male bone marrow, Y chromosomes were detected in the female Purkinje cells of both mouse and human brains (Fig. 10, see p. 149). To our knowledge, these examples constitute the first evidence of reprogramming of gene expression in stable binucleate *heterokaryons* existing *in vivo* in mammals. Based on these results, we hypothesize that the tissue culture phenomenon (changes in gene expression commensurate with nuclear reprogramming in heterokaryons) which we observed decades ago (1983), constitutes a mechanism by which complex, post-mitotic cells can receive aid from endogenous cells throughout life.

### *Reprogramming In Vivo*

Two different mechanisms have been proposed whereby BMDC contribute to non-hematopoietic tissues. (1) Reprogramming via extracellular signaling and (2) reprogramming via fusion and cytoplasmic mixing. Either mechanism ultimately involves nuclear reprogramming. Initial reports of 'plasticity' in adult stem cells suggested the first mechanism, i.e., that a developmentally immature BMDC could alter its typical course of differentiation to that of non-hematopoietic tissues (Anderson, Gage *et al.* 2001; Blau, Brazelton *et al.* 2001; Raff 2003). Therefore, BMDC could function as stem cells for other tissues. This first mechanism implies a response to extracellular signals that are detected by the BMDC and result in an alteration of gene expression and differentiation along an alternative developmental pathway. Examples that have been reported include bone marrow derived kidney epithelium (Krause, Theise *et al.* 2001), pulmonary epithelium (Krause, Theise *et al.* 2001), pancreatic islet cells (Ianus, Holz *et al.* 2003) and muscle satellite cells (LaBarge and Blau 2002) (Fig. 4, see p. 143).

As evidence for a second mechanism, fusion was detected in humans and mice leading to reprogramming by the cytoplasm, as previously seen with *in vitro* heterokaryon formation. Initial reports of contribution of BMDC to non-hematopoietic tissues by fusion described the phenomenon as 'merely fusion' (Blau 2002) or 'random' (Camargo, Green *et al.* 2003). However, time has granted a new respect for fusion. Indeed, fusion of BMDC with other tissues appears to be a specific process limited to particular donor and recipient cells, such as BDMC and Purkinje cells of the cerebellum (BMDC do not fuse with other cells within the cerebellum) and only binucleate, not mononucleate or trinucleate cells are detected. I propose that the observed fusion of BMDC with other cells may represent a way for cells from different parts of the body with diverse histories to contribute to one another. For example, one could envision a situation where a post-mitotic cell in a complex tissue environment suffers a deleterious loss of function due to mutation or trauma and is rescued by expression of a reprogrammed, wild-type copy of the genome present in a donated nucleus.

### *Conclusion*

Beginning with amphibian research, in the past five decades we have been endowed with a large fund of information and extraordinary technical achievements. We have learned that: (1) genetic material is not lost



during development and differentiation, (2) the cytoplasm of the oocyte as well as somatic cells have the ability to reprogram gene expression, (3) most genes can be reactivated, even in terminally differentiated cells, (4) a single somatic cell nucleus has the replicative capacity to yield sufficient progeny to produce the tissues necessary for a whole new organism, and (5) in life cells change their phenotypes, and in some cases, nuclear gene expression is reprogrammed across lineages. These findings indicate that theoretical roadblocks based on long held dogma that might have precluded the use of cells from one source to repair another have already been overcome.

Why should there be two mechanisms by which BMDC or HSC derivatives can contribute to adult tissues? In the case of tissues in which proliferation is ongoing, BMDC may contribute, like tissue specific stem cells (e.g. HSC or satellite muscle cells), via mitosis and subsequent specialization in response to extracellular signals. On the other hand, cells in tissues without proliferative capacity may not have the option for achieving tissue renewal. Thus, the concept of rescuing a defective cell, especially one that cannot and does not need to divide, may serve as an alternative means of tissue repair. Consequently, nuclear donation and subsequent reprogramming may constitute an elegant solution used by nature, and one that could be capitalized upon therapeutically.

