

# Stem Cell Therapy for Neurologic Disorders: Therapeutic Potential of Adipose-Derived Stem Cells

Kristine M. Safford\* and Henry E. Rice

Department of Surgery, Duke University Medical Center, Durham, North Carolina, USA

**Abstract:** There is growing evidence to suggest that reservoirs of stem cells may reside in several types of adult tissue. These cells may retain the potential to transdifferentiate from one phenotype to another, presenting exciting possibilities for cellular therapies.

Recent discoveries in the area of neural differentiation are particularly exciting given the limited capacity of neural tissue for intrinsic repair and regeneration. Adult adipose tissue is a rich source of mesenchymal stem cells, providing an abundant and accessible source of adult stem cells. These cells have been termed adipose derived stem cells (ASC). The characterization of these ASCs has defined a population similar to marrow-derived and skeletal muscle-derived stem cells. The success seen in differentiating ASC into various mesenchymal lineages has generated interest in using ASC for neuronal differentiation. Initial *in vitro* studies characterized the morphology and protein expression of ASC after exposure to neural induction agents. Additional *in vitro* data suggests the possibility that ASCs are capable of neuronal activity. Progress in the *in vitro* characterization of ASCs has led to *in vivo* modeling to determine the survival, migration, and engraftment of transplanted ASCs.

While work to define the mechanisms behind the transdifferentiation of ASCs continues, their application to neurological diseases and injuries should also progress. The subject of this review is the capacity of adipose derived stem cells (ASC) for neural transdifferentiation and their application to the treatment of various neurologic disorders.

## INTRODUCTION

Reservoirs of stem and progenitor cells have been shown to exist in several types of adult tissue, including skin, muscle, bone marrow, and fat [1-7]. Growing evidence suggests that these cells may retain multilineage potential and are capable of giving rise to cell lineages other than those of the resident tissue. The reprogramming of cells from one mature tissue type to another phenotype is termed "transdifferentiation" [8, 9]. The possibilities raised by cell transdifferentiation are exciting for several reasons. First, the traditional concept that cells in adult tissue cannot change their developmental fate may not be absolute. Second, the use of adult stem cells would circumvent the ethical and logistic concerns associated with the use of embryonic stem cells. Third, adult stem cells present an easily accessible, abundant, and replenishable source of cells for use in cellular therapy applications.

Recent discoveries in the area of neural transdifferentiation are especially interesting given the limited capacity of neurons for regeneration [10, 11]. Neuronal transdifferentiation is difficult to demonstrate, and evidence of transdifferentiation should include immunocytochemistry for the presence of neuronal markers and the absence of non-neuronal markers, and demonstration of neuronal function of transdifferentiated cells [8, 12]. Initial work in the field of neuronal differentiation of mesenchymal cells was observed

with bone marrow stromal cells, first demonstrating *in vitro* expression of neural markers, followed by *in vivo* studies of transplantation and animal models of neural injuries and disease [13- 21, 99].

Adipose tissue is the most abundant and accessible source of adult stem cells [22]. The capacity of adipose derived stem cells (ASC) for neural transdifferentiation has been the subject of our recent work, opening up the possibility of using these cells in the treatment of various neurologic disorders. This review examines the recent studies on the isolation, characterization, and neural differentiation of adipose-derived stem cells.

## SOURCES OF STEM CELLS

A long standing pillar of developmental biology continues to be challenged, namely that a cell committed to a specific phenotype cannot change its destiny [23]. Recent studies have demonstrated the conversion of cells between lineages, even between different germ layers [24-26]. This concept of "transdifferentiation" has been challenged by explanations other than lineage switching, such as the presence of contaminating cells from a different lineage or cell fusion [17, 27]. To help develop new therapeutic options for neuronal diseases, it is essential to understand both the potentials as well as the limitations of various stem cell populations.

A stem cell is defined by its capability both to self-renew and to generate multiple differentiated progeny. The prototypical stem cell is the totipotent egg, and many critical advances in developmental biology have benefited from the

\*Address correspondence to this author at the Department of Surgery, Duke University Medical Center, Durham, North Carolina, USA; E-mail: saffo002@mc.duke.edu

study of embryonic stem cells (ES). ES cells can differentiate into various cell lineages *in vivo* and *in vitro*, including neuronal tissue [28, 101]. Similarly, both fetal and adult neuronal stem cells (NSC) can differentiate into neurons and glia *in vitro* and *in vivo* [30-41]. However, the clinical use of both ES and NSC cells is encumbered by numerous logistic and ethical constraints [5].