The goal of controlling nuclear reprogramming *in vivo* will likely be made using numerous approaches. Investigation of reprogramming in nuclear transplantation is lending insight into the epigenetic changes that affect reprogramming (Jaenisch and Bird 2003). Indeed, chromatin remodeling enzymes and embryonic transcription factors are emerging as key players. Further understanding of the proteins responsible for regeneration in tissues in simpler organisms such as newts and zebrafish may provide insights. Characterization of fusion molecules or secreted factors that recruit and induce BMDCs may allow the efficiency of this form of tissue repair to be increased. Investigation of reprogramming in both BMDCs *in vivo* and *in vitro* in cultured heterokaryons using novel technologies will shed light on these important control mechanisms.

If the existence of technological hurdles and questions of degree or 'low frequency' had stopped researchers, then many significant discoveries would not have materialized. The study of *in vivo* reprogramming of BMDC warrants vigor, rigor and persistence. The future use of adult stem cells in regenerative medicine will rely on thorough investigation by numerous talented scientists.

*Acknowledgements*

I wish to thank the members of my laboratory, especially Jason Pomerantz, M.D., for help with this manuscript. Funding for the research in my laboratory was provided by NIH grants AG020961, AG009521, HD018179, HL065572, Ellison Foundation AG-SS-0817, the McKnight Endowment Fund for Neuroscience, and the Baxter Foundation.

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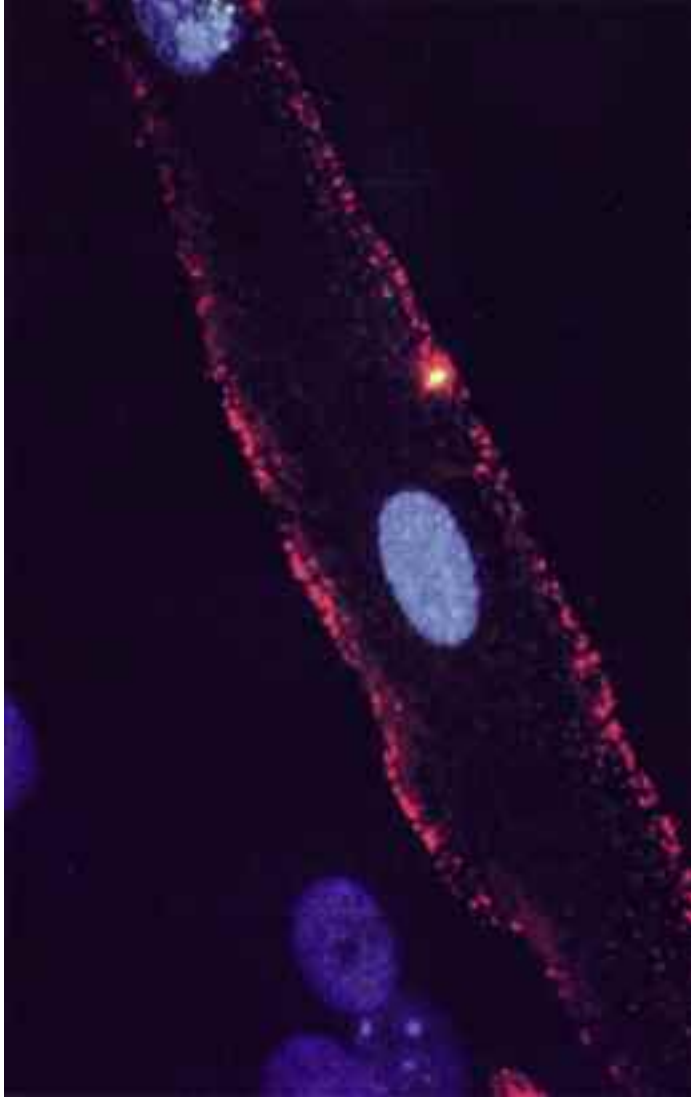


Figure 1. *Expression of a human muscle gene by a human hepatocyte. nucleus in a heterokaryon formed in tissue culture. Mouse muscle cells (blue punctate nuclei) and human nonmuscle cells (blue uniformly stained nuclei) are fused to form heterokaryons. Upon exposure to muscle cytoplasm, cells specialized for different tissues can be induced to express gene products characteristic of muscle (red immunofluorescence). From Blau *et al.*, *Science*, 230, pp. 758-766 (1985).*

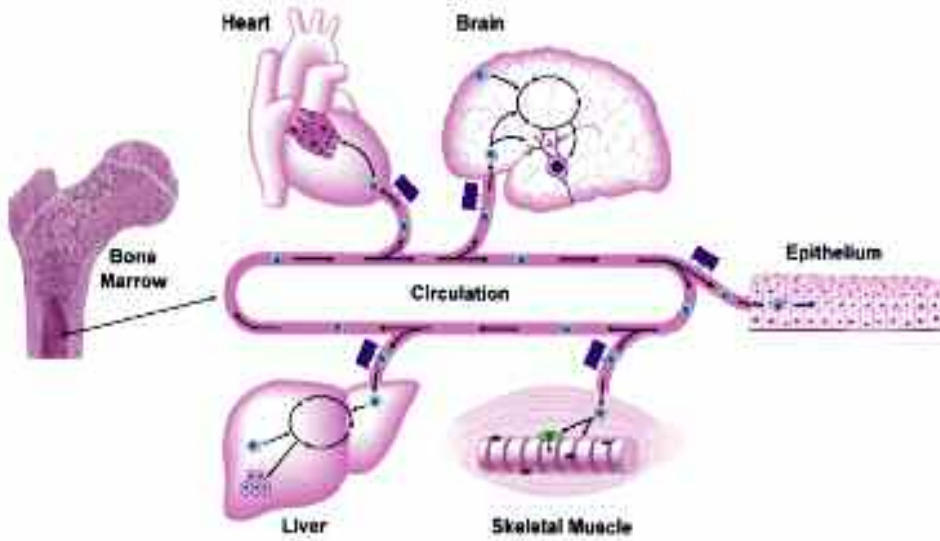


Figure 2. *Evolving concepts of stem cell plasticity.* Reported transitions in stem cell identity and differentiation are illustrated. In addition to localized tissue-specific stem cells, some stem cells may travel throughout the body via the circulation. The scheme also suggests that cell fate decisions may not be irreversible. Flexibility is the hallmark of this depiction allowing for regeneration and changes in cell fate in response to need.