### MESENCHYMAL STEM CELLS

Increasing recognition is being made of the plasticity of stromal cells within bone marrow, termed mesenchymal stem cells (MSC) [42]. MSCs can differentiate into multiple mesodermal lineages, including bone, fat, and cartilage, as well as cells that are not part of their normal repertoire, including skeletal and cardiac muscle and hepatocytes [25, 43-48]. In addition to mesenchymal lineages, recent reports have suggested that MSCs can differentiate into cells with neuronal characteristics *in vitro*. Verfaillie *et al.* have identified a rare cell within marrow, termed a multipotent adult progenitor cell, which can differentiate into all three germ layers, including neurons [15]. Both Eglitis and Woodbury have shown that MSCs can differentiate into neurons *in vitro* [16, 21].

Several common themes have emerged from these studies. First, the isolation of a homogeneous population of MSCs results from the adherence of stromal cells to plastic, which depletes hematopoietic progenitors and other cells. Second, culture cocktails used to induce neuronal differentiation generally require agents such as retinoic or valproic acid, growth factors, antioxidants, demethylating agents, or compounds which increase intracellular cAMP. Other studies have shown a role for genetic inducers of neuronal differentiation, including *Noggin* and *Notch* [49, 50]. The findings of differentiation induced by these approaches has been challenged recently [51].

In addition to *in vitro* studies, *in vivo* studies have suggested that MSCs are capable of neuronal differentiation. Following transplantation in animal models, MSCs assume a neuronal phenotype, migrate in CNS, and restore function following CNS injury [13, 14, 18, 21, 29, 52-56]. These findings raise intriguing questions about plasticity across lineage boundaries, and suggest that similar approaches may be applicable to other stem cell sources [57].

### ADIPOSE TISSUE AS AN ALTERNATIVE SOURCE OF STEM CELLS

Recently, *adult adipose tissue* has become recognized as a alternative and rich source of mesenchymal stem cells [58-67]. Observations of ectopic bone formation in patients suffering from progressive osseous heteroplasia and the expansion in adipocytes numbers seen in obesity both support the idea that a pool of multipotent cells exists within adipose tissue [2, 21, 68].

The isolation of a population of progenitor cells from adipose tissue was first described by Rodbell in 1964, whose work was done in rodents [69]. This procedure was adapted in later years to isolate progenitors from human adipose tissue [70-72]. In 2001, Halvorsen *et al.* published a modification of existing isolation methods using liposuction waste

as a starting material, demonstrating the potential of this abundant and replenishable source for future clinical therapies [61, 67].

Current methods for isolating ASCs from adipose tissue vary slightly among investigators, but generally rely on an enzyme digestion followed by centrifugal separation to isolate the stromal/vascular cells from primary adipocytes. Differential centrifugation separates floating mature adipocytes from the pellet of stromal/vascular cells. This pellet contains blood cells, fibroblasts, pericytes, endothelial cells, and ASCs [22, 70, 73]. This stromal/vascular fraction is then plated on plastic tissue culture dishes. Stromal cells adhere to plastic, and during culture non-adherent hematopoietic and other contaminating cells can be depleted. The final population of cells can be maintained in an undifferentiated state for extended periods. These cells have been called several names by various groups, including adipose-derived stromal cells, preadipocytes, and processed lipoaspirate cells [58, 60-62, 74, 75]. At a recent consensus conference, these cells have been termed adipose-derived stem (ASC) cells [76].

ASC cells display a fibroblast like morphology and lack intracellular lipid droplets seen in adipocytes. After expansion in culture, ASC cells display a distinct phenotype based on cell surface protein expression and cytokine expression [74]. This phenotype is similar to that described for marrow-derived stromal cells and skeletal muscle derived stem cells [66, 67, 74, 77, 98]. Adipose tissue is a rich source of stem cells, as the frequency of stem cells within adipose tissue range from 1:100-1:1500 adherent cells, which far exceeds the frequency of MSCs in bone marrow [78, 79].