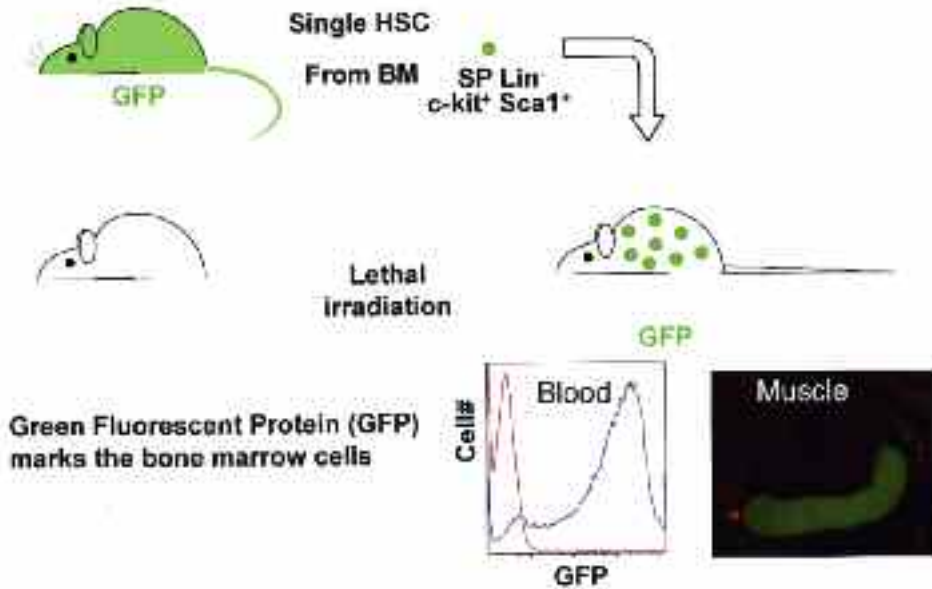


Figure 3. *General strategy for identifying cell fate transitions using bone-marrow derived cells.* Bone marrow cells from a genetically marked adult mouse are delivered intravascularly into isogenic, lethally irradiated, normal adult hosts. The bone marrow can derive either from transgenic donor mice that constitutively express green fluorescent protein (GFP) or  $\beta$ -galactosidase in all of their cells. Alternatively, cells from a male mouse can be used which, following transplantation into female mice, can be detected based on their Y-chromosome. Following irradiation at high doses, mice will die unless bone marrow is administered leading to reconstitution of all the lineages of the blood. The success of a bone marrow transplant can be ascertained by survival of the animal and the degree of chimerism in the blood, i.e., the proportion of the cells in the circulation of the recipient that express the genetic marker of the donor, determined either by microscopy or FACS. Four to eight weeks are usually required to reconstitute the blood in adult mice (8-10 weeks of age) and detection in the tissue of interest requires another 2-4 weeks.



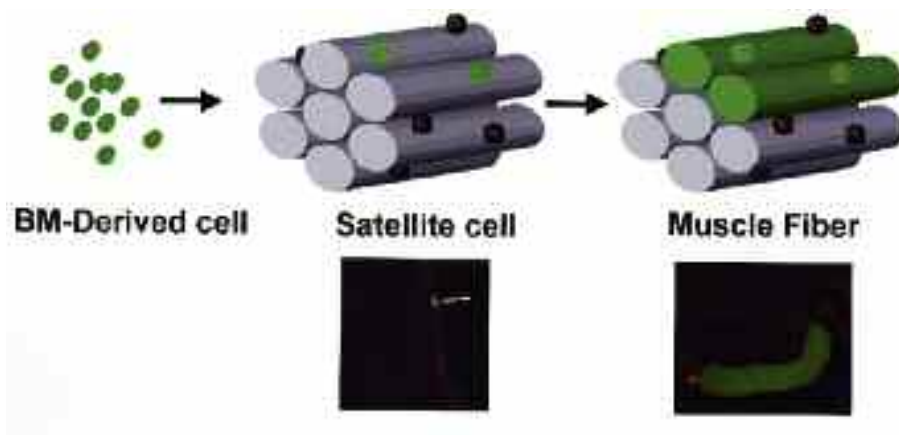


Figure 4. *Cells from the bone marrow give rise to muscle through a tissue-specific stem cells enroute to becoming mature muscle fibers.* Cells from the bone marrow, potentially hematopoietic stem cells or their progeny, contribute nuclei to multinucleate muscle fibers in adult mice that receive a bone marrow transplant. This schematic represents our hypothesis that the dominant mechanism for this contribution follows a series of differentiation steps from blood into muscle. First, a cell from the bone marrow differentiates into a muscle satellite cell. Muscle satellite cells are the entities in skeletal muscle that proliferate and fuse with muscle fibers during muscle regeneration. They are a biochemically and functionally diverse cohort of cells in adults and they may have multiple origins. Second, having adopted a muscle stem cell status, the bone marrow derived satellite cells are then activated by local cues to regenerate muscle, ultimately resulting in their fusion with existing and nascent muscle fibers in a manner typical of endogenous satellite cells.

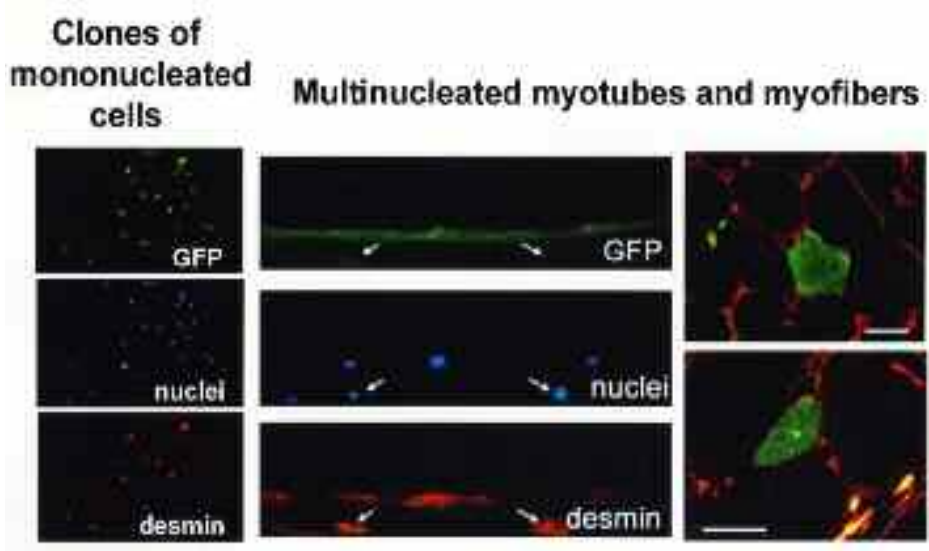


Figure 5. *The satellite cell niche (extracellular environment) causes reprogramming of nuclei of bone marrow derived cells and activation of muscle-specific genes.* A heritable myogenic phenotype is characteristic of bone marrow-derived satellite cells. The descendants of satellite cells, myoblasts, were isolated from the skeletal muscle of recipients of transplanted green fluorescent protein (GFP(+)) bone marrow in three independent experiments. Clones of mononucleate cells: clones that originate from single donor-derived myoblasts express *GFP*, exhibit *nuclei* stained with Hoechst 3342 and express the intermediate filament protein *desmin* (magnification x200). Multinucleated myotubes: when induced to differentiate, clones formed multinucleate myotubes in culture that expressed *GFP*, exhibited *nuclei* stained with Hoechst 3342 and express *desmin* (magnification x200). Arrows show nuclei of myoblasts outside the myotubes. Multinucleated myofibers: Bulk FACS-sorted donor-derived myoblasts were injected into the TA muscles of SCID mice where they fused with existing skeletal muscle fibers. Transverse sections of TA are shown with antibody staining GFP (green) and laminin (red) that are representative of two sections taken at 200 mm intervals showing that GFP(+) fibers span up to 200 mm. Scale bar represents 20 mm.