### DIFFERENTIATION OF ASC INTO NON-NEURAL LINEAGES

Under specific culture conditions, ASC cells can be induced to differentiate into various mesenchymal and endothelial lineages [60, 65, 67]. Under adipogenic conditions, ASC cells demonstrate perinuclear lipid droplets and expression of differentiation selective genes including *aP2*, *PPAR $\gamma$ 2*, and *C/EBP $\alpha$*  [58, 60, 61]. Under osteogenic conditions, ASC cells loaded onto a hydroxyapatite/tricalcium biomatrix form bone when implanted in mice. Under chondrogenic conditions, ASC cells express cartilage matrix molecules [67]. We have shown with Drs. Farsh Guilak and Jeff Gimble that human ASC-derived chondrocytes maintain a chondrogenic phenotype after implantation in nude mice, and these findings have been confirmed by others [63,64, 67, 80]. Expression of these lineage-specific proteins, transcription factors, and genes is not seen on ASC cells prior to *in vitro* exposure to supplemented media [22, 66, 81]. In addition to this *in vitro* characterization of the ASC differentiation, work is underway to explore tissue engineering applications for these cells.

### NEURONAL DIFFERENTIATION OF ASC CELLS

The success of mesenchymal differentiation of ASCs has led to our interest in ASCs for neuronal differentiation. Recent studies have reported the differentiation of ASCs into neuron-like cells, demonstrating differentiation towards a non-mesenchymal lineage. These findings are similar to

those seen with bone marrow-derived stromal cells [64, 66, 82, 83]. These findings suggest the existence of a replenishable source of stem cells for use in neurologic disorders and diseases, and are a promising alternative strategy for central nervous system cell therapy.

Neuronal differentiation of ASCs can be achieved by a variety of techniques, but generally involves exposing ASCs to a cocktail of induction agents. The induction media used by the two groups Safford *et al.* and Zuk *et al.* are similar, including the use of butylated hydroxyanisole, valproic acid, and forskolin [64, 66]. Butylated hydroxyanisole is an antioxidant, known to promote neural stem cell survival after ischemic injury. Valproic acid is a branch-chained fatty acid whose mechanism is not fully known. It is involved in the blockade of voltage-dependent sodium channels and the potentiation of GABAergic transmission. Forskolin is a neural stimulus involved in the regulation of neurotransmitter transporters and ion channels. These induction cocktails are modifications of previously published neuronal induction protocols developed for bone marrow-derived stromal cells [20, 21].

A recent study by Kang *et al.* employs a different induction protocol [82]. These researchers first expose the ASCs to 5-azacytidine, a demethylating agent capable of affecting gene expression [84]. Further differentiation is achieved by maintaining the cells in neurobasal medium containing B27 supplement. In another study, Ashjian *et al.* treated ASCs with indomethacin, isobutylmethylxanthine, and insulin to induce neural differentiation [80]. Indomethacin inhibits cyclooxygenase and has been shown to promote neural cell survival after ischemic injury. Isobutylmethylxanthine is a phosphodiesterase inhibitor, resulting in an elevation of intracellular cyclic adenosine monophosphate (cAMP). An increase in cAMP acts as a neural stimulus for several cell types, including mesenchymal stem cells [85-87].

Initial *in vitro* studies examined the neural differentiation of ASCs after exposure to induction agents, characterizing the morphology and protein expression of these cells [64, 75, 80, 83]. Within several hours of exposure of ASCs to neuronal induction media, many cells display changes in cellular morphology, with retraction of cytoplasm toward the nucleus and formation of compact cell bodies with cytoplasmic extensions. The majority of ASCs exposed to neuronal induction media became increasingly spherical and refractile, exhibiting a perikaryal appearance, suggestive of a primitive neuronal/glial phenotype. These morphological changes can be seen in ASCs as early as 5 hours after exposure to neuronal induction media [64].

Neuronal markers expressed by neuronally-induced ASCs, as determined by immunohistochemistry, include NeuN, MAP2, tau,  $\beta$ -tubulin, and NSE. Expression of glial markers including GFAP, vimentin, and S100 was also demonstrated by immunocytochemistry. Expression of the oligodendrocyte marker O4 has not been seen on neuronally-induced ASCs *in vitro*. The fraction of ASCs expressing neuronal phenotypic markers *in vitro* is relatively high, with some markers such as NeuN seen in approximately 80% of neuronally-induced ASCs. The high percentages of protein expression suggest that a majority of the cells in this

heterogeneous population are undergoing at least a degree of neural differentiation. Further, the co-expression of NeuN and GFAP in some cells suggests that at least in short-term culture, some ASCs may retain the potential for neuronal as well as glial development.

The expression of neuronal and glial markers on ASC cells has been examined further by Western blot and PCR analysis. Zuk *et al.* found nestin expression both in undifferentiated ASCs and ASCs exposed to neuronal induction media for nine hours [66]. No GFAP expression was found in either group of cells. Another group also found nestin expression in undifferentiated cells [82]. We found that after neuronal induction, ASCs displayed increased nestin expression, as well as MAP2 expression [83].