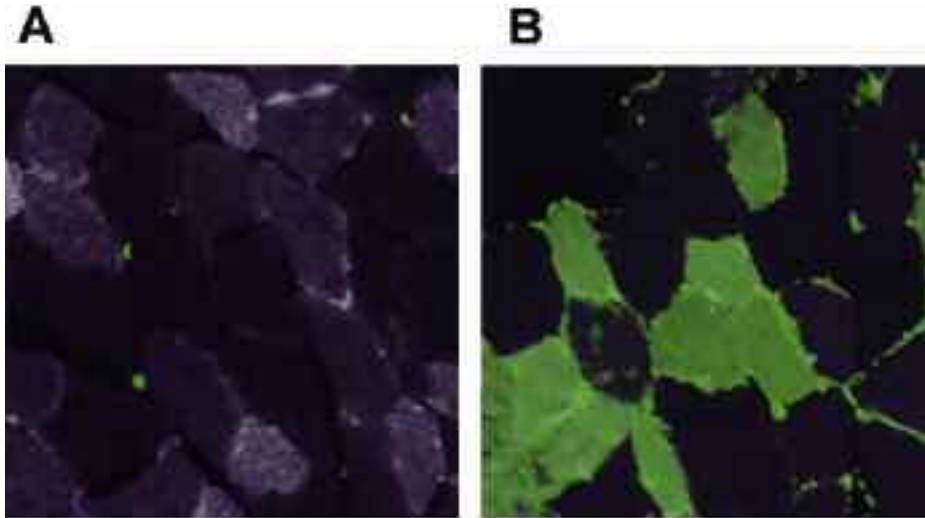


Figure 6. *Exercise causes damage which enhances the contribution of GFP(+) bone marrow-derived satellite cells to regenerating muscle. GFP(+) fibers in the Tibialis Anterior muscle are shown in unexercised (A) and exercised (B) mice. The background GFP(-) myofibers are shown in dark gray. GFP(+) muscle fibers were in clusters in the exercised mouse (B) suggesting that regeneration could have resulted from single GFP(+) satellite cell clones in these regions.*

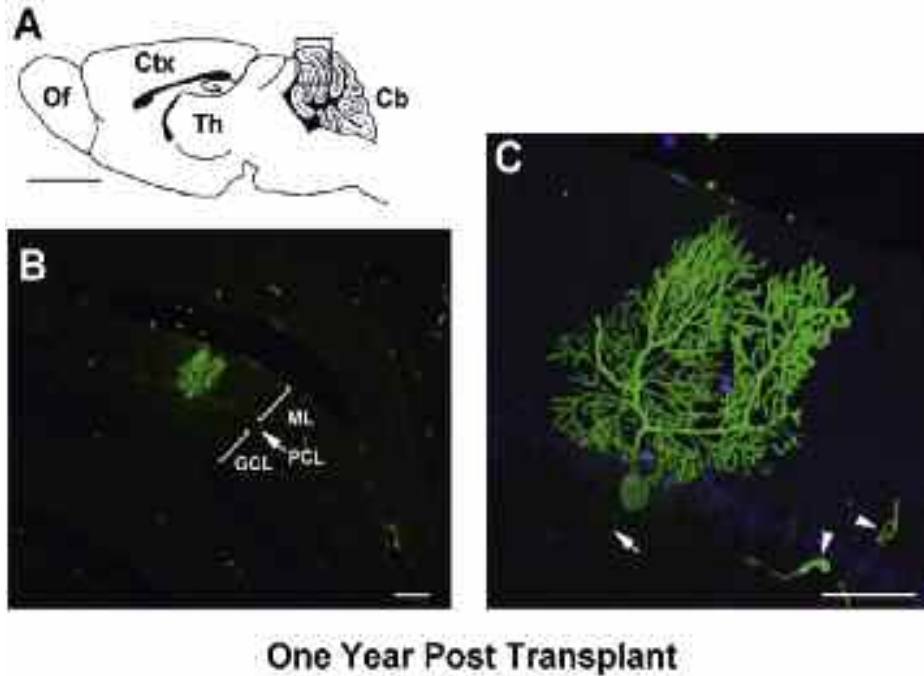


Figure 7. Fusion with Purkinje cells causes bone marrow derived cells results in GFP<sup>+</sup> Purkinje cells which and reprogramming of nuclei by intracellular signals. (A). Schematic of a mouse brain showing the anterior olfactory bulb (Of), cerebral cortex (Ctx), thalamus (Th), and the caudally located cerebellum (Cb). (B). In thick sections (45µm) cut from the cerebellum of a mouse post-bone marrow transplant, individual donor-derived GFP<sup>+</sup> Purkinje neurons are evident in the Purkinje cell layer (PCL). The dendrites from these cells extend into the cell sparse molecular layer (ML), while their axon projects through the granular cell layer (GCL) and is the only output connection from the cerebellum to the rest of the brain. Three lobes of the cerebellum in the box in (A) can be seen in (B). Note the many bone marrow derived (GFP<sup>+</sup>) cells in the parenchyma. (C). High power laser scanning confocal image of this cell show its many synaptic spines and single output axon (arrow). The 2 GFP<sup>+</sup> BMDC cells are probably microglia or macrophages in PCL and ML (arrowheads). Scale bars, (A): 2mm, (B): 100µm, (C): 50µm.