A more extensive profile of neuronal and glial markers in ASCs has been examined by Western blot analysis. Zuk *et al.* found expression of certain neuronal and glial markers in both undifferentiated and differentiated ASCs, including NSE, NeuN, trk-A, and vimentin. In contrast, we found expression of a broad panel of neuronal and glial markers only in ASCs exposed to neuronal differentiation media. Undifferentiated ASCs were only found to express synapsin I.

## FUNCTION OF ASC CELLS *IN VITRO*

Several groups have described a neuronal/glial protein expression profile of ASCs exposed to neuronal induction agents *in vitro*. However, the functional capabilities of ASCs have been more difficult to establish. Initial attempts at determining whether ASCs possess any functional potential of neurons involved staining neuronally-induced ASCs with markers against neurotransmitter, neurotransmitter precursor, and neural protein markers [83]. We found that select neuronally-induced ASCs express markers of both GABA and glutamate pathways. Also, select ASCs express markers of more mature neuronal tissues, including the expression of subunits of the glutamate NMDA receptor, synapsin I, the  $\alpha$ -1 calcium channel marker, or GAP-43. Select cells express both subunits of the glutamate NMDA receptor, suggesting that these cells may have the potential to respond to neuronal agonists [83].

Although this immunocytochemical data is significant, it does not show that ASCs are capable of functional activity. We have shown that neuronally-induced ASCs demonstrate an excitotoxic response to NMDA with a loss of cell viability suggesting that cells within this population may have formed functional NMDA receptors [83]. This data suggests that the exposure of ASCs to neuronal induction media induces a physiologic response to NMDA. However, we recognize that these findings are non-specific, and do not confirm the presence of neuronal tissue in these cultures. Zuk *et al.* have shown in limited studies that ASCs are capable of conducting an action potential using electrophysiology [66]. These researchers found that induced ASCs display a delayed rectifier  $K^+$  current, suggesting the presence of voltage-dependent  $K^+$  channels. They hypothesize that the expression of these channels, which precede  $Na^+$  and  $Ca^{2+}$  channels, correlates to the temporal development of ion channels in maturing neurons [80].

## IN VIVO MODELING USING ASC CELLS

The success and progress of the *in vitro* studies of ASCs has led to the fundamental question whether these cells are capable of survival and function after transplantation into the CNS. We have observed in preliminary experiments that ASCs can survive after transplantation into the CA1 hippocampus of the intact mouse brain. Preliminary results from this model suggest that neuronally induced ASCs, but not control undifferentiated ASCs, can survive *in vivo* to at least 12 weeks after transplantation. Surviving ASCs migrated up to 2.0 mm from the injection site similar to other studies of marrow stromal cells and adult neural progenitor cells, with migration along the corpus callosum and around the rostro-caudal axis within the striatum [13, 30, 75]. Select surviving ASC cells express both morphology as well as phenotypic markers of neuronal cells. These findings are similar to observations of adult and embryonic neural progenitor cells responding to local *in vivo* cues [88, 89]. Markers of mature neurotransmitter function were not seen. In these experiments, *in vitro* exposure of ASCs to induction agents appears to be necessary for their survival within a neuronal microenvironment.

In a comparable study, Kang *et al.* found survival and migration of human ASCs that were transplanted into rats [82]. This group cultured ASCs in neurobasal medium with B27 supplement, 5-azacytidine, and the growth factors BDNF, bFGF, and nerve growth factor. After transplantation into the lateral ventricle of the rat brain, ASCs survived and migrated to multiple areas of the brain. ASCs that were transplanted into animals with focal ischemia resulting from middle cerebral artery occlusion showed that ASCs migrated into the injured area of the cortex, suggesting that ischemia-induced factors facilitate donor cell migration [82]. Finally, behavioral testing demonstrated that ASCs improved functional recovery after MCA occlusion, with additional benefit seen when ASCs were transfected with BDNF prior to transplantation.

## TRANSDIFFERENTIATION

Much debate has centered on the mechanisms involved in transdifferentiation. The process is theorized to occur primarily through DNA transcriptional activation and repression from chromatin structure modifications, which are determined by intrinsic and extrinsic growth factors [90, 91]. No single unifying model describing this process has yet emerged, a result of the complexity of the mechanisms involved.

The *in vivo* ASC studies outlined above provide some insights to the potential of using ASCs for neuronal therapies, and raise additional questions about the transdifferentiation potential of adult stem cells. These *in vivo* studies demonstrate that ASCs are able to survive and migrate after transplantation, and may even improve behavioral outcomes after cerebral ischemia. However, it remains unclear if ASCs are able to express a full profile of neuronal and glial markers *in vivo*. Furthermore, it remains unclear the extent to which the phenotypic fate of ASCs is determined *in vitro* prior to transplantation, or whether the final fate is altered by the local microenvironment. Pretreatment of bone marrow

stromal cells with neurotrophic factors *in vitro* prior to transplantation has been shown to improve cell survival and neural differentiation *in vivo* [92]. It appears that a combination of *in vitro* pretreatment and transplantation within a neuronal microenvironment or an area of injury supports ASC differentiation toward a neuronal phenotype, even in a normally non-neurogenic area. The neuronal transdifferentiation process of ASCs may result from the interactions of groups of cells, cytokines provided by these cells, growth factors, and intercellular signals [90].

Can the findings of these *in vivo* studies can be explained by mechanisms other than transdifferentiation? Several reports have suggested that the transdifferentiation is the result of the fusion of donor and host cells *in vivo* [93-97]. These studies question the plasticity of adult-derived stem cells. In contrast to studies involving the transplantation of marrow stromal cells or embryonic stem cells into mitotically active host tissue, the limited proliferative capacity of the adult brain may limit the potential of fusion events. Also, the ASC culture systems in the studies outlined above are based on induction agents rather than the use of a feeder layer, eliminating the possibility of *in vitro* fusion events prior to transplantation. Kang *et al.* suggest that the percentage of engrafted donor cells expressing neural markers is too great to be explained by the low frequency of cell fusion events. Future studies clearly need to test for the possibility of cell fusion after transplantation.

Another criticism of ASC differentiation results from the ASC cell population itself. ASC isolation generally involves the use of an unpurified population of adherent stromal cells. Critics suggest that even a low fraction of contaminating hematopoietic stem cells could be the source of the differentiation seen in the ASC experiments. However, flow cytometry data refute that claim by demonstrating that ASCs cells do not express the hematopoietic markers CD11b or CD45 [64]. In addition, recent clonal analysis has demonstrated ASC multilineage differentiation, including neuronal differentiation, from clones derived from single cell clones of human ASCs [63].

Assuming that cell fusion or contamination are not the primary reasons for the results seen in the *in vivo* studies of ASCs, additional proof is needed before ASCs can be labeled as transdifferentiated. For instance, the behavioral improvement seen by Kang *et al.* in rats undergoing ASC transplantation after ischemic injury can be explained by other mechanisms other than the transdifferentiated of donor cells into neurons [8]. The transplanted cells may release cytokines and trophic factors that act on surviving host cells and produce functional improvement [8, 100]. In fact, the ASCs transfected with BDNF by Kang *et al.* result in increased behavioral benefits compared to ASCs alone [82]. As well, donor cells can be examined for the expression of molecules necessary for synaptic transmission, neurotransmitter synthesis, and neurotransmitter release [8]. More convincing evidence for the neuronal transdifferentiation of ASCs is required to establish this as mechanism, such as *in vivo* analysis of donor cell neuronal function, including electrophysiologic studies. However, even if all ASCs cannot fully differentiate, it is of great interest to see if ASCs can survive within the CNS, as repair processes may be

possible even without complete differentiation, as is seen with other cell types.

## ASC CELLS FOR THERAPIES IN NEURAL DISEASES AND INJURIES

Within the past few years, the discovery of neural stem cells (NSC) has redefined the previous belief that the nervous system was incapable of regeneration or repair [10, 27, 83]. The interaction of donor neuronal stem cells and the host CNS microenvironment has raised questions about the use of stem cells for gene therapies, cellular therapies, neuroprotection, and neural repair [83]. Equally as groundbreaking is the growing evidence that select cells within adult non-CNS tissues retain a certain amount of plasticity, allowing them to undergo a reprogramming from the lineage of their native tissue. Experiments using stem cells derived from bone marrow have already shown potential in models of stroke, traumatic brain injury, spinal cord injury, and Parkinsons disease [20]. Adipose tissue represents an accessible and replenishable source of multipotent progenitor cells with stem cell properties. While work to define the mechanisms behind the neuronal differentiation of ASC cells continues, their application to neurological diseases and injuries should also progress. The potential of using ASC cells for neural transdifferentiation has only begun to be investigated, but the promise of these cells for the treatment of neural diseases and injuries warrants continued exploration.

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