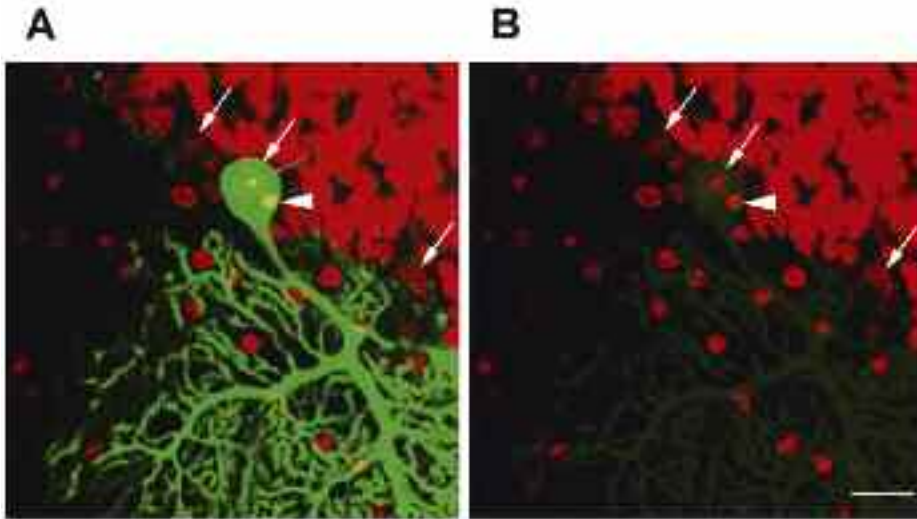


Figure 8. *100% of GFP<sup>+</sup> Purkinje cells are stably binucleate heterokaryons due to fusion.* Mice were bone marrow transplanted at 2 months of age and cerebella were collected and analyzed at various time points. (A and B) All of the GFP<sup>+</sup> Purkinje cells observed had 2 nuclei. (A) This Purkinje cell has a distinctive dendritic tree with many synaptic spines, and an axon exiting the soma at the left. One of the 2 nuclei in the cell is compact (arrow-head) and is the putative BMD nucleus. (B) The other nucleus has dispersed chromatin similar to other Purkinje neurons (arrows). In 752 control Purkinje neurons from transplanted and normal mice, no binucleated cells were observed. Scale bar: 20mm.

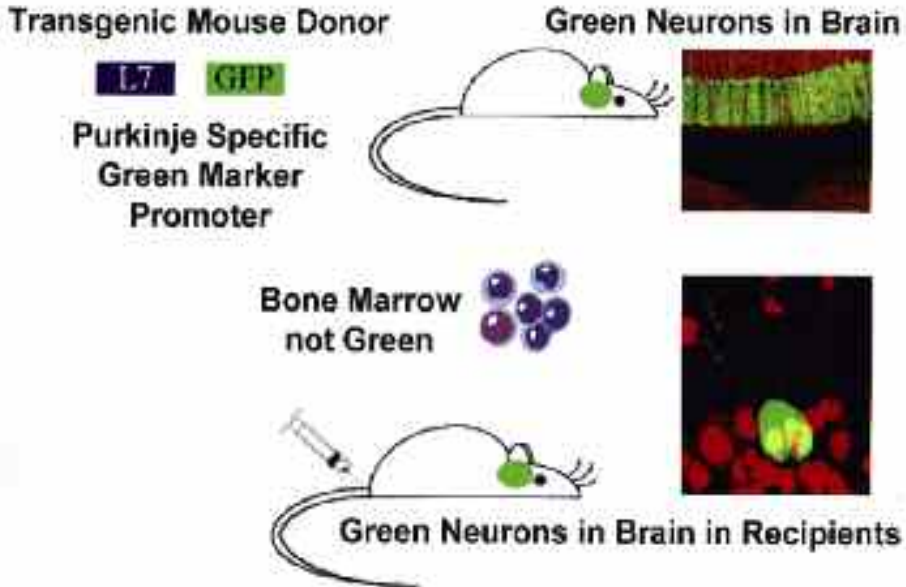
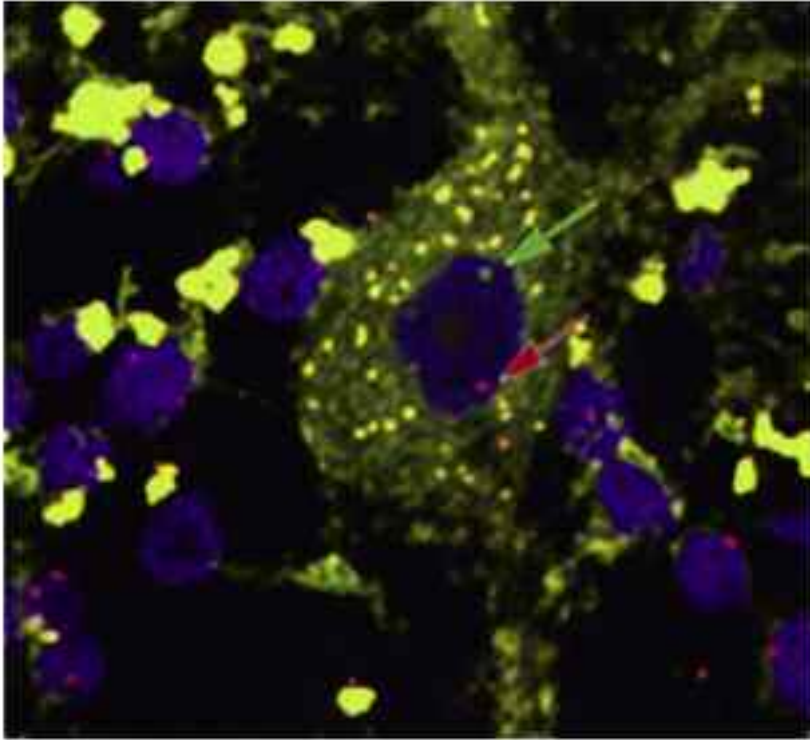


Figure 9. Schematic drawing of the experimental design demonstrating reprogramming of bone marrow derived cells (BMDCs) after transplantation into wild-type recipients. The upper panel illustrates, the Purkinje neuron-specific transgene, L7-EGFP-*pcp-2* construct, left and the micrograph, the expression pattern of L7-EGFP promoter in a 5mm optical coronal section of the cerebellum at the level of the 4th ventricle to the right. All Purkinje cells express the Purkinje specific promoter L7-EGFP (green), the section is counterstained with a nuclei dye, To-Pro3 (red).

The lower panel illustrates the injection of bone marrow, harvested from L7-EGFP tg-mice, into the tail vein of a wild-type isogenic recipient. The bone marrow does not express the Purkinje cell-specific transgene and hence is not green. The micrograph is a 1mm optical section showing the cell body of a Purkinje cell that express the L7-EGFP transgene. This reveals that a BMDC has fused with a Purkinje cell, forming a stable reprogrammed heterokaryon. Note the two nuclei in the Purkinje cell.



**Red - X chromosome**

**Green - Y chromosome**

**Blue - nuclear stain**

Figure 10. Evidence of *male* Y-chromosome in Purkinje neurons of *female* brains. The female-specific chromosomes (X) and the male-specific chromosomes (Y) in the cerebella were processed with X (red) and Y (green) probes, the nucleus counterstained with To-Pro-3 (blue) and imaged using a scanning confocal microscope at 1mm optical sections. The Purkinje neuron is clearly defined with a large nucleus surrounded by a large cytoplasmic region. The male bone marrow-derived nuclei (green arrow) can clearly be seen in the female (X-chromosome indicated by red arrow) Purkinje neuron.